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(71) Applicant: **AGENCY FOR SCIENCE, TECHNOLOGY AND RESEARCH** [SG/SG]; 1 Fusionopolis Way #20-10 Connexis, Singapore 138632 (SG).

(72) Inventors: **ARYA, Sunil Kumar**; c/o Industry Development, Institute of Microelectronics, 2 Fusionopolis Way, #08-02 Innovis Tower, Singapore 138634 (SG). **PARK, Mi Kyoung**; c/o Industry Development, Institute of Microelectronics, 2 Fusionopolis Way, #08-02 Innovis Tower, Singapore 138634 (SG). **KONGSUPHOL, Patthara**; c/o Industry Development, Institute of Microelectronics, 2 Fusionopolis Way, #08-02 Innovis Tower, Singapore 138634 (SG). **CHUNG, Jaehoon**; c/o Industry Development, Institute of Microelectronics, 2 Fusionopolis Way, #08-02 Innovis Tower, Singapore 138634 (SG).

(74) Agent: **VIERING, JENTSCHURA & PARTNER LLP**; P.O. Box 1088, Rochor Post Office, Rochor Road, Singapore 911833 (SG).

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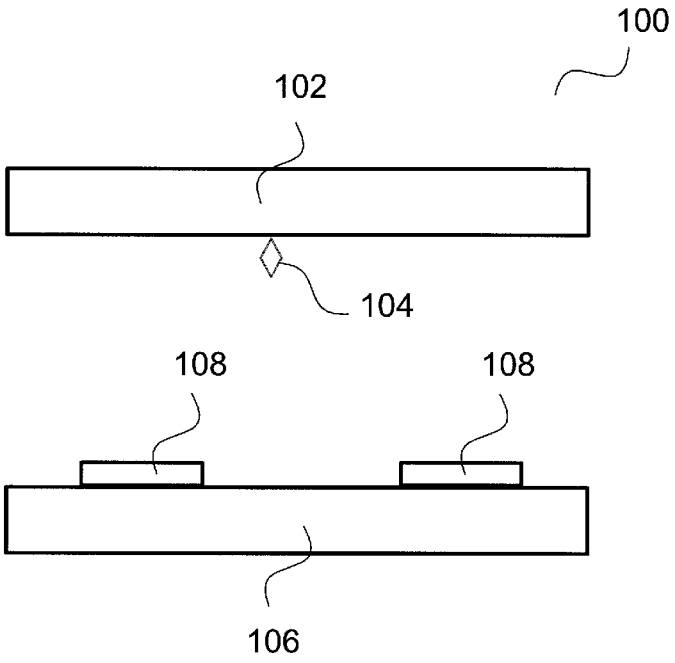
(54) Title: DEVICE FOR ELECTROCHEMICAL IMMUNOASSAY AND METHOD OF FABRICATING THE SAME

FIG. 1



(57) Abstract: Various embodiments provide a device for electrochemical immunoassay. The device includes a recognition surface comprising immobilized biomolecules wherein the immobilized biomolecules bind to a targeted analyte from a sample. The device further includes a sensor surface including a plurality of electrodes configured to detect the targeted analyte wherein the sensor surface and the recognition surface are arranged spaced apart from each other and facing each other.

WO 2016/167724 A1

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DEVICE FOR ELECTROCHEMICAL IMMUNOASSAY AND METHOD OF FABRICATING THE SAME

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of priority of Singapore application No. 10201502889P filed April 13, 2015, the contents of it being hereby incorporated by reference in its entirety for all purposes.

TECHNICAL FIELD

[0002] Various aspects of this disclosure relate to devices for electrochemical immunoassay and methods of fabricating the same.

BACKGROUND

[0003] The requirement for faster, real-time and reliable medical diagnosis has led to growing interest in development of new methods for biomolecular binding and systems that are capable of sensitive and specific detection of biomolecules. In this context, electrochemical biosensors, showing simplicity and capability in direct transduction of biomolecular recognition events into electronic signals, have shown promise.

[0004] However, continuing challenges to the wider use of biosensors include sensitivity, stability, nonspecific signals and false responses in real samples. Sadik et al. (O.A. Sadik, A.O. Aluoch, A. Zhou, Status of biomolecular recognition using electrochemical techniques, Biosensors and Bioelectronics, 24(2009) 2749-65) has recently reviewed the status of biomolecular recognition using electrochemical detection by analyzing the trends, limitations, challenges as well as existing commercial devices in the field of electrochemical biosensors. Integration of biomolecules is a crucial step in biosensor fabrication and is directly related to the biosensor performance. However, use of biomolecules in conventional biosensors often leads to the degradation of electrochemical properties of the sensor surface, which in turn cause variation in chip response and false signals.

SUMMARY

[0005] Various aspects of this disclosure provide a device for electrochemical immunoassay. The device may include a recognition surface including an immobilized

biomolecule. The immobilized biomolecule may be capable of binding to a targeted analyte from a sample. The device may further include a sensor surface including a plurality of electrodes configured to detect the targeted analyte. The recognition surface and the sensor surface may be arranged spaced apart from each other and facing each other.

[0006] Various aspects of the disclosure provide a method of fabricating a device for electrochemical immunoassay. The method may include immobilizing a biomolecule on a recognition surface of the device. The biomolecule may be capable of binding to a targeted analyte from a sample. The method may further include arranging the recognition surface and a sensor surface of the device spaced apart from each other and facing each other. The sensor surface may include a plurality of electrodes for detecting the targeted analyte.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

FIG. 1 shows a cross-sectional side view of a device for electrochemical immunoassay according to various embodiments.

FIG. 2A shows an overhead view of a three-dimensional matrix according to various embodiments.

FIG. 2B shows a cross-sectional side view of the three-dimensional matrix shown in FIG. 2A according to various embodiments.

FIG. 2C shows an overhead view of a two-dimensional membrane having non-uniform distribution of holes according to various embodiments.

FIG. 2D shows an overhead view of a two-dimensional membrane having uniform distribution of holes according to various embodiments.

FIG. 2E shows an overhead view of a two-dimensional membrane with uniform and defined distribution of holes according to various embodiments.

FIG. 2F shows an image of an electrochemical chip according to various embodiments.

FIG. 2G shows a magnified image of sensor surface.

FIG. 2H shows a lower cross-sectional view the electrochemical chip with sensor surface having electrodes.

FIG. 2I shows a middle cross-sectional view of the electrochemical chip.

FIG. 2J shows an upper cross-sectional view of the electrochemical chip.

FIG. 2K shows a lower cross-sectional view the electrochemical chip with sensor surface having electrodes.

FIG. 2L shows a first middle cross-sectional view of the electrochemical chip.

FIG. 2M shows a second middle cross-sectional view of the electrochemical chip including membrane with uniform and defined distribution of holes.

FIG. 2N shows an upper cross-sectional view of the electrochemical chip.

FIG. 3A shows a process of immobilizing or binding biomolecules on a recognition surface according to various embodiments.

FIG. 3B shows a process of conducting a bioassay according to various embodiments.

FIG. 4A shows a cross-sectional side view of an electrochemical chip according to various embodiments.

FIG. 4B is a magnified side view of the area shown in FIG. 4A.

FIG. 4C is an magnified perspective view of the area shown in FIG. 4A.

FIG. 5A shows a cross-sectional side view of an electrochemical chip according to various embodiments.

FIG. 5B is a magnified side view of the area shown in FIG. 5A.

FIG. 6A is a plot of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device with polymethyl methacrylate (PMMA) 3 dimensional (3D) matrix integrated on sensor surface according to one embodiment.

FIG. 6B is a plot of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device with polymethyl methacrylate (PMMA) 3 dimensional (3D) matrix integrated on sensor surface according to one embodiment.

FIG. 6C is a plot of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device with polymethyl methacrylate (PMMA) 3 dimensional (3D) matrix integrated on sensor surface according to one embodiment.

FIG. 7A is a plot of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor

alpha (TNF-α) in undiluted serum in a device with parylene membrane matrix integrated on sensor surface according to one embodiment.

FIG. 7B is a plot of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device with parylene membrane matrix integrated on sensor surface according to one embodiment.

FIG. 7C is a plot of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device with polycarbonate (PC) matrix integrated on sensor surface according to one embodiment.

FIG. 7D is a plot of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device with polycarbonate (PC) matrix integrated on sensor surface according to one embodiment.

FIG. 8 shows a schematic of a device for electrochemical immunoassay according to various embodiments.

FIG. 9A is a plot of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device without nanoparticles but with the second antibody between the recognition surface and the sensor surface according to one embodiment.

FIG. 9B is a plot of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device with second antibody tagged gold nanoparticles between the recognition surface and the sensor surface according to one embodiment.

FIG. 10 is a schematic showing the use of a device with a removable sensor surface according to various embodiments.

FIG. 11 is a flowchart showing the method of fabricating the device for electrochemical assay according to various embodiments.

DETAILED DESCRIPTION

[0008] The following detailed description refers to the accompanying drawings that show, by way of illustration, specific details and embodiments in which the invention may be practiced. These embodiments are described in sufficient detail to enable those skilled in the art to practice the invention. Other embodiments may be utilized and structural, and logical changes may be made without departing from the scope of the invention. The various embodiments are not necessarily mutually exclusive, as some embodiments can be combined with one or more other embodiments to form new embodiments.

[0009] In various embodiments, a device for electrochemical immunoassay may be provided. FIG. 1 shows a cross-sectional side view of a device 100 for electrochemical immunoassay according to various embodiments. The device 100 may include a recognition surface 102 including an immobilized biomolecule 104. The immobilized biomolecule 104 may be capable of binding to a targeted analyte from a sample. The device 100 may further include a sensor surface 106 including a plurality of electrodes 108 configured to detect the targeted analyte. The recognition surface 102 and the sensor surface 106 may be arranged spaced apart from each other and facing each other.

[0010] In other words, the device 100 may include one surface 102 for attaching or binding a biomolecule 104. The device 100 may also include another surface 106, which is at a distance from the surface 102 and which faces surface 102. The surface 106 may include electrodes 108. During operation, a sample, which contains a targeted analyte may flow between the surface 102 and the surface 106, and the targeted analyte may be captured by the biomolecule 104 that is attached or bound to the surface 102.

[0011] The electrochemical immunoassay may be referred to as enzyme linked immunosorbant assay (ELISA). The sample may be a biological sample such as serum, urine or blood etc. The recognition surface 102 may be referred to as an off surface immunoassay matrix.

[0012] Various embodiments may lead to an improvement in detection signal. Unlike conventional biosensors which require modification of sensor surface, various embodiments use a separate surface 102 for binding the analyte. By using a separate surface 102 for binding the analyte (via a biomolecule), the sensor surface 106 may be kept pristine, leading to improved detection signals.

[0013] The high detection sensitivity may be contributed by the off surface immunoassay matrix 102 which kept the electrical sensor surface 106 clear of physical attachment with surface-modification chemicals/molecules, therefore enhancing the ability of the electrodes 108 to 'sense' effectively any electrical events happening on the sensor surface 106.

[0014] The device 100 may be an electrochemical chip or an electrochemical sensor or an apparatus including the electrochemical chip or the electrochemical sensor. The electrochemical immunoassay may be conducted on-chip.

[0015] The analyte may be or may include an antigen such as tumor necrosis factor alpha (TNF-α) or troponin (TnI). The biomolecule 104 may be or may include an antibody such as TNF-α antibody or TnI antibody. In various embodiments, the analyte may be any biological entity such as a cancer biomarker, a brain injury marker, a disease marker such as a heart/lung/kidney disease marker, any protein, a deoxyribonucleic acid (DNA) biomarker, or the like with specific recognition molecules or functional groups which can be bound directly or via a biomolecule 104 to the recognition surface 102. The biomolecule 104 may be any biological molecule which is able to bind specifically with the analyte, and is also able to be attached or be bound to the recognition surface 102.

[0016] During operation, the analyte may be introduced to a further biomolecule such as a further antibody. In one possible assay pathway, the analyte may bind to the biomolecule 104 immobilized on the surface 102 and the further biomolecule may bind with the bound analyte. A detection tag, such as an enzyme, e.g. alkaline phosphatase (ALP) may then bind with the further biomolecule. In another possible assay pathway, the analyte may bind or form a first complex with the further biomolecule. The first complex may then bind with the immobilized biomolecule 104 and an enzyme such as alkaline phosphatase (ALP)) to form a second complex of analyte, biomolecules and enzyme.

[0017] In both pathways, washing steps may be included to remove the analyte, biomolecules and enzymes that are not bound to the recognition surface 102 via the biomolecules 104. The enzyme may be capable of converting an electrochemically passive specie, such as p-aminophenylphosphate (pAPP) to an electrochemically active specie, such as p-aminophenol (pAP). An electrochemically active specie may be defined as an entity which exchanges electrons with a conductive surface, such as an electrode (and therefore may be detected by the conductive surface), while an electrochemically passive specie may be defined as an entity which does not exchange electrons with the conductive surface (an

therefore may not be detected by the conductive surface). The electrochemically active species (e.g. pAP) may be generated via reaction of the electrochemically passive specie (e.g. pAPP) with the enzyme (e.g. ALP) bound to the immobilized biomolecule 104. The electrochemically active specie may then be detected by the electrodes 108. The signal detected at the electrodes varies with the amount of electrochemical active species, which is dependent on the bound enzyme, which is in turn dependent on the amount of bound analyte. As such, by detecting the signal, the amount or concentration of analyte in a sample may be determined. In various embodiments, the enzyme horse reddish peroxide (HRP) may be used in conjunction with the electrochemically passive species hydroquinone. In various embodiments, the enzyme ALP may be used with electrochemically passive species p-aminophenylphosphate (pAPP), e.g. 4 aminophenylphosphate, or p-nitrophenylphosphate (pNPP), e.g. 4-nitrophenylphosphae. In various embodiments, the enzyme glucose oxidate may be used with electrochemically passive specie such as glucose. In various embodiments, redox molecules such as ferrocene tagged biomolecules may also be used as a detection tag / electrochemically passive specie pair.

[0018] In various embodiments, the recognition surface 102 and the sensor surface 106 may define opposing inner surfaces of a chamber in which the sample is received. In various other embodiments, the recognition surface 102, the sensor surface 106 or both the recognition surface 102 and the sensor surface 106 may be positioned within the chamber of the device 100.

[0019] The device 100 may include an inlet fluidically connected to the chamber, the inlet for introducing the sample containing the analyte and/or fluid containing electrochemically passive species and/or fluid containing detection tags or enzymes and/or fluids for washing and/or other entities required for assay and/or detection. The device 100 may further include an outlet fluidically connected to the chamber, the outlet for removing unbound biomolecules and/or unbound enzymes and/or unbound analyte and/or other products generated in assay.

[0020] The device 100 may include at least one spacer arranged between the recognition surface 102 and the sensor surface 106. Generally, a shorter distance between the recognition surface 102 and the sensor surface 106 may improve the detection sensitivity of the device 100. Detection sensitivity may improve with a thinner spacer as occurrence of immunoassay events may be at a closer proximity to the sensor surface 106. In various embodiments, the thickness of the spacer may be any value between 1 μm to about 500 μm, e.g. between 1 μm

to about 200 μm e.g. between 1 μm to about 50 μm. The spacer may be made of semiconductors such as silicon; or insulators such as silicate, aluminum silicate, porous aluminum oxide, and so on.

[0021] In various embodiments, the recognition surface 102 and the sensor surface 106 may be arranged spaced apart from each other at a distance that is dependent on at least one of layouts of the plurality of electrodes 108, sizes of the plurality of electrodes, characteristics of the targeted analyte, a binding capacity of the recognition surface, or a geometry of the recognition surface 102.

[0022] In various embodiments, the recognition surface 102 and the sensor surface 106 may be arranged spaced apart from each other at a distance ranging from about 1 μm to about 500 μm, e.g. between 1 μm to about 200 μm e.g. between 1 μm to about 50 μm.

[0023] The plurality of electrodes 108 may include a planar working electrode and a planar counter electrode. The plurality of electrodes 108 may further include a planar reference electrode. For example, the planar working electrode may include a first comb electrode structure having a first set of comb fingers, and the planar counter electrode may include a second comb electrode structure having a second set of comb fingers; and wherein the first comb electrode structure and the second comb electrode structure may be arranged adjacent to each other with the first set of comb fingers interlaced with the second set of comb fingers. The plurality of electrodes 108 may be made of a conducting material selected from the group consisting of a carbon based matrix, a metal, a semiconductor and a mixture thereof. In various embodiments, the plurality of electrodes 108 or any of the plurality of electrodes 108 may be made of gold or platinum. In various embodiments, the plurality of electrodes 108 or any of the plurality of electrodes may be made of indium tin oxide. The working electrode and/or the counter electrode and/or the reference electrode may be made of the same material or may be made of different materials.

