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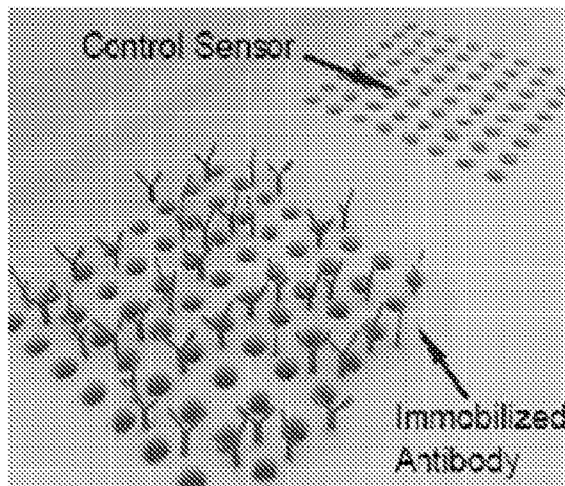
(54) **Title**: NANOSTRUCTURE BIOSENSORS AND SYSTEMS AND METHODS OF USE THEREOF



FIGURES 10A

(57) **Abstract**: A sensor scheme combining nano-photonics and nano-fluidics on a single platform through the use of free-standing photonic crystals and nanoplasmonic arrays is described. By harnessing nanoscale openings, both fluidics and light can be manipulated at sub-wavelength scales. The convective flow is actively steered through the nanohole openings for effective delivery of the analytes to the sensor surface, and refractive index changes are detected in aqueous solutions. Systems and methods using cross-polarization measurements to further improve the detection limit by increasing the signal-to-noise ratio are also described.

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NANOSTRUCTURE BIOSENSORS AND SYSTEMS AND METHODS OF USE THEREOF

FIELD OF THE INVENTION

[0001] The present invention relates generally to the field of biosensors, and systems and methods for overcoming mass transport limitations of on-chip biosensors with actively controlled, surface-targeted nanofluidics, methods of making biosensors, and apparatuses and methods for detection of biomolecular targets using nanostructure sensors.

CROSS REFERENCE TO RELATED APPLICATIONS

[0002] This application claims benefit under 35 U.S.C. §119(e) of U.S. Provisional Patent Application Serial No: 61/266,967 filed on 4 December 2009, and U.S. Provisional Patent Application Serial No: 61/288,101, filed on 18 December 2009, and U.S. Provisional Patent Application Serial No: 61/393,734 filed on 15 October 2010, the contents of which are incorporated herein by reference in their entirety.

GOVERNMENT SUPPORT

[0003] This invention was made with Government Support under Contract Nos. ECCS-0849603, ECCS-0954790 and EEC-0812056 awarded by the National Science Foundation, and Grant No. N0001410-1-0742 awarded by the Office of Naval Research. The Government has certain rights in the invention.

BACKGROUND

[0004] The ability to detect biological target molecules, such as DNA, RNA, and proteins, as well as nanomolecular particles such as virions, is fundamental to our understanding of both cell physiology and disease progression, as well as for use in various applications such as early and rapid detection of disease outbreaks and bioterrorism attacks. For example, early detection of infectious viral diseases is of great importance in terms of public health, homeland security, and the armed forces. A number of recent outbreaks of viral diseases (*e.g.*, H1N1 flu, H5N1 flu and SARS) in recent years have raised significant fears that such viruses could rapidly spread and turn into a pandemic, similar to 1918 Spanish flu that killed more than 50 million people[1].

[0005] Such detection, however, is limited by the need to use labels, such as fluorescent molecules or radiolabels, which can alter the properties of the biological target, *e.g.*, conformation, and which require additional, often time-consuming, steps, as well substantial equipment outlay. Traditional detection methods such as cell culturing, enzyme-linked immunosorbant assays (ELISA), and polymerase chain reaction (PCR) are not readily compatible with point-of-care use, without the

existence of extensive infrastructure[3,4]. Cell culturing is a time consuming, highly specialized and labor intensive process. In some cases, viruses cannot be cultured at all[5]. ELISA products require multiple steps and reagents, which can have a potential to create quenching interactions among each other[6]. PCR, another commonly used and powerful diagnostic tool, based on detection of nucleic fragments in samples, requires significant sample preparation, and can be confounded by inhibitors within a sample, such as a clinical sample[7]. In addition, PCR also provides only an indirect test of infections, as viral nucleic acid fragments can be present in the host organism after the infection has been "cleared" or effectively neutralized[8-10]. In addition, while PCR is a robust and accurate technique in detecting known strains, it is not always adaptable to newly emerged or highly divergent strains of an infections agent. One example is the description of a new strain of Ebola that was not identified in PCR-based diagnostics[11].

[0006] DNA and protein microarray technologies are actively being used by biologists and researchers today for high-throughput screening of biomarkers for drug discovery, disease research, and diagnosis, thereby converting the presence of target biomolecules to a measurable and quantifiable signal. The importance of high-throughput platforms has been demonstrated by the success of gene arrays in the analysis of nucleic acids, and to some degree, analysis of proteins. However, most detection systems available today for use in these high-throughput systems operate by the same guiding principle, whereby the surface of a microarray is scanned and fluorescence measured from labeled analytes or biomolecules. Fluorescent labeling is a costly and time-consuming step that sometimes proves to be prohibitively difficult and expensive for use in these technologies. In addition, detecting analytes through secondary probes is intrinsically complex, requiring multiple layers of interacting components that provide specificity without interfering with one another.

[0007] In recent years, label-free biosensors combined with innovative signal transduction methods have been proposed to push the detection limits down to femto-molar concentrations of analytes. Concurrently, researchers have also been integrating such sensitive and compact nanosensors with micro-fluidics for automated sample handling.

[0008] While micro-fluidics can enable portable and lab-on-a-chip systems, recent theoretical and numerical calculations indicate that the effects of various fluidic integration schemes must be taken into account because they can fundamentally limit the sensors performance. For nano-sensors embedded in conventional microfluidic channels, the detection limit is often determined by the analyte (mass) transport limitations as opposed to the detection capabilities of the sensors.

[0009] As the analytes are collected by the functionalized sensors, depletion zones form around the sensing area. Depletion zones, where the analytes transport diffusively, expand with time until the growth is halted by the convective flow. In micro-fluidic channels supporting laminar flow profile, the convective flow parallel to the surface is weaker close to the channel edge. Accordingly, the depletion zones extend significantly towards the center of the channel, causing dramatically lower amounts of analytes to reach the sensing surface per unit time. Consequently, if no method is

introduced to actively direct the convective flow towards the surface of the nano-micro size sensors, analytes at low concentrations may need weeks-to-years to diffuse due to mass (analyte) transport limitations imposed by the depletion zones.

[0010] Within the last decade, several highly sensitive optical label-free nano-sensors have been introduced, such as dielectric resonators supporting whispering-gallery modes, metallic nano-structures supporting localized/propagating surface plasmons, and photonic crystals (PhC) supporting cavity, waveguide and guided resonance modes. Among these, nanohole array based platforms are offering more freedom to manipulate the spatial extent and the spectral characteristics of the electromagnetic fields. Existing nanohole array-based platforms are formed using FIB lithography. FIB lithography, however, is operationally slow.

SUMMARY OF THE INVENTION

[0011] The following summary of the invention is included in order to provide a basic understanding of some aspects and features of the invention. This summary is not an extensive overview of the invention and as such it is not intended to particularly identify key or critical elements of the invention or to delineate the scope of the invention. Its sole purpose is to present some concepts of the invention in a simplified form as a prelude to the more detailed description that is presented below.

[0012] Described herein, in some aspects, are label-free, plasmonic nanostructure sensors that can directly detect biomolecular targets without the use of labels. The nanostructure sensor platforms described herein are based on extraordinary light transmission effects using plasmonic nanoelements, specifically nanoholes, and can utilize unlabeled capture agents, such as antibodies or fragments thereof, for detection of biomolecular targets. For example, the novel plasmonic nanostructure sensors, and systems and methods thereof described herein, can be used to detect intact viruses from biological media at clinically relevant concentrations with little to no sample preparation. The plasmonic nanostructure sensors, systems, and methods described herein are capable of detecting highly divergent strains of rapidly evolving viruses, as demonstrated herein by detection and recognition of small enveloped RNA viruses (*e.g.*, vesicular stomatitis virus and pseudo-typed Ebola), as well as enveloped DNA viruses (*e.g.*, vaccinia virus), within a dynamic range spanning at least three orders of magnitude. Remarkably, the quantitative detection methods described herein permit the detection of intact viruses at low concentration limits (10^5 PFU/ml), which enables not only sensing of the presence of virions in analyzed samples, but also the intensity of the infection process. Further, the non-destructive nature of the plasmonic nanostructure sensors and systems described herein allow the preservation of structural aspects of a biomolecular target being analyzed, such as a viral structure or a nucleic acid load (genome) for further studies. The plasmonic nanostructure sensors and systems described herein permit high signal:noise measurements without any mechanical

or optical isolation, and thus, open up opportunities for detection of a broad range of biomolecules, such as pathogens, in any biology lab or in a mobile setting.

[0013] High-throughput DNA and protein analysis technologies, such as microarray technologies, are actively being used by biologists and researchers today for high-throughput screening of biomolecules and analytes for drug discovery, disease research, and diagnosis. Most detection systems available operate by the same guiding principle, whereby they scan the surface of a microarray and measure fluorescence, or some other label, from biomolecules present on the array surface. Fluorescent labeling is a costly and time-consuming step that sometimes proves to be prohibitively difficult and expensive. Thus, the ability to rapidly detect biomolecular targets using label-free systems can have many practical applications and advantages. Further, label-free systems provide easier monitoring and quantification methods for detecting biomolecular interactions between such targets, such as antigen–antibody, receptor–ligand, virus–cell, and protein–DNA binding interactions.

[0014] Label-free biosensors have emerged as promising tools for detecting and analyzing biomolecules, such as diagnostics for cancer and infectious diseases[12-24]. Such sensors circumvent the need for fluorescence/radio-active tagging or enzymatic detection, and enable compact, simple, inexpensive, point-of-care diagnostics. Various sensing platforms based on optical[12-17], electrical[22,23], and mechanical[18-21] signal transduction mechanisms have been offered for applications ranging from laboratory research, to clinical diagnostics and drug development, to combating bioterrorism. Among these sensing platforms, optical detection platforms are particularly promising. Ideally, optical biosensors allow remote transduction of the biomolecular binding signal from the sensing volume without any physical connection between the excitation source and the detection channel[25,26]. Unlike mechanical and electrical sensors, they are also compatible with physiological solutions, and are not sensitive to the changes in the ionic strengths of the solutions[27,28]. However, a drawback of the most currently-used optical biosensors is that they require precise alignment of sensitive light coupling to the biodetection volume[15-17,24]. As a result these technologies are not particularly suitable for point-of-care type applications.

[0015] Plasmonic biosensors are distinctive among photonic sensors as they allow direct coupling of the perpendicularly incident light and constitute a robust sensing platform minimizing the alignment requirements for light coupling [12-14,29-32]. This capability also allows massive multiplexing in a ready manner[29]. In addition, the extraordinary transmission (EOT) signals in plasmonic nanohole arrays create an excellent detection window enabling spectral measurements with minimal background noise and high signal-to-noise ratios[33-35].

[0016] Demonstrated herein, in some aspects, are novel devices and approaches combining fluidics, optics, and plasmonic sensing in a single platform enabling both the resonant transmission of light and the active transport of fluidics through them[35]. With the newly developed fluidic nanostructure biosensor devices and systems described herein, higher sensitivities and faster sensor

response times were achieved as a result of lift-off free nanofabrication techniques in combination with the targeted analyte delivery scheme to the sensing surface of the nanostructure biosensor[35-37].

[0017] Accordingly, provided herein are plasmonic nanostructure sensors, fluidic biosensor devices, and systems and methods of use thereof.

[0018] In some aspects, provided herein are plasmonic nanostructure biosensors comprising a substrate and a metal film disposed upon the substrate. In such aspects, the metal film comprises one or more surfaces comprising a plurality of nanoelements arranged in a predefined pattern, where each nanoelement has a dimension less than one wavelength of an incident optical source to which the metal film produces surface plasmons, and where the metal film is activated with an activating agent.

[0019] In some embodiments of these plasmonic nanostructure biosensors, the predefined pattern of the plurality of nanoelements is a periodic pattern. In some embodiments, the plurality of nanoelements are separated by a periodicity of between 100-1000 nm. In some embodiments, the plurality of nanoelements are separated by a periodicity of between 400-800 nm. In some embodiments of these plasmonic nanostructure biosensors, the predefined pattern of the plurality of nanoelements is a non-periodic pattern, such as a pseudo-random pattern or a random pattern.

[0020] In some embodiments of these plasmonic nanostructure biosensors, the substrate comprises silicon dioxide, silicon nitride, glass, quartz, magnesium fluoride (MgF_2), calcium fluoride (CaF_2), ZnSe, germanium, or a polymer.

[0021] In some embodiments of these plasmonic nanostructure biosensors, the metal film produces surface plasmons to incident light in the UV-VIS-IR spectral range.

[0022] In some embodiments of these plasmonic nanostructure biosensors, the metal film comprises a Noble metal, a transition metal, or an alkali metal. In some embodiments of these plasmonic nanostructure biosensors, the metal film comprises a metal selected from the group consisting of gold, rhodium, palladium, silver, osmium, iridium, platinum, titanium, aluminum, or any combination thereof.

[0023] In some embodiments of these plasmonic nanostructure biosensors, the metal film is between 50-500 nm thick. In some embodiments of these aspects, the metal film is between 75-200 nm thick.

[0024] In some embodiments of these plasmonic nanostructure biosensors, the plurality of nanoelements is a plurality of nanoholes. In some embodiments of these plasmonic nanostructure biosensors, at least one dimension of the nanohole is between 10-1000 nm. In some embodiments of these plasmonic nanostructure biosensors, at least one dimension of the nanohole is between 50-300 nm.

[0025] In some embodiments of these plasmonic nanostructure biosensors, the nanoelements, such as nanoholes, are separated by a periodicity of between 100-1000 nm. In some embodiments of these plasmonic nanostructure biosensors, the nanoelements are separated by a periodicity of between 400-800 nm.

[0026] In some embodiments of these plasmonic nanostructure biosensors, the activating agent used to activate the metal film is a piranha solution.

[0027] In some embodiments of these aspects, the plasmonic nanostructure biosensors further comprise an adhesion layer between the metal film and the substrate. In some embodiments of these plasmonic nanostructure biosensors, the adhesion layer comprises titanium, chromium, or any combination thereof. In some embodiments of these plasmonic nanostructure biosensors, the adhesion layer is less than 50 nm. In some embodiments of these plasmonic nanostructure biosensors, the adhesion layer is less than 25 nm. In some embodiments of these plasmonic nanostructure biosensors, the adhesion layer is less than 15 nm.

[0028] In some embodiments of these plasmonic nanostructure biosensors, the activated metal film is further functionalized with one or more capture agents. In some embodiments of these plasmonic nanostructure biosensors, the capture agent is an antibody or antibody fragment thereof, a receptor, a recombinant fusion protein, a nucleic acid molecule, or any combination thereof. In some embodiments of these plasmonic nanostructure biosensors, the one or more capture agents comprise a first capture agent and a second capture agent, wherein the first capture agent is specific for the second capture agent, and the second capture agent is specific for one or more biomolecular targets. In some embodiments of these plasmonic nanostructure biosensors, the first capture agent is protein A/G. In some embodiments of these plasmonic nanostructure biosensors, the second capture agent comprises one or more antibodies or antibody fragments thereof.

[0029] Other aspects described herein provide a plasmonic nanostructure biosensor system for detecting one or more biomolecular targets comprising: (i) a plasmonic nanostructure biosensor as described herein; (ii) a device or a system for contacting one or more samples comprising one or more biomolecular targets to the metal film surface(s) of the plasmonic nanostructure biosensor; (iii) an incident light source for illuminating a surface of the metal film to produce the surface plasmons; and (iv) an optical detection system for collecting and measuring light displaced from the illuminated metal film, wherein the displaced light is indicative of surface plasmon resonance on one or more surfaces of the metal film. In some embodiments of these biosensor systems, the device for contacting one or more samples comprises a fluidic system, such as fluid inlets, fluid outlet, pumps, etc.

[0030] Other aspects described herein provide methods for detecting one or more biomolecular targets comprising:

(i) providing a plasmonic nanostructure biosensor system as described herein;

(ii) contacting one or more samples comprising one or more biomolecular targets to the metal film surface of the plasmonic nanostructure biosensor system;

(iii) illuminating one or more surfaces of the metal film of the plasmonic nanostructure biosensor with the incident light source to produce surface plasmons, before and after the contacting with the one or more samples;

(iv) collecting and measuring light displaced from the illuminated film with the optical detection system, before and after the contacting with the one or more samples; and

(v) detecting the one or more biomolecular targets based on a change or difference in the measurement of the light displaced from the illuminated film before and after the contacting with the one or more samples.

