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(54) **METHODS AND COMPOSITIONS FOR AMPLIFIED ELECTROCHEMILUMINESCENCE DETECTION**

VERFAHREN UND ZUSAMMENSETZUNGEN FÜR VERSTÄRKTE ELEKTROCHEMILUMINESZENZDETEKTION

PROCÉDÉS ET COMPOSITIONS POUR DÉTECTION AMPLIFIÉE D'UNE ÉLECTROCHIMILUMINESCENCE

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Description

BACKGROUND

[0001] A typical microarray system generally comprises biomolecular probes, such as DNA, proteins, or peptides, formatted on a solid planar surface like glass, plastic, or silicon chip, plus the instruments needed to handle samples (automated robotics), to read the reporter molecules (scanners) and analyze the data (bioinformatic tools). Microarray technology can facilitate monitoring of many probes per square centimeter. Advantages of using multiple probes include, but are not limited to, speed, adaptability, comprehensiveness and the relatively cheaper cost of high volume manufacturing. The uses of such an array include, but are not limited to, diagnostic microbiology, including the detection and identification of pathogens, investigation of anti-microbial resistance, epidemiological strain typing, investigation of oncogenes, analysis of microbial infections using host genomic expression, and polymorphism profiles.

[0002] Electrochemiluminescence or electrogenerated chemiluminescence (ECL) is a kind of luminescence produced during electrochemical reactions in solutions. In electrogenerated chemiluminescence, electrochemically generated intermediates undergo a highly exergonic reaction to produce an electronically excited state that then emits light upon relaxation to a lower-level state. This wavelength of the emitted photon of light corresponds to the energy gap between these two states. ECL excitation can be caused by energetic electron transfer (redox) reactions of electrogenerated species. Such luminescence excitation is a form of chemiluminescence where one/all reactants are produced electrochemically on the electrodes.

[0003] ECL is usually observed during application of potential (several volts) to electrodes of electrochemical cell that contains solution of luminescent species (polycyclic aromatic hydrocarbons, metal complexes, Quantum Dots or Nanoparticles) in aprotic organic solvent (ECL composition). In organic solvents both oxidized and reduced forms of luminescent species can be produced at different electrodes simultaneously or at a single one by sweeping its potential between oxidation and reduction. The excitation energy is obtained from recombination of oxidized and reduced species.

[0004] In aqueous medium which is mostly used for analytical applications simultaneous oxidation and reduction of luminescent species is difficult to achieve due to electrochemical splitting of water itself so the ECL reaction with the coreactants is used. In the later case luminescent species are oxidized at the electrode together with the coreactant which gives a strong reducing agent after some chemical transformations (the oxidative reduction mechanism).

[0005] There is a need for a platform which can simultaneously detect multiple analytes of varying concentrations. Typical ELISA based assays have 4 orders of magnitude and hence is restricted in detecting multiple analytes varying from ug/ml to fg/ml. Recent advances in electrochemiluminescence have pushed the limits of detection to pg/ml with a dynamic range up to 4- 5orders in log scale. For example, Yuan, L., et al., Integrated Tyramide and Polymerization-assisted Signal Amplification for a Highly Sensitive Immunoassay, Analytical Chemistry 84, 10737-10744 (2012), discloses an electrochemiluminescence method that can detect biomolecules using Quantum Dots. Sardesai, N.P., et al., A Microfluidic Electrochemiluminescent Device for Detecting Cancer Biomarker Proteins, Analytical and Bioanaytical Chemistry 405, 3831-3838 (2013) discloses an electrochemiluminescence method that can detect biomolecules using RuBYP silica nanoparticles. However, simultaneous detection of multiple analytes in varying concentration of 6 or more magnitudes has not been possible due to limitation on the tags used for electrochemiluminescence.

SUMMARY

[0006] The present disclosure encompasses, in several aspects formulations, substrates, and arrays. The invention includes methods of detecting analytes using the formulations, substrates, and arrays.

[0007] The invention comprises methods of detecting a target biomolecule comprising contacting a sample comprising said target biomolecule with a capture ligand, said capture ligand being immobilized at a defined location on a substrate and capable of specifically binding said target biomolecule thereby immobilizing said target biomolecule at said defined location on said substrate; contacting said immobilized target biomolecule with a detection ligand, said detection ligand capable of specifically binding to said immobilized target biomolecule and having peroxidase activity thereby forming an immobilized target biomolecule-detection ligand complex; contacting said complex with a tagging solution comprising an AECL tag under conditions that promote covalent binding of a plurality of AECL tags to said complex, wherein each of the AECL tags comprises a rare earth metal chelate and a tyramide bound to said metal chelate; washing said substrate to remove unbound AECL tag from said substrate; contacting said substrate with a detection solution that reacts with said bound AECL tag to generate luminescence when a voltage is applied to said defined location on said substrate; applying said voltage to said defined location on said substrate; and measuring luminescence from said defined location on said substrate thereby detecting said target biomolecule; wherein the method detects said target biomolecule in the sample at concentrations as low as 1 fg/mL.

[0008] In certain embodiments, the defined location on the substrate is a microarray feature or a plurality of microarray features. In certain embodiments, the features can have an edge dimension between 50 nm and 1 um, or between 50

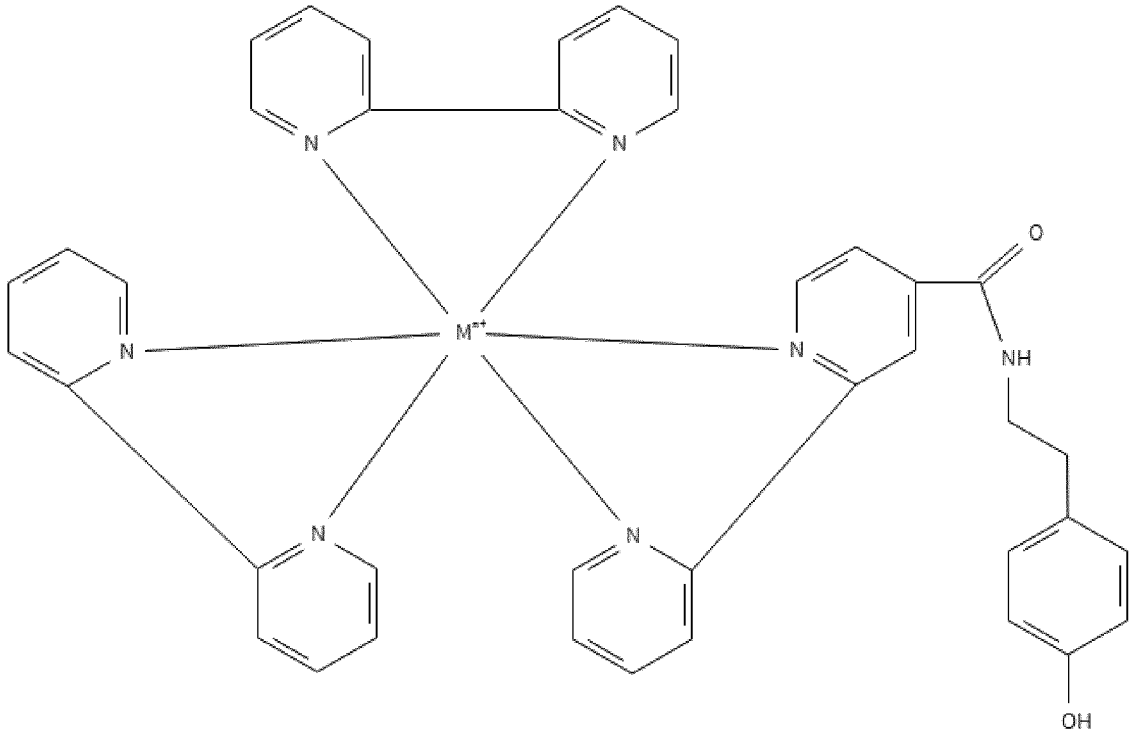
nm and 100 nm or between 50 nm and 75 nm. In certain embodiments, the capture ligands are covalently bound to the defined location via a COOH or an NH2 moiety provided on the substrate.

[0009] In certain embodiments, the capture ligand and detection ligand comprise antibodies, peptides, proteins, or antigen binding proteins.

[0010] In certain embodiments, the sample can comprise blood, serum, plasma, saliva, urine, feces or cerebrospinal fluid (CSF).

[0011] In certain embodiments, the sample is obtained from a human subject.

[0012] In certain embodiments, the AECL tag comprisesa ruthenium chelate or tris (bipyridine)ruthenium(II). In certain embodiments the AECL tag comprises



, wherein M^{n+} is Ru^{2+}.

[0013] The invention includes an AECL tag composition. The present disclosure also provides kits and solutions for binding said AECL tag compositions to target biomolecules and detecting their binding via an emitted luminescent signal.

[0014] In other aspects, the disclosure includes solid state microarrays and pillar assemblies for mounting the microarrays and performing AECL assays. In certain aspects of the disclosure, the solid state microarrays comprise chemically-functionalized surfaces comprising COOH or NH2 functional groups that can be covalently bound to capture ligands.

[0015] In some aspects, the solid state microarrays of the present disclosure have an electrical potential difference between at least one pair of working and counter electrodes that generates electrochemiluminescence from bound AECL tags.

[0016] In some aspects of the disclosure, the solid state microarrays comprise at least 2, 4, 8, 16, 32, 64, 100, 200, 500, 1000, 2000, 5000, 10,000, 15,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 1,000,000, 1, 500,000, 2,000,000 or more working electrodes.

[0017] In some aspects, the disclosure includes an assay plate for mounting a solid state microarray. The assay plate includes a pillar that includes a top surface and a bottom surface. The top surface includes a mounting surface to receive a solid state microarray of the invention as well as at least one working electrode and one counter electrode configured to contact and to be in electrical communication with a corresponding at least one working electrode and counter electrode on a bottom surface of the solid state microarray. The bottom surface of the pillar includes contacts for supplying power to the at least one pillar working electrode and pillar counter electrode. In some aspects of the disclosure, the assay plate includes a plurality of pillars, such as 24, 96, 384 or 1586 pillars. In some aspects of the disclosure, the pillars comprise at least 2, 4, 8, 16, 32, 64, 100, 200, 500, 1000, 2000, 5000, 10,000, 15,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 1,000,000, 1, 500,000, 2,000,000 or more working electrodes.

[0018] In some aspects, the disclosure includes an assembly that includes an assay plate with a microarray mounted on the surface of the assay plate pillar such that the corresponding pillar and microarray working and counter electrodes

are in electrical contact. In yet other aspects, the assemblies of the disclosure further include an assay cap, that provides pillar walls mounted on struts that slidably engage grooves on the pillar mounts. When the cap and the assay plate are engaged, the pillar walls provide barriers for a reservoir that can hold assay fluid in contact with the microarray.

[0019] In yet other embodiments, the AECL assays of the invention have improved detection limits, such that concentrations of target biomolecules in a sample can be detected at limits on the order of 1 fg/mL.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0020] These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, and accompanying drawings, where:

Figure 1 shows the structure of an amplified electrochemiluminescent (AECL) tag comprising a metal chelate ester attached to tyramide.

Figure 2 shows the prior art process of tyramide signal amplification catalyzed by horseradish peroxidase (HRP) attached to an antibody. The tyramide is bound to a fluorescent marker. The HRP localized to the secondary antibody catalyzes the binding of tyramide to electron rich moieties (predominantly tyrosine residues) in a target.

Figure 3 shows a prior art sandwich ELISA to detect a captured target biomolecule using an ECL-tagged secondary antibody and application of voltage in the presence of tripropyl amine (TPA) to produce a detectable light signal that can be used to quantitate captured target biomolecule.

Figure 4 illustrates a sandwich ELISA embodiment of the present invention to detect a captured target biomolecule using a secondary antibody - horseradish peroxidase (HRP) conjugate. Upon addition of an AECL tag in the presence of hydrogen peroxide, the HRP catalyzes attachment of the AECL tag's tyramide moiety to electron rich targets (predominantly tyrosine residues) that are proximate to the bound secondary antibody, thus labeling the captured target biomolecule complex with multiple AECL tags. Application of voltage in the presence of TPA produces an amplified light signal that can be used to quantitate captured target biomolecule.

Figure 5 illustrates a cross-sectional view, top view, and bottom view of the electrode and well configuration of an array. In an embodiment, the array is used with the electrochemiluminescence detection methods described herein.

Figure 6 shows a top and bottom view for chips (including an embodiment of electrode and well configurations) comprising 1 feature group, 4 feature groups, or 16 feature groups.

Figure 7 shows top and bottom view for chips comprising 1 pillar group, 4 pillar groups, or 16 pillar groups.

Figure 8 shows a detailed diagram of an AECL pillar mount (top left) onto which four AECL microarray assay chips are mounted. An AECL assay cap (top right) is used to cover the AECL assay plate (bottom right) which as shown, includes 9 separate pillar mounts. The assay cap includes pillar walls mounted on struts that engage grooves on the sides of the assay plate pillars. In connection with the top surface of the pillar mount, the pillar walls form a reservoir that retains the AECL assay solution (*see* side view, bottom left).

Figure 9 shows a diagram of AECL detection of analytes on a single pillar on an assay plate.

Figures 10A and 10B show steps in the AECL chip manufacturing process.

Figures 11 A and 11B respectively show steps in the AECL pillar mount manufacturing process and a top view of an AECL pillar mount according to an aspect of the disclosure.

Figure 12 compares results of ECL and AECL biochip assays for TNF-alpha. Y-axis is luminescence in arbitrary units, X-axis is amount of TNF-alpha/mL in assayed sample solution. Thus 100 ng on X-axis corresponds to a TNF-alpha concentration of 100 ng/mL in the assayed sample solution.

DETAILED DESCRIPTION

[0021] Terms used in the claims and specification are defined as set forth below unless otherwise specified.

[0022] As used herein the term "wafer" refers to a slice of semiconductor material, such as silicon or a germanium crystal generally used in the fabrication of integrated circuits. Wafers can be in a variety of sizes from, e.g., 25.4 mm (1 inch) to 300 mm (11.8 inches) along one dimension with thickness from, e.g., 275μm to 775μm.

[0023] As used herein the terms "biomolecule," "polypeptide," "peptide," or "protein" are used interchangeably to describe a chain or polymer of amino acids that are linked together by bonds. Accordingly, the term "peptide" as used herein includes a dipeptide, tripeptide, oligopeptide, and polypeptide. The term "peptide" is not limited to any particular number of amino acids. In some aspects, a peptide contains about 2 to about 50 amino acids, about 5 to about 40 amino acids, or about 5 to about 20 amino acids. A molecule, such as a protein or polypeptide, including an enzyme, can be a "native" or "wild-type" molecule, meaning that it occurs naturally in nature; or it may be a "mutant," "variant," "derivative," or "modification," meaning that it has been made, altered, derived, or is in some way different or changed from a native molecule or from another molecule such as a mutant.

[0024] As used herein the term "microarray" refers to a substrate on which different probe molecules of proteins (e.g.,

antibodies, antibody fragments, or other polypeptide sequences) or specific DNA binding sequences have been affixed at separate locations in an ordered manner thus forming a microscopic array. Specific probes are present in large copy number (e.g., 10^6) within an array unit called a feature. An array can be characterized by the feature density (e.g., # features/cm^2), the total number of features, the length of a feature edge, a feature area, or the separation between features (sometimes referred to as the array's "pitch").

[0025] As used herein the term "microarray system" refers to a system usually comprised of biomolecular probes formatted on a solid planar surface like glass, plastic or silicon chip plus the instruments needed to handle samples (automated robotics), to read the reporter molecules (scanners) and analyze the data (bioinformatic tools).

[0026] As used herein, the terms "immunological binding" and "immunological binding properties" refer to the type of non-covalent interactions that occurs between an immunoglobulin molecule (or variant thereof such as an scFv) and an antigen for which the immunoglobulin is specific.

[0027] As used herein the term "biological sample" refers to a sample derived from biological tissue or fluid that can be assayed for an analyte(s) of interest. Such samples include, but are not limited to, sputum, amniotic fluid, blood, blood cells (e.g., white cells), tissue or fine needle biopsy samples, urine, peritoneal fluid, and pleural fluid, or cells therefrom. Biological samples may also include sections of tissues such as frozen sections taken for histological purposes. Although the sample is typically taken from a human patient, the assays can be used to detect analyte(s) of interest in samples from any organism (e.g., mammal, bacteria, virus, algae, or yeast) or mammal, such as dogs, cats, sheep, cattle, and pigs. The sample may be pretreated as necessary by dilution in an appropriate buffer solution or concentrated, if desired.

[0028] As used herein, the term "assay" refers to a type of biochemical test that measures the presence or concentration of a substance of interest in solutions that can contain a complex mixture of substances.

[0029] The term "subject" as used herein may refer to a human or any other animal having a disorder for testing, diagnosis or treatment.

[0030] The term "antigen" as used herein refers to a molecule that triggers an immune response by the immune system of a subject, e.g., the production of an antibody by the immune system and/or activation of the cellular arm of the immune system (e.g., activation of phagocytes, natural killer cells, and antigen-specific cytotoxic T-lymphocytes, along with release of various cytokines in response to an antigen). Antigens can be exogenous, endogenous or auto antigens. Exogenous antigens are those that have entered the body from outside through inhalation, ingestion or injection. Endogenous antigens are those that have been generated within previously-normal cells as a result of normal cell metabolism, or because of viral or intracellular bacterial infection. Auto antigens are those that are normal protein or protein complex present in the host body but can stimulate an immune response.