[0024] During operation, the electrochemically active specie may come into contact with or be bound to the working electrode. A potential difference between the working electrode and the counter electrode depending on electrochemical species generate current which may be measured. The amount of current generated may depend on concentration of electrochemically active specie, which depends on concentration of analyte.

[0025] It should be appreciated that in various embodiments, the size, structure and design of the working electrode along with other electrodes may be different from the examples

described herein, depending on sensor design, size, required sensitivity, type of signal required during electrochemical process, and so on. Furthermore, the sensor surface may be in a 2-electrode, 3-electrode or 4-electrode format based on sensor requirements. In other words, any shape and size of the sensor surface (e.g., electrochemical sensor) may be suitable to be used for various embodiments of the present invention.

[0026] The recognition surface 102 may be or may include a three-dimensional (3D) matrix. The three-dimensional matrix may be made of semiconductors such as silicon; conductors such as metals, carbon materials, and so on; insulators such as silicate, aluminum silicate, porous aluminum oxide, and so on; polymers and polymeric membranes such as polymethyl methacrylate, polyethylene, polystyrene, polyethylene terephthalate, polycarbonate, polyvinylidene difluoride (PVDF), nitrocellulose, polypyrrole, polystyrene, and so on. Further, any high surface area matrix including nanocomposites or any porous material may be used.

[0027] In other embodiments, the matrix (three-dimensional matrix) may include a functionalized material such as aminated (NH_2) polymer or glass; or other functional groups such as carboxylic, aldehyde, epoxy, succinimide and so on for direct binding of biomolecules.

[0028] In various embodiments, the three-dimensional matrix may include a pillar structure including a base arranged substantially parallel to the sensor surface, and a plurality of pillars extending from the base and toward the sensor surface. The three-dimensional matrix may include a functionalized material or a functional group for direct binding of a biomolecule thereon to define the immobilized biomolecule.

[0029] In various embodiments, the three-dimensional matrix may include one or more nanostructures, such as nanoparticles, nanorods, nanopillars etc.

[0030] In various embodiments, the recognition surface may be or may include a porous membrane. The porous membrane may be made of, but is not limited to polycarbonate, parylene, nitrocellulose, polyvinylidene difluoride (PVDF), polymethyl methacrylate, polyethylene, polystyrene, polyethylene terephthalate, polycarbonate, polypyrrole, and polystyrene.

[0031] In various embodiments, the device may further include one or more nanostructures spaced apart from the recognition surface 102 and the detection surface 106. The one or more nanostructures may be between the recognition surface 102 and the

detection surface 106. The one or more nanostructures may be nanoparticles, nanorods, nanopillars etc. The nanostructures may protrude into the space between the recognition surface 102 and the sensor surface 106, therefore resulting in occurrence of immunoassay events at closer proximity to the sensor surface 106, which may improve detection sensitivity.

[0032] The recognition surface 102 may further include a crosslinker, the crosslinker binding a biomolecule 104 onto the recognition surface 102 to define the immobilized biomolecule 104. An example of a crosslinker is 4-fluoro-3-nitroazidobenzene (FNAB), which may be used to bind anti-TNF. The crosslinker to be used may depend on the analyte and/or the recognition surface. Different crosslinkers with different functional groups may be used depending on the analyte and/or the recognition surface. Other examples of crosslinkers may include glutaraldehyde, epoxy group containing molecules, dithiobis sucinimide (DSP) etc or any other molecule which can facilitate binding between two different molecules.

[0033] In various embodiments, the recognition surface 102 may further include at least one of a block agent, e.g. PBS T20 blocker, for blocking an unoccupied site or a stabilizing agent for stabilizing the immobilized biomolecule 104 on the recognition surface 102. Blocking may be done using various block agents. Block agents may include commercial blocking agents like starting blocker PBST20, TBST20, caseinate solution, bovine serum albumin (BSA) solutions at various concentrations, milk proteins, ethanol amines, mercapto hexanol, serum proteins, synthetic proteins etc.

[0034] The sensor surface 106 may be reversibly attachable to the device 100. The sensor surface 106 may be detachable from the device 100, for instance when use of the sensor surface is not required. In various embodiments, the device 100 may further include an attachment mechanism configured to fix the recognition surface 102 and the sensor surface 106 at a distance from each other so that the recognition surface 102 and the sensor surface 106 are arranged spaced apart at the fixed distance from each other. The attachment mechanism may only serve to fix the distance between the recognition surface 102 and the sensor surface 106 while still allowing the recognition surface 102 and the sensor surface 106 to move relative to each other. For instance, the attachment mechanism may be a slotting mechanism in which the sensor surface 106 may be slotted into the device 100, e.g. through grooves present in the device 100.

[0035] The attachment mechanism may further be configured that the sensor surface 106 may be moved away from the recognition surface 102 to a separation distance beyond the

fixed distance. In other words, the sensor surface 106 and the recognition surface 102 may be separated to a separation distance beyond the fixed distance, for instance when no detection is required. By restricting the sensor surface 106 from being exposed to samples, analytes, chemicals, biomolecules etc. and only introducing the sensor surface 106 when the electrochemically active species (e.g. immunoassay molecules such as ALP-pAPP products, i.e. pAP) are generated for detection by electrodes 108, exposure of the sensor surface 106 to contamination (e.g. fouling molecules) may be reduced, thus enhancing the detection sensitivity.

[0036] Various embodiments relate to a chip including a surface matrix modified with capturing biomolecule for on-chip electrochemical ELISA (enzyme linked immunosorbant assay). The off surface matrix may be solid 3D matrix or may be flexible porous membrane placed in very close vicinity of an electrode surface. The chip may further include a fluidic channel for reagent flow. The off surface matrix may be modified with capturing biomolecule separately using matrix and biomolecule compatible chemistry and may be integrated over sensor chip to prevent sensor chip surface from fouling during functionalization.

[0037] Use of the off surface matrix may provide the opportunity to electrochemically sense biomarkers sensitively to ng/ml range with negligible nonspecific binding and false signal in undiluted serum. Application of off surface matrix for on chip electrochemical ELISA based electrochemical biosensor may have potential to replace standard optical ELISA systems for large variety of biomarkers detection.

[0038] Various embodiments may provide a simple and new off surface biomolecule modified chip for on-chip electrochemical biosensing.

[0039] FIG. 2A shows an overhead view of a three-dimensional matrix 202a according to various embodiments. FIG. 2B shows a cross-sectional side view of the three-dimensional matrix 202a shown in FIG. 2A according to various embodiments. In various embodiments, the three-dimensional matrix 202a may be made of polymethyl methacrylate (PMMA). The matrix 202a may include a grid or mesh 210 attached to a planar substrate 214. Fluidic ports (inlet 212a and outlet 212b) are in fluidic communication with the matrix 202a.

[0040] FIG. 2C shows an overhead view of a two-dimensional membrane 202c having non-uniform distribution of holes according to various embodiments. FIG. 2D shows an overhead view of a two-dimensional membrane 202d having uniform distribution of holes according to various embodiments. FIG. 2E shows an overhead view of a two-dimensional

membrane 202e with uniform and defined distribution of holes according to various embodiments.

[0041] In various embodiments, the recognition surface may be the matrix shown in FIGS. 2A-B, or the membrane shown in FIG. 2B, FIG. 2D or FIG. 2E. In various embodiments, the off chip 3D matrix (which may include a polymer such as PMMA) or membrane (which may include polycarbonate, parylene etc.) may be modified with biomolecules in a separate location and then integrated on the electrochemical chip or sensor for on-chip electrochemical ELISA testing.

[0042] FIG. 2F shows an image of an electrochemical chip 200a according to various embodiments. The electrochemical chip 200a may include sensor surface 206 with electrodes 208. FIG. 2G shows a magnified image of sensor surface 206. FIG. 2G shows that the electrodes 208 may be comb-shaped.

[0043] FIGS. 2H-J shows an image of an electrochemical chip 200b according to various embodiments. FIG. 2H shows a lower cross-sectional view the electrochemical chip 200b with sensor surface 206 having electrodes 208. FIG. 2I shows a middle cross-sectional view of the electrochemical chip 200b. FIG. 2J shows an upper cross-sectional view of the electrochemical chip 200b. The electrochemical chip 200b may include 3D matrix 202a.

[0044] FIGS. 2K-N shows an image of an electrochemical chip 200c according to various embodiments. FIG. 2K shows a lower cross-sectional view the electrochemical chip 200b with sensor surface 206 having electrodes 208. FIG. 2L shows a first middle cross-sectional view of the electrochemical chip 200b. FIG. 2M shows a second middle cross-sectional view of the electrochemical chip 200b including membrane 202e with uniform and defined distribution of holes. FIG. 2N shows an upper cross-sectional view of the electrochemical chip 200c with membrane 202e.

[0045] FIG. 3A shows a process 300 of immobilizing or binding biomolecules 304 on a recognition surface 302 according to various embodiments. The biomolecules 304 may be antibodies, which may also be referred to as primary antibodies. A recognition surface 302 is provided. Cross-linking molecules 314, such as 4-fluoro-3-nitroazidobenzene (FNAB), may then be introduced. FNAB may be used for covalent immobilization of antibodies for tumor necrosis factor alpha (anti TNF-α) on matrix 302. The cross-linking molecules may also be referred to as cross linkers. The cross-linking molecules 314 may bind onto the recognition surface 302. The biomolecules 304, e.g. antibodies for tumor necrosis factor alpha (anti TNF-

α), may then be introduced. The cross-linking molecules 314 may bind biomolecules 304 onto the recognition surface 302 to define the immobilized biomolecule. It may also be envisioned that, in various embodiments, the biomolecules 304 may bind directly onto the surface 302 without requiring the cross-linking molecules 314. Azide chemistry FNAB may be granted in matrix 302 and fluoro group of FNAB layer may be utilized for covalent bonding of biomolecule 304. Covalent bonding may take place via nucleophilic substitution, in which the nucleophilic amino group of the biomolecule 304 attacks the FNAB molecule at a temperature e.g. about 37 °C, to remove the fluoro group and bond covalently with the phenyl ring. The immobilized biomolecules 304 may be bound covalently to the recognition surface 302. Washing may then be carried out to remove the nonspecifically adsorbed molecules. In various embodiments, each cross-linking molecule may only bind a single biomolecule 304 onto the recognition surface 302. In various embodiments, the biomolecules 304 may directly bind onto the recognition surface 302, without the need for cross-linking molecules.

[0046] Block agents 316 or stabilizing agents may also be introduced. The block agents 316, e.g. PBS T20 blocker, may block an unoccupied site. The blocking of unoccupied sites may reduce binding of other compounds, such as analytes, onto the recognition surface 302, thus improving accuracy of detection. Stabilizing agents may help stabilize the immobilized biomolecules 304 on the recognition surface 302.

[0047] FIG. 3B shows a process 350 of conducting a bioassay according to various embodiments. Process 350 may include an assay phase 350a and a detection phase 350b.

[0048] The recognition surface 302 with immobilized biomolecules 304 may be provided. FIG. 3B shows that a sensor surface 306 including electrodes 308 may be introduced in the assay phase. However, it may be envisioned that the sensor surface 306 is a reversibly attachable surface which is only arranged or positioned facing the recognition surface 302 during the detection phase. During the assay phase, the sensor surface 306 may be separated from the device.

[0049] The sample containing the analyte 318, which may also be referred to as detection molecule, may be flowed into the chamber of the device, for instance via an inlet. The analyte 318 may be an antigen such as tumor necrosis factor alpha (TNF-α). The recognition surface 302 may be incubated with detection molecules such as TNF-α in undiluted human serum. Further biomolecules 320 (e.g. further antibodies, which may also be referred to as secondary

antibodies) may also be introduced into the chamber. A secondary antibody may be a specific biomolecule normally with a tag, which specifically bind to the antigen, just like a primary antibody, but to a location on the antigen different from the location to which the primary antibody binds. The secondary antibody may be monoclonal or poly clonal from various host species and may have tags such as biotin, horseradish peroxidase (HRP), alkaline phosphatase (ALP) etc. Other further biomolecules that may be used in placed of secondary anitbodies may include aptamer or any other molecule or functional group which specifically bind to the antigen. The further biomolecules 320 may be the same as or may be different from the immobilized biomolecules 304.

[0050] In one assay pathway, the analyte 318 may first bind with the immobilized biomolecules 304 before binding with the further biomolecules 320. A detection tag, such as an enzyme 322 e.g. alkaline phosphatase (ALP), may then be introduced and the enzyme 322 may bind to the analyte 318 via further biomolecules 320. An electrochemically passive specie 324 (which may be referred to as detection target), such as p-aminophenylphosphate (pAPP), may then be introduced. Bound enzyme 322 reacts with electrochemically passive specie 324 to generate electrochemically active species 328 (e.g. pAP) via intermediate 326.

[0051] In another assay pathway, the analyte 318 may bind or form a first complex with the further biomolecule 320. The first complex may then bind with the immobilized biomolecules 304. The first complex may then bind with the immobilized biomolecule 104 and a detection tag, e.g. an enzyme 322 such as alkaline phosphatase (ALP)) to form a second complex of analyte 318, biomolecules 304, 320 and enzyme 322. The second complex may be capable of converting an electrochemically passive specie 324, such as p-aminophenylphosphate (pAPP) to an electrochemically active specie 328, such as p-aminophenol (pAP) via an intermediate 326. The electrochemically active species 328 (e.g. pAP) may be generated via reaction of the electrochemically passive specie 324 (e.g. pAPP) with the enzyme 322 (e.g. ALP) bound to the immobilized biomolecule 304.

[0052] The electrochemically active species 328 may then be detected by the electrodes 308. The signal detected at the electrodes varies with the amount of electrochemical active species 328, which is dependent on the bound enzyme, which is in turn dependent on the amount of bound analyte. As such, by detecting the signal, the amount or concentration of analyte in a sample may be determined. Detection of the signal may be done via differential

pulse voltammetry. The voltammetric (differential pulse voltammetric or DPV) signal may be recorded for analyte concentration estimation.

[0053] The detection process may be automated and may provide results of the analyte estimation in a real sample.

[0054] FIG. 4A shows a cross-sectional side view of an electrochemical chip 400 according to various embodiments. The electrochemical chip 400 includes sensor surface 406 and assay matrix (recognition surface) 402. The matrix 402 and sensor surface 406 are held together by an adhesive 432 such as epoxy or tape and define a chamber 430. Fluidic ports i.e. inlet 412a, outlet 412b are in fluidic communication with the chamber 430. An area indicated by box 434 is shown in FIGS. 4B and 4C.

[0055] FIG. 4B is a magnified side view of the area 434 shown in FIG. 4A. FIG. 4C is an magnified perspective view of the area 434 shown in FIG. 4A. As shown in FIGS. 4B and 4C, the matrix 402 may include a plurality of vertical pillars suspended over the sensor surface 406. The vertical pillars may extend from a base, which runs in a substantially perpendicular direction to the vertical pillars. The sensor surface 406 may include comb-shaped electrodes 408. Numerous biomolecules 404 are bound to the matrix 402 via crosslinkers 414. Each crosslinker may bind one biomolecule 404 to the matrix 402.

[0056] FIG. 5A shows a cross-sectional side view of an electrochemical chip 500 according to various embodiments. FIG. 5B is a magnified side view of the area 534 shown in FIG. 5A. The electrochemical chip 500 includes a cover 536, a sensor surface 506 and assay matrix (recognition surface) 502. The cover 536 and the sensor surface 506 define a chamber 530, with the assay matrix 502 between the cover 536 and the sensor surface 506. The matrix 502 may be adhered to both the sensor surface 506 and cover 536 using adhesive 532 such as epoxy or tape. The sensor surface 506 may include comb-shaped electrodes 508. The assay matrix 502 may have a first planar surface and a second planar surface opposite the first planar surface. The cover 536 may include fluidic ports, i.e. inlet 512a, outlet 512b, which are in fluidic communication with the chamber 530. Biomolecules 504 may be bound to the first planar surface or to the second planar surface or to both the first and second planar surfaces of the assay 502. Biomolecules 504 may be bound to the assay via crosslinkers 514.

[0057] Binding biomolecules and their applications in sensitive electrochemical biosensor fabrication for testing of biomarker in undiluted serum have been demonstrated by integration

of anti TNF-α modified PMMA based 3D matrix and anti TNF-α modified porous polycarbonate/parylene membrane for detection of TNF-α in undiluted serum.

