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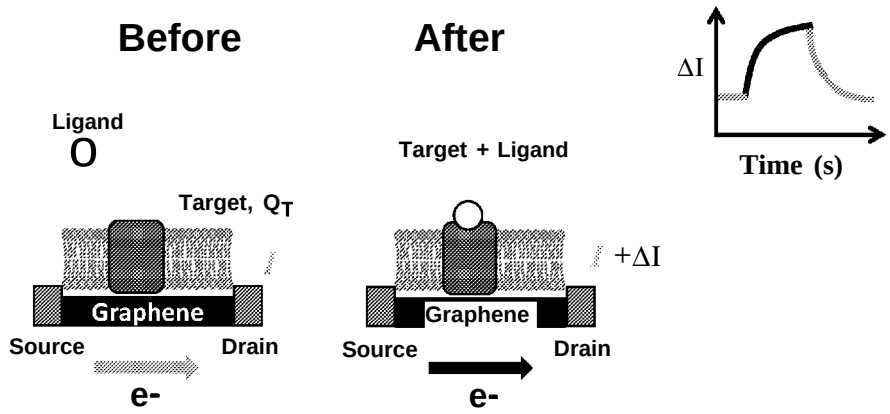

Figure 1

(57) Abstract: Disclosed herein are methods and devices for detection of biomolecules and chemical substances using a graphene bio-electronic sensor.

[Continued on next page]

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GRAPHENE BIO-ELECTRONIC SENSING TECHNOLOGY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/361,328, filed July 12, 2016, the disclosure of which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The invention relates to methods and devices for detecting biomolecules and chemical substances using graphene sensors comprising a functionalization layer interface on the graphene sensor surface.

BACKGROUND OF THE INVENTION

[0003] Graphene sensors have been used to detect biomolecules and chemical substances in academic research labs for the past several years; however, the hydrophobicity of graphene requires using conjugation chemistries that for the most part rely on pi-pi (stacking) non-covalent attachment directly to the hydrophobic graphene surface. This cumbersome approach prevents graphene sensors from leveraging decades of more mature surface chemistry methods, as well as integration of graphene sensors with supported lipid bilayers (SLBs). What is needed therefore, are graphene sensor devices compatible with mature covalent conjugation methods and supported lipid bilayers.

SUMMARY OF THE INVENTION

[0004] Various aspects disclosed herein may fulfill one or more of the above-mentioned needs. The systems and methods described herein each have several aspects, no single one of which is solely responsible for its desirable attributes. Without limiting the scope of this disclosure as expressed by the claims that follow, the more prominent features will now be discussed briefly. After considering this discussion, and particularly after reading the section entitled "Detailed Description," one will understand how the sample features described herein provide for improved systems and methods.

[0005] The instant invention is based, at least in part, on the coating of an insulating functionalization layer above or coated on the surface of a graphene sensor. As shown herein, the functionalization layer can be hydrophilic, allowing coating of a lipid bilayer onto its surface. Additionally, the functionalization layer can provide additional mechanisms for conjugation of a biomolecule within the detectable range of the graphene sensor. Therefore, in some embodiments, the methods and compositions disclosed herein enable detection of a binding event involving a single biomolecule conjugated to the functionalization layer. In some embodiments, the methods and compositions disclosed herein enable detection of a binding event involving a single and/or a plurality of biomolecules bound to a biological membrane (e.g., a lipid bilayer), wherein the biological membrane is coated on the surface of the functionalization layer.

[0006] Accordingly, in a first aspect, provided herein is a device, comprising: a support substrate; a graphene layer, the graphene layer deposited on the support substrate; a functionalization layer, comprising solid state material, the functionalization layer deposited continuously on at least a portion of the graphene layer; and electrodes in electrical contact with the graphene layer, the electrode adapted to detect an electrical signal from the graphene layer.

[0007] In various embodiments, the device further comprises a substrate insulating layer, wherein the substrate insulating layer deposited on the support substrate, wherein the graphene layer deposited on the substrate insulating layer.

[0008] In some embodiments, the electrodes are deposited on top of the graphene layer to generate an electrical connection. In some other embodiments, the graphene layer is deposited on top of the electrodes to generate an electrical connection.

[0009] In certain embodiments, the support substrate comprises a material selected from the group consisting of: silicon, glass, quartz, S1O2, silicon/SiCh, GaAs, GaN, polyethylene terephthalate (PET), and other polymer based materials. In some of these embodiments, the support substrate comprises silicon and/or S1O2. In certain embodiments, the support substrate has a thickness of 50 μm to 5000 μm.

[0010] In cetain embodiments, the substrate insulating layer comprises an oxide. In some of these embodiments, the oxide is selected from the group consisting of: S1O2, T1O2, AI2O3, **Ta2C"5, $Fe_3$04,** and ZrCh. In some embodiments, the substrate insulating layer has a thickness of 5 nm to 50000 nm. In some embodiments, the substrate insulating layer has a dielectric constant of 1 to 100.

[0011] In certain embodiments, the graphene layer is a graphene monolayer. In some of these embodiments, the graphene monolayer consists of one atomic layer of carbon atoms for at least 50% of its total area. In certain embodiments, the graphene layer is patterned. In various embodiments, the graphene layer has a thickness of 1 atomic layer to 3 atomic layers. In some embodiments, the graphene layer is in electrical contact with at least two electrodes.

[0012] In certain embodiments, the solid state material is an oxide, a nitride, or an oxinitride. In some of these embodiments, the oxide is selected from the group consisting of: $Si0_2$, T1O2, AI2O3, **Ta_2O5, Fe_3O4,** and $Zr0_2$. In some of these embodiments, the nitride or oxinitride is selected from the group consisting of: TiN, A1N, TiAIN, TiCN, TiOxNy, SiOxNy, SiN, and S13N4. In some embodiments, the functionalization layer has a thickness of 1 nm to 10 nm. In certain embodiments, the functionalization layer has a dielectric constant of 1 to 100. In certain embodiments, the product of the dielectric constant and the thickness of the functionalization layer is less than 1000 nm. In some embodiments, the functionalization layer further comprises a reactive group. In some of these embodiments, the reactive group is selected from the group consisting of: a carboxyl group, a hydroxyl group, an amine group, an epoxy group, an aldehyde group, and a sulfhydryl group. In certain embodiments, a functionalization molecule is bound to the functionalization layer. In some of these embodiments, the functionalization molecule is covalently bound to a reactive group of the functionalization layer. In certain embodiments, the functionalization molecule comprises silane. In various embodiments, the silane is selected from the group consisting of: 3-aminopropyltrimethoxysilane (APTMS, amino), 3-aminoproplyltriethoxy silane (APTES, amino), 3-isocyanatopropyltriethoxysilane (CYNP S, isocyanate), triethoxysilylbutyraldehyde (ALDPS, aldehyde), (3-glycidoxypropyl) trimethoxysilane (GPS, epoxy), 3-mercaptopropyltrimethoxysilane (MPS, sulfur), 7-octenyltrimethoxysilane (OTS, vinyl), 3-methacryloxypropyltrimethoxy silane (acrylate), 3,4-epoxycyclohexyltrimethoxy silane (ECPS, epoxy), 10-undecenyltrichlorosilane (V1 1TCS, vinyl), and carboxyl ethyl silanetriol. In some embodiments, the functionalization molecule has a size of 1 nm to 20 nm. In certain embodiments, the functionalization layer is homogenous. In some of these embodiments, the functionalization layer is homogenous as determined by a lipid bilayer applied to the surface of the functionalization layer having a fluidity of at least 0.5 μηι/cm^2.

[0013] In certain embodiments, the electrodes comprise a source and a drain. In some embodiments, the electrode is connected to an electrical power supply configured to generate an electrical potential on the graphene layer. In some embodiments, the electrode is

configured to measure a current or change in current through the graphene layer. In various embodiments, the electrode comprises a metal selected from the group consisting of: gold, titanium, aluminum, copper, silver, platinum, palladium, and combinations thereof. In certain embodiments, the electrode has a thickness of 10 nm to 1000 nm.

[0014] In various embodiments, device comprises a biological membrane layered on the functionalization layer. In some embodiments, the biological membrane is in native state. In some of these embodiments, the biological membrane comprises a target molecule capable of binding to a ligand molecule. In some embodiments, the target molecule is in native state. In certain embodiments, the target molecule is selected from the group consisting of: an integral membrane protein, a membrane associated protein, a glycoprotein, a phospholipid, and a glycolipid. In certain embodiments, the biological membrane is a lipid bilayer. In some embodiments, the lipid bilayer has a fluidity of at least 0.5 $\mu\eta/cm^2$. In certain embodiments, the biological membrane is derived from a cell.

[0015] In another aspect, provided herein is a method of synthesizing a graphene sensor device, comprising: providing a graphene field effect transistor comprising a graphene layer and a pair of electrodes in electrical contact with the graphene layer; forming a functionalization layer continuously on the graphene layer, wherein the functionalization layer comprises solid state material.

[0016] In certain embodiments, the functionalization layer comprises an oxide. In some of these embodiments, the oxide is selected from the group consisting of: $Si0_2$, T1O2, AI2O3, Ta_205, Fe_304, and $Zr0_2$. In certain preferred embodiments, forming the functionalization layer comprises depositing a silicon layer on the graphene layer, and placing the silicon layer in oxidizing conditions to form a layer of S1O2. In some embodiments, the functionalization layer has a thickness of 1 nm to 10 nm.

[0017] In certain embodiments, the method further comprises applying a lipid bilayer to the functionalization layer. In certain other embodiments, the method further comprises covalently binding a silane molecule to the functionalization layer. In some embodiments, the method further comprises covalently binding a target molecule to the silane molecule. In various embodiments, the target molecule is capable of binding to a ligand molecule. In some embodiments, the target molecule is in native state.

[0018] In another aspect, provided herein is a method of detecting a ligand molecule, comprising: providing the graphene sensor device; applying an electrical potential to the graphene layer; contacting the device with a sample suspected of comprising a ligand

molecule of interest; and collecting information generated by the device comprising changes in electrical current over time to determine whether or not the ligand molecule is present in the sample. In certain embodiments, the ligand molecule is capable of binding to a target molecule bound to the functionalization layer. In some embodiments, the target molecule is in native state. In some embodiments, the ligand molecule is label-free.

[0019] In another aspect, provided herein is a method of quantifying the concentration of a ligand molecule, comprising: providing the graphene sensor device; applying an electrical potential to the graphene layer; contacting the device with a sample suspected of comprising a ligand molecule of interest; and collecting information generated by the device comprising the magnitude of change in electrical signal before and after contacting the device with the sample, and comparing the data to a database comprising change in electrical signal from known concentrations of the ligand molecule. In certain embodiments, the ligand molecule is label-free.

[0020] In another aspect, provided herein is a method of quantifying the binding kinetics between a macromolecule and a ligand, comprising: providing the graphene sensor device; applying an electrical potential to the graphene layer; contacting the device with a sample suspected of comprising a ligand molecule of interest; and collecting information generated by the device comprising changes in electrical current over time to quantify the binding kinetics between the macromolecule and the ligand. In certain embodiments, the binding kinetics comprises the dissociation constant. In some embodiments, the macromolecule is in native state. In certain embodiments, the macromolecule is a receptor. In some embodiments, the ligand molecule is label-free.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] The foregoing and other objects, features and advantages will be apparent from the following description of particular embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead placed upon illustrating the principles of various embodiments of the invention. Provided also as embodiments of this disclosure are data figures that illustrate features by exemplification only, and not limitation.

[0022] Figure 1 is a schematic of graphene bio-electronic sensing technology (GBEST) for detection of ligand binding to a target molecule.

[0023] Figure 2 provides an example of addition of a sample comprising ligand molecules to a graphene sensor and a schematic of current over time when the sample is applied to and then removed from the sensor.

[0024] Figure 3 provides an example of the use of the graphene bio-electronic sensing technology to detect binding kinetics of EGF for EGFR.

[0025] Figure 4 shows an embodiment of the graphene chip wherein the electrodes are deposited on top of the graphene layer.

[0026] Figure 5 shows an embodiment of the graphene chip wherein the graphene layer is deposited on top of the electrodes.

[0027] Figure 6 depicts the method of making tethered supported membranes on the functionalization layer. The molecular weight of PEG can range from about 1000 to about 20000 (the value of n ranging from about 23 to about 460). DSPE can be replaced by DPPE, DOPE, or POPE.

[0028] Figure 7 shows a cushioned lipid bilayer attached to the functionalization layer of the sensor.

[0029] Figure 8 shows a fluorescence recovery after photobleaching (FRAP) assay of fluidity of lipid bilayer containing membrane-bound proteins layered on the surface of the functionalization layer.

[0030] Figure 9 depicts the results of an assay using the graphene bio-electronic sensor comprising a lipid bilayer to detect label-free cholera toxin subunit B (CTB) binding to ganglioside 1 (GMl) in the lipid bilayer. The figure shows the label-free binding kinetics of CTB and GMl at the concentration of 48 nM for the CTB ligand.

[0031] Figure 10 depicts the results of a label-free dose response assay to determine the sensitivity of the graphene bio-electronic sensor to different concentrations of CTB in the sample. The label-free CTB ligand concentrations displayed in this figure are 6 nM, 12 nM, 24 nM, and 48 nM.

[0032] Figure 11 depicts the label-free binding curve and dissociation constant of pentamer CTB binding to active GMl as determined by the graphene bio-electronic sensing technology. A comparison to labeled results from fluorescence assay and flow cytometry is also included in the figure.