[0031] As used herein the term "epitope" or "immunoactive regions" refers to distinct molecular surface features of an antigen capable of being bound by component of the adaptive immune system, e.g., an antibody or T cell receptor. Antigenic molecules can present several surface features that can act as points of interaction for specific antibodies. Any such distinct molecular feature can constitute an epitope. Therefore, antigens have the potential to be bound by several distinct antibodies, each of which is specific to a particular epitope.

[0032] As used herein the term "antibody" or "immunoglobulin molecule" refers to a molecule naturally secreted by a particular type of cells of the immune system: B cells. There are five different, naturally occurring isotypes of antibodies, namely: IgA, IgM, IgG, IgD, and IgE.

[0033] As used herein the term "immune-related molecule" refers to a biological molecule involved in the activation or regulation of an immune response. These include, for example, an antibody, T cell receptor, or MHC complex (e.g., human leukocyte antigen).

[0034] As used herein, the term "inflammatory response molecule" refers to molecules that signal or mediate an inflammatory response, e.g., cytokines such as interleukin and tumor necrosis factor. Inflammatory response molecules include, for example, pro-inflammatory molecules.

[0035] As used herein, the term "autoimmune disorder" refers to any of a large group of diseases characterized by abnormal functioning of the immune system that causes a subject's immune system to damage the subject's own tissues. Celiac disorder, lupus erythematosis, and rheumatoid arthritis are examples of autoimmune disorders. Autoimmune disorders may be induced by environmental factors.

[0036] The term "percent identity" or "percent sequence identity," in the context of two or more nucleic acid or polypeptide sequences, refer to two or more sequences or subsequences that have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned for maximum correspondence, as measured using one of the sequence comparison algorithms described below (e.g., BLASTP and BLASTN or other algorithms available to persons of skill) or by visual inspection. Depending on the application, the percent "identity" can exist over a region of the sequence being compared, e.g., over a functional domain, or, alternatively, exist over the full length of the two sequences to be compared.

[0037] For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer,

subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

[0038] Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection (see generally Ausubel et al., infra).

[0039] One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., J. Mol. Biol. 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information website.

[0040] It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

Compositions

AECL tags and secondary antibody.

[0041] Also disclosed herein are compositions for amplified electrochemiluminescent detection of biomolecules of interest on an array. In an embodiment, a compound is provided that covalently links an electrochemiluminescence (ECL) moiety with a signal amplification moiety to generate an amplified electrochemiluminescence (AECL) tag. **Figure 1.** The AECL tag generates detectable electromagnetic radiation (i.e., light) upon exposure to voltage in the presence of tripropyl amine (TPA). The ECL moiety is a rare earth metal chelate ester. In an embodiment the rare earth metal is Ruthenium (Ru). The signal amplification moiety is tyramide. The use of the tyramide as part of the AECL tag provides for a minimal background. The AECL tag is used with an enzyme-conjugated antigen binding protein (e.g., an HRP-conjugated antibody) resulting in highly localized enzyme-mediated AECL tag deposition to improve detection of bound target molecules. *See* **Fig. 4** and accompanying description below.

[0042] Prior art tyramide signal amplification assays result in covalent binding of labeled tyramide to tyrosine residues (e.g., on the secondary antibody, target biomolecule and primary antibody) in the presence of horseradish peroxidase (HRP) and hydrogen peroxide. The label can be a fluorescent tag, or a detectable reaction product e.g., an insoluble product produced by action of another enzyme such as alkaline phosphatase on a chromogenic substrate. *See* **Figure 2.**

[0043] Prior art ECL assays use an ECL tag comprising a metal chelate ester covalently bound to a detection ligand such as, e.g., a secondary antibody used in a sandwich ELISA format. The ECL tag emits light in the presence of tripropyl amine (TPA) when exposed to an electric field (e.g., by supplying a voltage difference across a working electrode in electrical communication with a binding complex comprising the captured target biomolecule and a counter electrode), a phenomena called electrochemiluminescence (**Figure 3**).

[0044] In some embodiments of the present invention, an antibody array is exposed to a sample comprising a biomolecule of interest. At least one primary antibody bound to the array surface binds to the biomolecule of interest. After washing the array, the array is exposed to a solution of secondary antibody conjugated to horseradish peroxidase (HRP), wherein the secondary antibody binds to the biomolecule of interest. After washing, the array is exposed to a solution comprising hydrogen peroxide and an AECL tag. AECL tags bind to complexes attached to the array which comprise primary antibodies bound to protein and secondary antibody conjugated to HRP. The AECL tags comprise tyramide which binds to tyrosine in the presence of HRP (conjugated to the secondary antibody). The array is then washed and exposed to tripropylamine (TPA), which reacts with the rare earth metal chelate of the AECL tag to activate it, thus causing it to generate chemiluminescence when exposed to a voltage potential (e.g., a voltage potential from the array). Thus, under applied voltage, the rare earth metal chelate ester generates an electrochemiluminescent (ECL) output (**Figure 4**). This method of AECL tagging improves the detection sensitivity by at least 10 fold to 1000 fold as compared to commercially available ECL.

Substrates

[0045] Also disclosed herein are substrates. In some aspects a substrate surface is planar (i.e., 2-dimensional). In some aspects a substrate surface is functionalized with free carboxylic acid groups. In some aspects, a substrate surface is functionalized with free amine groups. A surface that is functionalized with free amine groups may be converted to free carboxylic acid groups by reacting with activating the carboxylic acid groups of a molecule comprising at least two free carboxylic acid groups (e.g., converting the carboxylic acid group to a carbonyl group using carbodiimide) and reacting the molecule with the free amine groups attached to the surface of the substrate. In some aspects, the molecule

comprising multiple carboxylic acid groups is succinic anhydride, polyethylene glycol diacid, benzene-1,3,5-tricarboxylic acid, benzenehexacarboxylic acid, or carboxymethyl dextran.

[0046] In some aspects of the disclosure, a substrate can include a porous layer (i.e., a 3-dimensional layer) comprising functional groups for binding a first monomer building block. In some aspects of the disclosure, a substrate surface comprises pillars for peptide attachment or synthesis. In some aspects of the disclosure, a porous layer is added to the top of the pillars.

Porous Layer Substrates

[0047] Porous layers which can be used are flat, permeable, polymeric materials of porous structure which have a carboxylic acid functional group (which is native to the constituent polymer or which is introduced to the porous layer) for attachment of the first peptide building block. For example, a porous layer can be comprised of porous silicon with functional groups for attachment of a polymer building block attached to the surface of the porous silicon. In another example, a porous layer may comprise a cross-linked polymeric material. In some aspects of the disclosure, the porous layer may employ polystyrenes, saccharose, dextrans, polyacryloylmorpholine, polyacrylates, polymethylacrylates, polyacrylamides, polyacrylolpyrrolidone, polyvinylacetates, polyethyleneglycol, agaroses, sepharose, other conventional chromatography type materials and derivatives and mixtures thereof. In some aspects of the disclosure, the porous layer building material is selected from: poly(vinyl alcohol), dextran, sodium alginate, poly(aspartic acid), poly(ethylene glycol), poly(ethylene oxide), poly(vinyl pyrrolidone), poly(acrylic acid), poly(acrylic acid)-sodium salt, poly(acrylamide), poly(N-isopropyl acrylamide), poly(hydroxyethyl acrylate), poly(acrylic acid), poly(sodium styrene sulfonate), poly(2-acrylamido-2-methyl-1-propanesulfonic acid), polysaccharides, and cellulose derivatives. Preferably the porous layer has a porosity of 10-80%. In an aspect of the disclosure, the thickness of the porous layer ranges from 0.01 μm to about 1,000 μm. Pore sizes included in the porous layer may range from 2 nm to about 100 μm.

[0048] According to another aspect of the present disclosure there is provided a substrate comprising a porous polymeric material having a porosity from 10-80%, wherein reactive groups are chemically bound to the pore surfaces and are adapted in use to interact, *e.g.* by binding chemically, with a reactive species, *e.g.,* deprotected monomeric building blocks or polymeric chains. In an aspect of the disclosure the reactive group is a carboxylic acid group. The carboxylic acid group is free to bind, for example, an unprotected amine group of a peptide or polypeptide.

[0049] In an aspect of the disclosure, the porous layer is in contact with a support layer. The support layer comprises, for example, metal, plastic, silicon, silicon oxide, or silicon nitride. In another aspect of the disclosure, the porous layer may be in contact with a patterned surface, such as on top of pillar substrates described below.

AECL Chip Substrates

[0050] Semiconductor manufacturing processes can be used to generate AECL chips that have solid state electrode circuitry built into one surface of a silicon substrate and biomolecular features present, usually patterned as an array on the opposite face of the substrate on the working electrode surface. Any technique useful for patterning biomolecular features such as peptides or proteins can be used, including those for synthesizing peptides *in situ* in an N → C or C → N configuration, or for tethering whole proteins at defined locations using carbodiimide based chemistries such as those described in co-owned cases WO2013/119845 and WO2014/078606, and described below.

[0051] The AECL chip manufacturing process results in production of an integrated biochip sensor device that is attached to a controller used to drive voltage feeds to reference and working electrodes in order to excite a chemiluminescent signal, which, according to embodiments of the present invention, is amplified.

[0052] The controller can also be programmed and used to drive image acquisition and data storage for the assay results. Additional details relating to AECL chip substrate manufacture and the use of the resulting chips in AECL assays is described in greater detail in Examples 1-6, below.

Arrays

[0053] Also disclosed herein are arrays. In some aspects of the disclosure, the surface of the array is functionalized with free carboxylic acids. In some aspects, the free carboxylic acids are activated to bind to amine groups, e.g., during polypeptide synthesis on the surface of the array. In some embodiments, the surface density of free carboxylic acid groups on the array is greater than 10/cm^2, 100/ cm^2, 1,000/ cm^2, 10,000/ cm^2, 100,000/ cm^2, 1,000,000/ cm^2, or 10,000,000/ cm^2.

[0054] In some aspects, an array can be a three-dimensional array, *e.g.,* a porous array comprising features attached to the surface of the porous array. In some aspects, the surface of a porous array includes external surfaces and surfaces defining pore volume within the porous array. In some aspects, a three-dimensional array can include features attached to a surface at positionally-defined locations, said features each comprising: a collection of peptide chains of determinable

sequence and intended length. In an embodiment, within an individual feature, the fraction of peptide chains within said collection having the intended length is characterized by an average coupling efficiency for each coupling step of greater than 98%.

[0055] In some aspects, the average coupling efficiency for each coupling step is at least 98.5%. In some aspects, the average coupling efficiency for each coupling step is at least 99%. In some aspects, the average coupling efficiency for each coupling step is at least 90, 91, 92, 93, 94, 95, 96, 97, 98, 98.5, 98.6,98.7, 98.8, 98.9, 99.0, 99.1, 99.2, 99.3, 99.4, 99.5, 99.6, 99.7, 99.8, 99.9, or 100%.

[0056] In some aspects, each peptide chain is from 5 to 60 amino acids in length. In some aspects, each peptide chain is at least 5 amino acids in length. In some aspects, each peptide chain is at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 amino acids in length. In some aspects, each peptide chain is less than 5, at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, or greater than 60 amino acids in length. In some aspects, each peptide chain comprises one or more L amino acids. In some aspects, each peptide chain comprises one or more D amino acids. In some aspects, each peptide chain comprises one or more naturally occurring amino acids. In some aspects, each peptide chain comprises one or more synthetic amino acids.

[0057] In some aspects, an array can include at least 1,000 different peptide chains attached to the surface. In some aspects, an array can include at least 10,000 different peptide chains attached to the surface. In some aspects, an array can include at least 100, 500, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000, or greater than 10,000 different peptide chains attached to the surface (or any integer in between).

[0058] In some aspects, each of the positionally-defined locations is at a different, known location that is physically separated from each of the other positionally-defined locations. In some aspects, each of the positionally-defined locations is a positionally-distinguishable location. In some aspects, each determinable sequence is a known sequence. In some aspects, each determinable sequence is a distinct sequence.

[0059] In some aspects, the features are covalently attached to the surface. In some aspects, said peptide chains are attached to the surface through a linker molecule or a coupling molecule.

[0060] In some aspects, the features comprise a plurality of distinct, nested, overlapping peptide chains comprising subsequences derived from a source protein having a known sequence. In some aspects, each peptide chain in the plurality is substantially the same length. In some aspects, each peptide chain in the plurality is the same length. In some aspects, each peptide chain in the plurality is at least 5 amino acids in length. In some aspects, each peptide chain in the plurality is at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 amino acids in length. In some aspects, each peptide chain in the plurality is less than 5, at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, or greater than 60 amino acids in length. In some aspects, at least one peptide chain in the plurality is at least 5 amino acids in length. In some aspects, at least one peptide chain in the plurality is at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 amino acids in length. In some aspects, at least one peptide chain in the plurality is less than 5, at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, or greater than 60 amino acids in length. In some aspects, each polypeptide in a feature is substantially the same length. In some aspects, each polypeptide in a feature is the same length. In some aspects, the features comprise a plurality of peptide chains each having a random, determinable sequence of amino acids.

Carboxylic Acid Activation Solutions

[0061] Disclosed herein are activation formulations for activating carboxylic acid so that it reacts with a free amino group of a biomolecule, e.g., a peptide. An activation formulation can include components such as a carboxylic acid group activating compound and a solvent. In an aspect, the carboxylic acid group activating compound is a carbodiimide or a carbodiimide precursor. In some aspects, the carbodiimide is 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. In some aspects, the carboxylic acid group activating compound is *N*-Hydroxysuccinimide (NHS). In some aspects, the carboxylic acid group activating compound is selected from: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide [EDC], N-hydroxysuccinimide [NHS], 1,3-Diisopropylcarbodiimide [DIC], hydroxybenzotriazole (HOBt), (O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate) [HATU], benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate [PyBOP], and N,N-Diisopropylethylamine [DIEA]. In some aspects, the solvent is water. In some aspects, the solvent is N-methylpyrrolidone (NMP). In some aspects, the carboxylic acid group activating compound converts the carboxylic acid to a carbonyl group (i.e., carboxylic acid group activation). In some aspects, the carboxylic acid group is activated for 5, 10, 15, 20, 30, 45, or 60 minutes after exposure to an activation formulation.

[0062] In some aspects, the activation formulation comprises 4 % by weight of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 2 % by weight of N-hydroxysuccinimide (NHS) dissolved in deionized water. In some aspects, the activation formulation comprises 4 % by weight of 1,3-Diisopropylcarbodiimide (DIC) and 2 % by weight of hydroxyben-

zotriazole (HOBt) dissolved in NMP. In some aspects, the activation formulation comprises 4 % by weight of (O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate) (HATU) and 2 % by weight of N,N-Diisopropylethylamine (DIEA) dissolved in NMP. In some aspects, the activation formulation comprises 4 % by weight of Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and 2 % by weight of N,N-Diisopropylethylamine (DIEA) dissolved in NMP.

[0063] In some aspects, the carboxylic acid group activating compound is a carbodiimide precursor. In one aspect, the carbodiimide precursor is converted to a carbodiimide through exposure to radiation, e.g., ultraviolet radiation. In an aspect, the carbodiimide precursor is a thione. The carbodiimide precursor may also be referred to as a photoactivated carbodiimide. In an aspect, photoactivated carbodiimides are used to provide site-specific activation of carboxylic acid groups on an array by spatially controlling exposure of the photoactivated carbodiimide solution to electromagnetic radiation at a preferred activation wavelength. In some aspects, the preferred activation wavelength is 248 nm.

[0064] In an aspect, the carbodiimide precursor is a thione that is converted to carbodiimide via photoactivation. In one aspect, the thione is converted to a hydroxymethyl phenyl carbodiimide after exposure to electromagnetic radiation. In some aspects, the thione is 4,5-dihydro-4-(hydroxymethyl)-1-phenyl-1H-tetrazole-5-thione, 1-ethyl-4-dimethylaminopropyl tetrazole 5-thione, 1,3-Bis(2,2-dimethyl-1,3-dioxolan-4-ylmethyl)-5-thione, 4-cyclohexyl-1H-tetrazole-5(4H)-thione, or 1-phenyl-4-(piperidinomethyl)tetrazole-5(4H)-thione.

[0065] In some aspects, the activation solution comprises a carbodiimide precursor, a solvent, and a polymer. In an aspect, the carbodiimide precursor is 4,5-dihydro-4-(hydroxymethyl)-1-phenyl-1H-tetrazole-5-thione, 1-ethyl-4-dimethylaminopropyl tetrazole 5-thione, or 1,3-Bis(2,2-dimethyl-1,3-dioxolan-4-ylmethyl)-5-thione. In some aspects, the carbodiimide precursor is present in the activation solution at a concentration of 2.5% by weight. In some aspects the carbodiimide precursor is present in the activation solution at a concentration of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3., 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, or 5.0% by weight of the total formulation concentration.

[0066] In some aspects, the solvent is water. In some aspects, the solvent is about 80-90% by weight of the total formulation concentration. In some aspects, the solvent is about less than 70, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or greater than 99% by weight of the total formulation concentration.

[0067] In some aspects, a polymer is a polyvinyl pyrrolidone and/or a polyvinyl alcohol. In some aspects, a polymer is about 0.5-5% by weight of the total formulation concentration. In some aspects, a polymer is about less than 0.1, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3., 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, or greater than 5.0% by weight of the total formulation concentration.

