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(71) Applicant: **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, 12th Floor, Oakland, California 94607 (US).

(72) Inventors: **PLAXCO, Kevin**; University of California, 1103 Chemistry, Mail Code 9510, Santa Barbara, California 93106 (US). **KANG, Di**; University of California, 1103 Chemistry, Mail Code 9510, Santa Barbara, California 93106 (US). **SUN, Shen**; University of California, 1103 Chemistry, Mail Code 9510, Santa Barbara, California 93106 (US). **KURNIK, Martin**; University of California, 1103 Chemistry, Mail Code 9510, Santa Barbara, California 93106 (US).

(74) Agent: **BERMAN, Matthew**; P.O. Box 2034, Winter Park, Colorado 80482 (US).

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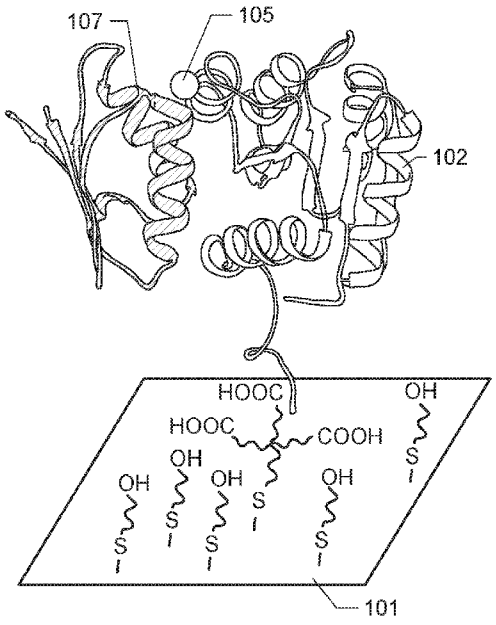

FIGURE 1B

(57) Abstract: The invention encompasses novel electrochemical sensors based on redox-reporter-modified polypeptides. The sensors detect faradic currents between the redox-reporter-modified polypeptides and an electrode substrate. Upon binding of the target species, steric interference modifies the signal in a concentration-dependent manner. The sensors design of the invention enables the use of protein-based probes to detect a wide range of chemical and biological targets. The sensors may advantageously be deployed in complex samples, such as whole blood, and may be deployed in vivo.

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Title: Single-step, reagentless detection by protein-based electrochemical biosensors using steric interference

[0001] CROSS-RELATED APPLICATIONS: This application claims the benefit of priority to United States Provisional Application Serial Number 62/372,558, entitled "Protein-Based Reagentless Electrochemical Sensors for Detecting Diverse Target Species," filed August 9, 2016, the contents of which are hereby incorporated by reference.

[0002] STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT: This invention was made with government support under grant number RO1AI107936 and R01GM59544 awarded by the National Institutes of Health. The government has certain rights in the invention

[0003] **BACKGROUND OF THE INVENTION**

[0004] Electrochemical DNA-based (EDNA) sensors are capable of reagentless, real-time detection of a wide range of molecular targets. These sensors comprise a conformation-changing nucleic acid "probe," such as an aptamer, that is covalently attached via one monomer to an electrode substrate and modified on a second (or more) with a redox reporter. Upon binding to its target molecule, the probe undergoes a conformational rearrangement or displacement of the redox reporter that generates an electrochemical signal in the form of a modulated redox current. Since this the signal generation mechanism is reagentless (e.g., label free), and the signal-generating change is rapid and reversible, the EDNA platform supports continuous, highly-time-resolved molecular detection.

[0005] EDNA sensors can provide continuous, real-time measurement of target molecules in complex samples such as serum or whole blood, and have been demonstrated to work in-vivo for extended periods of time. Accordingly, the EDNA concept potentially provides the art with a sensing platform that can be used in many important biomedical applications.

[0006] However, the EDNA concept has not been widely implemented. The major factor limiting the use of EDNA sensors is the availability of nucleic acids binding targets of interest with sufficient affinity and specificity. Currently, the ability to generate an aptamer to any selected target, for example, is limited. For many target species, a complementary aptamer is not available, is not readily generated, or may be impossible to generate due to constraints in nucleic acid chemistry.

[0007] Like aptamers, proteins can selectively bind a range of target species. The diversity of protein-ligand interactions in the natural world is enormous, with proteins capable of selectively binding to other proteins, nucleic acids, lipids, small molecules, and other species. If the high performance of EDNA sensors could be combined with the diversity of protein-based recognition, this would provide the art with a huge diversity of potential sensing applications. First, the binding interactions of proteins have been well studied, and for many biologically important proteins, the structure of the binding site for the target species is known. Furthermore, antibody generation technologies are quite well developed and often enable the straightforward generation of protein sequences (i.e., the variable region of the antibody) that can selectively bind a particular target species. Accordingly, it would be advantageous if the EDNA sensor design concept could be adapted for the use of proteins as probes.

[0008] The use of proteins as probes in electrochemical sensors has previously been contemplated, in the context of proteins that change conformation when a target ligand binds, in the manner of many aptamers. In aptamer-based sensors, conformation change upon target binding is typical and expected. However, in the case of ligands binding a protein, conformational change is not necessary, or indeed even typical, greatly reducing the number of potential targets that can be sensed using binding-induced conformational change. Accordingly, to the knowledge of the inventors of the present disclosure, the use of proteins in EDNA-like sensors has not been reported.

[0009] Accordingly, there remains a need in the art for sensor designs that combine the superior sensing abilities of EDNA sensors with the enormous diversity of

protein-ligand interactions and does not rely on the need for the protein to undergo a binding-induced conformational change.

[0010] SUMMARY OF THE INVENTION

[0011] The inventors of the present disclosure have advantageously developed novel electrochemical sensors using proteins as probes. The sensors of the invention do not require a binding-induced conformational change in the probe protein to generate a detectable signal. Instead, in the sensors of the invention, steric interference caused by target binding alters the efficiency of electron transfer between the reporter and electrode, providing a measurable signal of target binding.

[0012] In one aspect, the scope of the invention encompasses novel protein-based electrochemical sensors comprising redox reporter-modified proteins capable of selectively binding a target species. A plurality (e.g., thousands to billions) of these functionalized proteins are bound to an electrode substrate. When a target molecule binds to the protein, the close association of the target moiety to the protein probe interferes with charge transfer between the redox reporter and the electrode substrate. The result is the dampening or ablation of the signal observed in voltammetry or chronoamperometry, providing a concentration-dependent, measurable signal of target binding.

[0013] The sensors of the invention may be used for the detection of a wide array of target species, including proteins, lipids, carbohydrates, small molecules, pathogens, drugs, pollutants, and other targets. In another aspect, the scope of the invention encompasses novel methods of detecting such targets using steric-interference based protein sensors.

[0014] BRIEF DESCRIPTION OF THE DRAWINGS

[0015] **Fig. 1A and 1B.** Fig. 1A is a diagram depicting a sensing element of the invention comprising an electrode substrate (101) coated with a thiol monolayer (104). A recognition element polypeptide (102) comprising the bacterial chemotaxis protein CheY, is attached to the electrode by an anchoring moiety (103). The recognition element polypeptide is labeled with a redox reporter (105). In the absence of target species, charge transfer (106) between the redox reporter and the electrode is unimpeded. Fig. 1B depicts the recognition element with target species (107), CheY's naturally occurring binding partner CheA-P2, bound. Binding of the target species impedes charge transfer between the redox reporter (105) and the electrode (101).

[0016] **Fig. 2A, 2B, and 2C.** Fig 2A depicts cyclic voltammetry measurements for CheY-functionalized sensing element with and without Che-P2 present. Fig 2B depicts cyclic voltammetry measurements for CheY-functionalized sensing element with and without FliM. In the presence of target, Fig. 2A and 2B show a decrease in peak faradaic current caused by steric interference. Fig. 2C depicts time response of peak faradic current when a CheY-functionalized sensing element is exposed to 100 μM of the target CheA-P2, with a time constant of 2.8±0.7 min^{-1}. The error bars reflect standard deviation of measurements performed using multiple independently fabricated sensors and largely reflects sensor-to-sensor fabrication variation.

[0017] **Fig. 3A and 3B.** Fig. 3A depicts signal change response to varying concentrations of CheY-P2 target for a for both phosphorylated CheY and non-phosphorylated CheY recognition elements (reporter at position 97). Fig. 3B depicts signal change response to varying concentrations of FliM target for a for both phosphorylated CheY and non-phosphorylated CheY recognition elements (redox reporter at position 91).

[0018] **Fig. 4.** Fig. 4 depicts a CheY recognition element (401) and the spatial location of various redox reporter cysteine attachment sites generated by site-directed mutagenesis in different variants of the recognition element: A80C (402) , T71C (403) , E37C (404), M17C (405), and E117C (407). Bound target CheA-p2 (409) creates steric interference between redox reporter and the electrode (electrode surface attachment

region denoted with arrow). Signal gain is dependent on the placement of the redox reporter relative to the target-binding and surface attachment sites with the greatest signal gain generally being observed when the reporter is positioned adjacent to the binding site.

