AMPEROMETRIC SENSOR

Cross-Reference to Related Application

[0001] This application claims the benefit of priority of United State of America Provisional Patent Application No. 61/534,007, filed 13 September 2011, the contents of which being hereby incorporated by reference in its entirety for all purposes.

Technical Field

[0002] The invention relates to an amperometric sensor, and in particular, to an amperometric sensor for hydrogen peroxide (H_2O_2) operating without application of an external electrical potential.

Background

[0003] Electrochemical sensors and biosensors detect analytes typically by monitoring electron flow through the sensor under an applied electrical potential which provides an equivalent power magnitude of $I.V$ (current.voltage) to drive the redox reaction between the analyte and electrode, mediator or electrocatalytic species including enzymes. A biofuel cell design, which measures the changes in the open-circuit potential in response to the biofuel concentration, utilizes biological catalysts as the biocathode and/or bioanode in which the reduction of oxygen and the oxidation of biofuel occurred. Subsequent works in similar direction have included analytes such as lactate, glucose, cyanide, and Hg^{+}.

Summary

[0004] A self-powered amperometric sensor for detecting analyte present in a sample is disclosed herein. The sensor derives current signal from the spontaneous reaction between hydrogen peroxide (H_2O_2) and the sensor in the absence of an externally applied electrical potential. The sensor does not rely on the open-circuit potential as the

responsive signal; rather, a complete closed circuit which allows electrons to flow is employed in the present sensor. Additions or depletion of H_2O_2, directly or indirectly, results in a change in the detected current flow, correlating to the amount of analyte present in the sample.

[0005] Thus, in a first aspect, there is provided an amperometric sensor for measuring the amount of hydrogen peroxide (H_2O_2) present in a sample. The sensor comprises:

a working electrode in contact with a sensing solution, wherein the working electrode comprises a porous membrane structure, wherein the sensing solution comprises the sample containing H_2O_2 or a precursor of H_2O_2, wherein H_2O_2 is reduced at the working electrode;

a counter electrode in contact with a reference solution, wherein the porous membrane structure of the working electrode provides a physical barrier between the sensing solution and the reference solution, wherein a reducer is oxidized at the counter electrode;

a connector to electrically connect the working electrode and the counter electrode to allow a current flow therethrough, wherein the current flow is formed by a flow of electrons from the electrode where oxidation has occurred to the electrode where reduction has occurred, with the proviso that the current flow is not induced by an externally applied electrical potential; and

a current detector to detect and measure the current flow.

[0006] According to a second aspect, there is provided a method for measuring the amount of H_2O_2 present in a sample. The method comprises:

providing an amperometric sensor of the first aspect;

contacting a sensing solution with a working electrode of the amperometric sensor, wherein the working electrode comprises a porous membrane structure, wherein the sensing solution comprises the sample containing H_2O_2 or a precursor of H_2O_2, wherein H_2O_2 is reduced at the working electrode;

contacting a reference solution with a counter electrode of the amperometric sensor, wherein the porous membrane structure of the working electrode provides a physical barrier between the sensing solution and the reference solution, wherein a reducer is oxidized at the counter electrode;

measuring the current flow between the working electrode and the counter electrode, wherein the current flow is formed by a flow of electrons from the electrode where oxidation has occurred to the electrode where reduction has occurred, with the proviso that the current flow is not induced by an externally applied electrical potential; and

correlating the measured current flow to the amount of H_2O_2 present in the sample.

Brief Description of the Drawings

[0007] In the drawings, like reference characters generally refer to the same parts throughout the different views. The drawings are not necessarily drawn to scale, emphasis instead generally being placed upon illustrating the principles of various embodiments. In the following description, various embodiments of the invention are described with reference to the following drawings.

[0008] **Fig. 1** shows a schematic of **(a)** the H_2O_2-powered sensor using a 2-compartment cell separated by a Prussian blue nanotubes membrane; **(b)** the H_2O_2-

powered virus sensor using a 2-compartment cell separated by a Prussian blue nanotubes membrane, showing the binding of virus to antibody molecules within the nanochannels of the porous membrane.

[0009] Fig. 2 shows the current-potential curves for solution oxygen reduction under ambient condition (— ·), oxygen reduction in air-saturated solution (----), and hydrogen peroxide (8 mM) reduction in nitrogen-saturated solution (—) at **(a)** porous PB-*nt* membrane (i.e. porous Pt membrane coated with PB); **(b)** porous Pt membrane. Conditions: 1mV s^{-1} scan rate; 0.5M KCl solutions.

[0010] Fig. 3 shows **(a)** the effect of adding H_2O_2 on the driving force measured from the potential difference between PB-*nt* and auxiliary electrode during closed-circuit condition when current flows through the cell; **(b)** the effect of adding H_2O_2 on the potential difference between PB-*nt* and counter electrodes under open-circuit condition when no current flows through the cell; **(c)** the plot of driving force obtained from **(a)** versus the logarithm of H_2O_2 concentration. Conditions: Sensing solution contains 1M TRIS buffer, reference solution contains 0.5M KCl.

[0011] Fig. 4 shows the dependence of the power density on the potential of the PB-*nt* membrane electrode vs auxiliary electrode in sensing solution containing 30mM H_2O_2. Conditions: Sensing solution contains 1M TRIS buffer, reference solution contains 0.5M KCl.

[0012] Fig. 5 shows the effect of increasing K^+ concentration in the reference or sensing solution during the addition of H_2O_2 to the sensing solution. The following three arrangements of cell solutions are used: **(a)** Pt|KCl||H_2O_2, KCl|PB; **(b)** Pt|1M TRIS||H_2O_2, KCl|PB; **(c)** Pt|KCl||H_2O_2, 1M TRIS|PB. A control containing 1M TRIS buffer without KCl in both reference and sensing solutions is added to compare with the effect of KCl, which gives linear response unlike the rest.

[0013] Fig. 6 shows the chemical reaction driven amperometric responses of **(a)** H_2O_2 sensor during successive additions of H_2O_2 (indicated by arrows); **(b)** glucose sensor during successive additions of glucose (indicated by arrows). Inset: Best fitted calibration plot of sensor signal toward H_2O_2 or glucose, using three different sensors with normalization to initial charges needed to charge PB-*nt* upon circuit connection but before addition of analyte. Conditions: Signal filtered at 1 Hz, **(a)** Sensing solution contains 1M TRIS buffer (pH = 7), reference solution contains 0.5M KCl; **(b)** Sensing solution contains 1M TRIS buffer (pH = 7) with 4mg/mL glucose oxidase, reference solution contains 0.1M KCl in 1M TRIS buffer (pH = 7).

[0014] Fig. 7 shows the chemical reaction driven amperometric responses of virus biosensor during successive additions of stock solution of virus (indicated by arrows). Conditions: Signal filtered at 1Hz, sensing solution contains 1M TRIS buffer (pH = 7), reference solution contains 0.5M KCl.

[0015] Fig. 8 illustrates the construction of a porous PB-*nanotube* membrane electrode. The PB-*nt* membrane is fabricated by sputtering a ~50 nm thick platinum layer on one side of a 60 μm thick nanoporous alumina membrane, followed by electrodeposition of PB onto the porous Pt membrane electrode.