[0031] In some embodiments of these methods, the biomolecular target is a eukaryotic cell, a eukaryotic cellular component, a prokaryote, a prokaryotic cellular component, a viral particle, a protein, an oligonucleotide, a prion, a toxin, or any combination thereof.

[0032] In some embodiments of these methods, the collected light comprises light in a transmission mode, in a reflection mode, or a combination thereof. In some embodiments of these aspects, the step of measuring displaced light comprises measuring light over a spectral range selected to comprise at least one plasmon band. In some embodiments of these methods, the change in the measurement of the displaced light before and after the contacting is a resonance peak shift, a change in a resonance peak intensity, a broadening of a resonance peak, a distortion in resonance of peak, or a change in refractive index.

[0033] In some aspects, provided herein are fluidic biosensor devices comprising: an upper chamber, where the upper chamber comprises a fluid inlet; a lower chamber, where the lower chamber comprises a fluid outlet; and a suspended nanostructure sensor placed between the upper chamber and the lower chamber, where the suspended nanostructure sensor comprises a plurality of nanoholes, where an analyte is configured to flow from the fluid inlet, through the nanoholes in the suspended nanostructure sensor and to the fluid outlet.

[0034] In some embodiments of these fluidic biosensor devices, the upper chamber comprises a glass surface. In some embodiments of these fluidic biosensor devices, the lower chamber comprises a glass surface. The fluidic biosensor device can, in some embodiments of these aspects, further comprise a light source to direct light through one of the glass surfaces and, in some embodiments, a light detector to detect the light through the other one of the glass surfaces.

[0035] In some embodiments of these aspects, the fluidic biosensor devices can also comprise a housing, such that the upper chamber, lower chamber, and photonic crystal sensor are enclosed by the housing. In some embodiments, the housing comprises polydimethylsiloxane (PDMS).

[0036] In some embodiments of these fluidic biosensor devices, the suspended nanostructure sensor comprises a photonic crystal.

[0037] In other embodiments of these fluidic biosensor devices, the suspended nanostructure sensor comprises a plasmonic nanostructure biosensor comprising a substrate and a metal film disposed upon the substrate. In such aspects, the metal film comprises one or more surfaces comprising a plurality of nanoholes arranged in a predefined pattern, where each nanohole has a

dimension less than one wavelength of an incident optical source to which the metal film produces surface plasmons, and where the metal film is activated with an activating agent.

[0038] In some embodiments of these fluidic biosensor devices, the predefined pattern of the plurality of nanoholes a periodic pattern. In some embodiments, the plurality of nanoholes are separated by a periodicity of between 100-1000 nm. In some embodiments, the plurality of nanoholes are separated by a periodicity of between 400-800 nm. In some embodiments of these fluidic biosensor devices, the predefined pattern of the plurality of nanoholes is a non-periodic pattern, such as a pseudo-random pattern or a random pattern.

[0039] In some embodiments of these aspects, the substrate comprises silicon dioxide, silicon nitride, glass, quartz, magnesium fluoride (MgF_2), calcium fluoride (CaF_2), ZnSe, germanium, or a polymer.

[0040] In some embodiments of these aspects, the metal film produces surface plasmons to incident light in the UV-VIS-IR spectral range.

[0041] In some embodiments of these fluidic biosensor devices, the metal film comprises a Noble metal, a transition metal, or an alkali metal. In some embodiments of these fluidic biosensor devices, the metal film comprises a metal selected from the group consisting of gold, rhodium, palladium, silver, osmium, iridium, platinum, titanium, aluminum, or any combination thereof.

[0042] In some embodiments of these fluidic biosensor devices, the metal film is between 50-500 nm thick. In some embodiments of these aspects, the metal film is between 75-200 nm thick.

[0043] In some embodiments of these fluidic biosensor devices, at least one dimension of the nanohole is between 10-1000 nm. In some embodiments of these aspects, at least one dimension of the nanohole is between 50-300 nm.

[0044] In some embodiments of these fluidic biosensor devices, the nanoholes are separated by a periodicity of between 100-1000 nm. In some embodiments of these aspects, the nanoholes are separated by a periodicity of between 400-800 nm.

[0045] In some embodiments of these fluidic biosensor devices, the activating agent used to activate the metal film is a piranha solution.

[0046] In some embodiments, the plasmonic nanostructure biosensors of the fluidic biosensor device further comprise an adhesion layer between the metal film and the substrate. In some embodiments of these fluidic biosensor devices, the adhesion layer comprises titanium, chromium, or any combination thereof. In some embodiments of these fluidic biosensor devices the adhesion layer is less than 50 nm. In some embodiments of these fluidic biosensor devices, the adhesion layer is less than 25 nm. In some embodiments of these fluidic biosensor devices, the adhesion layer is less than 15 nm.

[0047] In some embodiments of these fluidic biosensor devices, the activated metal film is further functionalized with one or more capture agents. In some embodiments of these fluidic biosensor devices, the capture agent is an antibody or antibody fragment thereof, a receptor, a

recombinant fusion protein, a nucleic acid molecule, or any combination thereof. In some embodiments of these fluidic biosensor devices, the one or more capture agents comprise a first capture agent and a second capture agent, wherein the first capture agent is specific for the second capture agent, and the second capture agent is specific for one or more biomolecular targets. In some embodiments of these fluidic biosensor devices, the first capture agent is protein A/G. In some embodiments of these aspects, the second capture agent comprises one or more antibodies or antibody fragments thereof.

[0048] In other aspects, described herein are methods of making a suspended plasmonic nanostructure sensor. In some aspects, the method comprises: depositing a silicon nitride film on a wafer; removing at least a portion of the silicon nitride film to form silicon nitride membranes; depositing positive e-beam resist over the wafer; performing e-beam lithography to transfer a nanohole pattern to the silicon nitride film through a dry etching process; and depositing at least one metal layer over the wafer.

[0049] In some embodiments of these methods, the wafer is silicon.

[0050] In some embodiments of these methods, the silicon nitride is deposited using Low Pressure Chemical Vapor Deposition (LPCVD).

[0051] In some embodiments of these methods, the at least a portion of the silicon nitride film can be removed using optical lithography, and one or more of dry and wet etching.

[0052] In some embodiments of these methods, the positive e-beam resist comprises poly(methyl methylacrylate) (PMMA). In some embodiments of these methods, the positive e-beam resist is removed using an oxygen plasma cleaning process.

[0053] In some embodiments of these methods, the at least one metal layer comprises is selected from a noble metal, a transition metal, or an alkali metal. In some embodiments of these methods, the depositing the at least one metal layer comprises depositing a Ti (titanium) metal layer and an Au (gold) metal layer.

[0054] In some embodiments of these methods, the at least one metal layer can define the nanohole pattern openings of the suspended plasmonic nanostructure sensor.

[0055] In other aspects, described herein are methods of making a biosensor that comprise depositing a positive e-beam resist over a substrate; and performing e-beam lithography to form an array of nanoholes in the substrate. In some embodiments of these aspects, the method also comprises depositing at least one metal layer over the substrate. In some embodiments of these methods, the at least one metal layer comprises is selected from a noble metal, a transition metal, or an alkali metal.

[0056] According to other aspects described herein, a biosensor device is disclosed that comprises a light source to generate light; a sensing structure comprising a first chamber, the first chamber comprising a fluid inlet, a second chamber, the second chamber comprising a fluid outlet, and a suspended nanostructure sensor between the first chamber and the second chamber, the suspended nanostructure sensor comprising a plurality of nanoholes, wherein an analyte is configured

to flow from the fluid inlet, through the nanoholes in the uspended nanostructure sensor and to the fluid outlet, where the suspended nanostructure sensor changes the refractive index of the light when the analyte flows through the nanoholes; and a detector to detect the changes to the refractive index. In some embodiments of these biosensor devices, the suspended nanostructure sensor is a photonic crystal sensor. In other embodiments of these biosensor devices, the suspended nanostructure sensor is a plasmonic nanostructure sensor.

[0057] In some embodiments of these biosensor devices, the upper chamber further comprises a glass surface, and the lower chamber further comprises a glass surface. In some embodiments of these biosensor devices, the sensor can further comprise a light source to direct light through one of the glass surfaces and, in some embodiments, a light detector to detect the light through the other one of the glass surfaces.

[0058] In some embodiments, the biosensor device further comprises a housing, where the upper chamber, lower chamber and suspended nanostructure sensor are enclosed in the housing. In some embodiments, the housing can comprise polydimethylsiloxane (PDMS).

Definitions

[0059] For convenience, certain terms employed herein, in the specification, examples and appended claims are collected here. Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. Unless explicitly stated otherwise, or apparent from context, the terms and phrases below do not exclude the meaning that the term or phrase has acquired in the art to which it pertains. The definitions are provided to aid in describing particular embodiments, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0060] The terms "nanostructure" and "nanomaterial," are used interchangeably herein and refer to any structure or device comprising a combination or association or plurality of one or more "nanoelements," as the term is used herein.

[0061] A "nanoelement," as the term is defined herein, has a dimension of a material at the atomic, molecular or macromolecular levels, in the length scale of approximately 1-3000 nm range, for example, in the range of about 1-2500 nm, in the range of about 1-2000 nm, in the range of about 1-1500 nm, in the range of about 1-1000 nm, in the range of about 10 nm to about 1000 nm, in the range of about 10 nm to about 750 nm, in the range of about 10 nm to about 500 nm, in the range of about 10 nm to about 250 nm, in the range of about 10 nm to about 100 nm, in the range of about 2 nm to about 100 nm, or in the range of about 2 nm to about 100 nm. Such a nanoelement, whether comprising a plasmonic material or a non-plasmonic material, can be present on, embedded (*e.g.*, depressed) within the surface of a substance or substrate, or as a void, hole, or aperture, present within a substance or substrate. A nanoelement can have one dimension of about 300 nm or less, about 250

nm or less, about 240 nm or less, about 230 nm or less, about 220 nm or less, about 210 nm or less, about 200 nm or less, about 190 nm or less, about 180 nm or less, about 170 nm or less, about 160 nm or less, about 150 nm or less, about 140 nm or less, about 130 nm or less, about 120 nm or less, about 110 nm or less, about 100 nm or less, about 90 nm or less, about 80 nm or less, about 70 nm or less, about 60 nm or less, about 50 nm or less, about 40 nm or less, about 30 nm or less, about 20 nm or less, or about 10 nm or less; and a second dimension of about 1500 nm or less, about 1400 nm or less, about 1300 nm or less, about 1200 nm or less, about 1100 nm or less, about 1000 nm or less, about 900 nm or less, about 800 nm or less, about 700 nm or less, about 600 nm or less, or about 500 nm or less. The nanoelements described herein can have a preselected shape and can be a nanotube, a nanowire, nanosphere, a nanorod, or any shape comprising the above-described dimensions (*e.g.*, triangular, square, rectangular, or polygonal shape in 2 dimensions, or cuboid, pyramidal, spherical, discoid, or hemispheric shapes in the 3 dimensions).

[0062] The term "predefined" as used with respect to "predefined pattern" refers to a pattern that is designed and selected to be used for the pattern of nanoelements in a nanostructure, such as an array of nanoparticles, or a pattern of nanoapertures. The pattern design can be selected so the nanoelements function in the pattern for collective excitation of plasmons and localized plasmon resonance.

[0063] The term "predefined" as used with respect to a "predefined shape" refers to a shape of a nanoelement that was determined and selected to be used for the shape of nanoelements in a nanostructure, or shape of the void of the nanoapertures of a nanostructure.

[0064] In some embodiments of the aspects described herein, a nanostructure can comprise a "photonic crystal." As used herein, a "photonic crystal" refers to a substance or material composed of periodic dielectric or metallo-dielectric nanoelements that affect the propagation of electromagnetic waves (EM). Essentially, photonic crystals contain regularly repeating internal regions of high and low dielectric constant. Photons (behaving as waves) propagate through this structure - or not - depending on their wavelength. Wavelengths of light that are allowed to travel are known as modes, and groups of allowed modes form bands. Disallowed bands of wavelengths are called photonic band gaps. This gives rise to distinct optical phenomena. The periodicity of the photonic crystal structure has to be of the same length-scale as half the wavelength of an incident EM wave, *i.e.*, the repeating regions of high and low dielectric constants have to be of this dimension. Accordingly, in some embodiments, a photonic crystal can be used as a nanostructure sensor in a biosensor device.

[0065] The term "periodicity," as used herein, refers to a recurrence or repetition of nanoelements on or within a nanostructure at regular intervals by their positioning on the nanostructure. The term "periodic" as used herein therefore refers to the regular predefined pattern of nanoelements with respect to each other.

[0066] The term "non-periodic," as used herein, refers to a pattern of nanoelements which are in a pattern, which is not a periodic pattern, or is not a lattice or other repeating unit configuration.

[0067] The term "unit cell" refers to a collection of nanoelements in a predefined pattern, where an organized arrangement of a number of unit cells forms a "lattice." Each nanoelement of a unit cell that belongs to one or more other unit cells in the lattice is referred to as a unit mode.

[0068] The term "lattice," as used herein, refers to a repeating or reoccurring pattern of a unit or unit cell, where the unit cell can comprise one or more nanoelements. Typically, a unit cell comprising one or more nanoelements has the nanoelements in an organized predefined pattern with respect to each other. In some embodiments, the term "lattice" as referred to herein refers to the order or the type of partially ordered set. In some embodiments the lattice can be a discrete subgroup, which refers to a discrete subgroup of a topological group of nanoelements with finite covolume. In some embodiments, the lattice is a group lattice, which refers to a repeating arrangement of nanoelements.

[0069] The term "controlled" as used herein refers to a non-random shape and/or non-random arrangement of nanoelements with respect to each other on, or depressed within a support or material of a nanostructure.

[0070] The term "geometric shape" refers to the 2D and/or 3D shape of a nanostructure.

[0071] A "nanoparticle," as described herein, refers to a nanoelement, whether comprising a plasmonic material or a non-plasmonic material, present on or embedded (*e.g.*, depressed) within the surface of a substance or substrate, having one dimension of about 300 nm or less, about 250 nm or less, about 240 nm or less, about 230 nm or less, about 220 nm or less, about 210 nm or less, about 200 nm or less, about 190 nm or less, about 180 nm or less, about 170 nm or less, about 160 nm or less, about 150 nm or less, about 140 nm or less, about 130 nm or less, about 120 nm or less, about 110 nm or less, about 100 nm or less, about 90 nm or less, about 80 nm or less, about 70 nm or less, about 60 nm or less, about 50 nm or less, about 40 nm or less, about 30 nm or less, about 20 nm or less, or about 10 nm or less; and a second dimension of about 1500 nm or less, about 1400 nm or less, about 1300 nm or less, about 1200 nm or less, about 1100 nm or less, about 1000 nm or less, about 900 nm or less, about 800 nm or less, about 700 nm or less, about 600 nm or less, or about 500 nm or less. The nanoparticles described herein can have a preselected shape and can be a nanotube, a nanowires, nanosphere, a nanorod, or any shape comprising the above-described dimensions (*e.g.*, triangular, square, rectangular, or polygonal shape in 2 dimensions, or cuboid, pyramidal, spherical, discoid, or hemispheric shapes in the 3 dimsensions).

[0072] A "nanohole" or "nanoaperture," as used interchangeably herein, refer to a nanoelement that is an opening or aperture in a plasmonic material, such as a metal film, or a non-plasmonic material, preferably a sub-wavelength opening, such as a hole, a gap or slit. As used herein, nanoholes include symmetric circular holes, spatially anistropic shapes, *e.g.*, elliptical shapes, slits, and also include any aperture of a triangular, square, rectangular, or polygonal shape. In some embodiments, a combination of different shaped nanoholes can be used. In addition, nanoholes can be "through nanoholes" that penetrate through a material, such as a metal film, or "non-through nanoholes" that penetrate a part of a material without completely penetrating through the material or

substrate. Preferably, a nanohole has a dimension of about 1500 nm or less, about 1400 nm or less, about 1300 nm or less, about 1200 nm or less, about 1100 nm or less, about 1000 nm or less, about 900 nm or less, about 800 nm or less, about 700 nm or less, about 600 nm or less, about 500 nm or less, about 450 nm or less, about 400 nm or less, about 350 nm or less about 300 nm or less, about 250 nm or less, about 240 nm or less, about 230 nm or less, about 220 nm or less, about 210 nm or less, about 200 nm or less, about 190 nm or less, about 180 nm or less, about 170 nm or less, about 160 nm or less, about 150 nm or less, about 140 nm or less, about 130 nm or less, about 120 nm or less, about 110 nm or less, about 100 nm or less, about 90 nm or less, about 80 nm or less, about 70 nm or less, about 60 nm or less, about 50 nm or less, about 40 nm or less, about 30 nm or less, about 20 nm or less, or about 10 nm or less.