[0033] Figure 12 shows a method of conjugating biomolecules to the functionalization layer.

[0034] Figure 13 presents the binding of ligand R715 to native bradykinin receptor B1 in cell membranes on the graphene bio-electronic sensor described herein.

DETAILED DESCRIPTION

[0035] Throughout this application, the text refers to various embodiments of the present devices, compositions, systems, and methods. The various embodiments described are meant to provide a variety of illustrative examples and should not be construed as descriptions of alternative species. Rather, it should be noted that the descriptions of various embodiments provided herein may be of overlapping scope. The embodiments discussed herein are merely illustrative and are not meant to limit the scope of the present invention.

[0036] Also throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure in their entireties.

[0037] As used herein, "support substrate" or "supporting substrate" refers to a substrate onto which the substrate insulating layer and the graphene layer can be deposited on. Nonlimiting examples of such substrates include: silicon, glass, quartz, S1O2, silicon/SiCh, GaAs, GaN, polyethylene terephthalate (PET), and other polymer based materials.

[0038] As used herein, the term "substrate insulating layer" refers to an electrically insulating layer between the graphene layer and the support substrate. The substrate insulating layer materials comprise but are not limited to oxide materials that are at least 3 nm in thickness. In certain embodiments, when the support substrate is an insulating material, no substrate insulating layer is needed.

[0039] As used herein, "graphene layer" refers to a layer of carbon atoms, typically one, two, three, or four atoms thick. There are at least 100 carbon atoms in the graphene layer. Monolayer graphene refers to a graphene sheet that is one carbon atom thick for at least 50% of the area of the graphene layer.

[0040] As used herein, the term "functionalization layer" refers to a 1 nm - 10 nm thin layer deposited or grown on the graphene sensor device to make the sensor device compatible with lipid bilayers and conventional surface chemistry. Examples of materials suitable for the functionalization layer include but are not limited to oxide, nitride, and oxinitride materials, such as S1O2, T1O2, AI2O3, Ta_20_5, Fe_3O_4, $Zr0_2$, TiN, A1N, TiAIN, TiCN, TiOxNy, SiOxNy, SiN, and S13N4. The functionalization layer comprises solid state materials. The

functionalization layer does not comprise a linker molecule that immobilizes a lipid or a protein onto the graphene layer. Such linker molecule preferably comprises a graphene binding moiety, such as an aromatic moiety, e.g. a pyrene group, and the graphene binding moiety preferably binding to graphene by pi-pi electron stacking.

[0041] As used herein, the term "solid state material" refers a material that is formed from densely packed atoms with the property of a solid substance. Solid state materials include but are not limited to crystalline, polycrystalline, and amorphous solid material.

[0042] As used herein, the term "functionalization molecule" refers to a molecule bound to the functionalization layer. In some embodiments, the functionalization molecule is covalently bound to a reactive group of the functionalization layer. The functionalization molecule can bind to a target molecule of interest.

[0043] As used herein, "electrode" refers to the electric conductor that is connected to the graphene layer. Nonlimiting examples of electrode materials include: gold, titanium, aluminum, copper, silver, platinum, palladium, and combinations thereof.

[0044] As used herein, the term "target molecule" refers to a biomolecule of interest that is embedded in the biological membrane, or is naturally bound within the biological membrane coated on the surface of the functionalization layer. "Target molecule" also refers to a biomolecule of interest that can be conjugated to a functionalization molecule bound to the functionalization layer. Nonlimiting examples of target molecules include: an integral membrane protein (IMP), a membrane associated protein, a glycoprotein, a phospholipid, and a glycolipid.

[0045] As used herein, the term "ligand molecule" refers to a molecule that can bind to the biomolecule of interest. Nonlimiting examples of ligand molecules include: an antibody, a hormone, a toxin, a neurotransmitter, a small molecule, a drug, a nanoparticle, a chemical substrate, and an ion.

[0046] As used herein, the term "native state" refers to a properly folded and/or assembled form of biomolecule. The native state of a biomolecule can possess all four levels of biomolecular structure, with the secondary through quaternary structure being formed from weak interactions along the covalently-bonded backbone. The native state is in contrast to the denatured or partially denatured state, in which the weak interactions are disrupted, such as by detergent treatment, leading to the loss of one or more higher orders of structure such as the secondary, tertiary, and/or quaternary structure. The native state is also in contrast to a

"mutated state" where the sequence of a biomolecule, such as a protein, is changed, which can alter its folding and/or other properties.

[0047] As used herein, "Kd" refers to "dissociation constant," which is a specific type of equilibrium constant that measures the propensity of a larger object to separate (dissociate) reversibly into smaller components, as when a complex falls apart into its component molecules. The dissociation constant is the inverse of the association constant. For a general reaction

$$A_xB_y \rightleftharpoons xA + yB$$

in which a complex A_xB_y breaks down into x A subunits and y B subunits, the dissociation constant is defined

$$K_d = \frac{[A]^x \times [B]^y}{[A_xB_y]}$$

where [A], [B], and $[A_xB_y]$ are the concentrations of A, B, and the complex A_xB_y, respectively.

Graphene Bio-Electronic Sensing

[0048] The present invention relates to the addition of a functionalization layer applied on the surface of or in close proximity above a graphene layer of a graphene field effect transistor to enable expanded biomolecular integration for sensing and detection. In some embodiments, the functionalization layer enables graphene sensors to be compatible with known covalent conjugation methods and other new mechanisms to link a biomolecule or other small molecule to the graphene sensor. In some embodiments, the functionalization layer enables the graphene sensors to interface with and detect binding events for biomolecules in supported lipid bilayers, e.g., integral membrane proteins.

[0049] Because of its high sensitivity and selectivity, graphene field effect transistors (GFETs) present ideal tools for sensing applications. When a ligand molecule binds to a target biomolecule on or near the graphene surface, the redistribution of electronic charge generates a change in the electric field across the graphene field effect transistor (GFET), which changes the electronic conductivity and device response. As shown in Figure 1, the detection of the binding event is accomplished by measuring the current and the current change of GFET.

[0050] A graphene field effect transistor (GFET) comprises a source electrode, a drain electrode, a gate, and a graphene channel region connecting the source and the drain electrodes. Graphene layer can be modified with biomolecule conjugates, which anchor biomolecules to the surface of the graphene layer. In some embodiments, the target biomolecule contains specific groups that can be recognized by a ligand molecule. As depicted in Figure 2, the binding of the ligand molecule to the target molecule is detected by measuring the current change.

[0051] The surface of the GFET channel can be functionalized with proteins, chemical compounds, and DNA molecules to make sensors for various applications. Figure 3 shows an example of a GFET channel functionalized with epidermal growth factor receptor (EGFR). Different concentrations of EGF can be applied to the EGFR-functionalized graphene surface. The binding affinity between EGF and EGFR can be determined by the dissociation constant (Kd).

Device

[0052] Aspects of the subject disclosure include graphene sensor devices that comprise a support substrate, a graphene layer, a functionalization layer, electrodes, and optionally a substrate insulating layer.

Support Substrate

[0053] Support substrate refers to a substrate onto which the insulating layer and the graphene layer can be deposited. In various embodiments, the support substrate is used to support graphene sensor devices. Nonlimiting examples of such substrates include: silicon, glass, quartz, SiO_2, silicon/SiCh, GaAs, GaN, polyethylene terephthalate (PET), and other polymer based materials. In some embodiments, the support substrate comprises silicon or S1O2. In certain embodiments, the support substrate comprises silicon. In some embodiments, the support substrate is a semiconducting substrate. In some other embodiments, the support substrate is an insulating substrate. In various embodiments, the thickness of the support substrate is from about 5 μm to about 10000 μπι, such as about 10 μm to about 5000 μπι, about 50 μm to about 2500 μπι, or about 100 μm to about 1000 μm.

Substrate Insulating Layer

[0054] Substrate insulating layer refers to an electrically insulating layer between the graphene layer and the support substrate. In various embodiments, the substrate insulating layer is deposited on the support substrate. In various embodiments, the graphene layer is placed directly onto the substrate insulating layer on the support substrate. In some embodiments, the substrate insulating layer comprises an oxide material. In some of these embodiments, the oxide of the substrate insulating layer is selected from the group consisting of: S1O2, T1O2, AI2O3, Ta_2O5, Fe_3O4, and ZrO_2. In some other embodiments, the substrate insulating layer comprises a nitride material. In various embodiments, the substrate insulating layer is at least 3 nm in thickness, such as at least 5 nm, at least 10 nm, at least 50 nm, or at least 100 nm in thickness. In some embodiments, the substrate insulating layer is about 3 nm to about 100000 nm in thickness, such about 5 nm to about 50000 nm, about 10 nm to about 10000 nm, or about 100 nm to about 1000 nm in thickness. In certain preferred embodiments, the substrate insulating layer is a 100 nm to 300 nm thick layer of thermally grown S1O2. In various embodiments, the substrate insulating layer has a dielectric constant of 1 to 100, such as 2 to 80, 5 to 60, 10 to 50, or 20 to 40. In certain embodiments, when the support substrate is an insulating material, no substrate insulating layer is needed.

Graphene Layer

[0055] Graphene layer refers to a layer of carbon atoms, typically one, two, three, or four atoms thick. In various embodiments there are at least 100 carbon atoms in the graphene substrate, such as at least 200 carbon atoms, at least 500 carbon atoms, or at least 1000 carbon atoms. In certain embodiments, the graphene layer is a graphene monolayer. In some embodiments, the graphene monolayer is one carbon atom thick for at least 50% of the area of the graphene layer.

[0056] In various embodiments, the graphene layer is transferred onto the support substrate and placed directly onto the insulating layer. In some embodiments, the electrodes are deposited on top of the graphene layer. In some other embodiments, the graphene layer is deposited on top of the electrodes.

[0057] In some embodiments, the graphene layer is unpatterned. In some other embodiments, the graphene layer is patterned. In some of these embodiments, the graphene layer is patterned using standard photolithography methods. In some embodiments, the standard photolithography methods comprise spincoating positive i-Line resist onto the

graphene substrate to about 1.3 μιη thickness, exposing, and chemically developing the positive i-Line resist to create resist/graphene features. In certain embodiments, the resist/graphene features are 1 μιη x 1 μπι, 2 μιη x 2 μπι, 5 μιη x 5 μπι, 10 μιη χ 10 μπι, 20 μm χ 20 μπι, 40 μιη χ 40 μπι, 80 μιη χ 80 μπι, or 100 μιη χ 100 μιη in area. In some embodiments, graphene features as small as 90 nm across are obtained using immersion photolithography. In some embodiments, graphene features as small as 10 nm across is achieved using electron beam lithography methods. In various embodiments, an O_2 RIE (Reactive Ion Eching) plasma etching system is used to etch away the graphene exposed by lithography. In some embodiments, a resist stripper solution is used to remove the protective resist covering the patterned graphene features. In some of these embodiments, the resist stripper solution is microposit 1165 or PRS3000.

Functionalization Layer

[0058] Functionalization layer refers to a layer deposited or grown on the graphene sensor device to make the sensor device compatible with lipid bilayers, conventional surface chemistry, and functional biochemistry. In various embodiments, the functionalization layer protects the sensing surface from ambient contamination and allows lipid bilayer assembly or chemical conjugation above the surface. In some embodiments, the thickness of the functionalization layer varies from about 1 nm to about 50 nm, such as about 1 nm to about 20 nm, about 1 nm to about 10 nm, or about 1 nm to about 5 nm. In some of these embodiments, the thickness of the functionalization layer is from about 1 nm to about 3 nm. In certain embodiments, the thickness of the functionalization layer is less than 10 nm, such as less than 9 nm, less than 8 nm, less than 7 nm, less than 6 nm, less than 5 nm, less than 4 nm, less than 3 nm, or less than 2 nm.

[0059] In various embodiments, the functionalization layer comprises solid state material deposited continuously on the graphene layer. In some embodiments, the solid state material is crystalline, polycrystalline, or amorphous solid material. In some embodiments, the functionalization layer provides a continuous hydrophilic surface on top of the graphene layer, allowing the formation of a biological membrane on the surface. In various embodiments, the thickness of the functionalization layer is adjusted to allow the optimal formation of the biological membrane and the fine-tuning of the device. In some embodiments, the functionalization layer protects the graphene layer from external comtamination and keep the graphene layer intact.

[0060] In various embodiments, the material for the functionalization layer is electrically insulating such that it does not short the sensor. In various embodiments, the materials for the functionalization layer is oxide, nitride and oxinitride materials. In certain embodiments, the functionalization layer comprises an oxide. The oxide can be S1O2, T1O2, AI2O3, Ta_2O5, Fe_3C"4, or ZrO_2. In certain embodiments, the functionalization layer comprises a nitride or an oxinitride. The nitride or oxinitride can be TiN, A1N, TiAIN, TiCN, TiOxNy, SiOxNy, SiN, or S13N4. In certain preferred embodiments, the material for the functionalization layer is S1O2.

[0061] In some embodiments, the functionalization layer has a dielectric constant in the range of 1 to 100, such as 2 to 80, 5 to 60, 10 to 50, or 20 to 40. In some embodiments, the product of the dielectric constant and the thickness of the functionalization layer is less than 1000 nm, such as less than 100 nm, less than 10 nm, or less than 1 nm.

