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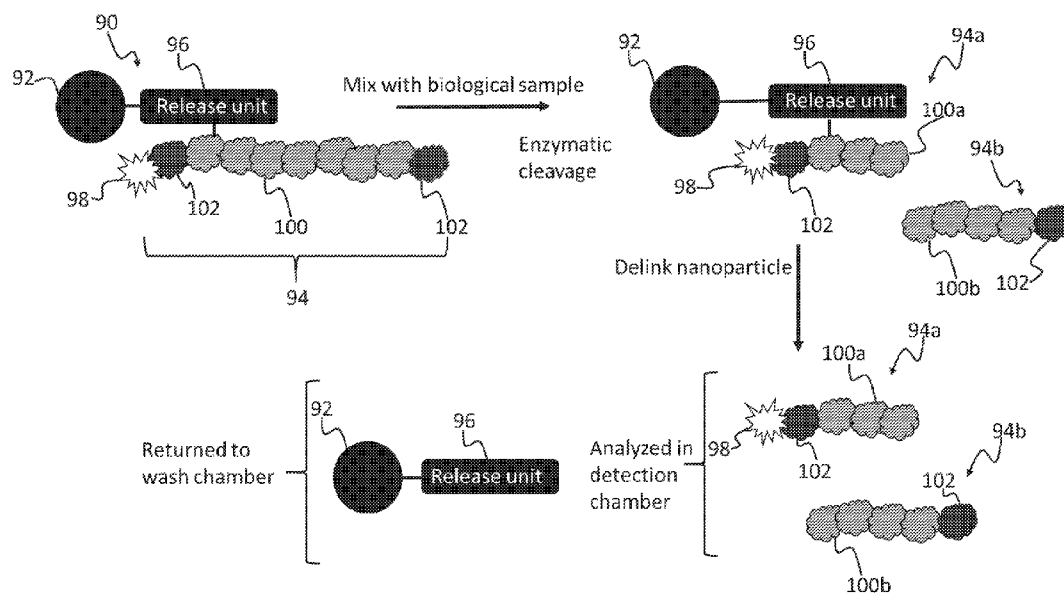


FIG. 4A

(57) **Abstract:** Microfluidic devices and nanobiosensors comprising a magnetic nanoparticle attached to a reporter molecule via a release unit for microfluidic-based detection of a target analyte in a biological sample. The nanobiosensor can be magnetically manipulated or guided through the microfluidics channels for incubation with the biological sample, concentration of the nanobiosensors, and detection of target analytes, without having to pump the entire initial sample through the microfluidic channel. The magnetic nanoparticles are separated from the reporter molecules before detection and can be re-used.



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- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

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MICROFLUIDICS-BASED NANOBIOSENSORS AND DEVICES

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the priority benefit of U.S. Provisional Patent Application
5 Serial No. 62/424,224, filed November 18, 2016, entitled MICROFLUIDICS-BASED
NANOBIOSENSORS, and Serial No. 62/455,853, filed February 7, 2017, entitled
MICROFLUIDIC ISOELECTRIC FOCUSING DEVICE, each of which is incorporated by
reference in its entirety herein.

SEQUENCE LISTING

The following application contains a sequence listing in computer readable format (CRF),
submitted as a text file in ASCII format entitled "Sequence Listing," created on November 17, 2017,
as 28 KB. The content of the CRF is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to nanobiosensors and microfluidics techniques for detecting
one or more target analyte(s) in a biological sample.

Description of Related Art

Molecular-based screening techniques and liquid-based biopsy approaches that can be used
to detect and quantify various biomarkers are promising techniques for non-invasive detection and
staging of cancer and various other diseases. For example, extracellular proteases are associated
with a variety of disease processes. In particular, increased activity of matrix metalloproteinases and
25 cathepsins have been reported for a variety of diseases and that makes them potential biomarker
candidates. Similarly, the protein kinase superfamily is dysregulated in cancer and other
malignancies. Furthermore, many specific kinases have functional roles in solid tumors and
numerous other inflammatory diseases. When these diseases are treated, kinome remodeling
modulates sensitivity to drugs, leading to drug resistance in numerous cancers.

30 While significant progress has been made for the diagnoses of various conditions, including
cancers, these techniques are inadequate to detect cancers at early stage of development mainly due
to their failure to detect molecular markers at the low concentrations associated with the disease
onset. Although molecular-based diagnostics can, principally, help distinguish early stage lung

cancer from benign nodules that are incidentally detected by a CT scan, the impact has been incremental reducing the number of false positives by 32%. Currently, there are no devices/sensing platforms that can be used in clinical diagnosis and/or point-of-care-detection to monitor the onset or recurrence of cancer utilizing biomarkers present in biofluid samples collected by minimally
5 invasive or non-invasive sampling techniques (e.g. peripheral blood, saliva, mucus etc.) with limits of detection (LODs) sufficient to detect solid tumor cancers at the 0 and 1 stages.

SUMMARY OF THE INVENTION

The present invention is broadly concerned with methods of microfluidic detection of a
10 target analyte in a biological sample collected from a subject.

The methods generally comprise providing a microfluidic device comprising a planar substrate comprising a sample inlet well positioned above a sample chamber in or on the substrate and a detection chamber in fluid communication with the sample chamber via a microfluidic channel extending from the sample chamber to the detection chamber. A washing or wash chamber
15 positioned along the microfluidic channel intermediate to the sample chamber and the detection chamber. The device also includes a magnet positioned external and adjacent to the microfluidic channel (e.g., generally underneath the channel), wherein the magnet is translatable along a plane parallel and to the plane of the microfluidic channel. In other words, the magnet is configured and positioned such that it can be slid back and forth underneath the microfluidic channel and
20 essentially follow the same linear/horizontal path as the channel. The magnet could also be laterally movable to follow the path of one or more inlet channels or outlet channels extending from the main microfluidic channel. The biological sample collected from the subject is incubated with a first nanobiosensor in the sample chamber for a time period and under conditions (e.g., ambient conditions or slightly elevated temperatures) sufficient for the target analyte (if present) in the
25 biological sample to interact with the first nanobiosensor. The first nanobiosensor comprises a magnetic nanoparticle releasably attached to a plurality of reporter molecules (which each contain peptide sequences specific for the target analyte). Thus, the magnet can be rotated or manipulated to magnetically manipulate and move the nanobiosensor. It will be appreciated that the magnet can be used to facilitate mixing of the nanobiosensor and biological sample during incubation.

30 The incubated sample and first nanobiosensor is then transferred to the wash chamber. This can be accomplished with a valve system or by using the magnet's attractive forces to "pull" the sample to the wash chamber. A wash fluid is introduced into the wash chamber to yield a first washed nanobiosensor. In general, this involves removing at least a portion of the biological

sample matrix and unreacted components from the system, such as through an outlet channel to a wash outlet chamber, so that only the washed nanobiosensors are left in the wash chamber (along with residual sample, cleaved reporter molecules, buffer, wash fluid, etc.). This step is facilitated by maintaining the magnet in a fixed position adjacent the wash chamber (e.g., underneath) to magnetically “hold” the nanobiosensors in a fixed position while the wash fluid moves through the wash chamber and exits the outlet channel into the waste chamber. It will also be appreciated that this step changes the relative concentration of the nanobiosensor in the biological sample, as a portion of the biological sample is removed, leaving a higher concentration of nanobiosensor behind for the subsequent detection step.

Next, the first washed nanobiosensor is magnetically transferred to the detection chamber. This step is facilitated by translating the magnet along a plane parallel to the microfluidic channel from a position adjacent the wash chamber to a position adjacent the detection chamber. In other words, this can involve sliding the magnet along a linear path underneath the microfluidic channel along the linear path followed by the channel, while the magnetic is in sufficient proximity to the channel so that the attractive forces of the magnetic will magnetically guide (or pull) the nanobiosensors from the wash chamber to the detection chamber.

In the detection chamber, the nanobiosensor is essentially cleaved apart so that the magnetic nanoparticle can be removed from the detection chamber. This generally involves separating or uncoupling the nanoparticle from its respective reporter molecules to yield a plurality of individual separated reporter molecules and a delinked or uncoupled nanoparticle.

The delinked (or uncoupled) nanoparticle is then magnetically removed from the detection chamber by translating the magnet along a plane parallel to the microfluidic channel away from the detection chamber. In other words, this can involve sliding the magnet backwards along the linear path underneath the microfluidic channel along the linear path followed by the channel, while the magnetic is in sufficient proximity to the channel so that the attractive forces of the magnetic will magnetically guide (or pull) the nanobiosensors from the detection chamber back into the detection chamber. It is envisioned that the magnet could also be translated laterally to move the nanoparticles out of the detection chamber via a lateral outlet channel extending from the detection chamber and/or the magnet could continue along the initial path of travel so that the nanoparticles would exit the distal end of the detection chamber (opposite from the wash chamber), instead of returning to the wash chamber.

Once the nanoparticles have been removed from the detection chamber, the presence or absence of reacted and/or unreacted separated reporter molecules that remain in the chamber is then

detected, wherein the presence and/or absence of a given reacted and/or unreacted reporter molecule is indicative of interaction (or presence/absence) of the target analyte with the reporter molecules.

It will be appreciated that the methods of the invention are particularly suited for detecting a plurality of target analytes in a single sample. This is achieved through the use of a plurality of
5 different types of nanobiosensors with different reporter molecules specific to a different target analytes. Further, detection techniques can generate a linearly spaced array of different reporter molecules at respective locations in the detection chamber, which can be “read” similar to a bar code reader.

Embodiments of the invention are also concerned with microfluidic devices for detection of
10 a target analyte in a biological sample. The microfluidic devices generally comprise a planar substrate comprising a sample inlet well positioned above a sample chamber in or on the substrate and a detection chamber in fluid communication with the sample chamber via a microfluidic channel extending from the sample chamber to the detection chamber; and a washing chamber positioned along the microfluidic channel intermediate to the sample chamber and the detection
15 chamber; and a magnet positioned external and adjacent to the microfluidic channel, wherein the magnet is translatable along a plane parallel and to the plane of the microfluidic channel. For IEF detection, the device further comprises in fluid communication with the detection chamber: an ampholyte inlet configured to introduce a high-resolution ampholyte mixture into the detection chamber, a catholyte inlet configured to introduce a catholyte mixture into the detection chamber,
20 an anolyte inlet configured to introduce a high-resolution anolyte mixture into the detection chamber, and a pressure fill inlet valve. The detection chamber can further comprise one or more inlets for applying an electrical current to the detection chamber.

Embodiments described herein are also concerned with nanobiosensors for detection of a target analyte in a biological sample. The nanobiosensors comprise a magnetic nanoparticle
25 releasably attached to a reporter molecule via a release unit, wherein the reporter molecule is an oligopeptide comprising a target analyte recognition sequence, detectable label, and one or more optional pI tag(s).

BRIEF DESCRIPTION OF THE DRAWINGS

30 FIG. 1A is a schematic side view of a microfluidic device in use accordance with one embodiment of the present invention;

FIG. 1B is a schematic side view of a microfluidic device in use accordance with one embodiment of the present invention;

FIG. 1C is a schematic side view of a microfluidic device in use accordance with one embodiment of the present invention;

FIG. 1D is a schematic side view of a microfluidic device in use accordance with one embodiment of the present invention;

5 FIG. 2 is a schematic top view of the microfluidic device of FIG. 1;

FIG. 3 is a schematic diagram of an optical detector apparatus in accordance with one embodiment of the present invention;

FIG. 4A illustrates a reaction mechanism for a enzymatic cleavage-based nanobiosensors in accordance with embodiments of the present invention;

10 FIG. 4B illustrates a reaction mechanism for posttranslational modification and supramolecular recognition (binding) based nanobiosensors in accordance with embodiments of the present invention;

FIG. 5 is a graph showing HPLC results following trypsin digestion at different time periods;

15 FIG. 6 is an annotated photograph of a microfluidic device fabricated in Example 3;

FIG. 7 is a graph showing isoelectric focusing results following trypsin digestion at different time periods;

FIG. 8 is a graph showing isoelectric focusing results following disulfide reduction; and

FIG. 9 is a graph showing isoelectric focusing results following disulfide reduction.

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DETAILED DESCRIPTION

The present invention is concerned with nanobiosensors and microfluidics techniques for detecting one or more target analyte(s) in a biological sample. The invention leverages the use of nanobiosensors comprising magnetic nanoparticles bound to a reporter molecule via a release unit.

25 In one or more embodiments, each nanobiosensor comprises a plurality of reporter molecules attached to a single central nanoparticle via respective release units. In particularly preferred embodiments, a plurality of a single type of reporter molecule may be attached to each nanoparticle. In other embodiments, however, a plurality of different types of reporter molecules may also be attached to each nanoparticle. Regardless a plurality of different types of reporter molecules are

30 preferably used so that the assays can analyze a plurality of different types of target analytes in a single biological sample.

In general, a biological sample is collected from a subject and prepared for analysis. The nanobiosensors are contacted with a biological sample for a period of time sufficient for the target

analyte (if present in the sample) to interact with the reporter molecules in the nanobiosensors. The nanobiosensors can advantageously be directly added to raw samples (biospecimens). The reacted sample is then washed with a wash solution, and the washed nanobiosensors are then transferred to a detection chamber. In the detection chamber, the reporter molecule is released from the nanobiosensor, and the nanoparticles are transferred out of the detection chamber (e.g., back to the wash chamber), while the reacted and unreacted reporter molecules remain in the detection chamber. Any reacted reporter molecules can then be detected in the detection chamber, and the presence and/or absence of reacted/unreacted reporter molecules at designated locations in the detection chamber (and their relative intensities) can be correlated with a prognosis or diagnosis if desired.

The nanoparticle facilitates transport of the nanobiosensor out of the sample matrix after reaction, which reduces matrix effects and thus noise in the final optical readout. In addition, the signal is further enhanced as the concentration of the nanobiosensors are increased $\sim 10^6$ (1,000,000) fold from the starting sample volume (e.g., ~ 1 to about 5 mL) into a smaller concentrated volume (e.g., $\sim$ nL) focused band as explained below. The nanoparticles can be manipulated (mixed and transported) by moving or rotating an external magnet, thus varying the external magnetic field, which can also be used to mix the nanobiosensor with the sample. While the nanoparticles can assist with mixing and transporting the peptide substrate they will hinder separation due to their charge and size dispersion and thus need to be separated from the reporter molecule before separation and detection. The release unit allows the reporter molecules, both uncleaved/cleaved or unmodified/modified to be delinked from the nanoparticle before the detection technique is applied to the assay.

Magnetic nanoparticle

The term “nanoparticle” as used herein refers to nanocrystalline particles that can optionally be surrounded by a metal and/or nonmetal nanolayer shell. The nanoparticles are preferably metal nanoparticles: metal, metal alloy, metal oxide, or core/shell metal nanoparticles (e.g. Fe_2O_3 , Fe_3O_4), and specifically ferromagnetic metal nanoparticles. Thus, the metal nanoparticles comprise any type of ferromagnetic metal (including elemental metal) or metal alloy: iron (Fe), nickel (Ni), cobalt (Co), certain alloys of rare-earth metals, and the oxides (e.g., FeO , Fe_3O_4 , Fe_2O_3 , Fe_xO_y (non-stoichiometric iron oxide), NiO), hydroxides, sulfides, selenides, and tellurides of the foregoing, and combinations thereof.