[0073] "Surface plasmon resonance," as used herein, refers to the physical phenomenon in which incident light is converted strongly into electron currents at the metal surface for planar surfaces, and the term "localized surface plasmon resonance (LSPR)" can also be used for surface plasmon resonance of nanometer-sized structures. The oscillating currents produce strong electric fields in the (non-conducting) ambient medium near the surface of the metal. The electric fields, in turn, induce electric polarization in the ambient medium. Electric polarization is well known to cause the emission of light at wavelengths characteristic of the medium, *i.e.*, the "Raman wavelengths." Additional background information regarding this phenomenon may be found in Surface Enhanced Raman Scattering, ed. Chang & Furtak, Plenum Press, NY (1982), the entire disclosure of which is incorporated herein by reference. As used herein, the term "Raman scattering" is intended to encompass all related physical phenomena where an optical wave interacts with the polarizability of the material, such as Brillouin scattering or polariton scattering.

[0074] As used herein, "surface plasmons," "surface plasmon polaritons," or "plasmons" refer to the collective oscillations of free electrons at plasmonic surfaces, such as metals. These oscillations result in self-sustaining, surface electromagnetic waves, that propagate in a direction parallel to the metal/dielectric (or metal/vacuum) interface. Since the wave is on the boundary of the metal and the external medium (air or water for example), these oscillations are very sensitive to any change of this boundary, such as, for example, the adsorption of a biomolecular target to the metal surface. Subsequently, the oscillating electrons radiate electromagnetic radiation with the same frequency as the oscillating electrons. It is this re-radiation of light at the same incident wavelength that is referred to as "plasmon scatter." These oscillations can also give rise to the intense colors of solutions of plasmonic nanoparticles and/or intense scattering. In the case of metallic plasmonic nanoparticles, excitation by light results in localized collective electron charge oscillations, *i.e.*, "localized surface plasmon polaritions" (LSPRs). They exhibit enhanced near-field amplitude at the resonance wavelength. This field is highly localized at the nanoparticle and decays rapidly away from the plasmonic nanoparticle/dieletric interface into the dielectric background, though far-field scattering by the particle can also enhanced by the resonance. LSPR has very high spatial resolution at

a subwavelength level, and is determined by the size of plasmonic nanoparticles. "Plasmon absorption," as used herein, refers to the extinction of light (by absorption and scattering) caused by metal surface plasmons.

[0075] As used herein, a "plasmonic material" refers to a material that exhibits surface plasmon resonance when excited with electromagnetic energy, such as light waves, even though the wavelength of the light is much larger than the size of the material. In some embodiments of the aspects described herein, plasmonic materials refer to metallic plasmonic materials. Such metallic plasmonic materials can be any metal, including noble metals, alkali metals, transition metals, and alloys. Preferred plasmonic materials include, but are not limited to, gold, rhodium, palladium, silver, platinum, osmium, iridium, titanium, aluminum, copper, lithium, sodium, potassium, and nickel. A plasmonic material can be "optically observable" when it exhibits significant scattering intensity in the optical region (ultraviolet-visible-infrared spectra), which includes wavelengths from approximately 100 nanometers (nm) to 3000 nm. A plasmonic material can be "visually observable" when it exhibits significant scattering intensity in the wavelength band from approximately 380 nm to 750 nm, which is detectable by the human eye, *i.e.*, the visible spectrum.

[0076] As used herein, the term "plasmonic nanostructure" refers to any independent nanostructure, device, or system exhibiting surface plasmon resonance or localized surface plasmon resonance properties due to the presence, combination, or association of one or more plasmonic nanoelements, such as a nanoparticle or nanohole, as those terms are defined herein. For example, an array of nanoparticles or nanoholes is a plasmonic nanostructure. The plasmonic nanoelements can be arranged in any pattern that gives rise to a desired optical property for the nanostructure, such as periodic pattern or a non-periodic pattern, including pseudo-random and random patterns.

[0077] The term "plasmonic nanoelement," as used herein, refers to an individual, microscopic unit or nanoelement, as the term is defined herein, of a plasmonic material that exhibits surface plasmon resonance properties, having at least one dimension in the approximately 1-3000 nm range, for example, in the range of about 1-2500 nm, in the range of about 1-2000 nm, in the range of about 1-1500 nm, in the range of about 1-1000 nm, in the range of about 10 nm to about 1000 nm, in the range of about 10 nm to about 750 nm, in the range of about 10 nm to about 500 nm, in the range of about 10 nm to about 250 nm, in the range of about 10 nm to about 100 nm, in the range of about 2 nm to about 100 nm, or in the range of about 2 nm to about 100 nm. Such a unit or nanoelement of plasmonic material can be in the form of a nanoparticle, and present on or embedded within the surface of a substance or substrate, or can be in the form of a nanohole and present as an aperture within a plasmonic material, such as a metal film. In those embodiments where the nanoelement is a nanohole, the nanohole can cause or enhance the surface plasmon resonance properties of the plasmonic material in which it is present. Such a unit or nanoelement of plasmonic material can be disposed upon a substrate or material, such as a metal film, by which is meant the nanoelement of plasmonic material is in contact with, placed upon, etc. the substrate or material.

[0078] The term “support” refers to a conventional platform or scaffold in which to position the nanostructures comprising nanoelements in predefined patterns. As disclosed herein, supports can be conducting or non-conducting materials. Supports can also be planar supports, non-planar supports (e.g, curved supports), such as beads, particles, dipsticks, fibers, filters, membranes, cables, optical fibers and silane or silicate supports such as glass slides. Support can be flexible or non-flexible (*e.g.* solid supports). Supports can also be thin supports, elastic and/or strechable supports (e.g, for active tuning of resondance), and thick supports. Supports can be sticky and elastic, e.g, for use with nanostencil fabrication methods. A support can contact a nanostructure on one side or multiple sides, though preferably only on one side. In some embodiments of the aspects described herein, a nanostructure can be a “suspended nanostructure,” by which is meant that a support is provided on one side of a nanostructure at one or more ends on that side, such that at least some part of the nanostructure is not contacted with the support. In other embodiments, a support can contact a nanostructure at the entirety of one side.

[0079] As used herein, the term "resist" refers to both a thin layer used to transfer an image or pattern, such as a nanoelement pattern, to a substrate which it is deposited upon. A resist can be patterned via lithography to form a (sub)micrometer-scale, temporary mask that protects selected areas of the underlying substrate during subsequent processing steps, typically etching. The material used to prepare the thin layer (typically a viscous solution) is also encompassed by the term resist. Resists are generally mixtures of a polymer or its precursor and other small molecules (*e.g.*, photoacid generators) that have been specially formulated for a given lithography technology. Resists used during photolithography, for example, are called “photoresists.”

[0080] As used herein, “resist deposition” refers to the process whereby a precursor solution is spin-coated on a clean (*e.g.*, semiconductor) substrate, such as a silicon wafer, to form a very thin, uniform layer. The layer is baked at a low temperature to evaporate residual solvent, which is known as “soft bake.” This is followed by the “exposure” step, whereby a latent image is formed in the resist, *e.g.*, (a) via exposure to ultraviolet light through a photomask with opaque and transparent regions or (b) by direct writing using a laser beam or electron beam. Areas of the resist that have (or have not) been exposed are removed by rinsing with an appropriate solvent during the development step. This step is followed by the post-exposure bake step, which is followed by a step of processing through the resist pattern using, for example, wet or dry etching, lift-off, doping, or any combination thereof. The resist deposition process can then be ended via resist stripping.

[0081] As used herein, the process known as "lift-off" refers to the removal of residue of functional material adsorbed on the mask or stencil along with the template itself during template removal by, for example, dissolving it in a solvent solution.

[0082] The terms "analyte," "bioanalyte," “biomolecule,” "target," or "biomolecular target " refer to a molecule of interest that is to be analyzed, detected, and/or quantified in some manner. The analyte can be a biological species, including, but not limited to, nucleic acids (DNA, RNA, modified

oligonucleotides), proteins, carbohydrates, lipids, toxins, pathogens, bacterium cells, viral cells, cancer cells, normal cells, organisms, tissues. The analyte can be a Raman active compound or a Raman inactive compound. Further, the analyte can be an organic or inorganic molecule. Other types of biomolecular targets that can be detected by the nanostructure sensors described herein include low molecular weight molecules (*i.e.*, substances of molecular weight <1000 Daltons (Da) and between 1000 Da to 10,000 Da), and include amino acids, nucleic acids, lipids, carbohydrates, nucleic acid polymers, viral particles, viral components, cellular components, as well as materials chemically modified with biomolecules or small molecules. The analyte molecule can be a fluorescently labeled molecule, such as for example, DNA, RNA, or protein. Cellular components that can serve as biomolecular targets can include, but are not limited to, vesicles, mitochondria, membranes, structural features, periplasm, or any extracts thereof.Disease cells refer to cells that would be considered pathological by a person of ordinary skill in the art, such as a pathologist. Non-limiting examples of disease cells include tumor cells, gangrenous cells, virally or bacterially infected cells, and metabolically aberrant cells.

[0083] As used herein, the terms "sample," "biological sample" or "analyte" means any sample comprising or being tested for the presence of one or more biomolecular targets, including, but not limited to cells, organisms (bacteria, viruses), lysed cells or organisms, cellular extracts, nuclear extracts, components of cells or organisms, extracellular fluid, media in which cells or organisms are cultured *in vitro*, blood, plasma, serum, gastrointestinal secretions, homogenates of tissues or tumors, synovial fluid, feces, saliva, sputum, cyst fluid, amniotic fluid, cerebrospinal fluid, peritoneal fluid, lung lavage fluid, semen, lymphatic fluid, tears, pleural fluid, nipple aspirates, breast milk, external sections of the skin, respiratory, intestinal, and genitourinary tracts, and prostatic fluid. In addition, a sample can be a viral or bacterial sample, a sample obtained from an environmental source, such as a body of polluted water, an air sample, or a soil sample, as well as a food industry sample. In some embodiments, a "biological sample" also refers to a cell or population of cells or a quantity of tissue or fluid from a subject. Most often, a sample has been removed from a subject, but the term "biological sample" can also refer to cells or tissue analyzed *in vivo, i.e.*, without removal from the subject. Often, a "biological sample" will contain cells from a subject, but the term can also refer to non-cellular biological material, such as non-cellular fractions of blood, saliva, or urine, that can be used to measure protein phosphorylation levels. In some embodiments, a biological sample is from a resection, bronchoscopic biopsy, or core needle biopsy of a primary, secondary or metastatic tumor, or a cellblock from pleural fluid. In addition, fine needle aspirate biological samples are also useful. In some embodiments, a biological sample is primary ascite cells. Biological samples also include explants and primary and/or transformed cell cultures derived from patient tissues. A biological sample can be provided by removing a sample of cells from subject, but can also be accomplished by using previously isolated cells (*e.g.*, isolated by another person, at another time, and/or for another purpose). Archival tissues, such as those having treatment or outcome history may also be used.

Biological samples include, but are not limited to, tissue biopsies, scrapes (*e.g.* buccal scrapes), whole blood, plasma, serum, urine, saliva, cell culture, or cerebrospinal fluid. Biological samples also include tissue biopsies, cell culture. The sample can be obtained by removing a sample of cells from a subject, but can also be accomplished by using previously isolated cells (*e.g.* isolated by another person), or by performing the methods described herein, *e.g.*, using a nanostructure biosensor device a as a probe to measure a target molecule in the body of a subject, *e.g.*, a human subject.

[0084] "Tissue" is defined herein as a group of cells, often of mixed types and usually held together by extracellular matrix, that perform a particular function. Also, in a more general sense, "tissue" can refer to the biological grouping of a cell type result from a common factor; for example, connective tissue, where the common feature is the function or epithelial tissue, where the common factor is the pattern of organization.

[0085] As used herein, a "capture agent" refers to any agent having specific binding for a biomolecular target that can be immobilized on the surface of a plasmonic nanostructure, including, but not limited to, a nucleic acid, oligonucleotide, peptide, polypeptide, antigen, polyclonal antibody, monoclonal antibody, single chain antibody (scFv), F(ab) fragment, F(ab')$_2$ fragment, Fv fragment, small organic molecule, polymer, compounds from a combinatorial chemical library, inorganic molecule, or any combination thereof.

[0086] A "nucleic acid", as described herein, can be RNA or DNA, and can be single or double stranded, and can be, for example, a nucleic acid encoding a protein of interest, a polynucleotide, an oligonucleotide, a nucleic acid analogue, for example peptide- nucleic acid (PNA), pseudo-complementary PNA (pc-PNA), locked nucleic acid (LNA) etc. Such nucleic acid sequences include, for example, but are not limited to, nucleic acid sequence encoding proteins, for example that act as transcriptional repressors, antisense molecules, ribozymes, small inhibitory nucleic acid sequences, for example, but not limited to, RNAi, shRNAi, siRNA, micro RNAi (mRNAi), antisense oligonucleotides etc.

[0087] As used herein, the term "DNA" is defined as deoxyribonucleic acid. The term "polynucleotide" is used herein interchangeably with "nucleic acid" to indicate a polymer of nucleosides. Typically a polynucleotide of this invention is composed of nucleosides that are naturally found in DNA or RNA (*e.g.*, adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine) joined by phosphodiester bonds. However the term encompasses molecules comprising nucleosides or nucleoside analogs containing chemically or biologically modified bases, modified backbones, etc., whether or not found in naturally occurring nucleic acids, and such molecules may be preferred for certain applications. As used herein, a polynucleotide is understood to include both DNA, RNA, and in each case both single- and double-stranded forms (and complements of each single-stranded molecule). "Polynucleotide sequence" as used herein can refer to the polynucleotide material itself and/or to the sequence information (*i.e.*, the succession of letters used as abbreviations for bases) that biochemically characterizes a specific

nucleic acid. A polynucleotide sequence presented herein is presented in a 5' to 3' direction unless otherwise indicated.

[0088] The term " polypeptide" as used herein refers to a polymer of amino acids. The terms "protein" and "polypeptide" are used interchangeably herein. A peptide is a relatively short polypeptide, typically between about 2 and 60 amino acids in length. Polypeptides used herein typically contain amino acids such as the 20 L-amino acids that are most commonly found in proteins. However, other amino acids and/or amino acid analogs known in the art can be used. One or more of the amino acids in a polypeptide may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a phosphate group, a fatty acid group, a linker for conjugation, functionalization, etc. A polypeptide that has a nonpolypeptide moiety covalently or noncovalently associated therewith is still considered a "polypeptide." Exemplary modifications include glycosylation and palmitoylation. Polypeptides may be purified from natural sources, produced using recombinant DNA technology, synthesized through chemical means such as conventional solid phase peptide synthesis, etc. The terms "polypeptide sequence" or "amino acid sequence" as used herein can refer to the polypeptide material itself and/or to the sequence information (*i.e.*, the succession of letters or three letter codes used as abbreviations for amino acid names) that biochemically characterizes a polypeptide. A polypeptide sequence presented herein is presented in an N-terminal to C-terminal direction unless otherwise indicated.

[0089] "Receptor" is defined herein as a membrane-bound or membrane-enclosed molecule that binds to, or responds to something more mobile (the ligand), with high specificity.

[0090] "Ligand" is defined herein as a molecule that binds to another; in normal usage a soluble molecule, such as a hormone or neurotransmitter, that binds to a receptor. Also analogous to "binding substance" herein.

[0091] "Antigen" is defined herein as a substance inducing an immune response. The antigenic determinant group is termed an epitope, and the epitope in the context of a carrier molecule (that can optionally be part of the same molecule, for example, botulism neurotoxin A, a single molecule, has three different epitopes. See Mullaney *et al.*, Infect Immun October 2001; 69(10): 6511-4) makes the carrier molecule active as an antigen. Usually antigens are foreign to the animal in which they produce immune reactions.

[0092] As used herein, “antibodies” can include polyclonal and monoclonal antibodies and antigen-binding derivatives or fragments thereof. Well-known antigen binding fragments include, for example, single domain antibodies (dAbs; which consist essentially of single VL or VH antibody domains), Fv fragment, including single chain Fv fragment (scFv), Fab fragment, and $F(ab')_2$ fragment. Methods for the construction of such antibody molecules are well known in the art. As used herein, the term "antibody" refers to an intact immunoglobulin or to a monoclonal or polyclonal antigen-binding fragment with the Fc (crystallizable fragment) region or FcRn binding fragment of

the Fc region. Antigen-binding fragments can be produced by recombinant DNA techniques or by enzymatic or chemical cleavage of intact antibodies. "Antigen-binding fragments" include, inter alia, Fab, Fab', F(ab')2, Fv, dAb, and complementarity determining region (CDR) fragments, single-chain antibodies (scFv), single domain antibodies, chimeric antibodies, diabodies and polypeptides that contain at least a portion of an immunoglobulin that is sufficient to confer specific antigen binding to the polypeptide. The terms Fab, Fc, pFc', F(ab') 2 and Fv are employed with standard immunological meanings [Klein, Immunology (John Wiley, New York, N.Y., 1982); Clark, W. R. (1986) The Experimental Foundations of Modern Immunology (Wiley & Sons, Inc., New York); Roitt, I. (1991) Essential Immunology, 7th Ed., (Blackwell Scientific Publications, Oxford)].