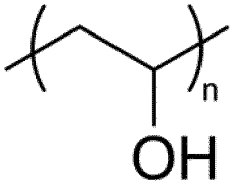
[0068] In some aspects, a coupling reagent is a carbodiimide. In some aspects, a coupling reagent is a triazole. In some aspects, a coupling reagent is 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. In some aspects, a coupling reagent is about 0.5-5% by weight of the total formulation concentration. In some aspects, a coupling reagent is about less than 0.1, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3., 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, or greater than 5.0% by weight of the total formulation concentration.

Linker Formulations

[0069] Also disclosed herein is a linker formulation. A linker formulation can include components such as a solvent, a polymer, a linker molecule, and a coupling reagent. In some aspects, the polymer is 1% by weight polyvinyl alcohol and 2.5% by weight poly vinyl pyrrolidone, the linker molecule is 1.25% by weight polyethylene oxide, the coupling reagent is 1% by weight 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and the solvent includes water. In some aspects, the polymer is 0.5-5% by weight polyvinyl alcohol and 0.5-5% by weight poly vinyl pyrrolidone, the linker molecule is 0.5-5% by weight polyethylene oxide, the coupling reagent is 0.5-5% by weight 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and the solvent includes water.

[0070] In some aspects, the solvent is water, an organic solvent, or a combination thereof. In some aspects, the organic solvent is N Methyl pyrrolidone, Di methyl formamide, Di chloromethane, Di methyl sulfoxide, or a combination thereof. In some aspects, the solvent is about 80-90% by weight of the total formulation concentration. In some aspects, the solvent is about less than 70, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or greater than 99% by weight of the total formulation concentration.

[0071] In some aspects, a polymer is a polyvinyl pyrrolidone and/or a polyvinyl alcohol. The general structure of polyvinyl alcohol is as follows, where n is any positive integer greater than 1:



[0072] In some aspects, a polymer is about 0.5-5% by weight of the total formulation concentration. In some aspects, a polymer is about less than 0.1, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3., 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, or greater than 5.0% by weight of the total formulation concentration.

[0073] A linker molecule can be a molecule inserted between a surface disclosed herein and peptide that is being synthesized via a coupling molecule. A linker molecule does not necessarily convey functionality to the resulting peptide, such as molecular recognition functionality, but can instead elongate the distance between the surface and the peptide to enhance the exposure of the peptide's functionality region(s) on the surface. In some aspects, a linker can be about 4 to about 40 atoms long to provide exposure. The linker molecules can be, for example, aryl acetylene, ethylene glycol oligomers containing 2-10 monomer units (PEGs), diamines, diacids, amino acids, and combinations thereof. Examples of diamines include ethylene diamine and diamino propane. Alternatively, linkers can be the same molecule type as that being synthesized (*e.g.,* nascent polymers or various coupling molecules), such as polypeptides and polymers of amino acid derivatives such as for example, amino hexanoic acids. In some aspects, a linker molecule is a molecule having a carboxylic group at a first end of the molecule and a protecting group at a second end of the molecule. In some aspects, the protecting group is a t-Boc protecting group or an Fmoc protecting group. In some aspects, a linker molecule is or includes an aryl acetylene, a polyethyleneglycol, a nascent polypeptide, a diamine, a diacid, a peptide, or combinations thereof. In some aspects, a linker molecule is about 0.5-5% by weight of the total formulation concentration. In some aspects, a linker molecule is about less than 0.1, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3., 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, or greater than 5.0% by weight of the total formulation concentration.

[0074] The unbound portion of a linker molecule, or free end of the linker molecule, can have a reactive functional group which is blocked, protected, or otherwise made unavailable for reaction by a removable protective group, *e.g.,* t-Boc or F-Moc as noted above. The protecting group can be bound to a monomer, a polymer, or a linker molecule to protect a reactive functionality on the monomer, polymer, or linker molecule. Protective groups that can be used include all acid and base labile protecting groups. For example, peptide amine groups can be protected by t-butoxycarbonyl (t-BOC or BOC) or benzyloxycarbonyl (CBZ), both of which are acid labile, or by 9-fluorenylmethoxycarbonyl (FMOC), which is base labile.

[0075] Additional protecting groups that can be used include acid labile groups for protecting amino moieties: tert-amyloxycarbonyl, adamantyloxycarbonyl, 1-methylcyclobutyloxycarbonyl, 2-(p-biphenyl)propyl(2)oxycarbonyl, 2-(p-phenylazophenylyl)propyl(2)oxycarbonyl, alpha,alpha-dimethyl-3,5-dimethyloxybenzyloxy-carbonyl, 2-phenylpropyl(2)oxycarbonyl, 4-methyloxybenzyloxycarbonyl, furfuryloxycarbonyl, triphenylmethyl (trityl), p-toluenesulfenylaminocarbonyl, dimethylphosphinothioyl, diphenylphosphinothioyl, 2-benzoyl-1-methylvinyl, o-nitrophenylsulfenyl, and 1-naphthylidene; as base labile groups for protecting amino moieties: 9 fluorenylmethyloxycarbonyl, methylsulfonylethyloxycarbonyl, and 5-benzisoazolylmethyleneoxycarbonyl; as groups for protecting amino moieties that are labile when reduced: dithiasuccinoyl, p-toluene sulfonyl, and piperidino-oxycarbonyl; as groups for protecting amino moieties that are labile when oxidized: (ethylthio)carbonyl; as groups for protecting amino moieties that are labile to miscellaneous reagents, the appropriate agent is listed in parenthesis after the group: phthaloyl (hydrazine), trifluoroacetyl (piperidine), and chloroacetyl (2-aminothiophenol); acid labile groups for protecting carboxylic acids: tert-butyl ester; acid labile groups for protecting hydroxyl groups: dimethyltrityl. (See also, Greene, T. W., Protective Groups in Organic Synthesis, Wiley-Interscience, NY, (1981)).

Arrays with electrodes for applying voltage to the surface of the array

[0076] Also described herein is a system for enhancing electrochemiluminescence on a silicon platform to provide a significant increase in the number of working electrodes/ counter electrodes that can be accommodated for a single assay pillar. **Figure 5** shows one aspect of the disclosure of a configuration of working electrodes and counter electrode across two wells on an array or pillar. In cross-sectional view (**Fig**. **5** top panel), it is a four layer integrated circuit. The top layer defines location of working electrode and counter electrode, which are isolated by dielectric material. Peptides or other capture ligands (e.g., antigen binding proteins such as antibodies, scFvs or the like) are synthesized *in situ* or otherwise coupled (e.g., using carbodiimide chemistry) on the surface of a working electrode. The middle two layers are metal interconnection layers to connect and group counter electrode or working electrode, which are also isolated by

dielectric material. The bottom layer includes the outputs of working electrode and counter electrode, which are connected to a power supply or control unit. In top view (**Fig**. **5**, middle panel), each array feature has its own working electrode and counter electrode, which are used to generate an electrical potential difference when the electrodes are powered. Bottom view (**Fig**. **5**, bottom panel) shows an example of electrode output. According to design choices for feature grouping, the electrode output layout will differ, as described below.

[0077] **Figure 6** shows a view of 16 features on a microarray chip according to 3 different aspects of the disclosure. In an aspect of the disclosure, 1 "feature group" is detected, allowing detection and quantitation of up to one biomolecule of interest (top left). In another aspect of the disclosure 4 "feature groups" are detected, allowing detection and quantitation of up to four biomolecules of interest (top middle). In another aspect of the disclosure 16 "feature groups" are detected, allowing detection and quantitation of up to 16 biomolecules of interest (top right). Working and counter electrode outputs are shown in the chip bottom view. In some aspects of the disclosure, the microarray chip comprises 2, 4, 8, 16, 32, 64, 100, 200, 500, 1000, 2000, 5000, 10,000, 15,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 1,000,000, 1, 500,000, 2,000,000 or more working electrodes or electrode pairs. In some aspects of the disclosure, the microarray chip includes multiple electrode pairs wherein each feature associated with these multiple electrode pairs includes the same capture ligand to capture the same analyte. In some aspects of the disclosure the microarray includes the same number of counter electrodes as working electrode and is configured to detect a single analyte or multiple distinct analytes on a chip. By having the same number of counter and working electrodes, the voltage applied to a feature can be precisely controlled. In some aspects of the disclosure, 500 or more analytes (e.g., for triplicate measuresments, one third the total of number of features, such as described above) that can be detected on one chip. Statistically robust data can be obtained by having multiple features having the same capture ligand.

[0078] Microarrays of the present disclosure can include features as small as 50 nm on edge because the amplified ECL tag system produces extremely high signal to noise ratios. In some embodiments, the features have an edge dimension between 50 nm and 1 um. In other embodiments, the features have an edge dimension between 50 nm and 100 nm. In yet other embodiments, the edge dimension is between 50 nm and 75 nm. We have demonstrated reliable and accurate detection of target biomolecules to AECL microarrays having features as small as 50 nm on edge notwithstanding the reduced number of capture ligands and bound targets as compared to larger features. Assuming constant capture ligand density, the number of capture ligands per feature is a function of feature area. Thus as compared to a square feature having an edge length of 100 nm, a 50 nm feature would have ¼ the number of capture ligands. By using the AECL approach and a 50 nm feature length, a 3 mm x 3 mm microarray chip can typically include anywhere from 200,000 to 2,000,000 features.

[0079] In **Figure 7,** an AECL assay plate array is shown in an aspect of the disclosure comprising a set of 16 pillars, each of which receives and supplies voltage to four separate AECL microarray chips. Three different pillar groupings are shown, in which the AECL assay plate is divided into 1 pillar group (left panel), 4 pillar groups (middle panel), or 16 pillar groups (right panel). The bottom view shows, according to each aspect of the disclosure, working and counterelectrode configurations for each number of pillar groups.

[0080] **Figure 8** shows a detailed view of an AECL assay plate, according to an aspect of the disclosure. The counter electrodes ("count electrode" in **Fig**. **8**) and working electrodes are attached to the array at the top of the pillar mount (top left panel). The array can be divided into multiple sections, with a counter electrode and working electrode attached to each. An AECL assay cap (top right panel) mounts onto an exemplary AECL assay plate (bottom right panel), that includes nine AECL pillar mounts (i.e., the structure shown in the top left panel), each of which, in this example, receives and supplies voltage to four separate AECL microarray chips. The assay cap includes pillar walls that contain the assay solution when the cap is mounted onto the assay plate. *See* **Fig. 8** bottom left (side view) and top right (assay cap). Bottom left panel (side view) shows AECL microarray ("chip") mounted on pillar and covered with AECL assay solution. The number of assay pillars included in an assay plate can be selected according to the number of different assays sought to be carried out. For example, the assay plate can include 24, 96, 384 or 1586 pillars in conformity with standard microtiter plate configurations.

[0081] **Figure 9** diagrams an exemplary system for detecting biomolecules bound to an AECL microarray chip, according to an embodiment of the invention. Voltage is applied to selected working and counter electrode leads array via a main controller attached to a power supplier controller. After applying voltage to the array, AECL tags illuminate features comprising bound target biomolecules of interest, as described in this specification. The chip is placed on a scanner stage and luminescence is optically detected.

[0082] In an embodiment, the system optics employ a wet (*i.e.,* immersion) microscope lens stepping and scanning at a very minimal distance from the pillar top (approx. 0.5mm) to increase the numerical aperture and reduces the loss of light from AECL. The control of the system can be completely automated such that individual electrodes can be turned on and off, at times precisely coinciding with optimal placement of array features with respect to the optics for image capture thus minimizing loss of signal from signal decay of light.

Methods of Manufacturing Arrays

Methods of attaching biomolecules to an array

[0083] Also disclosed herein are methods for manufacturing arrays. In some aspects, capture ligands positioned at predetermined locations on microarrays disclosed herein can be synthesized *in situ* on a surface, *e.g.,* a substrate disclosed herein. In some instances, the arrays are made using photolithography. For example, the substrate is contacted with a photoactive coupling solution. Masks can be used to control radiation or light exposure to specific locations on a surface provided with free linker molecules or free coupling molecules having protecting groups. In the exposed locations, the protecting groups are removed, resulting in one or more newly exposed reactive moieties on the coupling molecule or linker molecule. The desired linker or coupling molecule is then coupled to the unprotected attached molecules, *e.g.,* at the carboxylic acid group. The process can be repeated to synthesize a large number of features in specific or positionally-defined locations on a surface (see, for example, U.S. Pat. No. 5,143,854 to Pirrung et al., U.S. Patent Application Publication Nos. 2007/0154946 (filed on Dec. 29, 2005), 2007/0122841 (filed on Nov. 30, 2005), 2007/0122842 (filed on Mar. 30, 2006), 2008/0108149 (filed on Oct. 23, 2006), and 2010/0093554 (filed on June 2, 2008).

[0084] In some aspects, a method of producing a three-dimensional (*e.g.,* porous) array of features, can include obtaining a porous layer attached to a surface; and attaching the features to the porous layer, said features each comprising a collection of peptide chains of determinable sequence and intended length, wherein within an individual feature, the fraction of peptide chains within said collection having the intended length is characterized by an average coupling efficiency for each coupling step of at least about 98%. In some aspects, the features are attached to the surface using a photoactive coupling formulation, comprising a photoactive compound, a coupling molecule, a coupling reagent, a polymer, and a solvent. In some aspects, the features are attached to the surface using a photoactive coupling formulation disclosed herein. In some aspects, the photoactive coupling formulation is stripped away using water.

[0085] In an aspect of the disclosure, described herein is a process of manufacturing an array. A surface comprising attached carboxylic acid groups is provided. The surface is contacted with a photoactive coupling solution comprising a photoactive compound, a coupling molecule, a coupling reagent, a polymer, and a solvent. The surface is exposed to ultraviolet light in a deep ultra violet scanner tool according to a pattern defined by a photomask, wherein the locations exposed to ultraviolet light undergo photo base generation due to the presence of a photobase generator in the photoactive coupling solution. The expose energy can be from 1mJ/cm^2 to 100mJ/cm^2 in order to produce enough photobase.

[0086] The surface is post baked upon exposure in a post exposure bake module. Post exposure bake acts as a chemical amplification step. The baking step amplifies the initially generated photobase and also enhances the rate of diffusion to the substrate. The post bake temperature can vary between 75°C to 115°C, depending on the thickness of the porous surface, for at least 60 seconds and not usually exceeding 120 seconds. The free carboxylic acid group is coupled to the deprotected amine group of a free peptide or polypeptide, resulting in coupling of the free peptide or polypeptide to the carboxylic acid group attached to the surface. This surface can be a porous surface. The synthesis of peptides coupled to a carboxylic acid group attached to the surface occurs in an N→C synthesis orientation, with the amine group of free peptides attaching to carboxylic acid groups bound to the surface of the substrate. Alternatively, a diamine linker may be attached to a free carboxylic acid group to orient synthesis in a C→N direction, with the carboxylic acid group of free peptides attaching to amine groups bound to the surface of the substrate.

[0087] The photoactive coupling solution can now be stripped away. In some aspects, provided herein is a method of stripping the photoresist completely with DI water. This process is accomplished in a developer module. The wafer is spun on a vacuum chuck for, *e.g.,* 60 seconds to 90 seconds and deionized water is dispensed through a nozzle for about 30 seconds.

[0088] The photoactive coupling formulation may be applied to the surface in a coupling spin module. A coupling spin module can typically have 20 nozzles or more to feed the photoactive coupling formulation. These nozzles can be made to dispense the photoactive coupling formulation by means of pressurizing the cylinders that hold these solutions or by a pump that dispenses the required amount. In some aspects, the pump is employed to dispense 5-8 cc of the photoactive coupling formulation onto the substrate. The substrate is spun on a vacuum chuck for 15-30 seconds and the photoactive coupling formulation is dispensed. The spin speed can be set to 2000 to 2500 rpm.

[0089] Optionally, a cap film solution coat is applied on the surface to prevent the unreacted amino groups on the substrate from reacting with the next coupling molecule. The cap film coat solution can be prepared as follows: a solvent, a polymer, and a coupling molecule. The solvent that can be used can be an organic solvent like N methyl pyrrolidone, di methyl formamide, or combinations thereof. The capping molecule is typically acetic anhydride and the polymer can be Poly vinyl pyrrolidone, polyvinyl alcohol, polymethyl methacrylate, poly (methyl iso propenyl) ketone, or poly (2 methyl pentene 1 sulfone). In some aspects of the disclosure, the capping molecule is ethanolamine.

[0090] This process is done in a capping spin module. A capping spin module can include one nozzle that can be made to dispense the cap film coat solution onto the substrate. This solution can be dispensed through pressurizing the cylinder that stores the cap film coat solution or through a pump that precisely dispenses the required amount. In some

aspects, a pump is used to dispense around 5-8 cc of the cap coat solution onto the substrate. The substrate is spun on a vacuum chuck for 15-30 s and the coupling formulation is dispensed. The spin speed can be set to 2000 to 2500 rpm.

[0091] The substrates with the capping solution are baked in a cap bake module. A capping bake module is a hot plate set up specifically to receive wafers just after the capping film coat is applied. In some aspects, provided herein is a method of baking the spin coated capping coat solution in a hot plate to accelerate the capping reaction significantly. Hot plate baking generally reduces the capping time for amino acids to less than two minutes.