[0016] Fig. 9 shows (A) typical closed-circuit steady-state current response of a H_2O_2-powered sensor toward DENV-2 virus; (B) its calibration plot of normalized closed circuit steady-state current versus virus concentration; line is non-linear best fitted data; error bars are standard deviation of experiment data (points); (C) selective and non-selective responses of the H_2O_2-powered virus sensor toward DENV-2and DENV-3, added sequentially to the sensing solution. (A)-(C) use 200 nm pore size membranes as templates; (D) unstable closed-circuit current response of a H_2O_2-powered sensor toward DENV-2 virus, using a 20 nm pore size membrane.

[0017] **Fig. 10** shows a photograph and schematic of a standalone Nafion-filled membrane probe using the nanometer thick metal layer of the membrane at the air-electrolyte interface as the reference and counter electrode.

[0018] **Fig. 11** shows a closed-circuit current response of a dry membrane probe of **Fig. 10** immobilized with anti-dengue type II monoclonal antibody toward (A) DENV-2 and (B) DENV-3.

Description

[0019] The following detailed description refers to the accompanying drawings that show, by way of illustration, specific details and embodiments in which the invention may be practised. These embodiments are described in sufficient detail to enable those skilled in the art to practise the invention. Other embodiments may be utilized and structural, logical, and electrical changes may be made without departing from the scope of the invention. The various embodiments are not necessarily mutually exclusive, as some embodiments can be combined with one or more other embodiments to form new embodiments.

[0020] The spontaneous redox reactions between hydrogen peroxide (H_2O_2) and the present sensor is selected of interest because of the significance of H_2O_2 in treating waste in groundwater, in cell metabolism, signal transduction, as stress indicators of living cells, as clinical markers for diseases and is the product of several hundreds of oxidases enzymes, commonly used in many enzyme based biosensors.

[0021] In a first aspect, there is provided an amperometric sensor for measuring the amount of H_2O_2 present in a sample. The sensor comprises:

a working electrode in contact with a sensing solution, wherein the working electrode comprises a porous membrane structure, wherein the sensing

solution comprises the sample containing H_2O_2 or a precursor of H_2O_2, wherein H_2O_2 is reduced at the working electrode;

a counter electrode in contact with a reference solution, wherein the porous membrane structure of the working electrode provides a physical barrier between the sensing solution and the reference solution, wherein a reducer is oxidized at the counter electrode;

a connector to electrically connect the working electrode and the counter electrode to allow a current flow therethrough, wherein the current flow is formed by a flow of electrons from the electrode where oxidation has occurred to the electrode where reduction has occurred, with the proviso that the current flow is not induced by an externally applied electrical potential; and

a current detector to detect and measure the current flow.

[0022] According to a second aspect, there is provided a method for measuring the amount of H_2O_2 present in a sample. The method comprises:

providing an amperometric sensor of the first aspect;

contacting a sensing solution with a working electrode of the amperometric sensor, wherein the working electrode comprises a porous membrane structure, wherein the sensing solution comprises the sample containing H_2O_2 or a precursor of H_2O_2, wherein H_2O_2 is reduced at the working electrode;

contacting a reference solution with a counter electrode of the amperometric sensor, wherein the porous membrane structure of the working electrode provides a physical barrier between the sensing solution and

the reference solution, wherein a reducer is oxidized at the counter electrode;

measuring the current flow between the working electrode and the counter electrode, wherein the current flow is formed by a flow of electrons from the electrode where oxidation has occurred to the electrode where reduction has occurred, with the proviso that the current flow is not induced by an externally applied electrical potential; and

correlating the measured current flow to the amount of H_2O_2 present in the sample.

[0023] In the present context, an amperometric sensor refers to a galvanic electrochemical sensor for the detection of ions in a solution based on electric current or changes in electric current. In particular, changes in the concentrations of the H_2O_2 and of a reducer (or an oxidizer) at a respective electrode of the sensor enable a flow of electrons in a connector electrically connecting the respective electrodes of the sensor, thereby allowing an electric current or a change thereof to be detected. The detected current or a change thereof is then correlated to the amount of H_2O_2 present in the sample or the amount of H_2O_2 produced or consumed in the reaction. For the convenience of the present discussion, the H_2O_2 is described as being reduced at the working electrode. However, it is to be understood and appreciated that in certain embodiments, H_2O_2 is oxidzed at the working electrode and an oxidizer is reduced at the counter electrode.

[0024] In certain embodiments, the sample contains H_2O_2 as the analyte to be detected.

[0025] In other embodiments, changes in the concentration of H_2O_2 are correlated to the amount of an analyte present in the sample. For example, when analyte glucose is added to a solution comprising glucose oxidase (Gox), glucose is oxidized by Gox to gluconic

acid and H_2O_2 is produced in the reaction. The amount of H_2O_2 being produced during the reaction correlates to the amount of glucose being converted into gluconic acid. By determining the change in the concentration of H_2O_2 detected by the present amperometric sensor, the amount of analyte glucose added to the sensor can thus be determined accordingly. In other cases, H_2O_2 may be consumed during a reaction and likewise, the change in the concentration of H_2O_2 may be correlated to the analyte of interest.

[0026] In the present context, the amperometric sensor may also be termed as an amperometric biosensor if the analyte to be detected is a biomolecule. Hence, for the convenience of facilitating present discussion, an amperometric sensor, or sometimes simply termed as a sensor, is generally described herein and it is to be understood that it may also refer to an amperometric biosensor, when appropriate.

[0027] The terms "analyte" and "target molecule" as interchangeably used herein, refer to any substance that can be detected in an assay by binding to a binding or capture molecule, and which, in one embodiment, may be present in a sample. Therefore, the analyte can be, without limitation, any substance for which there exists a naturally occurring antibody or for which an antibody can be prepared. The analyte may, for example, be an antigen, a protein, a polypeptide, a nucleic acid, a hapten, a carbohydrate, a lipid, a cell or any other of a wide variety of biological or non-biological molecules, complexes or combinations thereof. Generally, the analyte will be a protein, peptide, carbohydrate or lipid derived from a biological source such as bacterial, fungal, viral, plant or animal samples. Additionally, however, the target may also be a small organic compound such as a drug, drug-metabolite, dye or other small molecule present in the sample.

[0028] The term "sample", as used herein, refers to an aliquot of material, frequently but not necessarily always, biological matrices, an aqueous solution or an aqueous suspension derived from biological material. Samples to be assayed for the presence of an analyte by the methods of the present invention include, for example, cells, tissues, homogenates, lysates, extracts, and purified or partially purified proteins and other biological molecules and mixtures thereof.

[0029] Non-limiting examples of samples typically used in the methods of the invention include human and animal body fluids such as whole blood, serum, plasma, cerebrospinal fluid, sputum, bronchial washing, bronchial aspirates, urine, semen, lymph fluids and various external secretions of the respiratory, intestinal and genitourinary tracts, tears, saliva, milk, white blood cells, myelomas and the like; biological fluids such as cell culture supernatants; tissue specimens which may or may not be fixed; and cell specimens which may or may not be fixed. The samples used in the methods of the present invention will vary based on the assay format and the nature of the tissues, cells, extracts or other materials, especially biological materials, to be assayed. Methods for preparing protein extracts from cells or samples are well known in the art and can be readily adapted in order to obtain a sample that is compatible with the methods of the invention. Detection in a body fluid can also be *in vivo*, i.e. without first collecting a sample.