[0062] In various embodiments, the functionalization layer further comprises a reactive group, such as a carboxyl group, a hydroxyl group, an amine group, an epoxy group, an aldehyde group, a sulfhydryl group, or other reactive groups used in surface chemistry methods well known to those skilled in the arts. In some embodiments, a functionalization molecule is bound to the functionalization layer. In some of these embodiments, the functionalization molecule is covalently bound to the reactive group of the functionalization layer. In certain embodiments, the functionalization molecule is covalently bound to the carboxyl group of the functionalization layer. In certain embodiments, the functionalization molecule is covalently bound to the hydroxyl group of the functionalization layer. In some of these embodiments, the functionalization molecule comprises silane. In various embodiments, the silane is 3-aminopropyltrimethoxy silane (APTMS, amino), 3-aminoproplyltriethoxysilane (APTES, amino), 3-isocyanatopropyltriethoxysilane (CYNPS, isocyanate), triethoxysilylbutyraldehyde (ALDPS, aldehyde), (3-glycidoxypropyl)trimethoxysilane (GPS, epoxy), 3-mercaptopropyltrimethoxysilane (MPS, sulfur), 7-octenyltrimethoxysilane (OTS, vinyl), 3-methacryloxypropyltrimethoxysilane (acrylate), 3,4-epoxycyclohexyltrimethoxysilane (ECPS, epoxy), 10-undecenyltrichlorosilane (VI 1TCS, vinyl), or carboxylethylsilanetriol. In some embodiments, the size of the functionalization molecule varies from 1 nm to 50 nm, such as 2 nm to 20 nm, or 5 nm to 10 nm.

[0063] The deposition of the functionalization layer can be done *via* physical/thermal deposition or *via* chemical methods. In some embodiments, the physical/thermal deposition is e-beam evaporation. In some embodiments, the chemical deposition is atomic layer

deposition. In certain embodiments, when the electrodes are deposited on top of the graphene layer, the functionalization layer is deposited on top of both the electrodes and the graphene layer. In certain other embodiments, when the graphene layer is deposited on top of the electrodes, the functionalization layer is deposited on top of the graphene layer.

[0064] In some embodiments, a lipid bilayer is immobilized on the functionalization layer. In certain embodiments, the fluidity of a lipid bilayer is measured by fluorescence recovery after photobleaching (FRAP) assay. In certain embodiments, the lipid bilayer applied to the surface of the functionalization layer has a fluidity of at least 0.5 μιη/cm^2, such as at least 1 μιη/cm^2, at least 2 μιη/cm^2, at least 5 μιη/cm^2, or at least 10 μιη/cm^2.

Electrodes

[0065] Electrode refers to the electrical conductor that is connected to the graphene layer. In some embodiments, the electrodes are deposited on top of the graphene layer. In some other embodiments, the graphene layer is deposited on top of the electrodes. In certain embodiments, after the graphene features are patterned, standard semiconductor processing techniques are used to generate the pattern for electrodes, with subsequent metal deposition and lift-off to expose the desired electrode pattern above the graphene pattern. In certain other embodiments, the electrodes are patterned first, and the graphene layer is transferred on top of the electrodes and subsequently patterned. In some embodiments, the electrode is deposited by thermal evaporation. In some embodiments, the excess electrode material is removed using a standard lift-off step.

[0066] In various embodiments, the electrode materials comprise metals such as gold, titanium, aluminum, copper, silver, platinum, palladium, and combinations thereof. In some embodiments, the electrode materials are titanium and gold. In some of these embodiments, titanium is used as an adhesion layer for gold. In various embodiments, the electrodes are about 10 nm to about 1000 nm in thickness, such as about 20 nm to about 800 nm, about 30 nm to about 500 nm, or about 40 nm to about 300 nm in thickness. In certain preferred embodiments, the electrodes are about 40 nm in thickness. In various embodiments, the electrodes are about 1 μm to about 100 μm in width, such as about 2 μm to about 80 μπι, about 5 μm to about 40 μπι, or about 10 μm to about 20 μm in width. In some preferred embodiment, the electrodes are about 10 μm to about 20 μm in width.

[0067] In some embodiments, the electrode comprises a source and a drain. In some embodiments, the electrode further comprises a gate. In some embodiments, the electrode is

in electrical contact with the graphene layer. In some embodiments, the electrode is connected to an electrical power supply configured to generate an electrical potential on the graphene layer. In some embodiments, the electrode is configured to measure a current or a change in current through the graphene layer.

Assembly of the Graphene Sensor Device

[0068] The graphene sensor devices can be fabricated through different sequences. In some embodiments, as shown in Figure 4, the graphene layer is transferred onto the supporting substrate and placed directly onto the substrate insulating layer. The transferred graphene sheet is patterned, and the electrodes are deposited above the graphene pattern. The functionalization layer is deposited or grown on the graphene sensor device after the deposition of the electrodes. In some other embodiments, as shown in Figure 5, the electrodes are patterned directly onto the substrate insulating layer above the supporting surface. The graphene layer is then transferred onto the supporting substrate and placed directly onto the substrate insulating layer and the electrodes. The functionalization layer is deposited or grown on the graphene sensor device after the deposition of the graphene layer.

[0069] Subsequently, the layers assembled can be protected with a photoresist, diced into individual chips, and mounted onto a printed circuit board (PCB), following methods well known to the skilled in the arts. In order to perform detection or sensing, the PCB mounted chip can be incorporated into a flow cell, connected to a vacuum or pressure source, and a buffered sample can be introduced to the sensor *via* a fluidic pathway.

[0070] In certain embodiments, a silicon wafer is used as the supporting substrate. Other semiconducting or insulating substrates such as AI2O3, GaAs, GaN, SiN, or polymer-based substrate can also be used as substrates to support graphene sensor devices.

[0071] The substrate insulating layer can comprise an oxide or nitride material that is at least 3 nm in thickness. In certain embodiments, a 100 nm to 300 nm thick layer of thermally grown S1O2 is used as the substrate insulating layer.

[0072] The transferred graphene sheet can be patterned using standard photolithography methods. In various embodiments, the graphene features are greater than 90 nm across. Features as small as 90 nm across can be obtained using immersion photolithograph. Features as small as 10 nm across can be achieved using electron beam lithography methods. In certain embodiments, the graphene sheet comprises a monolayer of carbon atoms. In certain embodiments, the graphene sheet comprises a finite multilayer of carbon atoms.

[0073] The demensions and lengths of the electrode can vary depending on the design of the sensor. Electrode materials include metals such as gold (Au), titanium/gold (Ti/Au), chrome/gold (Cr/Au), aluminum (Al), copper (Cu), silver (Ag), palladium (Pd), and combinations thereof. In certain embodiments, the electrode material is titanium (Ti) or gold (Au).

[0074] In various embodiments, the functionalization layer is deposited *via* physical/thermal deposition, such as e-beam evaporation, or *via* chemical methods, such as atomic layer deposition. The preferred thickness of the functionalization layer can vary from 1 nm to 10 nm. In some embodiments, the functionalization layer protects the sensing surface from ambient contamination and allows lipid bilayer assembly or chemical conjugation above the surface. In some embodiments, the functionalization layer is electrically insulating such that it does not short the sensor. Examples of materials suitable for the functionalization layer include but are not limited to oxide, nitride, and oxinitride materials (e.g. $Si0_2$, T1O2, AI2O3, Ta_2O_5, Fe_30_4, $Zr0_2$, TiN, A1N, TiAIN, TiCN, TiOxNy, SiOxNy, SiN, and Si_3N_4).

Mechanisms of Binding to the Functionalization Layer

[0075] In some embodiments, the graphene sensor devices further comprise a biological membrane layered on the functionalization layer. In some embodiments, the biological membrane is a lipid bilayer. In some embodiments, the lipid bilayer has a fluidity of at least 0.5 μηι/cm^2, such as at least 1 μιη/cm^2, at least 2 μιη/cm^2, at least 5 μιη/cm^2, or at least 10 μιη/cm^2. In certain embodiments, the fluidity of a lipid bilayer is measure by fluorescence recovery after photobleaching (FRAP) assay.

[0076] In various embodiments, the biological membrane is in native state. In some embodiments, the preparation of the biological membrane does not involve disruptive steps, such as detergent solublization and reconstitution. In some embodiments, the proteins and other macromolecules associated with the biological membrane are in native state. In some embodiments, the biological membrane is derived from a cell.

[0077] In some embodiments, the biological membrane comprises a target capable of binding to a ligand molecule. In some embodiments, the ligand molecule is labeled. In certain embodiments, the ligand molecule is labeled to measure the fluidity of the lipid bilayer. In certain embodiments, the ligand molecule is labeled with fluorescent tag.

[0078] In some embodiments, the ligand molecule is label-free. In various embodiments, the ligand molecule is a protein, a peptide, a nucleic acid, an antibody, a hormone, a toxin, a

neurotransmitter, a nanoparticle, a chemical substance, or other molecule of interest. In some embodiments, the ligand molecule is a small molecule, an ion, or a drug.

[0079] In some embodiments, the target molecule is embedded in the biological membrane coated on the surface of the functionalization layer. In some embodiments, the target molecule is naturally bound within the biological membrane coated on the surface of the functionalization layer. In some embodiments, the target molecule is a membrane protein. In various embodiments, the target molecule can be an integral membrane protein, a membrane associated protein, a glycoprotein, a glycolipid, a phospholipid, or a sugar. In some embodiments, the integral membrane protein is a G protein coupled receptor (GPCR). In some of these embodiments, the integral membrane protein is an ion channel protein, such as a chloride channel protein, a potassium channel protein, a sodium channel protein, or a calcium channel protein. In some embodiments, the glycolipid is ganglioside 1 (GM1). In some of these embodiments, the ligand to GM1 is cholera toxin subunit B (CTB).

[0080] In some embodiments, the graphene sensor devices further comprise a functionalization molecule. In some embodiments, the functionalization molecule comprises silane. In various embodiments, the silane can be 3-aminopropyltrimethoxysilane (APTMS, amino), 3-aminoproplyltriethoxy silane (APTES, amino), 3-isocyanatopropyltriethoxysilane (CYNP S, isocyanate), triethoxysilylbutyraldehyde (ALDPS, aldehyde), (3-glycidoxypropyl)trimethoxysilane (GPS, epoxy), 3-mercaptopropyltrimethoxysilane (MPS, sulfur), 7-octenyltrimethoxysilane (OTS, vinyl), 3-methacryloxypropyltrimethoxysilane (acrylate), 3,4-epoxycyclohexyltrimethoxysilane (ECPS, epoxy), 10-undecenyltrichlorosilane (V1 1TCS, vinyl), or carboxylethylsilanetriol. In some embodiments, the functionalization molecule is conjugated to the functionalization layer. In certain embodiments, the functionalization molecule is conjugated to the functionalization layer through a reactive group of the functionalization layer. In various embodiments, the reactive group is a carboxyl group, a hydroxyl group, an amine group, an epoxy group, an aldehyde group, a sulfhydryl group, or other reactive groups used in surface chemistry methods well known to those skilled in the arts. In some of these embodiments, when the functionalization layer comprises a carboxyl group, the functionalization molecule is covalently bound to the carboxyl group. In some of these embodiments, when the functionalization layer comprises a hydroxyl group, the functionalization molecule is covalently bound to the hydroxyl group. In various embodiments, a target molecule is conjugated to the functionalization molecule. In certain embodiments, the target molecule is a protein, a peptide, a nucleic acid, a lipid, or a sugar.

Methods of Use

[0081] Aspects of the subject disclosure include methods for using the graphene bio-electronic sensing devices to detect a binding event, to determine the label-free binding kinetics, and to quantify the concentration of a ligand molecule.

[0082] In some embodiments, the method comprises providing the graphene bio-electronic sensing device, applying an electrical potential to the graphene layer, contacting the device with a sample suspected of comprising a ligand molecule of interest, and collecting information generated by the device. In some of these embodiments, the information generated comprises changes in electrical current over time. In certain embodiments, the change in electrical current is analyzed to determine whether or not the ligand molecule is present in the sample. In some embodiments, the information generated comprises the magnitude of change in electrical signal before and after contacting the sensor with the sample. In some embodiments, the magnitude of change is compared to a database comprising changes in electrical signal from known concentrations of ligand molecules. In some of these embodiments, the concentration of the ligand molecule is quantified from the comparison. In some embodiments, the information generated comprises changes in electrical current over time for qualifying the label-free binding kinetics between the target molecule and the ligand molecule. In some embodiments, the label-free association and dissociation phases is measured systematically at different concentrations of the ligand molecule. In some embodiments, the label-free association and dissociation phases is measured systematically at different concentrations of the ligand and target molecules. In some embodiments, the association rate (k_{0n}) and the dissociation rate (k_{0ff}) is obtained. In some embodiments, the dissociation constant **(Kd)** is determined.

[0083] In various embodiments, the method comprises using the graphene bio-electronic sensing devices to detect the binding between a target molecule and a ligand molecule. In some embodiments, the target molecule is bound to the functionalization layer. In some embodiments, the target molecule is bound to a functionalization molecule conjugated to the functionalization layer. In some other embodiments, the target molecule is in a biological membrane. In some of these embodiments, the biological membrane is coated on the surface of the functionalization layer.

[0084] In various embodiments, the method comprises using the graphene bio-electronic sensing devices to detect the binding of a protein, a peptide, a nucleic acid, a lipid, a sugar, an

antibody, a hormone, a toxin, a neurotransmitter, a small molecule, an ion, a nanoparticle, a chemical substance, a drug, or other molecules of interest. In some embodiments, the ligand for the target molecule of interest is free of label.