[0092] The byproducts of the capping reaction are stripped in a stripper module. A stripper module can include several nozzles, typically up to 10, set up to dispense organic solvents such as acetone, iso propyl alcohol, N methyl pyrrolidone, Di methyl formamide, DI water, etc. In some aspects, the nozzles can be designated for acetone followed by iso propyl alcohol to be dispensed onto the spinning wafer. The spin speed is set to be 2000 to 2500 rpm for around 20 s.

[0093] This entire cycle can be repeated as desired with different coupling molecules each time to obtain a desired sequence.

[0094] In some aspects, an array comprising a surface of free carboxylic acids is used to synthesize polypeptides in an N->C orientation. In an aspect, the carboxylic acids on the surface of the substrate are activated (e.g., converted to a carbonyl) to allow them to bind to free amine groups on an amino acid. In an aspect, activation of carboxylic acids on the group of the surface can be done by addition of a solution comprising a carbodiimide or succinimide to the surface of the array. In some aspects, carboxylic acids can be activated by addition of a solution comprising 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide [EDC], N-hydroxysuccinimide [NHS], 1,3-Diisopropylcarbodiimide [DIC], hydroxybenzotriazole (HOBt), (O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate) [HATU], benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate [PyBOP], or N,N-Diisopropylethylamine [DIEA] to the surface of the array. The activation solution is washed away and the surface of the array is prepared for addition of an amino acid layer (i.e., one amino acid at each activated carboxylic acid group). Carboxylic acid groups remain activated for up to 2, 3, 4, 5, 6, 7, 8, 9, or 10 hours.

[0095] Addition of a solution comprising an amino acid with a free amine group to the activated carboxylic acid surface of the array results in binding of a single amino acid to each carboxylic acid group. In some aspects, the amino acid comprises an amino acid with protected amine groups. Using a photosensitive chemical reaction, the protecting group can be removed from the amine group of selected amino acids at site-specific locations using a reticle. For example, Fmoc-protected amino acids are mixed in a solution comprising a photobase. Upon exposure of the solution on the array to a specific frequency of light at site-specific locations, the photobase will release a base which will deprotect the amino acid, resulting in coupling of the amino acid to the activated carboxylic acid group on the surface of the array. Another method of generating a base is through the use of a photoacid generator. In some aspects of the disclosure, the photoacid generator is N-Boc-piperidine or 1-Boc-4-piperazine.

[0096] After a completed layer of amino acids is coupled, remaining uncoupled activated carboxylic acids are capped to prevent nonspecific binding of amino acids on subsequent synthesis steps. The steps of activation, addition of an amino acid layer, and capping are repeated as necessary to synthesize the desired polypeptides at specific locations on the array.

[0097] In an aspect of the disclosure, peptides synthesized in the N->C terminus direction can be capped with a diamine molecule to enhance binding properties of selected polypeptide sequences to a biological molecule, e.g., an antibody. In other aspects, peptides synthesized in the C->N direction can be capped with a dicarboxylic acid molecule to enhance binding properties of selected sequences to a biological molecule.

[0098] While synthesizing polypeptides in parallel on the surface of an array, the method described herein ensures complete activation of carboxylic acid on the surface of the array. Due to stability of the activated ester for an extended period of time, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more coupling cycles may be completed after a single activation step (e.g., to couple an entire layer of 2-25 or more different amino acids at different locations on the array). As the coupling occurs during hard bake and due to the presence of excess amino acid in the solution, complete 100% deprotection of Fmoc-protected amino acid may not be required for significantly high coupling yields. After addition of all amino acids and capping, all free activated carboxylic acids are either coupled or capped, thus resulting in high efficiency and accuracy of polypeptide synthesis.

[0099] In an apsect, proteins, polypeptides, or other molecules are attached to the activated carboxylic acid group on the surface of the array. After activation of carboxylic acid groups on the array, a solution comprising proteins, polypeptides, or other molecules with a free amine group are added to the surface of the array. The amine group binds to the activated carboxylic acid group, thus attaching the protein, polypeptide, or other molecule to the array. In an aspect, this method is used to attach antibodies to the surface of the array. In one aspect, the amine groups are protected, and subsequently deprotected on the surface of the chip. In an aspect, the deprotection occurs at specified locations on the chip using light shined through a reticle to interact with a photolabile compound, e.g., a photobase or photoacid, which deprotects the protected amine group.

EXAMPLES

[0100] 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.

Example 1: AECL wafer processing

[0101] Steps 1-7 are described with reference to **Fig. 10A;** steps 8-14 are described with reference to **Fig. 10B.**

Step 1: Silicon wafers were obtained from University wafers. 1000A silicon dioxide was deposited using thermal oxide deposition in an oxidation chamber.
Step 2: P5107 (photoresist) obtained from Rohm and Haas were coated on the wafers using a RF3S Sokudo coater. Using a working electrode photo mask, these wafers were exposed in a Nikon S205 DUV at 18mj/cm2. The wafers were developed in a developer for 60s. Oxide etch was performed using Hydrofluoric acid (HF) bath for 30s to remove 1000A oxide. Photoresist was stripped using Acetone wash followed by Isopropanol wash for 30s each in a coater. All solvents and HF were obtained from Sigma Aldrich.
Step 3: Uniform thickness of 1500A Gold was deposited on top of this wafer substrate by sputtering.
Step 4: The wafers were polished in a chemical mechanical planarization (CMP) polisher until oxide layer was reached.
Step 5: P5107 photoresist obtained from Rohm and Haas were coated on the wafers using a RF3S Sokudo coater. Using a counter electrode photo mask, these wafers were exposed in a Nikon S205 DUV at 18mj/cm2. The wafers were developed in a developer for 60s. Oxide etch was performed using Hydrofluoric acid (HF) bath to remove 1000A oxide. Photoresist was stripped using Acetone wash followed by Isopropanol wash for 30 s each in a coater.
Step 6: Uniform thickness of 1500A copper was deposited on top of this wafer substrate by sputtering.
Step 7: The wafers were polished in a CMP polisher until oxide layer was reached.
Step 8: Thermal oxide of 1000A was grown on top of the wafers in an oxidation chamber. P5107 photoresist obtained from Rohm and Haas were coated on the wafers using a RF3S Sokudo coater. Using a interconnect photo mask, these wafers were exposed in a Nikon S205 DUV at 18mj/cm2. The wafers were developed in a developer for 60s. Oxide etch was performed using Hydrofluoric acid (HF) bath to remove 1000A oxide. Photoresist was stripped using Acetone wash followed by Isopropanol wash.
Step 9: Uniform thickness of 500A Aluminum interconnect was deposited on top of this wafer substrate by sputtering.
Step 10: The wafers were polished in a CMP polisher until the electrodes layer was reached.
Step 11: P5107 photoresist obtained from Rohm and Haas was coated on the wafers using a RF3S Sokudo coater. Using an output photo mask, these wafers were exposed in a Nikon S205 DUV at 18mj/cm2. The wafers were developed in a developer for 60s. Oxide etch was performed using Hydrofluoric acid (HF) bath to remove 1000A oxide. Photoresist was stripped using Acetone wash followed by Isopropanol wash.
Step 12: Uniform thickness of 500A copper was deposited on top of this wafer substrate by sputtering.
Step 13: The wafers were polished in a CMP polisher until oxide layer was reached.
Step 14: The wafer was then flipped and photoresist was coated on the wafer backside. Using a photo mask, these wafers were exposed in a Nikon S205 DUV at 18mj/cm2. The wafers were developed in a developer for 60s. Silicon etch was performed using Hydrofluoric acid (HF) bath to remove silicon until the contacts were reached. Photoresist was stripped using Acetone wash followed by Isopropanol wash.

Example 2: Functionalization of Wafer and Dicing into Chips Production

[0102] Wafers comprising electrodes as shown in **Figs. 10A and B** for supplying voltage to array features were provided according to Example 1. A COOH-functionalized surface was formed as follows on an AECL wafer:

[0103] 11-Mercaptoundecanoic acid and Acetic Acid were obtained from Sigma Aldrich. Ethanol, Hydrogen Peroxide, Sulfuric Acid are obtained from VWR.

[0104] To functionalize with COOH groups, the AECL wafers of Example 1, having gold working electrodes, were cleaned with piranha solution which comprises 50 weight % of pure Sulfuric acid and 50 weight % of Hydrogen Peroxide for 60 minutes. The wafers were then rinsed with DI Water continuously for 5 minutes followed by rinsing with Ethanol for 5 minutes. The wafers were washed with a mixture of 50% Ethanol and 50% DI Water for 10 minutes. The wafers were then contacted with a solution containing 2.5 weight % of 11-Mercaptoundecanoic acid and 97.5 weight % of Pure Ethanol for 12 hours under mild shaking conditions. After 10-12 hours, wafers were then rinsed for Ethanol and isopropanol (IPA) for 5 minutes each. This was followed by washing the wafers with DI Water for 10 minutes and hot acetic acid

solution which was prepared by mixing 10 weight % of Acetic acid in 90 weight % of DI Water at 60C for 45 minutes. Finally, the wafers was rinsed with DI Water and IPA for 5 minutes each and blown dry under nitrogen. Following this step, the wafers were diced into chips of 3.0 mm x 3.0 mm.

Example 3: Chip Activation and anti-TNF-alpha Antibody Coupling

[0105] 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide [EDC], N-hydroxysuccinimide [NHS], Ethanolamine and Phosphate Buffer Saline (PBS) buffer were obtained from Sigma Aldrich. Primary and secondary anti TNF-alpha antibodies and TNF-alpha were obtained from ABCAM. An activation solution of EDC and NHS was prepared by dissolving 4% by weight of EDC and 2% by weight of NHS in deionized water. The activation solution was then applied to the surface of the wafer at room temperature for 10 minutes. The chips were then washed with deionized water for 3 minutes.

[0106] The primary anti-TNF alpha antibody was coupled to the chip by adding a solution of 10ug/ml of antibody in PBS buffer to the surface of the wafer with activated COOH groups for 30 mins, resulting in binding of the COOH groups to free amine of the primary antibody. This was followed by capping of unreacted carboxylic acid groups on the surface with 5 weight % Ethanolamine in 95 weight % DI water for 10 minutes followed by washing the wafer in DI water for 10 minutes.

Example 4: Prototype Pillar Mount for AECL Biochip

[0107] **Fig. 11A** shows steps for preparing a pillar mount for an AECL biochip.

Step 1: Silicon wafers were obtained from University wafers. 1000A silicon dioxide was deposited using thermal oxide deposition in an oxidation chamber.
Step 2: P5107 (photoresist) obtained from Rohm and Haas were coated on the wafers using a RF3S Sokudo coater. Using an AECL pillar working electrode photo mask, these wafers were exposed in a Nikon S205 DUV at 18mj/cm2. The wafers were developed in a developer for 60s. Oxide etch was performed using Hydrofluoric acid (HF) bath for 30s to remove 1000A oxide. Photoresist was stripped using Acetone wash followed by Isopropanol wash for 30s each in a coater. All solvents and HF were obtained from Sigma Aldrich.
Step 3: Uniform thickness of 1500A Gold was deposited on top of this wafer substrate by sputtering. The wafers were polished in a chemical mechanical planarization (CMP) polisher until oxide layer was reached.
Step 4: P5107 photoresist obtained from Rohm and Haas were coated on the wafers using a RF3S Sokudo coater. Using an AECL pillar counter electrode photo mask, these wafers were exposed in a Nikon S205 DUV at 18mj/cm2. The wafers were developed in a developer for 60s. Oxide etch was performed using Hydrofluoric acid (HF) bath to remove 1000A oxide. Photoresist was stripped using Acetone wash followed by Isopropanol wash for 30 s each in a coater.
Step 5: Uniform thickness of 1500A copper was deposited on top of this wafer substrate by sputtering. The wafers were polished in a CMP polisher until oxide layer was reached.

[0108] **Fig. 11 B** shows a top view of a pillar mount used for an AECL biochip, prepared according to the steps outlined above in this example. To test the performance of the AECL-TNF alpha chips, an AECL biochip was mounted on the pillar as follows:

[0109] The AECL biochip's working and counter electrodes were picked and placed over the AECL pillar mount. Positional correspondence and electrical contact between the pillar mount and chip working and counter electrodes were stabilized using conductive tape obtained from 3M. The AECL pillar mount working and counter electrodes were connected via copper clips to a model XP-100 voltage controller from Elenco which supplies from 1.5 to 12V.

Example 5: Preparation of an AECL tag

[0110] This example describes preparation of an amplified electrochemiluminescent tag. In this example, ruthenium bis(2,2 bipyridine) bis (2,2 dicarboxylic acid ester) is the electrochemiluminescent moiety, and tyramide is the signal amplification moiety (**Figure 1**) through which a plurality of AECL tags bind to target molecules in the vicinity of peroxidase activity (e.g., HRP enzyme) and an oxidizing agent (e.g., hydrogen peroxide).

[0111] 50ul of 0.01M of tyramine.HCL and 50ul of 0.01M of Ruthenium bis(2,2 bipyridine) bis (2,2 dicarboxylic acid) ester were mixed in DI water with the presence of 5ul of N,N-Diisopropylethylamine (DIEA). The mixture was shaken on a rotary mixer set at 400 rpm for 2 hours, followed by addition of 1ul of ethanolamine and then shaken again for an additional 10 minutes. TLC was used to purify the solution and the resulting solution was desalted and lyophilized to obtain 0.56 mg of the AECL tag shown in **Fig. 1.** The AECL tag was dissolved in volume of PBS buffer to generate a 0.5 mg/mL stock solution.

Example 6: TNF-alpha AECL Assay

[0112] TNF alpha was dissolved in varying concentration of 1 fg/mL to 100 ng/mL in PBST (PBST contains 3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, 0.05% Tween® 20, pH 7.4) and was added to the AECL chips on the pillar substrate. This was incubated for 30 mins at 37c. After this, the chips were washed with PBST buffer for 5 mins. A secondary TNF- alpha Ab - HRP conjugate obtained from ABcam, was added in a dilution of 1:1000 in PBST and incubated for 15 mins at 37c. Then a tag solution was made that included a 1:10 dilution in PBS of the AECL tag stock solution and 0.003 weight % hydrogen peroxide. This was added to the chips resulting in binding of multiple AECL tags to the captured TNF alpha/antibody-HRP complexes. Tripropylamine (TPA) was added to the chip in a concentration of 0.1M in an 0.02% sodium acetate buffer with Tween 20. The electrical potential at working electrode was ramped from 0 to 3.5V. The intensity of AECL was read by a CCD camera at 620nm. A similar assay was also conducted that differed by using an ECL tag obtained from Meso Scale Diagnostics. The data from both assays are shown in **Fig. 12.** The AECL tag can clearly detect TNF alpha in sub-picogram/mL ranges whereas an ECL tag can only detect pictogram/mL level ranges.

[0113] While the invention has been particularly shown and described with reference to a preferred embodiment and various alternate embodiments, it will be understood by persons skilled in the relevant art that various changes in form and details can be made therein without departing from the scope of the invention.

Claims

1. A method of detecting a target biomolecule, comprising
contacting a sample comprising said target biomolecule with a capture ligand, said capture ligand being immobilized at a defined location on a substrate and capable of specifically binding said target biomolecule thereby immobilizing said target biomolecule at said defined location on said substrate;
contacting said immobilized target biomolecule with a detection ligand, said detection ligand capable of specifically binding to said immobilized target biomolecule and having peroxidase activity thereby forming an immobilized target biomolecule-detection ligand complex;
contacting said complex with a tagging solution comprising an amplified electrochemiluminescent (AECL) tag under conditions that promote covalent binding of a plurality of AECL tags to said complex, wherein each of the AECL tags comprises a rare earth metal chelate and a tyramide bound to said metal chelate;
washing said substrate to remove unbound AECL tag from said substrate;
contacting said substrate with a detection solution that reacts with said bound AECL tag to generate luminescence when a voltage is applied to said defined location on said substrate;
applying said voltage to said defined location on said substrate; and
measuring luminescence from said defined location on said substrate thereby detecting said target biomolecule;
wherein the method detects said target biomolecule in the sample at concentrations as low as 1 fg/mL.

2. The method of claim 1, wherein said defined location on said substrate comprises a microarray feature or a plurality of microarray features.

3. The method of claim 2, wherein said feature or features have an edge dimension between 50 nm and 1 μm, between 50 nm and 100 nm, or between 50 nm and 75 nm.

4. The method of claim 1, wherein said capture ligand is covalently bound to said defined location on said substrate via a COOH moiety provided on said substrate, or via an NH2 moiety provided on said substrate.