[0019] **Fig. 5A, 5B, 5C, and 5D.** Fig. 5A depicts signal response for sensors comprising redox-reporter-modified Green Fluorescent Protein (GFP) or CheY as the recognition element when exposed to anti-GFP antibodies at 10 ng/ml or control anti-FLAG antibodies. Fig. 5B depicts the monotonic response to anti-GFP antibodies at various concentrations obtained when the recognition element is GFP modified with a methylene blue on random lysine residues. Fig. 5C depicts signal change in response to anti-hepatitis B surface antigen antibodies at varying concentrations for sensors comprising hepatitis B surface antigen (HBsAg) recognition element modified with methylene blue on random cysteines, compared to a control sensor comprising a CheY recognition element, when the sample is buffer. Fig. 5D depicts the same sensors as in Fig. 5C, when sample is 20% blood serum. In Fig. 5A, 5B, 5C, and 5D, current changes were measured using square wave voltammetry.

[0020] **DETAILED DESCRIPTION OF THE INVENTION**

[0021] The scope of the invention encompasses electrochemical sensors comprising redox-reporter-modified polypeptides as a probes which selectively bind a target molecule. In one implementation, the invention encompasses a sensing element, comprising

an electrode; and

a plurality of recognition element polypeptides attached to the electrode;

wherein each recognition element polypeptide is capable of selectively binding one or more target species;

wherein each recognition element polypeptide is functionalized with one or

more redox reporters, wherein charge transfer (e.g., faradic current) occurs between the one or more redox reporters and the electrode; and

wherein binding of the target species to the recognition element causes a detectable change in the charge transfer between the the one or more redox reporters and the electrode.

[0022] The sensing elements of the invention may be incorporated into sensor assemblies or sensor systems for the detection of target species by various electrochemical interrogation techniques. The various elements of the invention are next described in detail.

[0023] **Targets**. The sensing elements of the invention are directed to the detection of a target species. The target species may comprise any inorganic or organic molecule, for example: a small molecule drug, a metabolite, a hormone, a peptide, a protein, a carbohydrate, a nucleic acid, a lipid, or any other composition of matter. The target species may comprise a drug. The target species may comprise a chemical entity. The target species may comprise a naturally occurring factor, for example a hormone, metabolite, growth factor, neurotransmitter, nutrients, and pollutants, pathogen-induced or pathogen-derived factors, *etc*.

[0024] In addition to molecules, the target species may comprise large and complex targets such as pathogenic cells or immune system elements such as antibodies or immune cells.

[0025] Target size may affect the magnitude of the signal change detected upon binding to the recognition element polypeptide. Typically, larger target species will have a greater steric hindrance on the redox- electrode interactions.

[0026] **Electrodes**. The sensors of the invention will comprise one or more working electrodes to which recognition elements functionalized with redox reporters are bound. The one or more electrodes may comprise various materials and configurations. The electrode may comprise any suitable electrode material for electrochemical sensing, including, for example: gold or any gold-coated metal or

material, titanium, tungsten, platinum, carbon, aluminum, copper, palladium, mercury films, silver, oxide-coated metals, semiconductors, graphite, carbon nanotubes, and any other conductive material upon which biomolecules can be conjugated.

[0027] The electrode may be configured in any desired shape or size, including discs, strips, paddle-shaped electrodes, rectangular electrodes, electrode arrays, screen-printed electrodes, and other configurations. For *in vivo* measurements, a thin wire configuration is advantageous, as the low-profile wire may be inserted into cells, veins, arteries, tissue or organs and will not impede blood flow in blood vessels or cause substantial damage in tissues, for example, a wire having a diameter of 1 to 500 μm.

[0028] **Recognition element polypeptides**. The novel electrochemical sensors of the invention depart from the prior art EDNA design in the use of polypeptides as the recognition probe, i.e., as the unit that selectively binds the target. The recognition probe comprises a polypeptide wherein a portion of the polypeptide will selectively bind to one or more target species.

[0029] The recognition probe polypeptide may comprise a polypeptide, i.e., a chain of two or more amino acids. The polypeptide may comprise any combination of amino acids, including the natural amino acids, modified amino acids, non-natural amino acids or amino acid analogs. The polypeptide may comprise bound co-factors, carbohydrate moieties (e.g., the product of glycosylation processes), and other non-amino acid components. The polypeptide may comprise a polymeric protein, e.g., a protein having multiple subunits. The polypeptide may comprise a natural protein isolated from biological materials or cells, or may comprise a chemically synthesized or recombinant polypeptide.

[0030] The size of the recognition probe may vary, from a few amino acids to thousands of amino acids. In one embodiment, the recognition element polypeptide comprises a whole protein, i.e. the entire native sequence of a protein as translated. In one embodiment, the recognition probe comprises a subsequence of a protein, i.e., a truncated form or fragment of a complete native protein sequence. The polypeptide

may also comprise a mutant or engineered form of a protein or protein domain.

[0031] The recognition probe polypeptide may comprise any amino acid sequence having a binding affinity for one or more target species, with sufficient specificity to prevent non-specific interactions. Binding affinity, as used herein, means, for example, polypeptides having a picomolar to millimolar dissociation constant for the target ligand may be used.

[0032] The binding of the target species will occur at a binding site within the recognition probe. The binding site, as used herein, will refer to any amino acid residues of a polypeptide that facilitate or are necessary for the binding of the target species to the recognition probe.

[0033] In one embodiment, the polypeptide comprises a receptor. In one embodiment, the polypeptide comprises an extracellular domain. In one embodiment, the polypeptide comprises an epitope. In one embodiment, the polypeptide selectively binds a biomarker of a condition, biological process, or disease state.

[0034] In one embodiment, the polypeptide comprises an antibody or fragment thereof. Advantageously, antibodies can be generated against a broad array of target species, resulting in a variable region having high affinity and specificity for the target species. In one embodiment, the recognition probe comprises a whole antibody. In another embodiment, the recognition probe comprises an antibody fragment comprising one or more antigen-binding regions derived from an antibody.

[0035] In one embodiment, the recognition element polypeptide does not substantially change conformation when target is bound, i.e., there is no measurable change in faradic current attributable to the conformation change. In an alternative embodiment, there is a conformational change upon target binding which, independent of steric effects, increases or decreases the interactions between the redox reporter and the electrode by changing the position of the redox reporter with respect to the electrode. In one embodiment, this conformation effect is smaller than the change in signal caused by steric interference, for example accounting for 1-20% of signal

change. In another embodiment, this conformation effect causes a major change in signal reduction that is augmented by steric effects on faradic current. In alternative embodiments, upon target binding there is a conformational change that enhances faradic current, which such effect is cancelled or obscured by a greater reduction in faradic current caused by steric interferences.

[0036] **Anchoring Chemistry**. The recognition elements are bound to the surface of the electrode. The recognition element may be conjugated to or otherwise associated with the electrode surface by any appropriate chemistry, for example by covalent bonding to the electrode or to a monolayer on the electrode, or via chemisorption or adsorption.

[0037] In one embodiment, the polypeptide comprising the recognition element may be modified at one terminal end (i.e., C-terminus or N-terminus) with an anchoring moiety. The anchoring moiety may comprise a species that is capable of directly conjugating to the electrode surface. Alternatively, the anchoring species may be capable of conjugation to a complementary functional group with which the electrode surface has been modified or decorated. Anchoring moieties may comprise elements that form self-assembled monolayers on the electrode surface.

[0038] In one embodiment, polypeptides may be thiolated or activated on their carboxy or amino terminal ends for bonding to the electrode surface, using chemistries known in the art. Amide linkages to lysine residues and thio-ether bond formation to cysteine residues may also be used. In one embodiment, the electrode surface (e.g. gold) is functionalized with nickel-nitrilotriacetic acid. These moieties can bind to histidines from His-tag functionalized proteins using copper or zinc or nickel, forming stable complexes. In one embodiment, the recognition element comprises a carboxy-terminal or amino-terminal hexahistidine tag. In one embodiment, the electrode (for example, a gold electrode) is coated with an alkane thiol self-assembled monolayer doped with a small fraction of copper chelating nitrilotriacetic acid (NTA) head groups, to enable site-specific attachment of proteins to the electrode surface. In another embodiment, the electrode (for example, a metal oxide electrode) is coated with a self-

assembled silane monolayer to which polypeptides may be attached by chemistries known in the art.

[0039] The anchoring moiety may comprise a click chemistry group, as known in the art, which is capable of forming bonds with complementary click chemistry groups conjugated to the electrode surface. In one embodiment, non-natural amino acids are incorporated at specific positions in the protein to provide sites for surface tethering, for example, amino-acid analogs with azide side chains that enable surface attachment through click-chemistry. Alternatively, the anchoring moiety may be an activated silane, as known in the art, which is capable of forming bonds to many oxide surfaces. In another implementation, the anchoring moiety may contain a ligand, which can bind to the surface via coordination bond.

[0040] The recognition element polypeptides may be deposited on the electrode surface at any desired density, for example, in the range of $1x10^{9}$ to $1x10^{12}$ molecules/cm^{2}.