[0030] "Peptide" generally refers to a short chain of amino acids linked by peptide bonds. Typically peptides comprise amino acid chains of about 2-100, more typically about 4-50, and most commonly about 6-20 amino acids. "Polypeptide" generally refers to individual straight or branched chain sequences of amino acids that are typically longer than peptides. "Polypeptides" usually comprise at least about 20 to 1000 amino acids in length, more typically at least about 100 to 600 amino acids, and frequently at

least about 200 to about 500 amino acids. Included are homo-polymers of one specific amino acid, such as for example, poly-lysine. "Proteins" include single polypeptides as well as complexes of multiple polypeptide chains, which may be the same or different.

[0031] Multiple chains in a protein may be characterized by secondary, tertiary and quaternary structure as well as the primary amino acid sequence structure, may be held together, for example, by disulfide bonds, and may include post-synthetic modifications such as, without limitation, glycosylation, phosphorylation, truncations or other processing.

[0032] Antibodies such as IgG proteins, for example, are typically comprised of four polypeptide chains (i.e., two heavy and two light chains) that are held together by disulfide bonds. Furthermore, proteins may include additional components such associated metals (e. g., iron, copper and sulfur), or other moieties. The definitions of peptides, polypeptides and proteins includes, without limitation, biologically active and inactive forms; denatured and native forms; as well as variant, modified, truncated, hybrid, and chimeric forms thereof.

[0033] The terms "contact" or "contacting" as used herein refer generally to providing access of one component, reagent, analyte or sample to another. For example, contacting can involve mixing a solution comprising an analyte binding protein or conjugate thereof with a sample. The solution comprising one component, reagent, analyte or sample may also comprise another component or reagent, which facilitates mixing, interaction, uptake, or other physical or chemical phenomenon advantageous to the contact between components, reagents, analytes and/or samples.

[0034] The term "detecting" and associated term “detection”, as used herein, refer to a method of verifying the presence of a given molecule. The technique used to accomplish this is an electrochemical detection method involving generating an electric

current signal based on changes in the concentration of H_2O_2 as described above. The term "electrochemical detection" as used herein refers to the utilization of electrochemical means to indicate the presence or absence, either qualitatively or quantitatively, of an analyte, i.e. include correlating the detected signal with the amount of analyte.

[0035] The term "hapten" as used herein, refers to a small proteinaceous or non-protein antigenic determinant which is capable of being recognized by an antibody. Typically, haptens do not elicit antibody formation in an animal unless part of a larger species. For example, small peptide haptens are frequently coupled to a carrier protein such as keyhole limpet hemocyanin in order to generate an anti-hapten antibody response.

[0036] "Antigens" are macromolecules capable of generating an antibody response in an animal and being recognized by the resulting antibody. Both antigens and haptens comprise at least one antigenic determinant or "epitope", which is the region of the antigen or hapten which binds to the antibody. Typically, the epitope on a hapten is the entire molecule.

[0037] The term "capture molecule" as used herein refers to any molecule capable of binding to an analyte or target molecule of choice so as to form a complex consisting of the capture molecule and the target molecule. Preferably, this binding is specific so that a specific complex is formed.

[0038] "Specifically binding" and "specific binding" as used herein mean that the capture molecule binds to the target molecule based on recognition of a binding region or epitope on the target molecule. The capture molecule preferably recognizes and binds to the target molecule with a higher binding affinity than it binds to other compounds in the sample. In various embodiments, "specifically binding" may mean that an antibody or other biological molecule, binds to a target molecule with at least about a 10^6-fold

greater affinity, preferably at least about a 10^7-fold greater affinity, more preferably at least about a 10^8-fold greater affinity, and most preferably at least about a 10^9-fold greater affinity than it binds molecules unrelated to the target molecule. Typically, specific binding refers to affinities in the range of about 10^6-fold to about 10^9-fold greater than non-specific binding. In some embodiments, specific binding may be characterized by affinities greater than 10^9-fold over non-specific binding. The binding affinity may be determined by any suitable method. Such methods are known in the art and include, without limitation, surface plasmon resonance and isothermal titration calorimetry. In a specific embodiment, the capture molecule uniquely recognizes and binds to the target molecule.

[0039] The capture molecule may be a proteinaceous molecule, such as an antibody, for example a monoclonal or polyclonal antibody, which immunologically binds to the analyte at a specific determinant or epitope. The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies as well as antibody variants or fragments (e.g., Fab, $F(ab')_2$, scFv, Fv diabodies and linear antibodies), so long as they exhibit the desired binding activity.

[0040] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized by the

hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. The monoclonal antibodies can include "chimeric" antibodies and humanized antibodies. A "chimeric" antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

[0041] Monoclonal antibodies may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to the hybridoma technique of Koehler and Milstein (U. S. Patent No. 4,376,110), the human B-cell hybridoma technique, and the EBV-hybridoma technique. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb may be cultivated in vitro or in vivo. Production of high titres of mAbs *in vivo* makes this a very effective method of production.

[0042] "Polyclonal antibodies" are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals such as rabbits, mice and goats, may be immunized by injection with an antigen or hapten-carrier conjugate optionally supplemented with adjuvants.

[0043] Alternatively, techniques described for the production of single chain antibodies (U. S. Patent No. 4,946,778) can be used to produce suitable single chain antibodies. Single chain antibodies are typically formed by linking the heavy and light chain

fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

[0044] Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragments that can be produced by pepsin digestion of the antibody molecule and the Fab fragments that can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

[0045] The capture molecule may also be any other proteinaceous scaffold that has been adapted or mutated to bind a given ligand with sufficient binding affinity. Examples of useful scaffolds include those scaffolds described in US patent application 2005/0089932 or US Patent 6,682,736. Another example of suitable scaffolds are members of the lipocalin protein family as described in the international patent applications WO 99/16873, WO 00/75308, WO 03/029471, WO 03/029462, WO 03/029463, WO 2005/019254, WO 2005/019255 or WO 2005/019256, for instance.

[0046] In accordance with the above, scaffolds besides members of the lipocalin family include, but are not limited to, a EGF-like domain, a Kringle-domain, a fibronectin type I domain, a fibronectin type II domain, a fibronectin type III domain, a PAN domain, a G1a domain, a SRCR domain, a Kunitz/Bovine pancreatic trypsin inhibitor domain, tendamistat, a Kazal-type serine protease inhibitor domain, a Trefoil (P-type) domain, a von Willebrand factor type C domain, an Anaphylatoxin-like domain, a CUB domain, a thyroglobulin type I repeat, LDL-receptor class A domain, a Sushi domain, a Link domain, a Thrombospondin type I domain, an immunoglobulin domain or a an immunoglobulin-like domain (for example, domain antibodies or camel heavy chain antibodies), a C-type lectin domain, a MAM domain, a von Willebrand factor type A

domain, a Somatomedin B domain, a WAP-type four disulfide core domain, a F5/8 type C domain, a Hemopexin domain, an SH2 domain, an SH3 domain, a Laminin-type EGF-like domain, a C2 domain, Kappabodies, Minibodies, Janusins, a nanobody, a adnectin, a tetranectin, a microbody, an affilin, an affibody or an ankyrin, a crystallin, a knottin, ubiquitin, a zinc-finger protein, an ankyrin or ankyrin repeat protein or a leucine-rich repeat protein, an avimer; as well as multivalent avimer proteins evolved by exon shuffling of a family of human receptor domains.