[0093] "Polyclonal antibody" is defined herein as an antibody produced by several clones of B-lymphocytes as would be the case in a whole animal, and usually refers to antibodies raised in immunized animals. "Monoclonal antibody" is defined herein as a cell line, whether within the body or in culture, that has a single clonal origin. Monoclonal antibodies are produced by a single clone of hybridoma cells, and are therefore a single species of antibody molecule. "Single chain antibody (Scfv)" is defined herein as a recombinant fusion protein wherein the two antigen binding regions of the light and heavy chains (Vh and Vl) are connected by a linking peptide, which enables the equal expression of both the light and heavy chains in a heterologous organism and stabilizes the protein. "F(Ab) fragment" is defined herein as fragments of immunoglobulin prepared by papain treatment. Fab fragments consist of one light chain linked through a disulphide bond to a portion of the heavy chain, and contain one antigen binding site. They can be considered as univalent antibodies. "F(Ab')$_2$ Fragment" is defined herein as the approximately 90 kDa protein fragment obtained upon pepsin hydrolysis of an immunoglobulin molecule N-terminal to the site of the pepsin attack. Contains both Fab fragments held together by disulfide bonds in a short section of the Fe fragment. "Fv Fragment" is defined herein as the N-terminal portion of a Fab fragment of an immunoglobulin molecule, consisting of the variable portions of one light chain and one heavy chain.

[0094] As used herein, the term "small molecule" refers to a chemical agent including, but not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, aptamers, nucleotides, nucleotide analogs, organic or inorganic compounds (*i.e.*, including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

[0095] As used herein, the term "polymer" is used in the broad sense and is intended to include a wide range of biocompatible polymers, for example, but not limited to, homopolymers, co-polymers, block polymers, cross-linkable or crosslinked polymers, photoinitiated polymers,

chemically initiated polymers, biodegradable polymers, nonbiodegradable polymers, and the like. In other embodiments, the prevascularized construct comprises a polymer matrix that is nonpolymerized, to allow it to be combined with a tissue, organ, or engineered tissue in a liquid or semi-liquid state, for example, by injection. In certain embodiments, the prevascularized construct comprising liquid matrix may polymerize or substantially polymerize "in situ." In certain embodiments, the prevascularized construct is polymerized or substantially polymerized prior to injection. Such injectable compositions are prepared using conventional materials and methods know in the art, including, but not limited to, Knapp *et al.*, Plastic and Reconstr. Surg. 60:389 405, 1977; Fagien, Plastic and Reconstr. Surg. 105:362 73 and 2526 28, 2000; Klein *et al.*, J. Dermatol. Surg. Oncol. 10:519 22, 1984; Klein, J. Amer. Acad. Dermatol. 9:224 28, 1983; Watson *et al.*, Cutis 31:543 46, 1983; Klein, Dermatol. Clin. 19:491 508, 2001; Klein, Pedriat. Dent. 21:449 50, 1999; Skorman, J. Foot Surg. 26:511 5, 1987; Burgess, Facial Plast. Surg. 8:176 82, 1992; Laude *et al.*, J. Biomech. Eng. 122:231 35, 2000; Frey *et al.*, J. Urol. 154:812 15, 1995; Rosenblatt *et al.*, Biomaterials 15:985 95, 1994; Griffey *et al.*, J. Biomed. Mater. Res. 58:10 15, 2001; Stenburg *et al.*, Scfand. J. Urol. Nephrol. 33:355 61,1999; Sclafani *et al.*, Facial Plast. Surg. 16:29 34, 2000; Spira *et al.*, Clin. Plast. Surg. 20:181 88, 1993; Ellis *et al.*, Facila Plast. Surg. Clin. North Amer. 9:405 11, 2001; Alster *et al.*, Plastic Reconstr. Surg. 105:2515 28, 2000; and U.S. Pat. Nos. 3,949,073 and 5,709,854.

[0096] As used herein, the term "drug" or "compound" refers to a chemical entity or biological product, or combination of chemical entities or biological products, administered to a person to treat or prevent or control a disease or condition. The chemical entity or biological product is preferably, but not necessarily a low molecular weight compound, but may also be a larger compound, for example, an oligomer of nucleic acids, amino acids, or carbohydrates including, without limitation, proteins, oligonucleotides, ribozymes, DNAzymes, glycoproteins, siRNAs, lipoproteins, aptamers, and modifications and combinations thereof.

[0097] The terms "label" or "tag", as used herein, refer to a composition capable of producing a detectable signal indicative of the presence of the target in an assay sample. Suitable labels include radioisotopes, nucleotide chromophores, enzymes, substrates, fluorescent molecules, chemiluminescent moieties, magnetic particles, bioluminescent moieties, and the like. As such, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means.

[0098] The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.*, at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element. Thus, in this specification and the appended claims, the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, reference to a pharmaceutical composition comprising "an agent" includes reference to two or more agents.

[0099] As used herein, the term "comprising" means that other elements can also be present in addition to the defined elements presented. The use of "comprising" indicates inclusion rather than limitation. The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment. As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention. Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used in connection with percentages can mean ±1%.

[00100] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art to which this disclosure belongs. It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such can vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims. Definitions of common terms in immunology, and molecular biology can be found in The Merck Manual of Diagnosis and Therapy, 18th Edition, published by Merck Research Laboratories, 2006 (ISBN 0-911910-18-2); Robert S. Porter *et al.* (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8); Immunology by Werner Luttmann, published by Elsevier, 2006. Definitions of common terms in molecular biology are found in Benjamin Lewin, Genes IX, published by Jones & Bartlett Publishing, 2007 (ISBN-13: 9780763740634); Kendrew *et al.* (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), Maniatis *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (1982); Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (2 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (1989); Davis *et al.*, Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (1986); or Methods in Enzymology: Guide to Molecular Cloning Techniques Vol.152, S. L. Berger and A. R. Kimmerl Eds., Academic Press Inc., San Diego, USA (1987); Current Protocols in Molecular Biology (CPMB) (Fred M. Ausubel, *et al.* ed., John Wiley and Sons, Inc.), Current Protocols in Protein Science (CPPS) (John E. Coligan, et. al., ed., John Wiley and Sons, Inc.) and Current Protocols in Immunology (CPI) (John E. Coligan, et. al., ed. John Wiley and Sons, Inc.), which are all incorporated by reference herein in their entireties.

BRIEF DESCRIPTION OF THE DRAWINGS

[00101] The accompanying drawings, which are incorporated in and constitute a part of this specification, exemplify the embodiments of the present invention and, together with the description, serve to explain and illustrate principles of the invention. The drawings are intended to illustrate major features of the exemplary embodiments in a diagrammatic manner. The drawings are not intended to depict every feature of actual embodiments nor relative dimensions of the depicted elements, and are not drawn to scale.

[00102] **FIGURES 1A-1D** illustrate a biosensor according to one embodiment of the invention. Illustration of the actively controlled flow scheme is shown in **FIGURE 1A**. The nanohole arrays are used as sensing structures as well as nanofluidic channels. This is contrary to the conventional approach in which the convective flow stream passes over the sensor (**FIGURE 1B**). **FIGURES 1C** and **1D** show steady state velocity distribution for the actively (FIGURE 1A) and the passively (FIGURE 1B) controlled convective flow schemes.

[00103] **FIGURES 2A-2D** illustrate a method of making the biosensor according to one embodiment of the invention using a lift-off free fabrication process 200. E-beam lithography is shown in **FIGURE 2A**. A nanohole pattern (with hole diameters of approximately 220nm and a periodicity of approximately 600nm) is transferred to the suspended SiNx film through a dry etching process. The e-beam resist is then removed with an oxygen plasma cleaning process leaving only a patterned SiNx film with air on both sides. Only a small shrinking in nanohole diameter (<4%) is observed after gold deposition due to slight coverage of the metal layers on the nanohole sidewalls, as shown in **FIGURES 2B-2D**.

[00104] **FIGURES 3A-3B** demonstrate experimental implementation of a sensor comprising square lattice SiN_x PhC slabs. **FIGURE 3A** shows the transmission spectra of a specific design calculated by three dimensional finite-difference time-domain (3D-FDTD) method in three different media: air (refractive index n = 1), water (n = 1.33), and an IPA-chloroform mixture (n = 1.43). A normally incident plane wave source (corresponding to the □-point in the dispersion diagram) excites the eigenmodes of the system. For each case, two modes are observed within the given spectral range. **FIGURE 3B** shows the intensity distribution of the lowest (first) order mode when the structure is in air.

[00105] **FIGURES 4A-4D** show video images of the perpendicular convective flow, captured in a microscope with a CCD camera. FIGUREs 4A-4D show the merge of IPA to the top channel only through the openings, confirming the active steering of the liquid flow. No damage or breakage of the membrane due to the applied pressure is observed.

[00106] **FIGURES 5A-5C** show a comparison of transmitted spectra of PhCs to experimentally evaluate the sensing response of the different flow schemes by launching a collimated and unpolarized light at normal incidence.

[00107] **FIGURES 6A-6B** demonstrate testing of bulk sensitivity of PhCs by successively applying five different solutions through the directed flow scheme: DI-water, acetone, IPA and two IPA-chloroform mixtures with refractive indices of 1, 1.33, 1.356, 1.377, 1.401 and 1.424, respectively. As shown in **FIGURE 6A**, with increasing refractive index the resonances red-shift and the line-widths become narrower. **FIGURE 6B** shows shifts of the 1st resonant peaks in wavelength versus the surrounding refractive index change. Resonance peak positions found in experiments (stars) match very well with the simulation results (circles). The line is a linear fitting to the experimental results.

[00108] **FIGURES 7A-7B** compare a cross-polarization spectrum with a regular one. The spectra are taken when the structure is in air. Cross-polarization measurements clearly isolate two distinct resonance features from the background (**FIGURE 7A**). A single Lorentzian with 7 nm line-width fits very well with the second order mode resonance (**FIGURE 7B**). On the other hand, two Lorenztians are needed to fit the lowest order mode (**FIGURE 7B**). This indicates a potential resonance splitting for the lowest order mode, which could be due to a slight non-uniformity in fabrication. The addition of three Lorentzians (dashed curve in **FIGURE 7B**) matches very well with the experimentally measured spectrum.

[00109] **FIGURES 8A-8D** show targeted delivery of analytes to a sensor surface. **FIGURE 8A** shows bulk refractive index sensitivity of plasmonic nanohole arrays obtained in different solutions. **FIGURE 8B** demonstrates resonance shifts for the passively and actively controlled mass transport schemes compared after running IPA (analyte) for 10 min at 20 μm/min flow rate. Microfluidic simulations demonstrate low transfer rates for the passive transport scheme due to the weaker perpendicular flow of the analytes (**FIGURE 8C**), while **FIGURE 8D** demonstrates much more efficient mass transport toward the surface observed for the targeted delivery scheme.

[00110] **FIGURE 9** demonstrates efficiencies of the passive (triangles) and targeted (squares) delivery of the analytes compared in real time measurements. A 14-fold improvement in mass transport rate constant is observed for the targeted delivery scheme.

[00111] **FIGURES 10A-10D** show 3-D renderings (not drawn to scale), and experimental measurements illustrating a detection scheme using optofluidic-plasmonic biosensors based on resonance transmissions due to extraordinary light transmission effect. **FIGURE 10A** shows detection (immobilized with capturing antibody) and control sensors. **FIGURE 10B** demonstrates that VSV only attaches to the antibody immobilized sensor. **FIGURE 10C** demonstrates that no observable shift is detected for the control sensor after the VSV incubation and washing. **FIGURE 10D** demonstrates accumulation of VSV due to capture by immobilized antibodies. A large effective refractive index increase results in strong red-shifting of the plasmonic resonances (~100 nm).

[00112] **FIGURES 11A-11F** summarize a fabrication process. **FIGURE 11A** shows free standing membranes spin coated with positive e-beam resist, and e-beam lithography performed. **FIGURE 11B** shows that a nanohole pattern is transferred to a SiNx membrane through RIE

processes. **FIGURE 11C** shows that an oxygen cleaning process results in a free standing photonic crystal like structure. **FIGURE 11D** demonstrates that metal deposition results in a free standing optofluidic-nanoplasmonic biosensor with no clogging of the holes. **FIGURE 11E** shows scanning electron microscope images of patterned SiNx membranes before gold deposition. **FIGURE 11F** demonstrates that gold deposition results in suspended plasmonic nanohole sensors without any lift-off process. No clogging of the nanohole openings is observed (*inset*).

[00113] **FIGURES 12A-12B** depict a representative immunosensor function. **FIGURE 12A** shows a schematic of an immunosensor surface functionalization. Anti-viral immunoglobulins are attached from the Fc region to the surface through a protein A/G layer. **FIGURE 12B** shows sequential functionalization of the bare sensing surface (dark line) for the optofluidic-nanohole sensors with a sensitivity of FOM0. Immobilization of the protein A/G (medium line) and viral antibody layer (light line to the right) results in the red shifting of the EPT resonance by 4 nm and 14 nm.

[00114] **FIGURES 13A-13D** demonstrate detection of PT-Ebola viruses and vaccinia viruses. Detection of PT-Ebola virus (**FIGURE 13A**) and vaccinia viruses (**FIGURE 13C**) are shown in spectral measurements at a concentration of 10^8 PFU/ml. **FIGUREs 13B** and **13D** demonstrate repeability of the measurements obtained from multiple sensors (dark). Minimal shifting due to non-specific bindings are observed in reference spots (light).

[00115] **FIGURES 14A-14B** demonstrate applicability of inventors' optofluidic plasmonic nanostructure detection platforms in biologically relevant systems shown by virus detection measurements performed in cell culturing media. **FIGURE 14A** shows non-specific binding to control spots results in a 1.3 nm red-shifting of plasmonic resonances. Measurements are also obtained for control spots after each incubation process, although control sensor surfaces are not functionalized with protein A/G and antibody. **FIGURE 14B** demonstrates that a resonance shift of 4 nm is observed for the detection of sensor resonance showing that the specific capturing of intact viruses at a low concentration of 10^6 PFU/ml is clearly distinguishable at the antibody functionalized sensors.

[00116] **FIGURE 15** depicts exemplary processes present in living organisms, and that biomolecular recognition processes are important for understanding and tracking biomolecular functions. Further, association and dissociation rates (k_A and k_D) are set by affinities between two different biomolecules, which controls the likelihood of a biomolecular recognition event.

[00117] **FIGURE 16** depicts how in a surface biosensor, an analyte or biomolecules are captured by ligands attached on a detection surface, and how once an analyte is captured, this can be reported by the surface biosensor as a change in physical characteristics of the detection surface (*e.g.*, optical, mechanical, electrical, etc.).

[00118] **FIGURE 17** depicts how in a surface biosensor the likelihood of a biomolecular recognition event, in addition to being controlled by association/dissociation rates of ligand/analyte

pairs, is also dependent on availability of a diffused analyte towards the surface. Diffusion rate (k_{diff}). of an analyte to a ligand attached to a sensing surface controls the likelihood or rate of biomolecular recognition events, in addition to association/disassociation rates of the complexes.

[00119] **FIGURE 18** is an adaptation from P.E. Sheehan and L.J. Whitman (Nano Lett., 5(4), p. 803-807, 2005) illustrating how diffusion processes can be a limiting factor of the sensitivity of a biosensor, which is termed herein as the "mass transport limitation problem." Such mass transport limitation problems can result in impractically long detection times due to the need for extended accumulation times of enough biomolecules for detectable signal levels. As illustrated in Figure 18, depending on a biosensor's dimensions, months to years can be needed for detectable amount of biomolecules to be collected per unit area. While bigger sensors can allow analytes to be collected faster due to larger surface areas, the amount of molecules per detection area does not improve significantly with increasing dimensions. Accordingly, extended periods of accumulation time are needed due to mass transport limitation.

[00120] **FIGURE 19** is an adaptation from David G. Myszka *et al.* (Biophysical Journal 75, p. 583-594, 1998) depicting that using microfluidic channels to replenish analytes in a biosensor does not improve mass transport limitations either, since laminar flow effects in a microchannel cause fluid to move most rapidly in the middle of the channel, such that replenishment of the analytes close to the sensing surface is minimal. Thus, as shown herein, without methods to actively direct biomolecules to a sensor surface, biosensors are subject to mass transport limitations, resulting in impractical time scales for detection.

[00121] **FIGURE 20** demonstrates, as described herein, direct targeting of analytes to a sensing surface by connecting two different layers of microfluidic channels through nanohole openings in suspended plasmonic nanohole and photonic crystal biosensors.

[00122] **FIGURE 21** illustrates a multi-inlet/multi-outlet fluidic platform that allows fluidic flow in three dimensions to be actively controlled through plasmonic nanohole openings. Fabricated arrays are mounted in a custom designed multilayered multifluidic channel system based on poly(dimethylsiloxane) (PDMS). Connnective flow is steered perpendicularly towards the plasmonic sensing surface by allowing flow only through one inlet/outlet on either side of the plasmonic sensor. Flow can be directed, for example, from top-to-down and down-to-top directions by enabling flow between 1 to 4 and 3 to 2 respectively.

