



- (51) **International Patent Classification:**
C12Q 1/68 (2006.01) *G01N 27/22* (2006.01)
- (21) **International Application Number:**
PCT/US2016/053786
- (22) **International Filing Date:**
26 September 2016 (26.09.2016)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
62/222,947 24 September 2015 (24.09.2015) US
- (71) **Applicant:** CASE WESTERN RESERVE UNIVERSITY [US/US]; 10900 Euclid Avenue, Cleveland, Ohio 44106 (US).
- (72) **Inventors:** SECKLER, James; 10900 Euclid Avenue, Cleveland, Ohio 44106 (US). GOLDSMITH, Neil Austin; 10900 Euclid Avenue, Cleveland, Ohio 44106 (US). LEWIS, Stephen; 10900 Euclid Avenue, Cleveland, Ohio 44106 (US). SMITH, Corey; 10900 Euclid Avenue, Cleveland, Ohio 44106 (US).

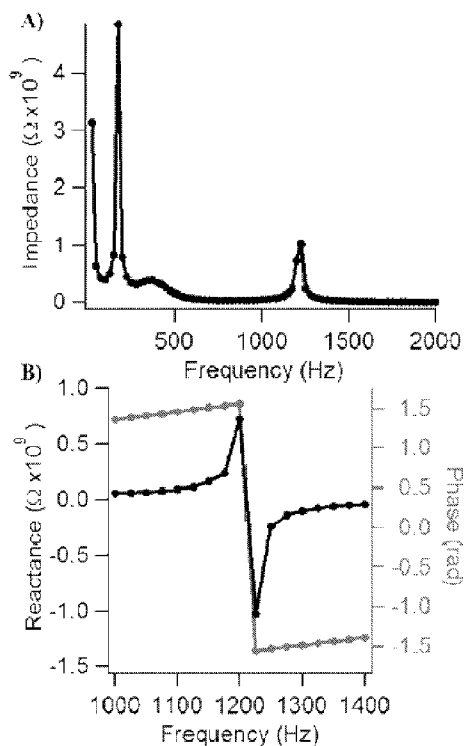
(74) **Agent:** SUTKUS, Richard A.; 1300 E. 9th Street, Suite 1700, Cleveland, Ohio 44114 (US).

(81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,

[Continued on next page]

(54) **Title:** SENSOR FOR DETECTION OF ANALYTES



Figs. 1A-B

(57) **Abstract:** A sensor for the detection of an analyte in a fluid includes an electrode having a detection surface, a polydopamine layer adhered to the electrode detection surface; and receptor chemically functionalized to the polydopamine of the detection surface of the electrode. The receptor selectively binds to the analyte of interest and the analyte once bound is detectable by measuring the change of capacitance of the electrode.



SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, —
GW, KM, ML, MR, NE, SN, TD, TG).

*before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments (Rule 48.2(h))*

Published:

— *with international search report (Art. 21(3))*

SENSOR FOR DETECTION OF ANALYTES

RELATED APPLICATION

[0001] This application claims priority from U.S. Provisional Application No. 62/222,947, filed September 24, 2015, the subject matter of which is incorporated herein by reference in its entirety.

GOVERNMENT FUNDING

[0002] This invention was made with government support under Grant No. R01-GM102191 awarded by The National Institutes of Health/National Institute of General Medicine Sciences. The United States government has certain rights to the invention.

BACKGROUND

[0003] A biosensor can determine the existence or the concentration of a certain analyte in a sample by translating molecular recognition of the analyte ultimately into an electrical signal by means of a translation system. Biosensors can be used for any kind of analyte that can be detected by biological means. Analytes that can be detected and quantified include metabolites, drugs, proteins, antigen-antibody interactions. For example, glucose can be detected in a diabetes patient's blood, life-threatening micro-organisms can be detected in food to enhance food safety, pollutants like CO, herbicides, chemicals and heavy metals can be detected to find and decontaminate polluted areas.

[0004] Biosensors can be catalogued in different groups, depending on their biological recognition system and the translation system.

[0005] Biological recognition system can include enzymatic sensors (based upon the reaction of a substrate catalysed by an enzyme, immunosensors (based upon the affinity between antibodies (or parts thereof) and antigenic determinants, *e.g.*, ELISA test) and genosensors (based *e.g.*, upon recognition of complementary RNA and/or DNA single strand molecules, and DNA-probes).

[0006] Translation systems can include electrochemical biosensors (amperometric, potentiometric, capacitive or impedimetric), optical biosensors (*e.g.*, Surface Plasmon Resonance (SPR), ellipsometric, fluorescence, . . .), gravimetric biosensors (measuring a difference in mass by measuring a change in resonance frequency of a quartz crystal when

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the analyte binds or adsorbs to the crystals), and calorimetric biosensors (measuring the reaction enthalpy released when the analyte binds to a substrate).

[0007] When using recognition biomolecules, such as antibodies, enzymes, oligonucleotides or nucleic acids, these molecules need to be fixed to a carrier surface in order to be able to perform their detection function in a reproducible way. Several possible techniques have been devised to perform the immobilisation.

[0008] One technique that is used is immobilisation of the biomolecule between two selectively permeable membranes. Another technique relies on physical adsorption to a fixed carrier surface. A third technique is based upon bifunctional reagents that can couple molecules to each other. Another technique is covalent binding to a substrate.

SUMMARY

[0009] Embodiments described herein relate to capacitive or impedimetric sensors or biosensors that are capable of providing analysis of various analytes or biomolecules in a fluidic sample or solution, such as biological or bodily fluids, using chemical or biological recognition elements. The biosensor can produce a signal that is related to the presence or quantity of the analytes being detected in a biological sample, such as a bodily fluid. In some embodiments, the biosensor can be used to detect the presence proteins, polypeptides, cytokines, micorganisms, polynucleotides (mRNA, DNA, cDNA, mRNA, etc.) that are present in a biological sample, such as a bodily fluid (*e.g.*, serum, blood, plasma, saliva, urine, mucous, etc). The biosensors can advantageously be used *in vivo* or *ex vivo* to provide a cost-effective means for simple point-of-care, real time assessment of analytes in biological samples, such as bodily fluids obtained by non-invasive or minimally invasive means, or for *in vivo* diagnostic purposes.

[0010] In some embodiments, the sensor includes a sensing electrode and a ligand receptor for a ligand, such as an analyte or biological molecule of interest. The ligand receptor is functionalized or chemically functionalized to a sensor active region of the electrode using a polydopamine semiconductive polymeric layer.

[0011] During operation, the biosensor can be placed in a fluid, such as a bodily fluid, that includes an analyte or biomolecule of interest. An altered dielectric at the electrode's surface active region caused by binding or complexing of the analyte or biomolecule in the fluid with the receptor can be used to detect the presence of the analyte or biomolecule.

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Binding of the analyte or biomolecule to the receptor can push high dielectric water in the fluid farther away the receptor on the sensor active region and replace it with the lower dielectric of the ligand, decreasing capacitance. Additionally, ligand binding can change the conformation of the polymeric layer, altering the capacitance further. This change in capacitance can be measured to determine the presence and/or concentration of the analyte or biomolecule in the fluid.

[0012] In other embodiments, the sensor can include a reference electrode with a polydopamine coating having a thickness, which turns it into a semiconductor. The sensing and reference electrodes can be charged using a Silver/Silver Chloride pellet when provided in a biological solution of interest. The direct current injection charges the sensing and reference electrode and both electrodes are allowed the discharge with their maximum discharge current when the electrodes are charged with a positive current and with a negative current. The combination of these two discharge currents serves as the signal. The sensing and reference electrode signals are collected and amplified independently of each other and then the difference is taken digitally to determine the presence of nonspecific binding to the electrodes. The asymmetry in discharge from a negative and positive current charge takes advantage of the gating potential of the polydopamine/electrode junction which the sensors at rest sit close to. The signal is derived from the fact the charge brought by the analyte binding moves the polydopamine/electrode junction closer to its gating potential and hence produces an asymmetric response. This provides a time domain signal, which can be used to determine both the presence of attomolar concentrations of an analyte as well as determine its concentration, by taking advantage of the slow on-rate of the ligand receptor to the analyte.

[0013] The use of a direct current injection into both a sensing and reference electrode, then employing separate signal amplification provides a novel technique for sensing the analyte. The signal itself comes from a novel mechanism by means of examining the distance from the junction potential by measuring the charge asymmetry upon the sensor discharging after experiencing a positive or a negative current.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figs. 1(A-B) illustrate plots showing naïve carbon fibers display a distinct anti-resonance peak at high frequencies. Frequency response was gathered from 25 – 2000 Hz at 25 Hz increments and 32 cycles per frequency. (A) Impedance response of the naïve carbon

fiber in a TBS solution. (B) The reactance of the impedance peak between 1 and 1.4 kHz and the phase angle at corresponding frequencies.

[0015] Figs. 2(A-B) illustrate plots showing dopamine electrodeposition alters the anti-resonance frequency of the electrode. (A) Electrodeposition of polydopamine on the tip of a 5 μm carbon fiber electrode. Representative deposition curve showing the dopamine oxidation current. (B) Frequency response of three electrodes before (filled circles) and after (filled triangles) dopamine deposition, gathered from 1100 – 1450 Hz at 10 Hz increments and 78 cycles per frequency.

[0016] Fig. 3 illustrates plots showing anti-resonance-like peaks are altered in response to ligand treatment. Frequency response of three electrodes before and after 10 nM PACAP treatment, gathered from 1300 – 1450 Hz at 10 Hz increments and 78 cycles per frequency.

[0017] Figs. 4(A-B) illustrate plots and graphs showing variance increases when functionalized electrodes are exposed to their antigen. (A) Variance response to baseline, 10 nM Met-Enkephalin, and 10 nM PACAP. (B) Variance difference between negative and positive controls ($n = 3$). Normalized to variance while perfusing with TBS. Variance was measured during the last 2 minutes of perfusion. There was a significant difference between negative and positive controls (Student's t-test, $p < .01$) Variance from a single electrode ran three times with 1XTBS, 10 nM Met-Enkephaline, and then TBS.

[0018] Figs. 5(A-C) schematically illustrate sensor production and use. Cyclic voltammetry is used to deposit dopamine tip of a carbon fiber electrode (black rectangle) that is then incubated in a basics solution with a PACAP antibody. The functionalized antibody is then presented with PACAP and a change in signal variance is measured.

[0019] Fig. 6 illustrates a graph showing the measurement of the release of Enkephalin in response to nerve stimulation with a 200 μAmp step function at 5 Hz using a sensor in accordance with an embodiment described herein.

[0020] Figs. 7(A-C) illustrate a schematic representation of the impedimetric antibody-based detection technique.

[0021] Figs. 8(A-B) illustrate plots showing that the capacitance at the electrode – solution interface was sampled by a step depolarization in the electrode – ground circuit (V_c = voltage command).

[0022] Fig. 9 illustrates plots showing example data obtained from an electrode bound with anti-enkephalin antibodies.

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[0023] Figs. 10(A-B) illustrates a graph and plot showing Increasing concentrations of enkephalin increased the current measured in the enkephalin electrode (grey bars) but not the control GAPDH electrode (white bars).

[0001] Fig. 11 illustrates a plot of the combined current versus time curve after the sensor was charged using either a positive or a negative current. This accentuates the asymmetry of the capacitance. The Baseline curve represents the sensor incubated in Tris buffered saline. The sensor was then infused with fresh buffer to produce the Tris curve. The sensor was then incubated in 4.78 aM SNOC for 10 minutes, washed with buffer and a reading was taken. Finally, the sensor was incubated with 478 aM SNOC for 10 minutes, washed with buffer and a reading was taken.

[0024] Fig. 12 illustrates a plot of the combined current versus time curve after the sensor was charged using either a positive or a negative current. This accentuates the asymmetry of the capacitance. The Baseline curve represents the sensor incubated in Tris buffered saline. The sensor was then infused with fresh buffer to produce the Tris curve. The sensor with incubated with SNOC or brain homogenate.

[0025] Fig. 13 illustrates a graph showing antibodies at the sensor's surface bound with ligand.

DETAILED DESCRIPTION

[0026] Unless specifically addressed herein, all terms used have the same meaning as would be understood by those of skilled in the art of the subject matter of the application. The following definitions will provide clarity with respect to the terms used in the specification and claims.

[0027] The term "bodily sample" refers to a sample that may be obtained from a subject (*e.g.*, a human) or from components (*e.g.*, tissues) of a subject. The sample may be of any biological tissue or fluid with which biomarkers described herein may be assayed. Frequently, the sample will be a "clinical sample", *i.e.*, a sample derived from a patient. Such samples include, but are not limited to, bodily fluids, *e.g.*, saliva, breath, urine, blood, plasma, or sera; and archival samples with known diagnosis, treatment and/or outcome history.

[0028] The term "biological sample" denotes a substance that contains the bio-molecules to be analyzed (for instance, blood plasma, saliva, urine, food, samples, etc., usually after pre-processing). The biological sample can encompass any material derived by

processing the bodily sample. Processing of the bodily sample may involve one or more of, filtration, distillation, extraction, concentration, inactivation of interfering components, addition of reagents, and the like.