[0047] The capture molecule may be a mutein of the member of the lipocalin protein family. In some of these embodiments, the open end of the β-barrel structure of the lipocalin fold (which encompasses the natural ligand binding site of the lipocalin family) is used to form the analyte binding site. Members of the lipocalin family of proteins include, but are not limited to the bilin binding protein of *Pieris brassicae* (SWISS-PROT Data Bank Accession Number P09464), human tear lipocalin (SWISS-PROT Data Bank Accession Number M90424), human apolipoprotein D (SWISS-PROT Data Bank Accession Number P05090), the retinol binding protein (RBP) (for example of human or porcine origin, SWISS-PROT Data Bank Accession Number of the human RBP: P02753, SWISS-PROT Data Bank Accession Number of the porcine RBP P27485), human neutrophil gelatinase-associated lipocalin (hNGAL, SWISS-PROT Data Bank Accession Number P80188), rat α_2-microglobulin-related protein (A2m, (SWISS-PROT Data Bank Accession Number P31052), and mouse 24p3/uterocalin (24p3, (SWISS-PROT Data Bank Accession Number P11672), Von Ebners gland protein 2 of *Rattus norvegicus* (VEG protein 2; SWISS-PROT Data Bank Accession Number P41244), Von Ebners gland protein 2 of *Sus scrofra* (pig) (LCN1; SWISS-PROT Data Bank Accession Number P53715), the Major allergen Can fl precursor of dog (ALL 1, SWISS-PROT Data Bank Accession Number O18873), and

insecticyanin A or insecticyanin B of the tobacco hawkmoth *Manducta sexta* (SWISS-PROT Data Bank Accession Number P00305 and Q00630, respectively).

[0048] The capture molecule may also be a binding protein, receptor or extracellular domain (ECD) thereof capable of forming a binding complex with a ligand, typically a polypeptide or glycopeptide ligand.

[0049] Those skilled in the art will recognized that the non-limiting examples given above describing various forms of antibodies as capture molecules can also be extended to other proteinaceous receptors such as recombinant, chimeric, hybrid, truncated etc., forms of non-antibody receptors.

[0050] The capture molecule can also be a non-proteinaceous receptor, such as for example a nucleic acid based molecule, such as an Aptamer or Spiegelmer (Aptamer made of L-ribonucleotides).

[0051] The working electrode comprises a porous membrane structure. In various embodiments, the working electrode comprises a porous alumina membrane coated with a conducting element provided on a surface thereon and the working electrode is in contact with a sensing solution. The conducting element facilitates the flow of electrons generated from or received at the working electrode. In embodiments where the working electrode comprises a conducting porous membrane, a separate conducting element may not be necessary. The sensing solution comprises the sample containing H_2O_2 or a precursor of H_2O_2. H_2O_2 is reduced or oxidized at the working electrode, depending the electrochemistry of the sensing solution and the working electrode, which will be elaborated in greater details in later paragraphs below.

[0052] The conducting element may be, but is not limited to, a platinum or gold layer. The thickness of the conducting element may be less than 100 nm such as ~90 nm, ~80 nm, ~70 nm, ~60 nm, ~50 nm, ~40 nm, or less.

[0053] In various embodiments, the working electrode comprises an electrocatalyst for the reduction of H_2O_2 at the working electrode. The electrocatalyst speeds up the rate of electro-reduction of H_2O_2 at the working electrode, thereby reducing the response time for current generation and detection.

[0054] In certain embodiments, the electrocatalyst is comprised in the pores of the porous membrane structure. The electrocatalyst may also be comprised, or further comprised in addition to the pores of the porous membrane structure, at a surface of the working electrode, including a portion of the surface having a conducting element thereon. The electrocatalyst for the reduction of H_2O_2 at a surface of the working electrode and/or in the pores of the porous membrane structure of the working electrode may be electro-deposited prior to contacting the sensing solution with the working electrode.

[0055] In various embodiments, the electrocatalyst comprises a hexacyanoferrate-derived material or Prussian blue (PB). PB is known to possess high activity for H_2O_2 electrocatalysis and may thus be used to enhance the electrochemical activity of the porous membrane towards H_2O_2. PB may be deposited onto the porous membrane using potential cycling method which forms nanotubes (*nt*) structures embedded within the channels or pores of the porous membrane. The working electrode thus-formed may be termed a PB-*nt* membrane electrode. **Fig. 8** illustrates the construction of the porous PB-*nt* membrane electrode by sputter-coating a 50 nm thick platinum layer on one side of a nanoporous 60 μm thick alumina membrane (step (A)), followed by electrodeposition of PB onto the porous Pt membrane electrode (step (B)). The thus-formed PB-*nt* is then employed as the working electrode in this embodiment.

[0056] PB has been widely used as an important pigment for dyes, paints, inks and others because of its intense and durable blue colour. Prussian blue is a face-centered

cubic crystal lattice with a unit cell constant of about 10.2Å and is a three dimensional polymer which consisted of alternating high-spin Fe^{3+} and low-spin Fe^{II} ions in which Fe^{II} ions are surrounded octahedrically by the carbon atoms of cyano ligands while Fe^{3+} ions are linked to the nitrogen atoms of cyano ligands. Besides Prussian blue, analogues of Prussian blue may also be suitable for use as the material for the working electrode. For example, suitable analogues of Prussian blue include, but are not limited to, Prussian blue analogues incorporating Fe and Cu or Fe and Ni.

[0057] Fig. 1 shows various embodiments of a schematic of **(a)** the H_2O_2-powered sensor using a 2-compartment cell separated by a Prussian blue nanotubes membrane; and **(b)** the H_2O_2-powered virus sensor using a 2-compartment cell separated by a Prussian blue nanotubes membrane, showing the binding of virus to antibody molecules within the nanochannels of the porous membrane. The porous membrane separates the sensing solution from the reference solution which contains the reference and/or counter electrodes. The working electrode is in contact with the sensing solution and facilitates transfer of electrons. A counter electrode is in contact with the reference solution, wherein the porous membrane structure of the working electrode provides a physical barrier between the sensing solution and the reference solution. A reducer is oxidized at the counter electrode. In various embodiments, the sensing solution is H_2O_2, KCl, a buffer, or a mixture thereof. The KCl solution may have a concentration of about 1 mM to 1 M. The buffer may be, but is not limited to Tris or phosphate buffer. For example, the sensing solution may be a mixture of H_2O_2 and Tris. The counter electrode works to balance the electrons added or removed by the working electrode. Optionally, a third electrode may be used to act as a reference electrode which acts as a reference in measuring and controlling the working electrode's potential.

[0058] In one embodiment, glucose is added to the sensing solution comprising glucose oxidase and is oxidized by oxygen to gluconic acid, and produces H_2O_2 during the reaction at the working electrode. At the working electrode, H_2O_2 undergoes reduction (O^- in peroxide to O^{2-} in water) to form water while at the counter electrode, water undergoes oxidation (OH^- or H_2O to O_2) to form oxygen gas and electrons are released. Electrons released from the oxidation reaction at the counter electrode flows via an electrically connecting connector to working electrodes where the electrons are received to form the reduction reaction at the working electrode, thereby generating an electrical current flow between the working and counter electrodes. The sensor design comprises the nanoporous PB-*nt* membrane to separate the two solutions in a 2-compartment sensor cell. H_2O_2 oxidizes PB-*nt* in the sensing solution, followed by Galvanic current flow between the porous PB-*nt* membrane electrode in the sensing solution and counter electrode in the reference solution, resulting in the sensing signal detected due to changes in the H_2O_2 concentration. A current detector is provided to detect and measure the current flow. The present sensor design thus allows a current flow to be not induced by an externally applied electrical potential.