[00123] **FIGURE 22** depicts an exemplary single-run scheme in which biological solution is wasted after running through a microfluidics platform. In such a single-run scheme, flow can be top-to-down or down-to-top.

[00124] **FIGURE 23** depicts an exemplary continuous recycle run scheme in which biological solution or sample is recycled after running through a microfluidics platform, so that any analytes in the solution are washed. In such a continuous recycle run scheme, after closing valve #3 and #2, fow can be circulated from input line #1 to #4 through a parasitic pump for continuous running of the

analyte. Such a scheme can be rearranged, for example, so that flow comes from bottom valve #3 and recycled back to parasitic pump through valve #2.

[00125] **FIGURES 24A-24C** depict using targeted delivery in other surface sensors. As shown herein, using nanoholes for targeted delivery is not limited to only photonic crystal/plasmonic based sensors and can be employed by other surface sensors. For example, a nanohole platform can be used as a fluorescence based sensor for ELISA analyses. Instead of using a plain glass surface a nanohole platform can be used to transfer a sample through nanoholes for more efficient antigen + antibody binding. As shown in the steps depicted in **FIGURES 24A-24B**, in a convention ELISA set-up, a sample or standard is incubated on an antibody immobilized surface. Target molecules present in the sample reach the capture agents, *i.e.*, antibodies, on the device surface mainly by diffusion processes. The sample is then washed to remove unbound molecule(s) that do not have a corresponding antibody (circles). In consecutive steps, the presence of captured target molecules from the sample is read out by labeled antibodies through enzymatic reactions. **FIGURE 24C** depicts using a surface patterned with nanohole arrays instead of a plain surface, as described herein. A sample or standard is incubated with the surface patterned with nanohole arrays, and then flow through the nanoholes. In such a manner, target molecules in a sample can be actively directed towards capture molecules on the device surface.

DETAILED DESCRIPTION

[00126] Described herein are label-free nanostructure sensors, such as plasmonic nanostructure sensors, and systems, devices, and methods of use thereof for targeting and detection of a variety of biomolecular targets. The sensing platforms described herein are based on extraordinary light transmission effect in suspended nanoholes, such as suspended plasmonic nanoholes. Also provided herein are sensing platforms or systems comprising a multilayered microfluidics scheme for contacting a sample to a plasmonic nanostructure sensor that allows three-dimensional control of fluidic flow by connecting layers of microfluidic channels through plasmonic nanoholes. This scheme, in some embodiments, comprises a hybrid biosensing system that merges plasmonics and fluidics into a single sensing platform or system using nanoholes. The nanoholes of the plasmonic nanostructure act as nanofluidic channels connecting fluidic chambers on both sides of the sensors. Embodiments described herein result in a fourteen-fold improvement in mass transport rate constants. These improvements results in superior analyte delivery to the biosensor surface at low concentrations. Another exemplary advantageous feature is an extra degree of freedom in microfluidic circuit engineering by connecting separate layers of microfluidic circuits through plasmonic nanostructure biosensors. These approaches make it possible to create "multilayered lab-on-chip systems" allowing three dimensional control of the fluid flow.

[00127] To fabricate the nanostructures sensors of the sensor devices, a lift-off free plasmonic device fabrication technique based on positive resist electron beam lithography (EBL) can be used. The simplicity of this fabrication technique allows fabrication of nanostructures with extremely high yield/reproducibility and minimal surface roughness.

[00128] An aspect of the invention is described herein in detail with reference to FIGURES 1A and 1B. A sensor device comprising free-standing PhCs (photonic crystals) sealed in a chamber or housing is provided such that only the nanohole arrays of the photonic crystals enable the flow between the top and the bottom channels. Illustration of the actively controlled flow scheme is shown in FIGURE 1A. Solution directed to the structure surface goes through the nanoholes of the arrays and flows to the bottom channel. Accordingly, in such aspects, the nanohole arrays are used as sensing structures as well as nanofluidic channels. This is contrary to the conventional approach in which the convective flow stream passes over the sensor (FIGURE 1B).

[00129] In some embodiments of these aspects, the housing of the sensing platform comprises sidewalls made of polydimethylsiloxane (PDMS), an upper surface made of glass, and a lower surface made of glass. In some embodiments of these aspects, the nanostructure sensor structure is suspended between the upper and lower glass surfaces. The housing also comprises, in some embodiments, a fluid inlet/outlet in at least one of the chambers, and at least one fluid inlet/outlet in the other one of the chambers. It will be appreciated that both of the chambers can comprise two or more fluid inlet/outlets. In some embodiments, valves, an air regulation system, one or more controllers, or any combination thereof can be used to control the flow in the sensing structure. Analytes that are delivered through the fluidic inlet flow through one chamber (upper chamber or lower chamber) over the sensing structure and through the nanoholes and leave the nanostructure sensor structure through the outlet in the other chamber (lower chamber or upper chamber). This offers an extra degree of freedom in microfluidic circuit engineering by connecting separate layers of microfluidic circuits through biosensors comprising nanoholes.

[00130] It will be appreciated that, in some embodiments, a optical source is provided that generates light and directs it toward the sensing membrane (*e.g.*, through the glass surface of the upper chamber). It will also be appreciated that a detector is also provided, in some embodiments of these aspects, to sense the refractive changes in the nanostructure sensor.

[00131] In some embodiments of the aspects described herein, in order to implement the proposed scheme, photonic crystal structures are used on or supported on free-standing membranes as nanostructure sensors. In some embodiments, the membranes are mechanically robust Low Pressure Chemical Vapor Deposition (LPCVD) silicon nitride (SiNx) films. In addition, LPCVD SiNx films that can be used are transparent in the visible/near-infrared regime with high refractive index. In some embodiments, the films can then be coated with one or more metals, such as titanium (Ti) or gold (Au).

[00132] As shown herein, the flow profile using the novel nanostructure sensor platforms described herein was compared to the flow profile with the conventional approach by numerically solving Navier-Stokes equations using finite element method in COMSOL™. The simulations are done in two-dimensions using incompressible isothermal fluid flow. In the model, two microfluidic channels (on top and bottom) with 200 μm in length and 50 μm in height were used. A row of ten rods spaced by 0.6 μm represents the nanohole arrays. The opening at the top left side of the microfluidic channel is used as the inlet to flow the solution (water) to the chamber at a velocity of 10^{-6} m/s. The openings at the bottom and the top right side with no pressure applied are used as an outlet for the actively controlled and the conventional fluidic flow schemes, respectively. The spacing between the rods is defined as continuous boundary which allows the solution to flow through, while the other boundaries are treated as no slip walls.

[00133] As illustrated in FIGURES 1A-1B, this multi-inlet/outlet fluidic nanostructure sensor platform allows for active control of the fluidic flow in three dimensions through the nanohole openings. Convective flow over different surfaces of the plasmonic nanostructure sensor is realized by running the solutions in between input-output lines on the same side, such as 1 →2/3→4 (FIGURE 1A). The convective flow in separate channels is nearly independent. In the actively controlled (targeted) delivery scheme shown herein, the convective flow is steered perpendicularly towards the plasmonic nanostructure sensor surface by allowing the flow only through one inlet/outlet on either side of the plasmonic nanostructure sensor (FIGURE 1B). Flow could be directed from top-to-down and down-to-top directions by enabling flow between 1 →4 and 3→2, respectively.

[00134] FIGURES 1C and 1D show steady state velocity distribution for the actively (FIGURE 1A) and the passively (FIGURE 1B) controlled convective flow schemes. Flow profiles around photonic crystal regions are shown in detail (insets). For the passively controlled scheme (FIGURE 1D), as the viscous forces in the fluid dominate over the inertial forces, the formation of laminar flow profile was observed. The convective flow is fast close to the center of the channel but becomes very slow near the edges. This indicates that using such a conventional scheme in an immunoassay based sensing applications, the depletion zones will extend further from the sensor surface causing ever slower analyte transport for detection of a biomolecular target. One can increase the convective flow rate to shrink the depletion zones. However, such a passive (indirect) control only results in moderate improvements in mass transport rates.

[00135] One approach described herein, according to the illustrated embodiment of FIGURE 1A, overcomes the mass transport limitation by steering convective flow directly towards the sensing surface. This is demonstrated in a microfludic simulation in FIGURE 1C where the convective flow is still very strong around the sensing surface and turbulences (*i.e.*, stirring of the solution) are generated around the holes. Such a directed flow can strongly improve the delivery of the analytes or samples to a sensor surface. This scheme also helps to overcome the surface tension of highly viscous solution and guarantees that the fluidic nanostructure sensor devices described herein can be totally

immersed in solution. In this way, as both sides of the structure are exposed to the solution, the sensitivity is further enhanced. The nanofluidic channels also create turbulences and stir the solution as it passes through the nanostructure sensor structure, further increasing the mass transport.

[00136] Targeted delivery of analytes to the sensing surface has been demonstrated using spectral measurements as shown in FIGURES 8A-8D. Initially, both the top and the bottom channels are filled with a low refractive index liquid, deionized (DI) water (n_{DI} = 1.333), at a high flow rate (550μL/min). Once the channels are filled with DI water completely, the plasmonic resonance shifts from $\lambda_{air} = 679nm$ (air on both sides) to $\lambda_{DI} = 889nm$ (DI on both sides). This corresponds to a bulk refractive index sensitivity of $\Delta\lambda/\Delta n = 630nm / RIU$. As plasmons at the Ti/SiN interface are suppressed by the losses, this shift only reflects the response of the plasmons on the gold surface to the changing refractive index in the top channel.

[00137] The spectrum obtained once the channels are filled with DI-water is used as a background for further measurements. To quantify the analyte transport efficiency of both delivery schemes, a lower viscosity analyte solution (IPA) with higher refractive index was introduced from the bottom inlet. The plasmonic nanostructure sensor responsds only to the refractive index change due to the perpendicularly diffused or actively delivered IPA solution depend on the scheme. In the diffusive transport scheme, IPA solution is pumped into the bottom channel and collected from the bottom side at a flow rate of 20μL/min (top outlet is kept open). For targeted delivery of the convective current to the surface, IPA can be directed from a down-to-top direction by enabling flow between 3→2. In this case, a much larger red shifting ($\Delta\lambda = 10nm$) of the plasmonic resonance from DI-water background is obtained after flowing IPA solution for 10min at the same flow rate (20μL/min). This clearly shows that the targeted delivery scheme in the fluidic biosensor devices comprising plasmonic nanostructure sensor platforms described herein transports an analyte to the sensor surface more efficiently and improves the sensor performance.

[00138] A lift-off free fabrication process 200, according to one aspect of the invention, is illustrated in FIGURES 2A-2D. The fabrication process 200 is based on single layer e-beam lithography and reactive ion etching (RIE). It will be appreciated that the process can include fewer or additional steps.

[00139] The fabrication process 200 begins by coating a silicon wafer with a Low Pressure Chemical Vapor Deposition (LPCVD) silicon nitride (SiNx) film, in one embodiment. The process continues by forming free standing SiNx membranes (*e.g.*, approximately 50nm thick) using optical lithography and dry/wet etching methods. The membranes are then covered with positive e-beam resist (PMMA). E-beam lithography is then performed, as shown FIGURE 2A. A nanohole pattern (with hole diameters of *e.g.*, approximately 220 nm and a periodicity of approximately 600 nm) is transferred to the suspended SiNx film through a dry etching process. The e-beam resist is then removed with an oxygen plasma cleaning process leaving only a patterned SiNx film with air on both sides. A directional e-beam metal deposition tool can be used to deposit one or more metallic layers,

such as Ti (5nm) and Au (125nm) metal layers, which define the suspended nanostructure sensors with plasmonic nanohole openings. This deposition process is advantageous because it is extremely reliable - large areas of nanoholes covered with gold are repeatedly obtained without clogging the openings. Only a small shrinking in nanohole diameter (<4%) is observed after gold deposition due to slight coverage of the metal layers on the nanohole sidewalls.

[00140] Nanostructure sensors, such as photonic crystals (PhCs), offer unique opportunities to tailor the spatial extent of the electromagnetic field and control the strength of the light-matter interaction. Guided resonances that are delocalized in the plane and tightly confined in the vertical direction can be used. The periodic index contrast of the nanostructures enables the excitation of the guided resonances with a plane-wave illumination at normal incidence and their out-coupling into the radiation modes. Such a surface normal operation eliminates the alignments of sensitive prism/waveguide/fiber coupling schemes needed by other optical nanosensors. The ease of resonance excitation by surface normal light is particularly advantageous for high-throughput micro-array applications. The incident light is transmitted by photonic crystals slabs through two different pathways. One of them is the direct pathway, where a portion of the electromagnetic field goes straight through the slab. The other is the indirect pathway, where the remaining portion couples into the guided resonances before leaking into the radiation modes. These two pathways interfere with each other and result in resonances with sharp Fano-type asymmetric line-shapes. The spectral location of the resonances is highly sensitive to the refractive index changes occurring within the surroundings of photonic crystal slabs. The index change due to the accumulation of bio-molecules or variations in the bulk solution can be detected optically in a label-free fashion, as shown herein.

[00141] To experimentally implement the nanostructure sensor, a square lattice SiN_x photonic crystal slab (inset in FIGURE 3A) was used. FIGURE 3A shows the transmission spectra of a specific design calculated by three dimensional finite-difference time-domain (3D-FDTD) method in three different media: air (refractive index n = 1), water (n = 1.33), and an IPA-chloroform mixture (n = 1.43). A normally incident plane wave source (corresponding to the Γ-point in the dispersion diagram) excites the eigenmodes of the system. For each case, two modes are observed within the given spectral range. FIGURE 3B shows the intensity distribution of the lowest (first) order mode when the structure is in air. The field has four-fold symmetry as the lattice and well confined within the slab in the vertical direction. Within the plane, the field extends into the holes, which is crucial in increasing the field overlap with the surrounding media for higher sensitivity. The bulk sensitivity (in units of nm/RIU) was calculated using the shift of the resonance position in wavelength versus the refractive index change in the surrounding environment. To optimize the structure for higher sensitivity, the effects of the slab thickness and the hole radius were studied by varying the thickness d from $0.1a$ to $0.3a$ and the radius r from $0.3a$ to $0.45a$ (a is the periodicity). For all the analyzed structures, the resonant wavelength of the lowest order mode in air was scaled to 670 nm. The calculated sensitivities and the parameter sets for each case are shown in Table 1.

[00142] Table 1: Sensitivity results with different hole radius and slab thickness (in unit of nm/RIU)

d \ r	$0.3a$	$0.35a$	$0.4a$	$0.45a$
$0.1a$	405	485	490	560
$0.15a$	317	351	422	535
$0.2a$	236	344	370	500
$0.3a$	230	281	307	388

[00143] As shown herein, the sensitivity improves as the size of the holes increases and the slab thickness decreases. When r=0.45a and d=0.1a, the sensitivity reaches 560 nm/RIU. As the sensitivity scales with wavelength, shifting the resonances to the longer wavelength (such as 1550 nm range) can increase the sensitivity even further (well above 1000 nm/RIU), in some embodiments.

[00144] The optimized photonic crystal structures can be fabricated on free standing SiN_x membranes according to the process flow described in FIGURES 2A-2D. SEM images indicate that the diameter and the periodicity, in the embodiments described herein, are 540 nm and 605 nm, respectively. Ellipsometer measurements are taken on the unpatterned area of the membrane to confirm that the slab thickness is ~90 nm. These numbers are quite close to the optimized design with r/a=0.45 and d/a=0.15. For a photonic crystal with a periodicity of 605 nm, the resonance peak in air is located at ~670nm.

[00145] To carry out the flow tests, as shown herein, the nanostructures are integrated in a chamber with two inlets/outlets both on the top and the bottom channels fabricated in polydimethylsiloxane (PDMS). To implement the laminar flow scheme, where the convective flow is parallel to the surface (FIGURES 1B and 1D), the inlet/outlet of the bottom channel was blocked. To steer the convective flow actively towards the sensing surface, one of the openings of the both channels was blocked (FIGURES 1A and 1C). The photonic crystal slab is sealed perfectly to ensure the flow is only through the openings. Video images of the perpendicular convective flow, captured in a microscope with a CCD camera, are shown in FIGURES 4A-4D. Here, the IPA solution is pumped into the bottom channel by a syringe at a rate of 80μL/s. The video recording starts when the bottom channel is almost filled-up. FIGURES 4A-4D show the merge of IPA to the top channel only through the openings, confirming the active steering of the liquid flow. No damage or breakage of the membrane due to the applied pressure is observed.