[0085] In some embodiments, the method comprises using the graphene bio-electronic sensing devices to detect the binding between a protein of interest and an antibody against the protein of interest. In certain embodiments, the target molecule is a protein of interest. In some of these embodiments, the ligand molecule is an antibody against the protein of interest. In certain other embodiments, the target molecule is an antibody against a protein of interest. In some of these embodiments, the ligand molecule is the protein of interest. In certain embodiments, the target molecule is prostate-specific antigen (PSA). In some of these embodiments, the ligand molecule is an anti-PSA antibody. In certain embodiments, the target molecule is an anti-PSA antibody. In some of these embodiments, the ligand is PSA.

[0086] In some embodiments, the method comprises using the graphene bio-electronic sensing devices to detect the binding between a hormone and a hormone receptor. In certain embodiments, the target molecule is a hormone receptor. In some of these embodiments, the ligand molecule is a hormone that binds to the hormone receptor. In certain other embodiments, the target molecule is a hormone that binds to a hormone receptor. In some of these embodiments, the ligand molecule is the hormone receptor.

[0087] In some embodiments, the method comprises using the graphene bio-electronic sensing devices to detect the binding between a nucleic acid and a protein. In certain embodiments, the target molecule is a nucleic acid. In some of these embodiments, the ligand molecule is a protein that binds to the nucleic acid. In certain other embodiments, the target molecule is a protein. In some of these embodiments, the ligand molecule is a nucleic acid that binds to the protein.

[0088] In some embodiments, the method comprises using the graphene bio-electronic sensing devices to detect the binding between a biomolecule and a small molecule. In some of these embodiments, the small molecule is an organic small molecule. In some other of these embodiments, the small molecule is an inorganic small molecule. In some embodiments, the method comprises using the graphene bio-electronic sensing devices to detect the binding between a biomolecule and a drug.

[0089] In some embodiments, the method comprises using the graphene bio-electronic sensing devices to detect the binding between a ligand molecule and a G protein coupled receptor (GPCR). In some of these embodiments, the GPCR is an ion channel protein, such as

a chloride channel protein, a potassium channel protein, a sodium channel protein, or a calcium channel protein. In certain embodiments, the GPCR is in a biological membrane coated on the surface of the functionalization layer. In some of these embodiments, the ligand molecule is a small molecule. In some of these embodiments, the ligand molecule is a protein, such as an antibody or a peptide.

[0090] In some embodiments, the method comprises using the graphene bio-electronic sensing devices to detect the binding between ganglioside 1 (GM1) and cholera toxin subunit B (CTB). In certain embodiments, the GM1 is in a biological membrane coated on the surface of the functionalization layer.

[0091] In some embodiments, the method comprises using the graphene bio-electronic sensing devices to detect the binding between a target of interest in a biological membrane coated on the surface of the functionalization layer and a ligand. In some of these embodiments, as shown in Figure 7, the biological membrane is made into a cushioned supported membrane to allow proteins to move more fluidly. In some of these embodiments, the biological membrane is raised by polyethylene glycol (PEG) and 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine (DSPE). In certain embodiments, DSPE is replaced by DPPE, DOPE, or POPE.

Equivalents and Scope

[0092] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments in accordance with the invention described herein. The scope of the present invention is not intended to be limited to the above Description, but rather is as set forth in the appended claims.

[0093] In the claims, articles such as "a," "an," and "the" may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

[0094] Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and

understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

[0095] In addition, it is to be understood that any particular embodiment of the present invention that falls within the prior art may be explicitly excluded from any one or more of the claims. Since such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded even if the exclusion is not set forth explicitly herein. Any particular embodiment of the compositions of the invention *(e.g.,* any nucleic acid or protein encoded thereby; any method of production; any method of use; *etc.)* can be excluded from any one or more claims, for any reason, whether or not related to the existence of prior art.

[0096] All cited sources, for example, references, publications, databases, database entries, and art cited herein, are incorporated into this application by reference, even if not expressly stated in the citation. In case of conflicting statements of a cited source and the instant application, the statement in the instant application shall control.

[0097] Section and table headings are not intended to be limiting.

EXAMPLES

[0098] Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Efforts have been made to ensure accuracy with respect to numbers used *(e.g.,* amounts, temperatures, *etc.),* but some experimental error and deviation should, of course, be allowed for.

[0099] The practice of the present invention will employ, unless otherwise indicated, conventional methods of biophysics, surface chemistry, nanotechnology, protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. *See, e.g.,* T.E. Creighton, Proteins: Structures and Molecular Properties (W.H. Freeman and Company, 1993); A.L. Lehninger, Biochemistry (Worth Publishers, Inc., current addition); Sambrook, *etal,* Molecular Cloning: A Laboratory Manual (2nd Edition, 1989); Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); Remington's Pharmaceutical Sciences, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); Carey and Sundberg Advanced Organic Chemistry 3rd Ed. (Plenum Press) Vols A and B(1992).

Example 1: Fabrication of graphene bio-electronic sensor.

[00100] The details of making two embodiments of graphene bio-electronic sensor are shown below.

[00101] A silicon wafer was used as the supporting substrate for the device fabrication. A 100 nm to 300 nm thick layer of thermally grown S1O2 served as the electrically insulating layer between the graphene device and the silicon. As shown in Figure 4a, a monolayer of graphene was transferred onto the supporting substrate and placed directly onto the substrate insulating layer. Detailed methods and materials for transfer of CVD (chemical vapor deposition) graphene sheets onto substrates are described in the literature. See *Saltzgaber, G. et al. Scalable Graphene Field-Effect Sensors for Specific Protein Detection. Nanotechnology 24, 355502 (2013).*

[00102] The transferred graphene sheet was patterned using standard photolithography methods. Briefly, positive i-Line resist (OiR 906-12) was spincoated onto the graphene substrate to ~1.3 μιη in thickness, exposed, and chemically developed (OPD 4262) to create resist/graphene features that were 20 μιη x 20 μm, 40 μιη x 40 μιτι, and 80 μιη x 80 μιη in area. An O2 RIE plasma etching system was used to etch away the graphene exposed by photolithography. A resist stripper solution (microposit 1165 or PRS3000) was used to remove the protective resist covering the patterned graphene features. After graphene features were patterned, as shown in Figure 4b, standard semiconductor processing techniques were used to generate the pattern for electrodes, with subsequent metal deposition, and lift-off to expose desired electrode pattern above the graphene pattern. Ti and Au were deposited onto the substrates by thermal evaporation, where Ti was used as an adhesion layer for Au. After the evaporation was complete, the resist and excess Ti/Au was removed using a standard lift-off step. The electrode dimensions obtained for the proof-of-concept sensors were 40 nm in thickness and 10 μιη to 20 μιη in width. The lengths varied depending on the design of the sensors.

[00103] To make the sensor device compatible with lipid bilayers, conventional surface chemistry, and functional biochemistry, a 1-3 nm thin layer of silicon oxide (S1O2) was deposited or grown on the devices, as shown in Figure 4c. Briefly, silicon (Si) was deposited by electron-beam evaporation up to an estimated thickness of about 15 Angstroms. Such

ultra-thin layer of Si oxidized within 30 minutes under ambient pressure, forming a S1O2 functionalization layer with a thickness in the range of 20 to 25 Angstroms.

[00104] Finally, the wafer was protected with a photoresist, diced into individual chips, mounted onto a printed circuit board (PCB), using methods well known to the skilled in the arts. In order to perform detection or sensing, the PCB mounted chip was incorporated into a flow cell, connected to a vacuum or pressure source, and a buffered sample was introduced to the sensor *via* a fluidic pathway.

[00105] In an alternative embodiment, a silicon wafer was used as the supporting substrate for device fabrication. As shown in Figure 5a, a 100 nm to 300 nm thick layer of thermally grown S1O2 served as the electrically insulating layer between the graphene device and the silicon.

[00106] In this embodiment, as shown in Figure 5b, the electrodes were patterned first. Ti and Au were deposited onto the substrates by thermal evaporation, where Ti was used as an adhesion layer for Au. After evaporation was complete, the resist and excess Ti/Au was removed using a standard lift-off step. The electrode dimensions obtained for the proof-of-concept sensors were 40 nm in thickness and 10 μm to 20 μm in width. The lengths varied depending on the design of the sensors.

[00107] As shown Figure 5c, a monolayer of graphene was transferred onto the supporting substrate and placed directly onto the substrate insulating layer. Detailed methods and materials for transfer of CVD graphene sheets onto substrates are described in the literature. See *Saltzgaber, G. et al. Scalable Graphene Field-Effect Sensors for Specific Protein Detection. Nanotechnology 24, 355502 (2013).* The graphene sheet comprised a monolayer of carbon atoms or a finite multilayer of carbon atoms. The graphene sheet was patterned using resist materials and photolithography methods such that portions of the graphene features were in direct contact with the electrodes.

[00108] To make the sensor device compatible with lipid bilayers, conventional surface chemistry, and functional biochemistry, a 1-3 nm thin layer of silicon oxide (S1O2) was deposited or grown on the devices, as shown in Figure 5d. Briefly, silicon (Si) was deposited by electron-beam evaporation up to an estimated thickness of about 15 Angstroms. Such ultra-thin layer of Si oxidized within 30 minutes under ambient pressure, forming a S1O2 functionalization layer with a thickness in the range of 20 to 25 Angstroms.

[00109] Finally, the wafer was protected with a photoresist, diced to individual chips, mounted onto a PCB, using methods well known to the skilled in the arts. To perform

detection or sensing, the PCB mounted chip was incorporated into a flow cell, connected to a vacuum or pressure source, and a buffered sample was introduced to the sensor *via* a fluidic pathway.

Example 2: Label-free binding assay of pentamer CTB to active GM1 using graphene bio-electronic sensing technology.

Applying the lipid bilayer with integral membrane-bound targets to the functionalization layer

[00110] Figure 6 shows the process of making tethered supported membrane. The functionalization layer on the sensor surface was first functionalized with a monolayer of APTES by vapor deposition, using methods well known to the skilled of the art. NHS-mPEG (95%) and NHS-PEG-PE (5%) were mixed and dissolved the mixture in DMF containing 100 mM Triethylamine to get 0.3 mM of NHS-mPEG. The sensor surface was incubated in the DMG/PEG solution overnight at room temperature, followed by rinse with isopropanol and dried under N_2 gas. The desired lipids dissolved in chloroform were mixed. The lipid was dried under N_2 while vortexing to form a dry film, then dissolved in IPA to reach 1.5 mg/mL. The lipid solution was spin coated on top of a PEGylated sensor surface with 500 rpm for 10 s then 3000 rpm for 120 s. The slide was dried under vacuum for at least 1 hr. To rehydrate the PEG layer, the slide was placed in a flow cell and exposed the surface to water at least 300 μL/miη flow rate for 1.5 hr with a 60 ml syringe. A tethered supported membrane was formed. To incorporate a transmembrane protein, the surface was incubated with proteoliposomes containing the protein of interest in PBS buffer containing 20 mM EDTA (or 8K PEG) over the sample for 1 hr.

Making a cushioned supported membrane

[00111] A cushioned supported membrane can be made to allow proteins to move more fluidly in the membrane. The functionalization layer was cleaned with RTE. Proteoliposomes were first incubated on the sensor surface for 15 min. Unbound proteoliposomes were then washed away by gentle flow. SUV (small unilamellar vesicles) containing 0.1% - 5% PEG-PE and 95% - 99.9% POPC were prepared using methods well known in the art. The surface was then incubated with the SUV in PBS buffer containing 20 mM EDTA for lhr. Excess

SUVs were washed by gentle flow. A schematic picture of a cushioned lipid bilayer attached to the functionalization layer of sensor is shown in Figure 7.

Fluidity of lipid bilayer on the SiO_2 functionalization layer

[00112] The GFET sensor can be covered with a supported lipid bilayer (SLB) and fluidity measurements of the lipid bilayer can be performed using fluorescence recovery after photobleaching (FRAP). If the graphene surface of the GFET sensor was not homogenously covered by the SiO_2 functionalization layer, the supported lipid bilayer would not be fluid. On the other hand, if the SLB were to demonstrate fluidity greater than 0.5 $\mu\eta/cm^2$, then it was concluded that indeed the supported lipid bilayer had formed above the protected graphene surface due to the oxide functionalization interface.

[00113] As shown in Figure 8, lipid bilayer fluidity was measured by fluorescence recovery after photobleaching (FRAP) by incorporating a fluorescently labeled lipid component. A ~50 μm spot was first photobleached by high intensity light. The recovery of fluorescence was recorded as a function of time. The recovery curve was then fitted with the diffusion equation to obtain the diffusion coefficient.

[00114] These data evidence that the methodologies described in detail herein were capable of supporting a fluid lipid bilayer comprising macromolecules capable of binding to ligands of interest. The data also show that the S1O2 functionalization layer was uniformly applied across the graphene layer.

Label-free association & dissociation of pentamer CTB to active GMl

[00115] A bio-electronic measurement was made to confirm that the conductance and sensing capability of the GFET was retained. In Figure 9A, a SLB containing membrane-bound ganglioside 1 (GMl) was formed on the functionalization layer of the GFET surface. Then, cholera toxin subunit B (CTB) was used as the analyte partner that binds to multiple fluid GMls on the top of the SLB.

[00116] Small unilamellar vesicles (SUVs) containing 5% GMl and 95% egg-PC was formed by standard methods. The sensor surface was cleaned by RIE and exposed to SUVs for 30 min. Excess vesicles were washed away by gentle flow. Cholera toxin subunit B (CTB) in 5 mM HEPES buffer was slowly flowed through the sensor surface and the change of current was recorded as a function of time. After the label-free binding reached a steady state, the solution was switched to one without CTB and dissociation of CTB was recorded.