Suitable nanoparticles preferably have a diameter of from about 1 nm to about 100 nm, more

preferably from about 10 nm to about 50 nm, and even more preferably from about 20 nm to about 40 nm. The nanoparticles may also include an additional metal, so long as the magnetic property of the nanoparticle is not impaired, including methods selected from the group consisting of gold (Au), silver (Ag), copper (Cu), palladium (Pd), platinum (Pt), rhodium (Rh), iridium (Ir), iron (Fe), ruthenium (Ru), osmium (Os), manganese (Mn), rhenium (Re), scandium (Sc), titanium (Ti),
5 vanadium (V), chromium (Cr), zinc (Zn), yttrium (Y), zirconium (Zr), niobium (Nb), molybdenum (Mo), technetium (Tc), cadmium (Cd), lanthanum (La), lutetium (Lu), hafnium (Hf), tantalum (Ta), tungsten (W), actinium (Ac), lawrencium (Lr), rutherfordium (Rf), dubnium (Db), seaborgium (Sg), bohrium (Bh), Hassium (Hs), meitnerium (Mt), darmstadtium (Ds), roentgenium (Rg), ununbium
10 (Uub), selenium (Se), and the oxides (e.g., CuO, Ag₂O, Mn₂O₃), hydroxides, sulfides, selenides, and tellurides of the foregoing, and combinations thereof.

In some embodiments, metal nanoparticles will be bi- or para-magnetic and comprise a metal or metal alloy core and a metal shell. Core/shell metal nanoparticles preferably comprise a metal or metal alloy core and a metal shell. Preferred cores are selected from the group consisting
15 of Au, Ag, Cu, Co, Fe, and Pt. Even more preferably, the metal nanoparticles feature a strongly paramagnetic Fe core. Preferred shells are selected from the group consisting of Au, Ag, Cu, Co, Fe, Pt, the metal oxides (e.g., FeO, Fe₃O₄, Fe₂O₃, Fe_xO_y (non-stoichiometric iron oxide), CuO, Cu₂O, NiO, Ag₂O, Mn₂O₃) thereof, and combinations thereof. Particularly preferred metal core/shell combinations are selected from the group consisting of Fe/Au, Fe(0)/Fe₃O₄, and
20 Au/Fe₂O₃. A particularly preferred metal nanoparticle is a superparamagnetic Fe/Fe₃O₄ core shell nanoparticle. More preferably, the nanoparticles feature an iron(0) core, which is more magnetic than iron oxide, based upon coercivity. This means that smaller nanoparticles can be used (diameter less than about 10 nm), which have the same or greater magneticity than larger iron oxide nanoparticles (diameter of about 200nm).

25 In one or more embodiments, the core of the metal nanoparticle preferably has a diameter of from about 5 nm to about 100 nm, more preferably from about 20 nm to about 50 nm and more preferably from about 30 nm to about 40 nm. The metal shell of the core/shell nanoparticle preferably has a thickness of from about 1 nm to about 10 nm, and more preferably from about 2 nm to about 5 nm. The nanoparticles preferably have a Brunauer-Emmett-Teller (BET)
30 multipoint surface area of from about 20 m²/g to about 500 m²/g, more preferably from about 50 m²/g to about 350 m²/g, and even more preferably from about 60 m²/g to about 80 m²/g. The nanoparticles preferably have a Barret-Joyner-Halenda (BJH) adsorption cumulative surface area of pores having a width between 17.000 Å and 3000.000 Å of from about 20 m²/g to about 500 m²/g,

and more preferably from about 50 m²/g to about 150 m²/g. The nanoparticles also preferably have a BJH desorption cumulative surface area of pores having a width between 17.000 Å and 3000.000 Å of from about 50 m²/g to about 500 m²/g, and more preferably from about 50 m²/g to about 150 m²/g. The nanoparticle population is preferably substantially monodisperse, with a very narrow
5 size/mass size distribution. More preferably, the nanoparticle population has a polydispersity index of from about 1.2 to about 1.05. It is particularly preferred that the nanoparticles used in the inventive nanoplateforms are discrete particles. That is, clustering of nanocrystals (i.e., nanocrystalline particles) is preferably avoided.

The nanoparticles can be stabilized or non-stabilized. Stabilized nanoparticles preferably
10 comprise an organic monolayer surrounding the nanoparticle core. The term “stabilized” as used herein means the use of a ligand shell or monolayer to coat, protect (e.g., from bio-corrosion), or impart properties (e.g., water solubility) to, the nanoparticle. The monolayer can be comprised of several of the same ligands (i.e., homoligand) or of mixed ligands. Various techniques for attaching ligands to the surface of various nanoparticles are known in the art. For example, nanoparticles
15 may be mixed in a solution containing the ligands to promote the coating of the nanoparticle. Alternatively, coatings may be applied to nanoparticles by exposing the nanoparticles to a vapor phase of the coating material such that the coating attaches to or bonds with the nanoparticle. Preferably, the ligands attach to the nanoparticle through covalent bonding. The number of ligands required to form a monolayer will be dependent upon the size of the nanoparticle.

The ligands comprise functional groups that are attracted to the nanoparticle’s metal surface.
20 Preferably, the ligands comprise at least one group selected from the group consisting of thiols, alcohols, nitro compounds, phosphines, phosphine oxides, resorcinarenes, selenides, phosphinic acids, phosphonic acids, sulfonic acids, sulfonates, carboxylic acids, disulfides, peroxides, amines, nitriles, isonitriles, thionitriles, oxynitriles, oxysilanes, alkanes, alkenes, alkynes, aromatic
25 compounds, and seleno moieties. Preferred organic monolayers are selected from the group consisting of alkanethiolate monolayers, aminoalkylthiolate monolayers, alkylthiolsulfate monolayers, and organic phenols (e.g., dopamine, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylpropionic acids and homologues/derivatives thereof). The thickness of the organic monolayer is preferably less than about 10 nm, and more preferably less than about 5 nm.
30 Particularly preferred stabilized nanoparticles are selected from the group consisting of trioctylphosphin oxide-stabilized nanoparticles, amine-stabilized nanoparticles, carboxylic acid-stabilized nanoparticles, phosphine-stabilized nanoparticles, thiol-stabilized nanoparticles, aminoalkylthiol-stabilized nanoparticles, and organic phenol-stabilized nanoparticles.

For attachment to the release unit, the preferred ligands will preferably readily react with the thiol group of a terminal cysteine. The nanoparticle surface will preferably be essentially completely covered with ligands. That is, at least about 70%, preferably at least about 90%, and more preferably about 100% of the surface of the nanoparticle will have attached ligands. The number of ligands required to form a monolayer will be dependent upon the size of the nanoparticle (and monolayer), and can be calculated using molecular modeling or ligand modeling methods.

Various techniques for attaching ligands to the surface of various nanoparticles are known in the art. For example, nanoparticles may be mixed in a solution containing the ligands to promote the coating of the nanoparticle surface. Alternatively, coatings may be applied to nanoparticles by exposing the nanoparticles to a vapor phase of the coating material such that the coating attaches to or bonds with the nanoparticle. Preferably, the ligands attach to the nanoparticle through covalent bonding. Preferred nanoparticles are characterized as 3,4-dihydroxyphenylacetic acid--covered, water-dispersible iron/iron oxide core/shell nanoparticles.

Release unit

The nanoparticle is attached to the reporter molecule via a release unit, which can be any suitable linkage between the reporter molecule and nanoparticle, so that the nanoparticle can be separated from the reporter molecule after exposure to the biological sample (and washing) and can be removed from the detection chamber. It will be appreciated that a variety of approaches can be used to releasably attach the reporter molecule to the magnetic nanoparticle, provided that the release mechanism is not detrimental to the functionality of the reporter molecule (e.g., specificity of the reporter molecule for the target analyte). Further, the release unit itself should not interact with or be modified by the biological sample itself (or any of its analytes, e.g., as an enzyme substrate). That is, as will be appreciated below, even after incubation with the biological sample and/or washing, the release unit (and thus, the linkage between the reporter molecule and nanoparticle) remains intact, such that the reporter molecule is not released until after the nanobiosensor is transferred to the detection chamber.

A number of exemplary approaches for various release units are described and demonstrated herein.

In one or more embodiments, the reporter molecule is attached to the nanoparticle via a reducible disulfide bridge. In one or more embodiments, the reporter molecule and nanoparticle can be joined to the nanoparticle by a disulfide bridge via respective cysteines residues. Disulfide bonds can be reductively cleaved upon addition of, for example, reducing agents, such as

dithiothreitol (DTT), water-soluble phosphorous (III) reagents (e.g. THPP (tris-hydroxypropyl-phosphine), mercaptoethanol, mercaptoethylamine, or tris(2-carboxyethyl)phosphine (TCEP), and the like.

In one or more embodiments, the reporter molecule is attached to the nanoparticle via a pH-sensitive linkage. For example, certain bonds become unstable at certain pH ranges, such as esters (pH > 9-10), hydrazones (pH > 6-8), or acetals (pH < 5), etc. In contrast, the reporter molecule includes oligopeptides featuring stable amide bonds in their backbones, which are essentially stable in the pH-range from 1 to 12.4. Thus, the reporter molecule will not be affected by changes in pH in the detection chamber.

In one or more embodiments, the reporter molecule is attached to the nanoparticle via a photosensitive linkage. Photorelease of the reporter molecule is an alternative to chemical transformations, which occur in the dark. Thus, in one or more embodiments, the release unit is a “photorelease unit” that absorbs one (or several) photon(s) upon exposure to activating radiation (e.g., light source), which triggers a chemically defined release reaction, resulting in cleavage of the linkage between the nanoparticle and reporter molecule. Although, principally, mono- or higher photonic processes can be used for photorelease purposes, monophotonic processes are preferred, because they possess distinctly higher release quantum yields (0.01 to unity). Exemplary photorelease units can be attached to the nanoparticle via a -NH-, -CONH-, -O-, -S-, or -C_nH_{2n}- bond. Boron-Dipyrromethene (BODIPY)-mediated photorelease is one example, where BODIPY serves as an organic bridging unit between the magnetic nanoparticle and reporter molecule that releases the C-terminal end of the reporter molecule oligopeptide upon monophotonic excitation with red laser light. Another example is 8-cyano-7-hydroxyquinoliny (CyHQ)-mediated release, which contains a photoremovable protecting group that releases N- or C-terminal histidine groups upon monophotonic excitation with blue light or bi-photonic NIR excitation. Another example is ruthenium-polypyridyl-mediated photorelease, which reacts under mono- or diphotonic excitation. Accessible ligand-centered states lead to photodissociation reaction of pyridine-type ligands upon exposure to blue or NIR laser diodes.

In one or more embodiments, the reporter molecule is attached to the nanoparticle via a thermosensitive linkage. Thermal release of the reporter molecule occurs after photons are absorbed by a plasmonic nanoparticle, or after thermal heating, A/C, or radiofrequency hyperthermia has been applied. The heat that is generated in the nanoparticle through light absorption will be able to facilitate thermal release reactions, such as the cleavage of thermally unstable bonds or adducts (e.g. imines or Diels-Alder products etc.) or strand separation (“melting”)

of DNA double-strands or supramolecular LEGO peptide sequences and phenylalanine-isoleucin “zippers” that may form a portion of the release unit. Exemplary thermal release units can be attached to the nanoparticle via a -NH-, -CONH-, -O-, -S-, -(CH₂CH₂-O)-, or -C_nH_{2n}- bond.

5 *Reporter molecule*

The reporter molecule is a peptide probe or sensing peptide sequence comprising a recognition sequence, detectable label, and optional pI tag(s). The recognition sequence is typically an oligopeptide sequence that has specificity for and is recognized by the target analyte, such as, for example, an enzyme substrate sequence. As used here, the phrase “specificity for” is intended to
10 differentiate the recognition sequences from non-specific binding or reactions between molecules, and means that the set of specific target analytes for which the recognition sequence can interact is limited, and in some cases even exclusive, such that neither binding nor enzymatic cleavage occurs at an appreciable rate with any other molecule except for the target analyte. Short oligopeptide sequences are preferably used for the recognition sequences including sequence segments with high
15 specificity for the target analyte. Exemplary oligopeptides are preferably less than 50 amino acid residues in length, and more preferably from about 5 to about 20 amino acid residues.

In one or more embodiments, the recognition sequence comprises, consists essentially, or even consists of a “protease consensus sequence,” which refers to an enzyme substrate (peptide sequence) that undergoes enzymatic cleavage in the presence of the target analyte (i.e., protease,
20 esterase, etc.). Such consensus sequences are described, for example, in U.S. Patent No. 8,969,027 and U.S. Patent No. 9,216,154, U.S. Publication No. 2017/0219548, each incorporated by reference herein to the extent not inconsistent with the present disclosure. In one or more embodiments, the recognition sequence comprises, consists essentially, or even consists of a recognition sequence for detection of enzymatic posttranslational modification described in WO 2016/149637, incorporated
25 by reference herein to the extent not inconsistent with the present disclosure. In the presence of the target enzyme, the enzyme modifies the recognition sequence (without cleavage), and more particularly modifies both the primary structure (amino acid residues and metabolic products) and the secondary structure of the recognition sequence. Such “modification” or “posttranslational modification” as used herein, refers to conversion or transformation of the amino acid residues (and
30 particularly the side chains) in the substrate sequence to a different form and/or different residue. This, in turn, extends, “unfolds,” or “unravels” the secondary structure and/or increases and/or decreases the mobility of the recognition sequence. In one or more embodiments, the recognition sequence comprises, consists essentially, or even consists of a supramolecular recognition sequence

to detect physical binding without chemical modification or enzymatic cleavage. In particular, the target protein binds to the supramolecular recognition sequence linearly, such that the initial folded secondary structure, such as an alpha-helix and/or beta-sheet structure, is modified and linearly extends or unfolds. In each instance, interaction of the target analyte (e.g., protein, enzyme, etc.) with the recognition sequence gives rise to a detectable change in the nanobiosensor, which is indicative of the presence of the target analyte.

Exemplary recognition sequences are listed in Table 1 below along with their corresponding target analyte.

Table 1. Recognition sequence examples

	Recognition Sequence(s)	SEQ ID NO:	Accession No.
Cytokine/Chemokine Targets			
CCL 2	CQEQFWW	1	P13500
MCP-1 ^a	PYFPRGSSYQGWN	2	P13500
CCL 3	CCIQNQ	3	P10147
CCL 4	AWYQPQFE	4	P13236
CCL 21	EQQKRN	5	O00585
CXCL 2	CNHGKFYC	6	P19875
CXCL 5	NIYCNIA Y	7	P42830
CXCL 8 (IL-8)	KAYRWEFI	8	P10145
CXCL 9	IQNSGAPCH	9	Q07325
HSP 27	WQEAKNANQM	10	Q5S1U1
HSP 70	RHQKTYSF	11	P0DMV8
HSP 90	XLPPHWAGAL	12	P02829
MIF	XLPPHWAFAL	13	P14174
Calprotectin	LTELEKALNSIIDVYHKYSLIKGNFHAV	14	S100A9
CCL20 ^b	GESMNFSDVFDSSDYFVSVNTSYYSVDSE	15	P78556
	GTQWWVVCQQFG	16	
GCSF ^c	PGHWSDWSPS	17	P09919
IL-6	YFPEPVTVSGAGTFPAVLGSGQPPGKGL	18	P05231

	TAVYYCANRAGWGMGDYWGQGTQVT TASNYGAGYSTNDRHS NRPAQAWMLG	19 20 21	
IL-13	AVYYCQQNNEDPRTFGGGTK AGDGYYPYAMDNW GWLPFGFILISAG YQQKPGQPPKL SVNWIRQPPGKALEWLAMIWGDGKIVYNS WLPFGFILIS	22 23 24 25 26 27	P35225
IL1RL1 ^d	TYYCQQWSGYPYTF	28	P14778
MIP-1 ^e	SHFPYSQYQFWKN	29	Q9BPZ7
OSTF1 ^f	DMSDTNWWKGTSKGRTGLIPSNYVAEQAE SIDNPL	30	Q92882
TIMP-1 ^g	IAGKLQSAGSALWTDQL	31	P01033
TSLP ^h	SSPKHVRFSWHQDAVTVC AKCCPCQQWW	32 33	Q960D9
Enzyme Consensus Sequence			
ADAM 17	LAQAVVSS	34	P78536
ADAM 33	GSQHIRAE	35	Q9BZ11
Cathepsin B	SLLKSRMVPNFN	36	P07858
Cathepsin D	GDSGLGRA	37	P07339
Cathepsin E	EVALVALK	38	P14091
Cathepsin K	LGLEGANL	39	P43235
Cathepsin L	AALGSAPG	40	P07711
Cathepsin S	SLLIFRSWANFN	41	P25774
Granzyme B	VEPNSLEE	42	P10144
NE	GEPLSLLP	43	P08246
MMP 1	IPVSLRSG	44	P03956
MMP 2	IPVSLRSG	44	P08253
MMP 3	RPFSMIMG	45	P08254

MMP 7	VPLSLTMG	46	P09237
MMP 8	GPSGLRGA	47	P22894
MMP 9	VPLSLYSG	48	P14780
MMP 11	GAANLVRG	49	P24347
MMP 12	GVPLSLTMG	50	P34960
MMP 13	PQGLAGQRGIV	51	P33435
MMP 14	GPAGLRLA	52	P50281
ALK	RDIYAAPFFRK	53	Q9UM73
AURKB	AMERRRTSAARRSY	54	Q96GD4
CDK2	KARAAVSPQKRKA	55	P24941
PKD2	RARKRRLSAPPLASGD	56	Q504Y2
MAPK1	AKAGPPLSPRPPHVH	57	P28482
JAK2	DLFIPDNYLKMKPAP	58	O60674
PLK1	AELDPEDSMDMDMAP	59	P53350
PLK3	EDEAEELSDEDEELK	60	Q9H4B4
ERK1/MAPK3	AAGPAPLSPVPPVVH	61	P27361
JunK2/MAPK9	DASRPPLSPLSPRA	62	P45984
ARG I/II	GRRRRRRRG	63	P05089 (ARG1) P78540 (ARG2)
uPA	SGRSA	64	

^aMonocyte Chemoattractant Protein-1; ^bChemokine (C-C motif) ligand 20 (CCL20) or liver activation regulated chemokine (LARC) or Macrophage Inflammatory Protein-3 (MIP3A); ^cGranulocyte colony-stimulating factor; ^dInterleukin-1-receptor-like type 1; ^eMacrophage Inflammatory Protein; ^fOsteoclast Stimulating Factor-1; ^gMetalloproteinase Inhibitor 1; ^hThymic stromal lymphopoietin.