[0041] **Linkers.** In some implementations, a short linker is present between the electrode surface and the recognition element polypeptide. In one embodiment, the linker comprises a nucleic acid sequence, for example a DNA or RNA sequence. Previously described electrochemical sensors, for example as in White et al., 2011, "Wash-free, Electrochemical Platform for the Quantitative, Multiplexed Detection of Specific Antibodies," Anal. Chem. 2012, 84, 1098–1103) have used polypeptide recognition elements tethered to double stranded nucleic acids, for example 10-30 nucleotide sequences of DNA, PNA, or RNA, wherein one strand is functionalized with a redox reporter. In such sensors, binding of the target to the peptide moiety changes the conformational flexibility of the reporter-labeled nucleic acid linker, resulting in a signal change. Unlike such previous sensors, the sensors of the present invention do not require the additional nucleic acid elements and do not rely on conformational change of the reporter element to generate signal.

[0042] **Redox Reporters and Conjugation to the Polypeptide**. Each

recognition element polypeptide is functionalized with one or more redox reporters. The redox reporter is any composition of matter that interacts with the selected electrode material creating a faradic current. Exemplary redox species include methylene blue, ferrocene, viologen, anthraquinone or any other quinones, daunomycin, organo-metallic redox reporters, for example porphyrin complexes or crown ether cycles or linear ethers, ruthenium, bis-pyridine, tris-pyridine, bis-imidizole, cytochrome c, plastocyanin, and ethylenetetracetic acid.

[0043] The redox reporter may be attached to the recognition element by any appropriate chemistry. In one embodiment, a particular conjugation chemistry is selected for conjugating the redox reporter to the polypeptide at a "compatible residue," i.e. a residue suitable for conjugation of a redox reporter using a selected linkage chemistry. Any system comprising appropriate chemistry and compatible residue may be utilized for attachment of the redox reporter to the recognition element polypeptide.

[0044] In one embodiment, the redox reporter is conjugated to a cysteine residue within the recognition element polypeptide. In one embodiment, the redox reporter is functionalized with maleimide and is conjugated to a cysteine residue in the recognition element polypeptide by thio-maliemide linkage. For example, in one embodiment, the redox reporter is a maleimide-functionalized methylene blue. In alternative embodiments, the redox reporter is conjugated to cysteines using iodacetamides, alkynes, or other conjugation reagents known in the art.

[0045] In another embodiment, the redox reporter may be conjugated to the polypeptide at a lysine residue, using linkage chemistries such as N-hydroxysuccinimidyl ester, isocyanate, or benzoyl fluoride conjugation, as known in the art.

[0046] In another embodiment, conjugation of the redox reporter to the polypeptide is achieved by incorporation of one or more non-natural amino acids in the recognition element, which such non-natural amino acids are suitable for conjugation chemistries, e.g., click chemistry. For example, in one embodiment, azidohomoalanine

or propargyl-derivatized lysine are incorporated into the polypeptide at selected sites for conjugation to the redox reporter using copper-catalyzed azide-alkyne cycloaddition.

[0047] **Redox Reporter Placement**. The detection capability of the sensors of the invention is based on steric interference, such that binding of the target species will impede electron transfer between the redox reporter and the electrode. Within the limits of the sensor design, the detected change in signal will be monotonically related to the concentration of the target species in the sample.

[0048] Advantageously, the steric-interference based sensors of the invention are highly sensitive, and often the binding of target to the recognition element creates sufficient steric interference to create a measurable signal change regardless of the placement of the one or more redox reporters on the recognition element polypeptide. Accordingly, in one embodiment, random residues of the recognition element polypeptide may be modified with redox reporter. For example, if multiple compatible residues (e.g., lysine or cysteine) are present in the polypeptide sequence, these may be functionalized with redox reporter in a reaction that randomly distributes one or more redox reporter moieties among the compatible residues. For example, in one embodiment, random lysines throughout the polypeptide are modified. For example, in one embodiment, epsilon amine group of random lysines may be modified methlyene blue NHS-ester.

[0049] While random labeling with redox species is effective, the inventors of the present disclosure have determined that signal gain is maximized when the redox reporter is placed in proximity to the target-binding site present on the recognition element polypeptide. Accordingly, in one implementation, redox reporters are placed in proximity to the target-binding site. In one embodiment, a modifiable residue in proximity to the binding site is selected for modification with the redox reporter and an appropriate chemistry for conjugation is utilized. In another embodiment, site-directed mutagenesis is used to create a compatible residue in proximity to the binding site. The reporter moiety may be placed in proximity to the binding site, for example, being within

1-30 angstroms from the binding site.

[0050] **Sensor Fabrication**. The sensors of the invention may be fabricated based on methods of fabricating EDNA sensors, as known in the art, with appropriate modification for the attachment of recognition element polypeptides to the electrode. For example, sensors may be prepared by analogy to previously described sensors, for example as described in: Xiao, Y., Rowe, A. A., and Plaxco, K. W. (2007) Electrochemical detection of parts per billion lead via an electrode-bound DNAzyme assembly. *J. Am. Chem. Soc. 129,* 262–263; United States Patent Number 8,003,374 by Heeger, Fan, and Plaxco; Ferguson et al., "Real-time, aptamer-based tracking of circulating therapeutic agents in living animals," *Sci Transl Med*. 2013 November 27; 5(213): 213ra165; and Swensen et al., "Continuous, Real-Time Monitoring of Cocaine in Undiluted Blood Serum via a Microfluidic, Electrochemical Aptamer-Based Sensor," *J Am Chem Soc*. 2009 April 1; 131(12): 4262–4266.

[0051] **Sensor Assemblies**. The electrochemical sensing elements of the invention may be configured in various assemblies to make fully functional sensing systems. As used herein, a sensor assembly will comprise a collection of elements that may operate together to perform various operations of the sensing process. For example, in one embodiment, the invention comprises a sensor assembly comprising a sensing element and a reference electrode, for example an Ag/AgCl electrode, or other reference electrode known in the art. The sensing assemblies of the invention may further comprise an auxiliary or counter electrode, for example, a platinum auxiliary electrode.

[0052] The sensor assemblies of the invention may be configured in a three-electrode cell system. The three-electrode cell system will comprise one or more sensing, reference, and auxiliary electrodes, appropriately configured for performing electrochemical interrogation measurements. The three-electrode cell system may comprise a mixing chamber or other vessel wherein the electrodes are present and are contacted with the sample.

[0053] The sensor assemblies of the invention may further comprise or be in connection with appropriate electronic components for performing electrochemical measurements. The electronic components may comprise two or more devices in electrical and/or network connection with one another, or may comprise a single integrated device.

[0054] Electronic components may include potientiostats or other voltage sources and voltage controllers. The system may further comprise appropriate circuitry for reading sensor outputs, and storing such outputs or routing the outputs to other devices. The systems of the invention may further comprise data processing means, for example, a general-purpose computer or other data processor capable of carrying out the various calculations utilized in the methods of the invention. The scope of the invention further encompasses non-transitory computer-readable recording media having stored thereon an encoding program that causes a computer to execute a process, the process comprising one or more data processing calculations for readout and interpretation of signals from a sensing element.

[0055] In some embodiments, the sensor assembly comprises a tabletop lab apparatus. In other embodiments, the sensor assembly comprises a hand-held device. In other embodiments, the sensor assembly comprises a microfluidic biochip.

[0056] In one embodiment, the sensor assembly of the invention is configured as an *in vivo* sensor. An "*in vivo*" sensor means a sensor configured to sample fluids within the body of a living organism. When an *in vivo* sensor is in use, the sensing element is inserted, implanted, or otherwise placed within the body of a living organism such that the sensing element is exposed to *in-vivo* fluids, e.g., blood. For *in vivo* measurements, a sensing assembly comprising a thin wire configuration is advantageous, as the low-profile wire may be inserted into veins, arteries, tissue or organs and will minimally impede blood flow in blood vessels or will cause minimal damage in the sampled area. For example, a wire having a diameter of 1-500 μm, for example, 100 μm, may be used. In one embodiment, the sensing assemblies are housed in a needle, catheter, or cannula that may be inserted into a vein, blood vessel,

organ, tissue, or interstitial space in order to place the sensor in the target environment. The needle, catheter, or cannula may be porous, comprising a plurality of holes or channels distal to the tip in order to allow the flow of blood over the sensor assembly.

[0057] **Methods**. The scope of the invention encompasses methods of using the afore-described sensors for the detection of target species in a sample. The basic method of the invention encompasses the steps of:

exposing a sensing element of the invention to a sample;

wherein the sensing element comprises an electrode functionalized with a plurality of redox-label modified recognition element polypeptides capable of selectively binding a target species, such that binding of the target species alters the flow of electrons between the electrode and the redox reporter by steric interference;

wherein the sensing element is in connection with and/or proximity to elements that enable interrogation of faradic current between the redox reporter-modified recognition probes and the electrode;

interrogating the faradic current between the redox reporter-modified recognition probes and the electrode; and

determining the presence or abundance of the target species in the sample by the observed faradic current between the redox reporter-modified recognition probes and the electrode.