The label-free binding kinetics of CTB and GM1 is presented in Figure 9B. As shown in Figure 10, the experiments were repeated with different concentrations of CTB: 6 nM, 12 nM, 24 nM, and 48 nM. As shown in Figure 11, the change of current at each steady state was used to plot the saturation binding curve and a multivalent Langmuir isotherm was used to fit the curve to obtain Kd value. A comparison to labeled results from fluorescence assay and flow cytometry is also included in Figure 11.

Example 3: Conjugation of biomolecules to functionalization layer and binding assay.

Conjugating biomolecules to the functionalization layer

[00117] To effectively conjugate silanes (e.g. APTES) to the functionalization layer, the devices were processed under oxygen plasma for a very limited time (e.g. 1 minute) to generate hydroxyl radicals on the surface. The APTES was then conjugated using a vapor evaporation process or a solution absorption process. Time and temperature are critical parameters of these processing steps (e.g. 150 °C for 2 hours). Once the silane was conjugated to the protective layer, it was characterized by fluorescent microscopy and/or atomic force microscopy (AFM) methods to quantify its homogeneity.

[00118] Next we conjugated amine linkers (e.g. BS3) to the silane (e.g. APTES) to create an activated surface for covalent attachment of amine-containing molecules and macromolecules such as proteins and/or antibodies. Proper density of the amine linkers and the covalently conjugated proteins were controlled using time and concentration as the main parameters. The chip surface was exposed to 25 mM to 50 mM BS3 at room temperature for 30 min and then washed with phosphate buffer. Subsequently, the surface was exposed to protein concentrations between 50 ug/ml to 500 ug/ml at room temperature for 60 min. The process of conjugating biomolecules to the functionalization layer is shown in Figure 12.

Detection of binding to the biomolecules with the graphene sensor

[00119] Once target molecules, proteins, and/or antibodies were conjugated onto the sensor surface, ligands were injected or absorbed onto the sensing surface to acquire label-free real time association, steady-state, and dissociation results of molecular interactions. The ligand of interest binds to the conjugated target protein (e.g. membrane protein or antibody) on the surface at a specific rate of association and dissociation. Ligand concentrations were measured. Kinetic information and kinetic constants were acquired due to the label-free

intrinsic nature of the measurement method. The affinity (Kd) of the ligand to the conjugated protein, which is in direct relation with the association **(kon)** and dissociation **(koff)** constants, was also determined.

Example 4: Binding of ligand R715 to native bradykinin receptor Bl in cell membranes.

[00120] R715 is a peptide that specifically binds the Bradykinin receptor Bl (BDKR Bl) with very high affinity (KD = ~ 1 nM). BDKR Bl is a G-protein coupled receptor (GPCR). GPCRs and other integral membrane receptors are unstable when purified from the cell membrane, thus hampering efforts to measure kinetic binding of drug candidates to this important class of therapeutic targets.

[00121] In this example we demonstrate the direct measurement of kinetic binding of peptide R715 to BDKR Bl using the GBEST sensor described herein without the need for receptor purification. Binding of drug candidates to receptors were measured in their native conformation in the cell membrane.

[00122] Native cell membrane vesicles (NMVs) were prepared by methods well known in the art, from CHO cells expressing BDKR Bl on the cell membrane. NMVs were used to prepare a cushioned supported membrane on a GBEST sensor as described in Example 2. After membrane formation on the GBEST sensor, samples containing different concentrations of peptide R715 were flowed over the sensors at 25 μL/ηιη and binding response was monitored in real time. Fig 13 shows the binding response for R715 at 10 nM and 100 nM concentrations.

OTHER EMBODIMENTS

[00123] It is to be understood that the words which have been used are words of description rather than limitation, and that changes may be made within the purview of the appended claims without departing from the true scope and spirit of the invention in its broader aspects.

[00124] While the present invention has been described at some length and with some particularity with respect to the several described embodiments, it is not intended that it should be limited to any such particulars or embodiments or any particular embodiment, but it is to be construed with references to the appended claims so as to provide the broadest possible interpretation of such claims in view of the prior art and, therefore, to effectively encompass the intended scope of the invention.

[00125] All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, section headings, the materials, methods, and examples are illustrative only and not intended to be limiting.

CLAIMS

1. A device, comprising:

a support substrate;

a graphene layer, said graphene layer deposited on said support substrate;

a functionalization layer, comprising solid state material, said functionalization layer deposited continuously on at least a portion of said graphene layer; and

electrodes in electrical contact with said graphene layer, said electrode adapted to detect an electrical signal from said graphene layer.

2. The device of claim 1, further comprising a substrate insulating layer, wherein said substrate insulating layer deposited on said support substrate, wherein said graphene layer deposited on said substrate insulating layer.

3. The device of claim 1 or 2, wherein said electrodes are deposited on top of said graphene layer to generate an electrical connection.

4. The device of claim 1 or 2, wherein said graphene layer is deposited on top of said electrodes to generate an electrical connection.

5. The device of any one of claims 1 to 4, wherein said support substrate comprises a material selected from the group consisting of: silicon, glass, quartz, **S1O2,** silicon/SiCh, GaAs, GaN, polyethylene terephthalate (PET), and other polymer based materials.

6. The device of claim 5, wherein said support substrate comprises silicon and/or **S1O2.**

7. The device of any one of claims 1 to 6, wherein said support substrate has a thickness of 50 μιη to 5000 μιη.

8. The device of any one of claims 2 to 7, wherein said substrate insulating layer comprises an oxide.

9. The device of claim 8, wherein said oxide is selected from the group consisting of: **S1O2, T1O2,** AI2O3, Ta_2Os, Fe_30_4, and $Zr0_2$.

10. The device of any one of claims 2 to 9, wherein said substrate insulating layer has a thickness of 5 nm to 50000 nm.

11. The device of any one of claims 2 to 10, wherein said substrate insulating layer has a dielectric constant of 1 to 100.

12. The device of any one of claims 1 to 11, wherein said graphene layer is a graphene monolayer.

13. The device of claim 12, wherein said graphene monolayer consists of one atomic layer of carbon atoms for at least 50% of its total area.

14. The device of any one of claims 1 to 13, wherein said graphene layer has a thickness of 1 atomic layer to 3 atomic layers.

15. The device of any one of claims 1 to 14, wherein said graphene layer is patterned.

16. The device of any one of claims 1 to 15, wherein said graphene layer is in electrical contact with at least two electrodes.

17. The device of any one of claims 1 to 16, wherein said solid state material is an oxide, a nitride, or an oxinitride.

18. The device of claim 17, wherein said oxide is selected from the group consisting of: SiO_2, T1O2, AI2O3, Ta_2Os, Fe_3O_4, and ZrO_2.

19. The device of claim 17, wherein said nitride or oxinitride is selected from the group consisting of: TiN, A1N, TiAIN, TiCN, TiOxNy, SiOxNy, SiN, and Si_3N_4.

20. The device of any one of claims 1 to 19, wherein said functionalization layer has a thickness of 1 nm to 10 nm.

21. The device of any one of claims 1 to 20, wherein said functionalization layer has a dielectric constant of 1 to 100.

22. The device of any one of claims 1 to 21, wherein the product of the dielectric constant and the thickness of said functionalization layer is less than 1000 nm.

23. The device of any one of claims 1 to 22, wherein said functionalization layer further comprises a reactive group.

24. The device of claim 23, wherein said reactive group is selected from the group consisting of: a carboxyl group, a hydroxyl group, an amine group, an epoxy group, an aldehyde group, and a sulfhydryl group.

25. The device of claim 23 or 24, wherein a functionalization molecule is bound to the functionalization layer.

26. The device of claim 25, wherein the functionalization molecule is covalently bound to a reactive group of the functionalization layer.

27. The device of claim 25 or 26, wherein said functionalization molecule is silane.

28. The device of claim 27, wherein said silane is selected from the group consisting of: 3-aminopropyltrimethoxy silane (APTMS, amino), 3-aminoproplyltriethoxy silane (APTES, amino), 3-isocyanatopropyltriethoxysilane (CYNPS, isocyanate), triethoxysilylbutyraldehyde (ALDPS, aldehyde), (3-glycidoxypropyl)trimethoxysilane (GPS, epoxy), 3-mercaptopropyltrimethoxysilane (MPS, sulfur), 7-octenyltrimethoxysilane (OTS, vinyl), 3-methacryloxypropyltrimethoxysilane (acrylate), 3,4-epoxycyclohexyltrimethoxysilane (ECPS, epoxy), 10-undecenyltrichlorosilane (V1 1TCS, vinyl), and carboxylethylsilanetriol.

29. The device of any one of claims 23 to 28, wherein said functionalization molecule has a size of 1 nm to 20 nm.

30. The device of any one of claims 1 to 29, wherein said functionalization layer is homogenous.

31. The device of claim 30, wherein said functionalization layer is homogenous as determined by a lipid bilayer applied to the surface of said functionalization layer having a fluidity of at least 0.5 $\mu m/cm^2$.

32. The device of any one of claims 1 to 31, wherein said electrodes comprise a source and a drain.

33. The device of any one of claims 1 to 32, wherein said electrode is connected to an electrical power supply configured to generate an electrical potential on said graphene layer.

34. The device of any one of claims 1 to 33, wherein said electrode is configured to measure a current or change in current through said graphene layer.

35. The device of any one of claims 1 to 34, wherein said electrode comprises a metal selected from the group consisting of: gold, titanium, aluminum, copper, silver, platinum, palladium, and combinations thereof.

36. The device of any one of claims 1 to 35, wherein said electrode has a thickness of 10 nm to 1000 nm.

37. The device of any one of claims 1 to 36, wherein said device comprises a biological membrane layered on said functionalization layer.

38. The device of claim 37 or 38, wherein said biological membrane is in native state.

39. The device of claim 37, wherein said biological membrane comprises a target molecule capable of binding to a ligand molecule.

40. The device of claim 39, wherein said target molecule is in native state.

41. The device of claim 39 or 40, wherein said target molecule is selected from the group consisting of: an integral membrane protein, a membrane associated protein, a glycoprotein, a phospholipid, and a glycolipid.

42. The device of any one of claims 37 to 41, wherein said biological membrane is a lipid bilayer.

43. The device of claim 42, wherein said lipid bilayer has a fluidity of at least 0.5 μιη/cm^2.

44. The device of any one of claims 37 to 43, wherein said biological membrane is derived from a cell.

45. A method of synthesizing a graphene sensor device, comprising:

a. providing a graphene field effect transistor comprising a graphene layer and a pair of electrodes in electrical contact with said graphene layer;

b. forming a functionalization layer continuously on said graphene layer, wherein said functionalization layer comprises solid state material.

46. The method of claim 45, wherein said solid state material is an oxide.

47. The method of claim 46, wherein said oxide is selected from the group consisting of: SiO_2, T1O2, AI2O3, Ta_2Os, Fe_3O_4, and ZrO_2.

48. The method of any one of claims 45 to 47, wherein forming said functionalization layer comprises depositing a silicon layer on said graphene layer, and placing said silicon layer in oxidizing conditions to form a layer of S1O2.

49. The method of any one of claims 45 to 48, wherein said functionalization layer has a thickness of 1 nm to 10 nm.

50. The method of any one of claims 45 to 49, further comprising applying a lipid bilayer to said functionalization layer.

51. The method of any one of claims 45 to 50, further comprising covalently binding a silane molecule to said functionalization layer.

52. The method of claim 51, further comprising covalently binding a target molecule to said silane molecule.

53. The method of claim 52, wherein said target molecule is capable of binding to a ligand molecule.

54. The method of claim 52 or 53, wherein said target molecule is in native state.

55. A method of detecting a ligand molecule, comprising

a. providing the device of any one of claims 1-44;

b. applying an electrical potential to said graphene layer;

c. contacting said device with a sample suspected of comprising a ligand molecule of interest; and

d. collecting information generated by said device comprising changes in electrical current over time to determine whether or not said ligand molecule is present in said sample.

56. The method of claim 55, wherein said ligand molecule is capable of binding to a target molecule bound to said functionalization layer.

57. The method of claim 55 or 56, wherein said target molecule is in native state.

58. The method of any one of claims 55 to 57, wherein said ligand molecule is label-free.

59. A method of quantifying the concentration of a ligand molecule, comprising:

a. providing the device of any one of claims 1-44;

b. applying an electrical potential to said graphene layer;

c. contacting said device with a sample suspected of comprising a ligand molecule of interest; and

d. collecting information generated by said device comprising the magnitude of change in electrical signal before and after contacting said device with said sample, and comparing said data to a database comprising change in electrical signal from known concentrations of the ligand molecule.

60. The method of claim 59, wherein said ligand molecule is label-free.

61. A method of quantifying the binding kinetics between a macromolecule and a ligand, comprising:

a. providing the device of any one of claims 1-44;

b. applying an electrical potential to said graphene layer;

c. contacting said device with a sample suspected of comprising a ligand molecule of interest; and

d. collecting information generated by said device comprising changes in electrical current over time to quantify the binding kinetics between the macromolecule and the ligand.