[0058] FIGS. 6A-C show the differential pulse voltammetric (DPV) responses of a device with polymethyl methacrylate (PMMA) 3 dimensional (3D) matrix integrated on sensor surface according to one embodiment for estimation of tumor necrosis factor alpha (TNF-α) in undiluted serum.

[0059] FIG. 6A is a plot 600a of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device with polymethyl methacrylate (PMMA) 3 dimensional (3D) matrix integrated on sensor surface according to one embodiment. 602a is the DPV response for undiluted serum, 602b is the DPV response for 1 nanogram (ng) TNF-α per milliliters (ml) of undiluted serum, 602c is the DPV response for 10 nanograms (ng) TNF-α per milliliters (ml) of undiluted serum, and 602d is the DPV response for 100 nanograms (ng) TNF-α per milliliters (ml) of undiluted serum.

[0060] FIG. 6B is a plot 600b of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device with polymethyl methacrylate (PMMA) 3 dimensional (3D) matrix integrated on sensor surface according to one embodiment. 604a is the DPV response for undiluted serum, 604b is the DPV response for 0.5 nanogram (ng) TNF-α per milliliters (ml) of undiluted serum, 604c is the DPV response for 5 nanograms (ng) TNF-α per milliliters (ml) of undiluted serum, and 604d is the DPV response for 50 nanograms (ng) TNF-α per milliliters (ml) of undiluted serum.

[0061] FIG. 6C is a plot 600c of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device with polymethyl methacrylate (PMMA) 3 dimensional (3D) matrix integrated on sensor surface according to one embodiment. 606a is the DPV response for undiluted serum, and 606b is the DPV response for 0.5 nanogram (ng) TNF-α per milliliters (ml) of undiluted serum.

[0062] FIGS. 7A-D show the differential pulse voltammetric (DPV) responses of a device with membrane based porous 2 dimensional (2D) matrixes integrated on sensor surface according to one embodiment for estimation of tumor necrosis factor alpha (TNF-α) in undiluted serum.

[0063] FIG. 7A is a plot 700a of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device with parylene membrane matrix integrated on sensor surface according to one embodiment. 702a is the DPV response for undiluted serum, 702b is the DPV response for 1 nanogram (ng) TNF-α per milliliters (ml) of undiluted serum, and 702c is the DPV response for 100 nanograms (ng) TNF-α per milliliters (ml) of undiluted serum.

[0064] FIG. 7B is a plot 700b of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device with parylene membrane matrix integrated on sensor surface according to one embodiment. 704a is the DPV response for undiluted serum, and 704b is the DPV response for 1 nanogram (ng) TNF-α per milliliters (ml) of undiluted serum.

[0065] FIG. 7C is a plot 700c of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device with polycarbonate (PC) matrix integrated on sensor surface according to one embodiment. 706a is the DPV response for undiluted serum, 706b is the DPV response for 1 nanogram (ng) TNF-α per milliliters (ml) of undiluted serum, and 706c is the DPV response for 100 nanograms (ng) TNF-α per milliliters (ml) of undiluted serum.

[0066] FIG. 7D is a plot 700d of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device with polycarbonate (PC) matrix integrated on sensor surface according to one embodiment. 708a is the DPV response for undiluted serum, and 708b is the DPV response for 1 nanogram (ng) TNF-α per milliliters (ml) of undiluted serum.

[0067] As seen from FIGS. 6A-C, 7A-D, the voltammetric response is a function of TNF-α concentrations. The response exhibits a high detection sensitivity down to single digit ng/ml and low nonspecific response. Thus, off surface biomolecule modified matrix for on-chip electrochemical ELISA may provide a new platform for development of sensitive electrochemical detection of various biological analytes in real samples such as in undiluted serum. Such a biosensor platform may have potential to replace most commonly used optical

ELISA based detection platforms. Also, different types of biomolecules may be immobilized selectively in array format to achieve multimarker detection and best performance.

[0068] FIG. 8 shows a schematic of a device 800 for electrochemical immunoassay according to various embodiments. The device 800 may include a recognition surface 802 including an immobilized biomolecule 804, e.g. a primary antibody. The immobilized biomolecule 804 may be capable of binding to a targeted analyte 818, e.g. a target protein, from a sample.

[0069] The device 800 may further include a sensor surface 806 including a plurality of electrodes 808 configured to detect the targeted analyte 818. The recognition surface 802 and the sensor surface 806 may be arranged spaced apart from each other and facing each other.

[0070] The device 800 may further include one or more nanoparticles 838. The one or more nanoparticles 838 may be arranged spaced apart from the sensor surface 806 and the recognition layer. The one or more nanoparticles 838 may be between the sensor surface 806 and the recognition layer 802.

[0071] The one or more nanoparticles 838 may be introduced into the space between the sensor surface 806 and the recognition layer 802 by a fluid or may be held between the sensor surface 806 and the recognition layer 802 by a support, such as a porous membrane. While FIG. 8 shows a plurality of nanoparticles 838, the nanoparticles 838 may be substituted by other nanostructures such as nanorods, nanowires, nanopillars etc. In various embodiments, the device 800 may include different types of nanostructures.

[0072] The nanoparticles 838 and/or other nanostructures may provide a 3D structured matrix. For example, the relatively large nanoparticles 838 may protrude into the space between the immunoassay matrix 802 and the sensor surface 806, therefore resulting in occurrence of immunoassay events at a very close proximity to the sensor surface 806, thus improving detection sensitivity.

[0073] A nanoparticle 838 or nanostructure may bind to an analyte 818 via a further biomolecule 820, such as a secondary antibody. The further biomolecule 820 may be the same as or may be different from the biomolecule 820. A detector tag, such as enzyme 322 may bind to the surface of the nanoparticle 838 or nanostructure. The enzyme 322 may be alkaline phosphatase (ALP).

[0074] The nanoparticles 838 may include or may be made of semiconductors such as silicon; conductors such as metals (e.g. gold, silver etc), carbon materials, and so on;

insulators such as silicate, aluminum silicate, porous aluminum oxide, and so on; polymers and polymeric membranes such as polymethyl methacrylate, polyethylene, polystyrene, polyethylene terephthalate, polycarbonate, polyvinylidene difluoride (PVDF), nitrocellulose, polypyrrole, polystyrene, and so on.

[0075] The enzyme 822 may convert an electrochemically passive specie 824 such as p-aminophenylphosphate (pAPP), which is introduced into the space between the sensor surface 806 and the recognition layer 802, to an electrochemically active specie 828 such p-aminophenol (pAP) for detection by the plurality of electrodes 808.

[0076] FIGS. 9A and 9B demonstrate that utilization of nanoparticles may improve detection sensitivity. Immunoassay based on ELISA procedure was performed for detection of tumor necrosis factor alpha (TNF-α) from non-diluted human serum using the AuNPs and a second antibody. Gold surface electrical sensors may be employed for signal detection in which the signals detected were pAP, products of the ALP-pAPP reactions.

[0077] FIGS. 9A-B show the differential pulse voltammetric (DPV) responses of a device with nanoparticles between a sensor surface and a recognition surface according to one embodiment for estimation of tumor necrosis factor alpha (TNF-α) in undiluted serum.

[0078] FIG. 9A is a plot 900a of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device without nanoparticles but with the second antibody between the recognition surface and the sensor surface according to one embodiment. 902a is the DPV response for undiluted serum, 902b is the DPV response for 100 picogram (pg) TNF-α per milliliters (ml) of undiluted serum, 902c is the DPV response for 1 nanogram (ng) TNF-α per milliliters (ml) of undiluted serum, and 902d is the DPV response for 5 nanograms (ng) TNF-α per milliliters (ml) of undiluted serum.

[0079] FIG. 9B is a plot 900b of current (μA) against potential difference (V) showing the differential pulse voltammetric (DPV) responses of different concentrations of tumor necrosis factor alpha (TNF-α) in undiluted serum in a device with second antibody tagged gold nanoparticles between the recognition surface and the sensor surface according to one embodiment. 904a is the DPV response for undiluted serum, 904b is the DPV response for 100 picogram (pg) TNF-α per milliliters (ml) of undiluted serum, 904c is the DPV response for 1 nanogram (ng) TNF-α per milliliters (ml) of undiluted serum, and 904d is the DPV response for 5 nanograms (ng) TNF-α per milliliters (ml) of undiluted serum.

[0080] FIG. 9B shows the results of the device with the nanoparticle while FIG. 9A shows the results of the control device.

[0081] The results show that in control experiment where normal 2nd antibody without gold nanoparticles (AuNPs) was utilized, 5 ng/ml limit of detection (LOD) was achieved (FIG. 9A) whereas when 2nd antibody tagged with AuNPs was utilized, 100 pg/ml LOD was achieved (FIG. 9B).

[0082] These data confirm that the 3D nanoparticles may improve detection sensitivity. In addition to the shortened distance between the immunoassay events and the electrical sensor surface, the use of AuNPs may also increase signal surface area. The increase in surface area may also contribute to the improvement in the detection sensitivity.

[0083] FIG. 10 is a schematic showing the use of a device 1000 with a removable sensor surface according to various embodiments. The device 1000 may include a recognition surface 1002 and a bare surface 1040 spaced apart from the recognition surface and facing the recognition surface 1002. Biomolecules 1004, such as primary antibodies, may be bound to the recognition surface 1002. During the assay phase, analyte 1018 and further biomolecules 1020, such as secondary antibodies, may be introduced into the device 100. The analyte 1018 may bind to the immobilised biomolecules 1004 and the further biomolecules 1020 may bind to the analyte. Further, detector tags 1022 may be introduced into the device 100. The detector tags 1022 may bind to the further biomolecules 1020. As seen in FIG. 10, the bare surface 1040 may be contaminated by analyte 1018, the further biomolecules 1020 and the detector tags 1022 that are not specifically bound.

[0084] After the assay phase and before the detection phase, the sensor surface 106 including a plurality of electrodes 1008 may be attached or arranged onto the bare surface 1040. In various embodiments, the device 1000 may include an attachment mechanism configured to fix the recognition surface 1002 and the sensor surface 1006 at a distance from each other so that the recognition surface 1002 and the sensor surface 1006 are arranged spaced apart at the fixed distance from each other. The attachment mechanism may be further configured so that the sensor surface 1006 may be removed or separated to a distance beyond the fixed distance from the recognition surface 1002. In various embodiments, the device 100 may include a detachable or reversibly attachable sensor surface 1006.

[0085] During the detection phase, electrochemically passive species 1024 such as pAPP may be introduced into the device 1000. The detection tags 1022 may convert the

electrochemically passive species 1024 to electrochemically active species 1028, such as pAP, which are detected by the electrodes 1028.

[0086] By restricting electrical sensor surface 1006 from being exposed to all analytes and chemicals applied to the immunoassay matrix and allowing the electrical sensor surface 1006 to be exposed to the immunoassay signal molecules 1028 e.g. ALP-pAPP products only during detection phase and not in the assay phase, detection sensitivity may be improved.

[0087] By keeping the electrical sensor surface 1006 out of the device 100 and introducing the electrical sensor surface 1006 only when signal is ready to be measured, the sensor surface 1006 may be kept free from any fouling molecules that may be introduced during the immunoassay procedure. In this manner the detection sensitivity may be enhanced.

[0088] In various embodiments, a method of fabricating a device for electrochemical immunoassay may be provided. FIG. 11 is a flowchart 1100 showing the method of fabricating the device for electrochemical assay according to various embodiments. The method may include, in 1102, immobilizing a biomolecule on a recognition surface of the device, wherein the biomolecule is capable of binding to a targeted analyte from a sample. The method may further include, in 1104, arranging the recognition surface and a sensor surface of the device spaced apart from each other and facing each other, wherein the sensor surface comprises a plurality of electrodes for detecting the targeted analyte.

[0089] In other words, a method of fabricating a device for electrochemical assay may include providing a recognition surface and a sensor surface separate from the recognition surface and facing the recognition surface. Biomolecules are attached to only the recognition surface while the sensor surface includes electrodes.

[0090] The step of arranging the recognition surface and the sensor surface spaced apart from each other and facing each other may include arranging the recognition surface and the sensor surface to define opposing inner surfaces of a chamber in which the sample is received. At least one spacer may be arranged between the recognition surface and the sensor surface. The recognition surface and the sensor surface may be arranged spaced apart from each other at a distance that is dependent on at least one of layouts of the plurality of electrodes, sizes of the plurality of electrodes, characteristics of the targeted analyte, a binding capacity of the recognition surface, or a geometry of the recognition surface.

[0091] The recognition surface and the sensor surface may be arranged spaced apart from each other at a distance ranging from about 1 μm to about 500 μm.

[0092] The method may further include further include introducing a nanostructure, wherein the nanostructure are capable of binding to the analyte. The nanostructure may bind the analyte directly or via a further biomolecule, e.g. a further or secondary antibody. The nanostructure may include or may be a nanoparticle. The nanostructure may be spaced apart from the recognition surface and the detection surface.

[0093] The method may include forming the plurality of electrodes on the sensor surface. The plurality of electrodes may include a planar working electrode and a planar counter electrode. The plurality of electrodes further may include a planar reference electrode. The plurality of electrodes may be made of a conducting material selected from the group consisting of a carbon based matrix, a metal, a semiconductor and a mixture thereof. The plurality of electrodes may be made of gold or platinum. The plurality of electrodes may be made of indium tin oxide.

[0094] The recognition surface may be or may include a three-dimensional matrix. The three-dimensional matrix may be made of a polymer or polymeric membrane selected from the group consisting of polymethyl methacrylate, polyethylene, polystyrene, polyethylene terephthalate, polycarbonate, polyvinylidene difluoride (PVDF), nitrocellulose, polypyrrole, and polystyrene.

[0095] The three-dimensional matrix may include a pillar structure comprising a base arranged substantially parallel to the sensor surface, and a plurality of pillars extending from the base and toward the sensor surface. The three-dimensional matrix may include nanostructures, such as nanoparticles.

[0096] The three-dimensional matrix may include a functionalized material or a functional group for direct binding of a biomolecule thereon to define the immobilized biomolecule.

[0097] The recognition surface may include a porous membrane. The porous membrane may be made of a material selected from the group consisting of polycarbonate, parylene, nitrocellulose, PVDF, polymethyl methacrylate, polyethylene, polystyrene, polyethylene terephthalate, polycarbonate, polypyrrole, and polystyrene.

[0098] The method may further include introducing a crosslinker, the crosslinker binding a biomolecule onto the recognition surface to define the immobilized biomolecule. In other words, immobilizing the biomolecule may include introducing a crosslinker so that the biomolecule may be bound to the recognition surface via the crosslinker.

[0099] The method may also wherein introducing at least one of a block agent for blocking an unoccupied site on the recognition surface or a stabilizing agent for stabilizing the immobilized biomolecule on the recognition surface.

[00100] In various embodiments, the sensor surface may be reversibly attachable. During detection, the sensor surface may be secured at a fixed distance from the recognition surface. At other times, such as during assay, the sensor surface may be detached and/or moved to a distance beyond the fixed distance from the recognition surface.

[00101] There are various reports of electrochemical ELISA studies; however most of them involve sensor surface modification, which reduce the sensor chip surface sensitivity and may bring variation in result especially for real sample analysis. Gan et al. (N. Gan, L. Jia, L. Zheng, A Novel Sandwich Electrochemical Immunosensor Based on the DNA-Derived Magnetic Nanochain Probes for Alpha-Fetoprotein, Journal of Automated Methods and Management in Chemistry, 2011(2011) 7) described the sandwich ELISA based on DNA derived magnetic nanochain probes. The process of sensor fabrication involved modification of chip surface and for analysis in serum 50 to 100 times dilution was required to reduce the matrix effect. In another study, Wang et al. (H. Wang, X. Wu, P. Dong, C. Wang, J. Wang, Y. Liu, et al., Electrochemical Biosensor Based on Interdigitated Electrodes for Determination of Thyroid Stimulating Hormone, International Journal of Electrochemical Science, 9(2014) 12-21), described the electrochemical ELISA for thyroid stimulating hormones. In their approach they modified surface between electrode, however such attempt also result in some modification on sensor electrode surface, which can result in passivation of active surface or can result in variation. Patent applications for electrochemical ELISA include "Electrochemical immunoassay methods" from Andcare, Inc., Durham, N.C (US005391272A); "Apparatus and methods for analyte measurement immunoassay" from Abbott Point of Care Inc. (US 2013/0224775 Al) and "Electrochemical immunoassays using colloidal metal markers" from Centre National de la Recherche Scientifique (CNRS), Paris (US 7045364 B2). However, all these involve the modification of sensor surface for electrochemical sensing.