[0059] The channels of PB crystal were found to be crucial for diffusion of small hydrated molecules such as oxygen and hydrogen peroxide through the crystal so that PB could act as a three dimensional electrocatalyst. The main advantage of PB-modified electrodes when compared to conventional platinum electrodes is the availability of low operating potential which could be as low as 0.0V vs Ag/AgCl when used for H_2O_2 detection and for biosensor applications.

[0060] In addition, since nanoporous membrane is used as template for the fabrication of PB-*nt* membrane electrode, the self-powered amperometric sensor exhibits a better electrochemical and mechanical stability because PB-*nt* is retained from leaking and at

the same time, the porous layers and channels can load a large amount of PB-*nt* compared to conventional solid electrodes.

[0061] Moreover, PB acts as a selective electron transfer mediator which catalyzes the electro-reduction or electro-oxidation of H_2O_2 because the channels of PB crystal lattice only selectively allowed low molecular weight molecules to penetrate through while blocking the molecules with higher molecular weight.

[0062] It is well known that PB can be reduced to the Everitt's salt (ES) at $E^{0'} \sim +0.24V$ (vs. Ag/AgCl, 1M KCl) or oxidize to Berlin green (BG) at more positive potential of $E^0 \sim +0.90V$ (vs Ag/AgCl, 1M KCl). These two half-cell reactions could be combined with the half-cell reactions for the oxidation of water or reduction of oxygen at the counter electrode to give a cell reaction with theoretical driving force of 0.55V.

[0063] In various embodiments, the working electrode comprises a capture molecule for capturing a target molecule, wherein the binding reaction between the capture molecule and the target molecule produces or consumes H_2O_2. Alternatively, H_2O_2 is added to the sensing solution prior to the addition of the target molecule. The response to the H_2O_2 in the sensing solution is measured before and after the capture of the target molecule and changes in the concentration of H_2O_2 during the binding reaction is thus determined. The capture molecule may be comprised in the pores of the porous membrane structure, such as immobilized onto the pore or channel surface. Immobilization of the capture molecule onto the pore or channel surface may include, but is not limited to, interaction with coupling molecules bound to the surface of the pores of channels. In other embodiments, immobilization of the capture molecules can be carried out on the surface by any suitable physical or chemical interaction. These interactions include, for example, hydrophobic interactions, van der Waals interactions, or ionic (electrostatic) interactions as well as covalent bonds. This further means that a capture molecule can

directly be immobilized on the surface of the pores or channels by hydrophobic interaction, van der Waals interactions or electrostatic interaction or through covalent coupling. In some embodiments, the capture molecules may carry at least one functional group, such as an amine, a hydroxyl, an epoxide or thiol group, which allow direct immobilization on the surface through chemical interaction, for example formation of a covalent bond. In various embodiments, the working electrode comprises a target molecule bound to the capture molecule. Alternatively or additionally, the capture molecule may be immobilized on a surface of the working electrode.The capture molecule, the target molecule and the binding relationship between the capture molecule and the target molecule have been described in earlier paragraphs above. For example, the capture molecule may be, but is not limited to, an antibody and fragment or variant thereof, antibody-like molecule, binding protein, protein receptor, extracellular domain (ECD) thereof, or a mixture thereof. For example, the target molecule may be, but is not limited to, a protein, peptide, lipid, nucleic acid, small organic molecule, organic polymer, carbohydrate, hapten, or a mixture thereof.

[0064] In order that the invention may be readily understood and put into practical effect, particular embodiments will now be described by way of the following non-limiting examples.

[0065] Examples

[0066] In the following examples, a hydrogen peroxide amperometric sensor design constructed from Prussian blue nanotubes coupled to a Galvanic cell which gives the sensing signal derived from the Galvanic current flow is illustrated. The amperometric sensor operates without input of an external electrical potential.

[0067] Subsequently, this self-powered amperometric sensor design is extended to a virus biosensor in which labeling of the target is not required for detection (i.e. enzyme-

labelling is not required). Dengue virus is chosen as the target in this example because dengue is one of the infectious diseases that is widely spread over global regions with yearly occurrence of epidemics and could be deadly in some cases.

[0068] Methods and Materials

[0069] Chemicals

[0070] Nanoporous alumina membranes (Anodisc™, 13mm diameter, 0.02μm pore size) from Whatman (Maidstone, Kent, UK). 37% HCl from Analar Normapur, 35% H_2O_2 and KCl from Scharlau, potassium hexacyanoferrate (III) and D(+)-Glucose anhydrous from Merck, anhydrous ferric chloride from GCE Laboratories, 1M Tris buffer pH 7.0 from 1st Base. Enzyme glucose oxidase (GOx) from *Aspergillus niger* (EC 1.1.3.4, ~200 units/mg), was purchased as lyophilized powder from Sigma and stored at -20°C. Stock solutions of glucose in 1M Tris buffer were left overnight and then stored at 4^0C before used. All solutions were prepared in Ultrapure water (Sartorius Ultrapure Water System).

[0071] Instrumentation

[0072] JEOL JFC-1600 Auto Fine Coater was used to prepare platinum-coated porous membrane electrode. Electrochemical experiments were studied with a CHI750D electrochemical workstation, an e-corder 401 (eDAQ) and a potentiostat (eDAQ EA161) and performed in a 2-compartment cell with 3-electrode system with porous PB-nanotubes (*nt*) membrane electrode as the working electrode, a Ag/AgCl (1M KCl) reference electrode and a platinum mesh as the counter electrode were used.

[0073] Example 1

[0074] Sensor Fabrication

[0075] The Prussian blue nanotubes sensor was fabricated as briefly described as follows. Conductive platinum layer sputtered on one side of the membrane was

subsequently electrodeposited with a Prussian blue layer to give the porous PB-*nt* membrane. The electrodeposition of PB-*nt* was achieved by a potential sweep from -0.5 to +0.6V at 50mV s^{-1} for 30 cycles in a solution containing 5.0mM $K_3Fe^{III}(CN)_6$, 5.0mM $FeCl_3$, 0.1M KCl and 0.01M HCl. The porous PB-*nt* membrane was subsequently rinsed with ultrapure water and dried overnight at room temperature.

[0076] Determination of H_2O_2 and Glucose and Dengue Virus

[0077] 35% H_2O_2 solution was used to prepare the stock hydrogen peroxide solutions. A sensing solution refers to the solution that was in direct contact with the porous PB-*nt* membrane while a reference solution refers to the solution where both the reference and the counter electrodes were placed in. Stock solution of glucose in 1M Tris was added successively into a 0.1M KCl in 1M Tris solution containing 4.0mg/mL glucose oxidase for glucose sensing (**Fig. 1(a)**). For virus determination. 20μL of 0.2mg/mL Immunoglobulin G (IgG) dengue II was adsorbed on the electrode surface for an hour. Stock solution of heat-inactivated dengue virus type II was added successively to the sensing solution containing 1M Tris and H_2O_2. H_2O_2 solution is added prior to the virus detection. No external electrical potential was applied during the amperometric detection of H_2O_2, glucose and the dengue virus type II was not labelled.