[0029] The terms "control" or "control sample" refer to one or more biological samples isolated from an individual or group of individuals that are normal (*i.e.*, healthy). The term "control", "control value" or "control sample" can also refer to the compilation of data derived from samples of one or more individuals classified as normal.

[0030] The term "biological molecules" or "biomolecules" may particularly denote any molecules, which play a significant role in biology or in biological or biochemical procedures, such as DNA, RNA, proteins, enzymes, cells, bacteria, virus, etc.

[0031] The term "sensor" may particularly denote any device, which may be used for the detection of the presence/absence or even the concentration of analytes or biomolecules.

[0032] The term "biosensor" may particularly denote any device, which may be used for the detection of an analyte comprising biological molecules, such as DNA, RNA, proteins, enzymes, cells bacteria, virus, etc. A biosensor may combine a biological component (for instance capture molecules or receptors or ligand receptors at a sensor active surface capable of detecting molecules) with a physiochemical or physical detector component (for instance a capacitor having a capacitance which is modifiable by a sensor event).

[0033] The term "sensor active region" may particularly denote an exposed region of a sensor which may be brought in interaction with a fluidic sample so that a detection event may occur in the sensor active region. In other words, the sensor active region may be the actual sensitive area of a sensor device, in which area processes take place which form the basis of the sensing.

[0034] The term "subject" refers to a human or another mammal, which can be afflicted with a neural injury, such as a traumatic brain injury, but may or may not have such an injury. Typically, the terms "subject" and "patient" are used herein interchangeably in reference to a human individual.

[0035] The term "substrate" may denote any suitable material, such as a semiconductor, glass, plastic, etc. According to an exemplary embodiment, the term "substrate" may be used to define or support generally the elements for sensor. Also, the substrate may be any other base on which a electrode is formed or provided within.

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[0036] The term "fluidic sample" may particularly denote any subset of the phases of matter. Such fluids may include liquids, gases, plasma and, to some extent, solids, as well as mixtures thereof. Examples for fluidic samples are DNA-containing fluids, blood, interstitial fluid in subcutaneous tissue, muscle or brain tissue, urine or other body fluids. For instance, the fluidic sample may be a biological substance. Such a substance may comprise proteins, polypeptides, nucleic acids, DNA strands, etc.

[0037] The term "receptor" or "ligand receptor" may particularly denote a molecule that can capture specific target biomolecule or analyte.

[0038] Embodiments described herein relate to capacitive or impedimetric sensors or biosensors that are capable of providing analysis of various analytes or biomolecules in a fluidic sample or solution, such as biological or bodily fluids, using chemical or biological recognition elements. The biosensor can produce a signal that is related to the presence or quantity of the analytes being detected in a biological sample, such as a bodily fluid. In some embodiments, the biosensor can be used to detect the presence proteins, polypeptides, cytokines, micromolecules, polynucleotides (mRNA, DNA, cDNA, mRNA, etc.) that are present in a biological sample, such as a bodily fluid (*e.g.*, serum, blood, plasma, saliva, urine, mucous, etc.). The biosensors can advantageously be used *in vivo* or *ex vivo* to provide a cost-effective means for simple point-of-care, real time assessment of analytes in biological samples, such as bodily fluids by non-invasive or minimally invasive means.

[0039] The sensor includes a sensing electrode and a ligand receptor for a ligand, such as an analyte or biological molecule of interest. The ligand receptor is functionalized or chemically functionalized to a sensor active region of the electrode using a polydopamine semiconductive polymeric layer. The term "functionalized" or "chemically functionalized," as used herein, means addition of functional groups onto the surface of a material by chemical reaction(s). As will be readily appreciated by a person skilled in the art, functionalization can be employed for surface modification of materials in order to achieve desired surface properties, such as biocompatibility, wettability, and so on. Similarly, the term "biofunctionalization," "biofunctionalized," or the like, as used herein, means modification of the surface of a material so that it has desired biological function, which will be readily appreciated by a person of skill in the related art, such as bioengineering.

[0040] During operation, the biosensor can be placed in a fluid, such as a bodily fluid, that includes an analyte or biomolecule of interest. An altered dielectric at the electrode's

surface active region caused by binding or complexing of the analyte or biomolecule in the fluid with the receptor can be used to detect the presence of the analyte or biomolecule. Binding of the analyte or biomolecule to the receptor can push high dielectric water in the fluid farther away the receptor on the sensor active region and replace it with the lower dielectric of the ligand, decreasing capacitance. Additionally, ligand binding can change the conformation of the polymeric layer, altering the capacitance further. This change in capacitance can be measured to determine the presence and/or concentration of the analyte or biomolecule in the fluid.

[0041] As the size of the electrode decreases, capacitances of the electrode decrease accordingly, including a change in capacitance at the surface active region of the electrode upon ligand binding. This creates a lower limit on electrode size possible that still provides adequate signal to noise fidelity. In order to overcome this limitation, the polydopamine semiconductive polymeric layer can be used to chemically functionalize the ligand receptor to the surface active region of the electrode. This can provide a capacitive sensor with field effect contributions, a hybrid between a purely capacitive and field-effect sensor. What signal is lost in detection surface area can be gained in field-effect.

[0042] Advantageously, polydopamine or dopamine films have been shown to form electronic-ionic hybrid conductors, similar to p-type semiconductors. Dopamine contains a catechol and an amine group. In a basic environment it will form a polymer that is adherent to most surfaces. The catechol group of polydopamine can be reactive towards thiol, amino, and imidazole groups under a basic environment. This makes polydopamine an ideal immobilization polymer to bind a wide range of ligand receptors to the electrode surface as any protein in solution will bind with the polydopamine layer.

[0043] Coating a sensor active region of the electrode with a polydopamine layer can make a capacitive sensor with a semiconductor-like region that can be easily functionalized with a large variety of ligand receptors. The electrode can then be functionalized with a ligand receptor, such as an antibody (*e.g.*, pituitary adenylate cyclase activating protein (PACAP) IgG antibody) to sense a ligand (*e.g.*, PACAP ligand) in solution. Upon ligand binding, resulting changes in capacitance of the electrode can be detected using, for example, a variance analysis method that adopts a software lock-in approach. This approach provides a simple and sensitive method for detection of the analyte or biomolecule in solution. The

sensitivity of the sensor can be dependent on the binding receptor, in this instance an IgG antibody, and provide adequate signal when presented with ligand in the nanomolar range.

[0044] In some embodiments, the electrode can have a surface active region that is defined by an insulator or dielectric that covers at least a portion of the electrode. The surface active region can have an area comparable to that of the cross-section of a carbon microfiber. Small physical dimensions may be advantageous to achieving nanomolar-molecule resolution. The smaller the electrode size, the higher the relative capacitance change as a result of a molecule capture. The footprint area of a captured ligand on the electrode area may determine the corresponding capacitance change. All electrode area that is not covered by the ligand receptor may in fact act as a parasitic capacitance in parallel to the capacitance change due to the single-molecule capture. That is why the electrode area should be as small as possible. A specifically appropriate electrode is as small as a molecule, provided the electrode pitch is small enough to ensure a reasonable surface coverage. The detectability of single molecules is a matter of achieving high-enough signal-to-noise ratio.

[0045] In some embodiments, the electrode may be a micron or sub-micron electrode. In other words, the electrode may have linear dimensions in the order of magnitude of several micrometers or less. Particularly, the electrode can be a microelectrode and, particularly, can have dimensions in a range of essentially one micrometer to ten micrometers.

[0046] The electrode may comprise an electrically conductive material, for instance, a material selected from the group consisting of gold, silver, platinum, palladium, carbon, indium tin oxide, alloys thereof, and composites thereof. In some embodiments, the electrode can include, carbon based materials, such as carbon fibers having micron dimensions.

[0047] In some embodiments, the electrode can be provided or formed on a substrate formed of electrically non-conductive material, such as glass, silica, alumina, ceramic based materials or a electrically non-conductive polymer, or a semi-conductive substrate, such as silicon. The electrode can be made using a thin film, thick film, and/or ink-jet printing technique, especially for the deposition of multiple electrodes on a substrate. The thin film process can include physical or chemical vapor deposition.

[0048] In other embodiments, the electrode can be formed from a carbon fiber with a cross-sectional diameter on a micron scale, *e.g.*, about 5 μm to about 10 μm . The carbon fiber can be encapsulated or ensheathed with an insulator so that the tip of fiber is exposed. The fiber tip can form a surface active region of carbon fiber electrode.

[0049] Exposed regions that define surface active regions of the electrode can be coated with a microlayer or nanolayer of polydopamine by immersing the exposed surface of the electrode in a solution in which dopamine or a derivative thereof is dissolved. In some embodiments, the solution containing dopamine or the derivative thereof dissolved therein may have a pH of about 7 to about 10. When the pH of the solution is within the range described above, the dopamine or the derivative thereof may be self-polymerized.

[0050] Specifically, dopamine can be used in a state of being dissolved in a distilled water-based buffer solution (*e.g.*, 10 mM Tris buffer solution, pH 8.5), which is inexpensive and environmentally friendly, instead of commonly used organic solvents, which are expensive and environmentally harmful. Dopamine is used in this state because the solution with dopamine dissolved therein needs to be maintained in a weak base state (*i.e.*, pH 8.5) to form a layer coated with polydopamine through self-polymerization of dopamine.

[0051] In other embodiments, polydopamine can be provided on a surface active region of the electrode using an electrochemical method. As described in the Example, the electrochemical method was used to deposit polydopamine on an uninsulated tip of a 5 μm carbon fiber electrode. Briefly, the surface active region of the electrode can be placed in a phosphate buffered dopamine solution. A sawtooth voltage waveform (*e.g.*, between 650 mV and -600 mV at 20 mV/sec) can then be applied to the electrode. The evoked current response is proportional to the amount of polydopamine deposited on the electrode. Polydopamine deposition can be calculated after every voltage cycle as the cumulative charge and current response. The deposition can be stopped when current response is about 80% of maximum current response, *i.e.*, prior to deposition of polydopamine on the electrode.

[0052] Polydopamine formed by the self-polymerization of dopamine may form a polydopamine layer on the surface active region of the electrode. The polydopamine layer may completely cover the surface active region of the electrode. In some cases, the polydopamine layer may partially cover the surface active region of the electrode. The polydopamine layer may have a thickness of 0.01 μm to 5 μm .

[0053] The ligand receptor, which is functionalized or chemically functionalized to the electrode using polydopamine, can be any molecule that binds selectively to an analyte of interest. A ligand receptor that binds selectively to an analyte is a molecule that binds preferentially to that analyte (*i.e.*, its binding affinity for that analyte is greater than its binding affinity for any other analyte). Its binding affinity for the analyte of interest may be

2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 40-fold, 50-fold, 100-fold or more than its binding affinity for any other analyte. In addition to its relative binding affinity, the receptor must also have an absolute binding affinity that is sufficiently high to efficiently bind the analyte of interest (*i.e.*, it must have a sufficient sensitivity). Receptors having binding affinities in the picomolar to micromolar range are suitable. Such interaction can be reversible.

[0054] The ligand receptor may be of any nature (*e.g.*, chemical, nucleic acid, peptide, polypeptide, lipid, combinations thereof and the like). The analyte too may be of any nature provided there exists a receptor that binds to it selectively and in some instances specifically. In some embodiments, the analyte can be a charged species or molecule.

[0055] It will be appreciated, the flexibility of the chemical functionalization using polydopamine makes the sensor useful for attaching essentially any ligand receptor or ligand having an affinity for analytes. Examples of analytes for which receptors or ligands having affinity therefor may be attached to the working electrode include, but are not limited to DNA, oligonucleotides, proteins, biotin, and streptavidin. The receptors for these analytes chemically functionalized to the electrode using polydopamine can include ligands, such as aptamers, oligomers, polymers, catalysts, cells, bacteria, viruses, enzymes, proteins, heptans, saccharides, lipids, glycogens, enzyme inhibitors, enzyme substrates, neurotransmitters, hormones, antigens, antibodies, DNA, and/or RNA. Proteins, including antibodies, generally have several primary amines in the side chain of lysine (K) residues and the N-terminus of each polypeptide can be available for linking to the catechol groups of the polydopamine. Chemical functionalization using polydopamine also enables the bioconjugation of DNA aptamers having an amino group. These aptamers could potentially bind small molecules and proteins.

[0056] In some embodiments, the receptor can be an antibody specific for an analyte of interest. Suitable antibodies for use in the sensors and methods described herein include monoclonal and polyclonal antibodies, immunologically active fragments (*e.g.*, Fab or (Fab)₂ fragments), antibody heavy chains, humanized antibodies, antibody light chains, and chimeric antibodies. Antibodies, including monoclonal and polyclonal antibodies, fragments and chimeras, may be prepared using methods known in the art (see, for example, R. G. Mage and E. Lamoyi, in "Monoclonal Antibody Production Techniques and Applications ", 1987, Marcel Dekker, Inc.: New York, pp. 79-97; G. Kohler and C. Milstein, Nature, 1975, 256:

495-497; D. Kozbor et al., J. Immunol. Methods, 1985,81: 31-42; and R. J. Cote et al., Proc. Natl. Acad. Sci. 1983, 80: 2026-203; R. A. Lerner, Nature, 1982, 299: 593-596; A. C. Nairn et al., Nature, 1982,299: 734-736; A. J. Czernik et al., Methods Enzymol. 1991, 201: 264-283; A. J. Czernik et al., Neuromethods: Regulatory Protein Modification: Techniques & Protocols, 1997, 30: 219-250; A. J. Czemik et al., NeuroNeuroprotocols, 1995, 6: 56-61; H. Zhang et al., J. Biol. Chem. 2002, 277: 39379-39387; S. L. Morrison et al., Proc. Natl. Acad. Sci., 1984, 81: 6851-6855; M. S. Neuberger et al., Nature, 1984,312: 604-608; S. Takeda et al., Nature, 1985, 314: 452-454). Antibodies to be used in the methods of the invention can be purified by methods well known in the art (see, for example, S. A. Minden, "Monoclonal Antibody Purification ", 1996, IBC Biomedical Library Series: Southbridge, Mass.). For example, antibodies can be affinity purified by passage over a column to which a protein marker or fragment thereof is bound. The bound antibodies can then be eluted from the column using a buffer with a high salt concentration.