[00146] To experimentally evaluate the sensing response of the different flow schemes described herein, transmission spectra of photonic crystals can be obtained by launching a collimated and unpolarized light at normal incidence. The transmitted signal is collected with a 0.7 numerical aperture objective lens and coupled into a spectrometer for spectral analysis. A comparison of the transmitted spectra is shown in FIGURES 5A-5C. Dark curve is the transmission spectrum taken in

air, which clearly shows the excitation of the lowest and the next higher order modes at 667 nm and 610 nm, respectively. The other dark and the light curves are the responses in the solution (DI-water) for both flow schemes, as indicated by the arrows. When the convective flow is parallel to the surface (light curve), no leakage to the bottom surface is observed due to the large surface tension of the DI-water. On the other hand, when the convective flow is actively directed through the openings, the photonic crystal sensor surface is totally immersed in DI-water. This results in a larger refractive index change and more than 40 nm additional resonance shift. This observation is also confirmed by numerical simulations. 3D-FDTD calculations are performed for the photonic crystals in air and totally immersed in water. The slab parameters are obtained from SEM images and ellipsometer measurements. FIGURES 5B and 5C show the simulation results overlaid directly with the experimental measurements without any shifting. Near perfect match between the resonance locations and the line-widths are observed for both modes. There is a slight distortion in the resonance shape of the first mode in air, which could be due to fabrication disorder. Simulation for the case in which water fills only the top channel was also performed (such that the holes and the bottom channel are still in air). The calculated resonance position for the lowest order mode is nearly same with the experimental result. This indicates that due to the large surface tension, solutions cannot penetrate through the nanoholes if no steering method is employed. It was observed that the widths of the resonance peaks are significantly narrower when the structure is immersed in solution. This is due to the reduction of the index contrast within the photonic crystal sensor resulting in less efficient coupling with the radiation continuum. With reduced index contrast (which could be, without wishing to be bound or limited by theory, due to immersion in solution or reduction of hole size), guided resonances asymptotically turns into fully confined slab modes (with infinite Q factor and narrow line-width).

[00147] FIGURE 5A shows an experimental comparison of transmission spectra for two different flow schemes. Actively controlled flow scheme (dark curve with arrow) shows better sensitivity and narrower linewidth compared to the conventional scheme (light curve with arrow). FIGURE 5B shows experimentally measured transmission spectrum in air (thicker line) overlaid with the simulation result (thinner line). FIGURE 5C shows experimentally measured transmission spectrum in water (thicker line) overlaid with the simulation result (thicker line).

[00148] Bulk sensitivity of the photonic crystals described herein were tested by successively applying five different solutions through the directed flow scheme: DI-water, acetone, IPA and two IPA-chloroform mixtures with refractive indices of 1, 1.33, 1.356, 1.377, 1.401 and 1.424, respectively. The refractive indices of all the liquids were initially measured using a commercial refractometer. The measurements were performed by slowly pumping the solution to the chamber at 50 μL/s pumping rate. Prior to each measurement, it was ensured that the former solution is entirely replaced by the new one. As shown in FIGURE 6A, with increasing refractive index the resonances red-shift and the line-widths become narrower. The linewidth of the resonance in DI-water is

measured to be ~10 nm. FIGURE 6B shows the shift in resonance wavelength versus the refractive index of the liquid. The agreement between the experimental data and the theoretically predicted shifts is excellent. The experimentally measured sensitivity of the sensor, 510 nm/RIU for operation around 850 nm in wavelength.

[00149] FIGURE 6A shows experimentally measured transmission spectra of a photonic crystal slab using actively controlled delivery scheme in air (dashed line), water (dotted line), IPA (thin solid line) and an IPA-chloroform mixture (thick solid line). FIGURE 6B shows shifts of the 1st resonant peaks in wavelength versus the surrounding refractive index change. Resonance peak positions found in experiments (stars) match very well with the simulation results (circles). The line is a linear fitting to the experimental results.

[00150] With the nanostructure sensor devices and systems provided herein, refractive index changes can be effectively detected by tracking the resonance shifts with a spectrometer. On the other hand, in some embodiments, detecting the index change by a laser/CCD system through intensity modulation offers advantages for highly multiplexed sensing. In such a read-out setting, however, it is crucial to have sharp resonances with large signal-to-noise ratios. This can be achieved by using cross-polarization measurements. As mentioned above, the transmission spectra result from interference of two optical paths: one is the direct transmission while the other is through the guided resonances. When an unpolarized light is employed and all the light transmitted through the photinc crystal sensor is collected, both pathways contributes to the detected signal. However, if a polarized light is launched and the signal after an analyzer oriented perpendicular to the polarizer is collected, only the scattering from the guided resonances contributes. This results in dramatic suppression of the background and isolation of the resonances with large signal-to-noise ratios. In addition, the cross-polarization measurements result in purely Lorentzian-shape resonance profiles with narrower line-widths. FIGURE 7A compares the cross-polarization spectrum (thick line) with the regular one (thin line). The spectra are taken when the structure is in air. Cross-polarization measurements clearly isolate two distinct resonance features from the background. A single Lorentzian with 7 nm line-width fits very well with the second order mode resonance (FIGURE 7B). On the other hand, two Lorenztians are needed to fit the lowest order mode (FIGURE 7B). This indicates a potential resonance splitting for the lowest order mode, which could be due to a slight non-uniformity in fabrication. The addition of three Lorentzians (dashed curve in Figure 7B) matches very well with the experimentally measured spectrum.

[00151] It will be appreciated that in certain circumstances, minute amounts of biomolecular targets from small quantities of analytes or biological samples can result in very small resonance peak shifts. In such circumstances, narrow resonances with large signal-to-noise ratios should be used. This can be achieved, in some embodiments, by using cross-polarization measurements. As mentioned above, the transmission spectra result from interference of two optical paths: one is the direct transmission while the other is through the guided resonances. When an unpolarized light is

employed and all the light transmitted through the photonic crystal sensor is collected, both pathways contribute to the detected signal. However, a polarized light is launched and the signal is collected after an analyzer oriented perpendicular to the polarizer, only the scattering from the guided resonances contributes. This results in dramatic suppression of the background and isolation of the resonances with large signal-to-noise ratios. In addition, the cross-polarization measurements result in purely Lorentzian-shape resonance profiles with narrower line-widths. FIGURE 7A compares the cross-polarization spectrum (Line 1) with the regular one (Line 2). The spectra are taken when the structure is in air. Cross-polarization measurements clearly isolate two distinct resonance features from the background. A single Lorentzian with 7 nm line-width fits very well with the second order mode resonance (FIGURE 7B). On the other hand, two Lorenztians are needed to fit the lowest order mode (FIGURE 7B). This indicates a potential resonance splitting for the lowest order mode, which could be due to a slight non-uniformity in fabrication. The addition of three Lorentzians (dashed curve in FIGURE 7B) matches very well with the experimentally measured spectrum.

[00152] Novel nanostructure sensors combining nanophotonics and nanofluidics on a single platform are described herein. By using nanoscale openings in photonic crystals and plasmonic nanoarrays both light and fluidics can be manipulated on chip. Compared to the laminar flow in conventional fluidic channels, active steering of the convective flow results in the direct delivery of the stream to the nanohole openings. This leads to enhanced analyte delivery to the sensor surface by overcoming the mass transport limitations. As described herein, this method can be applied to detect refractive index changes in aqueous solutions. Bulk measurements show that actively directed convective flow results in better sensitivities. The sensitivity of the sensor reaches 510 nm/RIU for resonance located around 850 nm with a line-width of ~10 nm in solution. In addition, a cross-polarization measurement can be employed to further improve the detection limit by increasing the signal-to-noise ratio.

Plasmonic Nanostructure Sensors and Detection of Biomolecular Targets

[00153] Described herein are rapid, sensitive, simple to use, and portable plasmonic nanostructure biosensors that are useful for a variety of applications involving the detection of biomolecular targets in samples and analytes, ranging from research and medical diagnostics, to detection of agents used in bioterrorism. Such targets include, but are not limited to, polynucleotides, peptides, small proteins, antibodies, viral particles, and cells. Furthermore, the biosensors described herein have the ability to simultaneously quantify many different biomolecular interactions and formation of biomolecular complexes with high sensitivity for use in pharmaceutical drug discovery, proteomics, and diagnostics. Such biomolecular complexes include, for example, oligonucleotide interactions, antibody-antigen interactions, hormone-receptor interactions, and enzyme-substrate interactions.

[00154] The ability to detect biological target molecules, such as DNA, RNA, and proteins, as well as nanomolecular particles, such as virions, is fundamental to understanding both cell physiology and disease progression, as well as for use in various applications such as the early and rapid detection of disease outbreaks and bioterrorism attacks. Such detection, however, is limited by the need to use labels, such as fluorescent molecules or radiolabels, which can alter the properties of the biological target, *e.g.*, conformation, and which can add additional, often time-consuming, steps to a detection process.

[00155] The direct detection of biochemical and cellular binding without the use of a fluorophore, a radioligand or a secondary reporter, using the plasmonic nanostructure biosensors and methods described herein, removes the experimental uncertainty induced by the effect of a label on, for example, molecular conformation, the blocking of active binding epitopes, steric hindrance, inaccessibility of the labeling site, or the inability to find an appropriate label that functions equivalently for all molecules or targets in a sample. The plasmonic nanostructure sensors and detection methods described herein greatly simplify the time and effort required for assay development, while removing experimental artifacts that occur when labels are used, such as quenching, shelf life, and background fluorescence.

Detection of Sub-Cellular Biomolecular Targets

[00156] The plasmonic nanostructure biosensors, devices, systems, and methods of use thereof provided herein are suitable for the detection of a wide variety of biomolecular targets present in a sample or analyte. Such biomolecular targets include, but are not limited to, sub-cellular molecules and structures, such as polynucleotides and polypeptides present in a sample. Binding of one or more of these molecules to the surface of the plasmonic nanostructure biosensors described herein causes a change in the optical properties, relative to the optical properties of the plasmonic nanostructure sensor surface in the absence of binding, that can be measured by an optical detector, thus allowing the plasmonic nanostructure biosensor to indicate the presence of one or more binding events. In addition, the plasmonic nanostructure biosensors described herein can be designed to have immobilized capture agents bound to the sensor surface, such that a change in an optical property is detected by the biosensor upon binding of one or more biomolecular targets present in a sample to one or more of the immobilized capture agents present on the substrate surface. Such plasmonic nanostructure biosensors are useful for the detection of a variety of biomolecular interactions, including, but not limited to, oligonucleotide-oligonucleotide, oligonucleotide-protein, antibody-antigen, hormone-hormone receptor, and enzyme-substrate interactions.

[00157] The plasmonic nanostructure biosensors of the invention can be used, in some embodiments, to study one or a number of specific binding interactions in parallel, *i.e.*, multiplex applications. Binding of one or more biomolecules to their respective capture agents can be detected, without the use of labels, by applying a analyte or sample comprising one or more biomolecular targets to a plasmonic nanostructure biosensor that has one or more specific capture agents

immobilized on its surface. The plasmonic nanostructure biosensor is illuminated with an optical source, such as light source, and if one or more biomolecular targets in the sample specifically binds one or more of the immobilized capture agents, the surface plasmon resonance of the plasmonic nanostructure biosensor changes, causing a change in an optical property relative to the optical property when one or more biomolecular targets have not bound to the immobilized capture agents. In those embodiments where a plasmonic nanostructure biosensor comprises an array of one or more distinct locations comprising one or more specific capture agents, then the desired optical property can be detected from each distinct location of the plasmonic nanostructure biosensor.

[00158] Accordingly, in some aspects, provided herein are plasmonic nanostructure biosensors comprising a substrate and a metal film disposed upon the substrate. In such aspects, the metal film comprises one or more surfaces comprising a plurality of nanoelements arranged in a predefined pattern, where the plurality of nanoelements have a dimension less than one wavelength of an incident light source to which the metal film produces surface plasmons, and where the metal film is activated with an activating agent. The plurality of plasmonic nanoelements can be arranged in any predefined pattern that gives rise to a desired optical property for the plasmonic nanostructure biosensor, including both periodic patterns, such as lattice or arrayed patterns, and non-periodic patterns, such as pseudo-random and random patterns. Accordingly, in some embodiments of these aspects, the predefined pattern of the plurality of nanoelements is a periodic pattern. In some embodiments of these aspects, the plurality of nanoelements is separated by a periodicity of between 100- 1000 nm. In some embodiments of these aspects, the plurality of nanoelements is separated by a periodicity of between 400- 800 nm. In some embodiments of this aspect, the predefined pattern of the plurality of nanoelements is a non-periodic pattern, such as a pseudo-random pattern or a random pattern.

[00159] The metals used in the plasmonic nanostructures described herein, such as the plasmonic nanohole biosensors, are selected on the basis of their surface plasmon properties when an incident light source illuminates their surface. The metal used can be in the form of a metal film comprising a plurality of nanoelements, such as nanoholes of a desired diameter or dimension shorter than the wavelength of the incident light, or in the form of a plurality of metallic nanoparticles on the surface of a substrate. Accordingly, in some embodiments of these aspects, the metal used can be a Noble metal, an alkali metal, or a transition metal, or any metal selected from the group consisting of gold, rhodium, palladium, silver, osmium, iridium, platinum, titanium, aluminum, or any combination thereof. The plasmonic nanoelements, such as nanoparticles, in some embodiments, can comprise multiple metals.

[00160] In those plasmonic nanostructures comprising a metallic film, the thickness of the film used can vary. The thickness of the metal film is preferably between 50- 500 nm thick, between 50- 450 nm thick, between 50- 400 nm thick, between 50- 350 nm thick, between 50- 300 nm thick, between 50- 250 nm thick, between 50- 200, between 75-500 nm thick, between 50- 450 nm thick,

between 75- 400 nm thick, between 75- 350 nm thick, between 75- 300 nm thick, between 75- 250 nm thick, or between 75-200 nm thick.

[00161] Substrate materials or support materials refer to materials upon which a metallic film or plasmonic nanoelement is disposed. Examples of substrate materials for use in the plasmonic nanostructure biosensors described herein include, but are not limited to, silicon dioxide, silicon nitride, glass, quartz, MgF_2, CaF_2, or a polymer, such as a polycarbonate or Teflon.

[00162] Preferably, the metal film comprising one or more nanoelements used in the plasmonic nanostructure biosensors described herein produces surface plasmons to wavelengths of light in the UV-VIS-IR spectral range. Ultraviolet (UV) light wavlengths can range from approximately 10 nm to 400 nm. Preferably, the range of UV wavelengths that elicit surface plasmon resonance in the nanostructures described herein, such as the plasmonic nanohole biosensors, are from 100 nm to 400 nm. The visible spectrum of light ranges from approximately 380 nm to 750 nm. Wavelengths within the infrared spectrum of light can range from 750 nm to 100,000 nm. Preferably, the infrared wavelengths that elicit surface plasmon resonance in the nanostructures described herein, such as the plasmonic nanohole biosensors, range from 750 nm to 3000 nm, from 750 nm to 2000 nm, or from 750 nm to 1000 nm.

[00163] In order to elicit surface plasmon resonance in the nanostructures described herein, an incident optical source producing light having wavelengths within a range useful for eliciting surface plasmon resonance is required. Such an incident optical light source can be a polychromatic illumination device or a broad spectral light source, or a monochromatic light source, such as a laser or light emitting diode (LED) having emission spectrum of a desired wavelength(s). In some embodiments, an optical filter can be used to produce light of a desired wavelength. In some embodiments, an optical source may further comprise a modulator to shift the phase or polarization of the light, or an actuator to control the angle of the incident light source.

[00164] A nanoelement for use in the nanostructures described herein can be of a plasmonic material of any suitable shape or dimension that exhibits surface plasmon resonance properties. Such a unit of plasmonic material can be in the form of a nanoparticle and present on or embedded within the surface of a plasmonic substance or substrate, or can be in the form of a nanohole and present as an aperture within a plasmonic material. Preferably, a nanoelement, such as a nanoparticle or nanohole, has at least one dimension in the approximately 1-3000 nm range, for example, in the range of about 1-2500 nm, in the range of about 1-2000 nm, in the range of about 1-1500 nm, in the range of about 1-1000 nm, in the range of about 50 nm to about 200 nm, in the range of about 50 nm to about 100 nm, in the range of about 10 nm to about 1000 nm, in the range of about 10 nm to about 750 nm, in the range of about 10 nm to about 500 nm, in the range of about 10 nm to about 250 nm, in the range of about 10 nm to about 100 nm, in the range of about 50 nm to about 300 nm, in the range of about 5 nm to about 100 nm, or in the range of about 2 nm to about 50 nm.

[00165] In some embodiments of the aspects described herein, the nanoelement is a nanohole. In some such embodiments, the nanohole is a through nanohole that completely penetrates the metal film. In other embodiments, the nanohole is a non-through nanohole that does not completely penetrate the metal film. In some embodiments of these aspect, at least one dimension of the nanohole is between 10-1000 nm. In some embodiments of the aspect, at least one dimension of the nanohole is between 50-300 nm.