[0078] Results and Discussion

[0079] Oxygen and H_2O_2 Reduction

[0080] **Fig. 2(a)** and **2(b)** show that hydrogen peroxide reduction at the PB-*nt* commences at ~+0.05V (vs. Ag/AgCl, 1M KCl), with an overpotential of ~250mV compared to a control voltammetric experiment carried out using porous platinum (Pt) membrane electrode. In addition, the Pt membrane gives lower reduction currents towards H_2O_2 compared to the PB-*nt* membrane (**Fig. 2(b)**). These observations are consistent with the more rapid electrochemical rate constant for H_2O_2 reduction at PB

compared to Pt. Further, the absence of a chloride peak on Pt substrate positive of -0.1V vs. Ag/AgCl at the PB-*nt* membrane electrode indicates significantly reduced Pt activity when coated with PB (**Fig. 2(a)** and **2(b)**). Under ambient condition, a reduction current towards oxygen occurs at ~0V vs. Ag/AgCl is observed for the Pt membrane electrode (**Fig. 2(b)**). However, at the PB-*nt* membrane, the oxygen reduction current is negligible when compared to H_2O_2 reduction (**Fig. 2(a)**). Overall and importantly, the PB-*nt* do not suffer significant interferences from the underlying Pt layer nor reduction of solution oxygen under ambient condition.

[0081] Mechanism of the Sensor

[0082] PB can be reduced to the Everitt's salt (ES) as follows:

$$KFe^{III}Fe^{II}(CN)_6 + K^+ + e^- \rightleftharpoons K_2Fe^{III}Fe^{II}(CN)_6 \qquad (1)$$

PB ES ($E^{0'} \approx +0.24$V vs. Ag/AgCl, 1M KCl)

or can be oxidized to Berlin green (BG) as follows:

$$KFe^{III}Fe^{II}(CN)_6 \rightleftharpoons K_{1/3}(Fe^{III}(CN)_6)_{2/3}(Fe^{II}(CN)_6)_{1/3} + \tfrac{2}{3}K^+ + \tfrac{2}{3}e^- \qquad (2)$$

PB BG ($E^{0'} \approx +0.90$V vs. Ag/AgCl, 1M KCl)

[0083] H_2O_2 is known to undergo electrocatalytic reduction or oxidation at a PB modified electrode when the polymer exists in the ES or BG form, respectively at potentials less than 0.3V or larger than 0.85V (vs. Ag/AgCl).

[0084] To investigate if H_2O_2 is reduced to water by the oxidation of ES to PB according to:

$$H_2O_2 + 2H^+ + 2e^- \rightleftharpoons 2H_2O \qquad (3)$$

or is oxidized to oxygen by the reduction of BG to PB according to:

$$H_2O_2 \rightleftharpoons O_2 + 2e^- + 2H^+ \qquad (4)$$

it is determined the open-circuit potentials of the PB-*nt* membrane electrode measured against Ag/AgCl reference before and after the addition of 10mM H_2O_2. These values are ~0.21V and ~0.33V, respectively, which correspond closely to the formal potential for the redox conversion of the ES and PB forms. Clearly, this increase in the open circuit PB-*nt* membrane electrode from ~0.21V to ~0.33V after addition of 10mM H_2O_2 is due to the reduction of H_2O_2 and oxidation of ES to the PB form. The open-circuit potential of the PB-*nt* membrane electrode is found to decrease to ~0.10V (vs. Ag/AgCl) after current is discharged for 5 minutes through the close-circuit sensor cell in the presence of 10mM H_2O_2. These potentials are close to the half-cell potential in Eq. 1, clearly indicating that H_2O_2 is reduced by ES, as opposed to oxidation of H_2O_2 by BG.

[0085] The mechanism of the chemically driven sensor can be described by the following reactions. First, the analyte hydrogen peroxide is reduced:

$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O \qquad (5)$$

by the catalytic PB-*nt* in the sensing solution. At the PB-*nt* membrane, ES is oxidized to PB:

$$ES \rightarrow PB + K^+ + e^- \qquad (6)$$

[0086] The overall sensing mechanism comprises the following H_2O_2 reduction:

$$2ES + H_2O_2 + 2H^+ \xrightarrow{k_1} 2PB + 2H_2O + 2K^+ \qquad (7)$$

coupled to the inter-conversion between PB and ES (Eq. 1), regenerated by a spontaneous Galvanic cell reaction with the auxiliary electrode. k_1 is the bimolecular rate constant for H_2O_2 reduction by ES. Fast bimolecular rate constant k_1 of between 2 and $5x10^3$ M^{-1} s^{-1} indicates reaction between ES and H_2O_2 (Eq. 7) proceeds irreversibly.

[0087] The rate of this catalyzed reaction can be described using a bimolecular rate constant k_1 (in M^{-1} s^{-1}):

$$\text{Rate} = k_1[ES][H_2O_2] \qquad (8)$$

[0088] PB is subsequently converted back to ES via the Galvanic cell reaction which comprises a cathodic reaction at PB-*nt*:

$$PB + K^+ + e^- \xrightarrow{k_2} ES \qquad (9)$$

where k_2 is the electron transfer rate constant,

and an anodic reaction at the Pt auxiliary electrode in the reference solution:

$$2H_2O \rightarrow O_2 + 4H^+ + 4e^- \qquad (10)$$

[0089] The overall rate for this Galvanic cell reaction can be described by the electro-reduction of PB (Eq.9):

$$\text{Rate} = k_2[PB][K^+] \qquad (11)$$

[0090] Overall, the sensor reaction comprises the H_2O_2 reduction by PB-*nt* membrane in the sensing solution, coupled to the Galvanic cell reaction between PB-*nt* and Pt counter electrode.

[0091] Effect of Adding H_2O_2 on the Closed-circuit and Open-circuit Potential

[0092] K^+ selective and dual-ion PB-*nt* sensors show that the nanotubes potential is influenced by the ratio of PB to ES according to the Nernst relation. That is, the relation between the electrochemical potential of the PB-*nt* membrane and H_2O_2 concentration under steady-state condition can be related.

[0093] **Fig. 3(a)** shows the driving force between the PB-*nt* membrane and auxiliary electrode under closed-circuit condition, measured from the potential difference between the nanotubes and auxiliary electrode during closed-circuit condition and when

aliquots of H_2O_2 are added to the sensing solution. The incremental change in the driving force is evident of a second reaction that can regenerate the reduced form of PB even in excess amount of H_2O_2. In contrast, a control using the same sensor under open-circuit condition when no current flows between the working and auxiliary electrodes, shows a typical sigmoidal titration curve expected for exhaustive depletion of ES in increasing H_2O_2 concentration (**Fig. 3(b)**). In general, the driving force of the sensor reaches steady-state values in ~30 to 60s, upon addition of each aliquot of H_2O_2 as noted from the time needed to achieve plateau values of the potential-time curve (**Fig. 3(a)**, inset). Best fitted linear line for the change in driving force versus the logarithm of H_2O_2 concentration gives a slope of 29.7mV (**Fig. 3(c)**). This confirms the steady-state condition can be achieved when the sensor is challenged with concentrations of H_2O_2 ranging from 10μM to 5mM (**Fig. 3(c)**).

[0094] **Fig. 4** shows the power density of the cell potential in 1M TRIS buffer solution containing 30mM H_2O_2. The open circuit potential was determined to be ~0.29V vs. auxiliary electrode and the maximum power was 30.16μW cm^{-2} at V_{cell} = 0.105V vs. auxiliary electrode.