[0058] The sample may be any sample. The sample may comprise blood, serum, saliva, urine, interstitial fluid, spinal fluid, cerebral fluid, tissue exudates, macerated tissue samples, cell solutions, intracellular compartments, water, food, groundwater, or other biological and environmental samples. In the case of in-vivo measurements, the sample may be flowing whole blood, interstitial fluid, or other biological material. In one embodiment, the sample comprises the surrounding environment in which the sensing element is deployed, e.g., in water, in soil, in vivo, etc. Samples may be unaltered or may be pretreated prior to analysis, for example being filtered, diluted, concentrated, buffered, or otherwise treated. Advantageously, the sensors of the invention are

reagentless and have the capacity to operate in complex samples such as whole blood, greatly simplifying the sample preparation process.

[0059] The interrogation of faradic current may be accomplished by any means known in the art, for example by cyclic voltammetry, differential pulse voltammetry, alternating current voltammetry, square wave voltammetry, potentiometry or chronoamperometry. In one embodiment, the use of kinetic differential measurement techniques, as known in the art can be employed to improve signal to noise ratio.

[0060] Determination of target concentration may be accomplished by comparison to a standard curve or like reference, generated for the particular sensor being used, or for like sensors of a class or common manufacturing batch.

[0061] The sensors of the invention may be operated for extended periods of time, e.g., days, weeks, or months, for the continuous measurement of target species, for example, in the body of an organism (e.g., a human or test animal) or in the environment.

[0062] The sensors of the invention may also be used in ex-vivo applications. In one embodiment, the method of the invention comprises the steps of withdrawing a sample from a living organism, exposing a sensor of the invention that is directed to detection of a target species to the sample, and measuring the concentration of the target species in the sample. In one embodiment, the sample fluid is withdrawn continuously from the living organism and target species concentration is measured on a prolonged basis. In one embodiment, a single sample is analyzed. In one embodiment, the sample is blood. In one embodiment, the sensor is housed in a wearable or otherwise portable device.

[0063] In one embodiment, the sensors of the invention are employed in point of care testing methods. In such an application, a sample is withdrawn from an animal, subject, or patient and the concentration of a target species is measured using a sensor of the invention. For example, in one embodiment, the sample is a blood sample, for

example, a pin-prick or finger-prick blood sample, for example, a self-withdrawn pin-prick or finger-prick blood sample, or a urine or saliva sample. The sensors of the invention advantageously enable the immediate testing of small samples, obviating the need for processing blood or other samples prior to analysis.

[0064] The sensors of the invention may be utilized for various biological or environmental measurements. In one embodiment, the recognition element polypeptide selectively binds a drug. The sensors of the invention may be utilized to determine the concentration of a target drug in the body, for example in an in-vivo real time monitoring system. In one embodiment, the methods of the invention are performed for the detection of a drug. The target species may comprise a drug having significant side effects, such as a chemotherapeutic drug, or a drug having a narrow therapeutic index, wherein accurate measurement of blood levels would allow for safe dosing with minimal side effects. In one embodiment, the method of the invention is performed in the operation of a feedback controlled dosing system, as known in the art.

[0065] In one embodiment, the methods of the invention are performed for the detection of metabolite, hormone or other biomarker indicative of organ function, health, or disease status, for example, glucose, creatinine, cortisol, or A and B-type natriuretic peptides.

[0066] In one embodiment, the recognition element polypeptide selectively binds a biomarker of a condition, a biological process, or disease state and the methods of the invention are performed for the assessment of such condition, biological process, or disease state.

[0067] In one embodiment, the recognition element polypeptide comprises a receptor protein and the methods of the invention are performed for the detection of an activator, modulator, or repressor species.

[0068] In one embodiment, the recognition element polypeptide comprises an epitope to which antibodies, activated T-cells, or other immune system elements may bind and the methods of the invention are performed for the detection of such immune

system elements. Such sensing elements may be used, for example, to detect and quantify antibody titers against a selected target.

[0069] In one embodiment, the recognition element polypeptide selectively binds a pathogenic cell or a factor secreted by a pathogenic cell and the methods of the invention are performed for the diagnosis of infection or disease.

[0070] In one embodiment, the methods of the invention are performed for the detection of a pollutant or contaminant and the sample comprises an environmental sample or a food sample.

[0071] **EXAMPLES.** Among quantitative methods for measuring the levels of specific, diagnostically relevant proteins, only fluorescence polarization (also known as fluorescence anisotropy) has seen wide use in point-of-care applications. This approach, which reports on the presence of a specific protein-protein complex via binding-induced changes in the tumbling of an attached fluorophore, does not require washing to remove unbound reagents, rendering it one of the more convenient methods for quantifying the levels of specific proteins in clinical samples. Several limitations, however, significantly reduce its utility at the point of care. For example, when challenged with authentic clinical samples the approach requires considerable signal averaging and careful background subtraction. In part, this is due to its modest signal gain: the intensity difference between the two polarizations is typically of order ~15% (i.e., 150 millipolarization units) for an antibody-antigen complex, which must be measured against background polarizations of similar magnitude. Fluorescence polarization also requires fairly large sample volumes, necessitating venous blood draws that further reduce its utility at the point of care. Finally, fluorescence polarization is not easily multiplexed, rendering it ill-suited for the simultaneous monitoring of, for example, multiple antibodies diagnostic of a single pathogen or simultaneously monitoring for antibodies against multiple pathogens.

[0072] In response to the above arguments significant effort has gone into the development electrochemical sensing platforms that attempt to capture the generality of fluorescence polarization while avoiding its limitations. Electrochemical impedance spectroscopy (EIS), for example, employs an electrode-bound receptor such that the steric or electrostatic "bulk" of the target molecule impedes the ability of an added redox moiety (e.g., ferrpcyanide) to approach and generate a current. EIS suffers, however, from overwhelming background effects when challenged with realistically complex samples due its extreme sensitivity to non-specific adsorption. In previous work, an electrochemical sensing approach has been pursued, utilizing a double-stranded nucleic acid "scaffold" modified on one end to present both a protein-recognizing polypeptide or small molecule and a redox reporter and covalently attached to gold electrode via a flexible linker via the other. The binding of the sensor's target to this recognition element reduces the efficiency with which the attached redox reporter approaches the electrode (analogous to the change in tumbling seen in fluorescence polarization), producing an easily measured change in electron transfer efficiency (analogous to a change in fluorescence polarization). This strategy offers several potential advantages over other methods for detecting protein-polypeptide and protein-small-molecule interactions, including the reduced complexity associated with its reagentless, single-step, wash-free format and better performance in complex samples, such as undiluted blood serum and crude soil extracts. Here, the previous approach is extended by utilizing sensors that, rather than using a double-stranded DNA scaffold and a relatively low molecular weight recognition element (e.g., a polypeptide), instead employ full-length proteins as both the recognition element (receptor) and the scaffold, expanding the range of analytes that the approach can be used to detect.

[0073] To demonstrate the concept, CheY, a response regulator protein from the *E. coli* chemotaxis signal transduction system was selected. The structure and folding of CheY and its binding to its protein and peptide targets CheA-P2 and $FliM_{16}$ have seen extensive prior study, rendering them a convenient model system. To convert CheY into a single-step electrochemical sensor a family of CheY variants was generated containing a carboxy-terminal hexa-His-tag with each exposing a single cysteine side chain for conjugation to a maleimide-functionalized methylene blue. To generate the

sensors, copper complexation with the His-tag was used to attach each modified protein onto a gold electrode coated with an alkane thiol self-assembled monolayer doped with a small fraction of copper chelating nitrilotriacetic acid (NTA) head groups, an approach that has seen significant prior exploration as a means of site-specifically attaching proteins to surfaces. Copper, rather than nickel, was used as histidine-copper complexes are less kinetically labile.

[0074] The sensor architecture responds quantitatively when challenged with the appropriate target molecule. In the absence of either of CheY's binding partners the redox reporter is relatively free to collide with the electrode surface, producing a large faradaic current at the redox potential expected for methylene blue when the system is interrogated using cyclic voltammetry. This peak is reduced in the presence of the protein's binding partners. For example, for a CheY modified with the redox reporter at position 97, the current falls 22% upon the addition 100 μM of the ligand CheA-P2, a 74-residue protein that is part of the bacterial chemotaxis system, with a time constant of $2.8\pm0.7\ min^{-1}$. Upon titration, the observed signal change increases monotonically with increasing ligand concentration until it approaches saturation at a change of 24%. The resultant binding curve is well fitted with a Langmuir isotherm, producing a dissociation constant of 14 ± 4 μM, which is within error of the value previously reported for this interaction for the proteins when free in solution. As expected for electrochemical sensors of this class, the sensor also performs well when challenged in clinically realistic samples. For example, the sensor's gain is only slightly reduced, to 17% (reporter at position 97), when it is instead challenged in 20% blood serum.