The recognition sequences can include one or more spacer residues on the N- and/or C-terminal ends. For example, between 1 and 10 amino acids (any amino acids, naturally and non-naturally, L- and D-, or combinations thereof) can be used at one or both ends as spacers. Examples include N-terminal sequences such as GAG- and C-terminal sequences such as -AG.

The reporter molecule further includes a detectable label that generates a detectable signal which can be detected and correlated with presence (or absence) of the target analyte. Exemplary

detectable labels include detectable particles that generate a detectable signal (e.g., optical or spectroscopic), such as fluorescence or color change, which can be perceived visually or measured with an appropriate instrument. Chromophore/luminophores suitable for use in the inventive assays include any organic or inorganic dyes, fluorophores, phosphophores, light absorbing nanoparticles (e.g., Au, Ag, Pt, Pd), combinations thereof, or the non-magnetic metalated complexes thereof. Preferably, the chromophore/luminophores have a particle size (maximum surface-to-surface dimension, i.e., diameter) of less than about 100 nm.

Suitable organic dyes are selected from the group consisting of coumarins, pyrene, cyanines, benzenes, N-methylcarbazole, erythrosin B, N-acetyl-L-tryptophanamide, 2,5-diphenyloxazole, rubrene, and N-(3-sulfopropyl)acridinium. Specific examples of preferred coumarins include 7-aminocoumarin, 7-dialkylamino coumarin, and coumarin 153. Examples of preferred benzenes include 1,4-bis(5-phenyloxazol-2-yl)benzene and 1,4-diphenylbenzene. Examples of preferred cyanines include oxacyanines, thiacyanines, indocyanins, merocyanines, and carbocyanines. Other exemplary cyanines include ECL Plus, ECF, C3-Oxacyanine, C3-Thiacyanine Dye (EtOH), C3-Thiacyanine Dye (PrOH), C5-Indocyanine, C5-Oxacyanine, C5-Thiacyanine, C7-Indocyanine, C7-Oxacyanine, CypHer5, Dye-33, cyanines (Cy7, Cy7.5, Cy5.0, Cy5.5, Cy3Cy5 ET, Cy3B, Cy3.0, Cy3.5, Cy2), CBQCA, NIR1, NIR2, NIR3, NIR4, NIR820, SNIR1, SNIR2, SNIR4, Merocyanine 540, Pinacyanol-Iodide, 1,1-Diethyl-4,4-carbocyanine iodide, Stains All, Dye-1041, or Dye-304.

Cyanine dyes are particularly preferred organic dyes for use in the nanobiosensors. All cyanine dyes have an excitation maximum, which is blue shifted by 20-25 nm with respect to their emission maximum (typical for fluorescent singlet states). Exemplary emission spectra of: NS-Cy3.0 ($\lambda_{\text{ex}} = 538$, $\lambda_{\text{em}} = 560$), NS-Cy5.5 ($\lambda_{\text{ex}} = 639$, $\lambda_{\text{em}} = 660$), NS-Cy7.0 ($\lambda_{\text{ex}} = 740$, $\lambda_{\text{em}} = 760$) and NS-Cy7.5 ($\lambda_{\text{ex}} = 808$, $\lambda_{\text{em}} = 830$).

Suitable inorganic dyes are selected from the group consisting of metalated and non-metalated porphyrins, phthalocyanines, chlorins (e.g., chlorophyll A and B), and metalated chromophores. Preferred porphyrins are selected from the group consisting of tetra carboxy-phenyl-porphyrin (TCPP) and Zn-TCPP. Preferred metalated chromophores are selected from the group consisting of ruthenium polypyridyl complexes, osmium polypyridyl complexes, rhodium polypyridyl complexes, 3-(1-methylbenzimidazol-2-yl)-7-(diethylamino)-coumarin complexes of iridium(III), and 3-(benzothiazol-2-yl)-7-(diethylamino)-coumarin complexes with iridium(III).

Suitable fluorophores and phosphophores are selected from the group consisting of phosphorescent dyes, fluoresceines, rhodamines (e.g., rhodamine B, rhodamine 6G), and anthracenes (e.g., 9-cyanoanthracene, 9,10-diphenylanthracene, 1-Chloro-9,10-bis(phenyl-

ethynyl)anthracene).

The pI tag(s) are used to modify the normal isoelectric point (pI) of the reporter molecule to permit complete separation from one another so that a plurality of analytes can be targeted in the assay without overlapping. Thus, depending upon the technique used for detection of the nanobiosensor, one or more pI tags may be used. For example, when an electrophoresis-based approach is used, it may be desirable to increase, decrease, or adjust the spacing of the designated (or expected) detection location for each nanobiosensor along the microfluidics channel in the detection chamber (as discussed in more detail below). The nanobiosensors themselves can be designed and calibrated with the detection device, such that each type of nanobiosensor for a particular target analyte has a designated (or expected) position in the detection chamber for each of its reacted and unreacted sensing molecules. For example, the reporter molecules will each have a standard position in the detection chamber where each respective (expected) isoelectric point is reached for the given pH used in the channel. In some cases, reporter molecules comprising different recognition sequences (thus, targeting different analytes) may nonetheless have similar isoelectric points (in reacted and/or unreacted states), resulting in overlapping standard positions along the chamber. Therefore, one or more pI tags can be added to the reporter molecule to adjust the isoelectric point of a given reporter molecule and accordingly adjust the designated location of detection for that particular reporter molecule in the detection chamber, so that the presence of one reporter molecule (and thus one analyte) can be differentiated from the presence another type of reporter molecule (and thus another analyte). Thus, it will be appreciated that the invention allows for separation of the sample into a linearly spaced array of reporter molecules with detectable labels, that will be present (or absent) at designated locations along the channel, and can be “read” similar to a linear barcode. In one or more embodiments, at least 20 different reporter molecules (or bands) can be detected at a time (i.e., in a single assay), and preferably from about 20 to about 100 different reporter molecules (or bands) can be detected at a time (i.e., in a single assay) with a single detector/sensor (e.g., positioned behind the laser or other light source).

Exemplary reporter molecules are listed in Table 2 below, along with their target analyte.

Table 2.

Enzyme	Total Sequence	pI	SEQ ID NO:
ALK	FI-[A]RDIY*AAPFFRKC	9.31/8.35	65
AURKB	FI-[DD]AMERRRT*SAARRSYC	9.49/8.56	66
CDK2	FI-I[C]KARAAVS*PQKRKAD[D]C	9.68/7.51	67

PKD2	FI-[DDC]RARKRRLS*APPLASGDC	8.95/7.72	68
MAPK1	FI-AKAGPPLS*PRPPHVHC	6.91/5.63	69
JAK 2	FI-DLFIPDNY*LKMKPAPC	5.95/4.46	70
PIK1	FI-AELDPEDS*MDMDMAPC	3.25/3.08	71
PIK3	FI-EDEAEELS*DEDEELKC	3.65/3.38	72
ERK1	FI-AAGPAPLS*PVPPVVHC	6.73/5.50	73
JunK2	FI'-[D]ASRPPPLS*PLSPRAC	7.24/6.11	74
MMP 1	FI- <u>GAGC</u> [DSD]GPEG-LRGA[R]G	4.66/3.52	75
	FI-H ₆ CGPEG-LRGAE ₄	6.2	76
	FI-H ₆ CGPEG	7.14	77
MMP 2	FI'-[H] <u>GAGC</u> GPSA-LVSDG	3.94/4.31	78
MMP 3	FI-[H] <u>GAGC</u> GPA-G-LRGLG	7.42/5.01	79
	FI-H ₆ RCGPAG-LRGLE ₄	6.79	80
	FI-H ₆ RCGPAG	8.31	81
MMP 7	FI- <u>GAGC</u> RVPLS-LTMGDDG	4.18/8.15	82
	FI-H ₆ RHRCVPLS-LTMGE ₂	7.66	83
	FI-H ₆ RHRCVPLS	10.0	84
MMP 9	FI'- <u>GAGC</u> HHHVPLS-LYSGDG	5.14/6.21	85
	FI-H ₆ E ₃ CVPLS-LYSGK ₃	7.61	86
	FI-H ₆ E ₃ CVPLS	6.48	87
MMP11	FI-H ₆ CKCGA ₂ N-LVRG	9.64	88
	FI-H ₆ CKCGA ₂ N	8.12	89
MMP13	FI-H ₆ E ₅ CGPQGLA-GQRGIVE ₂	6.13	90
	FI-H ₆ E ₅ CGPQGLA	5.88	91
MMP14	FI-H ₆ CDEC GPAG-LRLA	7.09	92
	FI-H ₆ CDEC GPAG	6.77	93
CTS B	FI- <u>GAGC</u> RHGLAG-LAGAHHC	7.96/8.26	94
	FI-H ₆ CEDCSL ₂ RSR-MVPNFNC ₂	7.47	95
	FI-H ₆ RCSL ₂ RSR	7.56	96
CTS D	FI- <u>GAGC</u> LEVL-FVLGG	6.38/5.73	97
	FI-H ₆ KEKCSL ₂ IFR-SWANFNK	9.72	98
	FI-H ₆ KEKCSL ₂ IFR	9.26	99
CTS E	FI'- <u>GAGC</u> HKHKEVVL-VALARHG	7.61/6.56	100
CTS K	FI- <u>GAGC</u> HKRKRGLG-GADDRHG	9.17/9.96	101
	FI-H ₆ D ₇ CGPRAGR-R	6.23	102
	FI-H ₆ D ₇ CGPR	5.90	103

CTS L	Fl- <u>GAGCRRRRELLG</u> -SPPD[D] <u>G</u>	5.38/8.82	104
	Fl-H ₆ E ₇ CSGV ₂ IA-TVIVITK	5.50	105
	Fl-H ₆ E ₇ CSGV ₂ IA	5.08	106

Fl: Fluorophore; Fl': mono-sulfonated fluorophore; *: phosphorylated residue; Underlined residues: spacer; Residue(s) in brackets: amino acids added to change pI; bold C: cysteine to link to magnetic nanoparticles; ALK: Anaplastic lymphoma kinase; AURKB: Aurora kinase B; CDK2: Cyclin-dependent kinase 2; PKD2: Pyruvate dehydrogenase (acetyl transferring) kinase; MAPK1: Mitogen-activated protein kinase 1; JAK 2: Janus kinase 2; PIK1: Polo-like kinase 1; P1K3: Polo-like kinase 3; ERK 1: extracellular signal-regulated kinase 1; JunK2: c-Jun N-terminal kinase 2

The reporter molecule can be attached to the nanoparticle via a terminal end of the reporter molecule sequence, or alternatively at a position intermediate to either end of the sequence along the backbone. The detectable label is preferably attached on a terminal end (e.g., N-terminus) of the reporter molecule. For example, the N-terminus of the pI tag nearest the release unit can be labeled with a fluorophore.

Microfluidics Device

Embodiments of the invention are directed to a device for performing the analysis of biospecimens/biological samples using the nanobiosensors. The nanobiosensors are particularly suited for use with microfluidic devices. The microfluidic devices generally comprise a planar substrate comprising a sample inlet well positioned above a sample application region in or on the substrate and at least one detection region in fluid communication with the sample application region via a microfluidic channel extending from the sample application region to the detection region. A washing region may be positioned along the channel intermediate to the sample application region and the detection region. One or more side channels may also be formed in the device to facilitate introduction or removal of components, reagents, buffers, etc. from the main channel. For example, one or more inlet side channels can be formed to introduce a wash buffer into the wash region. Likewise, one more outlet side channels can be formed to remove waste material from the wash region. Similarly, one or more inlet channels may be formed adjacent the detection region to introduce reagents into the detection region and/or to supply electrical connection to the detection region to assist in the separation of the reporter substrates (through, for example, channels containing anolyte and catholyte for isoelectric focusing). The microfluidics channel regions may be separated by one or more valves. One or more valves (e.g., air or mechanical valves) may also be incorporated into the channel to provide forward and/or back

pressure. However, as will be appreciated from the additional information below, microfluidics devices in accordance with the invention do not require a pump to move the sample through the channel.

An exemplary device is shown in FIG. 1A-FIG. 1D (side view) and FIG. 2 (schematic top view), and its features and operation are described below. Device 10 generally comprises sample chamber 20, washing chamber 30, and detection chamber 40 positioned in fluid communication along a microfluidic channel 60. Device 10 further comprises a magnet 80 positioned on a translatable stage below channel 60 and configured to mix nanobiosensors 90 and a biological sample within sample chamber 20 and/or to translate nanobiosensors 90 into the various chambers along microfluidic channel 60. In certain embodiments, device 10 further comprises one or more valves 70 interposed within microfluidic channel 60. The operation of device 10 and its individual components may be manual or automated. The separate chambers can be separated from each other using one or more valves 70a-d. Any of a variety of types of valves can be used, so long as they are capable of being opened/closed to allow/prevent nanobiosensors 90 to be moved from one chamber to another. In certain embodiments, the valves are soft lithographic valves. Other exemplary valves include mechanical push-down valves or pinch valves.

Microfluidic channel 60 is generally provided as a central/main elongated void region cooperatively defined by opposing side walls, bottom wall, and optional opposing top wall. Microfluidic channel 60 has a channel length extending generally from the sample chamber 20 to the terminal end of the detecting chamber 40, and a channel width extending between the opposing sidewalls. The microfluidic channel 60 is generally linear, as shown in FIG. 2. However, in certain embodiments, microfluidic channel 60 may be non-linear, branched, or have a variety of other geometries. Microfluidic channel 60 may be a variety of sizes. In certain embodiments, channel 60 has a total channel length (i.e., the distance from sample chamber 20 to the terminal end of detection chamber 40) of less than about 10 cm, preferably less than about 5 cm, and more preferably, less than about 3 cm. In certain embodiments, channel 60 has a total length of from about 1 cm to about 10 cm, preferably from about 2 cm to about 5 cm. In certain embodiments, channel 60 has an average width along its length of less than about 1000 μm , preferably less than about 600 μm , and more preferably less than about 400 μm . In certain embodiments, channel 60 has an average width along its length of about 10 μm to about 1000 μm , preferably about 50 μm to about 800 μm , and more preferably 100 μm to about 500 μm . In certain embodiments, channel 60 has an average height (depth, as measured from the bottom wall to the top of the sidewall) along its length of less than about 200 μm , preferably less than about 100 μm , and more preferably less than

about 50 μm . In certain embodiments, channel 60 has an average height (depth) along its length of about 5 μm to about 75 μm , preferably about 10 μm to about 40 μm , and more preferably about 15 μm to about 30 μm .