5. The method of any of claims 1-2, wherein:

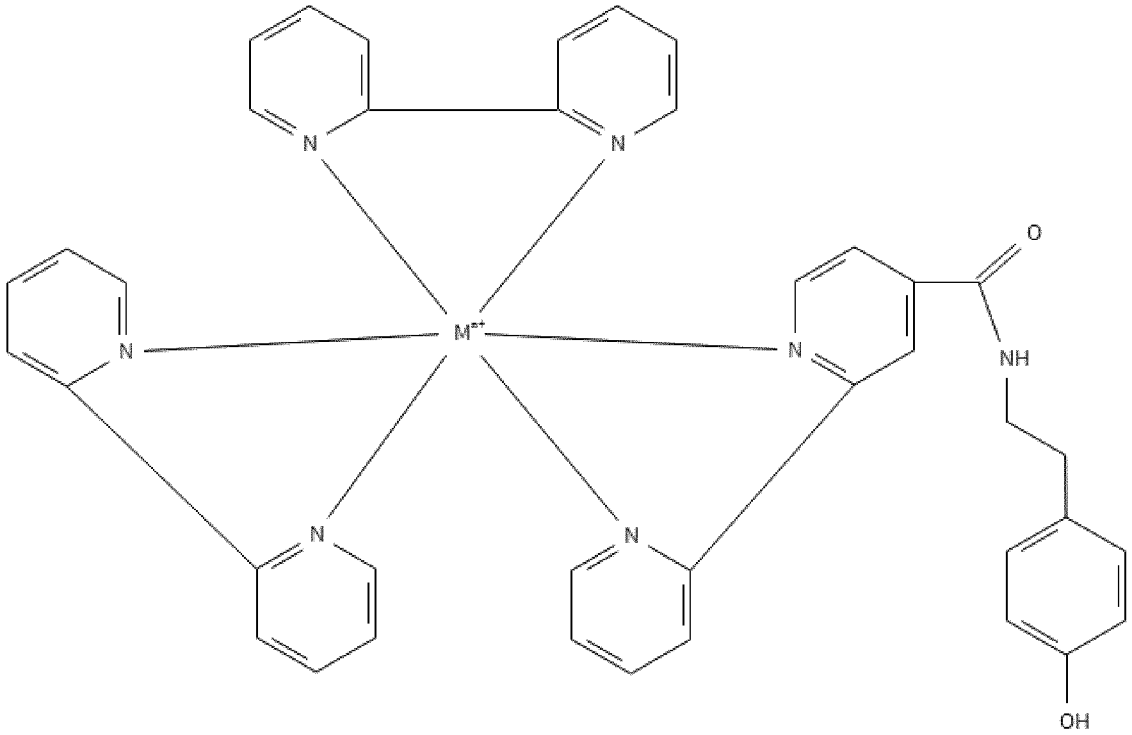
(i) said capture ligand comprises a peptide;
(ii) said capture ligand comprises a protein;
(iii) said target biomolecule comprises a peptide;
(iv) said target biomolecule comprises a protein, optionally wherein said protein is an antibody;
(v) said sample comprises, blood, serum, plasma, saliva, urine, feces or cerebrospinal fluid (CSF); or
(vi) said sample is obtained from a human.

6. The method of any of claims 1-2, wherein said detection ligand comprises an antibody-horseradish peroxidase conjugate.

7. The method of any of claims 1-2, wherein said rare earth metal is ruthenium.

8. The method of any of claims 1-2, wherein said AECL tag comprises tris (bipyridine) ruthenium(II).

9. The method of any of claims 1-2, wherein said AECL tag comprises:

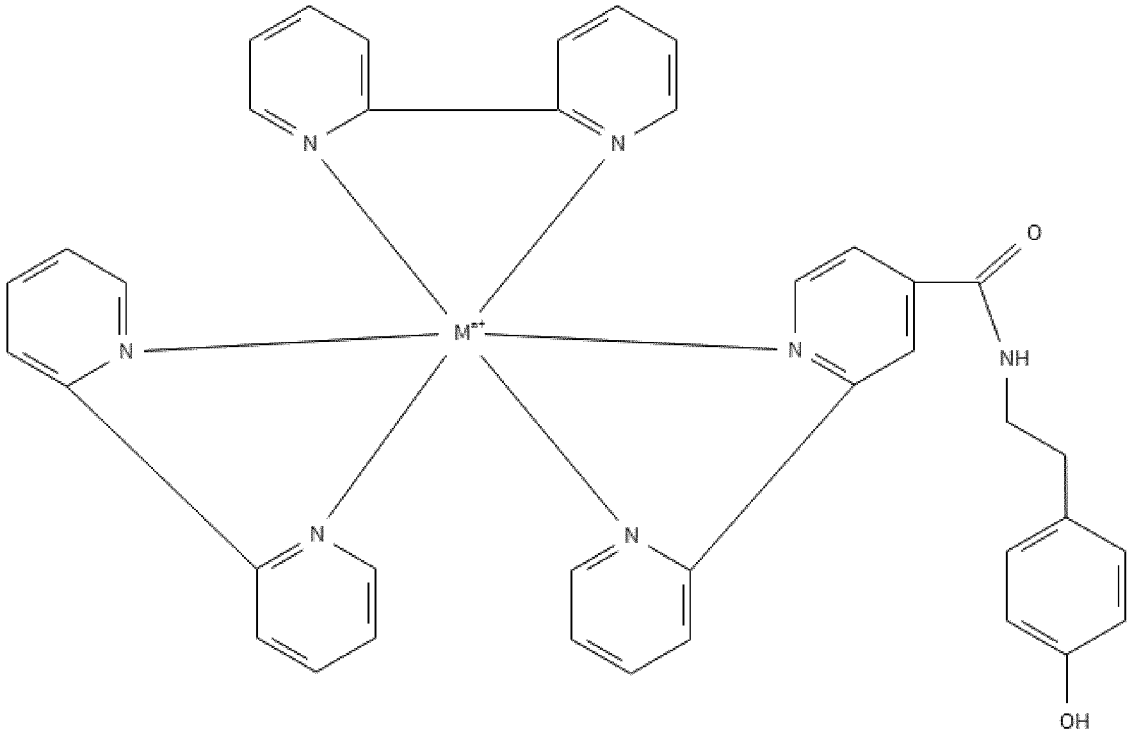


wherein M^{n+} is Ru^{2+}.

10. The method of any of claims 1-2, wherein said tagging solution comprises hydrogen peroxide, optionally wherein said detection solution comprises tripropylamine.

11. The method of any of claims 1-2, wherein said capture ligand and said detection ligand each comprise an antibody.

12. An AECL tag composition, comprising:



13. The AECL tag composition of claim 12, wherein M^{n+} is Ru^{2+}.

Patentansprüche

1. Verfahren eines Nachweisens eines Zielbiomoleküls, umfassend:

Inkontaktbringen einer Probe, umfassend das Zielbiomolekül, mit einem Abfangliganden, wobei der Abfangligand an einem definierten Ort auf einem Substrat immobilisiert ist und in der Lage ist, das Zielbiomolekül spezifisch zu binden, wodurch das Zielbiomolekül an dem definierten Ort auf dem Substrat immobilisiert wird;
Inkontaktbringen des immobilisierten Zielbiomoleküls mit einem Nachweisliganden, wobei der Nachweisligand in der Lage ist, spezifisch an das immobilisierte Zielbiomolekül zu binden und Peroxidase-Aktivität zu haben, wodurch ein immobilisierter Zielbiomolekül-Nachweisligand-Komplex gebildet wird;
Inkontaktbringen des Komplexes mit einer Markierungslösung, umfassend ein amplifiziertes elektrochemilumineszentes (AECL) Tag unter Bedingungen, die kovalente Bindung einer Vielzahl von AECL-Tags an den Komplex fördern, wobei jedes der AECL-Tags ein Seltenerdmetallchelat und ein Tyramid, das an das Metallchelat gebunden ist, umfasst;
Waschen des Substrats, um ungebundenes AECL-Tag aus dem Substrat zu entfernen;
Inkontaktbringen des Substrats mit einer Nachweislösung, die mit dem gebundenen AECL-Tag reagiert, um Lumineszenz zu erzeugen, wenn eine Spannung an den definierten Ort auf dem Substrat angelegt wird;
Anlegen der Spannung an den definierten Ort auf dem Substrat; und
Messen von Lumineszenz von dem definierten Ort auf dem Substrat, wodurch das Zielbiomolekül nachgewiesen wird;
wobei das Verfahren das Zielbiomolekül in der Probe in Konzentrationen so gering wie 1 fg/ml nachweist.

2. Verfahren nach Anspruch 1, wobei der definierte Ort auf dem Substrat ein Mikroanordnungsmerkmal oder eine Vielzahl von Mikroanordnungsmerkmalen umfasst.

3. Verfahren nach Anspruch 2, wobei das Merkmal oder die Merkmale ein Kantenmaß zwischen 50 nm und 1 μm, zwischen 50 nm und 100 nm oder zwischen 50 nm und 75 nm haben.

4. Verfahren nach Anspruch 1, wobei der Abfangligand kovalent an den definierten Ort auf dem Substrat über einen COOH-Teil, der auf dem Substrat bereitgestellt ist, oder über einen NH2-Teil, der auf dem Substrat bereitgestellt

ist, gebunden ist.

5. Verfahren nach einem der Ansprüche 1-2, wobei:

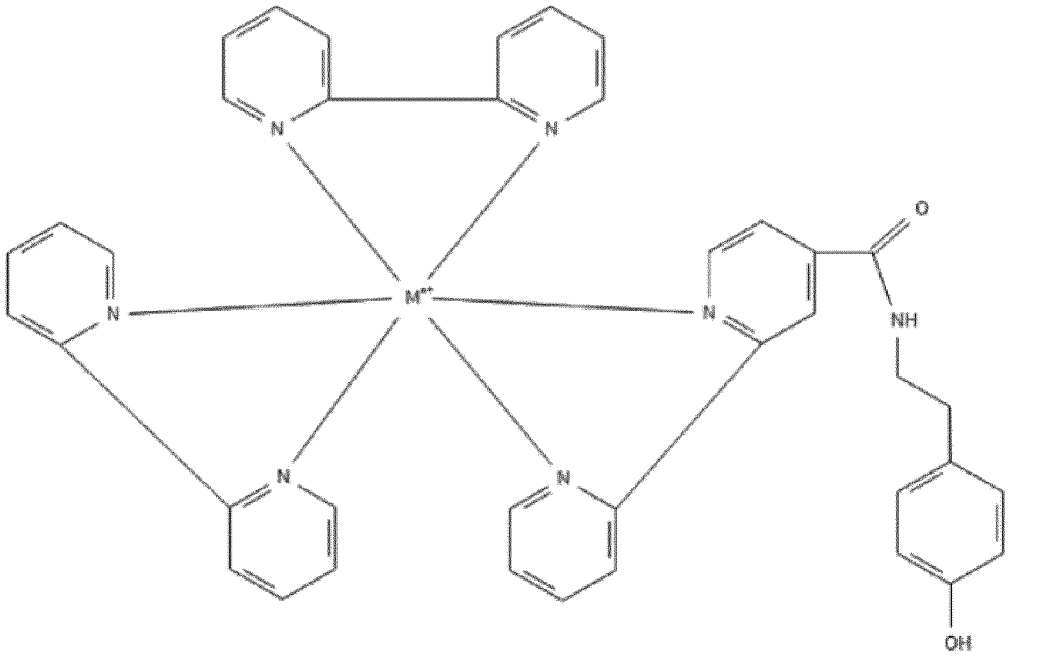
(i) der Abfangligand ein Peptid umfasst;
(ii) der Abfangligand ein Protein umfasst;
(iii) das Zielbiomolekül ein Peptid umfasst;
(iv) das Zielbiomolekül ein Protein umfasst, optional wobei das Protein ein Antikörper ist;
(v) die Probe Blut, Serum, Plasma, Speichel, Harn, Fäkalien oder Zerebrospinalflüssigkeit (CSF) umfasst; oder
(vi) die Probe von einem Menschen erhalten ist.

6. Verfahren nach einem der Ansprüche 1-2, wobei der Nachweisligand ein Antikörper-Meerrettichperoxidase-Konjugat umfasst.

7. Verfahren nach einem der Ansprüche 1-2, wobei das Seltenerdmetall Ruthenium ist.

8. Verfahren nach einem der Ansprüche 1-2, wobei das AECL-Tag Tris(bipyridin)ruthenium(II) umfasst.

9. Verfahren nach einem der Ansprüche 1-2, wobei das AECL-Tag Folgendes umfasst:



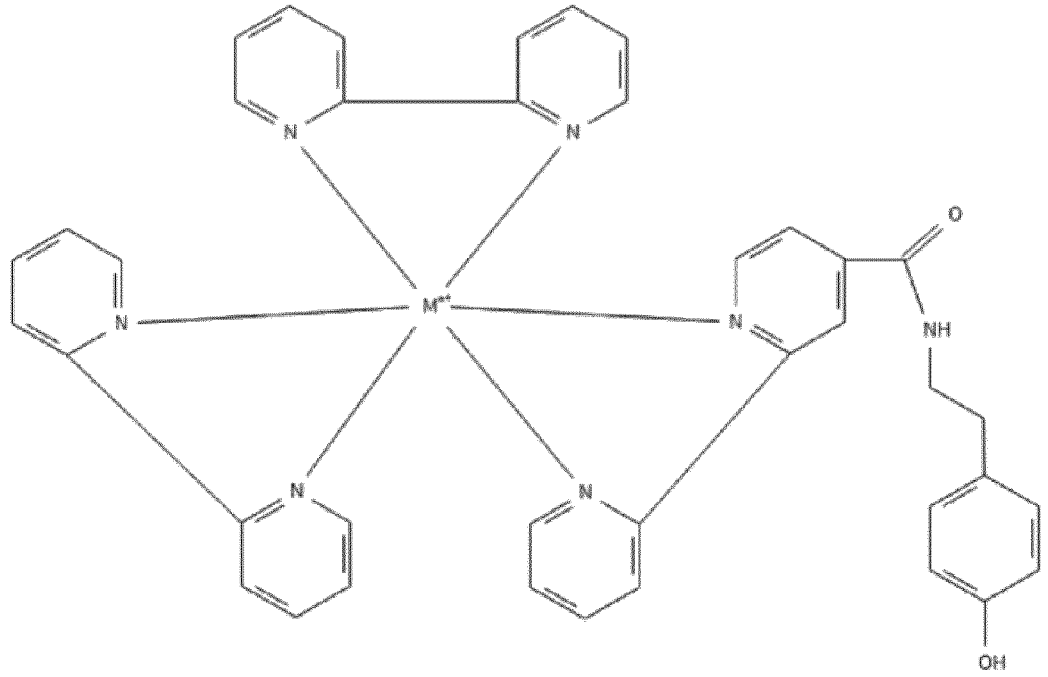
,

wobei M^{n+} Ru^{2+} ist.

10. Verfahren nach einem der Ansprüche 1-2, wobei die Markierungslösung Wasserstoffperoxid umfasst, optional wobei die Nachweislösung Tripropylamin umfasst.

11. Verfahren nach einem der Ansprüche 1-2, wobei der Abfangligand und der Nachweisligand jeweils einen Antikörper umfassen.

12. AECL-Tag-Zusammensetzung, umfassend:



13. AECL-Tag-Zusammensetzung nach Anspruch 12, wobei M^{n+} Ru^{2+} ist.

Revendications

1. Procédé de détection d'une biomolécule cible, comprenant
la mise en contact d'un échantillon comprenant ladite biomolécule cible avec un ligand de capture, ledit ligand de capture étant immobilisé à un emplacement défini sur un substrat et capable de se lier spécifiquement à ladite biomolécule cible, ainsi immobilisant ladite biomolécule cible audit emplacement défini sur ledit substrat ;
la mise en contact de ladite biomolécule cible immobilisée avec un ligand de détection, ledit ligand de détection étant capable de se lier spécifiquement à ladite biomolécule cible immobilisée et ayant une activité peroxidase, ainsi formant un complexe biomolécule cible immobilisée-ligand de détection ;
la mise en contact dudit complexe avec une solution de marquage comprenant un marqueur électrochimiluminescent amplifié (AECL) dans des conditions qui favorisent la liaison covalente d'une pluralité de marqueurs AECL audit complexe, dans lequel chacun des marqueurs AECL comprend un chélate de métal de terres rares et un tyramide lié audit chélate de métal ;
le lavage dudit substrat pour enlever un marqueur AECL non lié dudit substrat ;
la mise en contact dudit substrat avec une solution détection qui réagit avec ledit marqueur AECL lié pour générer une luminescence lorsqu'une tension électrique est appliquée sur ledit emplacement défini sur ledit substrat ;
l'application de ladite tension électrique sur ledit emplacement défini sur ledit substrat ; et
la mesure de la luminescence provenant dudit emplacement défini sur ledit substrat, ainsi détectant ladite biomolécule cible ;
dans lequel le procédé détecte ladite biomolécule cible dans l'échantillon à des concentrations pouvant atteindre un minimum de 1 fg/mL.

2. Procédé selon la revendication 1, dans lequel ledit emplacement défini sur ledit substrat comprend un dispositif microréseau ou une pluralité de dispositifs microréseaux.

3. Procédé selon la revendication 2, dans lequel ledit dispositif ou lesdits dispositifs ont une dimension de bord entre 50 nm et 1 μm, entre 50 nm et 100 nm, ou entre 50 nm et 75 nm.

4. Procédé selon la revendication 1, dans lequel ledit ligand de capture est lié de façon covalente audit emplacement défini sur ledit substrat par l'intermédiaire d'un groupe caractéristique COOH prévu sur ledit substrat, ou par l'intermédiaire d'un groupe caractéristique NH2 prévu sur ledit substrat.