[00102] Other than these, off chip based electrochemical ELISA has been described by Liu et al. (Q.-L. Liu, X.-H. Yan, X.-M. Yin, B. Situ, H.-K. Zhou, L. Lin, et al., Electrochemical Enzyme-Linked Immunosorbent Assay (ELISA) for α-Fetoprotein Based on Glucose Detection with Multienzyme-Nanoparticle Amplification, Molecules, 18(2013) 12675-86.)

for α-Fetoprotein detection; and ImmuDrop by DiagnoSwiss S. A. (DiagnoSwiss, http://www.diagnoswiss.com, ImmuDrop.). However, miniaturization and automation of such systems may not be easy and involve manual handling for testing.

[00103] Other than above mentioned systems, Yaku et al, from Osaka: Panasonic Corporation, Osaka also has a patent application "Chip for electrochemical immunoassay" (US 7,585,400 B2). However, in their system, bio-assay occurs in liquid phase at different location than sensor area. Also, it do not involve matrix for biomolecule immobilization, which may result in higher background signal and suitable mainly for enzymatic type system.

[00104] With all the prior efforts summarized above, a new biosensor platform based on off surface matrix for on-chip electrochemical ELISA may be desirable to fabricate common biosensor platform for efficient, sensitive, cost effective, and specific detection of analytes of interest in real samples.

[00105] Various embodiments describe a new platform for biosensor development, which is able to efficiently reduce the effect of background interference and enhance the detection sensitivity in undiluted serum samples. Various embodiments may relate to the concept of using off chip matrix for on-chip electrochemical ELISA assay. Optical ELISA has shown great success and is most used method of screening target proteins in real samples. However, due to its limitation in sensitivity, high cost of instrumentation and non-portability, electrochemical ELISA may be desirable. However, till date, electrochemical ELISA involves the binding of biomolecule and electrochemical signal recognition on same sensor surface. Binding on biomolecule on sensor surface may result in degradation of its electrochemical properties and application of input electrochemical signal may also affect sensing layer. To avoid these issues and to improve signal sensitivity with low background noise, various embodiments may relate to the concept of integrated sensor chip, where off chip matrix is employed for on chip electrochemical ELISA, have been described herein. The new platform fabrication process may involve the selection of matrix followed by its off-site modification with sensing biomolecules and its integration near electrochemical sensor surface. Electrochemical sensor or sensor surface may be made of any conducting material such as carbon based matrix, metals (Au, Pt etc), semiconductor (ITO etc) in desired shape, size and geometry based on application. The matrix or recognition surface may be made of any possible solid material capable of binding biomolecule directly or after some modification.

[00106] The following have been demonstrated: (i) 3D matrix of PMMA modified with FNAB and anti-TNF-α integrated on gold based sensor electrode and (ii) 2D porous membrane of track etched polycarbonate and lithographically made porous parylene membrane modified with FNAB and anti-TNF-α integrated on gold based sensor electrode. Electrochemical measurements, e.g. using DPV, have been performed using suitable electrochemical techniques.

[00107] Experimental results revealed that various embodiments may be successfully used to reduce or minimize the background noise and false signal in undiluted serum samples. Thus, use of biomolecule modified off surface matrix for on chip electrochemical ELISA may have the potential to provide a new biosensor platform for sensitive electrochemical detection of various biological analytes.

[00108] Various embodiments relate to a new biosensor platform that is able to reduce the effect of background interference and result in enhance detection sensitivity and specificity has been proposed. The platform may include a biomolecule-modified off surface matrix for on-chip electrochemical ELISA to sense biomarkers in presence of high level of undesired proteins in undiluted serum. As the recognition surface/matrix is separate and is not in direct contact of sensing surface, the sensor may be free of modification and may produce high sensitivity response with very low background. Further, various embodiments may be employed with any possible matrix used till date with suitable chemistry to modify them with recognition molecule. Thus, various embodiments may hold high potential to replace presently used optical ELISA for biomarker sensing.

[00109] While the invention has been particularly shown and described with reference to specific embodiments, it should be understood by those skilled in the art that various changes in form and detail may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. The scope of the invention is thus indicated by the appended claims and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced.

CLAIMS

1. A device for electrochemical immunoassay, the device comprising:

a recognition surface comprising an immobilized biomolecule, wherein the immobilized biomolecule is capable of binding to a targeted analyte from a sample; and

a sensor surface comprising a plurality of electrodes configured to detect the targeted analyte,

wherein the recognition surface and the sensor surface are arranged spaced apart from each other and facing each other.

2. The device of claim 1, wherein the recognition surface and the sensor surface define opposing inner surfaces of a chamber in which the sample is received.

3. The device of claim 1, further comprising at least one spacer arranged between the recognition surface and the sensor surface.

4. The device of claim 1, wherein the recognition surface and the sensor surface are arranged spaced apart from each other at a distance that is dependent on at least one of layouts of the plurality of electrodes, sizes of the plurality of electrodes, characteristics of the targeted analyte, a binding capacity of the recognition surface, or a geometry of the recognition surface.

5. The device of claim 1, wherein the recognition surface and the sensor surface are arranged spaced apart from each other at a distance ranging from about 1 μm to about 500 μm.

6. The device of claim 1, wherein the plurality of electrodes comprises a planar working electrode and a planar counter electrode.

7. The device of claim 6, wherein the plurality of electrodes further comprises a planar reference electrode.

8. The device of claim 1, wherein the plurality of electrodes is made of a conducting material selected from the group consisting of a carbon based matrix, a metal, a semiconductor and a mixture thereof.

9. The device of claim 1, wherein the recognition surface comprises a three-dimensional matrix.

10. The device of claim 9, wherein the three-dimensional matrix is made of a polymer or polymeric membrane selected from the group consisting of polymethyl methacrylate, polyethylene, polystyrene, polyethylene terephthalate, polycarbonate, polyvinylidene difluoride (PVDF), nitrocellulose, polypyrrole, and polystyrene.

11. The device of claim 9, wherein the three-dimensional matrix comprises a pillar structure comprising a base arranged substantially parallel to the sensor surface, and a plurality of pillars extending from the base and toward the sensor surface.

12. The device of claim 9, wherein the three-dimensional matrix comprises a functionalized material or a functional group for direct binding of a biomolecule thereon to define the immobilized biomolecule.

13. The device of claim 1, wherein the recognition surface comprises a porous membrane.

14. The device of claim 13, wherein the porous membrane is made of a material selected from the group consisting of polycarbonate, parylene, nitrocellulose, PVDF, polymethyl methacrylate, polyethylene, polystyrene, polyethylene terephthalate, polycarbonate, polypyrrole, and polystyrene.

15. The device of claim 1, further comprising one or more nanostructures spaced apart from the recognition surface and the detection surface.

16. The device of claim 1, wherein the recognition surface further comprises a crosslinker, the crosslinker binding a biomolecule onto the recognition surface to define the immobilized biomolecule.

17. The device of claim 1, wherein the recognition surface further comprises at least one of a block agent for blocking an unoccupied site or a stabilizing agent for stabilizing the immobilized biomolecule on the recognition surface.

18. The device of claim 1, further comprising an attachment mechanism configured to fix the recognition surface and the sensor surface at a distance from each other so that the recognition surface and the sensor surface are arranged spaced apart at the fixed distance from each other.

19. A method of fabricating a device for electrochemical immunoassay, the method comprising:

immobilizing a biomolecule on a recognition surface of the device, wherein the biomolecule is capable of binding to a targeted analyte from a sample;

arranging the recognition surface and a sensor surface of the device spaced apart from each other and facing each other, wherein the sensor surface comprises a plurality of electrodes for detecting the targeted analyte.

20. The method of claim 19, further comprising introducing a nanostructure, wherein the nanostructure is capable of binding to the analyte.