Device 10 may be manufactured using a variety of techniques and materials. In certain
5 embodiments, device 10 is fabricated from polydimethylsiloxane (PDMS), or other suitable material, on a glass slide or silicon substrate. The materials may be treated to render it hydrophilic. Microfluidic channel 60 may be formed in device 10 using a variety of techniques. In a particularly preferred embodiment, microfluidic channel 60 is formed in device 10 using soft lithography or 3D printing to create the shaped channel. For example, a mold for the PDMS channels can be patterned
10 using a mask and an appropriate light source. The mold may be created using a negative tone photoresist (e.g. SU-8). The negative tone photoresist in the exposed regions is cross-linked and the photoresist in the unexposed regions can be rinsed away using an organic liquid. This results in a mold against which the PDMS prepolymer can be cast. Once the PDMS has crosslinked, it can be peeled from the mask and annealed against a glass or silicon surface. Alternatively, the channels
15 can be etched in glass using well known state of the art techniques and then be sealed with a flat piece of PDMS or a piece of PDMS that contains channels that can connect various channels in the glass. Methods of forming microfluidics channels are known in the art, and include, without limitation, wax printing, wet or dry etching, thermoforming techniques such as hot/roll embossing and injection molding, polymer casting, and direct writing with micro machines or lasers.

20 Sample chamber 20 comprises an opening 22 and a reservoir or well 24 configured for receiving a biological sample. Sample chamber 20 is in fluid communication with washing chamber 30 via one or more optional valves 70. Washing chamber 30 may include an optional wash inlet 32, as shown in FIG. 2 for introducing a wash fluid, as well as an outlet for waste 34. Washing chamber 30 is in fluid communication via one or more optional valves 70 with detection
25 chamber 40.

In one or more embodiments, detection chamber 40 is particularly configured for microfluidic Isoelectric Focusing (IEF). In one aspect, IEF chamber 40 generally comprises ampholyte reservoir 42, catholyte reservoir 44, anolyte reservoir 46, and pressure fill inlet 48. Ampholyte reservoir 42 is configured to introduce a high-resolution ampholyte mixture into IEF
30 chamber 40. An exemplary commercially-available ampholyte is 39878 SIGMA by Sigma-Aldrich. As shown in FIG. 2, Valve 70c is positioned so as to allow the ampholyte mixture to flow into IEF chamber 40 when opened and prevent the flow of the ampholyte mixture when closed. Catholyte reservoir 44 comprises a catholyte solution, such as a sodium hydroxide solution. Anolyte reservoir

46 comprises an anolyte solution, such as a phosphoric acid solution. An electric field can be applied to IEF chamber 40 by supplying a current to electrodes 50. Additional components necessary for the IEF process (e.g., pI standards, electroosmotic flow (EOF) suppressor, reducing agent, and/or sacrificial ampholytes) can be supplied to IEF chamber 40 via pressure fill inlet 48, which may further comprise valve 70d.

In use, from about 0.1 mL to about 5 mL of biological sample (preferably from about 0.5 mL to about 5 mL and even more preferably from about 1 mL to about 5 mL) can be loaded into the sample chamber 20 reservoir or well 24, along with a plurality of nanobiosensors 90. Exemplary biological samples include, for example, blood, serum, saliva, sputum, or lung fluid (i.e., breath condensate), urine, sweat, and other bodily secretions. In one or more embodiments, the biological sample is not pre-treated, purified, concentrated or otherwise modified prior to mixing with nanobiosensors 90 or adding to sample chamber 20. Nanobiosensors 90 and the biological sample may be added to sample chamber 20 individually or pre-mixed prior to being added to sample chamber 20. The mixture comprising the biological sample and nanoparticles 90 is then incubated. Incubation times can vary depending upon the nanobiosensor and the target analyte, for example from about 1 second to about 1 hour, and preferably from about 1 minute to about 5 minutes. Incubation preferably occurs under ambient conditions (e.g., room temperature, $\sim 27^{\circ}\text{C}$), but can occur at elevated temperatures of from about 28°C to about 50°C .

In one or more embodiments, magnet 80 is initially positioned below sample chamber 20, as shown in FIG. 1A. From this position, magnet 80 can be rotated manually or automatically by varying an external magnetic field to mix nanobiosensors 90 and the biological sample within sample chamber 20. Magnet 80 may be any kind of magnet, so long as it is capable of moving nanobiosensors 90 within sample chamber 20 and along channel 60, as described herein. In certain embodiments, magnet 80 is a rare-earth magnet comprising an alloy of elements selected from the group consisting of lanthanides, scandium, yttrium, and mixtures thereof.

After incubation, nanobiosensors 90 are transferred to washing chamber 30 by laterally translating (e.g., sliding) magnet 80 from below sample chamber 20 to a position below washing chamber 30, as shown in FIG. 1B. The magnetic attraction between magnet 80 and nanobiosensors 90 causes the nanobiosensors 90 to move along the microfluidic channel 60 from sample chamber 20 to washing chamber 30, as the magnet 80 correspondingly moves along a parallel path below the channel 60. In other words, the magnet 80 pulls the nanobiosensors 90 along the channel and/or the nanobiosensors 90 follow the attractive force of the magnet 80. Valve 70a can be opened, while valve 70b remains closed, to allow for easy transfer into washing chamber 30. Once

nanobiosensors 90 have been transferred into washing chamber 30, valve 70a can be closed. Nanobiosensors 90 are washed within washing chamber 30 to the biological matrix, which reduces the matrix effects and thus noise during subsequent optical detection steps. In certain embodiments, as shown in FIG. 2, the washing step comprises introducing a wash fluid via wash inlet 32. The wash fluid may be pumped into the main channel 60 through wash inlet 32 so as to mix with nanobiosensors 90 within wash chamber 30, with the waste exiting via waste outlet 34. In one or more embodiments, the wash inlet 32 is positioned at the distal end of the wash chamber 30 (farther from the sample chamber 20), and when the wash fluid is introduced, it travels in a direction from the distal end of the wash chamber 30 towards the sample chamber 20 (i.e., “backwards” or “upstream”), and exits via the waste outlet 34, which is positioned at the proximate end of the wash chamber 30 (closer to the sample chamber 20). In one or more embodiments, the wash fluid comprises de-ionized water or a mixture of de-ionized water and a non-ionic surfactant. The washing step may comprise a single wash or a series of two or more washes. After washing, valve 70b can be opened, and nanobiosensors 90 are transferred into detection chamber 40 using magnet 80, as shown in FIG. 1C. It will be appreciated that the nanobiosensors 90 are moved in a direction of flow that is opposite of the wash fluid direction, from the wash chamber 30 to the detection chamber 40.

While magnetic nanoparticles 92 used in the nanobiosensors 90 are useful for mixing and transporting the peptide substrates, they can hinder detection techniques, such as IEF separation, due to their charge and size dispersion, and thus they should be separated from the reporter molecule 94. The release unit 96 allows the reporter molecules 94, both reacted/unreacted, to be delinked from nanoparticles 92 in the nanobiosensors 90 before the detection step is performed. In one or more embodiments, where disulfide release units are used, the disulfide bridges connecting the reporter molecules 94 (both cleaved/modified and uncleaved/unmodified) to the nanoparticles 92 are reduced, thereby releasing the reporter molecules 94 from the nanoparticles 92. Other release mechanisms are described in detail above regarding release unit alternatives. Regardless, once “de-linked,” the nanoparticles 92 are then removed from detection chamber 40 by translating the nanoparticles 92 out of the chamber 40, using magnet 80, as shown in FIG. 1D. Using magnet 80, nanoparticles 92 are returned to any of the upstream chambers (e.g., washing chamber 30 or sample chamber 20) or outlets to be cleaned and prepared for re-use in further nanobiosensors 90.

Once nanoparticles 92 are removed from IEF chamber 40, the released peptide substrates 94 are focused into discrete optical bands via IEF. IEF is an analytical technique that is unique compared to most separation methods in that analytes are simultaneously separated and

concentrated as they are focused at their pI (isoelectric) points along a pH gradient created through the addition of carrier ampholytes to the separation solution.

To perform IEF, the sample (i.e., the reporter molecules), carrier ampholytes (the pH gradient forming compounds), an electroosmotic flow (EOF) suppressor, and a reducing agent are loaded into the separation column (in this case, the detection chamber 40). Exemplary EOF suppressors include methylcellulose (MC), polyvinylpyrrolidone (PVP, MW 360,000), or hydroxymethylcellulose (HPMC). As shown in FIG. 2, an anolyte (e.g., phosphoric acid) is added to anolyte reservoir 46 and a catholyte (e.g., sodium hydroxide) catholyte reservoir 44. An electric field is applied and the current decreases as the reporter molecules (which may or may not be bound to a target analyte, or have been modified by a target analyte), and ampholytes reach their isoelectric points or points of zero (neutral) charge. Advantageously, IEF in chamber 40 of microfluidic channel 60 can focus the reporter molecules in less than about 30 minutes, preferably less than about 10 minutes, and more preferably less than about 5 minutes. This IEF process is robust and reproducible, and may produce peak capacities of at least about 20 different bands, preferably at least about 50, and more preferably at least about 80. In certain embodiments, the IEF process in accordance with embodiments of the present invention can produce peak capacities of up to about 100 different bands. The IEF process is able to increase concentration of the peaks (focused zones) by at least about 1,000 times, preferably at least about 1,000,000 times. Once focused, the reporter molecule bands are stable for several minutes due to the EOF suppressor being added to the ampholyte. The focused bands may visually resemble a “barcode” within IEF chamber 40, and the bands may be “read” similar to a barcode, as discussed in more detail below.

Once the various peptide substrate bands have been separated and focused within IEF chamber 40, an optical detector can be used to analyze the presence (or absence) of the various reporter molecules within IEF chamber 40. A number of techniques can be used to analyze the resulting peptide bands within IEF chamber 40, including optical detection techniques. Exemplary optical detection techniques are described below. However, it should be understood that other methods of analyzing the peptide bands are also within the scope of the present invention. In general, the methods involve exposing the reporter molecules in the detection chamber (and thus any attached detectable labels) to an appropriate energy source. The wavelength used will depend upon the detectable labels used in the nanobiosensors. Both the position/presence and concentration/intensity of the signal from the detectable label can then be sensed or detected with the appropriate sensing or detecting instrument (examples described below) for both quantitative and qualitative assessment of the reporter molecules. The detector generates an analog signal that is

sent to a decoder that interprets the signal, and converts it into an output that can be read to indicate the results of the assay.

In one embodiment, the imaging and detection is performed using an inverted microscope stage (e.g., Nikon TE-2000U) with a halogen excitation source, a fluorescein filter cube, and a camera (e.g., Princeton Instruments CCD). This embodiment provides flexibility to modify fluorescent tags and imaging areas easily while optimizing the separations. While the entire chamber cannot be imaged with the microscope at one time, the chamber can be scanned by sliding the stage along the length of the chamber (or vice versa). A 2048-element linear CCD line camera (2000:1 S/N ratio, 900Hz scan rate with a max sensitivity in the 500-600nm range) can be used to image the IEF results in IEF chamber 40. This embodiment should resolve pH differences as small as $\Delta\text{pH}/\text{pixels} = 7/2048 = 0.0034$ pH units, which is narrower than can be focused using IEF, and thus it should not be the peak resolution limiting factor. The excitation source for the focused fluorescently labeled reporter molecules is a solid-state diode pumped 488nm laser. The laser line is spread out into an excitation line using a cylindrical lens.

In another embodiment, an organic light emitting diode (OLED) array can be used to spatially scan the IEF chamber by turning on each row of diodes in the chamber sequentially. The emitted light from the focused fluorescently labeled substrate bands is detected using a photomultiplier tube (PMT). The excitation is performed, for example, using a SXGA120-R5 1280 X 1024 low power monochrome green xlt AMOLED microdisplay from Emagin with a thin fiber optic faceplate, or other similar instrument. The OLED array is positioned below IEF chamber 40. Between chamber 40 and the OLED, a thin excitation filter is positioned. The OLED array can then be manually or automatically driven in a scanning mode along the length of IEF chamber 40, preferably over a period of less than 5 seconds. Rescanning can be performed to improve the signal-to-noise (S/N) ratio, if necessary. The emission from the fluorescently labeled peptide substrates is then measured using a photomultiplier tube (e.g., Hamamatsu R-928 PMT) positioned behind an emission filter. The OLED array has pixel density such that the resolution should be $7/1024 = 0.0068$ pH units, which is which is narrower than can be focused using IEF, and thus it should not be the peak resolution limiting factor. Because the emission from the OLED is $520\pm55\text{nm}$, a more red-shifted dye than fluorescein should be used in this embodiment.

In another embodiment, detection is performed using a digital versatile disc (DVD) optical pickup head and stepper motor that moves the head. A schematic representation of this embodiment is shown in FIG. 3, which includes the laser diode package (laser diode and photodiode) and optics components. In this embodiment, the red (650nm) diode laser is controlled

using a constant current source. The stepper motor can be controlled through an H-bridge, for example, using LabVIEW. The photodiode used for autofocus and reading the pits in the conventional DVD is removed and replaced by a 100- μm multimodal fiber. The emitted light from the channel is focused onto the surface of the fiber using the optics integrated in the DVD optical pickup head. The fiber then transmits the light to a photocathode (e.g., Hamamatsu microPMT). A cylindrical lens is used to focus the light exiting the fiber onto the rectangular photocathode in the photocathode, and a bandpass (and/or holographic notch) filter is used to filter out the excitation light. The optical fiber serves as the spatial filter. The spatial resolution of the detector is governed either by the laser spot size or the smallest increment that the stepper motor can be moved, both of which are smaller than the most narrowly focused IEF bands in chamber 40.

The device and methods in accordance with embodiments of the present invention have a number of advantages over the prior art. For example, the use of the magnetically translatable nanoparticles in the nanobiosensors allows for a pre-concentration of the peptides prior to the IEF step. Using this pre-concentration, the signal is further enhanced as the concentration of the nanobiosensors are increased 10^6 -fold, for example from a $\sim 1\text{mL}$ sample volume into a $\sim \text{nL}$ focused band. One potential issue with IEF is the limited sample volume that can be loaded. For a typical microfluidic IEF device, the volume of sample that can be loaded is defined by the IEF channel/column volume which is usually on the order of $\sim 180\text{nL}$ (for a $3\text{ cm} \times 300\text{ }\mu\text{m} \times 20\text{ }\mu\text{m}$ channel). The final focused bands, based on a $\sim 0.05\text{ pH}$ resolution, are $\sim 200\mu\text{m}$, giving a preconcentration ratio of about 75 if $\frac{1}{2}$ the initial column volume is loaded with sample. If the nanobiosensors were placed in a 100 nL sample volume with the enzymes present at a concentration of $\sim 10^{-15}\text{ M}$, there would only be ~ 60 enzyme molecules in the analyzed sample. This would be very difficult to detect. For traditional plate readers where the sample volume is $\sim 10\text{-}100\text{ }\mu\text{L}$, the number of enzymes is $\sim 6000\text{-}60,000$.