[0057] Instead of being prepared, antibodies to be used in the methods described herein may be obtained from scientific or commercial sources.

[0058] It will be appreciated that the receptors are not limited to antibodies or antigen binding fragments and that other receptors to other biomarkers associated with other diseases, disorders, conditions, or pathologies which can be detectable in a bodily sample can also be functionalized or chemically functionalized to the electrode.

[0059] In some embodiments, the electrode can be attached to a detection means, such as an external circuit, for detecting a change in an impedance/capacitance of electrode caused by binding of an analyte of interest to the receptor. For convenient attachment, electrode can be electrically connected to conducting pads formed by methods, such as evaporation, soldering, chemical vaporization and the like.

[0060] A voltage can be applied to the electrodes at a specific frequency and the presence of the target analytes of interest can be sensed by monitoring the capacitance of the electrodes using, for example, variance analysis methods. Upon ligand or analyte binding to the receptors, resulting changes in the equivalent circuit are detected using a variance analysis methods adopted from a software lock-in approach. Sensitivities are dependent on the binding receptor and are demonstrated here to provide adequate signal when presented with ligand in the nanomolar range.

[0061] The sensor may be adapted as a biosensor, particular as a single molecule biosensor, which is able to detect even the presence of nanomolar concentrations of select molecules in a biological sample, such as biological fluid. In some embodiments, the biosensor can be used *in vivo* to detect the presence of a biomolecule or analyte in a subject. In other embodiments, the biosensor can be used *ex vivo* to detect a biological molecule of a biological sample obtained from a subject.

[0062] By way of example, Fig. 6 shows the results of an *ex vivo* experiment measuring the release of Enkephalin in response to nerve stimulation with a 200 μ Amp step function at 5 Hz using a sensor in accordance with an embodiment described herein. In this Example, rat kidneys, a portion of the spine, splanchnic nerve, ribs, and the adrenal gland are removed from the rat and placed in Tris-buffered saline. The adrenal glands are cut to expose the adrenal medulla to the bath. A sensor is positioned close to this cut to measure any release that may be evoked when the splanchnic nerve is stimulated with a 200 μ Amp step function at 5 Hz.

[0063] Advantageously, the analyte or ligand captured by the receptor can be repelled by increasing the frequency and potential of electrode to allow the electrode to be effectively tuned or to be reused for subsequent applications. As frequency increases and as potential increases across the electrode, a dielectric force repelling the analyte or ligand is increased. This is due to the high dielectric constant of water (80 vs. 2-3 for peptides). Water is more attracted and displaces the analyte or ligand. Altering the sinusoidal potential alters the force opposing the natural diffusion of these analyte or ligand in solution. At high potentials or frequencies, the effective concentration of any analyte or ligand near the electrode would be zero. By altering the potential, the concentration of the analyte or ligand near the electrode is can be altered and the electrode can be effectively tuned to a concentration range of choosing.

[0064] In some embodiments, a plurality of capacitive biosensors can be provided on a surface of a substrate to provide a biosensor array. The capacitive biosensor array can be configured to detect analyte concentration changes in a host of chemical and/or biological processes (chemical reactions, cell cultures, neural activity, nucleic acid sequencing processes, etc.) occurring in proximity to the array. The capacitive biosensor array includes a plurality biosensors arranged in a plurality of rows and a plurality of columns. Each biosensor comprises on electrodes configured to provide at least one output signal representing the presence and/or concentration of an analyte proximate to a surface of the

array. For each column of the plurality of columns or for each row of the plurality of rows, the array further comprises column or row circuitry configured to provide source voltage to respective electrodes in the column or row. Each electrode in the row or column can potentially detect a different analytes.

[0002] In other embodiments, the sensor can also include a reference electrode with a polydopamine coating having a thickness, which turns it into a semiconductor. The sensing and reference electrodes can be charged using a Silver/Silver Chloride pellet when provided in a biological solution of interest. The direct current injection charges the sensing and reference electrode and both electrodes are allowed the discharge with their maximum discharge current when the electrodes are charged with a positive current and with a negative current. The combination of these two discharge currents serves as the signal. The sensing and reference electrode signals are collected and amplified independently of each other and then the difference is taken digitally to determine the presence of nonspecific binding to the electrodes. The asymmetry in discharge from a negative and positive current charge takes advantage of the gating potential of the polydopamine/electrode junction, which the sensors at rest sit close to. The signal is derived from the fact the charge brought by the analyte binding moves the polydopamine/electrode junction closer to its gating potential and hence produces an asymmetric response. This provides a time domain signal, which can be used to determine both the presence of attomolar concentrations of an analyte as well as determine its concentration, by taking advantage of the slow on-rate of the ligand receptor to the analyte.

[0003] The use of a direct current injection into both a sensing and reference electrode, then employing separate signal amplification provides a novel technique for sensing the analyte. The signal itself comes from a novel mechanism by means of examining the distance from the junction potential by measuring the charge asymmetry upon the sensor discharging after experiencing a positive or a negative current.

[0065] In some embodiments, a plurality of capacitive biosensors can be provided on a surface of a substrate to provide a biosensor array. The sensor itself relies on the reference electrode which has no ligand receptor attached to it which allows us to determine the signal given by nonspecific binding of analytes or biomolecules in biological fluids. This reference can be used with multiple electrodes at a time. Hence, a sensor, which has any number of sensing electrodes and a single reference can be readily designed. This allows us the simultaneous testing of a fluid for several compounds at once. This is done by injecting a

single current into a bath and measuring the restoring current produced by amplifiers attached to each electrode. These currents can be recorded and the single reference will be digitally subtracted from each of the signals separately. In this way, almost any number of electrodes can be provided in a single biological sample.

Examples

Example 1

[0066] We set out to adapt an analytically similar variance analysis to measure peptides in solution using a novel capacitive biosensor. Experimental conditions were designed for eventual application of the new device and signal processing strategy in a physiological context, defined by a relatively strong saline solution to match blood serum.

[0067] A capacitive biosensor functions on the principle of an altered dielectric at the electrodes surface to detect the presence of a biological molecule. In this approach, a ligand receptor is immobilized by binding to a polymer surface deposited at the tip of the electrode. Ligand binding to the surface receptor pushes the high dielectric water layer farther away and replaces it with the lower dielectric of the ligand, decreasing capacitance. Additionally, ligand binding can change the conformation of the polymeric layer, altering the capacitance further. This makes the immobilization technique important to the overall sensitivity of the sensor.

[0068] The capacitive biosensor can be modelled as three capacitors in series, the electrode surface, the biological layer and the electronic double layer capacitance. The capacitances add inversely so the total capacitance is dominated by the smallest capacitance in the series, ideally the capacitance of the biological layer. As the size of the electrode decreases, all these capacitances decrease accordingly, including the change in capacitance at the biological layer upon ligand binding. This creates a lower limit on electrode size possible that still provides adequate signal to noise fidelity. One possible way to overcome this limitation is an immobilization layer on the electrode that is also semiconductive. This would then be a capacitive sensor with field effect contributions, a hybrid between a purely capacitive and field-effect sensor. What signal is lost in detection surface area could be gained in field-effect. A semiconductive immobilization polymer that fulfills this criteria is polydopamine.

[0069] Dopamine contains a catechol and an amine group. In a basic environment it will form a polymer that is adherent to most surfaces. This polymer, polydopamine, has a quinone functional group that is a target for nucleophilic groups such as primary amines, forming a covalent bond with them. This makes polydopamine an idea immobilization polymer to bind antibodies to the electrode surface as any protein in solution will bind with the polydopamine layer. Additionally, dopamine belongs to a large family of molecules called melanins. Melanin films have been shown to form electronic-ionic hybrid conductors, similar to p-type semiconductors.

[0070] Coating an electrode with a polydopamine layer can make a capacitive sensor with a semiconductor-like tip that can be easily functionalized with a large variety of potential proteinaceous ligand receptors. We describe the use of an electrochemical approach to deposit polydopamine on the tip of a 5 μm carbon fiber electrodes. These electrodes are then further functionalized with a pituitary adenylate cyclase activating protein (PACAP) IgG antibody to sense PACAP ligand in solution. Upon ligand binding, resulting changes in the equivalent circuit are detected using a variance analysis methods adopted a software lock-in approach. The approach described here provides a simple and sensitive method for protein detection in solution. Sensitivities are dependent on the binding receptor, in this instance an IgG antibody, and are demonstrated here to provide adequate signal when presented with ligand in the nanomolar range. Finally, the general sensing strategy provided here holds promise for expansion to a large number of applications beyond immune-based sensing.

Experimental Section

Chemicals and Materials

[0071] Dopamine hydrochloride, pituitary adenylate cyclase activating protein (PACAP) and Met-Enkephalin acetate were purchased from Sigma-Aldrich (St. Louis, MO). Parylene insulated 5 μm carbon fiber electrodes were purchased from ALA Scientific (Farmingdale, NY). Goat α -PACAP antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). Sodium Phosphate, dibasic anhydrous (Na_2HPO_4) was used to make the 10 mM phosphate buffer. Tris base (Tris (hydroxymethyl) aminomethane) was used to make the 10 mM Tris buffer and Tris-buffered saline (TBS). All solutions were brought to the appropriate pH with HCl.

[0072] Voltammetric polydopamine deposition was performed using a Dagan ChemClamp headstage amplifier (Dagan, Minneapolis, MN). Data acquisition was performed using IGOR Pro (Wavemetrics, Lake Oswego, OR) software controlling an ITC-1600 (Heka Elektronik, Bellmore, NY) data acquisition system. Frequency response and sensing data was gathered using the same software and data acquisition system controlling a custom frequency-boosted VA-10X amplifier with capacitance compensation (NPI, Hauptstrasse, Germany).

Electrode Functionalization

[0073] Polydopamine deposition was performed on a carbon fiber electrode with a freshly cut tip to expose a clean, conductive surface. Briefly, a sawtooth voltage waveform was applied between 650 mV and -600 mV at 20 mV/sec to an electrode superfused with 10 mM Phosphate buffer, pH - 6.5 (Fig. 5A). The evoked current response is proportional to the amount of polydopamine deposited on the electrode and was sampled at 500 Hz and filtered through a 3 kHz, 3-pole Bessel low-pass filter. The current response during the first 5 cycles provided a baseline before superfusing with a phosphate buffer with 10 mM dopamine added. Polydopamine deposition was calculated after every voltage cycle as the cumulative charge and the deposition was stopped when the target charge was achieved.

[0074] Binding of PACAP IgG antibody was accomplished by mixing antibody (1 mg/mL) with 10 mM Tris buffer, pH - 8.5 (Fig. 5B). The polydopamine coated electrode was tip-submerged in this solution and incubated in a 4⁰C overnight.

Electrode characterization

[0075] Frequency response was measured by applying a 10 mV sinusoid over a range of frequencies, with 10 to 50 msec gaps between each frequency transition. Each frequency was allowed to run between 32 and 78 cycles. The current response was sampled at 100 kHz and filtered through an 8 kHz, 2-pole Bessel low pass filter. This process was repeated and averaged 50 to 100 times while superfusing with Tris buffered biological saline (40 mM Tris base, 132 mM NaCl, 4.2 KCl, 11.2 Glucose, 2 CaCl₂, 0.7 MgCl₂, pH - 7.25).

[0076] A software lock-in algorithm was used to determine the real and imaginary components of the evoked signal at each frequency. Equations 1 and 2 were used to calculate the real and imaginary components of the signal sinusoid. P is the total number of points in

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the averaged region. To avoid possible edge effects the first and last four cycles were not included in the average. The time and current at point p are t_p and $I(p)$ respectively.

$$1) I_{imaginary} = \frac{2}{p} \sum_{p=0}^{p-1} I(p) * \cos(\omega * t_p)$$

$$2) I_{Real} = \frac{2}{p} \sum_{p=0}^{p-1} I(p) * \sin(\omega * t_p)$$

[0077] The phase angle (ω) is calculated by taking the inverse tangent of the ratio of the imaginary and real current.

$$3) \tan^{-1} \left(\frac{I_{imaginary}}{I_{Real}} \right)$$

[0078] Impedance components are calculated by dividing the current component by the applied voltage. Equation 4 was used to calculate the magnitude of the impedance.

$$4) |Z| = \sqrt{(Z_{Real}^2 + Z_{imaginary}^2)}$$

Sensing Experiments

[0079] After characterizing the frequency response of each electrode, the frequency of the command potential sinusoid at which a particular electrode is most sensitive was determined. The electrode was driven with a 10 mV sinusoid at each electrodes characteristic frequency while superfusing with Tris buffered biological saline solution (Fig. 1C). The signal was sampled at 20 times the frequency of the command sinusoid and the lock-in algorithm was used to calculate average phase angle and amplitude over each sine cycle. Variance was calculated over every 1 second time period (Fig. 5D, Fig. 4A) and this variance was averaged over a two minute period at the end of a treatment condition for comparison between treatments (Figs. 4 B, C).