FIG. 1

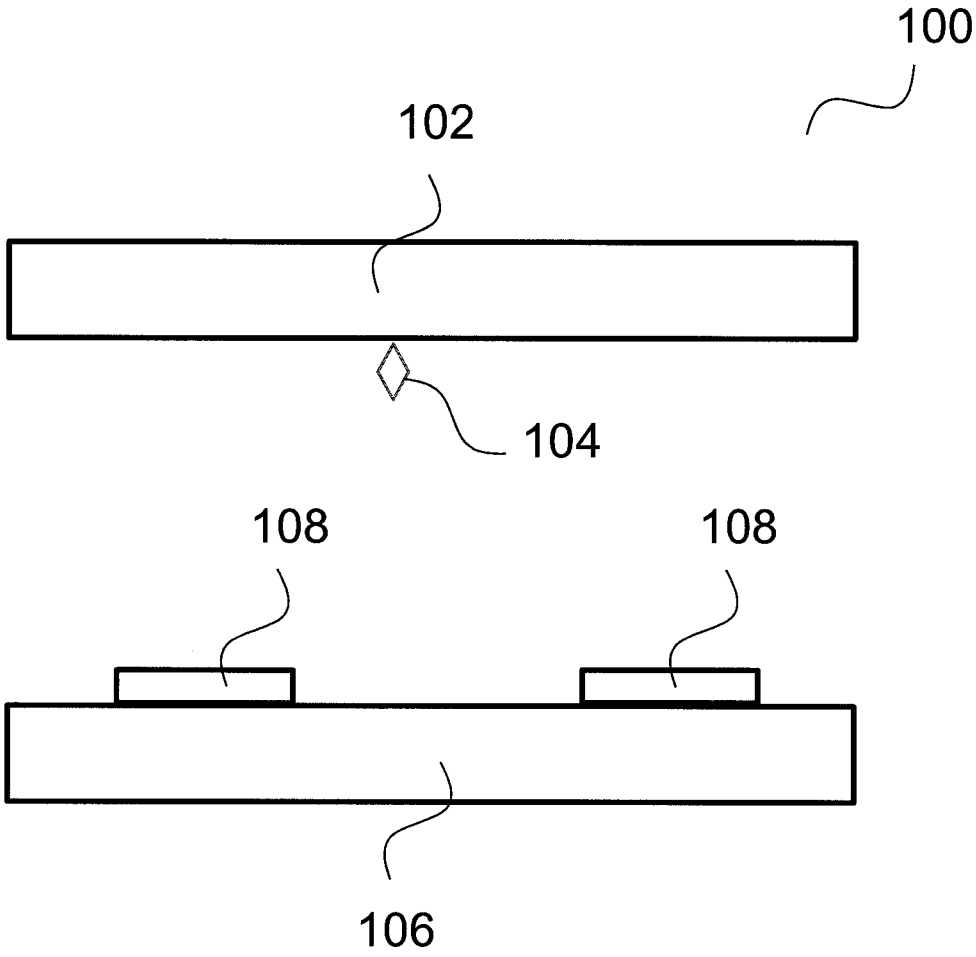


FIG. 2A

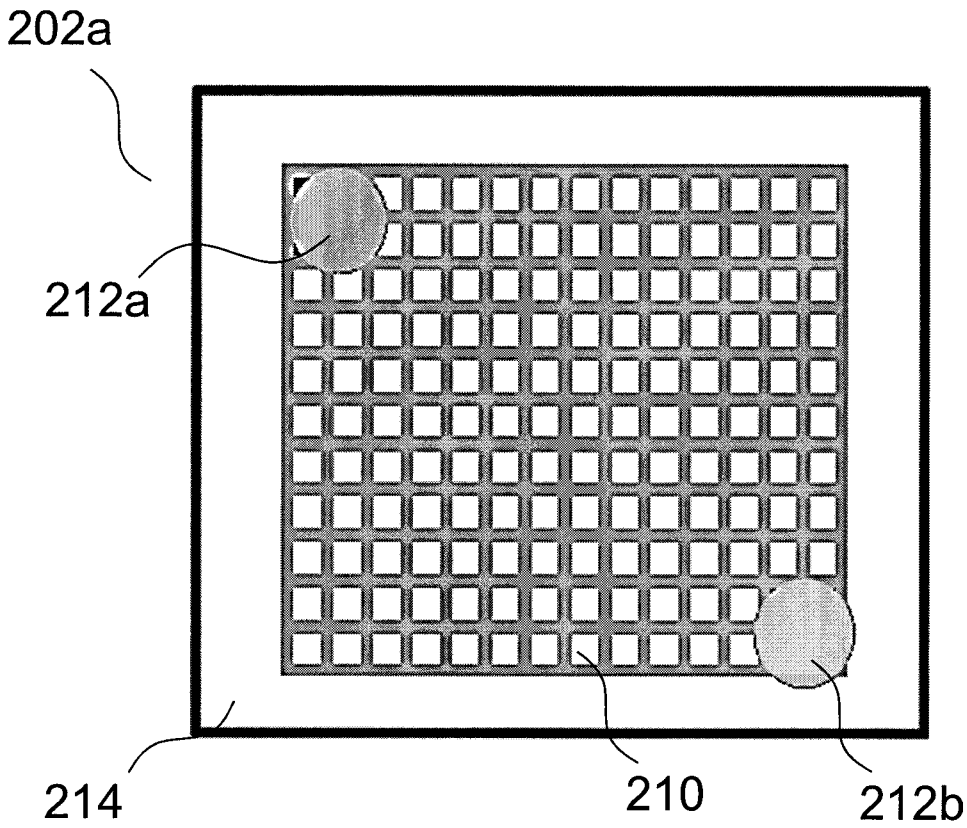


FIG. 2B

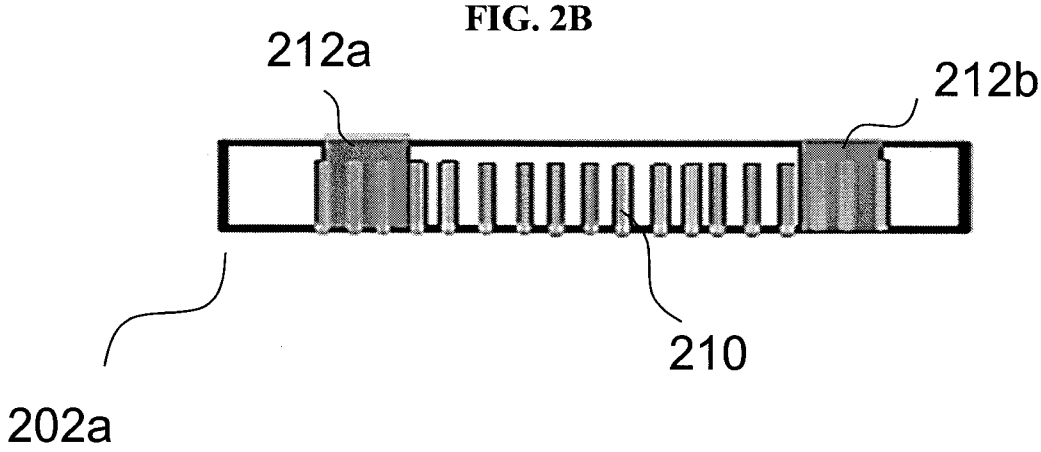


FIG. 2C

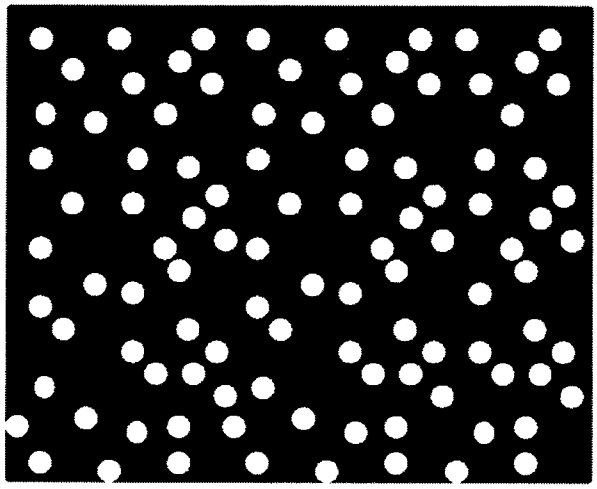


FIG. 2D

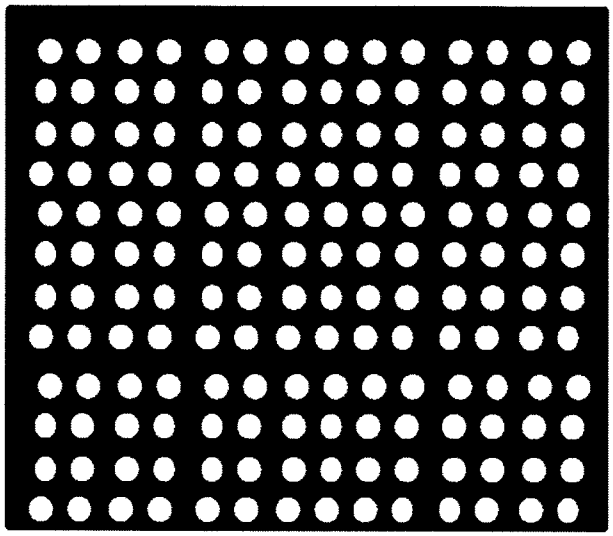


FIG. 2E

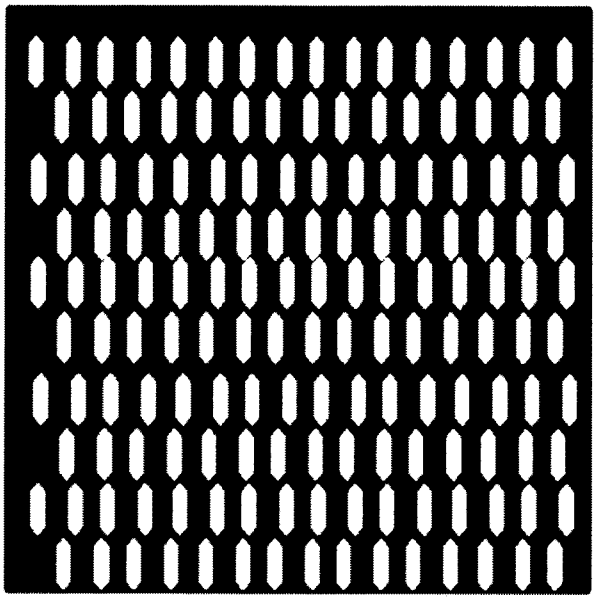


FIG. 2F

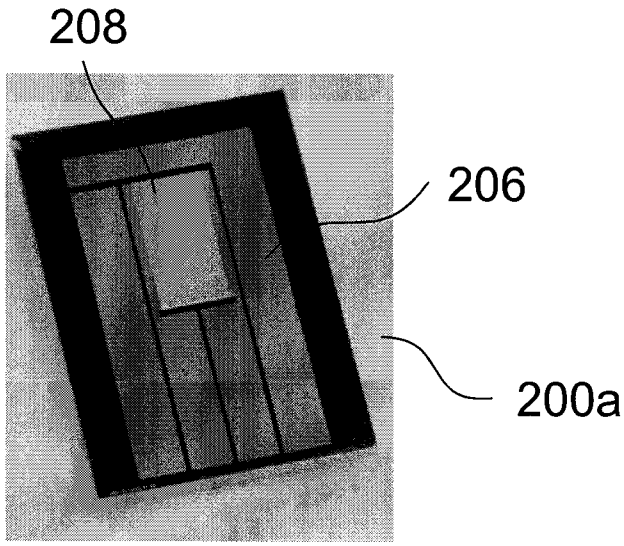


FIG. 2G

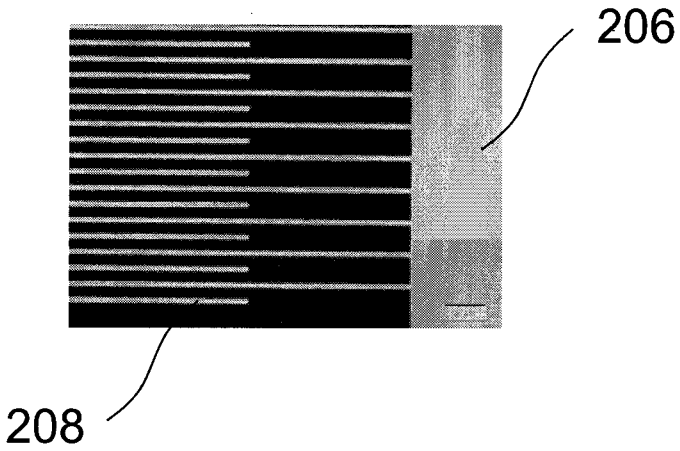


FIG. 2H

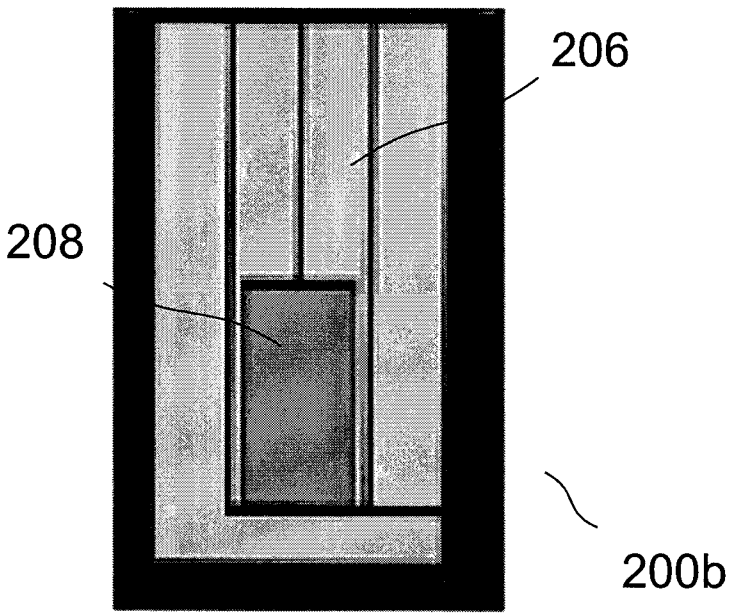


FIG. 2I

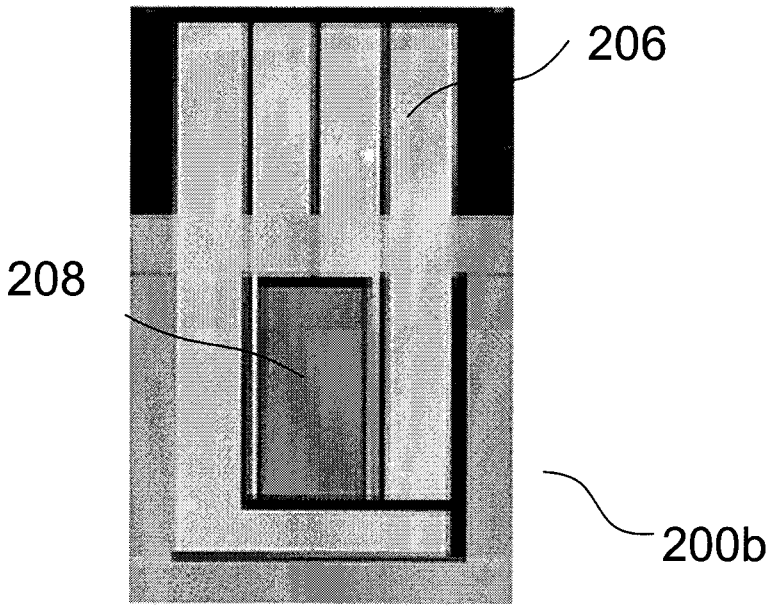


FIG. 2J

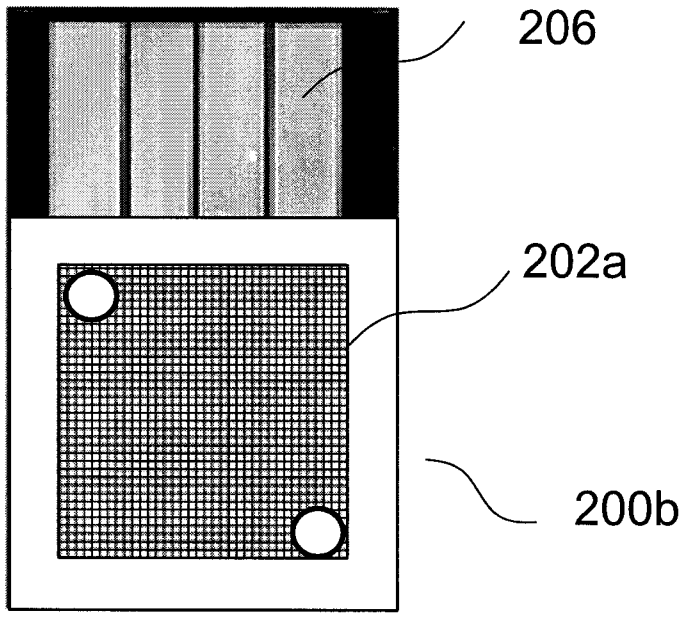


FIG. 2K

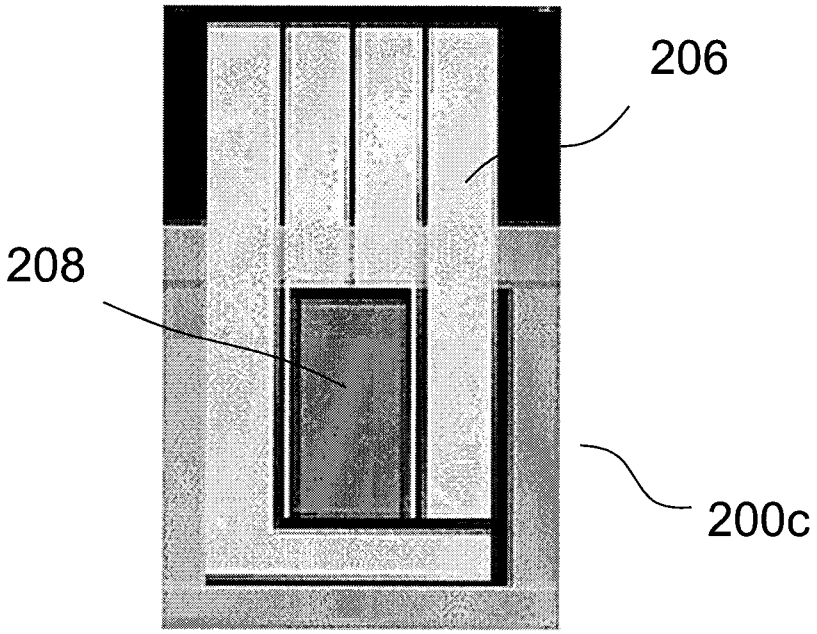


FIG. 2L

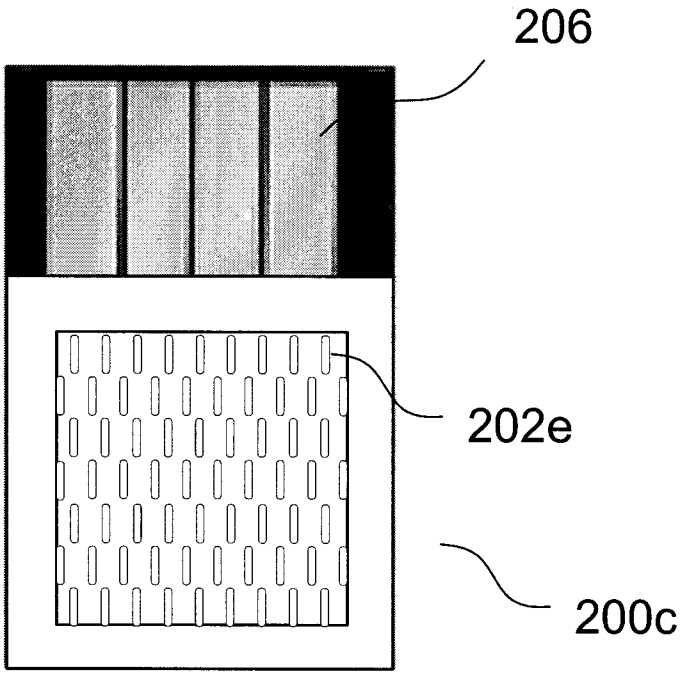


FIG. 2M

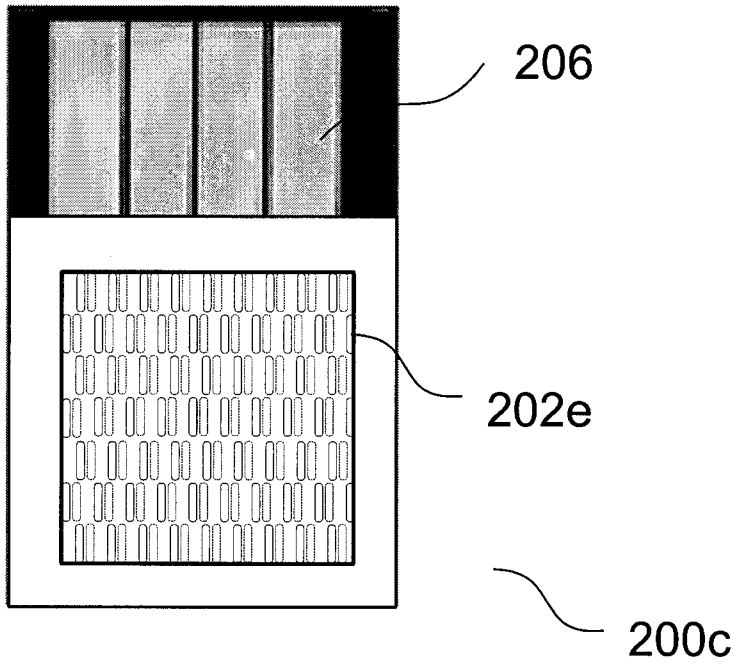


FIG. 2N

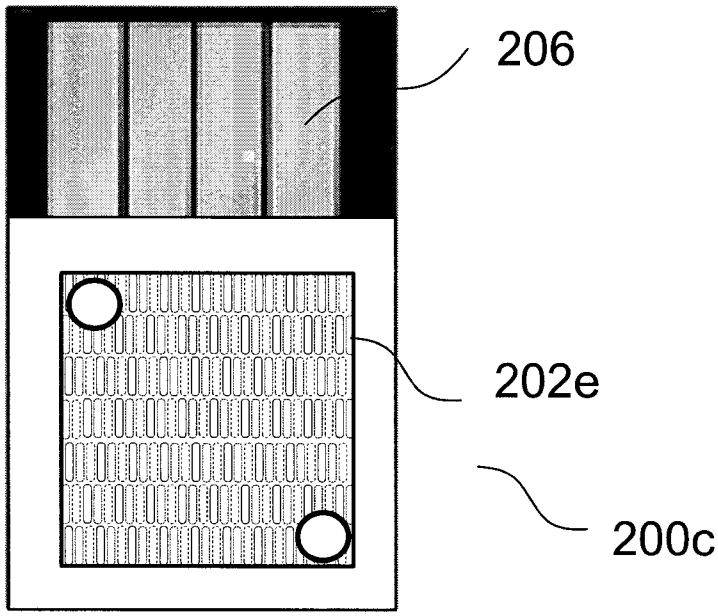
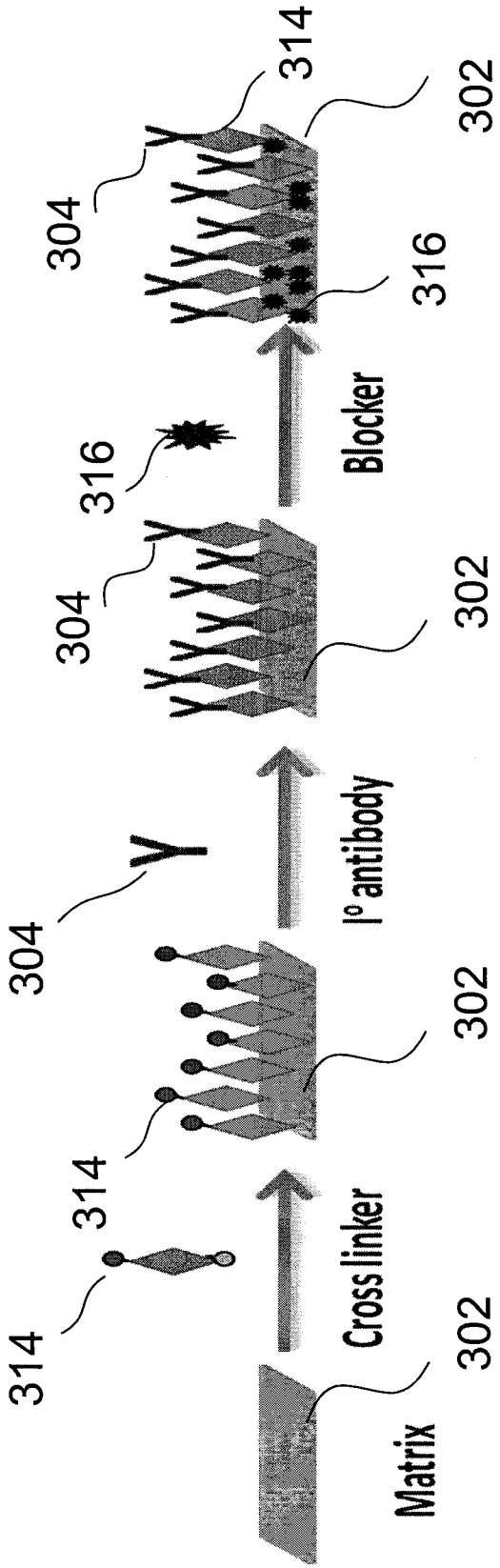