By adding the magnetic nanoparticle to the reporter molecules, the nanobiosensor can now be added to a larger initial volume of sample and later removed through the use of a magnet into a much smaller volume to be analyzed. For example, the nanobiosensors could be added to a sample volume of 1mL and then moved to the IEF region of an integrated microfluidic device. Reducing agents, such as DTT, are often used in IEF and can be added to the ampholyte mixture without affecting the formation of the pH gradient. Under such conditions the disulfide bond is reduced, releasing the uncleaved and cleaved peptide substrates from each particle. Because the final peptide substrate bands will be focused to a volume of $\sim 1\text{ nL}$ that results in a concentration enhancement of $\sim 10^6$. Because the focused bands are stable, multiple images of the final result can be signal

averaged to further improve the LOD.

Additionally, the peak capacity for the IEF chamber can be up to about 80, or even about 100, so a multiplexed sample of at least 20 cleaved/uncleaved or modified/unmodified reporter molecule pairs can be separated. One particular advantage of this technique is that amino acid sequences (pI tags) can be added to the ends of the substrates to alter their pIs so that none of the reporter molecules will overlap after they are focused.

Target analytes that could be detected in embodiments of the invention include protease biomarkers (e.g. matrix metalloproteinases (MMPs), serine proteases, and cysteine proteases), kinase biomarkers (Anaplastic lymphoma kinase (ALK) or CD246 (cluster of differentiation 246), Aurora family of kinases, Cell Cycle Kinases, Cyclin-dependent kinase 2 (CDK2), Protein Kinase D 2 (PKD2), Janus Kinase 2 (JAK2), Polo-Like Kinases, Mitogen-activated protein (MAP) kinases), other enzymes capable of posttranslational modifications, cytokines/chemokines, and the like. The protease biomarkers that are used to validate the exemplary microfluidics device have been shown to be elevated in level 1 stage breast, lung and pancreatic cancers, compared to the levels in apparently healthy human subjects. Thus, embodiments of the present invention are particularly suitable for detecting these markers in spiked commercially available heat-deactivated serum samples and model cell systems at clinically relevant levels for stage 1 and stage 0 detection of cancers.

Exemplary reaction mechanisms of the different types of nanobiosensors 90 are depicted in FIG. 4A and FIG. 4B. As described above, the nanobiosensors 90 comprise a magnetic nanoparticle 92 linked to a reporter molecule 94 by a release unit 96. The reporter molecule comprises a detectable label 98, a recognition sequence 100, and one or more optional pI tag(s) 102. The nanobiosensors 90 will have peptide sequences specific for a particular target analyte (e.g. cathepsin, kinase, matrix metalloprotease (MMP) or other enzyme that catalyzes peptide cleavage or a posttranslational modification). For example, as illustrated in FIG. 4A, if a protease is present and active, it will cleave the sequence at a specific position resulting in two reporter molecule products 94a and 94b with different isoelectric points (pIs). Similarly, as shown in FIG. 4B, if a kinase or other posttranslational enzyme (e.g. arginase) is present, it will biochemically modify its respective oligopeptide leading to a modified reporter molecule product 94', which may or may not include a bound analyte, but nonetheless will have a different pI from the unreacted/unmodified reporter molecule 94. The resulting cleaved or modified reporter molecule can then be detected and correlated with a disease condition based upon its position (i.e., presence of absence) at its designated detection region in the channel.

Additional advantages of the various embodiments of the invention will be apparent to those skilled in the art upon review of the disclosure herein and the working examples below. It will be appreciated that the various embodiments described herein are not necessarily mutually exclusive unless otherwise indicated herein. For example, a feature described or depicted in one embodiment
5 may also be included in other embodiments, but is not necessarily included. Thus, the present invention encompasses a variety of combinations and/or integrations of the specific embodiments described herein.

As used herein, the phrase "and/or," when used in a list of two or more items, means that any one of the listed items can be employed by itself or any combination of two or more of the
10 listed items can be employed. For example, if a composition is described as containing or excluding components A, B, and/or C, the composition can contain or exclude A alone; B alone; C alone; A and B in combination; A and C in combination; B and C in combination; or A, B, and C in combination.

The present description also uses numerical ranges to quantify certain parameters relating to
15 various embodiments of the invention. It should be understood that when numerical ranges are provided, such ranges are to be construed as providing literal support for claim limitations that only recite the lower value of the range as well as claim limitations that only recite the upper value of the range. For example, a disclosed numerical range of about 10 to about 100 provides literal support for a claim reciting "greater than about 10" (with no upper bounds) and a claim reciting "less than
20 about 100" (with no lower bounds).

EXAMPLES

The following examples set forth methods in accordance with the invention. It is to be understood, however, that these examples are provided by way of illustration and nothing therein
25 should be taken as a limitation upon the overall scope of the invention.

EXAMPLE 1

Prototype nanobiosensors comprise 5 principal components: a superparamagnetic Fe/Fe₃O₄ nanoparticle, a disulfide bridge, reporter molecule containing a protease specific peptide substrate, a
30 fluorophore, and 2 pI tags (FIG. 4A and FIG. 4B). Each pI tag (short peptide sequences), will be positioned on each end of the protease specific peptide substrate. These pI tags will be used to adjust the pI of the uncleaved and cleaved peptide substrates so that they can be completely separated from one another and the other peptide substrates used for the multiplexed analyses.

Between one of the pI tags and the enzyme substrate will be a cysteine that can be linked to another cysteine on a superparamagnetic Fe/Fe₃O₄ nanoparticle through a disulfide bridge (although the cysteine could also be at the end of the sensor followed by the pI tags, the reporter peptide, and 2nd pI tag). The N-terminus of the pI tag nearest the disulfide linkage will also be labeled with a fluorophore.

The pI tags for all of the nanobiosensors and their cleaved counterparts are designed so that they should all be resolved from one another by at least 0.1-0.2 pI units. All consensus sequences are designed based on known cleavage motifs.

Solid-supported peptide synthesis is utilized to prepare the reporter molecules. The fluorescent dye (here: 5-carboxy-fluorescein) is attached while the oligopeptide is still attached to the resin. Wang resins will be utilized, which permits the formation of a carboxylate group at the C-terminal end. The N-terminal end features an amine group after the oligopeptide is cleaved from the Wang resin. Depending on the lengths of the synthesized oligopeptides, the density of oligopeptides on the resin, as well as additional factors (e.g. steric hindrance, formation of beta-sheets etc.), the expected yields range from 95 to 65%. Therefore, the final step of dye-attached oligopeptide synthesis involves purifying the product after cleavage from the resin. Classically, this is achieved with HPLC.

The Fe/Fe₃O₄ core/shell nanoparticles are synthesized by thermal decomposition of Fe(CO)₅, followed by kinetic growth of the formed iron(0, bcc) nanocrystals. Once they reach a diameter of 35nm, the Fe(0) nanocrystals are passivated by treating with oxygen, which leads to a strongly adherent Fe₃O₄ layer. The latter is subsequently coated with 3,4-Dihydroxyphenylacetic acid to create nanoparticles that can be easily dispersed in various media (aqueous buffers, breath condensate, etc.). After attaching 3-mercaptopropionic acid through an amide bond to the dopamine layer, disulfide bonds between a cysteine group of the fluorescein-attached oligopeptides and the thiol-group of 3-mercaptopropionic amide can be formed. This bond can be reductively cleaved upon addition of THPP (tris-hydroxypropyl-phosphine) to delink the nanoparticles from the reporter molecule.

EXAMPLE 2

The nanobiosensors are mixed with heat-deactivated serum samples that are “spiked” with commercially available (recombinant) proteases and incubated. This is followed by a wash step and IEF focusing of the multiple nanobiosensors. The initial separation/detection column is fabricated to be 3 cm long and filled with a pH 3-10 range high resolution ampholyte solution (39878 Sigma),

dithiothreitol (DTT) or THPP, 0.4% methyl cellulose (MC) to suppress EOF, 1.7mM iminodiacetic acid, 40mM arginine, and the peptide substrate sample. After filling the channel, excess mixture is removed from the reservoirs and ~200mM H₃PO₄ added to the anolyte reservoir and ~300mM NaOH to the catholyte reservoir. Both reservoirs will also contain 2.5% methyl cellulose.

5 Preconditioning is performed by rinsing the channel with 1:1 methanol and 1M sodium hydroxide. After conditioning, the channel is filled with 0.4% methylcellulose and allowed to sit for 10min to create an EOF reducing coating. The sample is then loaded. The anolyte/catholyte wells/channels are rinsed 2x with ultrapure water then filled with the anolyte and catholyte. 300V is applied for ~5min to focus the peptides or until the current drops from 25uA to 2-4uA. The applied electric

10 field is optimized to minimize the focusing time and maximize resolution. The effectiveness of the different suppressors at various concentrations to suppress EOF is evaluated. The drift should be <0.05 pH units over the course of the detector scan time (at present this is about 30s). A peak capacity of 80 is the goal. Additionally, the resolution is maximized through adjustments to the ampholyte and reducing agent (e.g., THPP) concentrations using in-house synthesized fluorescently

15 labeled pI standards that have been calibrated against standard uv calibration standards in a cIEF system. Our initial experiments with a scanning bench scale device indicate that it can be cleaned, pretreated, and reused dozens of times.

EXAMPLE 3

20 Materials. The following chemicals were obtained from commercial sources: Tris(hydroxypropyl)phosphine (THPP), Methyl Cellulose, and Ampholyte high resolution, pH 3.0-10.0, were purchased from Sigma Aldrich (St. Louis, Mo). Thermo Scientific™ Pierce™ MS Grade Trypsin Protease, Acetonitrile (HPLC Grade), Trifluoroacetic Acid, Glacial Acetic Acid, Calcium Chloride, Phosphoric Acid, Sodium Hydroxide, and Brij 35 (Enzymatic Grade), were purchased

25 from Fisher Scientific (Waltham, MA). 1.5M Tris-HCl, pH 8 was purchased from BioRad (Hercules, CA). DL Arginine was purchased from Acros Organics (Geel, Belgium).

Methods.

Trypsin Digestion. 50mM Tris-HCl (pH 7.8), 1mM CaCl₂, 0.005mg/mL Trypsin, and 0.1mg/mL Trypsin Substrate were mixed and then incubated at 37°C with samples taken for

30 analysis at 0hr, 2hr, and 24hr. The digestion was stopped by adding acetic acid to adjust the pH below 4.

HPLC Testing. The digestions were tested on a Thermo Fisher Ultimate 3000 with a Variable Wavelength Detector and an Acclaim 300 C18 column. The parameters were 0.3mL/min

flow rate, with a gradient of 5% acetonitrile to 80% over 15min and a 5min hold at 80%. The data was analyzed with Igor Pro (WaveMetrics Inc., Lake Oswego, OR). The results are shown in FIG. 5.

Fabrication of PDMS Microfluidic Isoelectric Focusing Device. Soft photolithography was used to create the desired channel pattern, by using a positive photoresist on a Si-wafer substrate. PDMS prepolymer and curing agent (Sylgard 184, Dow Corning Inc., Midland, MI) were uniformly mixed at a ratio of 10:1, respectively, and degassed for 30min under vacuum. The PDMS was poured into a mold on Si wafer that contained the channel pattern. The PDMS was cured in an 80°C oven for 90 minutes. At the end of the curing process, the PDMS was carefully peeled from the glass substrate to become the bottom layer of the microchip. The reservoirs were created with a 3mm biopsy punch and then the channel layer was sealed to a glass slide. The microchannel was 3 cm in long, 300 μm wide, and 16 μm deep. A photograph of the device is shown in FIG. 6.

Isoelectric Focusing Testing. The digestions were also tested on the created device. The channel was cleaned with 50% 1M NaOH+ 50% Methanol for 10min. Then the channel was pretreated with the 0.4% methyl cellulose for 30min. The sample consisted of: 40mM Arginine (*High pI Sacrificial Ampholyte*), 0.05% Acetic Acid (*Low pI Sacrificial Ampholyte*), 0.11% Brij 35 (*Surfactant*), ~1-100 μM Rhodamine Tagged Peptide (*Sample*), 1% Carrier Ampholytes, and 0.4% Methyl Cellulose (*EOF Suppressor*).

The sample was loaded into the channel and then the reservoirs were filled with the anolyte (200mM H_3PO_4 + 2% methyl cellulose) and catholyte (300mM NaOH+ 2% methyl cellulose). The microfluidic chip was then aligned on the detector and the voltage was applied (200V/cm) until the current dropped to 10% of its initial value. While this was occurring, the channel was automatically scanned at 937 μm per second over the detection point and the fluorescence was detected using a μPMT (Hamamatsu Photonics, Japan). LabView (National Instruments, Austin, TX) programming was used to automatically control all motion, voltage, data collection, and saving. Data was analyzed using Igor Pro. The results are shown in FIG. 7.

Nanobiosensor Peptide Reduction Testing. 50mM Tris-HCl buffer @ pH=8 was mixed with THPP for a 1.3mM THPP solution. The nanoparticles were resuspended by swirling and then 50 μL were removed into a 1.5mL tube. A neodymium magnet was used to pull the particles to the side and remove the supernatant. The 1mL of 1.3mM THPP in 50mM Tris-HCl @pH 8 was added and then mixed and then placed on a rotator for 30min. After 30min the nanoparticles were pulled to the side by the magnet and then 100 μL of the sample was removed for testing in the IEF microfluidic chip.

In a first disulfide reduction test (results shown in FIG. 8), peptide (RhB-GCYKK, SEQ ID NO: 107) was attached via disulfide bond to cysteine coated 5nm nanoparticles with a dopamine coating to reduce aggregation. In a second disulfide reduction test (results shown in FIG. 9), Peptide (RhB-GCEHH, SEQ ID NO:108) was attached via disulfide bond to cysteine coated 35nm
5 Fe/Fe₃O₄ nanoparticles with a 3,4-diphenylacetic acid coating to reduce aggregation.

CLAIMS:

1. A method of microfluidic detection of a target analyte in a biological sample collected from a subject, the method comprising:
 - providing a microfluidic device, said device comprising:
 - a planar substrate comprising a sample inlet well positioned above a sample chamber in or on the substrate and a detection chamber in fluid communication with the sample chamber via a microfluidic channel extending from the sample chamber to the detection chamber; and a washing chamber positioned along the microfluidic channel intermediate to the sample chamber and the detection chamber; and
 - a magnet positioned external and adjacent to said microfluidic channel, wherein said magnet is translatable along a plane parallel and to the plane of said microfluidic channel;
 - incubating said biological sample with a first nanobiosensor in said sample chamber, wherein said first nanobiosensor comprises a magnetic nanoparticle releasably attached to a plurality of reporter molecules;
 - transferring said incubated sample and said first nanobiosensor to said wash chamber;
 - introducing a wash fluid into said wash chamber to yield a first washed nanobiosensor;
 - magnetically transferring said first washed nanobiosensor to said detection chamber by translating said magnet along a plane parallel to said microfluidic channel from a position adjacent said wash chamber to a position adjacent said detection chamber;
 - separating said nanoparticle and said reporter molecules to yield separated reporter molecules and a delinked nanoparticle,
 - magnetically removing said delinked nanoparticle from said detection chamber by translating said magnet along a plane parallel to said microfluidic channel away from said detection chamber;
 - detecting said separated reporter molecules in said detection chamber, wherein said reporter molecules are indicative of interaction of said target analyte with said reporter molecules.