[00166] The periodicity of the nanoelements can also play a role in increasing or enhancing surface plasmonic resonance effects in a nanostructure for use in the biosensors, devices, systems, and methods described herein. In some embodiments of these aspects, the nanoelements are separated by a periodicity of between 100-1000 nm, between 100-900 nm, 100-800 nm, 100-700 nm, between 100-600 nm, 100-500 nm, 100-400 nm, between 100-300 nm, or between 100-200 nm. In some embodiments, the periodicity is between 400-800 nm or between 500-700 nm.

[00167] The plasmonic nanostructure biosensors described herein can further comprise an adhesion later between the metal film and the substrate to help fix the metal film to the substrate it is disposed upon. In some such embodiments, the adhesion layer comprises titanium, chromium, or any combination thereof. The adhesion layer is preferably a thin layer, of a thickness less than that of the metal film. The thickness of the adhesion layer can be 50 nm or less, 45 nm or less, 50 nm or less, 35 nm or less, 30 nm or less, 25 nm or less, 20 nm or less, 15 nm or less, or 10 nm or less, 5 nm nm or less. In some embodiments, the thickness of the adhesion layer is in the range of 1 nm-20 nm, in the range of 1 nm-10 nm, in the range of 2 nm-9 nm, in the range of 3 nm-8 nm, or in the range of 4 nm-7 nm. In some embodiments, a through nanohole also completely penetrates the adhesion layer, for use, for example, with fludic systems.

[00168] It is also desirable, in some embodiments, to activate a surface of the metal of the plasmonic nanostructure using an activating agent. As used herein, "activating" the surface of a metal refers to treating it with an activating agent in order to allow, permit or enhance the binding of a capture agent. The activating agent can be chosen on the basis of the nature of the capture agent used with the plasmonic nanostructure, for example, whether the capture agent is a protein or a nucleic acid. Accordingly, in some embodiments, when the capture agent is a protein, the activating agent used to activate a metal surface is a piranha solution.

[00169] A metallic surface of a plasmonic nanostructure can also be functionalized using one or more specific capture agents. The metallic surface can be that of a nanoelement, such as a nanoparticle or nanohole (for example, along the side and/or bottom of a nanohole), on the surface of the metallic film comprising an array or periodic arrangement of nanoholes, or any combination thereof. Accordingly, as used herein, "functionalization" refers to adding to the surface of the metal of a plasmonic nanostructure described herein one or more specific capture agents. In some embodiments, the surface of a photonic crystal can be also be functionalized. In some embodiments, the metallic surface is first activated, then functionalized. In other embodiments, functionalization of a

metallic surface, such as a metallic film comprising one or more nanoholes, or a metallic nanoparticle, can be performed in the absence of activation.

[00170] The capture agent used to functionalize a plasmonic nanostructure should have specific binding properties for one or more biomolecular targets. As used herein, a "capture agent" refers to any of a variety of specific binding molecules, including, but not limited to, a DNA oligonucleotide, an RNA oligonucelotide, a peptide, a protein (*e.g.*, transcription factor, antibody or antibody fragment thereof, receptor, a recombinant fusion protein, or enzyme), a small organic molecule, or any combination thereof, that can be immobilized onto the surface of the plasmonic nanostructure described herein, such as a plasmonic nanohole array biosensor. In some embodiments, the capture agent is immobilized in a periodic fashion. For example, one or more specific immobilized capture agents can be arranged in an array at one or more distinct locations on the surface of the plasmonic nanostructure. In some such embodiments, capture agents specific for different biomolecular targets are immobilized at such distinct locations on the surface of a plasmonic nanostructure, such that the structure can be used to detect multiple biomolecular targets in a sample. In other embodiments, the capture agent is immobilized in a non-periodic or random fashion. For high-throughput applications, a plasmonic nanostructure can be arranged in an array of such arrays, wherein several biosensors comprising an array of specific capture agents on the plasmonic nanostructure are further arranged in an array.

[00171] Such functionalized biosensors are useful for the detection of biomolecular interactions, including, but not limited to, DNA-DNA, DNA-RNA, DNA-protein, RNA-RNA, RNA-protein, and protein-protein interactions. For example, a plasmonic nanostructure biosensor having a plurality of DNA oligonucleotides immobilized on the surface can be used to detect the presence of a protein, such as a transcription factor, present in a sample contacted with the substrate layer, that binds to one or more of the oligonucleotides.

[00172] Thus, in some embodiments, the metallic surface of a plasmonic nanostructure is functionalized with a capture agent comprising one or more of a plurality of immobilized DNA oligonucleotides. In some embodiments, the metallic surface of a plasmonic nanostructure is functionalized with a capture agent comprising one or more of a plurality of immobilized RNA oligonucleotides. In some embodiments, the metallic surface of a plasmonic nanostructure is functionalized with a capture agent comprising one or more of a plurality of immobilized peptides. In some embodiments, the metallic surface of a plasmonic nanostructure is functionalized with a capture agent comprising one or more of a plurality of immobilized proteins. In some such embodiments, the protein is an antigen. In other such embodiments, the protein is a polyclonal antibody, monoclonal antibody, single chain antibody (scFv), F(ab) fragment, $F(ab')_2$ fragment, or an Fv fragment, as these terms are defined herein. In other such embodiments, the protein is an enzyme, a transcription factor, a receptor, or a recombinant fusion protein.

[00173] The functionalization of the metallic surface of a plasmonic nanostructure can also occur in multiple steps using one or more specific capture agents, in order to provide greater specificity for one or more biomolecular targets. Thus, in some embodiments, a first capture agent and a second capture agent are used to functionalize a plasmonic nanostructure, such that the first capture agent is specific for the second capture agent, and the second capture agent is specific for one or more biomolecular targets. For example, a first capture agent specific for a common domain present in a variety of different second capture agents can be used to immobilize all capture agents having that common domain. Non-limiting examples of such common domains include constant regions of immunoglobulins or antibodies, DNA-binding domains of transcription factors, and the like. Accordingly, in one such embodiment, the first capture agent is protein A/G, and the second capture agent comprises one or more antibodies or antibody fragments thereof. In some such embodiments, the one or more antibodies or antibody fragments thereof are all specific for a particular class of biomolecular targets, for example, a family of related viruses. In other embodiments, the one or more antibodies or antibody fragments thereof have specificities for a variety of unrelated biomolecular targets.

[00174] A sample or analyte can be applied to or contacted with a plasmonic nanostructure, using fluidics or other methods known to one of skill in the art, in such a way to allow a biomolecular target present in the sample to bind to the plasmonic nanostructure or capture agent present on the plasmonic nanostructure. In some embodiments, the plasmonic nanostructure itself possesses nanofluidic properties using nanoholes present in the plasmonic nanostructure, as described and shown herein. In other embodiments, a sample or analyte can be directly applied to or contacted with the surface of the plasmonic nanostructure.

[00175] A sample or analyte can be any sample to be contacted with a plasmonic nanostructure as described herein, such as a plasmonic nanohole biosensor, for detection of one or more biomolecular targets, such as, for example, blood, plasma, serum, gastrointestinal secretions, homogenates of tissues or tumors, synovial fluid, feces, saliva, sputum, cyst fluid, amniotic fluid, cerebrospinal fluid, peritoneal fluid, lung lavage fluid, semen, lymphatic fluid, tears, prostatitc fluid, or cellular lysates. A sample can also be obtained from an environmental source, such as water sample obtained from a polluted lake or other body of water, or a liquid sample obtained from a food source believed to contaminated.

[00176] In some aspects, provided herein are plasmonic nanostructure biosensor devices or systems for detecting one or more biomolecular targets comprising: (i) any of the plasmonic nanostructure biosensors described herein; (ii) a device for contacting one or more samples comprising one or more biomolecular targets to the metal film surface(s) of the plasmonic nanostructure biosensor; (iii) an incident light source for illuminating a surface of the metal film to produce surface plasmons; and (iv) an optical detection system for collecting and measuring light

displaced from the illuminated metal film, where the displaced light is indicative of surface plasmon resonance on one or more surfaces of said metal film.

[00177] The device for contacting one or more samples for use in the plasmonic nanostructure biosensor systems described herein can be any device or mechanism by which a sample can be brought into contact with the detecting surface of the plasmonic nanostructure to allow a biomolecular target present in the sample to bind to the plasmonic nanostructure or capture agent present on the plasmonic nanostructure. For example, in some embodiments, a microfluidic device that can supply the sample along with a buffer and other reactants to the plasmonic nanostructure biosensor can be used. Such a device can provides a first microchannel or fluid inlet for the introduction of the sample onto the plasmonic nanostructure biosensor, and a second microchannel or fluid outlet for removing the compacted sample to a reservoir, such as a water reservoir. Additional microchannels can be provided for other purposes. In some embodiments, the plasmonic nanostructure itself can take advantage of possessing nanofluidic properties, as described herein, whereby the nanoholes of the plasmonic nanostructure are used as nanochannels to direct a sample supplied through, *e.g.*, a microfluidic device, below, through, and on the functionalized surface of the plasmonic nanostructure biosensor. Thus, detection of optical properties with and without microfluidics can occur. For example, in some embodiments, a sample or analyte can be directly applied to or contacted with the surface of the plasmonic nanostructure biosensor, for example, by applying the sample using a pipette, or by immersing the plasmonic nanostructure biosensor in the fluid sample, whereas in other embodiments, the plasmonic nanostructure biosensors are used in combination with a fluid flow device for contacting the sample(s).

[00178] The incident optical light source for use in such plasmonic nanostructure biosensor devices and systems described herein can be a polychromatic illumination device or a broad spectral light source, such as a gas discharge lamp (mercury lamps, sodium vapor lamps, xenon lamps, mercury-xenon lamps), a gar arced pulse lamp, an incandescent lamp, or a light emitting diode (LED) having a broad emission spectrum; a monochromatic light source, such as a laser or LED having emission spectrum of a desired wavelength(s), or any combination thereof. In some embodiments, an optical filter can be used to produce light of a desired wavelength. In some embodiments, an optical source may further comprise a modulator to shift the phase or polarization of the light, or an actuator to control the angle of the incident light source.

[00179] The optical detection system for collecting and measuring light displaced refers to any instrument that either processes light waves to enhance an image for viewing, or analyzes light waves (or photons) to determine one of a number of characteristic optical properties. Known optical detection system for determining optical properties include, but are not limited to, microscopes, cameras, interferometers (for measuring the interference properties of light waves), photometers (for measuring light intensity); polarimeters (for measuring dispersion or rotation of polarized light), reflectometers (for measuring the reflectivity of a surface or object), refractometers (for measuring

refractive index of various materials), spectrometers or monochromators (for generating or measuring a portion of the optical spectrum, for the purpose of chemical or material analysis), autocollimators (used to measure angular deflections), and vertometers (used to determine refractive power of lenses such as glasses, contact lenses and maginfier lens).

[00180] In some embodiments of the aspects described herein, the optical detection system is a spectrometer. A "spectrograph" or "spectrometer" refers to an optical instrument used to measure properties of light over a specific portion of the electromagnetic spectrum, typically used in spectroscopic analysis to identify materials. The variable measured is most often the light's intensity but could also, for instance, be the polarization state. The independent variable is usually the wavelength of the light, normally expressed as a fraction of a meter, but sometimes expressed as a unit directly proportional to the photon energy, such as wavenumber or electron volts, which has a reciprocal relationship to wavelength. If the region of interest is restricted to near the visible spectrum, the measurements are called spectrophotometry using a spectrophotometer.

[00181] In some embodiments of the aspects described herein, the optical detection system is a spectrophotometer. As defined herein, a "spectrophotometer" is a photometer (a device for measuring light intensity) that can measure intensity as a function of the color, or more specifically, the wavelength of light. There are many kinds of spectrophotometers. Among the most important distinctions used to classify them are the wavelengths they work with, the measurement techniques they use, how they acquire a spectrum, and the sources of intensity variation they are designed to measure. Other important features of spectrophotometers include the spectral bandwidth and linear range. There are two major classes of spectrophotometers; single beam and double beam. A double beam spectrophotometer measures the ratio of the light intensity on two different light paths, and a single beam spectrophotometer measures the absolute light intensity. Although ratio measurements are easier, and generally more stable, single beam instruments have advantages; for instance, they can have a larger dynamic range, and they can be more compact. Historically, spectrophotometers use a monochromator to analyze the spectrum, but there are also spectrophotometers that use arrays of photosensors. Especially for infrared spectrophotometers, there are spectrophotometers that use a Fourier transform technique to acquire the spectral information quicker in a technique called Fourier Transform InfraRed. The spectrophotometer quantitatively measures the fraction of light that passes through a given solution. In a spectrophotometer, a light from the lamp is guided through a monochromator, which picks light of one particular wavelength out of the continuous spectrum. This light passes through the sample that is being measured. After the sample, the intensity of the remaining light is measured with a photodiode or other light sensor, and the transmittance for this wavelength is then calculated. In short, the sequence of events in a spectrophotometer is as follows: the light source shines through the sample, the sample absorbs light, the detector detects how much light the sample has absorbed, the detector then converts how much light the sample absorbed into a number, the numbers are e are transmitted to a comparison module to be further manipulated (*e.g.*

curve smoothing, baseline correction). Many spectrophotometers must be calibrated by a procedure known as "zeroing." The absorbency of some standard substance is set as a baseline value, so the absorbencies of all other substances are recorded relative to the initial "zeroed" substance. The spectrophotometer then displays % absorbency (the amount of light absorbed relative to the initial substance). The most common application of spectrophotometers is the measurement of light absorption, but they can be designed to measure diffuse or specular reflectance.

[00182] The plasmonic nanostructure biosensor devices and systems described herein can also further comprise or be in communication with a controlling device, such as, for example, a computer or a microprocessor. The controlling device can determine, for example, the rate of fluids used for transferring the sample to the plasmonic nanostructure biosensor, and/or compile and analyze the optical properties detected by the optical detection system.

[00183] Accordingly, the novel technologies and plasmonic nanostructure biosensor devices and systems described herein are useful in applications where large numbers of biomolecular interactions are measured in parallel, particularly when molecular labels will alter or inhibit the functionality of the biomolecular targets under study. High-throughput screening of pharmaceutical drug compound libraries with protein biomolecular targets, and microarray screening of protein-protein interactions for proteomics are non-limiting examples of applications that require the sensitivity and throughput afforded by the systems and approaches described herein.

[00184] The structures and methods described herein can also be used to determine kinetic and affinity constants for molecular interactions between a biomolecular target in a sample and an immobilized molecule attached to the substrate, including association constants, dissociation constants, association rate constants, and dissociation rate constants. The structures and methods provided herein can also be used to determine the concentration of one or more biomolecular targets in a sample, such as viral concentration in a blood sample.

[00185] Some embodiments of the invention provide a method of detecting whether a biomolecular target inhibits the activity of an enzyme or binding partner, *i.e.*, "inhibition activity" of the biomolecular target. In one such embodiment, a sample comprising one or more biomolecular targets to be tested for having inhibition activity is contacted with a biosensor comprising one or more immobilized molecules. This is followed by adding one or more enzymes known to act upon at least one of the immobilized molecules on the biosensor substrate. Where the one or more enzymes have altered the one or more immobilized molecules on the substrate surface of the biosensor, for example, by cleaving all or a portion of an immobilized molecule from the surface of a biosensor, a shift in the interference pattern is detected by the biosensor. Thus, a sample comprising a biomolecular target having no inhibition activity allows the enzyme activity to occur unabated, such that the resonance pattern or refractive index changes upon addition of the enzyme(s); a biomolecular target with substantially complete inhibition activity halts the reaction substantially completely, such that no change in resonance pattern or refractive index is detected by the biosensor upon addition of the

enzyme(s); and a biomolecular target with partial inhibition halts the reaction partially, resulting in an intermediate shift in the resonance pattern or refractive index upon addition of the enzyme(s).

[00186] Further, in some embodiments, the plasmonic nanostructure biosensor devices and systems described herein can be used to detect a change in an optical property, such as a resonance pattern or refractive index at one or more distinct locations on a plasmonic nanostructure biosensor surface. For example, when the plasmonic nanostructure biosensor is used to identify biomolecular targets having enzymatic inhibition activity, the samples comprising one or more biomolecular targets is contacted with one or more distinct locations on the plasmonic nanostructure biosensor surface, and then one or more enzymes are contacted at these distinct locations. The desired optical property, such as the resonance pattern of the one or more distinct locations, is then detected and compared to the initial optical resonance pattern. In other embodiments, the sample comprising one or more biomolecular targets being tested for inhibitory activity is mixed with the one or more enzymes, which can be contacted to the one or more distinct locations, and the desired optical property is compared to the optical property obtained when no biomolecular targets are present in the sample.

Detection of Viral Biomolecular Targets

[00187] While some success had been achieved for detecting protein or nucleic acid molecules in a label-free fashion, viral targets have thus far eluded label-free detection strategies. The development of the plasmonic nanostructure biosensors and methods of use thereof described herein is useful for a variety of applications in which it was not previously possible, feasible, or practical to perform frequent or rapid testing for viruses, such as the fields of pharmaceutical discovery, diagnostic testing, environmental testing, bioterrorism, and food safety. A virus is a small infectious agent that can replicate only inside the living cells (host cells) of other organisms. Most viruses are too small to be seen directly with a light microscope. Additionally, many viruses cannot be cultured as appropriate host cells cannot be cultured. Early and rapid detection of viruses or viral particles is important for detecting contaminations in food supplies, and in protection against bioterrorism threats, as current detection methods, such as electron microscopy, are time-consuming, non-portable, and expensive.