Results and Discussion

Characterizing the Naïve Carbon Fiber Electrode

[0080] The frequency response of each naïve carbon fiber electrode had four characteristic impedance peaks (Fig. 1A). At 25 Hz, a high impedance is expected, and seen, as the electrode is designed to be predominantly capacitive. The next impedance peak is due to from a change in reactance, the imaginary component of the impedance, due to inductive and capacitive elements within the circuit. The peak nearest 500 Hz is due to a change in resistance. The highest frequency impedance peak is due to a change in reactance.

[0081] We chose the highest frequency impedance peak to analyze for ligand detection. This feature is dominated by the reactive component of the impedance (Fig. 1B) and a change in the reactance of the system is expected upon a change in the electrostatic environment at the electrode tip. Reactive elements, mainly capacitance, from the rest of the electrode should be minimal and constant due to the insulated coating of the electrode. Additionally, higher frequencies evoke a greater magnitude, which improves the signal-to-noise ratio.

[0082] The reactance appears discontinuous at the high frequency impedance peak (Fig. 1B). As the frequency increases to 1.2 kHz, a positive and increasing reactance was observed (Fig. 1B). A transition then occurs between 1.2 kHz and 1.225 k Hz where the phase and the reactance abruptly transitions to negative values. The large phase shift and increased impedance is characteristic of an anti-resonance, a region of destructive interference between oscillating elements of the circuit. The phase shift comes from moving from a positive reactance dominated region, typically attributed to an inductance, to a negative reactance dominated region associated with a capacitance.

The Anti-Resonance Feature is Sensitive to the Environment at the Electrode Tip

[0083] After characterizing the naïve electrode we adopted a protocol for polydopamine deposition at the electrode tip. We used integrated electrode current in response to a sawtooth command potential as an index of dopamine deposition. The deposition protocol included 5 cycles recorded in regular Tris buffer to provide a baseline. The solution was then changed to contain dopamine and resulted in an initial increase in recorded current for approximately 7 cycles, representing the wash-in of dopamine and oxidative polymerization of polydopamine on the conductive electrode tip. (Fig. 2A, 0-1500s). During the eighth

cycle the oxidative current began to decrease, consistent with a thickening coat of polydopamine on the carbon fiber tip (Fig. 2A, 1500s to end).

[0084] Integration of the oxidative current is useful as a guide but should not be considered a quantitative measure of the amount of dopamine on the tip. A confounding effect emerges as polydopamine is deposited. A background subtraction method is used to determine oxidative current but this does not account for a change in the baseline reduction of buffer. Deposition of polydopamine on the tip will reduce the oxidation of dopamine but will also inhibit the reduction of phosphate to phosphine. This is the dominant current in the baseline and without it the baseline will shift more positive and give a falsely decreased integral. One possible solution to this is to switch to a constant potential dopamine deposition protocol.

[0085] The carbon fiber electrodes show inherent variability (Fig. 2B, Naïve electrodes). High frequency anti-resonance peaks have been seen, in the naïve electrodes, as low as 1100 Hz and as high as 1450 Hz. This variability is believed to be due to the cutting of the fiber before each deposition to provide a fresh surface (see Chemicals and Materials). Rinsing the cutting blades with ethanol before cutting and rinsing the electrode with ethanol after cutting appears to decrease this variability. The same electrode undergoing the same deposition protocol can exhibit different characteristics after re-cutting. This limitation requires normalization of signals to the variance under baseline conditions to allow for comparison.

[0086] Dopamine deposition shifts the anti-resonance feature to a higher frequency (Fig. 2B). Three different total oxidative charges were considered with no correlation seen between the amount of charge and the frequency shift. One feature that did appear is an increase in cycle-to-cycle variance at the highest charge. The fiber with the greatest cumulative oxidative charge showed an increase in cycle-to-cycle variance while the two with a lesser cumulative oxidative charge showed a decrease (data not shown). If, as has been reported for melanin films, polydopamine has semiconductive properties, it would increase the cycle-to-cycle variance of the electrode due to an increase in the real part of the admittance. A semiconductive tip should increase sensitivity as you would no longer be measuring purely a change in capacitance upon ligand binding, but the resistance of the semiconductive tip would also be altered by field effects from any charge in the ligand. This could possibly lead to a change in the variance of the signal.

α -PACAP Functionalized Carbon Fiber Electrodes Show Increased Variance when Exposed to Ligand

[0087] Initially, frequency response experiments were run to determine if the anti-resonance peak was shifted in response to ligand. The frequency response should be more sensitive due to the signal averaging and the ability to test over a range of frequencies. This approach is advantageous in that it provides a greater signal to noise ratio, but comes at the cost of temporal resolution. Fig. 3 shows the frequency response recorded in three separate α -PACAP functionalized electrodes. A change is seen in the anti-resonance frequency in response to PACAP presentation.

[0088] A trend towards a more robust response to PACAP is noted as frequency of the anti-resonance features, defined by the most negative value, increases (Fig. 3). The 1.41 kHz feature (Fig. 3, top) has a minimum of $-4.486 \times 10^9 \Omega$. With 10 nM PACAP in the bath, the maximum increases to $51.828 \times 10^9 \Omega$, representing a change of greater than an order of magnitude change. The 1.39 kHz feature (Fig. 3, middle) shifts upward by approximately $2.8 \times 10^9 \Omega$. Finally, the 1.35 kHz peak shifts downward less than $0.5 \times 10^9 \Omega$. It is unclear at this time if these results indicate a change in the shape of the anti-resonance feature, or a shift in the position of the feature. Based on this data, we only used electrodes with an anti-resonance feature at a frequency greater than 1.3 kHz.

[0089] Fig. 4A shows the variance of an α -PACAP functionalized sensor with a 1.35 kHz, 10 mV potential applied while perfusing with TBS, 10 nM Met-Enkephalin, and 10 nM PACAP, respectively. While a trend may appear, no significant increase in variance occurred during the Met-Enkephalin treatment (Fig. 4C) while a much larger, significant, increase in variance was measured with PACAP treatment (Fig. 4B). To control for variance increase due to non-specific interactions, a single electrode was run three times in succession with buffer, 10 nM Met-Enkephalin, and then buffer alone (Fig. 4C). Due to inherent differences in frequency response between electrodes we normalized to the variance during the ligand-free baseline. Sensors were perfused with TBS, 10 nM Met-Enkephalin, and then 10 nM PACAP. The change in the normalized variance from TBS is presented in Fig. 4B. The PACAP treatment doubled the signals variance over the negative control and this difference was significant ($p < .01$).

[0090] We have demonstrated a capacitive immunosensor able to detect 10 nM PACAP in a physiological saline solution using variance analysis. A high frequency anti-resonance

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feature of the sensor's frequency response was identified that was sensitive to polydopamine coating at the tip. Attachment of a PACAP IgG antibody to the polydopamine layer allowed us to measure a change anti-resonance feature in response to PACAP presentation. We used this anti-resonance feature to determine a sinusoid frequency at which to do sensing experiments. A lock-in algorithm allowed us to measure a change in the signal variance in response to PACAP presentation at the sinusoid frequency in a biological saline solution.

[0091] We chose a frequency domain analysis, but it is theoretically possible to use the same sensor while applying a step potential. The current in response to the step function decays as the capacitor charges, this is measurement of an RC circuit. Changes in the capacitor, change the RC circuit, and thus the nature of the current decay. This has the disadvantage of having less time resolution, but in situations where time resolution is not required, it can be used.

Example 2

Impedimetric antibody-based enkephalin detection

[0092] Figs. 7(A-C) illustrate a schematic representation of the impedimetric antibody-based detection technique. An electrode (carbon fiber or platinum) is functionalized by electrodeposition of polydopamine to which antibodies are covalently bound. Presentation with a non-specific ligand for the bound antibody does not result in tight binding and is ineffective at altering the capacitance formed and the electrode - solution interface. Presentation of the ligand to the bound antibody results in high affinity binding. This displaces high dielectric water and presents an immobile charged moiety to the tip of the electrode, altering the capacitance at the electrode – solution interface.

[0093] In this example, parylene-insulated carbon fiber electrodes were dipped into a TRIS-buffered saline with dopamine in solution. Step depolarizations were applied to the electrode to electrodeposit polydopamine onto the fiber tip. The electrode was then tip-dipped into a TRIS-buffered solution containing antibody for several hours. The electrode was then mounted to the input of a differential amplifier (1 channel for experimental and a second channel for a negative-control reference antibody expected to deliver no specific signal, GAPDH in this case) and exposed to a solution containing potential ligand. The equivalent circuit of the electrode was measured in a time-domain approach by stepping the electrode with alternating commands (V_c) to +10 and -10 mV. Resulting currents were

recorded and digitized at 20 KHz and stored. Analysis consists of subtraction of the current measured through the reference electrode from the experimental electrode and measure of the resulting difference.

[0094] Figs. 8(A-B) show that the capacitance at the electrode – solution interface was sampled by a step depolarization in the electrode – ground circuit (V_c = voltage command). The current injected into the circuit in response to the voltage step decays with kinetics defined by the RC (resistance and capacitance) of the circuit – a parameter altered by the antibody – ligand binding. Example data obtained from an electrode bound with anti-enkephalin antibodies shows increased signal amplitude in response to 50 pM enkephalin versus non-specific bovine serum albumin (BSA) (Fig. 9).

In Vitro calibration of enkephalin detection

[0095] Two electrodes were prepared as described above; one with enkephalin antibodies and another with antibodies against Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Enkephalin serves as the positive condition and GAPDH as the negative control. Signal was measured in solution containing no (TRIS) or increasing concentrations of enkephalin. The measured current was normalized to the initial TRIS currents measured from each electrode. Fig. 10A shows Increasing concentrations of enkephalin increased the current measured in the enkephalin electrode (grey bars) but not the control GAPDH electrode (white bars). Fig. 10B shows currents measured in A are plotted against concentration in a semi-log plot and show that the currents follow typical pharmaco-kinetics.

Example 3

L-CSNO sensor

[0096] We developed an S-nitroso-L-cysteine (L-CSNO) sensor in order to measure the presence and concentration of L-CSNO in blood. To accomplish this, we begin with insulated carbon fiber electrodes (ALA id CFE-2) and coated the tip with a thin layer of polydopamine. Before passivation, polydopamine is an electrophile and nucleophiles will covalently bond to it. We functionalized the sensor tip. We passivated the sensor tip in a solution of polyclonal rabbit anti-L-CSNO antibody in a solution of high pH (pH 8.2) Tris buffer. This ensures the sensor is coated with Tris and L-CSNO antibody. When L-CSNO binds to the antibody, it changes the local charge environment of the sensor's tip changing the

signal. We then create a reference electrode by coating another insulated carbon fiber with polydopamine and passivating it with pure high pH Tris buffer.

[0097] The bath was charged by applying a potential step to the Ag/AgCl pellet and the response to each electrode was recorded. The maximum response current from the sensing electrode is dependent upon the fraction of anti-L-CSNO antibodies binding L-CSNO. This sensor showed a significant change in maximum response in the presence of 4 pM L-CSNO and 40 μ M SNO-Albumin and Albumin, but no significant change in response to other ligands similar to L-CSNO (SNO-Cysteamine, SNO-Glutathione, and L-Cysteine) (Fig. 11).

[0098] For each experiment, three sensing runs were conducted by charging the sensing and reference electrode with a 1 second, +50 mV direct current injection, and the allowing the electrodes to discharge for 1 second before charging the electrodes with a -50 mV direct current injection and again allowing them to discharge. The difference between the first 10 ms of the discharge current between when the probe was positively charged and negatively charged and this served as the signal for the sensor. We then collected three sensing experiments with the electrodes in 10 mM Tris buffered saline, pH 7.4 (Running Buffer) to serve as a baseline signal. We then perfused in 50 mL of Running Buffer and took an additional three readings to assess electrode stability. Finally, we incubated the sensing and reference electrodes in 10 mL of running buffer mixed with ligand or biological fluid and allowed it to incubate for 10 minutes. Afterwards the sensor was washed with 50 mL of Running Buffer and a final three experiments were performed to determine the signal we obtained from our solution.

Blood assays using the probe

[0099] Venous blood was drawn from the antecubital fossa and arterial blood from the radial artery of normal, non-smoking, healthy volunteers on no medications. Blood was drawn into a heparinized syringe and immediately diluted 1:7 in Tris buffer (above). It was placed in a Petrie dish and analysed using the L-CSNO sensor relative to the reference electrode (see above). Phlebotomy was performed in accordance with an institutional review board protocol.