FIG. 3A


300
314
314
Cross linker
302
Matrix
304
I° antibody
302
304
316
Blocker
316
304
314
302

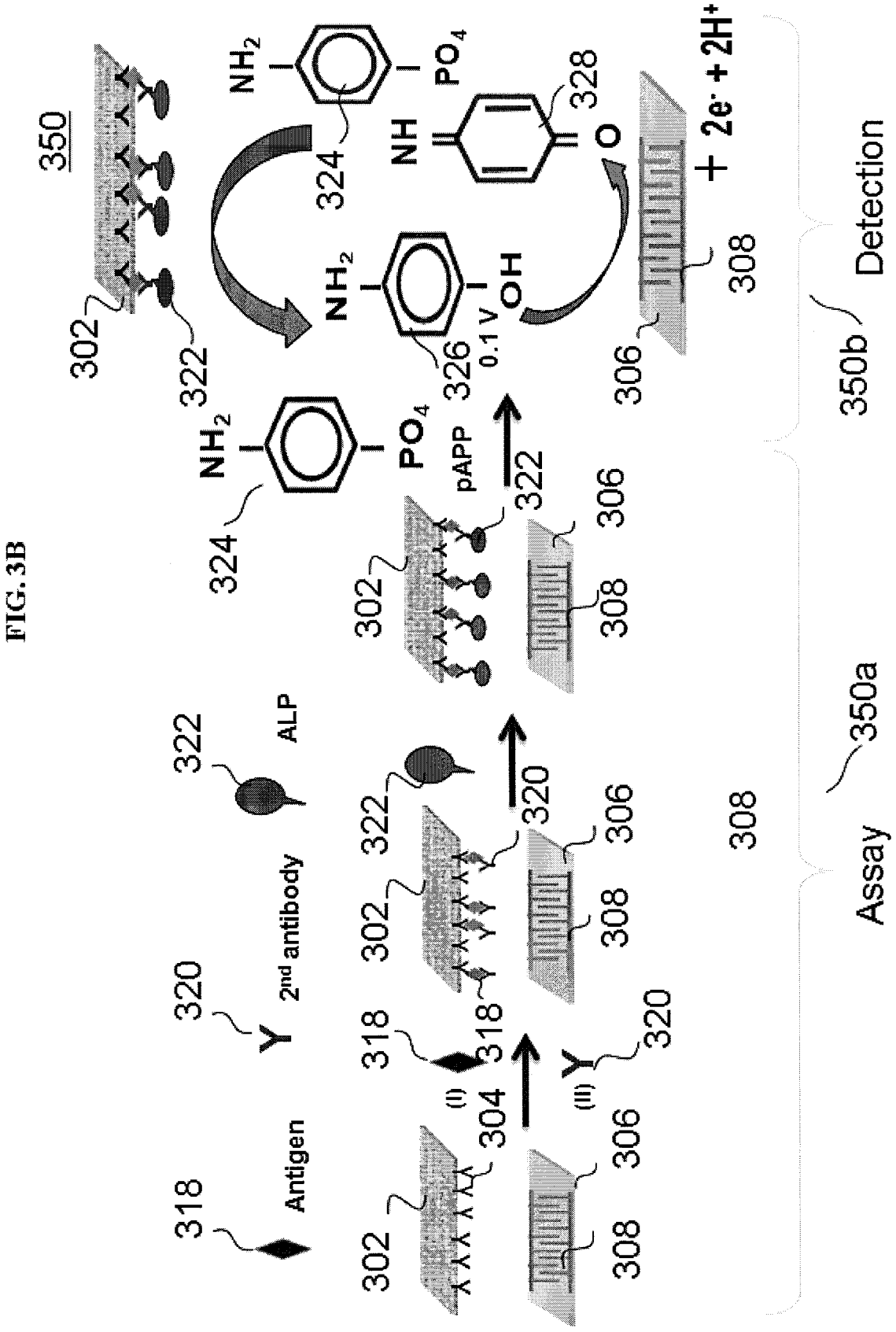

Assay
Detection
350a
350b
308
318
Antigen
320
2nd antibody
322
ALP
302
304
306
(I)
(II)
pAPP
324
326
328
350
0.1 V
+ 2e⁻ + 2H⁺
NH2
PO4
OH
NH
O

FIG. 3B

FIG. 4A

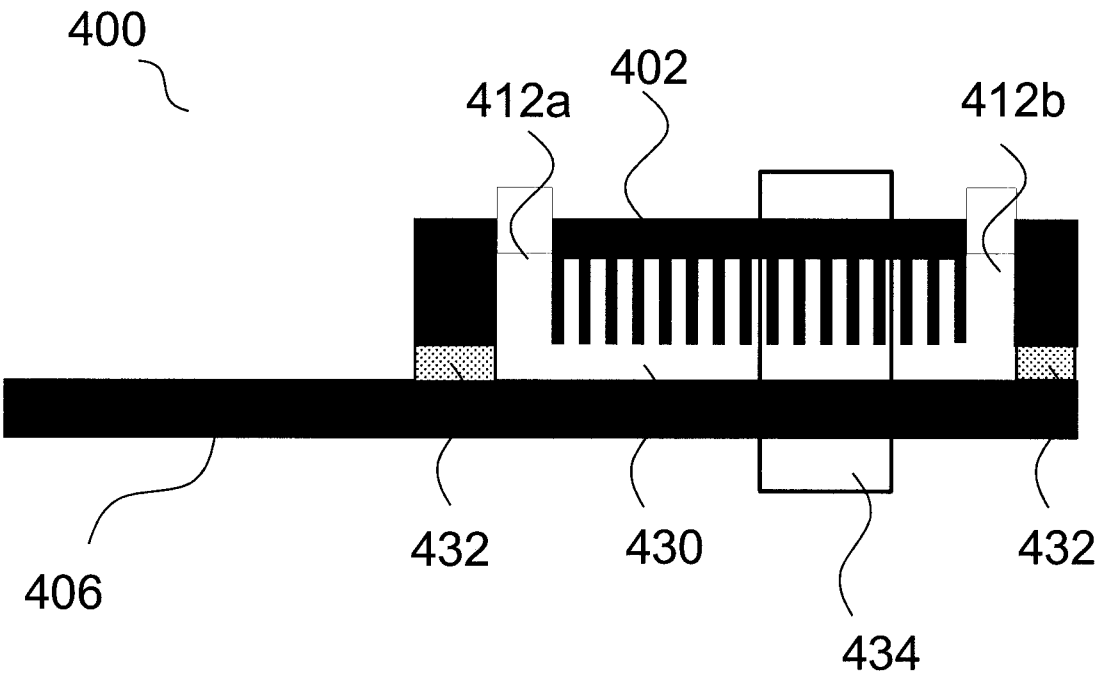


FIG. 4B

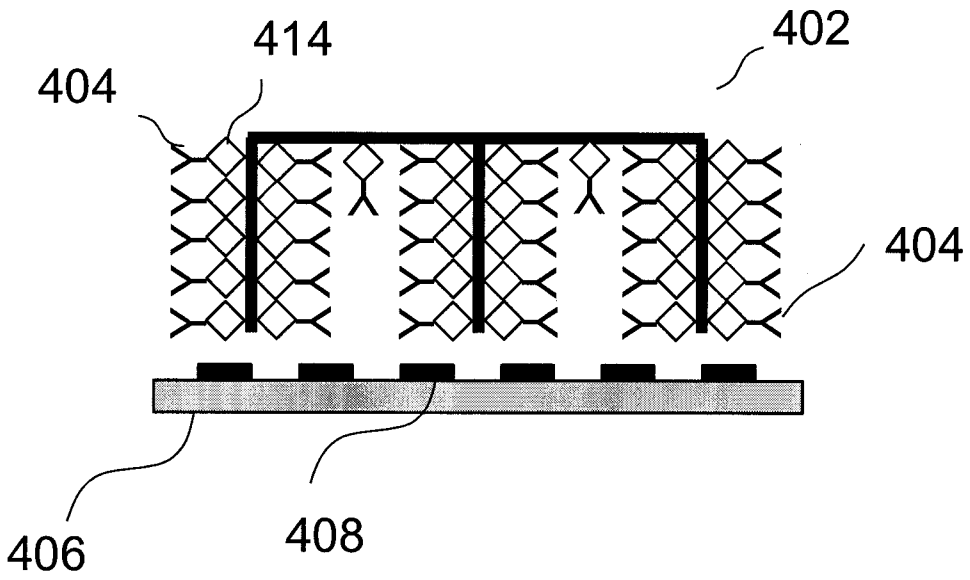


FIG. 4C

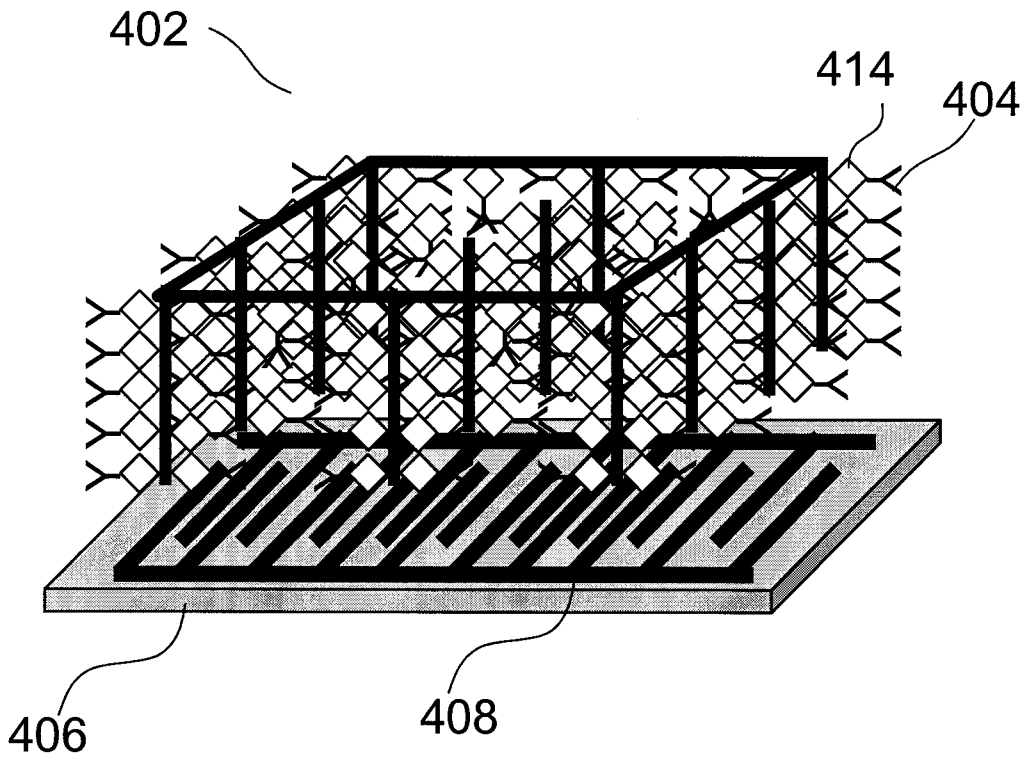


FIG. 5A

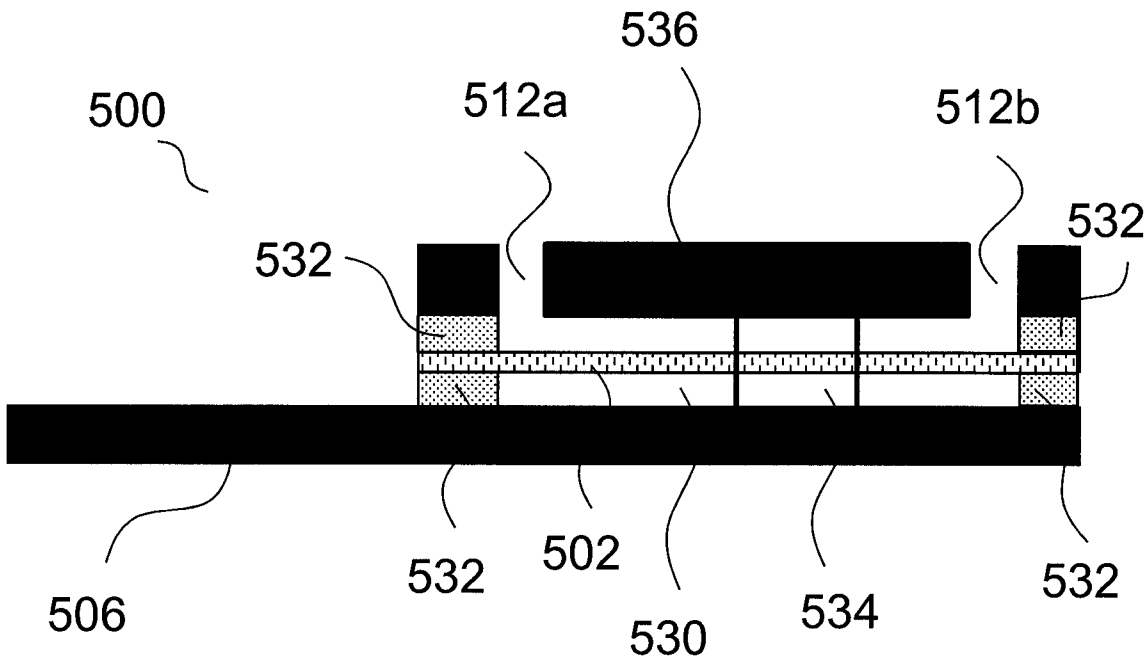


FIG. 5B

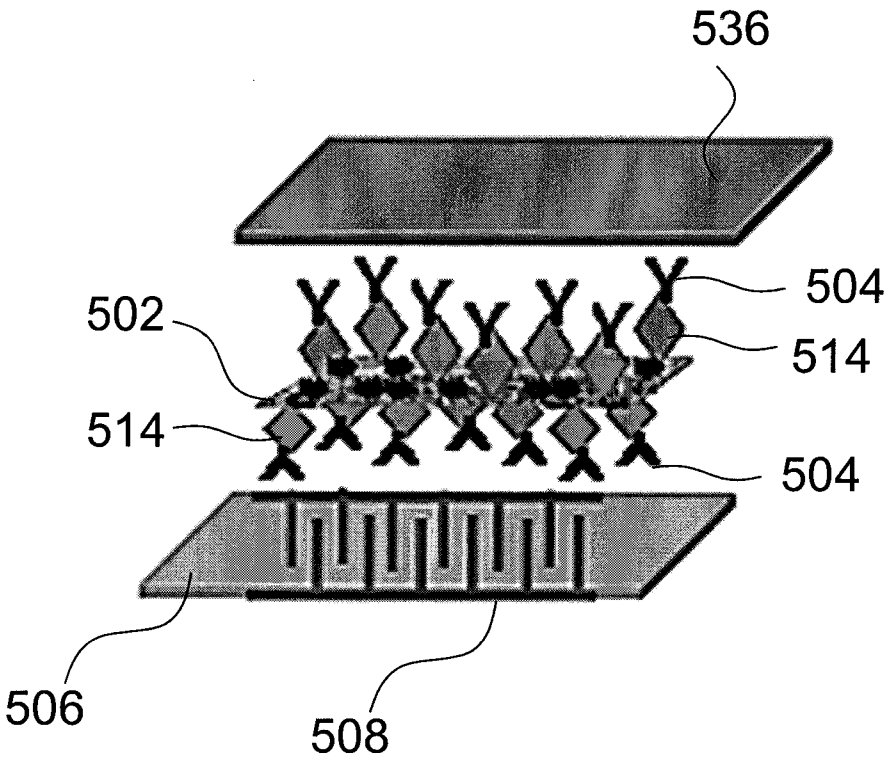


FIG. 6A

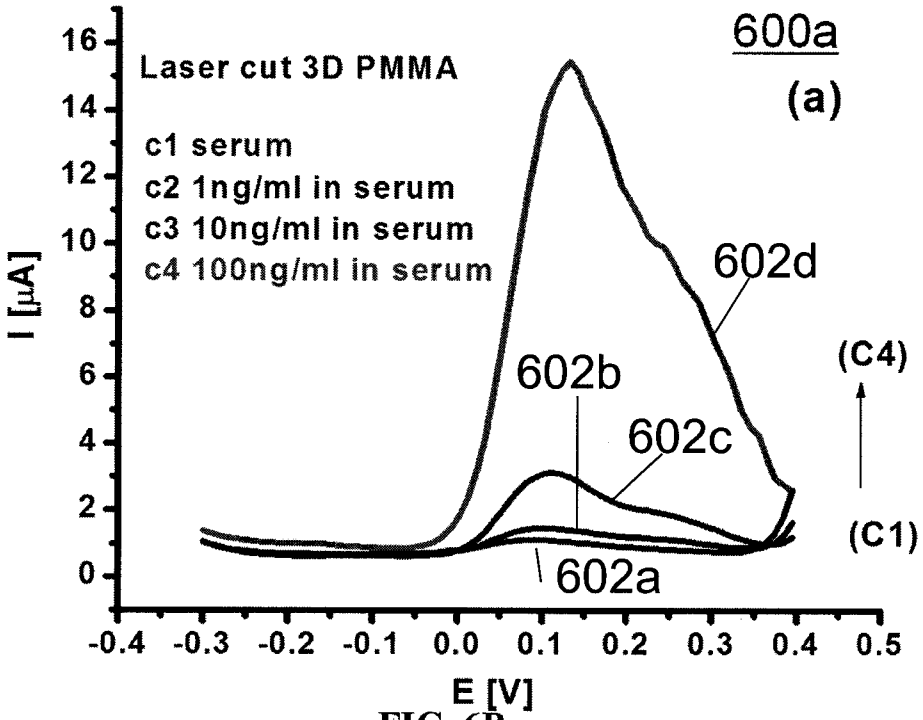

600a
(a)
Laser cut 3D PMMA
c1 serum
c2 1ng/ml in serum
c3 10ng/ml in serum
c4 100ng/ml in serum
602d
602b
602c
602a
(C4)
(C1)
I [μA]
E [V]
16
14
12
10
8
6
4
2
0
-0.4 -0.3 -0.2 -0.1 0.0 0.1 0.2 0.3 0.4 0.5