5. Procédé selon l'une quelconque des revendications 1 et 2, dans lequel :

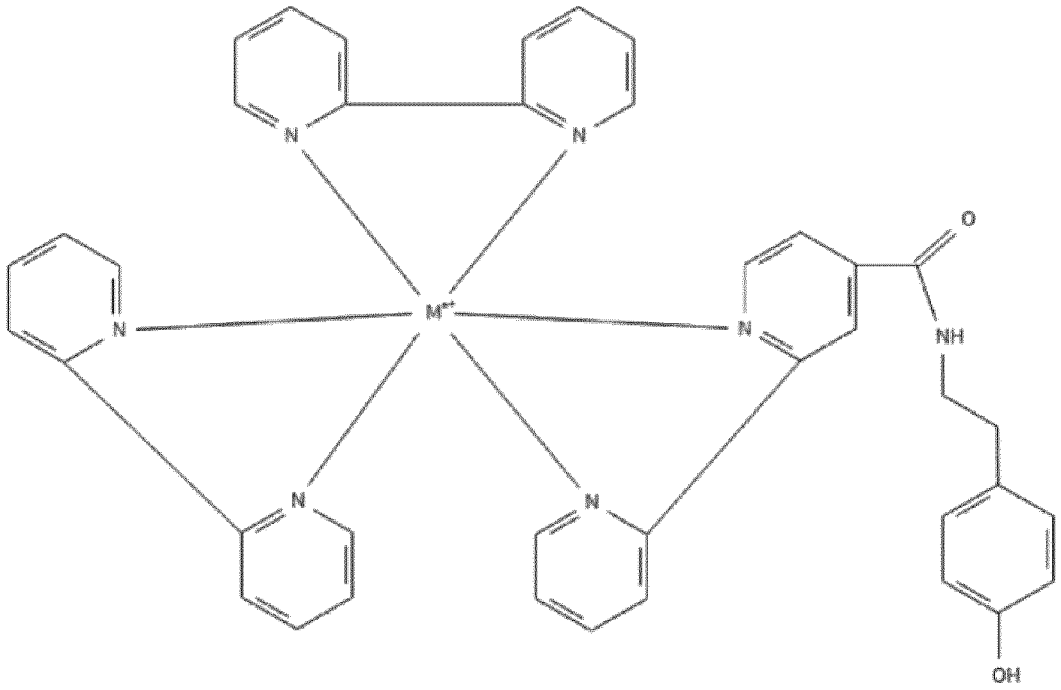
(i) ledit ligand de capture comprend un peptide ;
(ii) ledit ligand de capture comprend une protéine ;
(iii) ladite biomolécule cible comprend un peptide ;
(iv) ladite biomolécule cible comprend une protéine, optionnellement dans lequel ladite protéine est un anticorps ;
(v) ledit échantillon comprend du sang, du sérum, du plasma, de la salive, de l'urine, des fèces ou du liquide céphalorachidien (CSF) ; ou
(vi) ledit échantillon est obtenu d'un humain.

6. Procédé selon l'une quelconque des revendications 1 et 2, dans lequel ledit ligand de détection comprend un conjugué anticorps-peroxidase de raifort.

7. Procédé selon l'une quelconque des revendications 1 et 2, dans lequel ledit métal de terres rares est du ruthénium.

8. Procédé selon l'une quelconque des revendications 1 et 2, dans lequel ledit marqueur AECL comprend du tris (bipyridine) ruthénium(II).

9. Procédé selon l'une quelconque des revendications 1 et 2, dans lequel ledit marqueur AECL comprend :

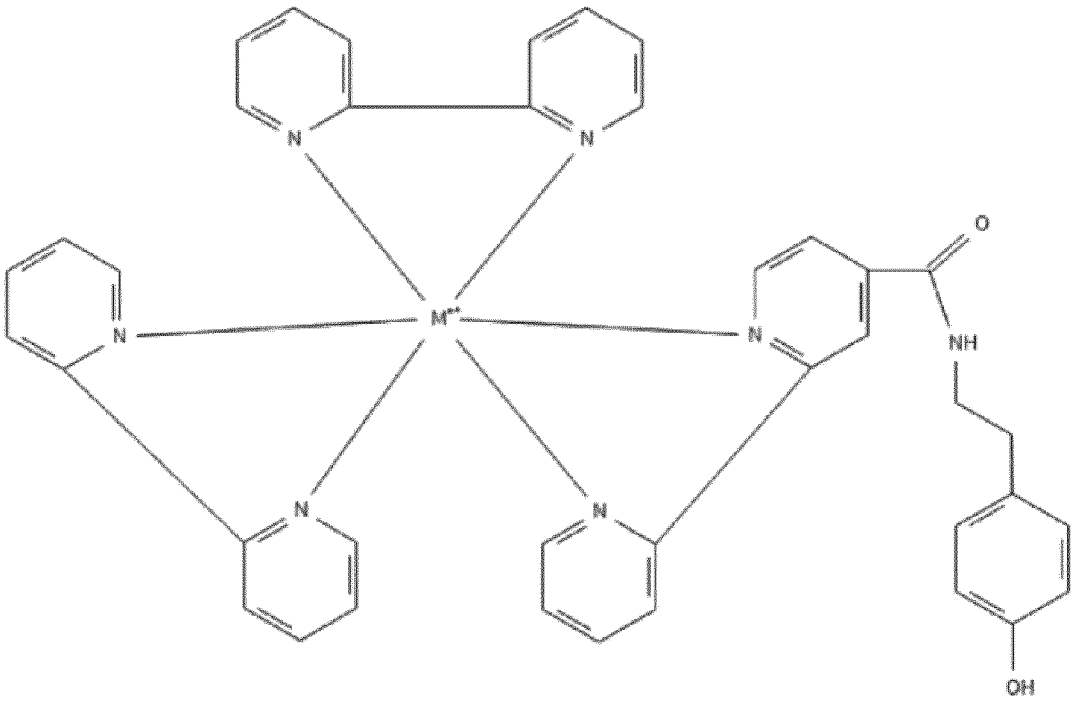


dans lequel M^{n+} est Ru^{2+}.

10. Procédé selon l'une quelconque des revendications 1 et 2, dans lequel ladite solution de marquage comprend du peroxyde d'hydrogène, optionnellement dans lequel ladite solution de détection comprend de la tripropylamine.

11. Procédé selon l'une quelconque des revendications 1 et 2, dans lequel ledit ligand de capture et ledit ligand de détection comprennent chacun un anticorps.

12. Composition de marqueur AECL, comprenant :



13. Composition de marqueur AECL selon la revendication 12, dans laquelle M^{n+} est Ru^{2+}.

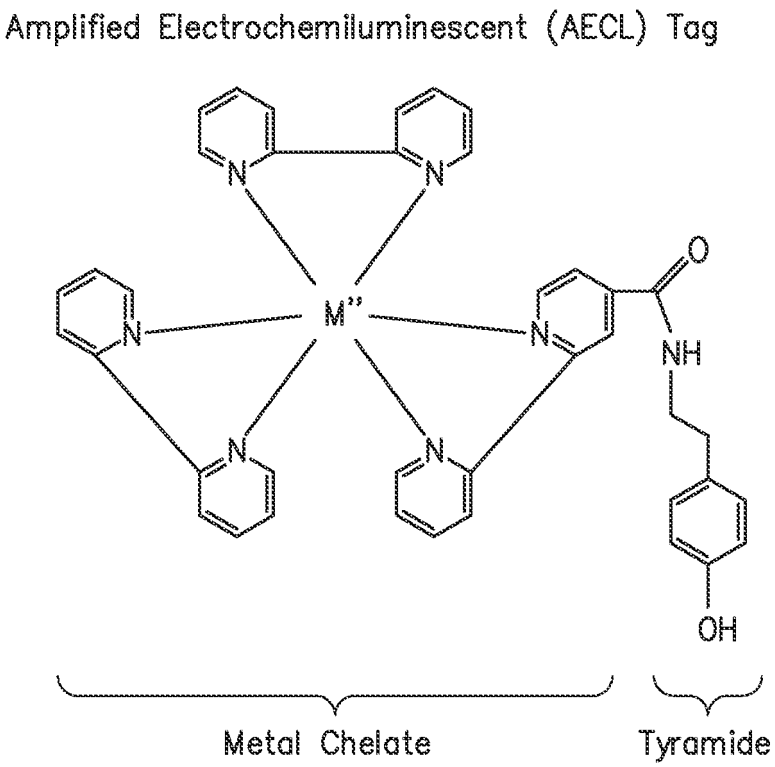

Amplified Electrochemiluminescent (AECL) Tag
N
N
M''
N
N
N
N
O
NH
OH
Metal Chelate
Tyramide

Figure 1

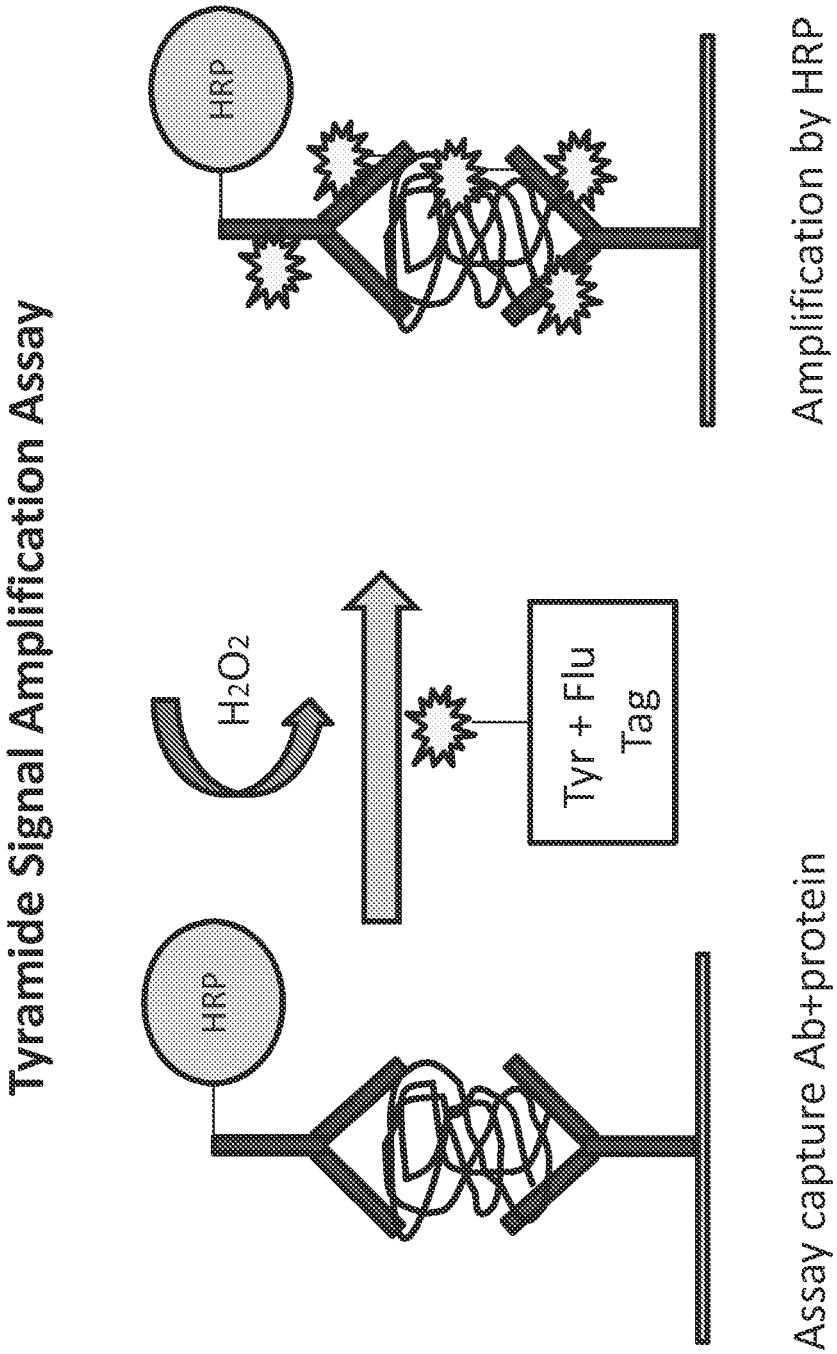

Tyramide Signal Amplification Assay
HRP
H_2O_2
Tyr + Flu
Tag
HRP
Assay capture Ab+protein
Sec Ab with HRP
Amplification by HRP

Figure 2

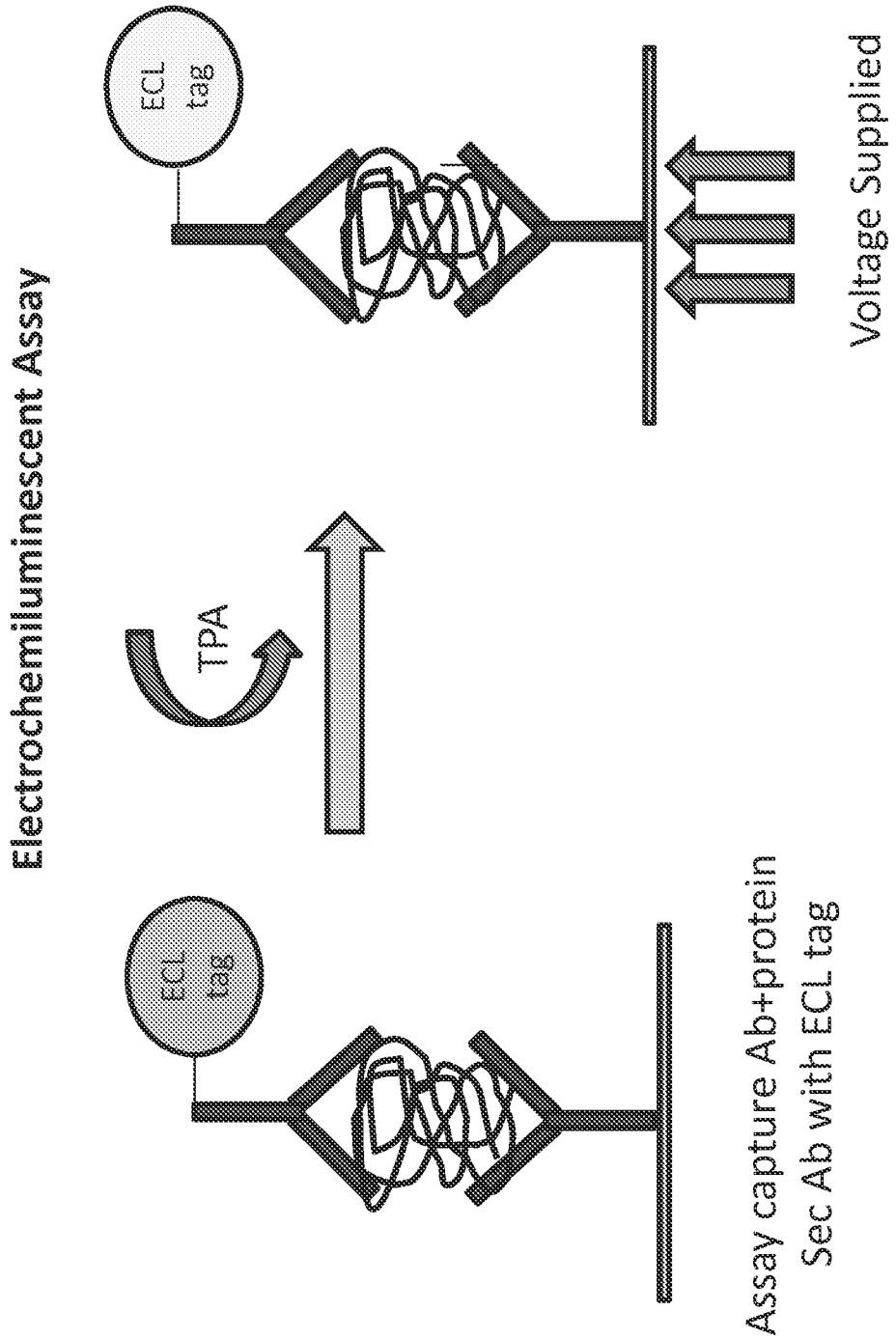

Electrochemiluminescent Assay
ECL tag
TPA
Assay capture Ab+protein
Sec Ab with ECL tag
ECL tag
Voltage Supplied