[00100] We extracted brains from male C57B6 mice and dissected those brains into their component parts. We then weighed and homogenized the brain tissue in 400 μ l of 10 mM Tris buffered saline, pH 7.4 (Running Buffer), before further diluting the brain homogenate to

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10 ml with running buffer. The sensing and reference electrodes were allowed to relax in pure running buffer and a baseline reading and two readings with fresh running buffer were taken to establish a stable baseline. Afterwards, the reference and sensing electrodes were incubated in the brain homogenate for 10 minutes before the sensor was cleaned with running buffer and a new reading was taken. The signal we are recording is marked by a fast rise in the charging of the sensing and reference electrode. This fast rise reaches its maximal amount in the first 10 milliseconds (Fig. 12). The maximal charging current experienced by the sensor is proportional to the percentage of antibodies at the sensor's surface bound with ligand (Fig. 13).

[00101] While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims. All patents, publications and references cited in the foregoing specification are herein incorporated by reference in their entirety.

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Having described the invention, we claim:

1. A capacitive sensor for the detection of an analyte in a fluid comprising:
an electrode having a detection surface;
a polydopamine layer adhered to the electrode detection surface; and
a receptor chemically functionalized to the polydopamine of the detection surface of the electrode, the receptor selectively binding to the analyte of interest and the analyte once bound being detectable by measuring the change of capacitance of the electrode.
2. The sensor of claim 1, wherein the electrode comprises a carbon or platinum fiber.
3. The sensor of claim 2, wherein electrode include a surface active region that is defined by an insulator that covers at least a portion of the electrode.
4. The sensor of claim 1, wherein the receptor comprises at least one of a aptamer, oligomer, polymer, catalyst, cell, bacteria, virus, enzyme, protein, heptan, saccharide, lipid, glycogen, enzyme inhibitor, enzyme substrate, neurotransmitter, hormones, antigen, antibody, DNA, or RNA.
5. The sensor of claim 1, wherein presentation of a non-specific ligand to the bound receptor is ineffective at altering the capacitance of the electrode and presentation of a ligand for the receptor to the receptor results in binding of the ligand to the receptor and presents an immobile charged moiety to the surface active region of the electrode, altering the capacitance at the electrode–fluid interface.
6. The sensor of claim 1, wherein the electrode comprises a carbon fiber that is insulated to define a surface active region at a tip of the carbon fiber, the tip of the carbon fiber being covered with polydopamine.
7. The sensor of claim 1, being capable of quantifying an analyte in vivo when placed in a subject.

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8. The sensor of claim 1, the receptor comprising an antibody or antigen binding fragment thereof.

9. The sensor of claim 1, wherein the antibody or antigen binding fragment binds enkephalin or S-nitroso-L-cysteine.

10. The sensor of claim 1, further comprising a reference electrode having a polydopamine layer adhered to a reference electrode detection surface.

11. A method of detecting an analyte of interest in a fluid, the method comprising; positioning a capacitive sensor in the fluid, the capacitive sensor including an electrode having a detection surface, a polydopamine layer adhered to the electrode detection surface; and
stepping the electrode with alternating voltages; and
recording the changes in current in response to the stepped voltage.

12. The method of claim 11, wherein the electrode comprises a carbon or platinum fiber.

13. The method of claim 1, wherein electrode include a surface active region that is defined by an insulator that covers at least a portion of the electrode.

14. The method of claim 11, wherein the receptor comprises at least one of a aptamer, oligomer, polymer, catalyst, cell, bacteria, virus, enzyme, protein, heptan, saccharide, lipid, glycogen, enzyme inhibitor, enzyme substrate, neurotransmitter, hormones, antigen, antibody, DNA, or RNA.

15. The method of claim 11, wherein presentation of a non-specific ligand to the bound receptor is ineffective at altering the capacitance of the electrode and presentation of a ligand for the receptor to the receptor results in binding of the ligand to the receptor and presents an immobile charged moiety to the surface active region of the electrode, altering the capacitance at the electrode–fluid interface.

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16. The method of claim 11, wherein the electrode comprises a carbon fiber that is insulated to define a surface active region at a tip of the carbon fiber, the tip of the carbon fiber being covered with polydopamine.

17. The method of claim 11, being capable of quantifying an analyte in vivo when placed in a subject.

18. The method of claim 11, the receptor comprising an antibody or antigen binding fragment thereof.

19. The method of claim 11, wherein the antibody or antigen binding fragment binds enkephalin or S-nitroso-L-cysteine.

20. The method of claim 11, further comprising a reference electrode having a polydopamine layer adhered to a reference electrode detection surface.

21. A capacitive sensor for the detection of an analyte in a fluid comprising:
a sensing electrode having a detection surface a polydopamine layer adhered to the electrode detection surface; and a receptor chemically functionalized to the polydopamine of the detection surface of the electrode, the receptor selectively binding to the analyte of interest and the analyte once bound being detectable by measuring the change of capacitance of the electrode
a reference electrode having a polydopamine layer adhered to a reference electrode detection surface; and
a direct current injection source for charging the sensing and reference electrodes.

22. The sensor of claim 21, wherein the electrode comprises a carbon or platinum fiber.

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23. The sensor of claim 22, wherein electrode include a surface active region that is defined by an insulator that covers at least a portion of the electrode.

24. The sensor of claim 21, wherein the receptor comprises at least one of a aptamer, oligomer, polymer, catalyst, cell, bacteria, virus, enzyme, protein, heptan, saccharide, lipid, glycogen, enzyme inhibitor, enzyme substrate, neurotransmitter, hormones, antigen, antibody, DNA, or RNA.

25. The sensor of claim 21, wherein presentation of a non-specific ligand to the bound receptor is ineffective at altering the capacitance of the electrode and presentation of a ligand for the receptor to the receptor results in binding of the ligand to the receptor and presents an immobile charged moiety to the surface active region of the electrode, altering the capacitance at the electrode–fluid interface.

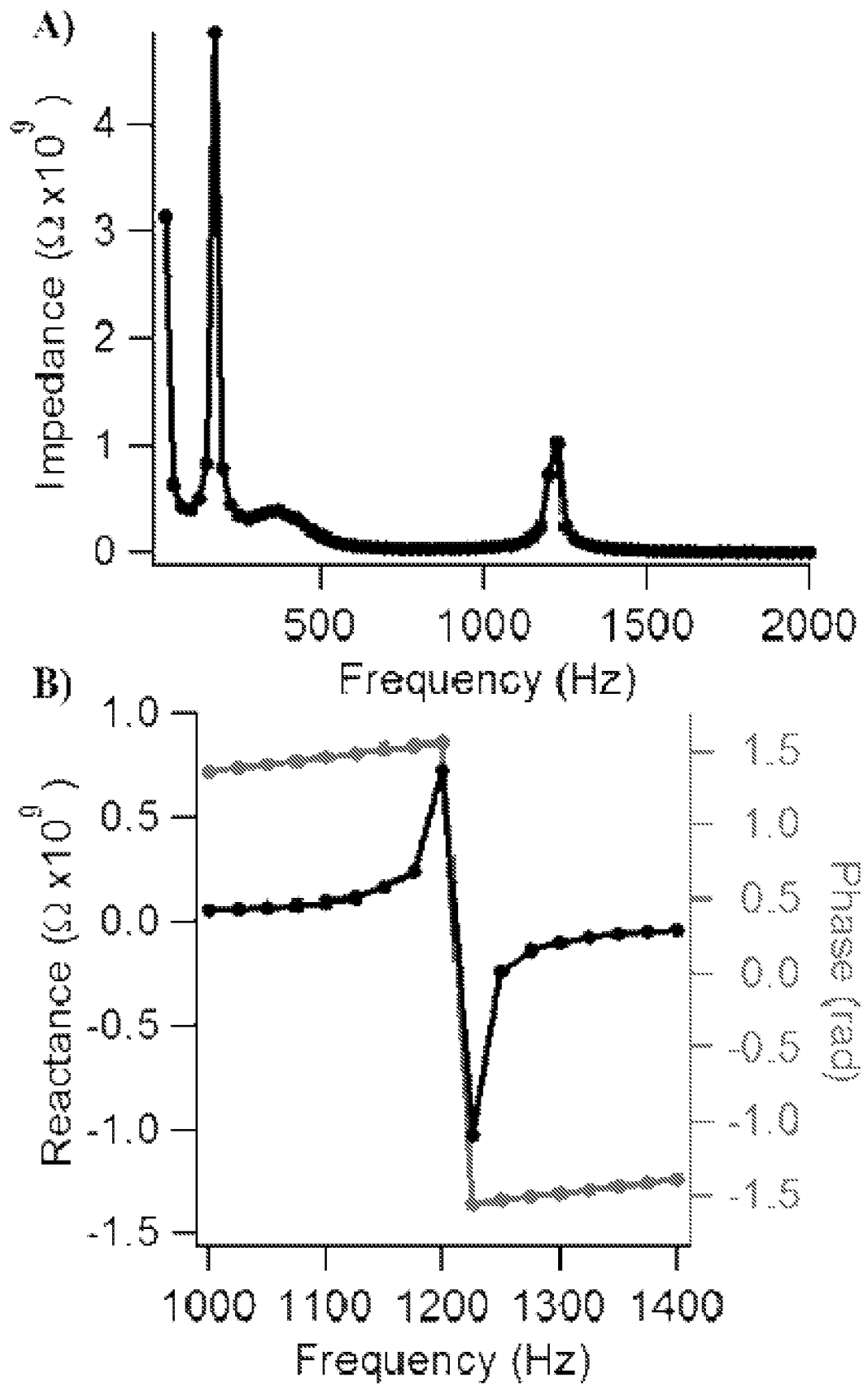
26. The sensor of claim 21, wherein the electrode comprises a carbon fiber that si insulated to define a surface active region at a tip of the carbon fiber, the tip of the carbon fiber being covered with polydopamine.

27. The sensor of claim 21, being capable of quantifying an analyte in vivo when placed in a subject.

28. The sensor of claim 21, the receptor comprising an antibody or antigen binding fragment thereof.

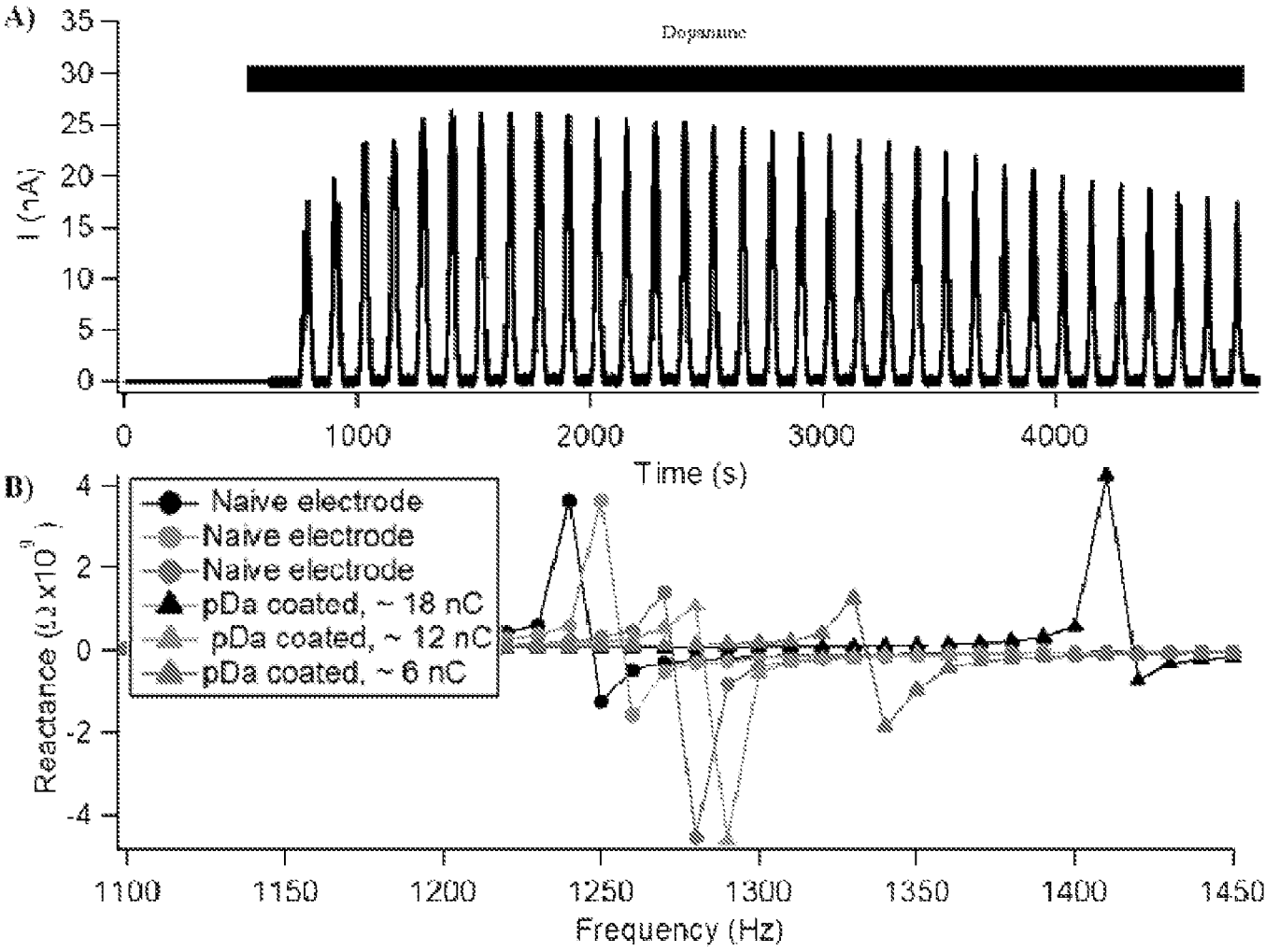
29. The sensor of claim 21, wherein the antibody or antigen binding fragment binds enkephalin or S-nitroso-L-cysteine.