[0075] The signal gain (the relative signal change seen upon the addition of saturating target) seen for this sensing architecture depends on both the attachment-position of the methylene blue and the structure of the target. To illustrate the former, seven sensors were fabricated differing only in the residue on which the methylene blue was attached using the variants M17C, E37C, T71C, A80C, G89C, K91C, K97C and E117. The signal gain observed for these when CheA-P2 was employed as the target ranged from 4% to 30%, with the later value larger than the signal change typically seen in fluorescence polarization assays. The largest gain is seen when the redox-reporter is

placed closest to the CheA-binding site (position 97), demonstrating that signal change arises due to steric blocking of the redox reporter by the target protein. Consistent with this, the gain of the sensor is abated when the reporter is positioned farther from the binding site (e.g., at E37C or A80C). The sensor's ability to detect a second naturally occurring binding CheY binding partner was also tested, the 16-residue peptide $FliM_{16}$, and behavior similar to that for the detection of CheA-P2 was observed. Specifically, a sensor modified with methylene blue near the binding site (at residue 91) exhibits the greatest gain (14%). The signal gain observed upon $FliM_{16}$ binding, however, is generally less than that observed upon CheA-P2 binding. This demonstrates the consequence of the larger bulk of CheA-P2, which hinders the approach of the reporter to the electrode.

[0076] This new sensor architecture readily detects changes in binding affinity associated with the phosphorylation of CheY. Phosphorylation induces allosteric communication between the phosphorylation site (D57) and the target-binding site, which in turn facilitates CheY's dissociation from CheA while strengthening its interactions with the flagellar motor switch protein FliM. Consistent with this, significant decreases and increases in affinity for CheA-P2 and $FliM_{16}$, respectively, were observed upon phosphorylation of the surface-bound CheY. Specifically, the phosphorylation of surface-bound CheY enhances its affinity for $FliM_{16}$ by a factor of ca. 15, which is consistent with the results of prior solution-phase studies and what has been observed via tryptophan fluorescence quenching experiments in free solution. The phosphoryation of surface-bound CheY likewise reduces its affinity for CheA-P2 by a factor of ca. 5. Also, introduction of the D57A mutation to the CheY single-cysteine variants disabled the phosphorylation of CheY. Consistent with expectations, the affinity of this variant is not altered by the presence of the phosphorylating agent acetyl phosphate.

[0077] The detection of specific antibodies via the inclusion of the relevant antigen as the recognition element was also tested. Green fluorescent protein (GFP) modified with methylene blue at random lysine epsilon amino groups or cysteine thiols was tested as a receptor for the detection of GFP-binding antibodies. Using polyclonal anti-GFP antibodies as the target, Langmuir isotherm binding was observed, with a

dissociation constant 53±8 ng/ml, which is the equivalent of ~0.3 nM for the mixed antibody concentration. As a second test of the ability to detect specific antibodies, a disease-related, clinically relevant antigen-antibody pair, the hepatitis B surface antigen (HBsAg) and anti-hepatitis antibodies (HBsAb) were tested. Using HBsAg modified with methylene blue at random lysine epsilon amino groups or at cysteine thiols as the recognition element, the antibody can be detected with a detection limit of a few nanograms per milliliter, a value that compares well with commercial approaches to this same end. A test of this sensor in 20% blood serum, a proxy for more authentic clinical conditions, renders its detection limit effectively unchanged.

[0078] Here are described a new class of reagentless, single-step sensors that are the electrochemical analog to optical fluorescence polarization assays. As proof of principle the approach was used to monitor the interaction of CheY with its two binding partners, the P2 domain of CheA and the 16-residue peptide $FliM_{16}$ and for the detection of both anti-GFP and anti-HBsAg antibodies. In all cases, binding induced signal changes were observed as large or larger than those typically seen in fluorescence polarization without the need for light sources, optics, or extensive signal averaging.

[0079] This new protein-based, electrochemical sensing platform provides an alternative means to fluorescence polarization for probing protein-macromolecular interactions and thus provides the art with a promising tool for point-of-care clinical applications. Fluorescence polarization assays have seen widespread adoption at the point of care for the measurement of markers of heart attacks and drug overdose despite producing signal changes of only 10-20%. The sensors of the invention produce similar signal gains and are similarly label-free, providing a system that is faster, simpler, and, less costly than ELISAs and western blots. In addition, the transition from optical to electrochemical read-outs offers potential advantages, including its relatively inexpensive supporting electronics, its ability to perform well in relatively high concentrations of blood serum, and the ready multiplexing of electrochemical approaches.

[0080] Materials and Methods. The receptor proteins used were obtained as follows. The gene encoding wild-type *E. coli* CheY (residues 1–129) was cloned into pET28a at the NcoI and XhoI sites in frame with the carboxy-terminal hexahistidine tag. Single-cysteine variants (M17C, E37C, T71C, A80C, G89C, K91C, K97C and E117) were generated with a site-directed mutagenesis kit and transformed into *E. coli* BL21(DE3). The mutagenesis results were checked via sequencing. Transformants were grown at 37°C in lysogeny broth (LB) medium and induced at OD_{600} = 0.4 by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture was harvested after 3 hr, resuspended in 50 mM sodium phosphate, 10 mM imidazole, 300 mM sodium chloride, pH 8.0 buffer and lysed by French pressure cell press. Cell debris was removed by centrifugation 30,000 g for 30 min before application of the supernatant to an immobilized metal ion (nickel) affinity chromatography column. The column was washed with 50 mM sodium phosphate, 300 mM sodium chloride and 35 mM imidazole, pH 8. His-tagged CheY was eluted with 150 mM imidazole in the same buffer. The collected fractions were dialyzed into 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.9 and concentrated to ~1 mM (CheY D57A variants were concentrated to ~ 0.5 mM). Purity of all variants was >95% as determined by SDS-PAGE electrophoresis. His-tagged emerald green fluorescent protein (GFP) was expressed from pRSET-EmGFP expression plasmid in *E. coli* BL21 (DE3) and purified via nickel ion affinity chromatography. The resin was washed with 8 column volumes of 50 mM sodium phosphate, 300 mM sodium chloride, and 20 mM imidazole, pH 8.0. The His-tagged GFP was then eluted with 1 column volume of 250 mM imidazole in the same buffer. GFP containing fractions were dialyzed and concentrated. Purity was >95% as determined by SDS-PAGE electrophoresis.

[0081] The target proteins and peptides were obtained as follows. The CheA P2 domain (residues 156-229) was expressed from the pTM22 plasmid in *E. coli* strain K38 and purified by ion-exchange chromatography and size-exclusion fast performance liquid chromatography. Purity was >95% as determined by SDS-PAGE electrophoresis.

[0082] The maleimide-functionalized methylene blue used as the redox reporter and was synthesized starting with 5 mg monocarboxy-methylene blue NHS ester

dissolved in 150 µL DMSO. 15 mg N-(2-aminoethyl) maleimide trifluoroacetate salt and 10 µL triethylamine were added followed by 12 hr stirring at room temperature. 50 µL 0.5 M sodium bicarbonate solution was added to this crude reaction mixture and incubated for 2 hr. The final mixture was dried under reduced pressure and purified using silica thin layer chromatography ($CHCl_3$: methanol 10:1).

[0083] The relevant receptor protein was modified with a methylene-blue redox reporter at either cysteine or lysine. For modification at cysteine, either single-cysteine variants of CheY or the wild-type cysteines of GFP and HBsAg were used. These were first reduced by treatment with 5 mM dithiothreitol for 2 hr prior to removal using a spin column) immediately followed by the addition of maleimide-modified methlyene blue (as 1-2% v/v solution in DMSO) at a 10:1 reagent-to-protein molar ratio. The CheY D57A variants were labeled with MB2 maleimide-modified methylene blue. The reaction mix was incubated at room temperature for 12 hr, and a spin column was then used to remove unreacted methylene blue and exchange the sample into 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.9. For other experiments, the receptor protein on the epsilon amine group of random lysines was modified. Wild-type protein was mixed with methlyene blue NHS-ester (as 1-2% v/v solution in DMSO) at a 2:1 reagent-to-protein molar ratio in 0.5 M sodium bicarbonate solution (pH 8.5) for 4 hr in dark at room temperature, and then a spin column was used to remove unattached methylene blue and exchange the sample into 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.9.

[0084] Sensors were prepared by analogy to previously described E-DNA sensors. In brief, prior to sensor fabrication, gold disk electrodes (2.0 mm diameter,) were cleaned both mechanically (by successively polishing with 1 µm diamond and 0.05 µm aluminum oxide slurries) and electrochemically (through successive scans in 0.5 M sulfuric acid and 0.1 M sulfuric acid 0.01M KCl). Proteins were grafted onto these electrodes using Cu-NTA/His-tag complexation as follows. NTA-thiol (N-[Nα,Nα-bis(carboxymethyl)-lysine]-12-mercaptodocanamide) was dissolved in 3 mM 6-mercapto-1-hexanol methanol solution at 10 µM (NTA-thiol/6-mercapto-1-hexanol ratio is 1:300). Freshly cleaned electrodes were incubated in this solution for 1 hr at room

temperature, rinsed with methanol, and then incubated in 10 mM 6-mercapto-1-hexanol in methanol overnight (~16 hr) at 4°C to form a continuous, mixed, self-assembled monolayer on the gold electrode surface. The NTA-modified electrodes were then rinsed with methanol and incubated with 100 µM copper sulfate in deionized water for 20 min. The electrodes were then incubated with the methylene blue (MB) modified protein for 30 min at room temperature, using 2 to 10 µM CheY (based on concentration determined by ultraviolet–visible spectroscopy immediately prior to modification with methylene blue) and GFP or 0.2 mg/ml HBsAg. The electrodes were then washed with 150 mM imidazole in 50 mM sodium phosphate, 500 mM sodium chloride, pH 7.0. The CheY coated electrode was rinsed with 50 mM sodium phosphate, 100 mM sodium chloride buffer, and incubated in the buffer for 30 min immediately prior to use. The GFP and HBsAg coated electrodes were washed with 2% bovine serum albumin (BSA) and 0.05% Tween 20.