Figure 3

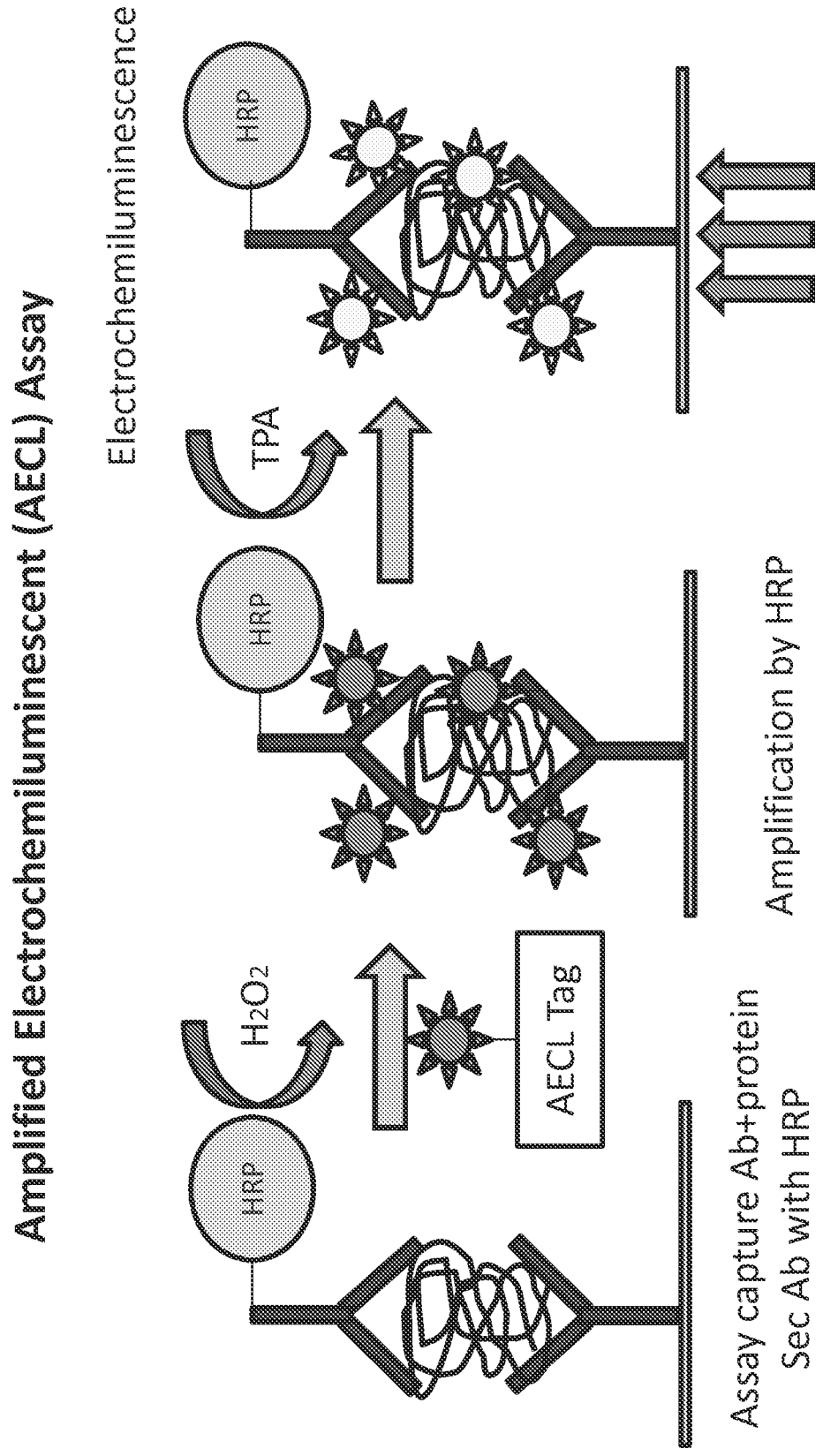

Amplified Electrochemiluminescent (AECL) Assay
Electrochemiluminescence
HRP
H_2O_2
AECL Tag
HRP
TPA
HRP
Assay capture Ab+protein
Sec Ab with HRP
Amplification by HRP
Voltage Supplied

Figure 4

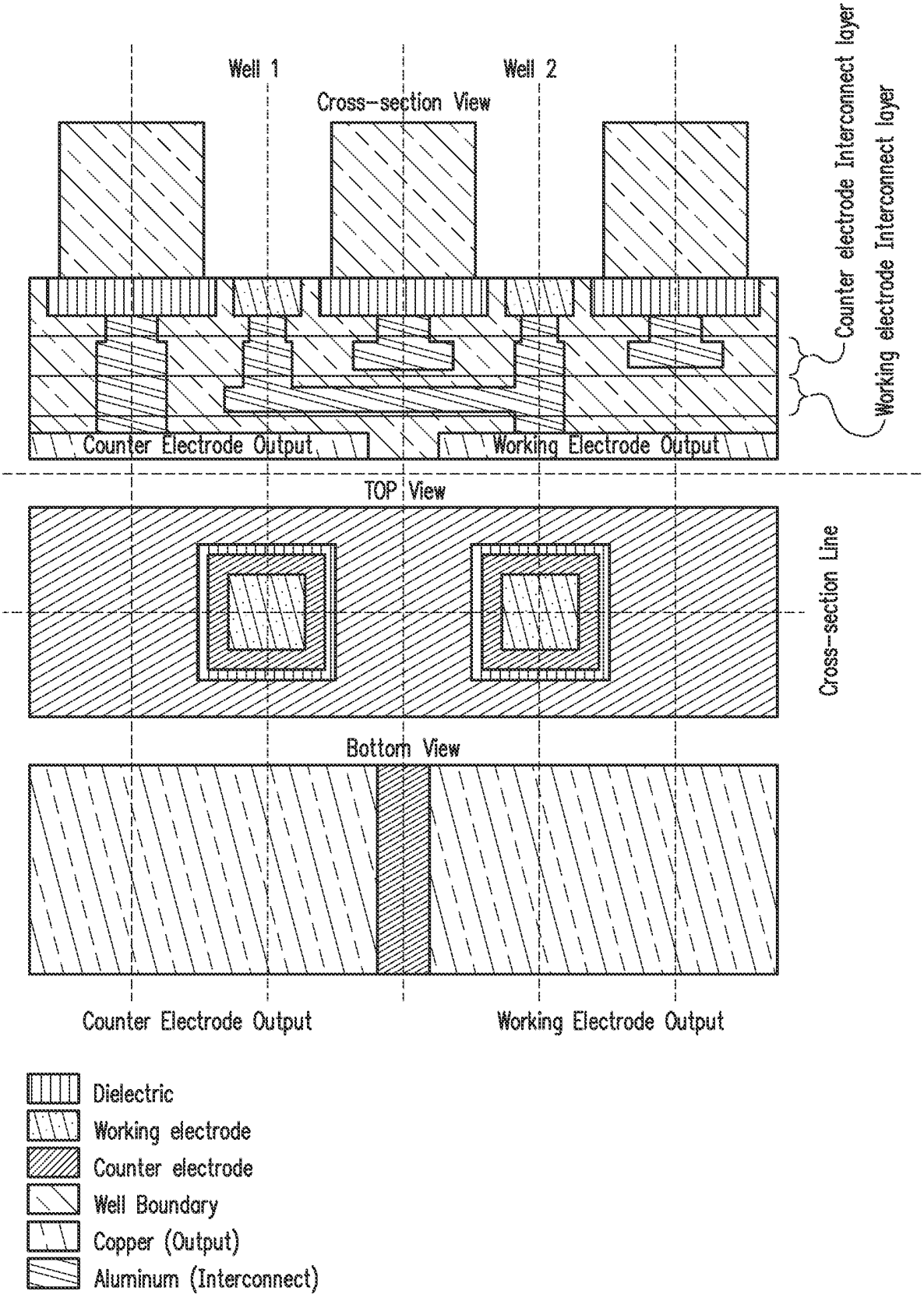

Well 1
Well 2
Cross-section View
Counter electrode Interconnect layer
Working electrode Interconnect layer
Counter Electrode Output
Working Electrode Output
TOP View
Cross-section Line
Bottom View
Counter Electrode Output
Working Electrode Output
Dielectric
Working electrode
Counter electrode
Well Boundary
Copper (Output)
Aluminum (Interconnect)

Figure 5

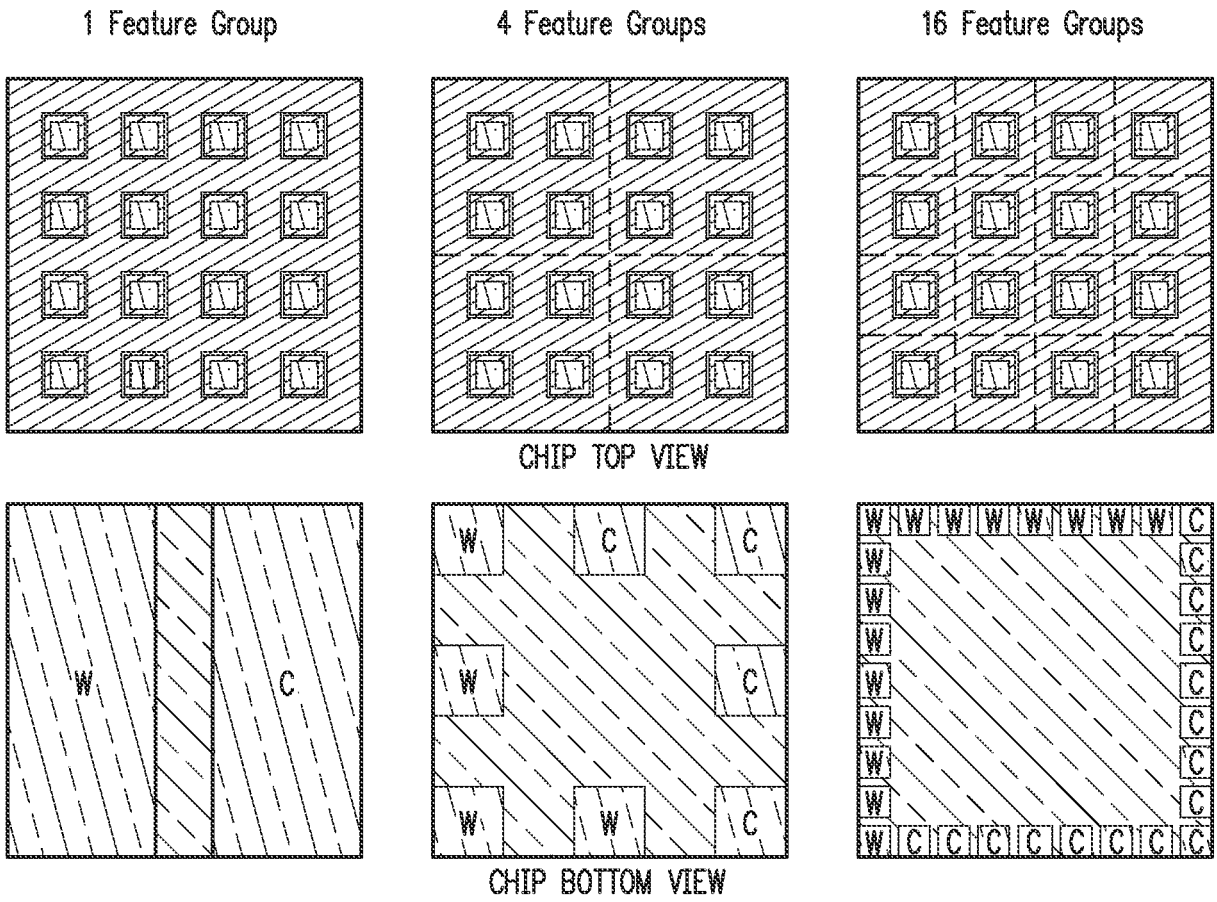

1 Feature Group
4 Feature Groups
16 Feature Groups
CHIP TOP VIEW
W
C
CHIP BOTTOM VIEW
Dielectric
Working electrode
Counter electrode
Well Boundary
Copper (Output)
W: Copper (working electrode)
C: Copper (counter electrode)

Figure 6

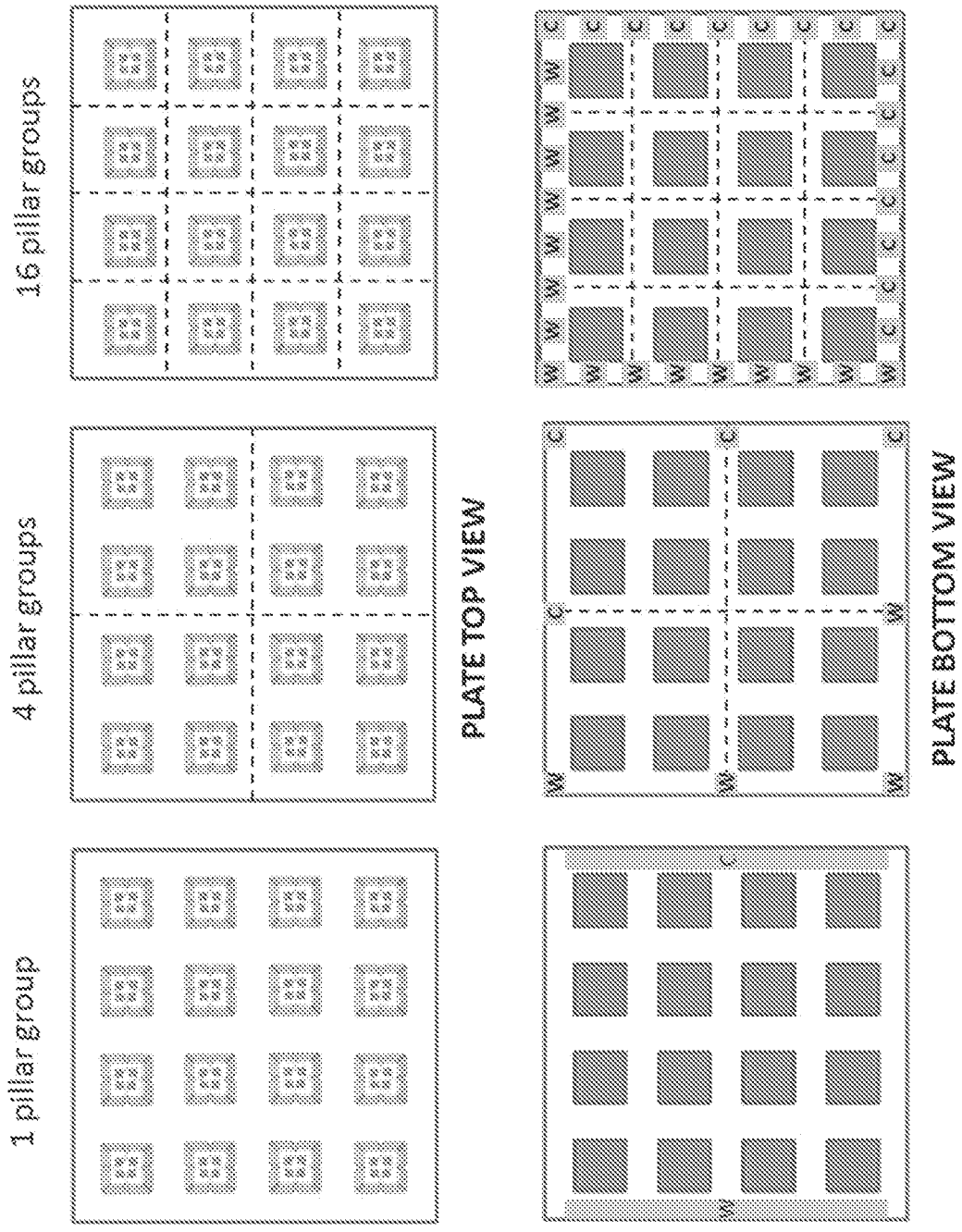

1 pillar group
4 pillar groups
16 pillar groups
PLATE TOP VIEW
PLATE BOTTOM VIEW

Figure 7

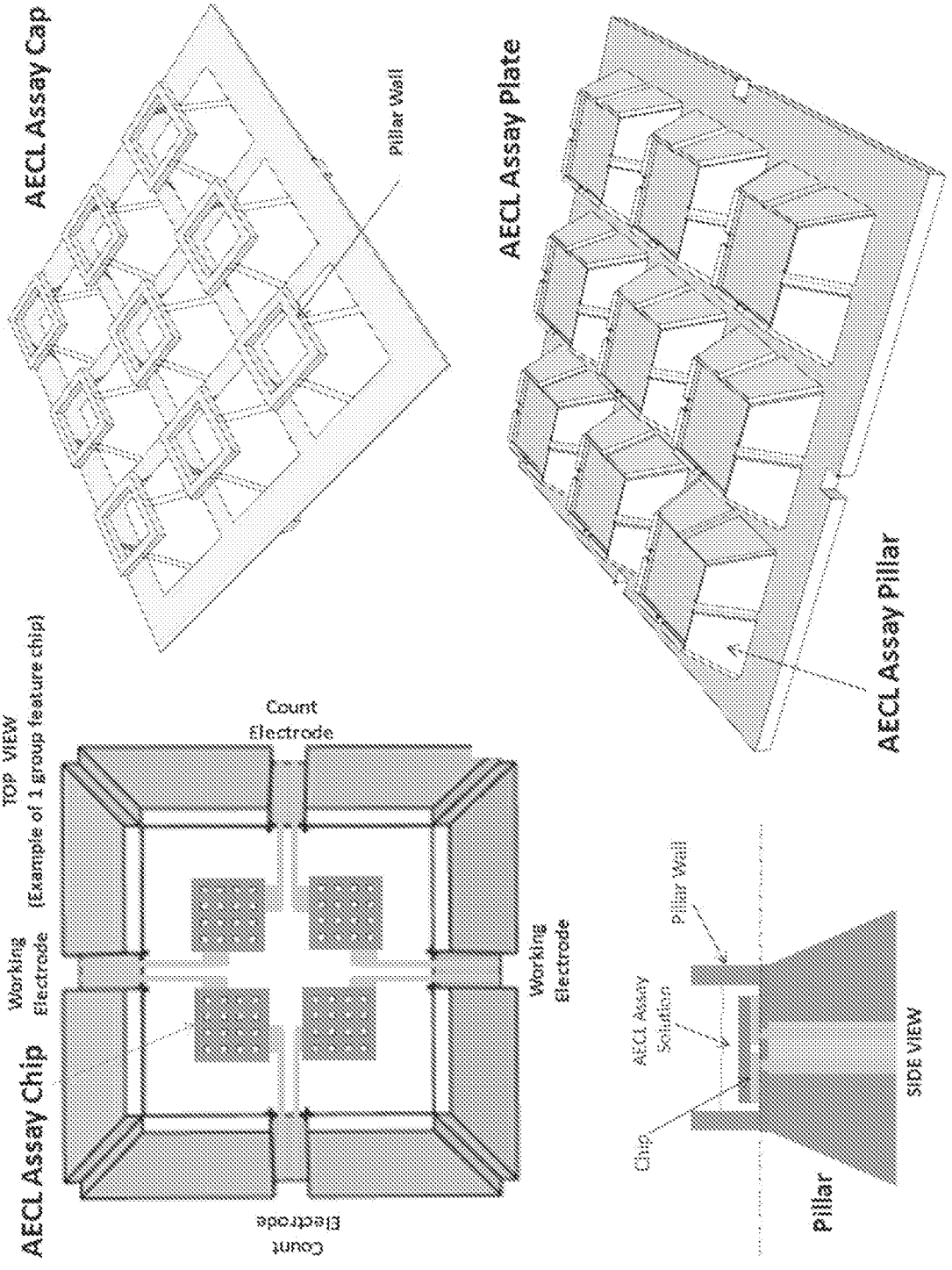

AECL Assay Cap
Pillar Wall
AECL Assay Plate
AECL Assay Pillar
AECL Assay Chip
TOP VIEW
(Example of 1 group feature chip)
Working Electrode
Count Electrode
Count Electrode
Working Electrode
Pillar Wall
AECL Assay Solution
Chip
Pillar
SIDE VIEW