30. The sensor of claim 21, further comprising a reference electrode having a polydopamine layer adhered to a reference electrode detection surface.



Figs. 1A-B

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Figs. 2A-B

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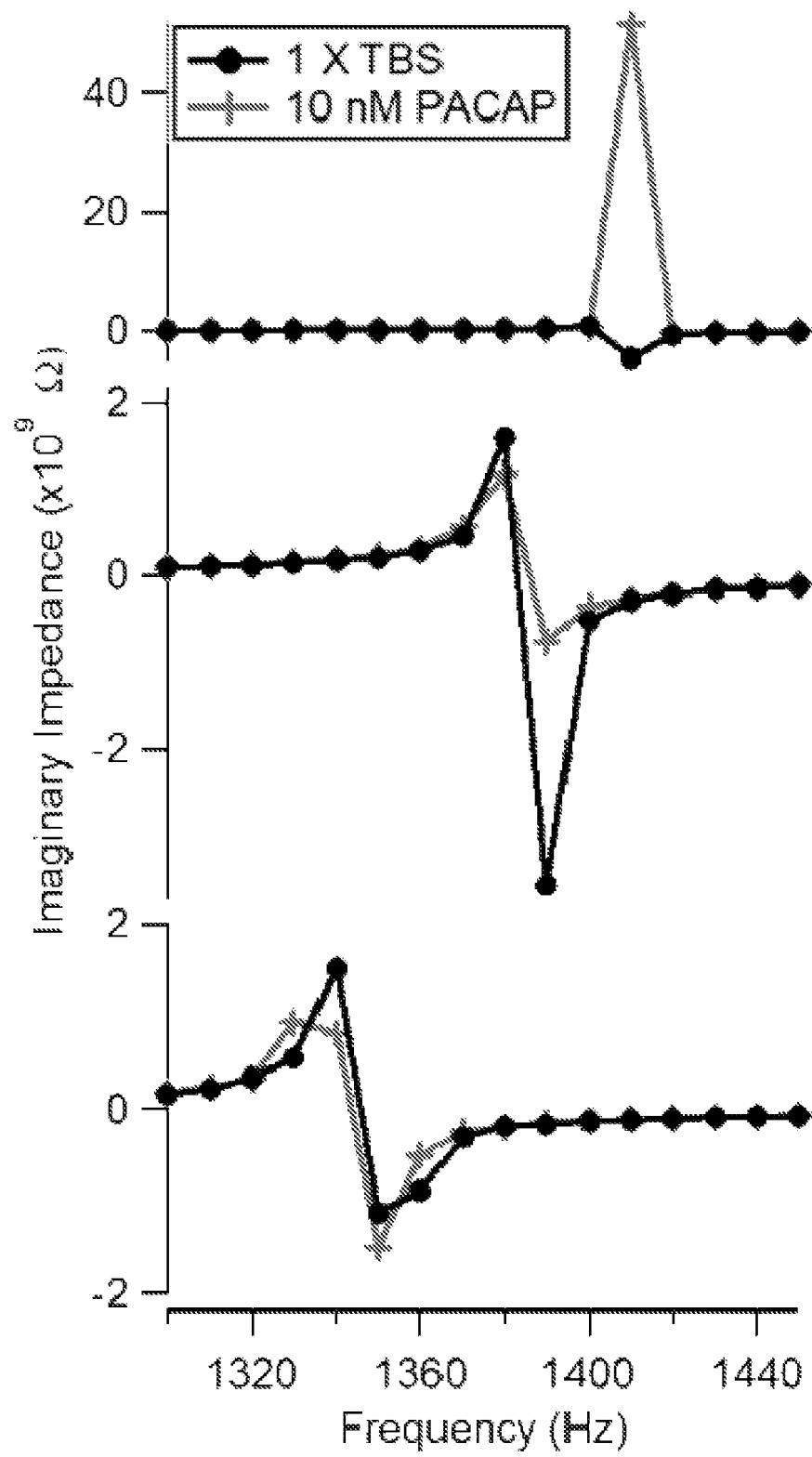
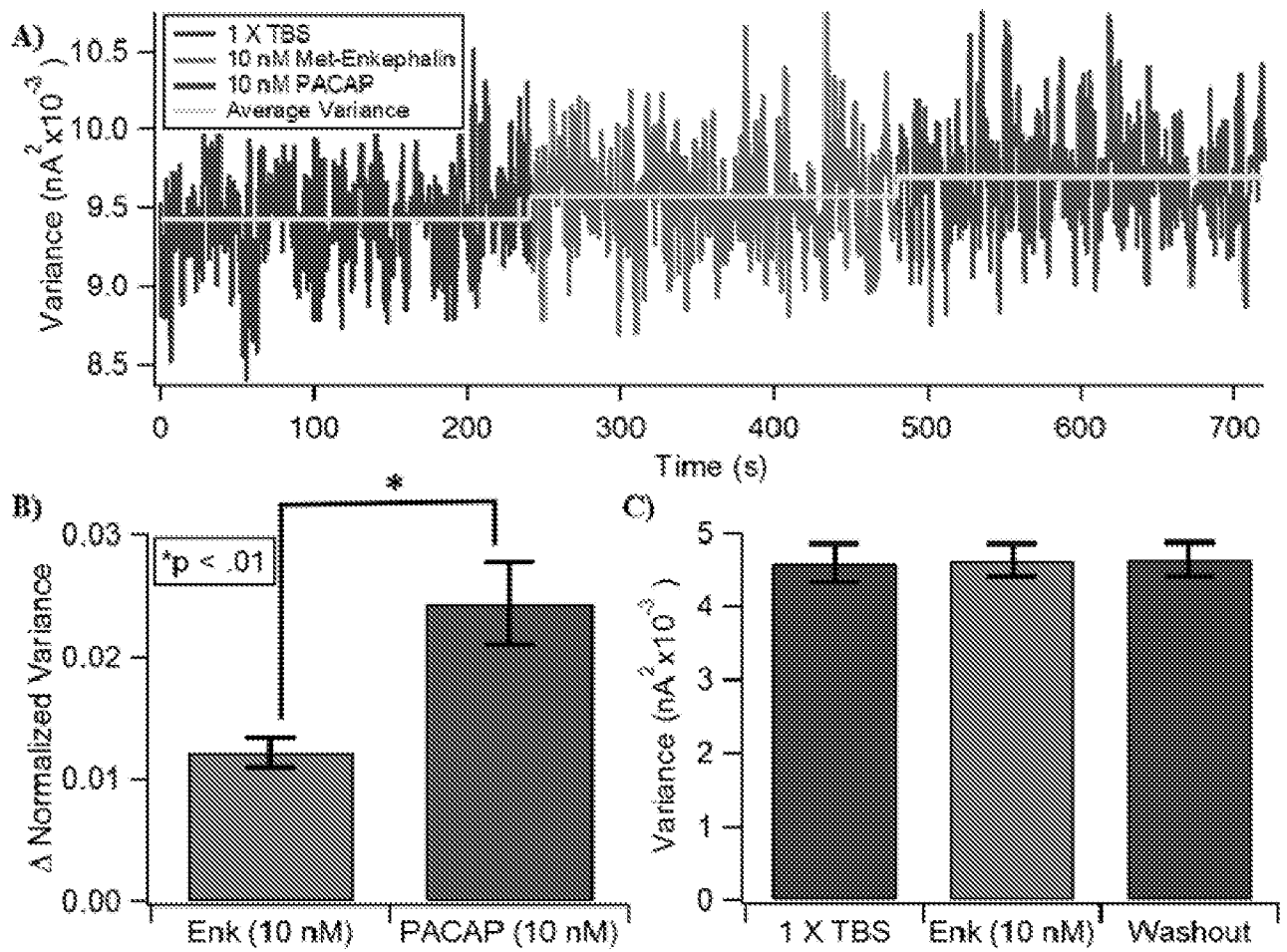
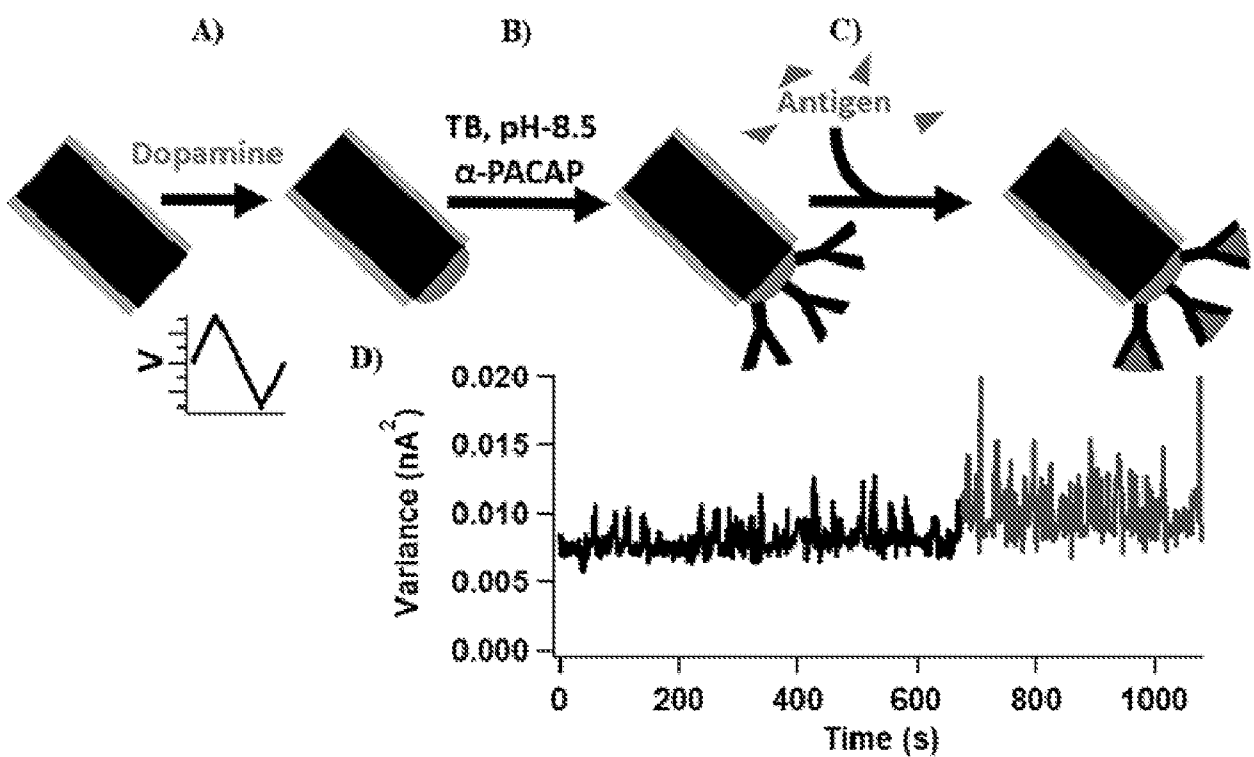