62. The method of claim 61, wherein said binding kinetics comprises the dissociation constant.

63. The method of claim 61 or 62, wherein said macromolecule is in native state.

64. The method of any one of claims 61 to 63, wherein said macromolecule is a receptor.

65. The method of any one of claims 61 to 64, wherein said ligand molecule is label-free.

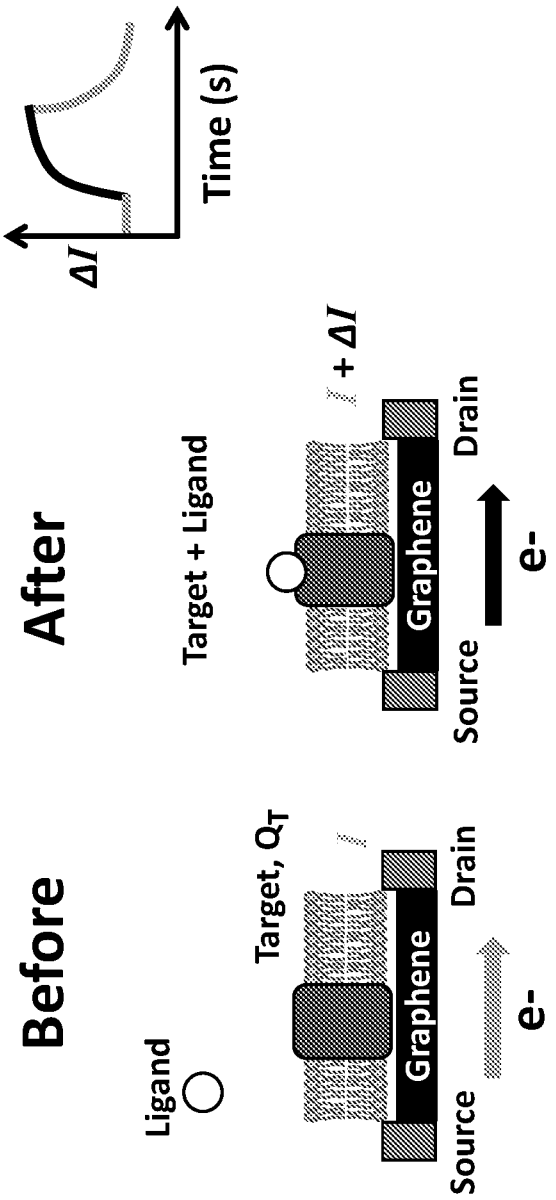

Before
Ligand
Target, Q_T
Graphene
Source
Drain
e-
After
Target + Ligand
$I + \Delta I$
Graphene
Source
Drain
e-
ΔI
Time (s)

Figure 1

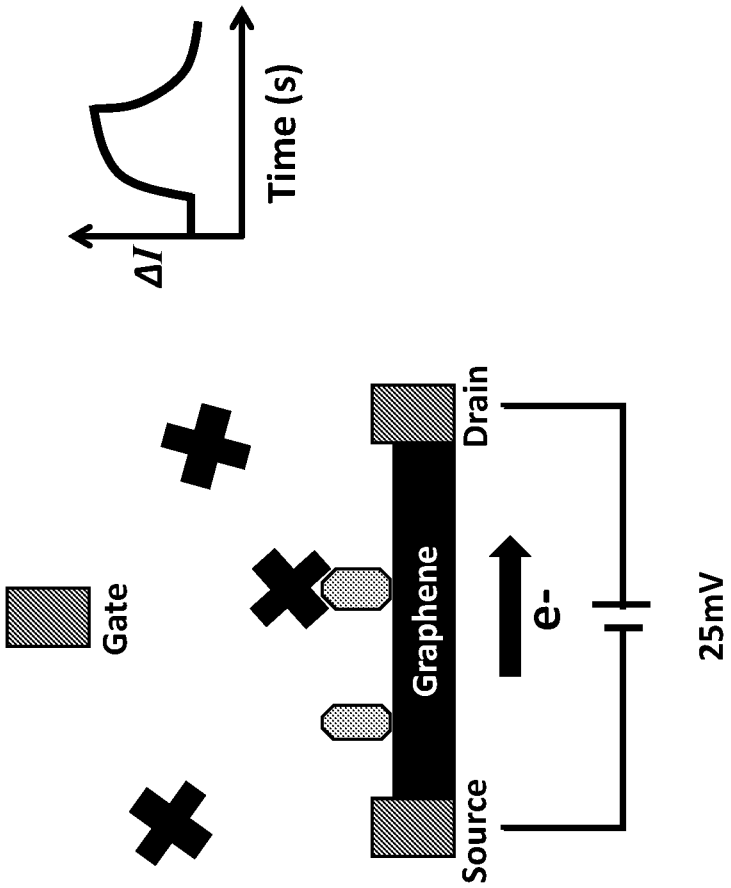


Figure 2

Technology	K_D (nM)
GBEST-device 1	29.2 ± 1.7
GBEST-device 2	31.1 ± 7.9
Biacore	25.9 ± 2.1

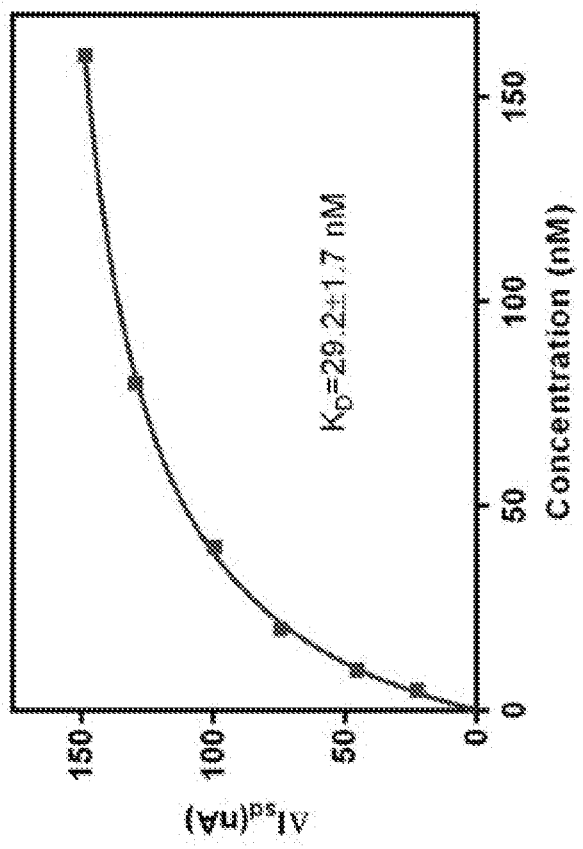
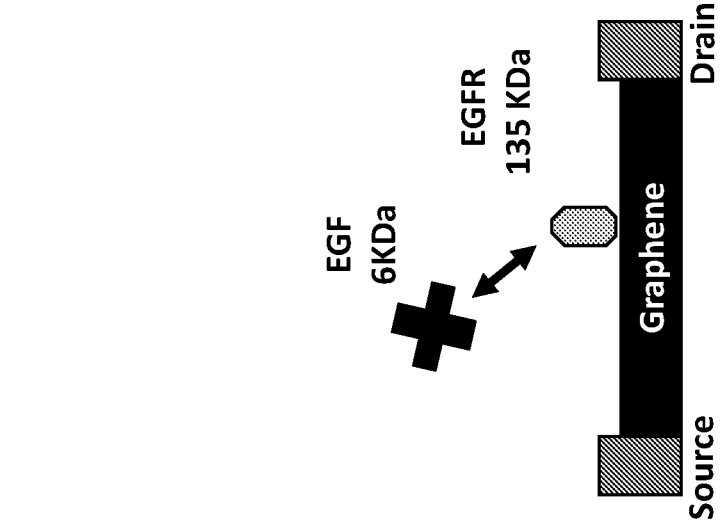


Figure 3



EGFR-EGF interaction

Layer/ Element
Key Code
Substrate
Insulating Layer
Transferred Graphene
Electrodes
Functionalization Layer
4a.
4b.
4c.

Figure 4

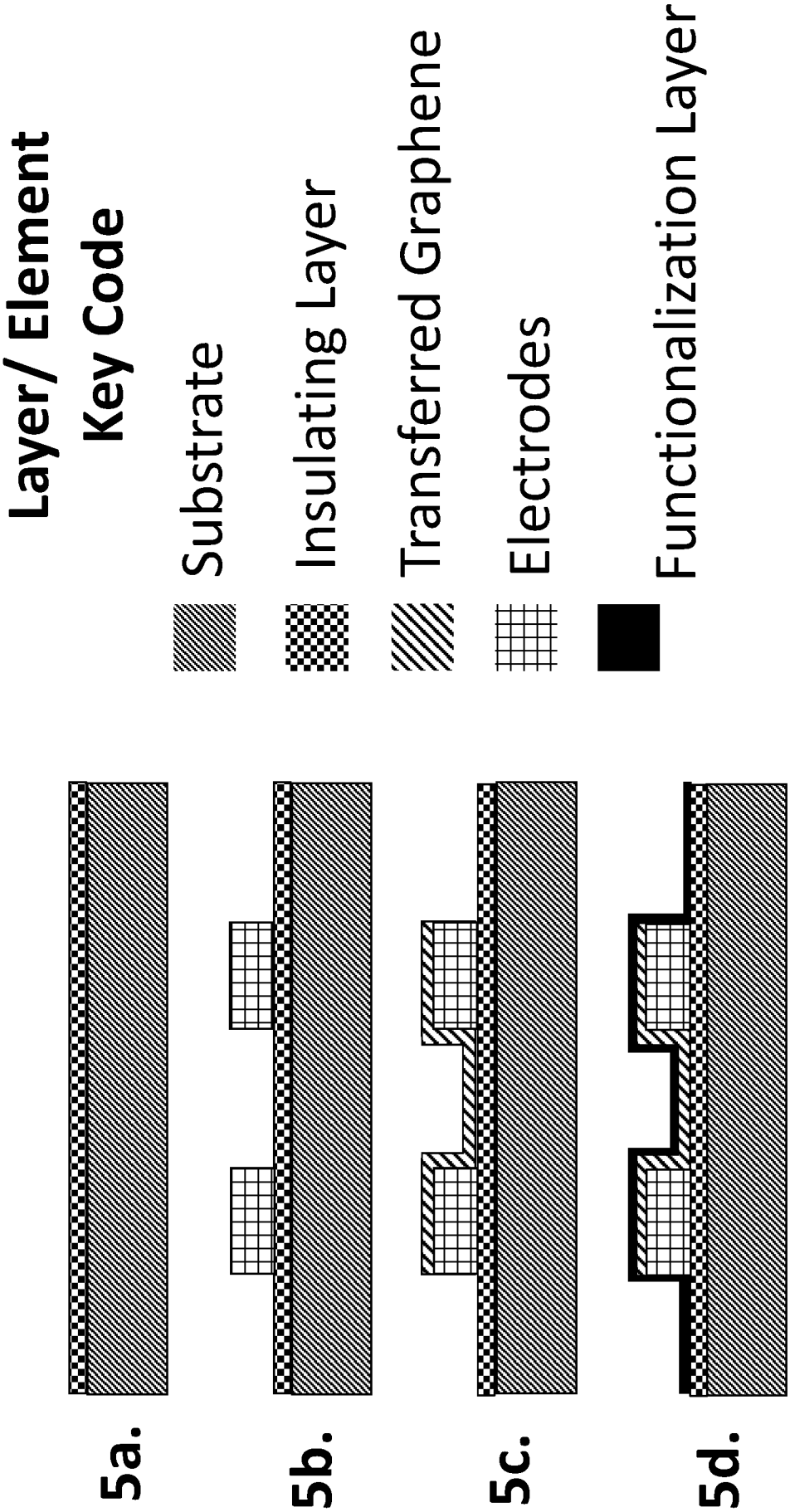

Layer/ Element
Key Code
Substrate
Insulating Layer
Transferred Graphene
Electrodes
Functionalization Layer
5a.
5b.
5c.
5d.