[0095] Effect of K^+ Ions Concentration on the Performance of the Sensor

[0096] A typical sensor response (**Fig. 5**) shows the increase in steady-state current towards increasing concentration of H_2O_2. In the absence of K^+, the sensor seems to give least sensitive response towards H_2O_2 due to the low activity of PB. At increasing K^+ concentration, the sensor sensitivity (response slope) improves significantly (**Fig. 5**). This sensitivity attenuation by K^+ ion can be explained by the K^+ dependent conversion reaction between PB and ES (Eq. 1) which influences its electrochemical reduction and oxidation rate (Eq. 11).

[0097] In general, the loading amount of PB nanotubes is determined to be ~$1x10^{-7}$mol cm^{-2}, which is 10 to 100 times higher than thin films of PB. From fitted results of **Fig. 5**, the maximum bimolecular rate constant for H_2O_2 reduction by ES is ~$1.75x10^3$ M^{-1} s^{-1}, lower than reported values using potential controlled amperometric measurements. This is attributed to the lower ratio of ES to PB in the present sensor, unlike potential controlled amperometric measurements where most of PB film exists as ES under strongly reducing potentials of $\leq$ 0V vs. Ag/AgCl.

[0098] The sensor gives similar increasing analytical signal responses when K^+ is added in either the reference or sensing solution (**Fig. 5**). Placement of high K^+ concentration in the reference solution is the more favourable cell arrangement to avoid the additional procedure of adding K^+ ion to the sample solution and to reduce interference from unknown amount of K^+ ion when present in some samples. This demonstration of a high sensitivity H_2O_2 sensor without the use of an applied electrical potential opens up the development of analytical sensors with low energy needs by a simple change from a single compartment cell to a two-compartment cell comprising a porous electrocatalytic electrode as a sensor and a counter anodic (or cathodic) element of a Galvanic cell.

[0099] It is also feasible to employ this self-powered amperometeric sensor in a one-compartment cell by selecting the anodic and cathodic reactions with suitable thermodynamic potentials with consideration of reactions at the sensing and auxiliary electrodes modified with appropriate mediators or catalysts. A two-compartment cell design is selected at this stage because of the side reaction of H_2O_2 and Pt auxiliary electrode, leading to a lower responsive signal.

[00100] Determination of H_2O_2, Glucose and Virus

[00101] Under optimized conditions, the amperometric signal towards H_2O_2 gives rapid response time of 30s, with excellent linearity at low H_2O_2 concentration

which has a low detection limit of 0.1 μM and sensitivity of 48mA M^{-1} cm^{-2} (**Fig. 6(a)**). Its detection limit is comparable to reported potential controlled PB sensors. Reasonably reproducible responses with standard deviation of ~20% can be obtained from three different sensors by normalizing the sensors' signals against the amount of charges passed during initial charging process of PB-*nt* upon closing the circuit in the absence of H_2O_2. For individual sensors, the typical standard errors are in the range of 1 to 3% and can be used up to four sets of 15 measurements each.

[00102] To demonstrate the general utility of this new sensor design as an enzyme biosensor, the reduction of H_2O_2 at PB-*nt* membrane is coupled to the glucose oxidase (GOx) catalysis of glucose, selected for its well-studied reaction, high stability of GOx and significance of glucose. **Fig. 6(b)** shows the enzyme sensor response during successive addition of glucose to an air-saturated 1M TRIS pH 7.0 buffer, 0.1M KCl solution containing 4.0mg/mL GOx enzyme. Apparent Michaelis Km (glucose) value of 25 (± 3.4) mM derived from non-linear curve fitting of the biosensor response is similar to reported value (33 mM) of the free enzyme, thus indicating that the sensor response is controlled by homogeneous enzyme kinetics. The sensor linear working range from 1 to 25mM and rapid response time of ~60s are typical of homogeneous GOx sensors for measuring physiological concentration of glucose in blood. Presumably, detection limits of such biosensors could be enhanced significantly for surface bound oxidase enzymes which yield significantly higher concentrations of redox products in the confined volumes of suitable immobilization matrices, in particular for micro-sized and nano-sized electrodes.

[00103] Conclusion

[00104] In conclusion, a chemical energy driven sensor for the detection of hydrogen peroxide and glucose based on a porous PB-*nt* membrane electrode is

described herein. The unique Galvanic cell sensor configuration does not utilize any external electrical potential, thus requiring minimal electrical energy input. Potential-controlled voltammetric experiments and measurements of the sensor electrochemical potentials indicate that the PB-*nt* are of similar characteristics as the usual PB films and that the sensing reaction proceeds via reduction of hydrogen peroxide. Excellent curve fits of the potential and current data to theory indicates the spontaneous reduction of hydrogen peroxide at the PB-*nt*, coupled to the Galvanic cell regeneration of the reduced form of PB, proceeds under steady-state condition. Outstanding analytical performance of the chemical energy driven sensor towards hydrogen peroxide is comparable to potential-controlled PB sensors. Proof-of-concept glucose biosensor or virus biosensor derived from the hydrogen peroxide sensor has been demonstrated in the presence of a homogeneous solution of glucose oxidase enzyme or adsorption of IgG, indicates the possibility for other oxidase enzyme-based and virus biosensors.

[00105] In order to detect the presence of antigens or antibodies in the sample, immunoglobulin M-antibody capture ELISA (MAC-ELISA) is the most common and widely used serological test. In MAC-ELISA, the detection of the immune-complexes formation is carried out by enzyme conjugated-monoclonal or polyclonal antibodies that will transform the colorless substrate into a colored product, measured with a spectrophotometer. Unlike ELISA which may require enzyme-labeling, in the present self-powered and energy-free amperometric biosensor, virus detection can be completed within two hours with very simple procedures and basic recorder to measure the current change, without the need for labeling of the enzymes.

[00106] **Fig. 7** shows the preliminary result of using the present self-powered amperometric biosensor in virus detection. It is observed that the current has changed after stock solutions of virus are added into the sensing solution containing H_2O_2.

[00107] **Example 2**

[00108] Sensor Fabrication

[00109] Prussian blue nanotubes membrane was prepared as follows: First, a ~50 nm thick platinum layer was sputtered on one side of the membrane (either 20 or 200 nm pore size). Then the conductive membrane was subjected to potential cycling from -0.5 V to +0.6 V at 50 mV s^{-1} for 30 cycles in a solution containing 5.0 mM $K_3Fe^{III}(CN)_6$, 5.0 mM $FeCl_3$, 0.1 M KCl and 0.01 M HCl, to give blue coloured membrane that indicates formation of the PB-nanotubes (**Fig. 8**).

[00110] All PB-*nt* membranes were immobilized with antibody before use as virus sensor as follows: Mouse anti-dengue type II or III virus monoclonal antibody stock was diluted to 0.2 μg mL^{-1} in 1 M Tris buffer, pH 7 and was stored in 4 °C before used. 50 μL of the 0.2 μg mL^{-1} antibody was applied onto the PB-*nt* side of the membrane and incubated at 4 °C for 45 min and subsequently at room temperature for further 15 min before use.