Fig. 3

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Figs. 4A-B



Figs. 5A-C

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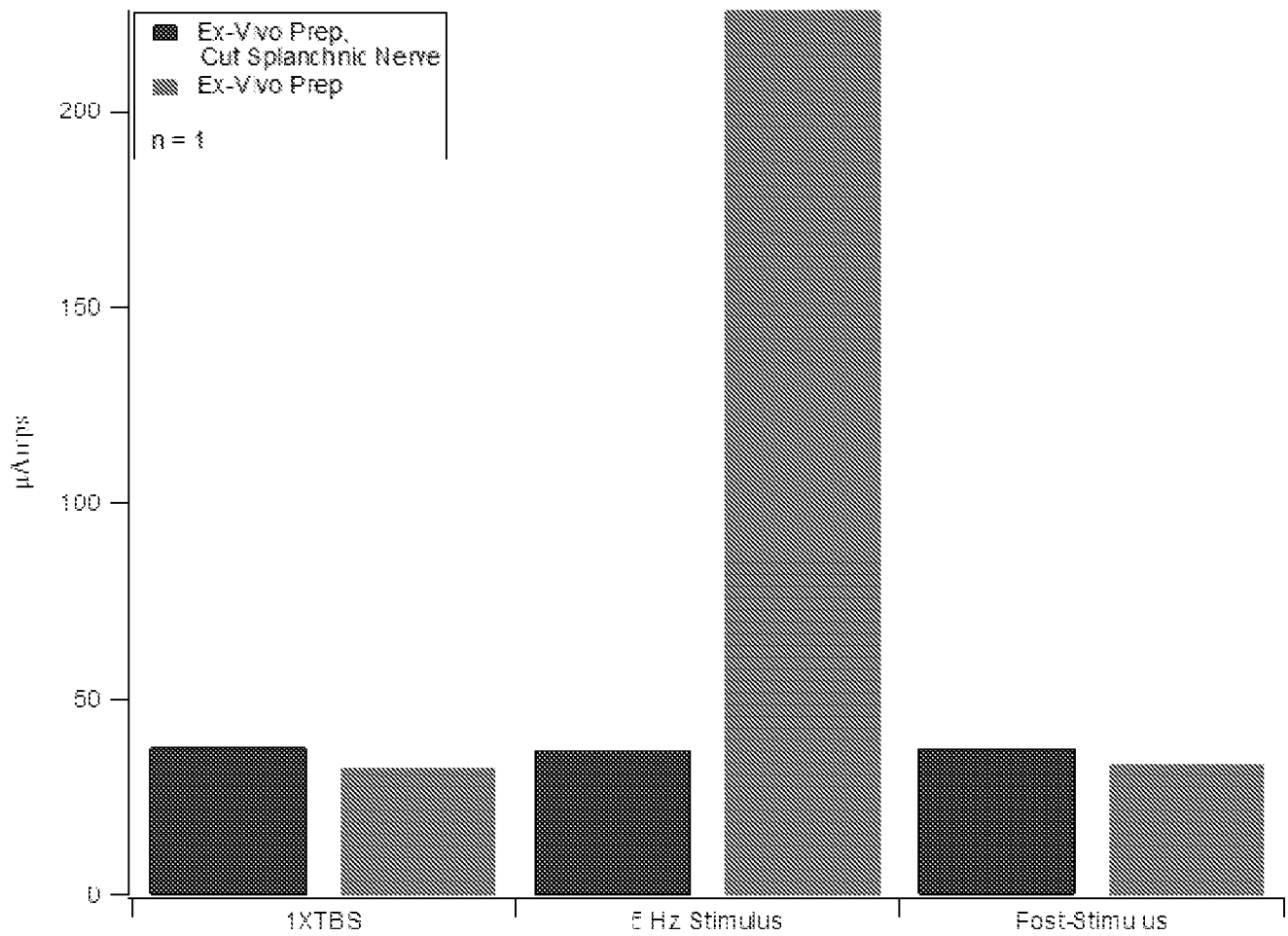
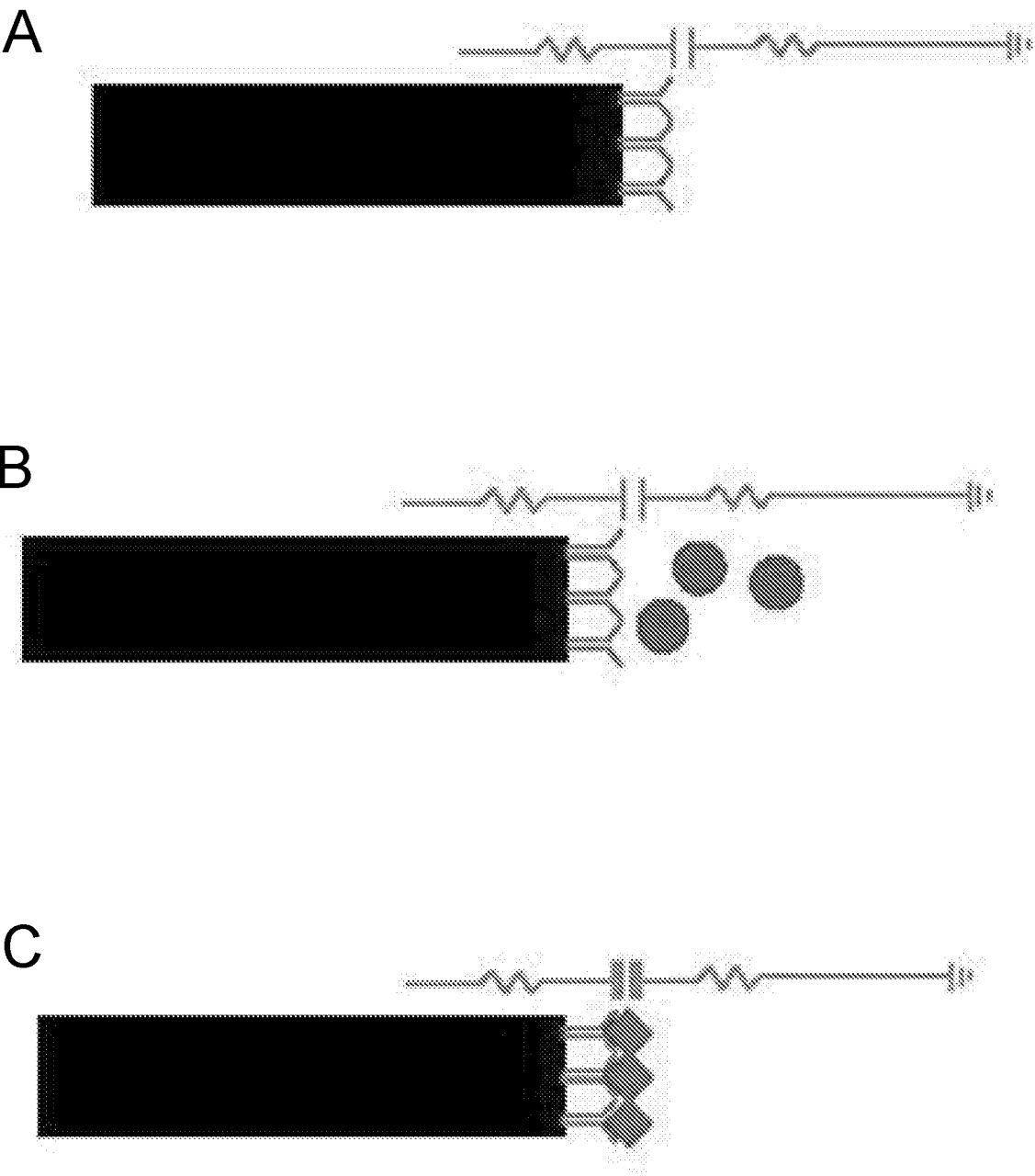
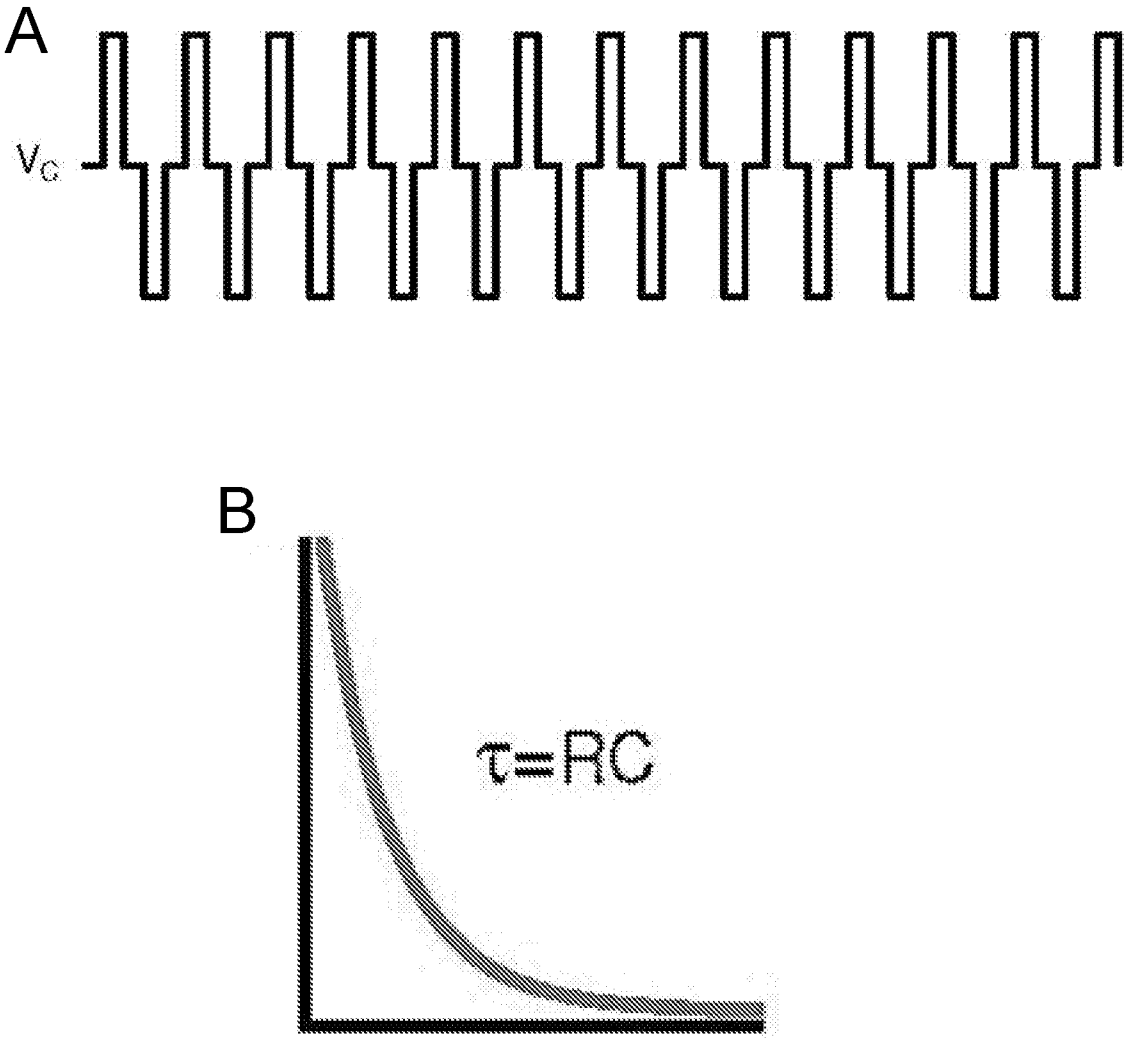


Fig. 6



Figs. 7A-C



Figs. 8A-B

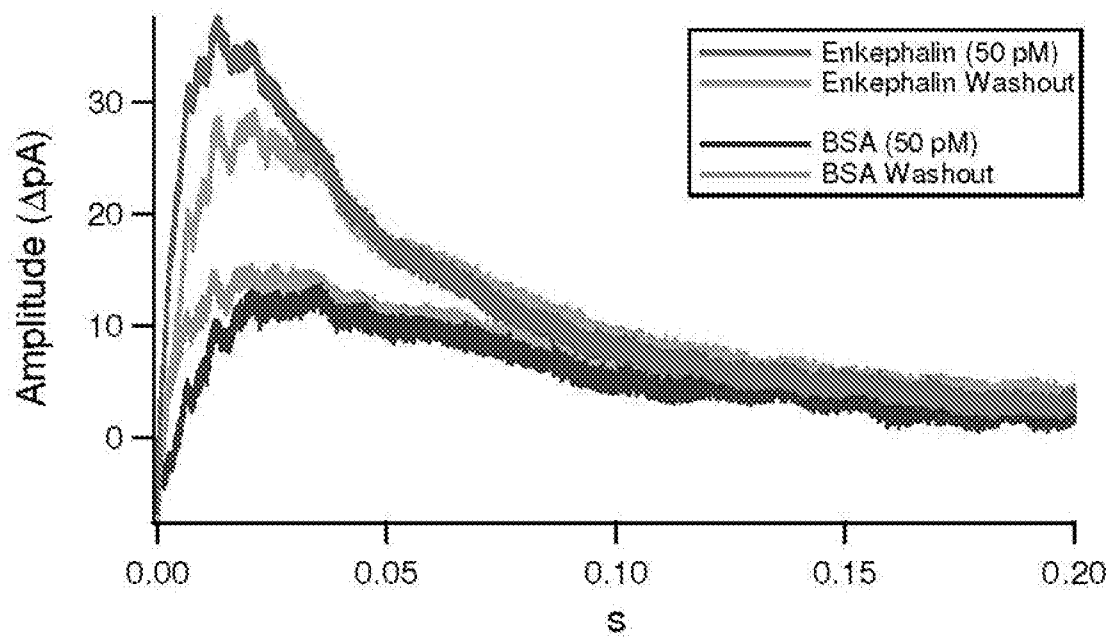
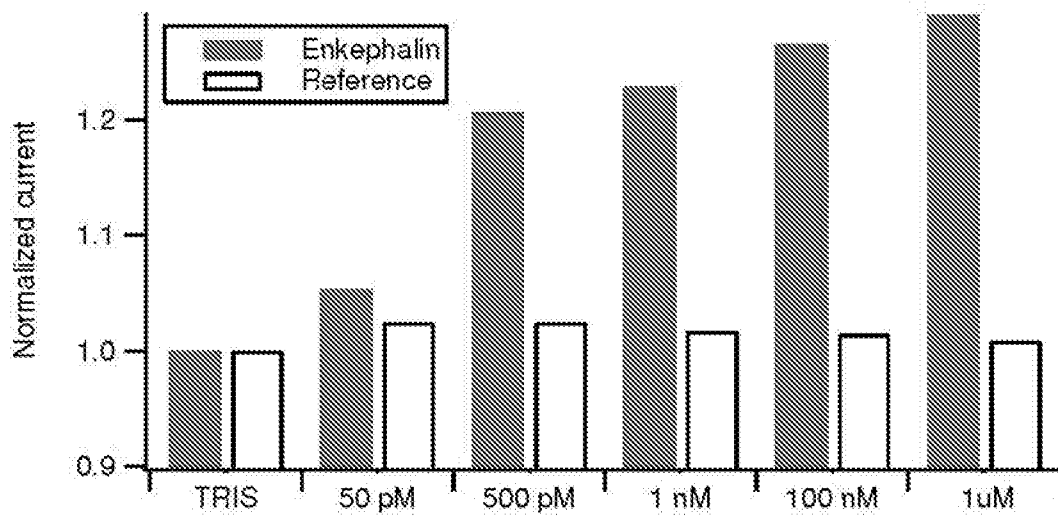


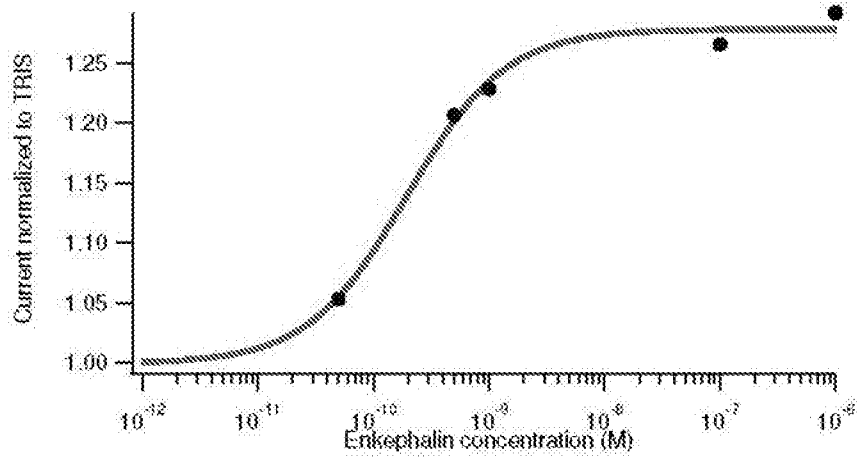
Fig. 9

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A.