[0085] Sensors were interrogated using either cyclic voltammetry (CV) with 10 V/s scan rate (CheY experiments) or square wave voltammetry (SWV) with a 50 mV amplitude signal at a frequency of 100 Hz (GFP and HBsAg experiments). Experiments performed in buffer employed 50 mM sodium phosphate, 100 mM NaCl, 20 mM imidazole. Experiments employing serum were performed in 50 mM sodium phosphate, 100 mM NaCl with 20% bovine serum. Signal gain was computed by the relative change in CV peak or SWV peak currents with respect to background current (CV peak or SWV peak current in the absence of target).

[0086] CheY phosphorylation was carried out using 10 mM acetyl phosphate in 50 mM sodium phosphate, 5 mM $MgCl_2$, 100 mM NaCl. The freshly prepared CheY electrodes were incubated in 10 mM acetyl phosphate in buffer for 30 min before test. CheY was phosphorylated by acetyl phosphate on the surface immediately prior to measuring binding.

[0087] The binding affinity of several CheY variants (wild type, E37C, K91C and E117C) was measured for the $FliM_{16}$ peptide using tryptophan fluorescence . In brief, fluorescence spectra were recorded on fluorimeter at 20ºC. 10 µM CheY variants were

titrated in solution of 50 mM sodium phosphate (pH 7.9) 150 mM NaCl with aliquots of 5 mM $FliM_{16}$ peptide in a 100 µL sub-micro quartz fluorometer cell. The excitation wavelength was 285 nm and emission was recorded at 340 nm. To test the binding affinity of phosphorylated CheY variants, 20 mM acetyl phosphate and 20 mM magnesium chloride were added to the sample of CheY in otherwise identical buffer.

[0088] All patents, patent applications, and publications cited in this specification are herein incorporated by reference to the same extent as if each independent patent application, or publication was specifically and individually indicated to be incorporated by reference. The disclosed embodiments are presented for purposes of illustration and not limitation. While the invention has been described with reference to the described embodiments thereof, it will be appreciated by those of skill in the art that modifications can be made to the structure and elements of the invention without departing from the spirit and scope of the invention as a whole.

CLAIMS

What is claimed is:

Claim 1. A sensing element for measuring the concentration of a target species in a sample, comprising

an electrode; and

a plurality of recognition element polypeptides attached to the electrode;

wherein each recognition element polypeptide is capable of selectively binding one or more target species;

wherein each recognition element polypeptide is functionalized with one or more redox reporters, wherein charge transfer occurs between the one or more redox reporters and the electrode; and

wherein binding of the target species to the recognition element causes a detectable change in the charge transfer between the the one or more redox reporters and the electrode.

Claim 2. The sensing element of Claim 1,

wherein no substantial change in the conformation of the recognition element polypeptides occurs upon target binding.

Claim 3. The sensing element of Claim 1, wherein

the recognition probe polypeptides are capable of selectively binding a target species selected from the group consisting of : a small molecule drug, a metabolite, a hormone, a peptide, a protein, a carbohydrate, a nucleic acid, a

lipid, growth factor, neurotransmitter, a pathogen; a pathogen-induced or pathogen-derived factor, a nutrient, a contaminant, and a pollutant.

Claim 4. The sensing element of Claim 1, wherein

each recognition probe polypeptide comprises a whole protein, protein fragment, or polymeric protein.

Claim 5. The sensing element of Claim 1, wherein

each recognition probe polypeptide comprises an antibody.

Claim 6. The sensing element of Claim 1, wherein

each recognition probe polypeptide comprises the antigen-binding region of an antibody.

Claim 7. The sensing element of Claim 1, wherein

each recognition probe polypeptide comprises a receptor.

Claim 8. The sensing element of Claim 1, wherein

each recognition probe polypeptide comprises the ligand binding domain of a receptor.

Claim 9. wherein

a linker is present between the electrode and each recognition probe polypeptide.

Claim 10. The sensing element of Claim 1, wherein

the electrode comprises a material selected from the group consisting of gold, gold-coated metal or material, titanium, tungsten, platinum, carbon, aluminum, copper, palladium, mercury films, silver, oxide-coated metals, semiconductors, graphite, and carbon nanotubes.

Claim 11. The sensing element of Claim 1, wherein

the electrode is functionalized with nickel-nitrilotriacetic acid and the recognition probe polypeptides include His-tags.

Claim 12. The sensing element of Claim 1, wherein

the recognition probe polypeptides are bound to the electrode by click chemistry components.

Claim 13. The sensing element of Claim 1, wherein

the one or more redox reporters is selected from the group consisting of methylene blue, ferrocene, viologen, anthraquinone or any other quinones,

daunomycin, organo-metallic redox reporters, for example porphyrin complexes or crown ether cycles or linear ethers, ruthenium, bis-pyridine, tris-pyridine, bis-imidizole, cytochrome c, plastocyanin, and ethylenetetracetic acid.

Claim 14. The sensing element of Claim 1, wherein

the one or more redox reporters is conjugated to the recognition element polypeptide by a linkage chemistry selected from the group consisting of: thio-maliemide, iodacetamides, an alkyne, N-hydroxysuccinimidyl ester, isocyanate, benzoyl fluoride and copper-catalyzed azide-alkyne cycloaddition.

Claim 15. The sensing element of Claim 1, wherein

the one or more redox reporters is conjugated to a lysine or cysteine residue.

Claim 16. The sensing element of Claim 1, wherein

the redox reporter is conjugated to the recognition element polypeptide at a location within 1-30 ångstroms from the binding site of the target species.

Claim 17. A sensing assembly, comprising

the sensing element of any of Claims 1-18;

further comprising a reference electrode.

Claim 18. The sensing assembly of Claim 17;

further comprising electronic components for the interrogation of faradic current between the electrode and the redox reporters of the recognition probes.

Claim 19. A method of detecting the presence and/or abundance of a target species in a sample, comprising

exposing the sensing element of any of Claims 1-18 to a sample, wherein the sensing element comprises an electrode functionalized with a plurality of redox-label modified recognition element polypeptides capable of selectively binding a target species, such that binding of the target species alters the flow of electrons between the electrode and the redox reporter by steric interference;

wherein the sensing element is in connection with and/or proximity to elements that enable interrogation of faradic current between the redox reporter-modified recognition probes and the electrode;

interrogating the faradic current between the redox reporter-modified recognition probes and the electrode; and

determining the presence or abundance of the target species in the sample by the observed faradic current between the redox reporter-modified recognition probes and the electrode.

Claim 20. The method of Claim 19, wherein

the sample comprises a material selected from the group consisting of: blood, serum, saliva, urine, interstitial fluid, spinal fluid, cerebral fluid, tissue exudates, macerated tissue samples, cell solutions, intracellular compartments, water, food,

and groundwater.

Claim 21. The method of Claim 19, wherein

the interrogation of faradic current is accomplished using a methodology selected from the following: cyclic voltammetry, differential pulse voltammetry, alternating current voltammetry, square wave voltammetry, potentiometry and chronoamperometry.

Claim 22. The method of Claim 19, wherein

the sensing element is deployed for an extended time period for the continuous measurement of the target species concentration in the sample.

Claim 23. The method of Claim 19, wherein

the sensing element is deployed in-vivo.

Claim 24. The method of Claim 19, wherein

the sensing element is deployed in a point-of-care system.

Claim 25. The method of Claim 19, wherein

the target species is a drug.

Claim 26. The method of Claim 19, wherein

the target species is the ligand of a biological receptor

Claim 27. The method of Claim 19, wherein

the target species is an antibody.

Claim 28. The method of Claim 19, wherein

the target species is a contaminant or pollutant.