2. The method of claim 1, wherein said magnetic nanoparticle is releasably attached to a plurality of said reporter molecules via respective release units, wherein said release units are selected from the group consisting of reducible disulfide bridge, pH-sensitive linkage, photosensitive linkage, and thermosensitive linkages.
3. The method of claim 1, wherein said magnetic nanoparticle comprises any type of ferromagnetic metal (including elemental metal) or metal alloy selected from the group consisting of iron (Fe), nickel (Ni), cobalt (Co), certain alloys of rare-earth metals, and the oxides, hydroxides, sulfides, selenides, and tellurides of the foregoing, and combinations thereof.
4. The method of claim 1, wherein said magnetic nanoparticle is a core/shell nanoparticle selected from the group consisting of Fe/Au, Fe(0)/Fe₃O₄, and Au/Fe₂O₃.
5. The method of claim 1, wherein said reporter molecules are peptide sequences comprising a target analyte recognition sequence, detectable label, and one or more optional pI tag(s).
6. The method of claim 5, wherein said recognition sequence is selected from the group consisting of a supramolecular recognition sequence, a protease consensus sequence, and a post-translationally modifiable sequence.
7. The method of claim 5, wherein said detecting comprises exposing said detection region to an energy source to generate a detectable signal from said detectable label.
8. The method of claim 1, wherein said incubating comprising mixing said biological sample and said nanobiosensor by rotating said magnet in a position adjacent to said sample chamber, thereby magnetically moving said nanobiosensors throughout said biological sample.
9. The method of claim 1, wherein said magnet is positioned on a translatable stage below said microfluidic channel.

10. The method of claim 1, wherein said microfluidic channel further comprises one or more valves to provide forward and/or back pressure in said channel.
11. The method of claim 1, wherein at least a portion of said biological sample is removed via a waste outlet prior to magnetically transferring said first washed nanobiosensor to said detection chamber.
12. The method of claim 11, wherein said first nanobiosensor is present in said biological sample in a first concentration in said sample chamber; and wherein said washed nanobiosensor is present in a second concentration in said detection chamber, wherein said second concentration is greater than said first concentration.
13. The method of claim 1, wherein said detection chamber further comprises one or more inlets for applying an electrical current into said detection chamber.
14. The method of claim 1, further comprising incubating a plurality of additional nanobiosensors with said biological sample and first nanobiosensor in said sample chamber, wherein said plurality of additional nanobiosensors each comprise respective reporter molecules, wherein said respective reporter molecules are specific for a different target analyte than said first nanobiosensor.
15. The method of claim 14, wherein said detecting comprises microfluidic Isoelectric Focusing of said separated reporter molecules and detecting the location of said separated reporter molecules in said detection chamber.
16. The method of claim 15, wherein said separated reporter molecules are configured in a linearly spaced array of reporter molecules at respective locations in said detection chamber.
17. The method of claim 16, further comprising exposing said array to a light source and optically detecting the configuration of said array.

18. The method of claim 17, further comprising detecting the intensity of a detectable signal from said separated reporter molecules.
19. A microfluidic device for detection of a target analyte in a biological sample, comprising:
a planar substrate comprising a sample inlet well positioned above a sample chamber in or on the substrate and a detection chamber in fluid communication with the sample chamber via a microfluidic channel extending from the sample chamber to the detection chamber; and a washing chamber positioned along the microfluidic channel intermediate to the sample chamber and the detection chamber; and
a magnet positioned external and adjacent to said microfluidic channel, wherein said magnet is translatable along a plane parallel and to the plane of said microfluidic channel.
20. The device of claim 19, further comprising an ampholyte inlet configured to introduce a high-resolution ampholyte mixture into said detection chamber, a catholyte inlet configured to introduce a catholyte mixture into said detection chamber, an anolyte inlet configured to introduce a high-resolution anolyte mixture into said detection chamber, and a pressure fill inlet valve.
21. The device of claim 20, wherein said detection chamber further comprises one or more inlets for applying an electrical current into said detection chamber.
22. A nanobiosensor for detection of a target analyte in a biological sample, said nanobiosensor comprising a magnetic nanoparticle releasably attached to a reporter molecule via a release unit, wherein said reporter molecule is an oligopeptide comprising a target analyte recognition sequence, detectable label, and one or more optional pI tag(s).