Figure 5

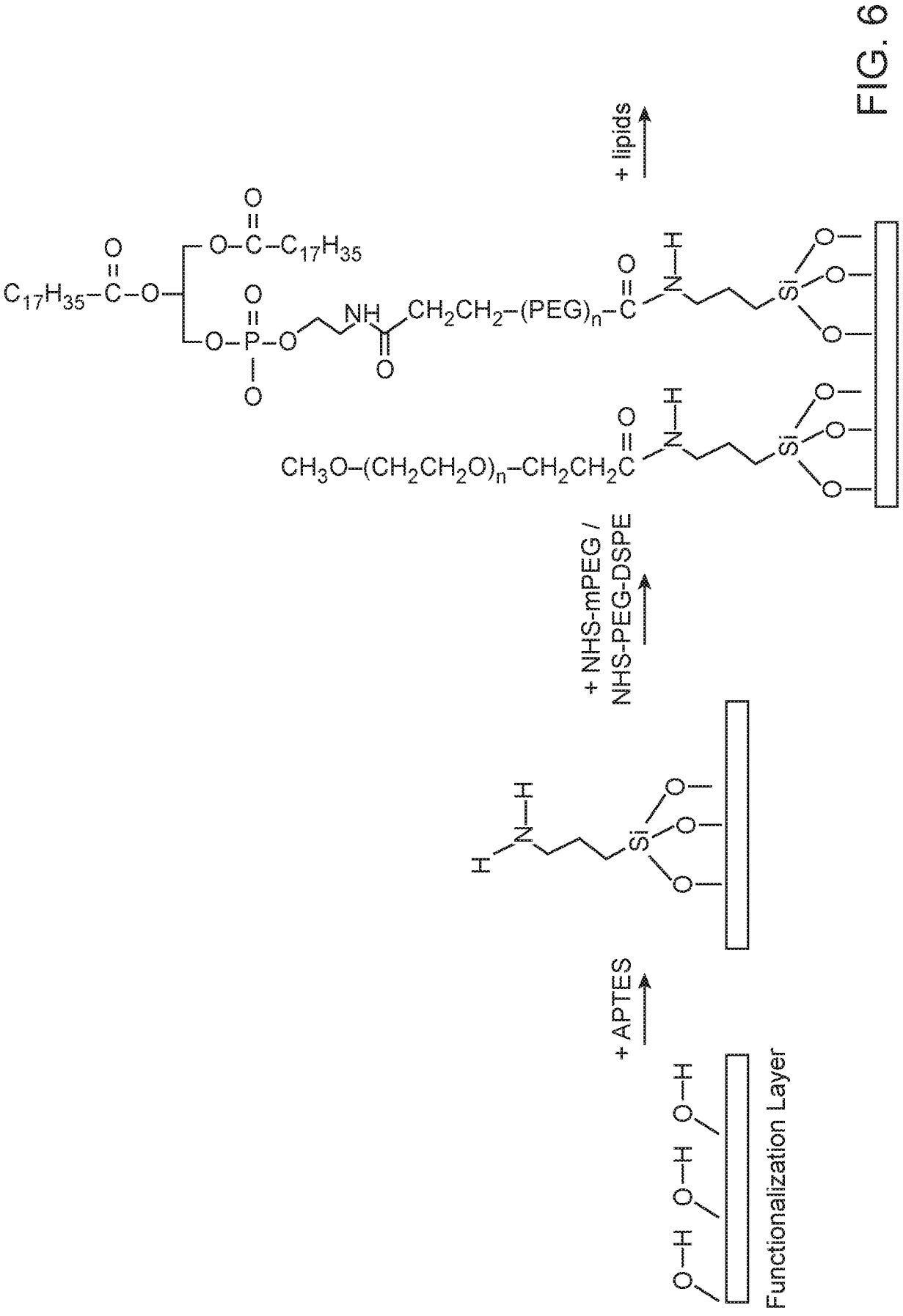

+ APTES
Functionalization Layer
+ NHS-mPEG / NHS-PEG-DSPE
+ lipids

FIG. 6

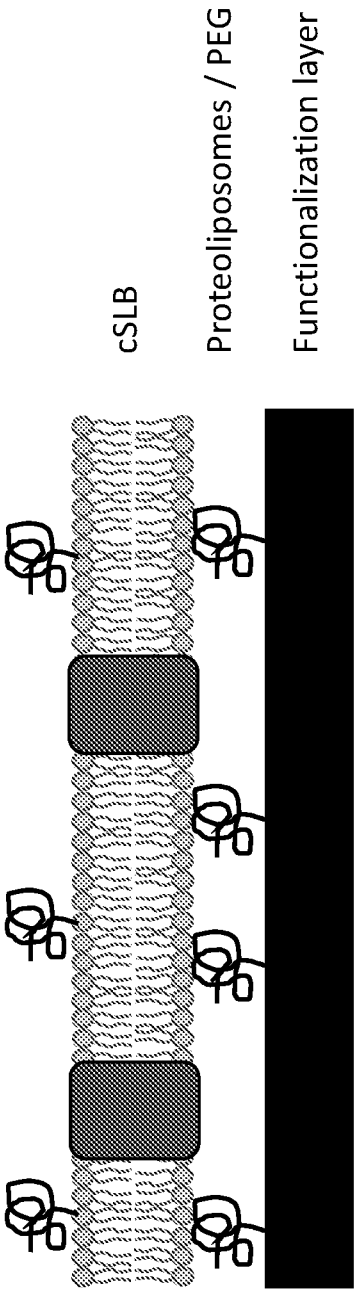


Figure 7

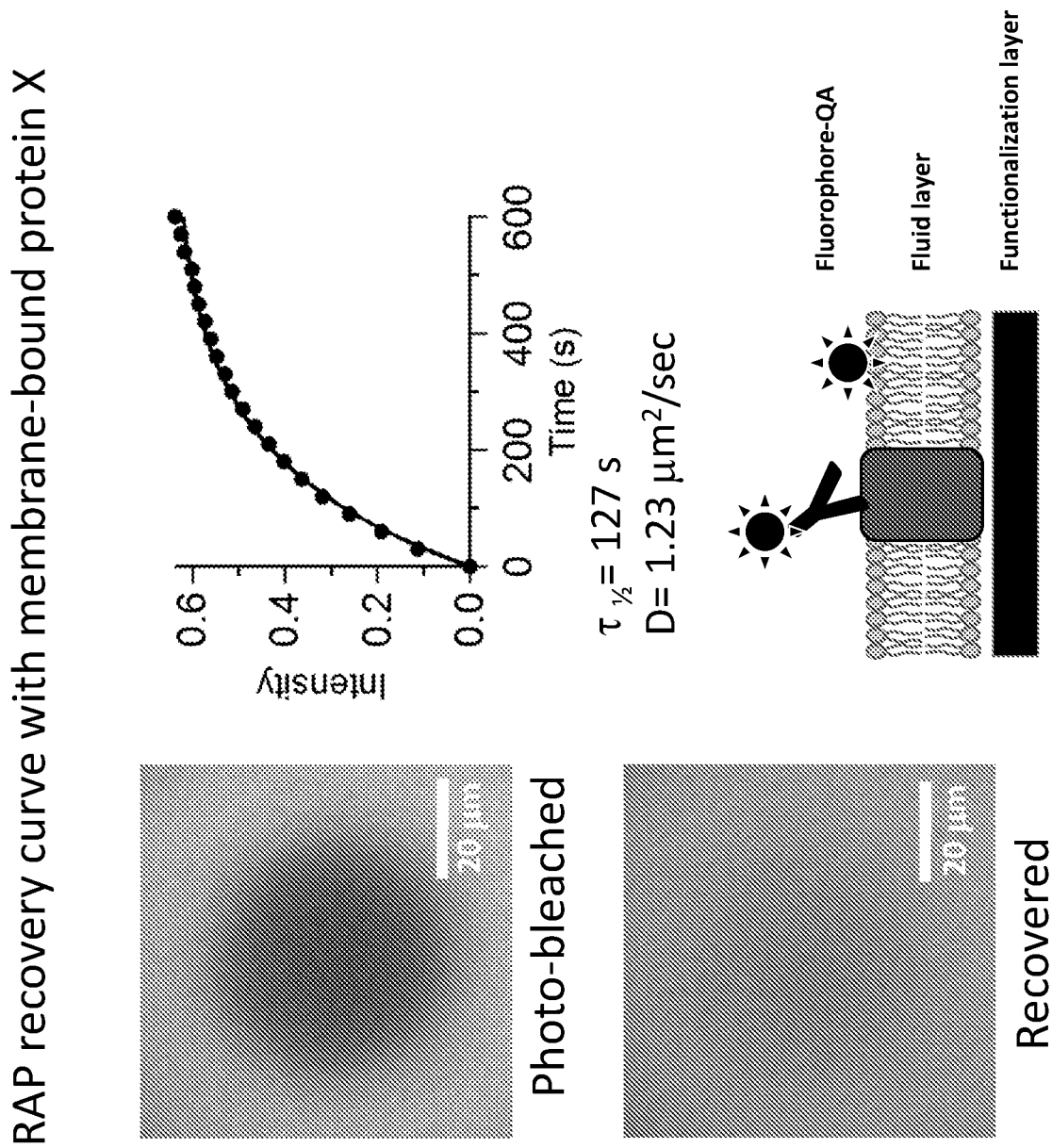

FRAP recovery curve with membrane-bound protein X
Intensity
0.0
0.2
0.4
0.6
0
200
400
600
Time (s)
τ ½= 127 s
D= 1.23 μm²/sec
Fluorophore-QA
Fluid layer
Functionalization layer
Photo-bleached
20 μm
Recovered
20 μm

Figure 8

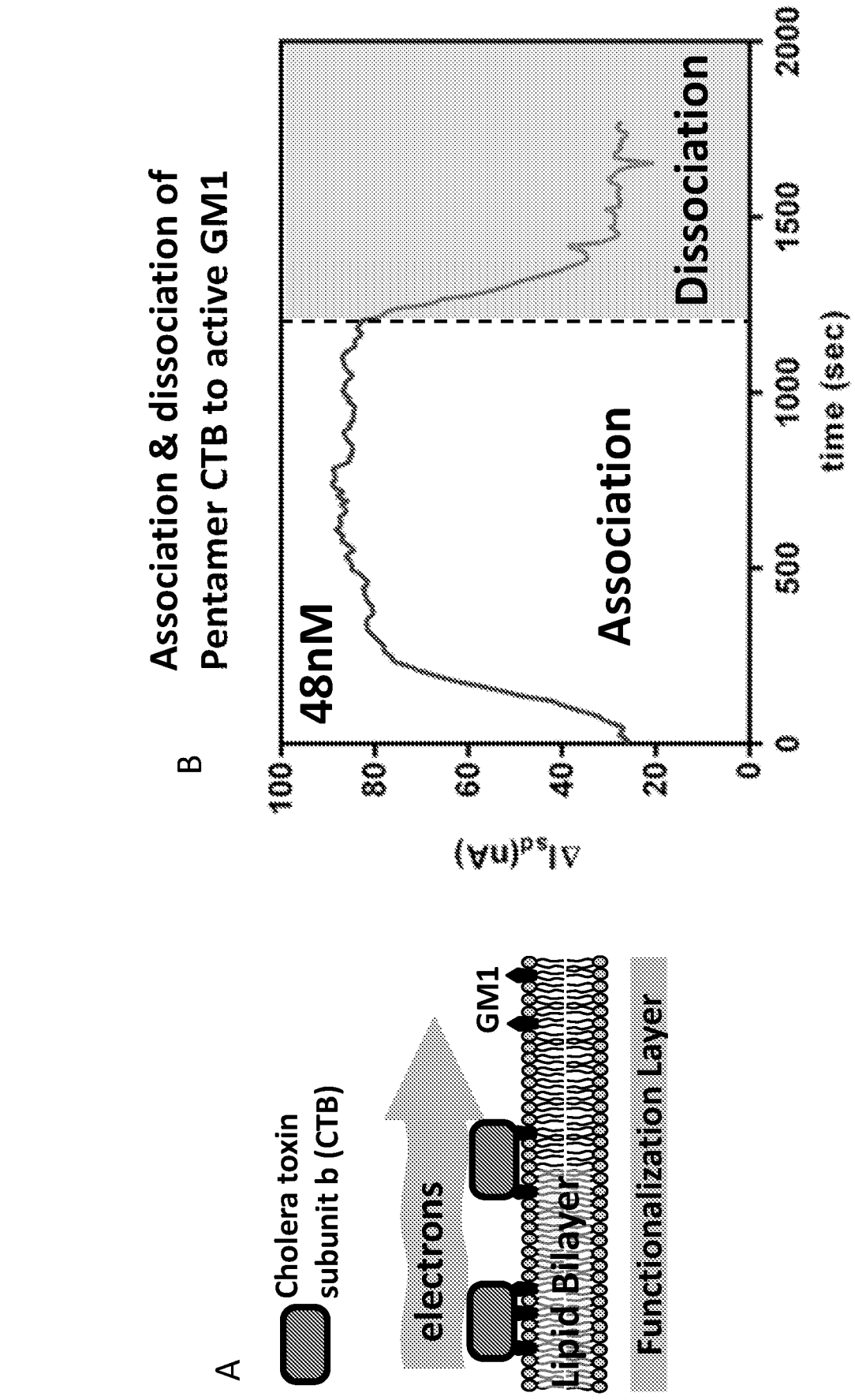

A
Cholera toxin subunit b (CTB)
electrons
GM1
Lipid Bilayer
Functionalization Layer
B
Association & dissociation of Pentamer CTB to active GM1
48nM
Association
Dissociation
ΔI_{sd}(nA)
time (sec)
0
20
40
60
80
100
0
500
1000
1500
2000