FIG. 6B

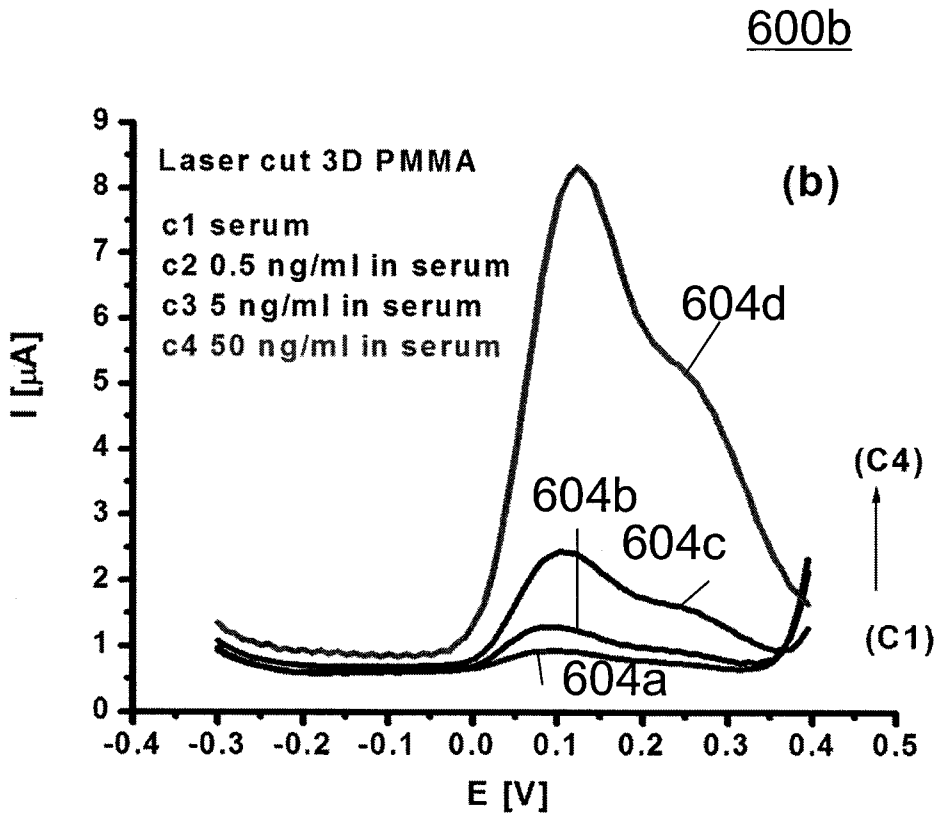

600b
(b)
Laser cut 3D PMMA
c1 serum
c2 0.5 ng/ml in serum
c3 5 ng/ml in serum
c4 50 ng/ml in serum
604d
604b
604c
604a
(C4)
(C1)
I [μA]
E [V]
9
8
7
6
5
4
3
2
1
0
-0.4 -0.3 -0.2 -0.1 0.0 0.1 0.2 0.3 0.4 0.5

FIG. 6C

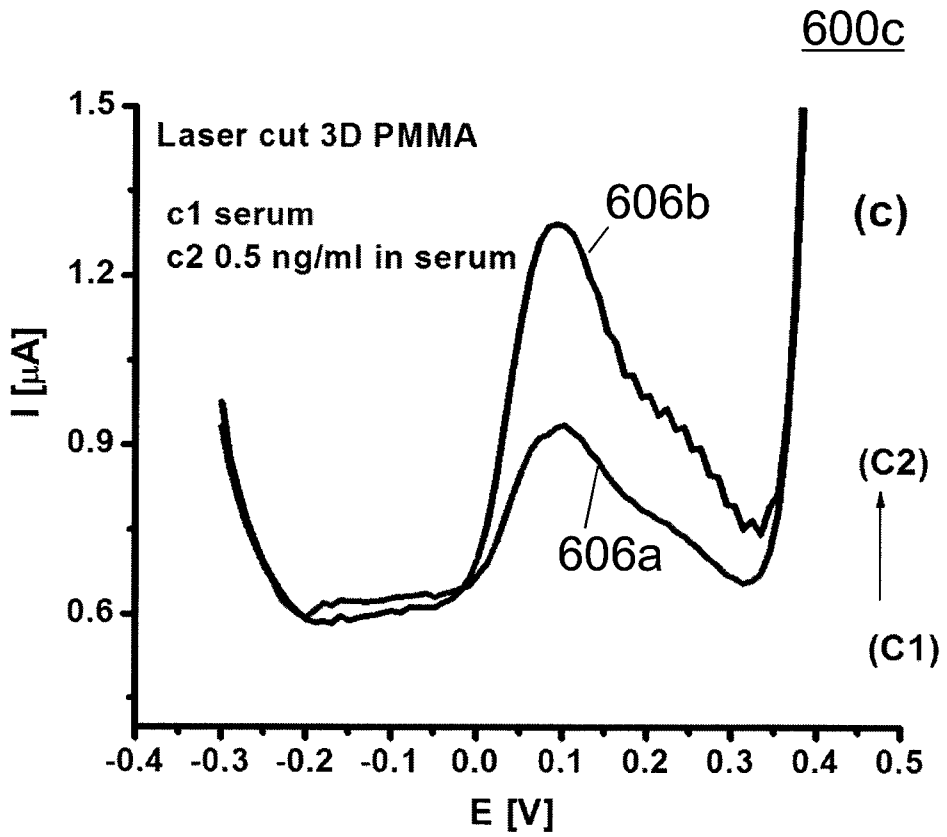

600c
Laser cut 3D PMMA
c1 serum
c2 0.5 ng/ml in serum
606b
606a
(c)
(C2)
(C1)
I [μA]
E [V]
1.5
1.2
0.9
0.6
-0.4
-0.3
-0.2
-0.1
0.0
0.1
0.2
0.3
0.4
0.5

FIG. 7A

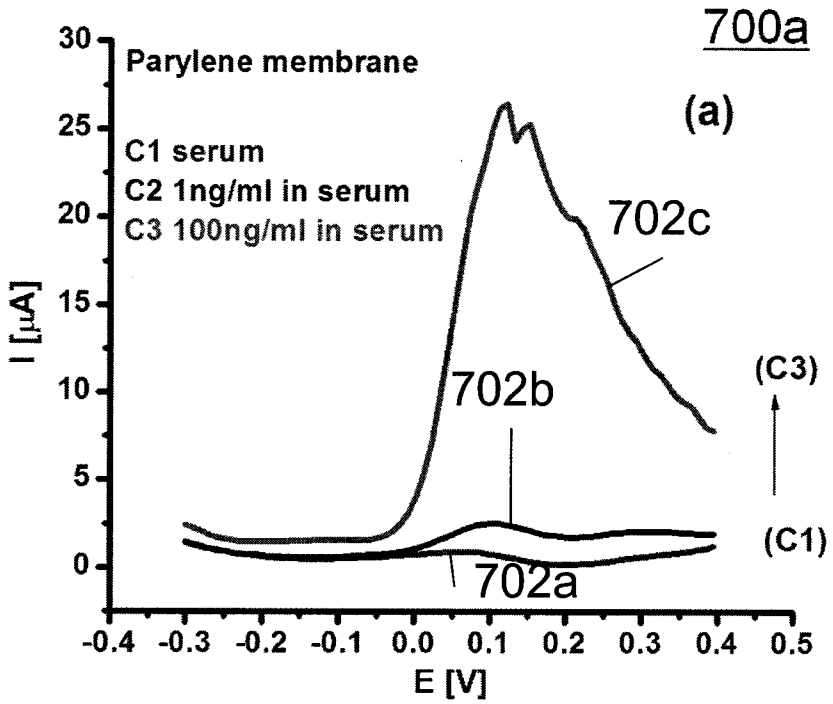

700a
Parylene membrane
(a)
C1 serum
C2 1ng/ml in serum
C3 100ng/ml in serum
702c
702b
(C3)
(C1)
702a
I [μA]
E [V]
30
25
20
15
10
5
0
-0.4 -0.3 -0.2 -0.1 0.0 0.1 0.2 0.3 0.4 0.5

FIG. 7B

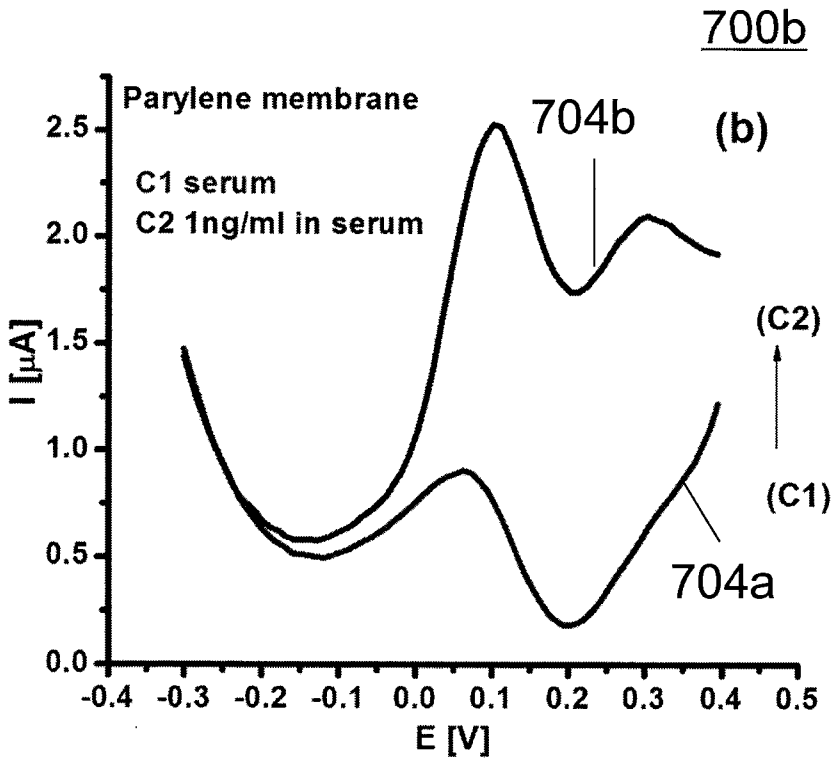

700b
Parylene membrane
704b
(b)
C1 serum
C2 1ng/ml in serum
(C2)
(C1)
704a
I [μA]
E [V]
2.5
2.0
1.5
1.0
0.5
0.0
-0.4 -0.3 -0.2 -0.1 0.0 0.1 0.2 0.3 0.4 0.5

FIG. 7C

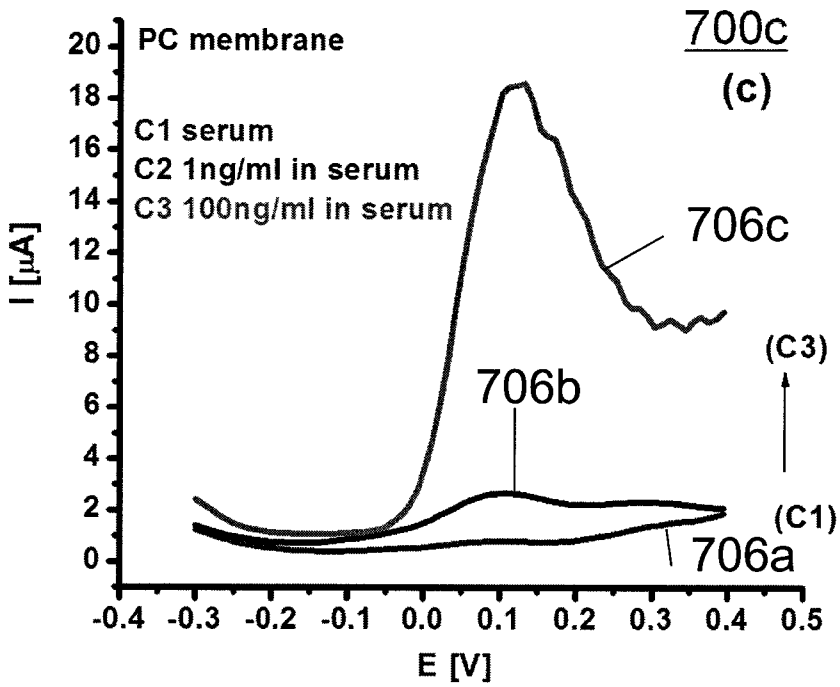

700c
(c)
PC membrane
C1 serum
C2 1ng/ml in serum
C3 100ng/ml in serum
706c
706b
706a
(C3)
(C1)
I [μA]
E [V]
20
18
16
14
12
10
8
6
4
2
0
-0.4 -0.3 -0.2 -0.1 0.0 0.1 0.2 0.3 0.4 0.5

FIG. 7D

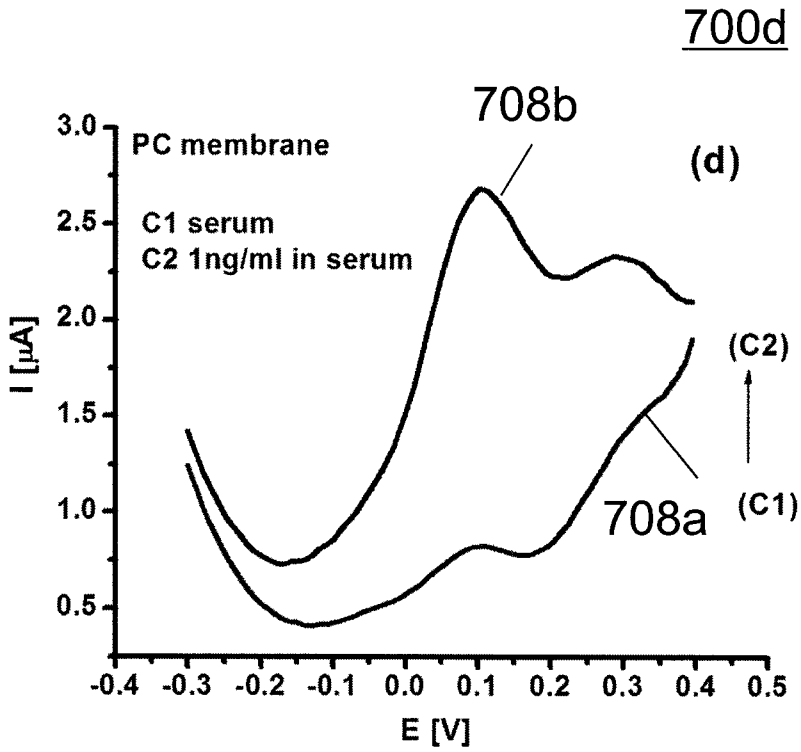

700d
708b
(d)
PC membrane
C1 serum
C2 1ng/ml in serum
708a
(C2)
(C1)
I [μA]
E [V]
3.0
2.5
2.0
1.5
1.0
0.5
-0.4 -0.3 -0.2 -0.1 0.0 0.1 0.2 0.3 0.4 0.5