Figure 8

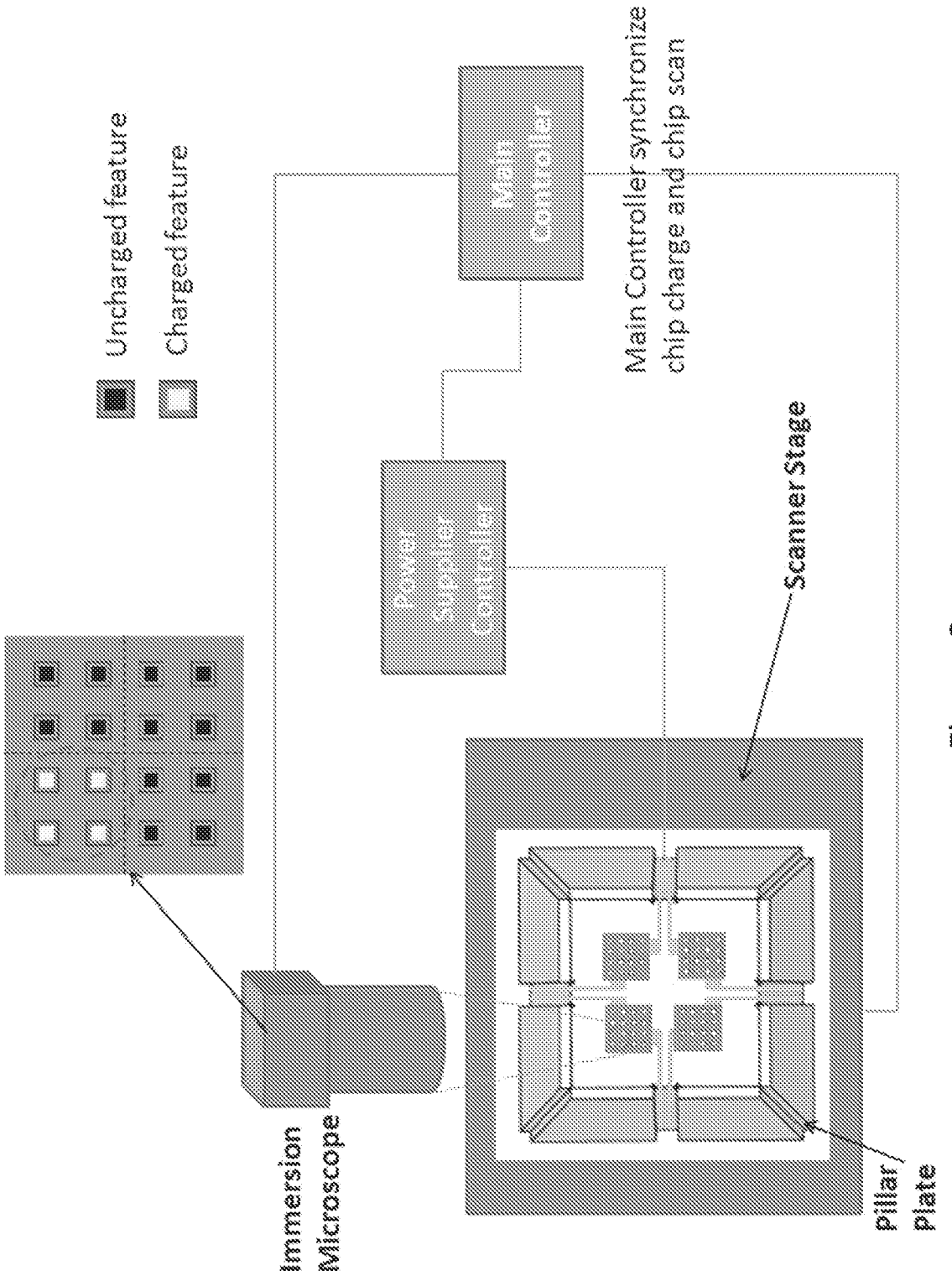
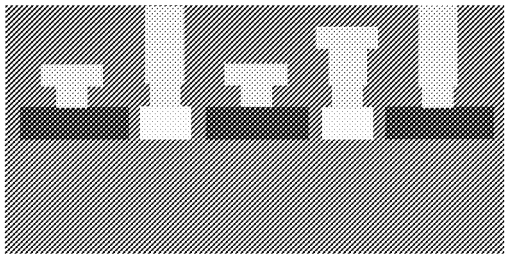

Uncharged feature
Charged feature
Main controller
Main Controller synchronize chip charge and chip scan
Power Supplier Controller
Scanner Stage
Immersion Microscope
Pillar plate

Figure 9

AECL Chip Manufacturing Process
Fig. 10 A

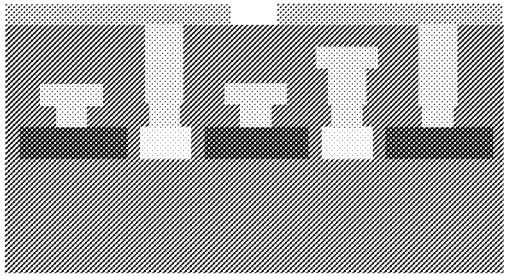
AECL Chip Manufacturing Process (cont.)
Fig. 10 B

Steps 8-10



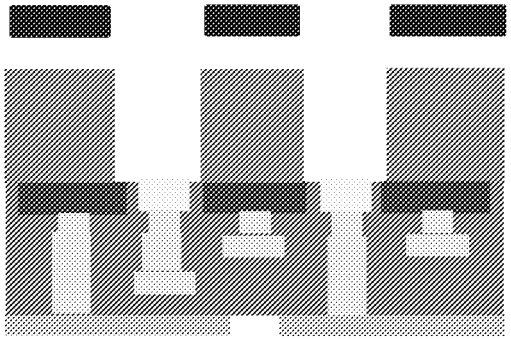
Repeat steps 1,2 and 3, except using different photomask to form Interconnection Layer

Steps 11-13

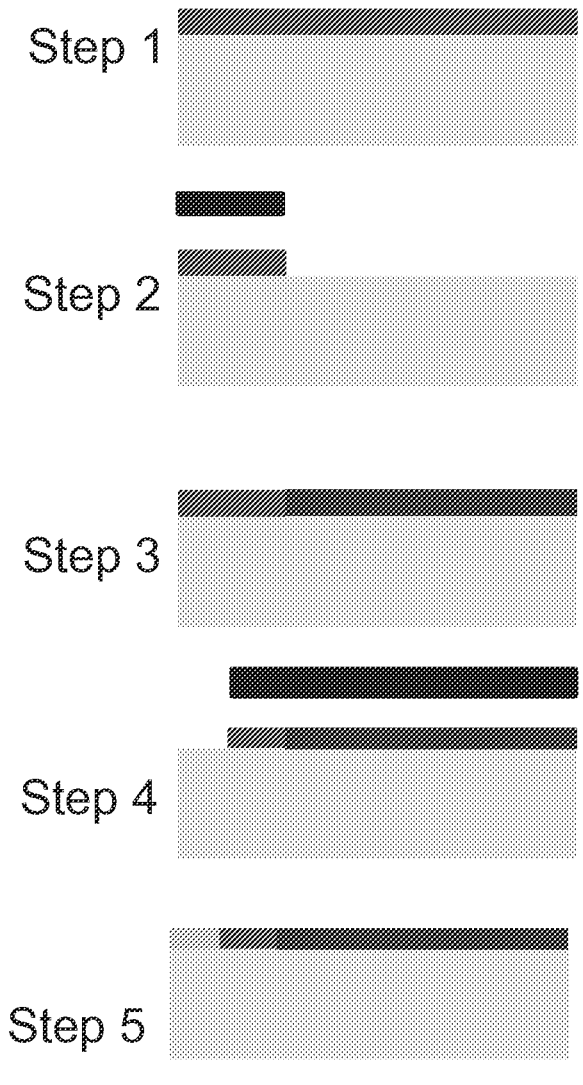


Repeat steps 2,3 and 4, except using different photomask to form Electrode Outputs

Step 14



FLIP THE WAFER
Pattern Transfer:
PR Coating, Exposing, Developing, Etching, Stripping



Silicon dioxide Deposition

Pattern Transfer Process:
Photoresist(PR) Coating, Exposing,
Developing, Etching, Stripping

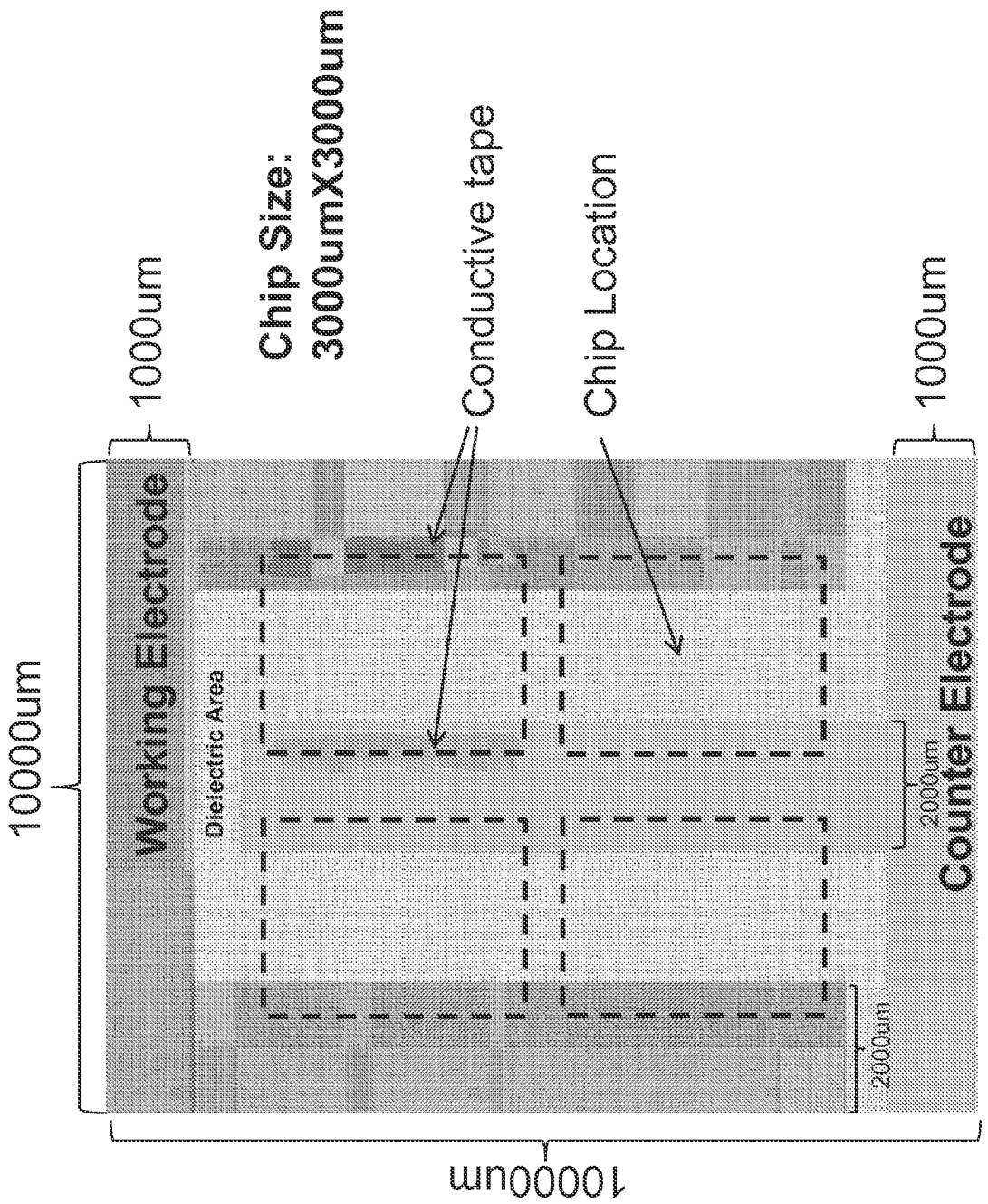
Physical Vapor Deposition (PVD)
Working Electrode Metal and
Chemical mechanical polishing

Pattern Transfer Process:
Photoresist(PR) Coating, Exposing,
Developing, Etching, Stripping

Physical Vapor Deposition (PVD)
Counter Electrode Metal and
Chemical mechanical polishing

AECL Pillar Preparation

Fig. 11 A



AECL Pillar (Top View)
Fig. 11 B

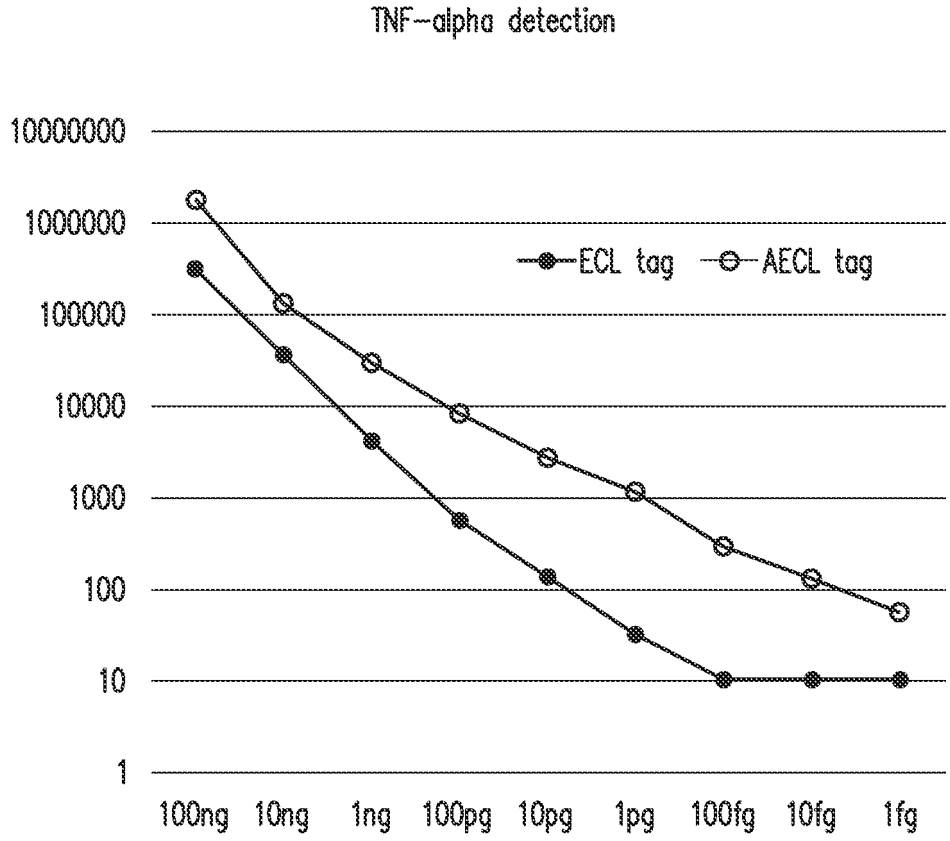

TNF-alpha detection
10000000
1000000
100000
10000
1000
100
10
1
ECL tag
AECL tag
100ng
10ng
1ng
100pg
10pg
1pg
100fg
10fg
1fg

Figure 12

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

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- US 20070122842 A **[0083]**
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- **SMITH ; WATERMAN.** *Adv. Appl. Math.,* 1981, vol. 2, 482 **[0038]**
- **NEEDLEMAN ; WUNSCH.** *J. Mol. Biol.,* 1970, vol. 48, 443 **[0038]**
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- **ALTSCHUL et al.** *J. Mol. Biol.,* 1990, vol. 215, 403-410 **[0039]**
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专利名称(译)	用于放大电化学发光检测的方法和组合物		
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申请(专利权)人(译)	VIBRANT控股有限责任公司		
当前申请(专利权)人(译)	VIBRANT控股有限责任公司		
[标]发明人	RAJASEKARAN JOHN J JAYARAMAN VASANTH WANG TIANHAO BEI KANG KRISHNAMURTHY HARI KRISHNAN		
发明人	RAJASEKARAN, JOHN J. JAYARAMAN, VASANTH WANG, TIANHAO BEI, KANG KRISHNAMURTHY, HARI KRISHNAN		
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其他公开文献	EP2956771B1 EP2956771A4		
外部链接	Espacenet		

摘要(译)

本文公开了制剂，底物和阵列。 本文还公开了用于制造和使用制剂，底物和阵列的方法。 还公开了鉴定可用于诊断和治疗疾病的肽序列的方法，以及使用该肽序列用于诊断和治疗例如乳糜泻的疾病的方法。 在某些实施方案中，基底和阵列包括用于合成和附着聚合物或生物分子的多孔层。