[00111] Determination of H_2O_2 and Dengue Virus

[00112] Before virus sensing, 14 μL of 0.1 M H_2O_2 was first added to the 1.4 mL 1 M Tris buffer sensing solution of the closed-circuited 2-compartment membrane cell and allowed to reach steady-state condition after ~10 min. After that, aliquots (in μLs) of 600 plaque forming unit per mL (pfu mL^{-1}) virus stock solution were successively added into the H_2O_2 solution (**Fig. 1(b)**).

[00113] For the dry membrane probe shown in **Fig. 10**, the electrolyte used was Nafion® perfluorinated resin, incorporated within the 60 μm thick membrane structure and the counter electrode consists of the ~50 nm thick platinum coating on the other side of the PB-*nt* membrane side exposed to surrounding air, in a two-electrode cell arrangement. During virus sensing, 80 μL of 0.5 mM H_2O_2 was first added onto the PB-

nt coated side of the dry membrane probe under closed-circuit condition and allowed to generate steady-state current values after ~7 min. After that, aliquots (2 μL) of 1000 pfu mL^{-1} virus stock solution were successively added into the H_2O_2 solution.

[00114] **Fig. 9** shows (A) typical closed-circuit steady-state current response of a H_2O_2-powered sensor toward DENV-2 virus; (B) its calibration plot of normalized closed circuit steady-state current versus virus concentration; line is non-linear best fitted data; error bars are standard deviation of experiment data (points); (C) selective and non-selective responses of the H_2O_2-powered virus sensor toward DENV-2and DENV-3, added sequentially to the sensing solution. (A)-(C) use 200 nm pore size membranes as templates; (D) unstable closed-circuit current response of a H_2O_2-powered sensor toward DENV-2 virus, using a 20 nm pore size membrane.

[00115] **Fig. 10** shows a photograph and schematic of a standalone Nafion-filled membrane probe using the nanometer thick metal layer of the membrane at the air-electrolyte interface as the reference and counter electrode. A standalone membrane probe, filled with Nafion® perfluorinated resin that allows it to be kept under dry ambient condition and activated in the presence of small amount of solution or solution droplets is provided. The Nafion® perfluorinated resin-incorporated dry membrane probe is designed with the air-platinum as the reference/counter and the PB-*nt* membrane as the working electrode. Nafion® provides the ionic conduction between the two electrodes. This detection strategy may be employed to distinguish virus sub-types in direct assays of air-borne (influenza) virus, dengue and influenza infected human serum and dengue infected vector samples, without the usual sample pre-treatment procedures.

[00116] **Fig. 11** shows a closed-circuit current response of a dry membrane probe of **Fig. 10** immobilized with anti-dengue type II monoclonal antibody toward (A) DENV-2 and (B) DENV-3.

[00117] By "comprising" it is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present.

[00118] By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present.

[00119] The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[00120] By "about" in relation to a given numberical value, such as for temperature and period of time, it is meant to include numerical values within 10% of the specified value.

[00121] The invention has been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[00122] Other embodiments are within the following claims and non- limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

Claims

1. An amperometric sensor for measuring the amount of hydrogen peroxide (H_2O_2) present in a sample, comprising:

a working electrode in contact with a sensing solution, wherein the working electrode comprises a porous membrane structure, wherein the sensing solution comprises the sample containing H_2O_2 or a precursor of H_2O_2, wherein H_2O_2 is reduced at the working electrode;

a counter electrode in contact with a reference solution, wherein the porous membrane structure of the working electrode provides a physical barrier between the sensing solution and the reference solution, wherein a reducer is oxidized at the counter electrode;

a connector to electrically connect the working electrode and the counter electrode to allow a current flow therethrough, wherein the current flow is formed by a flow of electrons from the electrode where oxidation has occurred to the electrode where reduction has occurred, with the proviso that the current flow is not induced by an externally applied electrical potential; and

a current detector to detect and measure the current flow.

2. The amperometric sensor of claim 1, wherein the working electrode comprises an electrocatalyst for the reduction of H_2O_2 at the working electrode.

3. The amperometric sensor of claim 2, wherein the electrocatalyst is comprised in the pores of the porous membrane structure.

4. The amperometric sensor of claim 2 or 3, wherein the electrocatalyst comprises a hexacyanoferrate-derived material (or Prussian blue) or a Prussian blue analogue.

5. The amperometric sensor of any one of claims 1 to 4, wherein the sensing solution comprises H_2O_2, KCl, a buffer, or a mixture thereof.

6. The amperometric sensor of any one of claims 1 to 5, wherein the working electrode comprises a capture molecule for capturing a target molecule, wherein the binding reaction between the capture molecule and the target molecule produces or consumes H_2O_2.

7. The amperometric sensor of claim 6, wherein the capture molecule is comprised in the pores of the porous membrane structure.

8. The amperometric sensor of claim 6 or 7, wherein the working electrode comprises a target molecule bound to the capture molecule.

9. The amperometric sensor of any one of claims 1 to 8, wherein the working electrode further comprises a conducting element provided on a surface thereon.

10. The amperometric sensor of any one of claims 6 to 9, wherein the capture molecule is an antibody and fragment or variant thereof, antibody-like molecule, binding protein, protein receptor, extracellular domain (ECD) thereof, or a mixture thereof.

11. The amperometric sensor of any one of claims 8 to 10, wherein the target molecule is a protein, peptide, lipid, nucleic acid, small organic molecule, organic polymer, carbohydrate, hapten, or a mixture thereof.

12. A method for measuring the amount of H_2O_2 present in a sample, comprising:

providing an amperometric sensor of any one of claims 1 to 11;

contacting a sensing solution with a working electrode of the amperometric sensor, wherein the working electrode comprises a porous membrane structure, wherein the sensing solution comprises the sample containing H_2O_2 or a precursor of H_2O_2, wherein H_2O_2 is reduced at the working electrode;

contacting a reference solution with a counter electrode of the amperometric sensor, wherein the porous membrane structure of the working electrode provides a physical barrier between the sensing solution and the reference solution, wherein a reducer is oxidized at the counter electrode;

measuring the current flow between the working electrode and the counter electrode, wherein the current flow is formed by a flow of electrons from the electrode where oxidation has occurred to the electrode where reduction has occurred, with the proviso that the current flow is not induced by an externally applied electrical potential; and

correlating the measured current flow to the amount of H_2O_2 present in the sample in the reaction.

13. The method of claim 12, comprising electrodepositing an electrocatalyst for the reduction of H_2O_2 at a surface of the working electrode and/or in the pores of the porous membrane structure of the working electrode prior to contacting the sensing solution with the working electrode.

14. The method of claim 12 or 13, further comprising immobilizing a capture molecule on a surface of the working electrode or in the pores of the porous membrane structure of the working electrode.

15. The method of claim 14, further comprising binding a target molecule to the immobilized capture molecule, wherein the binding reaction consumes or produces H_2O_2.

16. The method of any one of claims 13 to 15, wherein the electrocatalyst comprises a hexacyanoferrate-derived material (or Prussian blue) or a Prussian blue analogue.

17. The method of any one of claims 12 to 16, wherein the sensing solution comprises H_2O_2, KCl, a buffer, or a mixture thereof.

18. The method of any one of claims 14 to 17, wherein the capture molecule is an antibody and fragment or variant thereof, antibody-like molecule, binding protein, protein receptor, extracellular domain (ECD) thereof, or a mixture thereof.

19. The method of any one of claims 15 to 18, wherein the target molecule is a protein, peptide, lipid, nucleic acid, small organic molecule, organic polymer, carbohydrate, hapten, or a mixture thereof.