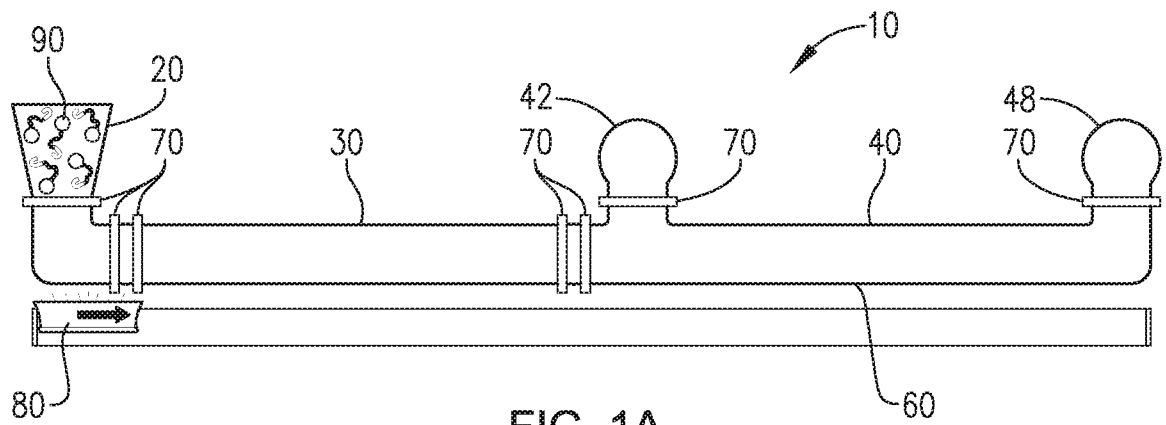


FIG. 1A

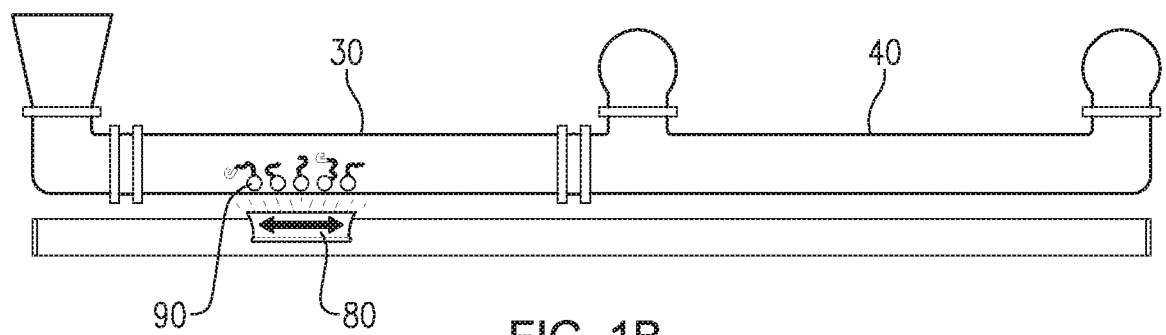


FIG. 1B

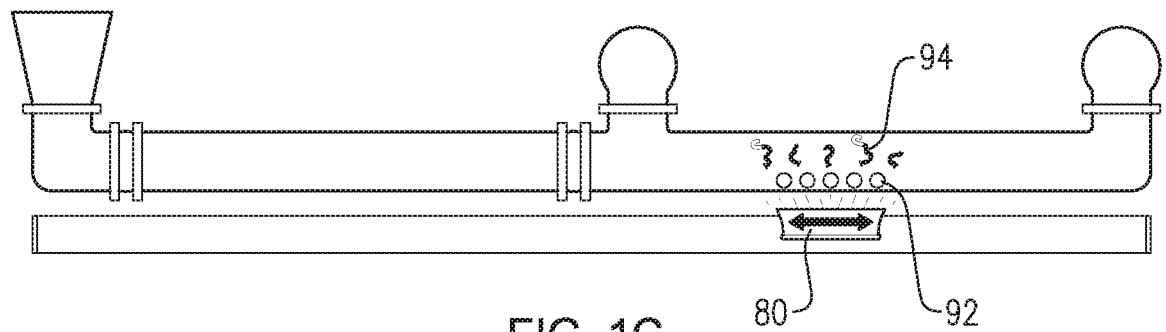


FIG. 1C

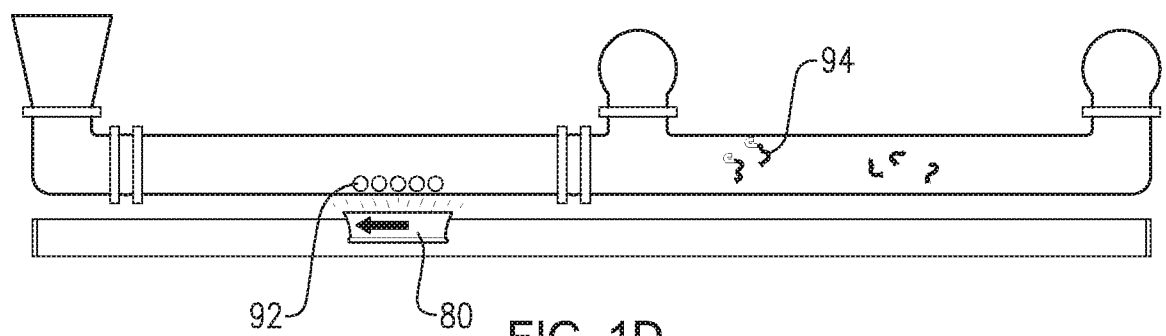


FIG. 1D

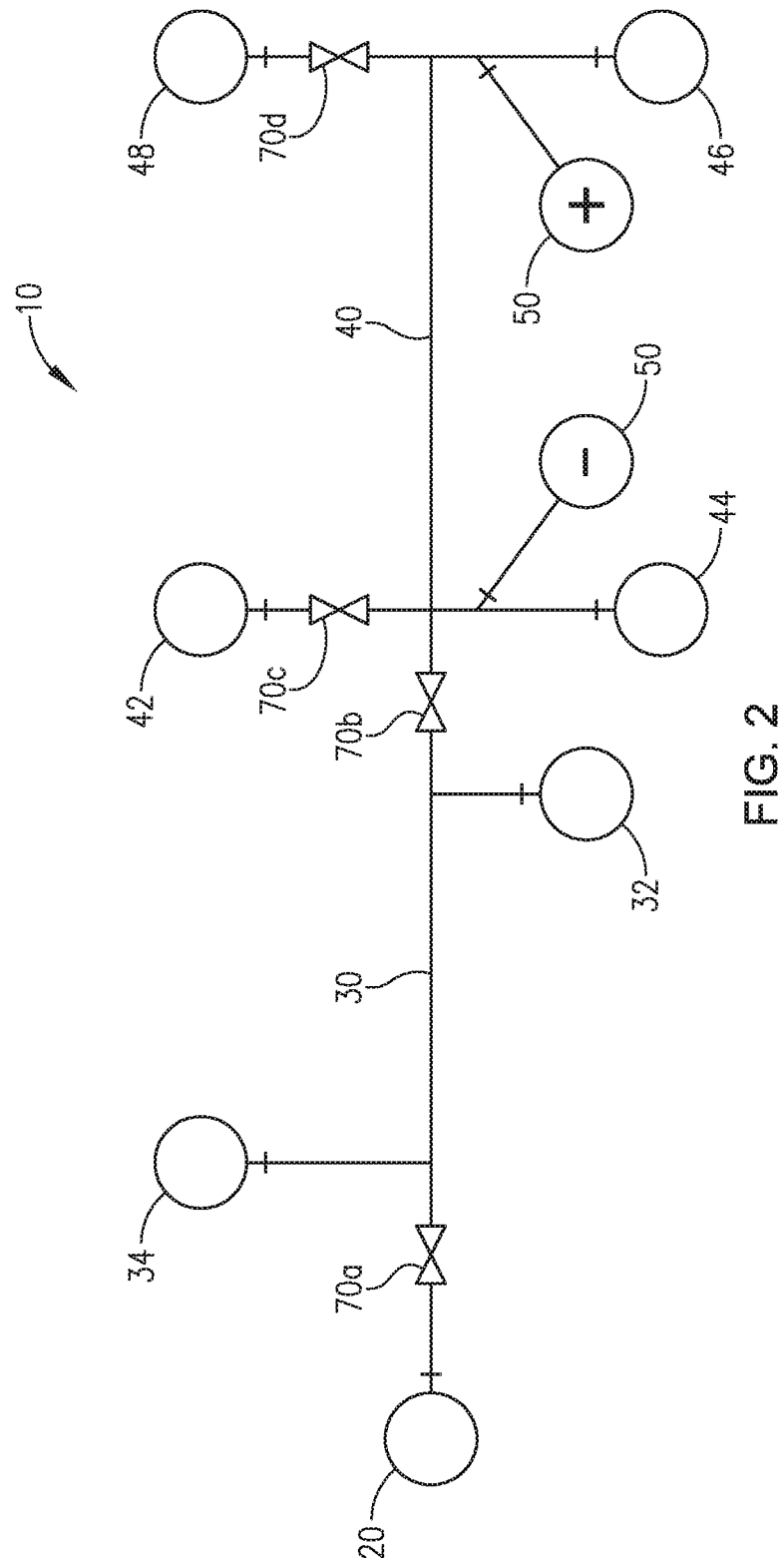


FIG. 2

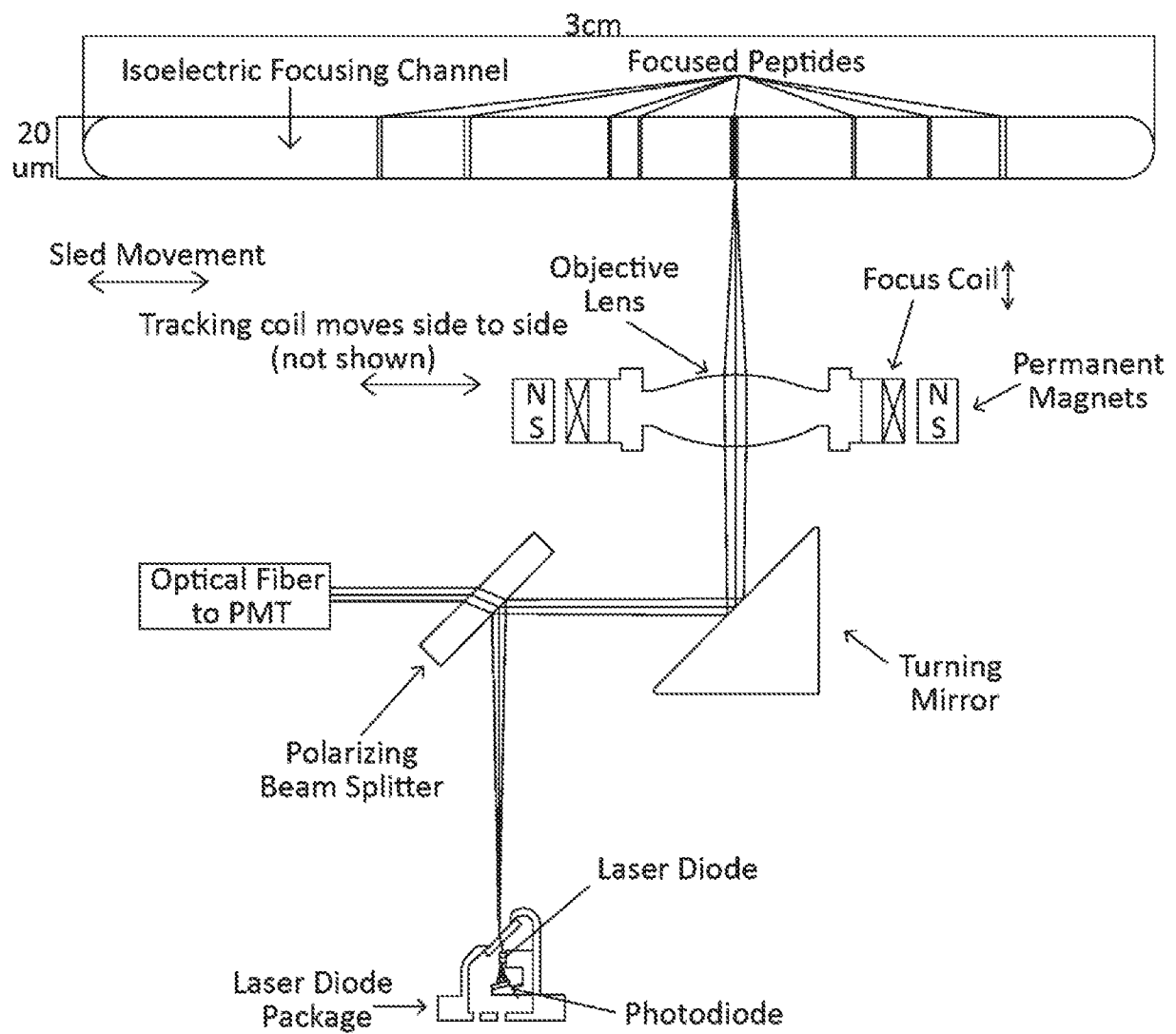


FIG. 3

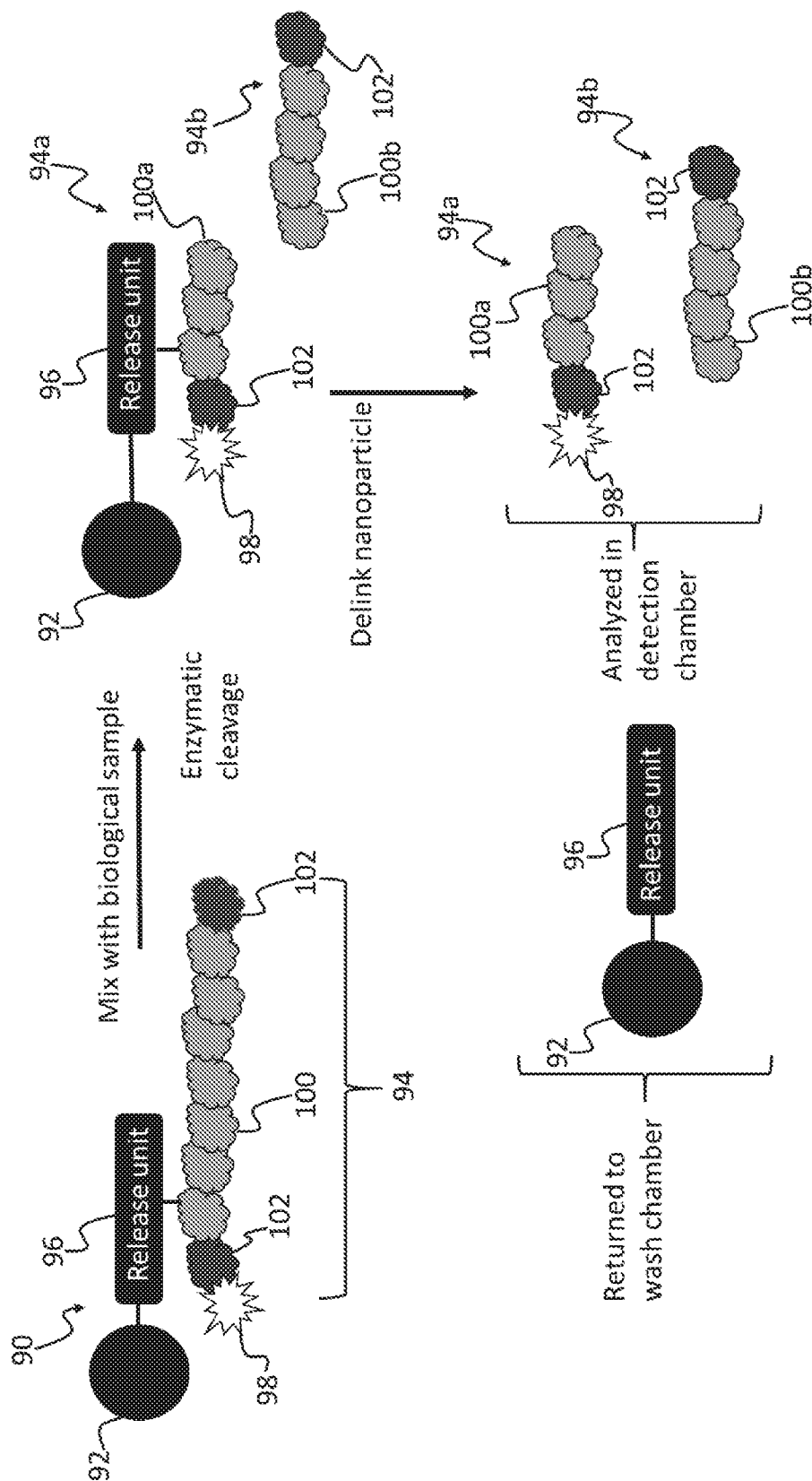


FIG. 4A

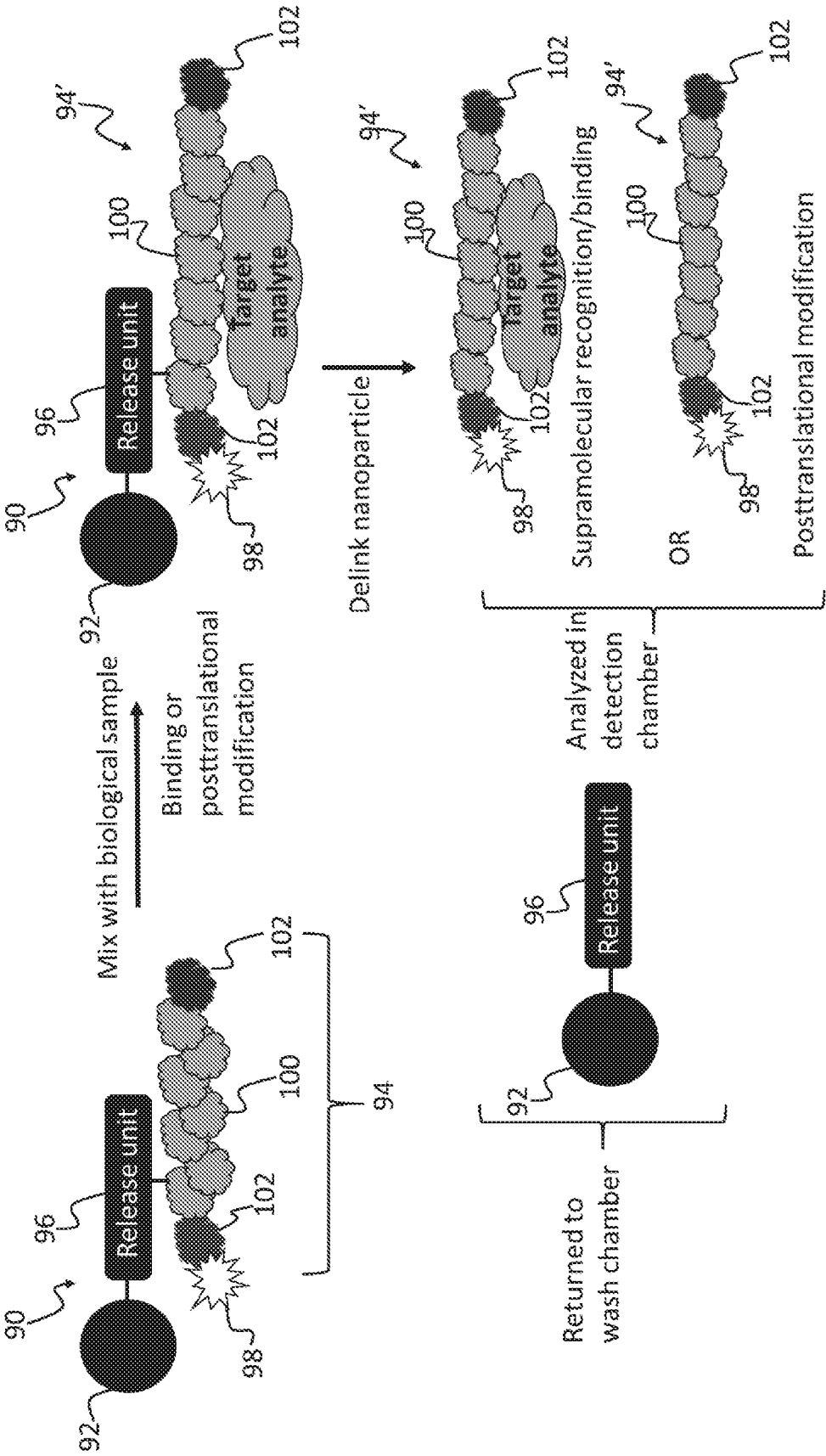


FIG. 4B

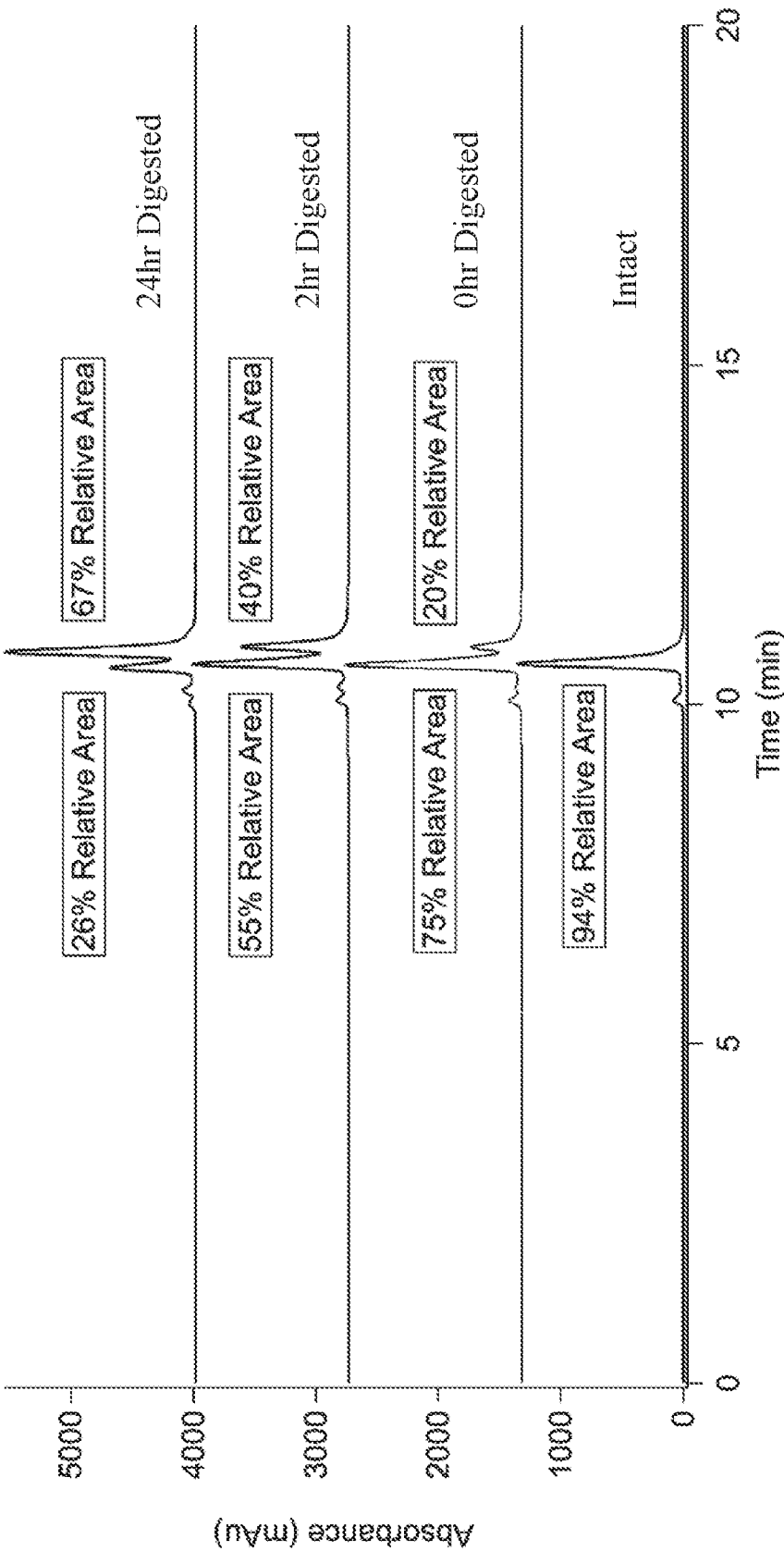


FIG. 5

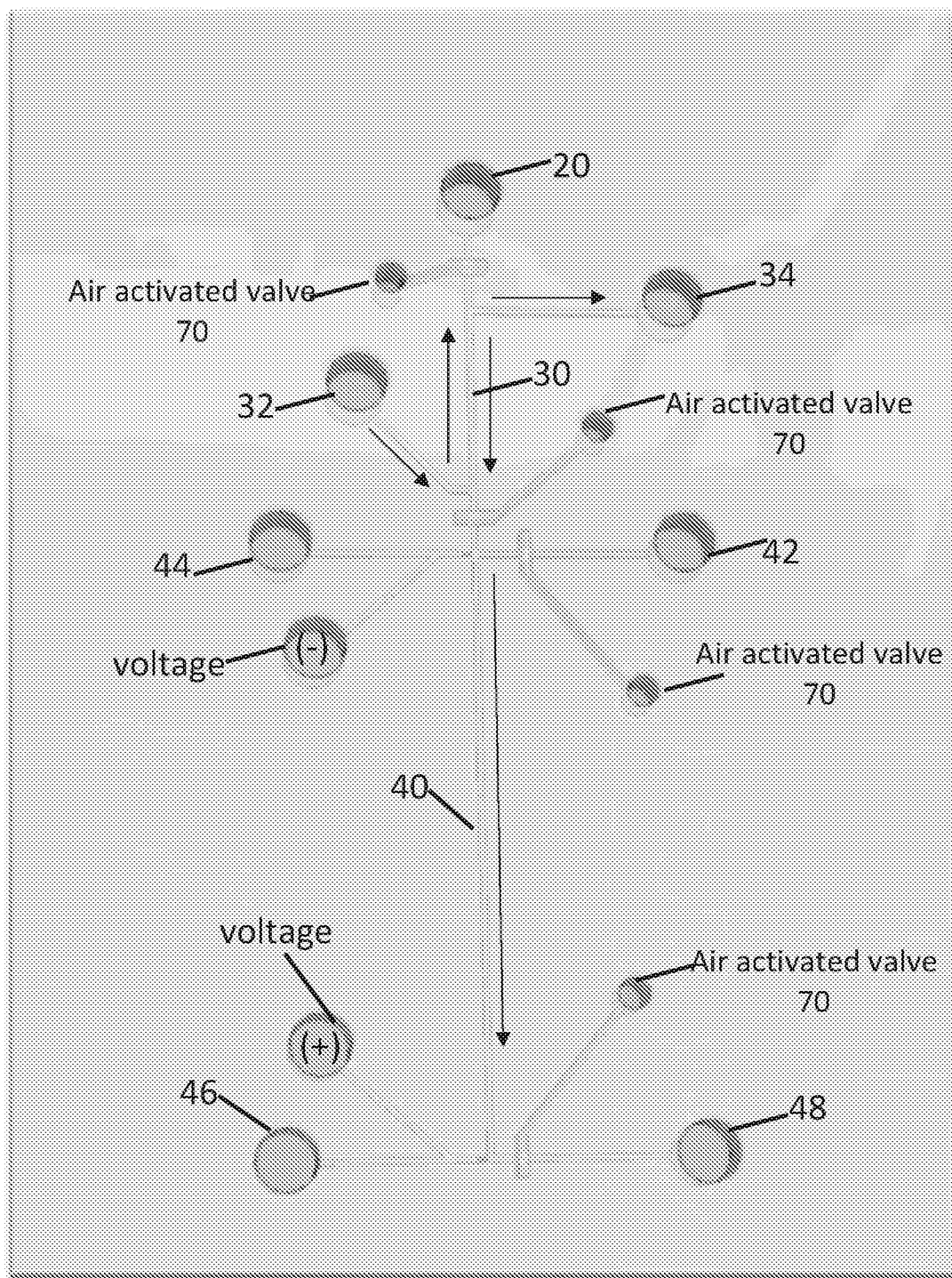


FIG. 6

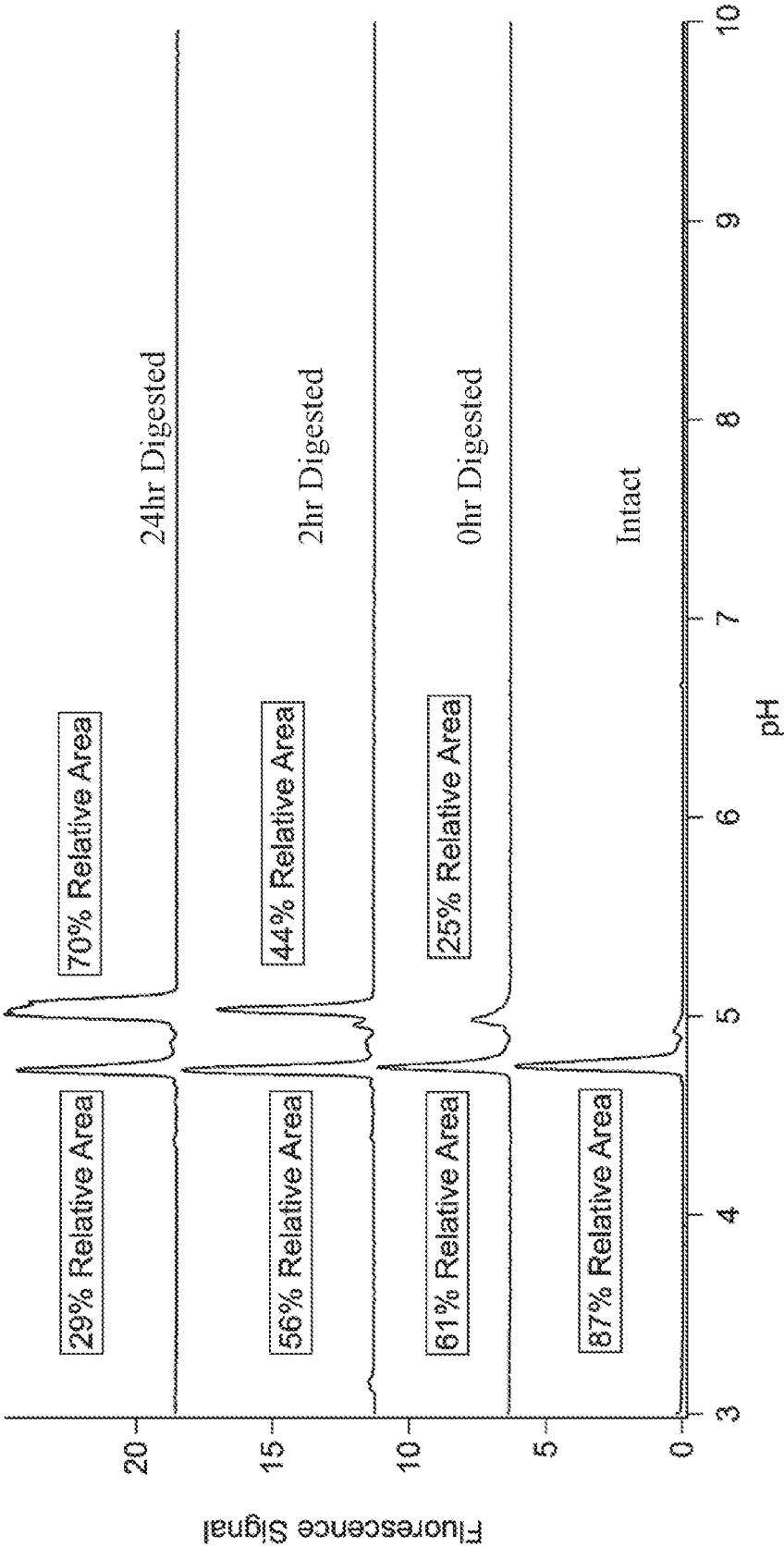


FIG. 7

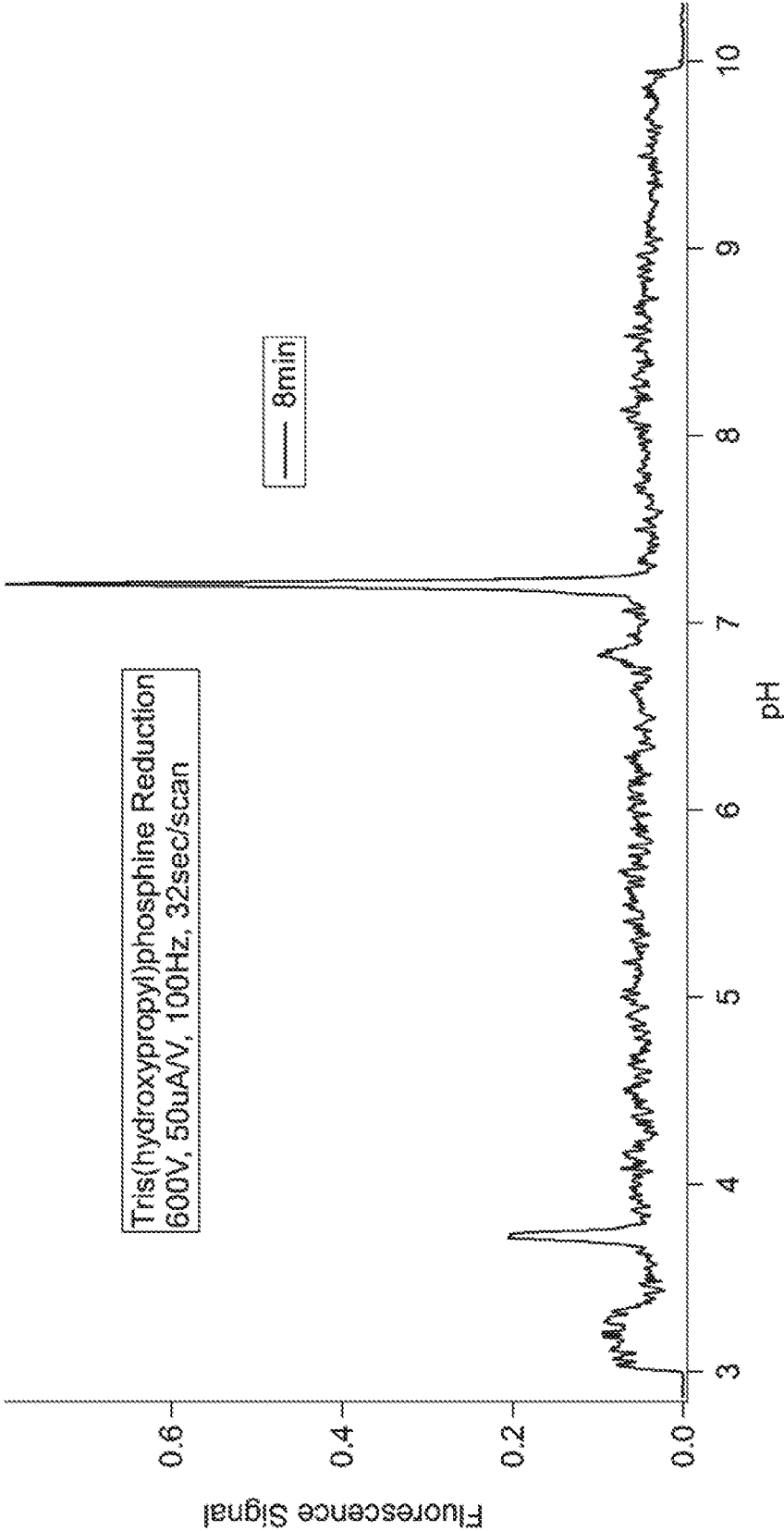


FIG. 8

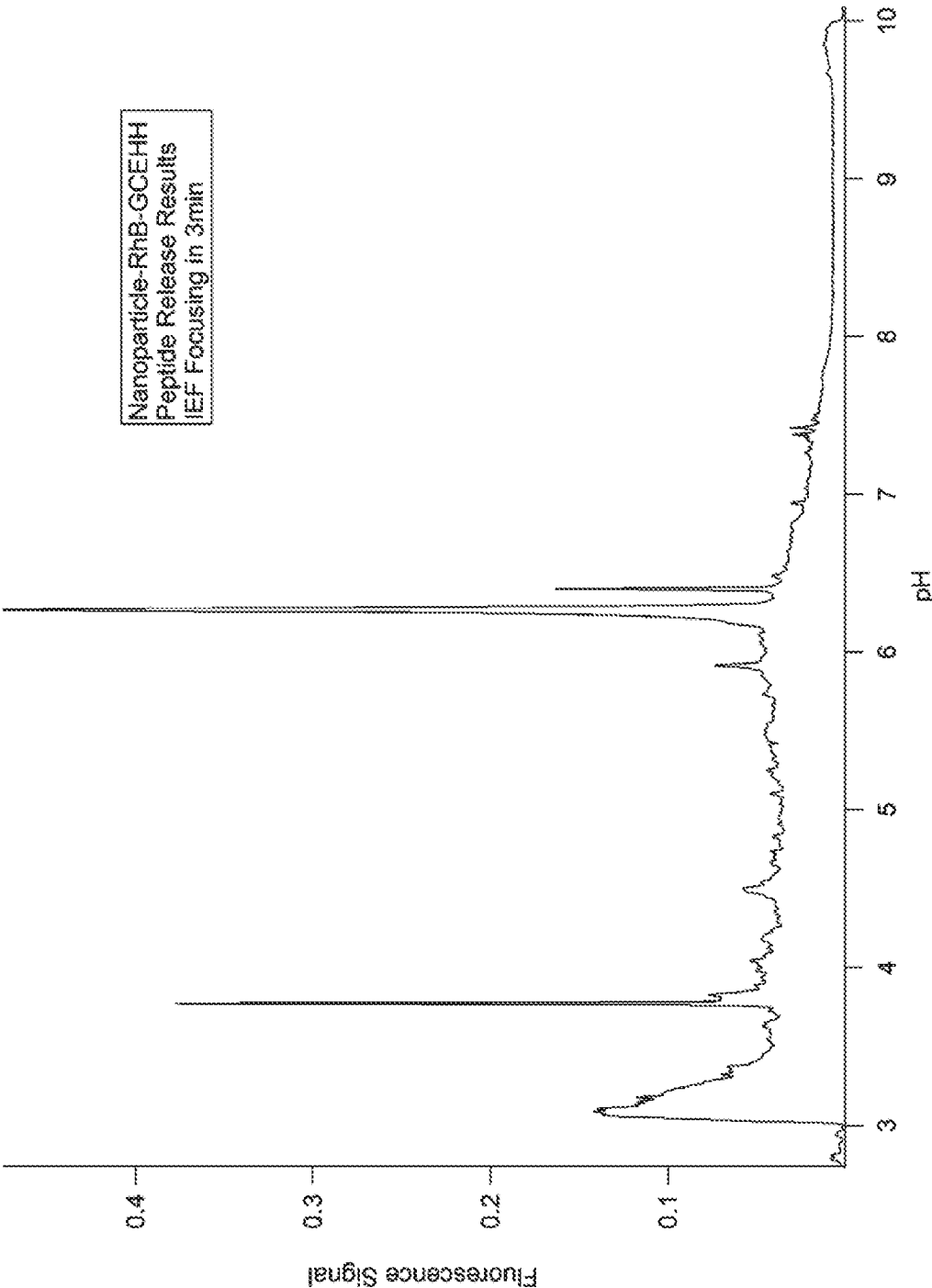


FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/62304

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - B82Y 30/00; C07K 1/28, G01N 1/34, B01L 3/00 (2018.01)

CPC - B82Y 30/00; C12N 15/1013; C07K 1/285; B01L 2200/0647, 2300/087, 2400/043, 2400/0487, 3/5023, 3/502784

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2015/0290639 A1 (Evtodienko) 15 October 2015 (15.10.2015). Especially para [0014], [0076], [0097].	1-18
Y	US 2014/0030717 A1 (The Regents of the University of California) 30 January 2014 (30.01.2014). Especially para [0005], [0020], [0040], [0042], [0052], [0055], [0062], [0066], [0072]	1-18
Y	US 2007/0054337 A1 (Ferring et al.) 8 March 2007 (08.03.2007). Especially claims 1, 18-20.	1-18
Y	US 2015 0357102 A1 (Toyota Motor Engineering & Manufacturing North America, Inc.) 10 December 2015 (10.12.2015). Especially claim 1.	4
Y	US 2006/0054504 A1 (Lee et al.) 16 March 2006 (16.03.2006). Especially abstract, claims 10-12.	13, 15-18

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

* Special categories of cited documents:

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

12 March 2018

Date of mailing of the international search report

27 MAR 2018

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/62304

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
----Go to Extra Sheet for continuation----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-18

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/62304

continuation of Box III: Observations where Lack of Unity is Lacking'

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

Group I: Claims 1-18, drawn to a method of microfluidic detection of a target analyte.

Group II: Claim 19-21, drawn to a microfluidic device.

Group III: Claim 22, drawn to a nanobiosensor for detection of a target analyte.

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

Special Technical Features:

Group I has the special technical feature of a method involving steps for detection of a target analyte, not required by Groups II or III.

Group II has the special technical feature of a microfluidic device, not required by Groups I or III.

Group III has the special technical feature of a nanobiosensor comprising a magnetic nanoparticle releasably attached to a reporter molecule.

Common Technical Features:

1. Groups I and II share the common technical feature of a planar substrate comprising a sample inlet well positioned above a sample chamber in or on the substrate and a detection chamber in fluid communication with the sample chamber via a microfluidic channel extending from the sample chamber to the detection chamber; and a washing chamber positioned along the microfluidic channel intermediate to the sample chamber and the detection chamber.

2. Groups I and II share the common technical feature of a magnet positioned external and adjacent to said microfluidic channel, wherein said magnet is translatable along a plane parallel and to the plane of said microfluidic channel.

3. Groups I and III share the common technical feature of a nanobiosensor comprising a magnetic nanoparticle releasably attached to a reporter molecule.

However, said common technical features do not represent a contribution over the prior art and are obvious over US 2015/0290639 A1 (Evtodienko), in view of US 2014/0030717 A1 to the Regents of the University of California (hereinafter "Univ California").

As to common technical feature #1, Evtodienko teaches (para [0097]; "One exemplary device is shown in FIG. 1. A cartridge (1) is shown, which houses the present device. The device comprises a sample chamber (2) having a sample entry port (11), and a detection channel (4) with an encapsulated lateral flow porous membrane (3). The lateral flow membrane (3) has an analyte capture zone (4). Inside the porous membrane is an analyte capture zone (5) to which is immobilized a capturing species. Also provided is a wash chamber (10)...A system of micro-fluidic channels (9) connects the different fluid chambers to the detection channel (3)"; see sheet 1 fig 1, sheet 3 figs 3-5).

As to common technical feature #2, Univ California teaches (para [0005]; "In yet another embodiment, the system further comprises a magnet for moving the magnetic beads through a fluid channel, the magnet positioned relative to the substrate to provide a magnetic field that manipulates the magnetic bead....the system further comprises a computer controller for moving the magnet relative to the substrate")

As to common technical feature #3, Univ California teaches (para [0005]; "a magnetic bead comprising an analyte binding molecule. In another embodiment, the analyte binding molecule comprises an oligonucleotide, peptide, polypeptide, antibody or nanoparticle"; para [0052]; For example, when the analyte is a single-stranded nucleic acid, the binding/targeting ligand (e.g., a magnetic bead) comprises a substantially complementary nucleic acid. Similarly, the analyte may be a nucleic acid binding protein and the capture binding ligand is either a single-stranded or double-stranded nucleic acid"; para [0072]; "A CE-LIF (laser induced fluorescence) method was applied to demonstrate the M2 chip-based selective binding and extraction of small RNAs").

As the common technical features were known in the art at the time of the invention, they cannot be considered common special technical features that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I-III lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.