Figure 9

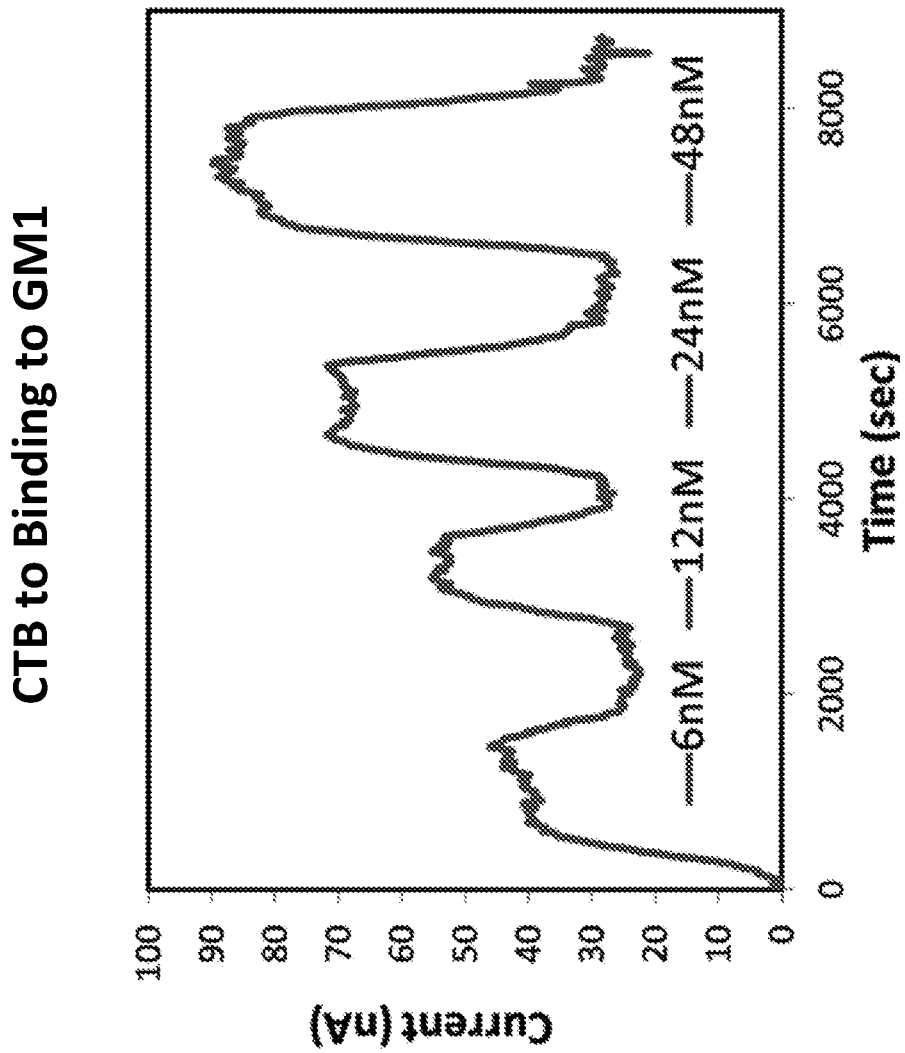
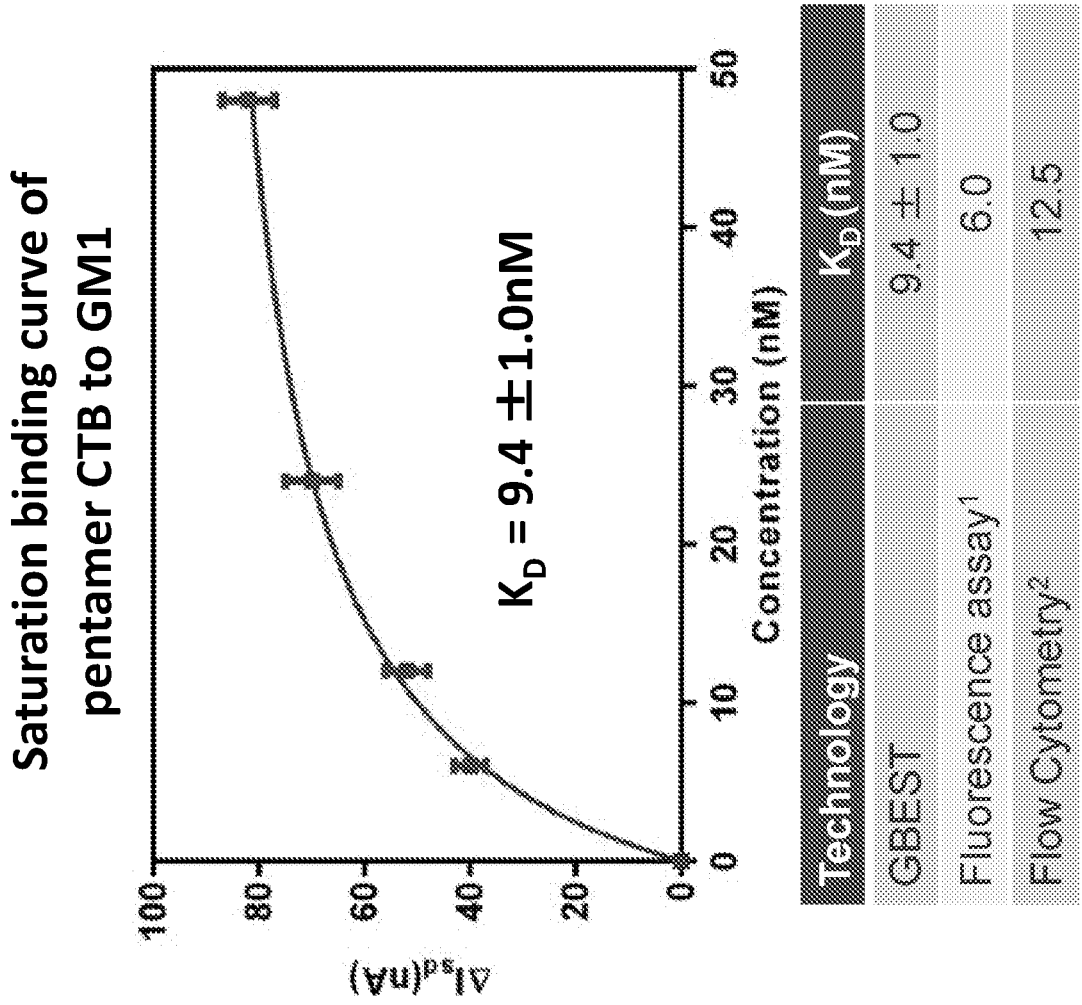

CTB to Binding to GM1
Current (nA)
Time (sec)
6nM
12nM
24nM
48nM
0
10
20
30
40
50
60
70
80
90
100
0
2000
4000
6000
8000

Figure 10



Technology	K_D (nM)
GBEST	9.4 ± 1.0
Fluorescence assay[1]	6.0
Flow Cytometry[2]	12.5

Figure 11

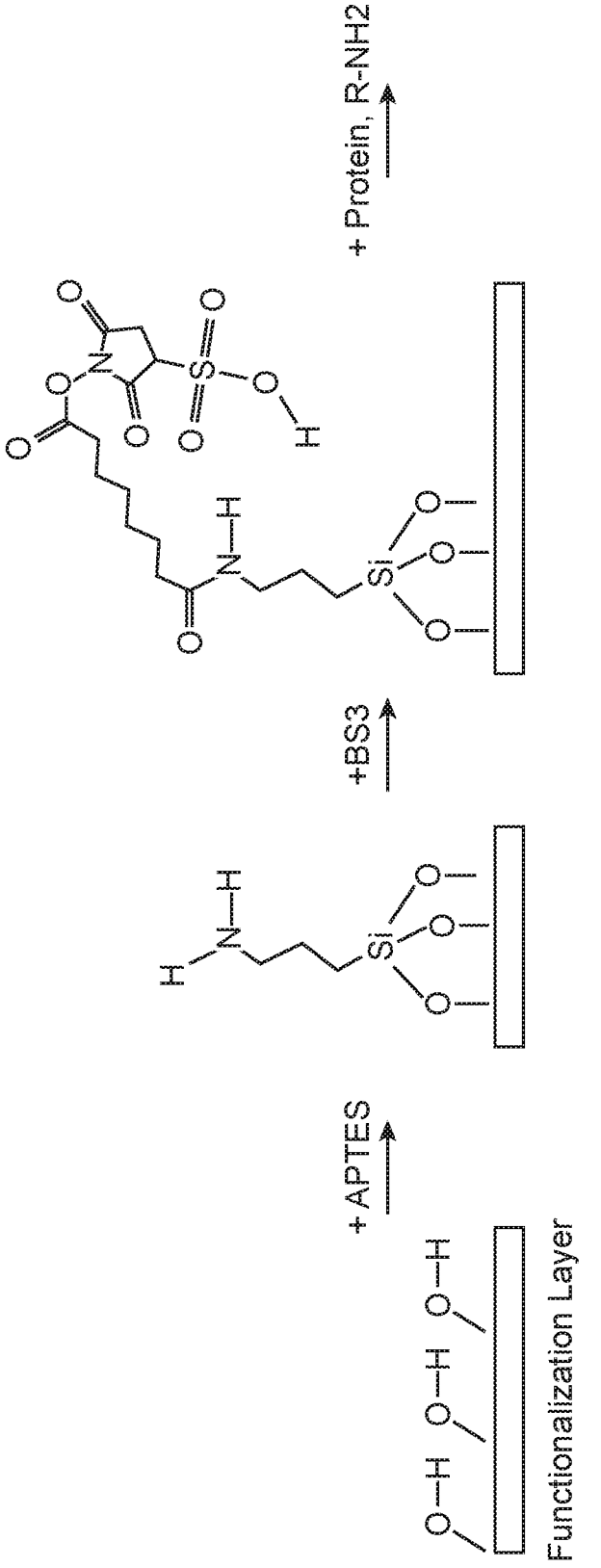


FIG. 12

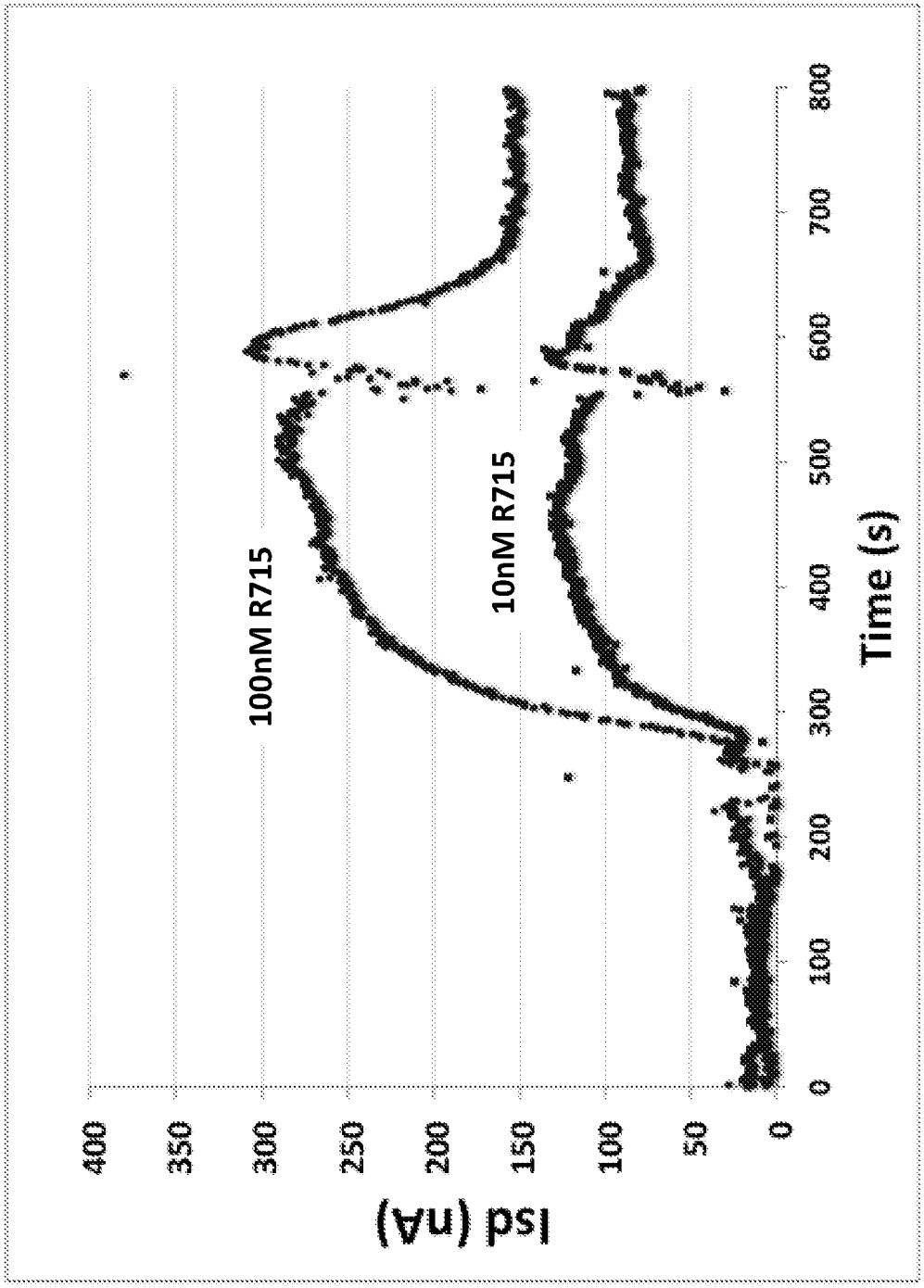

Isd (nA)
400
350
300
250
200
150
100
50
0
0
100
200
300
400
500
600
700
800
Time (s)
100nM R715
10nM R715

Figure 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US201 7/041 563

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - B82Y 15/00; B82Y 10/00; B82Y 40/00; C01B 31/00; G01 N 27/414 (201 7.01)

CPC - H01 L 29/1606; B82Y 15/00; B82Y 30/00; B82Y 40/00; C01B 32/1 94 (201 7.08)

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)

See Search History document

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

USPC - 257/29; 438/49; 438/478; 977/734 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 201 1/0089404 A1 (MARCUS et al) 21 April 201 1 (21 .04.201 1) entire document	1-4, 45-48
A	US 7,767,1 14 B2 (GORDON et al) 03 August 2010 (03.08.2010) entire document	1-4, 45-48
A	US 7,449,1 33 B2 (GRUNER et al) 11 November 2008 (11.11.2008) entire document	1-4, 45-48
A	US 201 1/001 7979 A1 (MERIC et al) 27 January 201 1 (27.01 .201 1) entire document	1-4, 45-48

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 September 2017	22 SEP 2017

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/041563

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.: 5-44, 49-65
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. Ill 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:

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.:

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.