FIG. 8

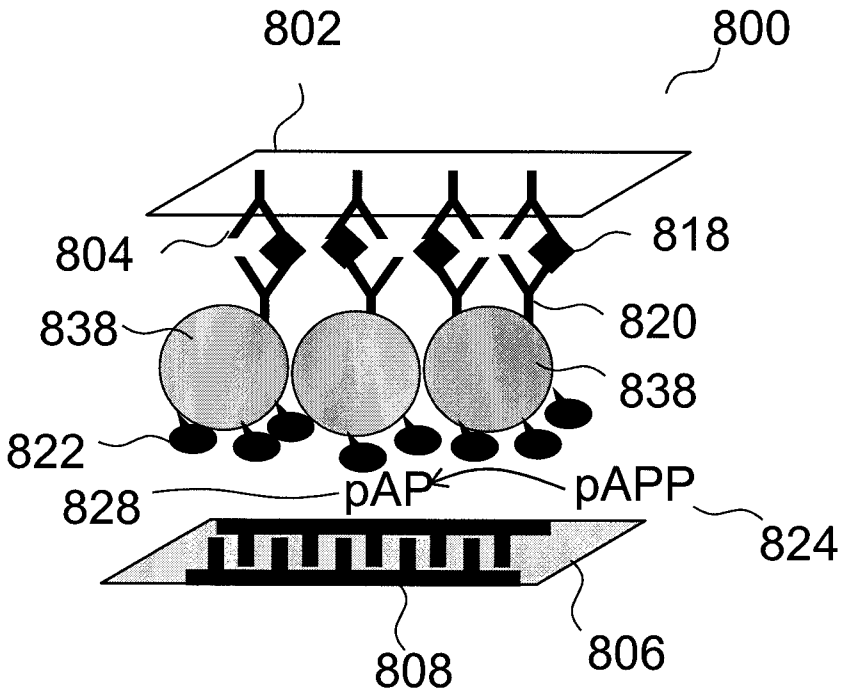


FIG. 9A

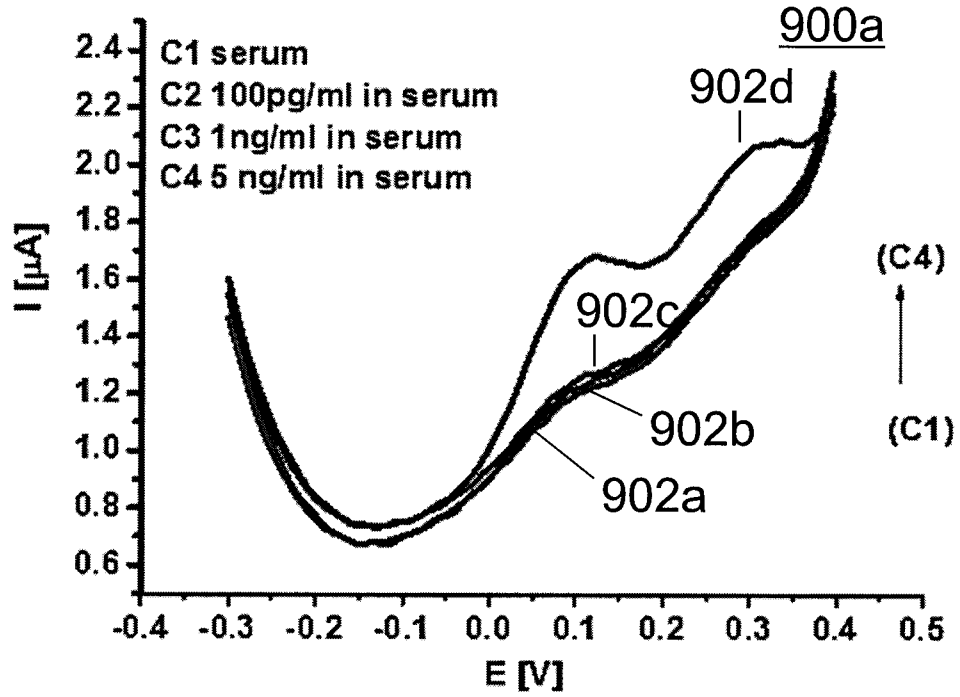
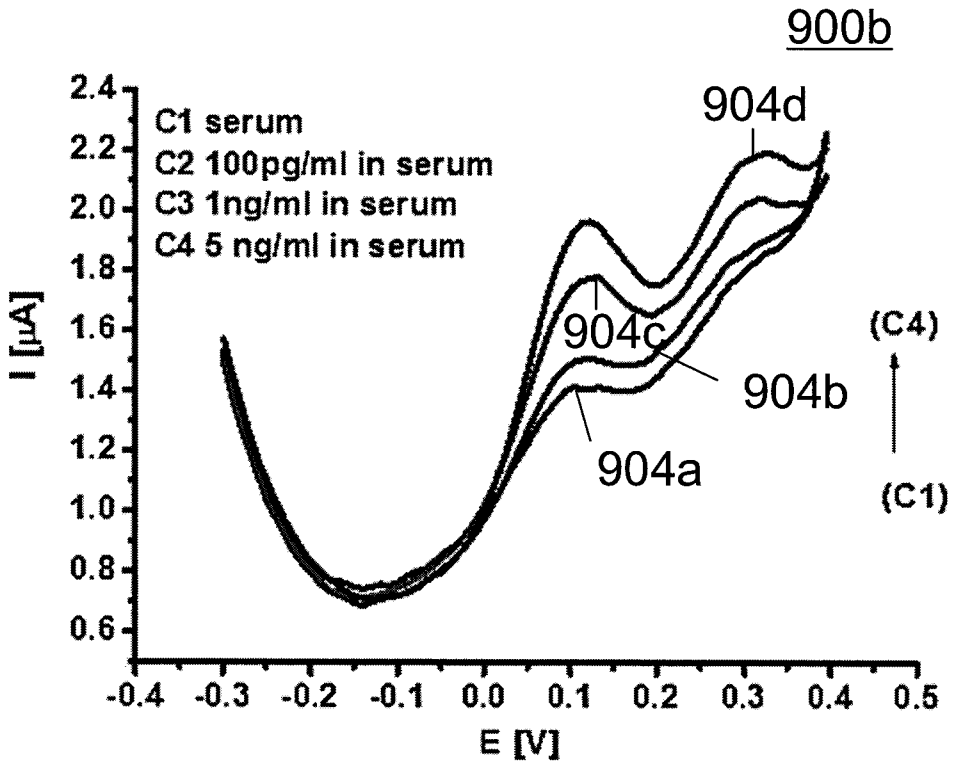

900a
C1 serum
C2 100pg/ml in serum
C3 1ng/ml in serum
C4 5 ng/ml in serum
902d
902c
902b
902a
(C4)
(C1)
I [μA]
E [V]
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
-0.4 -0.3 -0.2 -0.1 0.0 0.1 0.2 0.3 0.4 0.5

FIG. 9B


900b
C1 serum
C2 100pg/ml in serum
C3 1ng/ml in serum
C4 5 ng/ml in serum
904d
904c
904b
904a
(C4)
(C1)
I [μA]
E [V]
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
-0.4 -0.3 -0.2 -0.1 0.0 0.1 0.2 0.3 0.4 0.5

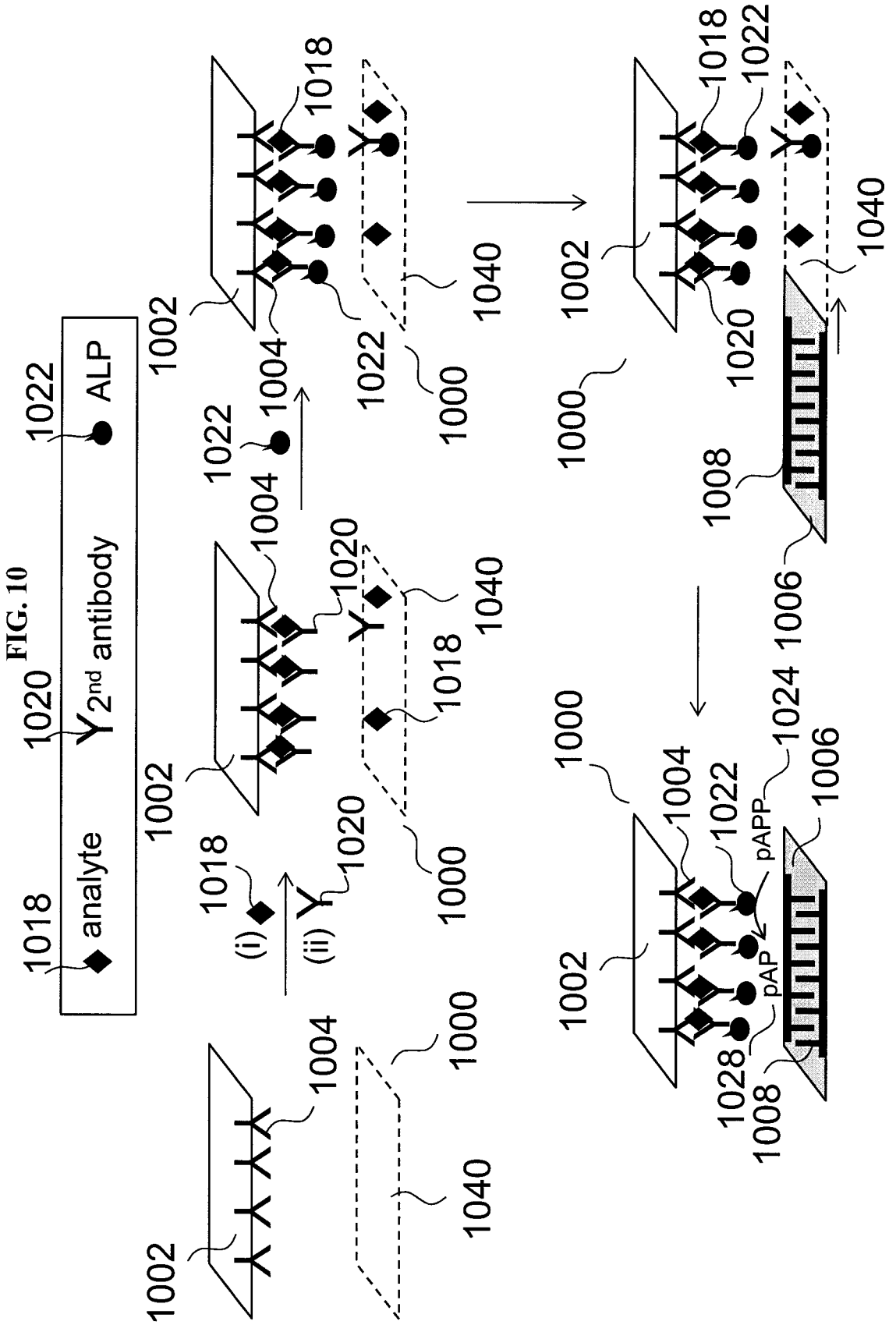
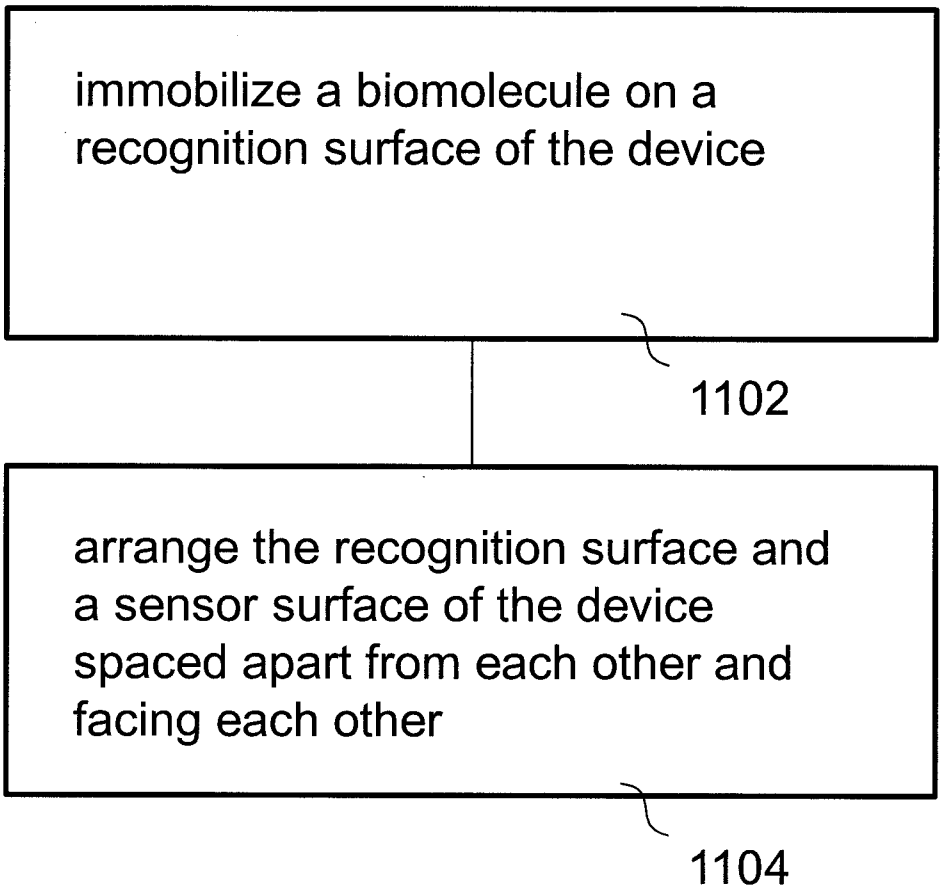

1018
analyte
1020
2nd antibody
1022
ALP
1002
1004
1000
1040
(i)
(ii)
pAP
pAPP
1024
1006
1008
1028

FIG. 10

FIG. 11


immobilize a biomolecule on a recognition surface of the device
1102
arrange the recognition surface and a sensor surface of the device spaced apart from each other and facing each other
1104

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2016/050174

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/543 (2006.01) G01N 33/535 (2006.01)

According to International Patent Classification (IPC)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

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

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

FAMPAT, BIOSIS, CAPLUS, MEDLINE, EMBASE, SCOPUS: Separated, different, apart, opposite, facing, distinct, surface, substrate, base, support, electrochemical, immunoassay, electrode and like terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 86/03837 A1 (IQ (BIO) LIMITED) 3 July 1986 Pg. 5, lines 2-22; pg. 8, line 21- pg. 9, line 13; pg. 9, line 23; pg. 17, line 11- pg. 18, line 22; examples 1, 3; fig. 4	1-20
X	WO 95/31725 A1 (CAMBRIDGE LIFE SCIENCES PLC ET AL.) 23 November 1995 Pg. 2, para. 2; pg. 3, paras. 1-2; pg. 4, para. 2; pg. 5-6; fig. 1	1-20
X	US 2003/0186274 A1 (LIMOGES B. ET AL.) 2 October 2003 Paras. [0028], [0030], [0032],[0036]; fig. 1	1-20
A	WO 2009/068862 A1 (THE SECRETARY OF STATE FOR INNOVATION, UNIVERSITIES AND SKILLS OF HER MAJESTY'S BRITANNIC GOVERNMENT) 4 June 2009 Whole document, see especially pg. 3, lines 6-11	-

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

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"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
04/07/2016 *(day/month/year)*	11/07/2016 *(day/month/year)*

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IPOS Customer Service Tel. No.: (+65) 6339 8616

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/SG2016/050174

Note: This Annex lists known patent family members relating to the patent documents cited in this International Search Report. This Authority is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 86/03837 A1	3/7/1986	AU 5310386 A	22/7/1986
		EP 0235153 A1	9/9/1987
		JPS 62501171 A	7/5/1987
WO 95/31725 A1	23/11/1995	CA 2189994 A1	23/11/1995
		EP 0760101 A1	5/3/1997
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		JPH 10500212 A	6/1/1998
US 2003/0186274 A1	2/10/2003	AT 306670 T	15/10/2005
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		AU 2008/328588 A1	4/6/2009
		AU 2009/269863 A1	14/1/2010
		CN 101971029 A	9/2/2011
		CN 102089661 A	8/6/2011
		EP 2220494 A1	25/8/2010
		EP 2316029 A1	4/5/2011
		GB 2462062 A	27/1/2010
		GB 2458420 A	23/9/2009
		JP 2011/504593 A	10/2/2011
		JP 2011/527431 A	27/10/2011
		NZ 585851 A	29/4/2011
		NZ 590219 A	31/5/2013
		US 2010/320092 A1	23/12/2010
		US 2012/034708 A1	9/2/2012