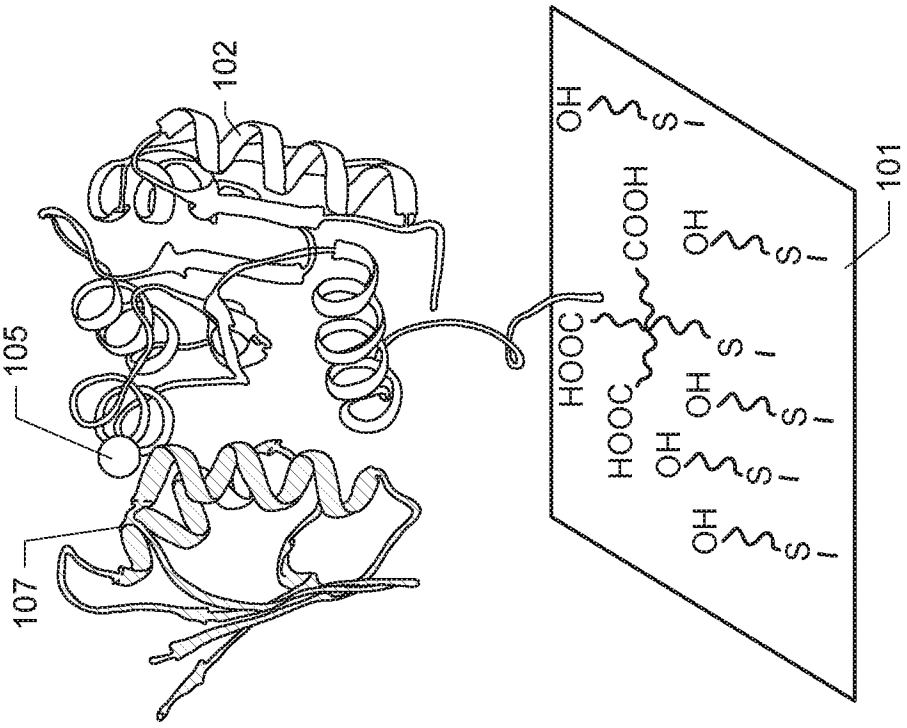

FIGURE 1B

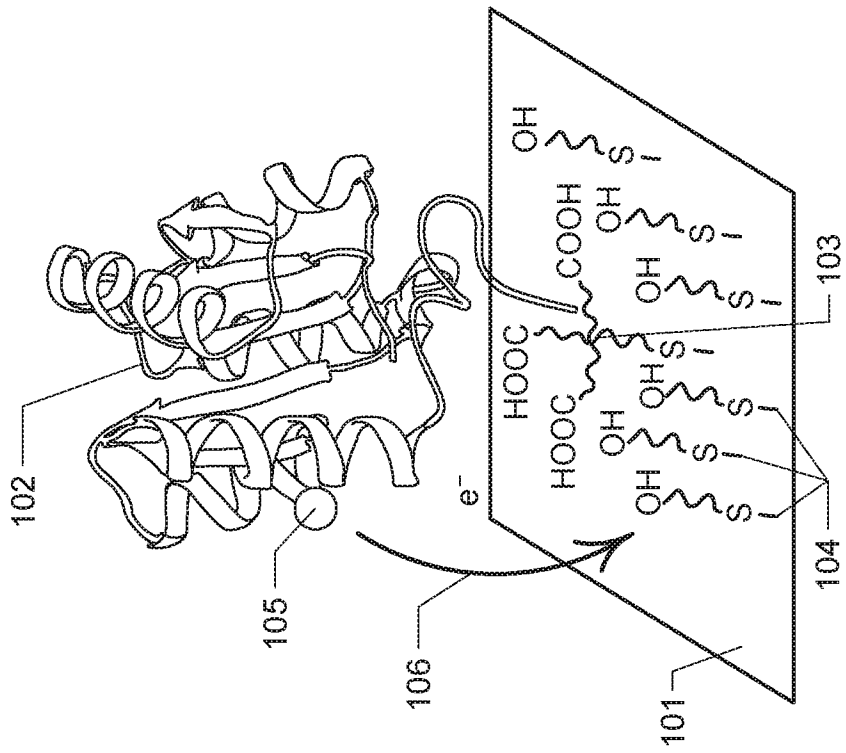

FIGURE 1A

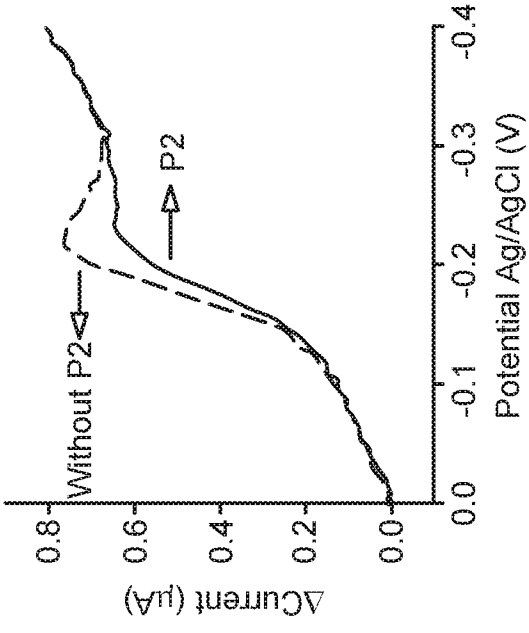

FIGURE 2A

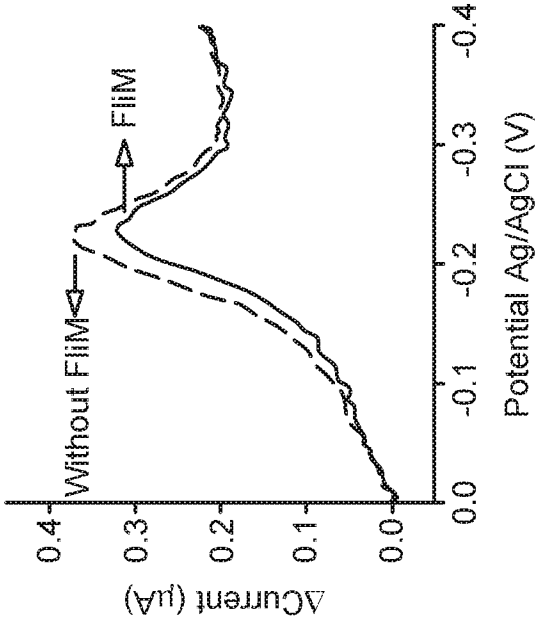

FIGURE 2B

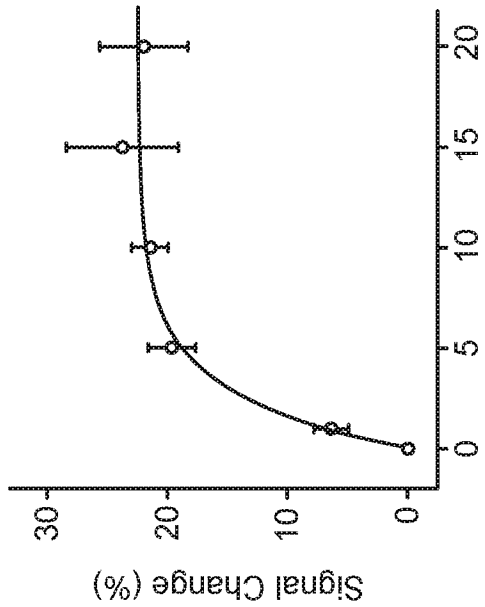

FIGURE 2C

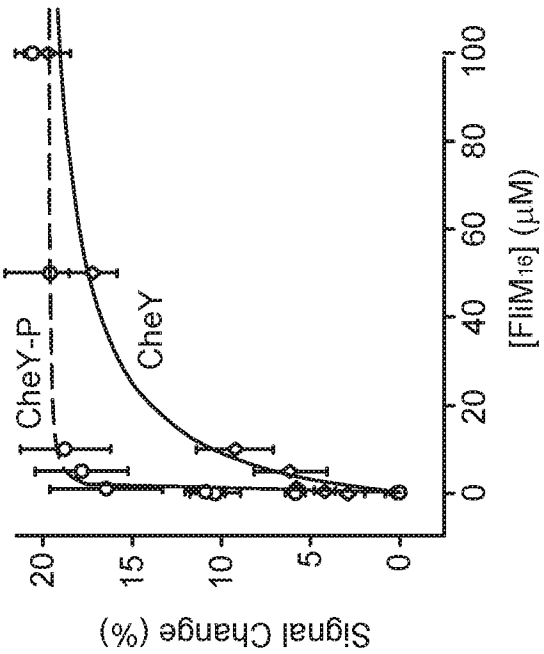

FIGURE 3B

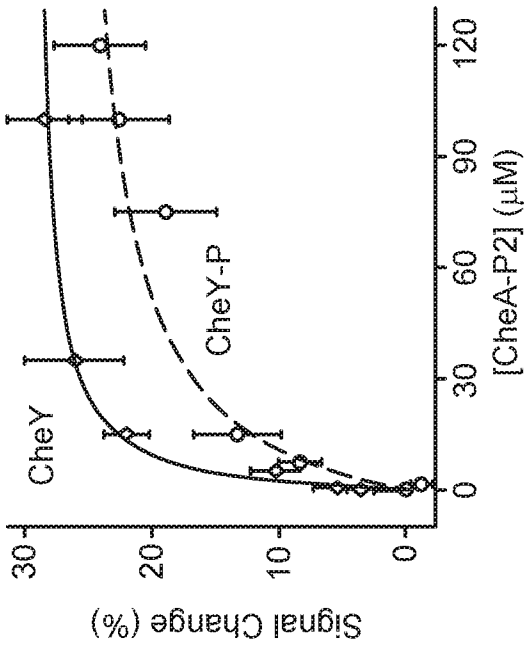

FIGURE 3A

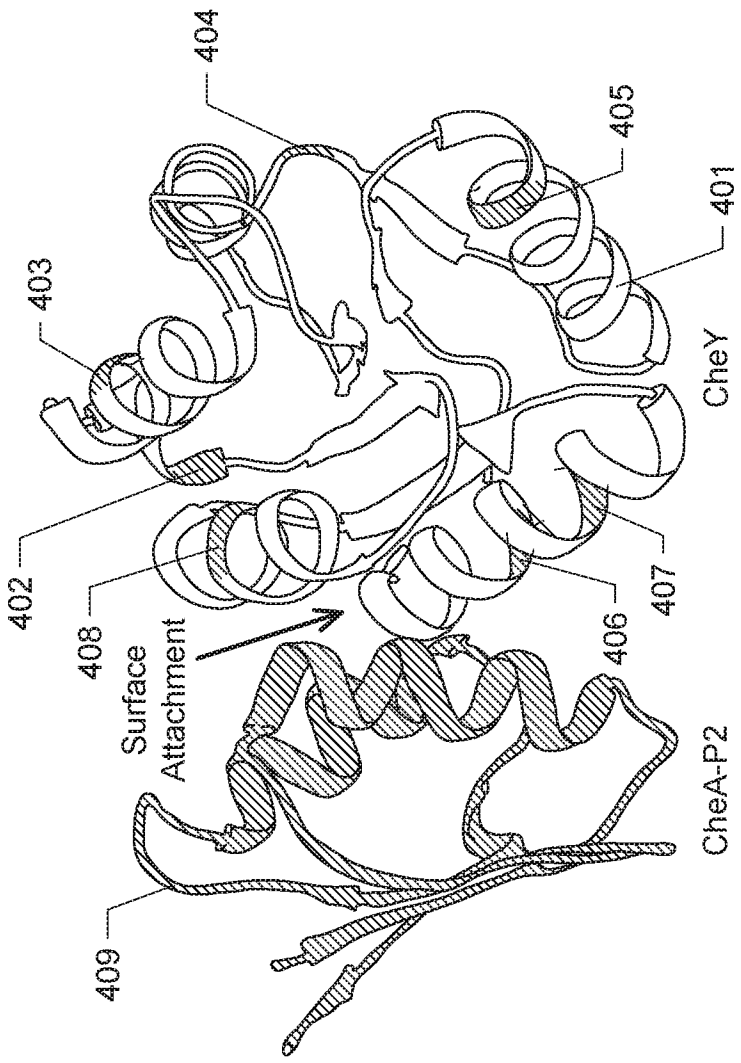
404
405
401
403
CheY
402
408
Surface
Attachment
406
407
CheA-P2
409

FIGURE 4

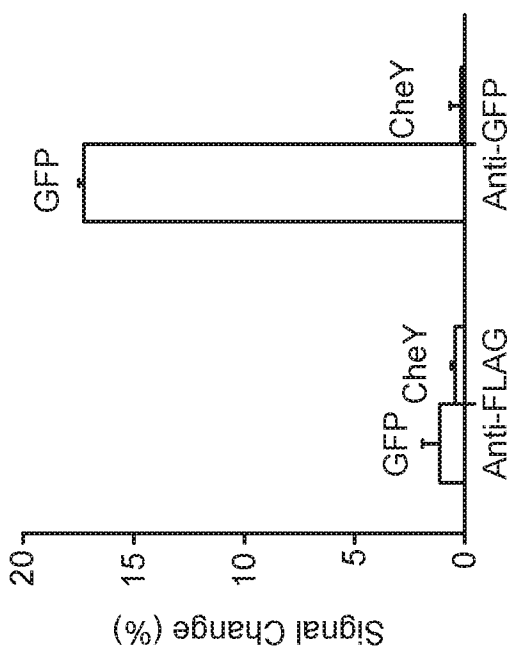

FIGURE 5A

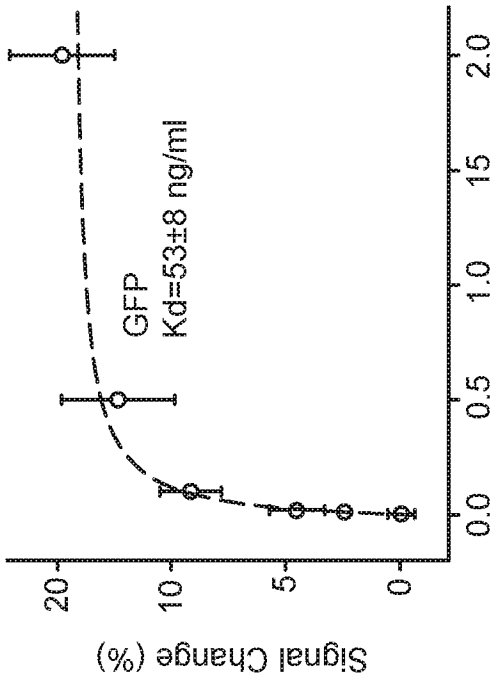

FIGURE 5B

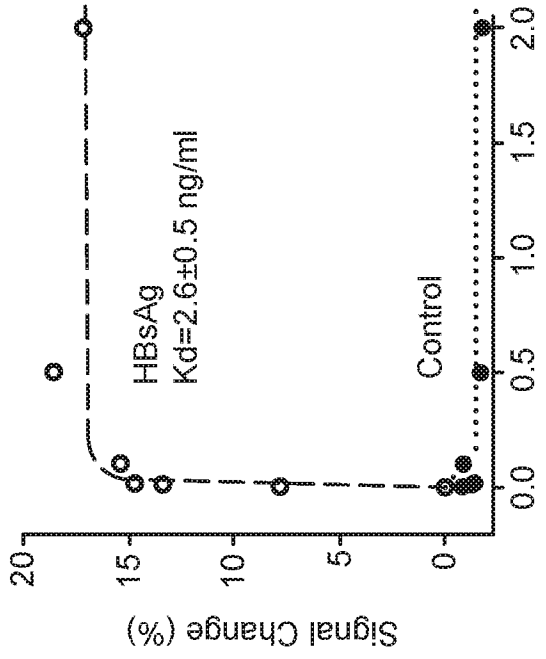

FIGURE 5C

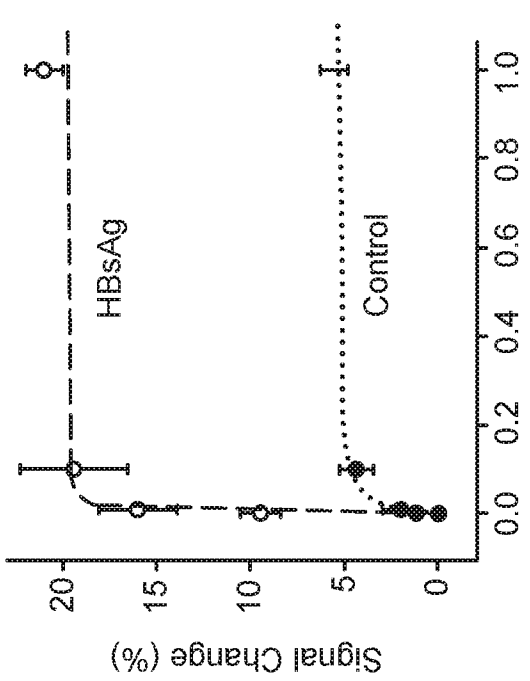

FIGURE 5D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/045810

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/68(2006.01)i, G01N 33/58(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N 33/68; C25B 11/04; C07H 21/02; B23P 11/00; G01N 27/327; C12Q 1/68; G01N 33/58

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: electrochemical biosensor, redox reporter, electrode, recognition probe polypeptide, steric interference

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011-0139636 A1 (LAI, REBECCA Y. et al.) 16 June 2011 See claims 5-18; paragraphs [0018], [0021]-[0026], [0031]-[0037], [0043]; and figures 7-8.	1,3-28
A		2
A	US 2011-0210017 A1 (LAI, REBECCA Y. et al.) 01 September 2011 See abstract; claims 1-12; paragraphs [0027], [0033]-[0046]; and figure 18.	1-28
A	MAHSHID, SAHAR SADAT et al., 'A highly selective electrochemical DNA-based sensor that employs steric hindrance effects to detect proteins directly in whole blood', Journal of the American Chemical Society, 2015, Vol.137, No.50, pp.15596-15599 See the whole document.	1-28
A	US 6221586 B1 (BARTON, JACQUELINE K. et al.) 24 April 2001 See the whole document.	1-28
A	KOUTSOUMPELI, ELENI et al., 'Probing molecular interactions with methylene blue derivatized self-assembled monolayers', Sensing and Bio-Sensing Research, 2015, Vol.6, internal pages 1-6 See the whole document.	1-28

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

☒ See patent family annex.

* Special categories of cited documents:
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Date of the actual completion of the international search	Date of mailing of the international search report
17 November 2017 (17.11.2017)	**17 November 2017 (17.11.2017)**

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea
Facsimile No. +82-42-481-8578

Authorized officer
KIM, Sun Hee
Telephone No. +82-42-481-5405

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2017/045810

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