B.



Figs. 10A-B

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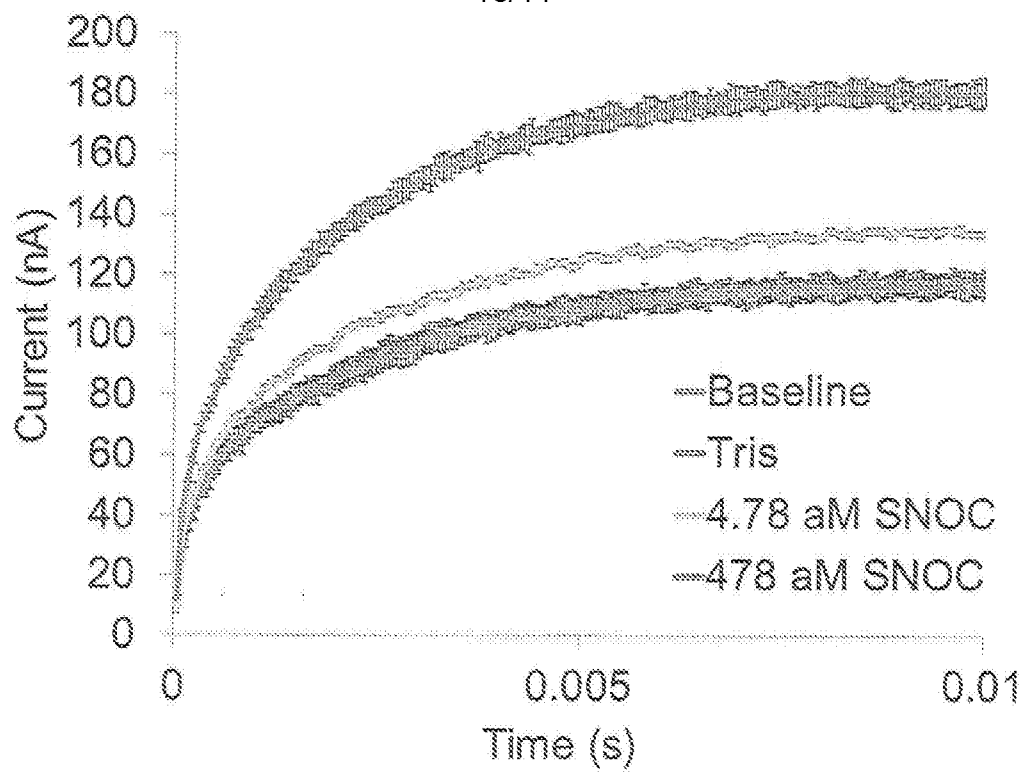


Fig. 11

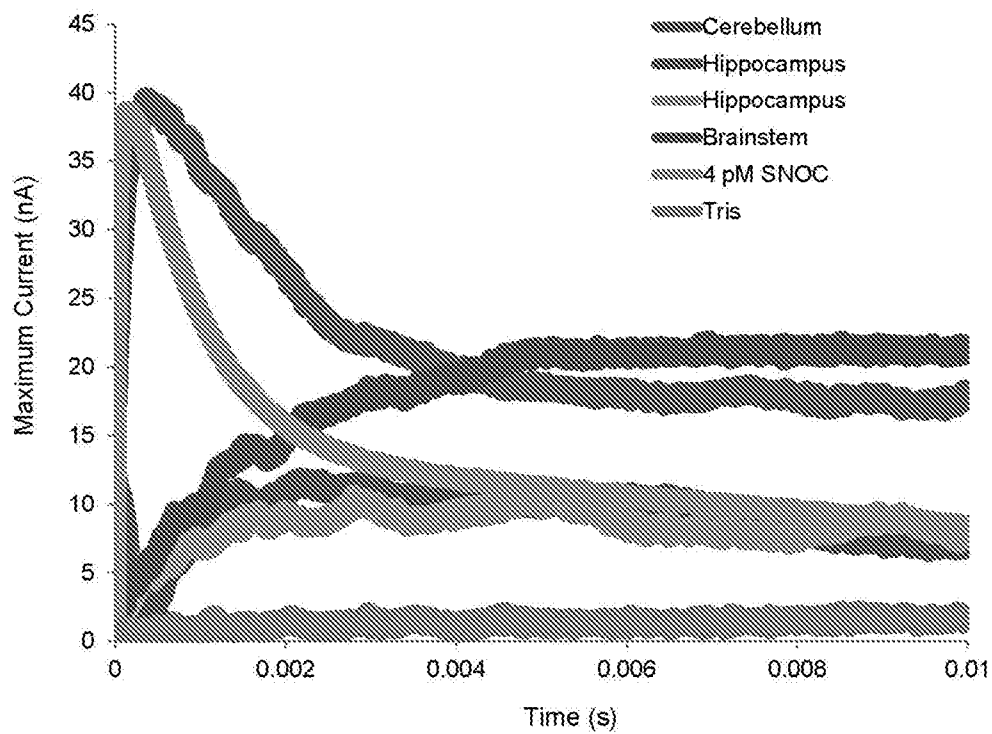


Fig. 12

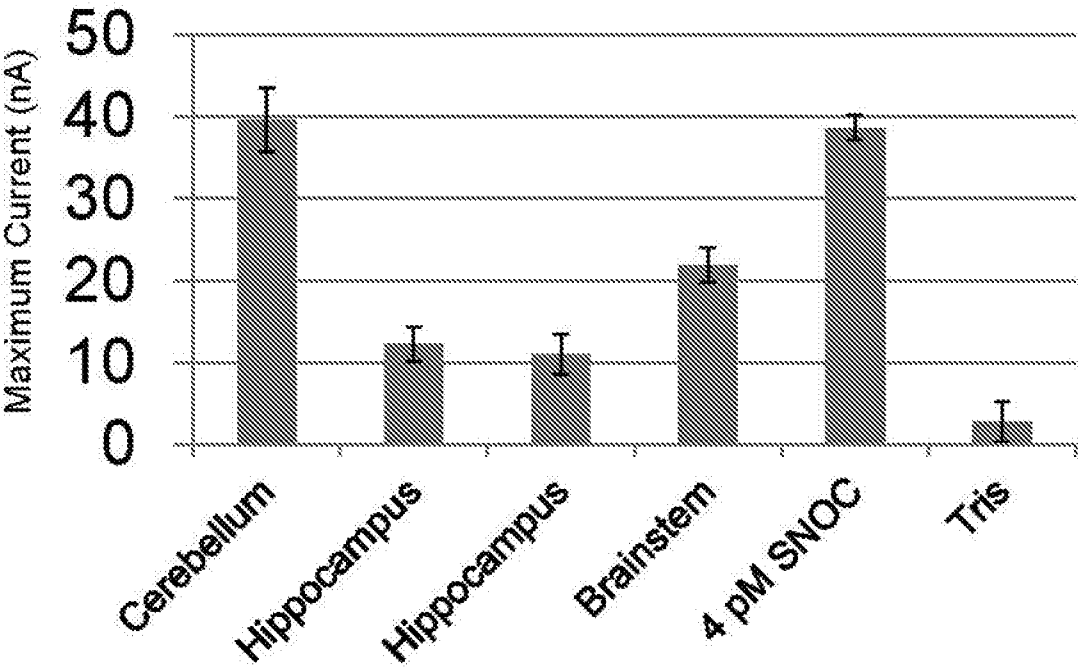


Fig. 13

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/53786

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/68, G01N 27/22 (2016.01)

CPC - G01N 27/221

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)

IPC(8)- C12Q 1/68, G01N 27/22 (2016.01)

CPC- G01N 27/221

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)

PatBase, Google Patents, Google Scholar (without Patents)

Keywords: capacitive sensor detection analyte electrode detection surface polydopamine layer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2012/0156688 A1 (McAlpine et al.) 21 June 2012 (21.06.2012) Abstract, para [0047], para [0085], para [00117], para [00161], para [0085], para [00164], para [0033], para [00100]	1-10,21-30
Y	~ CN 104820093 A (Univ. Shanghai) 05 August 2015 (05.08.2015) Abstract, para [0004], para [0010], para [0008]-[0025], and entire document	1-10,21-30
Y	~ CN 102914580 A (Univ Xinyang Normal) 06 February 2013 (06.02.2013) Abstract, pg 4 para 1; pg 1 para 2;	1-10,21-30
A	~ CN 102000658 A (Univ Jiaotong, Southwest) 06 April 2011 (06.04.2011) Abstract, Claims 1-3	1-10,21-30
A	US 2014/0336040 A1 (Yan et al.) 13 November 2014 (13.11.2014) Abstract;	1-10,21-30

☐ Further documents are listed in the continuation of Box C.


* 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

16 January 2017

Date of mailing of the international search report

09 FEB 2017

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/53786

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 supplemental box -----

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 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.:
1-10,21-30

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.

—continued from BOX III—

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-10 and 21-30 directed towards capacitive sensor for the detection of an analyte in a fluid comprising: an electrode having a detection surface; a polydopamine layer adhered to the electrode detection surface; and a receptor chemically functionalized to the polydopamine of the detection surface of the electrode, the receptor selectively binding to the analyte of interest and the analyte once bound being detectable by measuring the change of capacitance of the electrode.

Group II: Claims 11-20 directed towards a method of detecting an analyte of interest in a fluid, the method comprising: positioning a capacitive sensor in the fluid, the capacitive sensor including an electrode having a detection surface, a polydopamine layer adhered to the electrode detection surface; and stepping the electrode with alternating voltages; and recording the changes in current in response to the stepped voltage.

The inventions listed as Groups I-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I requires a receptor chemically functionalized to the polydopamine of the detection surface of the electrode, the receptor selectively binding to the analyte of interest and the analyte once bound being detectable by measuring the change of capacitance of the electrode, not required by groups II.

Group II requires a method of detecting an analyte of interest in a fluid, the method comprising: positioning a capacitive sensor in the fluid and stepping the electrode with alternating voltages; and recording the changes in current in response to the stepped voltage, not required by groups I

Shared Technical Features:

Groups I-II share the common feature of a capacitive sensor having a detection surface, a polydopamine layer adhered to the electrode detection surface. However, these shared technical features do not represent a contribution over prior art, because the shared technical feature is obvious over US 2012/0156688 A1 to Mcalpine et al. (hereinafter Mcalpine) in view of CN102914580 A to Univ Xinyang Normal (hereinafter Xinyang). Mcalpine discloses a capacitive electrodes (para [0047], interdigitated capacitive electrode) having a biosensor polypeptide adhered to the surface (para [0006], peptide immobilized on a sensing element) that is used for detection of analytes (para [0132], microcapacitive sensor detects impedance changes in the dielectric properties of an electrode surface upon analyte binding). Mcalpine does not specifically disclose a polydopamine layer adhered to the capacitive electrode. However, Xinyang discloses a silver-polydopamine-graphene nanocomposite modified electrochemical sensor (pg 1 para 2) for detecting analytes (pg 4 para 1; present invention provides a high sensitivity, simple, environmentally friendly and quickly detect adenine and guanine electrochemical sensor). As both Mcalpine and Xinyang disclose methods of preparing sensors for detecting analytes, it would have been obvious to a person having ordinary skill in the art to know that the peptide disclosed by Mcalpine can be a polydopamine disclosed by Xinyang depending on analyte to be detected through routine experimentation.

As the shared technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups. Therefore, Groups I-II lack unity under PCT Rule 13.