[00188] The novel plasmonic nanostructure biosensors and methods of use thereof described herein unexpectedly provide a new and rapid means by which to detect viral biomolecular targets, with minimal sample processing, and allow for detection of intact viral particles, even in the absence of uniform coating of a sample comprising a viral particle on the biosensor surface. The plasmonic nanostructure biosensors are designed to have optimal size and spacing (periodicity) of the nanoelements, such as the nanoholes, to allow for viral particles to bind to the functionalized surface of the biosensor. In some embodiments, the size and spacing of the nanoelements of a plasmonic nanostructure biosensor are designed to permit flow-through of a sample comprising a viral particle. Specificity for a viral biomolecular target can be modified by altering the functionalization of a biosensor surface. Different viral biomolecular targets can be differentiated on the basis of, for

example, size, shape, or a combination therein. The inventors have discovered that sufficiently high viral concentrations result in a resonance shift large enough to be detected by the human eye, without the use of an optical detection system. Thus, the plasmonic nanostructure biosensors and methods thereof are also useful in determining concentrations of viruses in a given sample.

[00189] The plasmonic nanostructure biosensors described herein can be used for multiplex applications whereby one or a number different viruses are studied in parallel. Binding of one or more specific binding viral biomolecular targets can be detected, without the use of labels, by applying a sample comprising one or more biomolecular targets to a plasmonic nanostructure biosensor that has one or more specific capture agents, such as virus-specific antibodies or fragments thereof, immobilized on the plasmonic nanostructure surface. The functionalized plasmonic nanostructure biosensor is illuminated with a light source before and after application of a sample. If one or more viral biomolecular targets in the sample specifically binds one or more of the capture agents, a shift in the resonance pattern or refractive index occurs relative to the resonance pattern or refractive index when one or more specific viral biomolecular targets have not bound to the immobilized capture agents. In those embodiments where a plasmonic nanostructure biosensor surface comprises an array of one or more distinct locations comprising the one or more specific immobilized virus-specific capture agents, then the resonance pattern or refractive index is detected from each distinct location of the biosensor.

[00190] Thus, in some aspects of the invention, a variety of specific capture agents, for example, antibodies or fragements thereof, can be immobilized in an array format onto the surface of a plasmonic nanostructure biosensor described herein. The biosensor is then contacted with a test sample of interest comprising potential viral biomolecular targets. Only the viruses that specifically bind to the capture agents immobilized on the biosensor remain bound to the biosensor.

[00191] In some embodiments of the aspect, a plasmonic nanostructure surface comprises one or more capture agents specific for different viruses, whereby different locations on the surface comprise capture agents specific for distinct viral species, such that changes in the optical resonance pattern or refractive index at different locations on the surface, upon contacting the sample with the surface, is indicative of the presence of distinct viral species in the sample (*e.g.*, smallpox, Ebola and Marburg viruses). In some embodiments, if the concentration of virus is high enough in the sample, visual detection is sufficient. In other embodiments, an optical detection system such as a spectrophotometer can be used to detect changes in the optical properties of the plasmonic nanostructure biosensor. Such a biosensor is useful, for example, in the rapid identification of agents used during a bioterrorist attack.

[00192] In some embodiments of the aspect, a plasmonic nanostructure biosensor is functionalized with one or more antibodies or antibody-fragments thereof specific for different influenza hemagglutinins, whereby different locations plasmonic nanostructure biosensor surface comprise antibodies specific for distinct hemagglutinins, such that changes in the optical resonance

patterns at different locations upon contacting a sample with the plasmonic nanostructure biosensor is indicative of the presence of distinct influenza species (*e.g.*, Influenza A, Influenza B, and Influenza C) in the sample. Such a plasmonic nanostructure biosensor can distinguish, for example, between the presence of different influenza serotypes in a sample, such as H1N1, H2N2, H3N2, H5N1, H7N7, H1N2, H9N2, H7N2, H7N3, and H10N7.

[00193] Exemplary viruses and viral families that can be detected using the plasmonic nanostructure biosensors, devices, systems, and methods described herein include, but are not limited to: *Retroviridae* (*e.g.*, human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III), HIV-2, LAV or HTLV-III/LAV, or HIV-III, and other isolates, such as HIV-LP; *Picornaviridae* (*e.g.*, polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); *Calciviridae* (*e.g.*, strains that cause gastroenteritis); *Togaviridae* (*e.g.*, equine encephalitis viruses, rubella viruses); *Flaviviridae* (*e.g.*, dengue viruses, encephalitis viruses, yellow fever viruses); *Coronaviridae* (*e.g.*, coronaviruses); *Rhabdoviridae* (*e.g.*, vesicular stomatitis viruses, rabies viruses); *Filoviridae* (*e.g.*, ebola viruses); *Paramyxoviridae* (*e.g.*, parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); adenovirus; *Orthomyxoviridae* (*e.g.*, influenza viruses); *Bungaviridae* (*e.g.*, Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); *Arena viridae* (hemorrhagic fever viruses); *Reoviridae* (*e.g.*, reoviruses, orbiviurses and rotaviruses, *i.e.*, Rotavirus A, Rotavirus B. Rotavirus C); *Birnaviridae*; *Hepadnaviridae* (Hepatitis A and B viruses); *Parvoviridae* (parvoviruses); *Papovaviridae* (papilloma viruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and 2, Human herpes virus 6, Human herpes virus 7, Human herpes virus 8, varicella zoster virus, cytomegalovirus (CMV), herpes virus; Epstein-Barr virus; Rous sarcoma virus; West Nile virus; Japanese equine encephalitis, Norwalk, papilloma virus, parvovirus B19; *Poxyiridae* (variola viruses, vaccinia viruses, pox viruses); and *Iridoviridae* (*e.g.*, African swine fever virus); Hepatitis D virus, Hepatitis E virus, and unclassified viruses (*e.g.*, the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=enterally transmitted; class 2=parenterally transmitted (*i.e.*, Hepatitis C); Norwalk and related viruses, and astroviruses).

Detection of Sub-Cellular and Cellular Changes

[00194] The plasmonic nanostructure biosensors described herein are also useful for applications involving the detection of changes in cellular and sub-cellular functions in a sample. Such applications include, but are not limited to, testing of pharmaceutical drug candidates on cellular functions, morphology, and growth.

[00195] Accordingly, in some aspects, the plasmonic nanostructure biosensors described herein are used in a method of conducting a cell-based assay of a sample comprising one or more cells, whereby a cellular function being measured by the cell-based assay results in a shift in the optical resonance pattern of the plasmonic nanostructure biosensor, as detected and measured by an

appropriate optical detection system. The resonance pattern detected and measured by the plasmonic nanostructure biosensor provides can be used to identify and detect, for example, internal and external changes to a cell or cells present in a sample. In some embodiments, the cell-based assay measures a cellular function. In some embodiments, the cellular function is selected from the group consisting of cellular viability, cellular growth or changes in size, phagocytosis, channel opening/closing, changes in intracellular components and organelles, such as vesicles, mitochondria, membranes, structural features, periplasm, or any extracts thereof, and protein distribution.

Other Applications

[00196] The plasmonic nanostructure sensors described herein can also be used in a variety of other applications. These applications include, but are not limited to, environmental applications (*e.g.*, the detection of pesticides and river water contaminants); detection of non-viral pathogens; determining the presence and/or levels of toxic substances before and following bioremediation; analytic measurements in the food industry (*e.g.*, determination of organic drug residues in food, such as antibiotics and growth promoters; detection of small molecules, such as water soluble vitamins; detection of non-organic chemical contaminants), and the detection of toxic metabolites such as mycotoxins.

[00197] This invention is further illustrated by the following examples which should not be construed as limiting. It is understood that the foregoing detailed description and the following examples are illustrative only and are not to be taken as limitations upon the scope of the invention. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims. Various changes and modifications to the disclosed embodiments, which will be apparent to those, skilled in the art, may be made without departing from the spirit and scope of the present invention.

[00198] Further, all patents, patent applications, and publications identified, as well as the figures and tables, are expressly incorporated herein by reference in their entireties, for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents are based on the information available to the applicants and do not constitute any admission as to the correctness of the dates or contents of these documents.

[00199] The present invention can be defined in any of the following numbered paragraphs:

1. A plasmonic nanostructure biosensor comprising a substrate and a metal film disposed on the substrate, wherein said metal film comprises one or more surfaces comprising a plurality of nanoelements arranged in a predefined pattern, wherein each said nanoelement has a

dimension less than one wavelength of an incident optical source to which said metal film produces surface plasmons, and wherein said metal film is activated with an activating agent.

2. The plasmonic nanostructure biosensor of paragraph 1, wherein the substrate comprises silicon, silicon dioxide, silicon nitride, glass, diamond, quartz, magnesium fluoride (MgF_2), calcium fluoride (CaF_2), ZnSe, germanium, or a polymer.

3. The plasmonic nanostructure biosensor of paragraphs 1 or 2, wherein the metal film produces surface plasmons to incident light in the UV-VIS-IR spectral range.

4. The plasmonic nanostructure biosensor of any of paragraphs 1-3, wherein the metal is a Noble metal, a transition metal, or an alkali metal.

5. The plasmonic nanostructure biosensor of any of paragraphs 1-4, wherein the metal is selected from the group consisting of gold, rhodium, palladium, silver, osmium, iridium, platinum, titanium, aluminum, or any combination thereof.

6. The plasmonic nanostructure biosensor of any of paragraphs 1-5, wherein the metal film is between 50-500 nm thick.

7. The plasmonic nanostructure biosensor of any of paragraphs 1-5, wherein the metal film is between 75-200 nm thick.

8. The plasmonic nanostructure biosensor of any of paragraphs 1-7, wherein the nanoelement is a nanohole.

9. The plasmonic nanostructure biosensor of paragraph 8, wherein at least one dimension of the nanohole is between 10-1000 nm.

10. The plasmonic nanostructure biosensor of paragraphs 8, wherein at least one dimension of the nanohole is between 50-300 nm.

11. The plasmonic nanostructure biosensor of any of paragraphs 1-10, wherein the predefined pattern is a non-periodic pattern.

12. The plasmonic nanostructure biosensor of any of paragraphs 1-10, wherein the predefined pattern is a periodic pattern.

13. The plasmonic nanostructure biosensor of paragraph 12, wherein the plurality of nanoelements are separated by a periodicity of between 100-1000 nm.

14. The plasmonic nanostructure biosensor of paragraph 12, wherein the plurality of nanoelements are separated by a periodicity of between 400-800 nm.

15. The plasmonic nanostructure biosensor of any of paragraphs 1-14, wherein the activating agent is a piranha solution.

16. The plasmonic nanostructure biosensor of any of paragraphs 1-15, further comprising an adhesion layer, wherein the adhesion layer is between the metal film and the substrate.

17. The plasmonic nanostructure biosensor of paragraph 16, wherein the adhesion layer comprises titanium, chromium, or a combination thereof.

18. The plasmonic nanostructure biosensor of paragraphs 16 or 17, wherein the adhesion layer is less than 50 nm thick.

19. The plasmonic nanostructure biosensor of paragraphs 16 or 17, wherein the adhesion layer is less than 25 nm thick.

20. The plasmonic nanostructure biosensor of paragraphs 16 or 17, wherein the adhesion layer is less than 15 nm thick.

21. The plasmonic nanostructure biosensor of any of paragraphs 1- 20, wherein the activated metal film is further functionalized with one or more capture agents.

22. The plasmonic nanostructure biosensor of paragraph 21, wherein the capture agent is an antibody or antibody fragment thereof, a receptor, a recombinant fusion protein, a nucleic acid molecule, or any combination thereof.

23. The plasmonic nanostructure biosensor of paragraphs 1-22, wherein the one or more capture agents comprise a first capture agent and second capture agent, wherein the first capture agent is specific for the second capture agent, and the second capture agent is specific for one or more biomolecular targets.

24. The plasmonic nanostructure biosensor of paragraph 23, wherein the first capture agent is protein A/G.

25. The plasmonic nanostructure biosensor of paragraphs 23- 24, wherein the second capture agent comprises one or more antibodies or antibody fragments thereof.

26. A plasmonic nanostructure biosensor system for detecting one or more biomolecular targets comprising:

i. a plasmonic nanostructure biosensor of any of paragraphs 1-25;
ii. a device for contacting one or more samples comprising one or more biomolecular target to the metal film surface(s) of the plasmonic nanostructure biosensor;
iii. an incident light source for illuminating a surface of said metal film to produce said surface plasmons; and
iv. an optical detection system for collecting and measuring light displaced from said illuminated metal film, wherein said displaced light is indicative of surface plasmon resonance on one or more surfaces of said metal film.

27. The plasmonic nanostructure biosensor system of paragraph 26, wherein the device for contacting one or more samples comprises a fluidic system.

28. A method for detecting one or more biomolecular targets comprising:

(i) providing a plasmonic nanostructure biosensor system of paragraphs 26 or 27;

(ii) contacting one or more samples comprising one or more biomolecular targets to the metal film surface of the plasmonic nanostructure biosensor;

(iii) illuminating one or more surfaces of the metal film of the plasmonic nanostructure

biosensor with the incident light source to produce surface plasmons, before and after the contacting with the one or more samples;

(iv) collecting and measuring light displaced from the illuminated film with the optical detection system, before and after the contacting with the one or more samples; and

(v) detecting the one or more biomolecular targets based on a change or difference in the measurement of the light displaced from the illuminated film before and after the contacting with the one or more samples.

29. The method of paragraph 28, wherein the biomolecular target is a eukaryotic cell, a eukaryotic cellular component, a prokaryotic cell, a prokaryotic cellular component, a viral particle, a protein, an oligonucleotide, a prion, a toxin, or any combination thereof.

30. The method of paragraphs 27- 29, wherein said collected light comprises light in a transmission mode, in a reflection mode, or a combination thereof.

31. The method of any of claims 27- 30, wherein the step of measuring displaced light comprises measuring light over a spectral range selected to comprise at least one plasmon band.

32. The method of any of paragraphs 27- 31, wherein the change in the measurement of the displaced light before and after the contacting is a resonance peak shift, a change in a resonance peak intensity, a broadening of a resonance peak, a distortion in resonance of peak, or a change in refractive index.

33. A fluidic biosensor device comprising:

an upper chamber, the upper chamber comprising a fluid inlet;

a lower chamber, the lower chamber comprising a fluid outlet; and

a suspended nanostructure sensor placed between the upper chamber and the lower chamber, the nanostructure sensor comprising a plurality of nanoholes, wherein an analyte is configured to flow from the fluid inlet, through the nanoholes in the suspended nanostructure sensor and to the fluid outlet.

34. The fluidic biosensor device of paragraph 33, wherein the upper chamber comprises a glass surface.

35. The fluidic biosensor device of paragraphs 33- 34, wherein the lower chamber comprises a glass surface.

36. The fluidic biosensor device of any of paragraphs 33- 35, further comprising a light source to direct light through one chamber, and a light detector to detect the light through the other chamber.

37. The fluidic biosensor device of any of paragraphs 33- 36, further comprising a housing, wherein the housing encloses the upper chamber, the lower chamber, and the suspended nanostructure sensor.

38. The fluidic biosensor device of paragraph 37, wherein the housing comprises polydimethylsiloxane (PDMS).

39. The fluidic biosensor device of any of paragraphs 33- 38, wherein the suspended nanostructure sensor comprises a photonic crystal sensor.

40. The fluidic biosensor device of any of paragraphs 33- 38, wherein the suspended nanostructure sensor comprises a plasmonic nanohole biosensor comprising a substrate and a metal film disposed on the substrate, wherein said metal film comprises one or more surfaces comprising a plurality of nanoholes arranged in a predefined pattern, wherein each said nanohole has a dimension less than one wavelength of an incident optical source to which said metal film produces surface plasmons, and wherein said metal film is activated with an activating agent.

41. The fluidic biosensor device of paragraph 40, wherein the substrate comprises silicon, silicon dioxide, silicon nitride, glass, diamond, quartz, magnesium fluoride (MgF_2), calcium fluoride (CaF_2), ZnSe, germanium, or a polymer.

42. The fluidic biosensor device of paragraphs 40 or 41, wherein the metal film produces surface plasmons to incident light in the UV-VIS-IR spectral range.

43. The fluidic biosensor device of any of paragraphs 40- 42, wherein the metal is a Noble metal, a transition metal, or an alkali metal.

44. The fluidic biosensor device of any of paragraphs 40- 43, wherein the metal is selected from the group consisting of gold, rhodium, palladium, silver, osmium, iridium, platinum, titanium, aluminum, or any combination thereof.

45. The fluidic biosensor device of any of paragraphs 40- 44, wherein the metal film is between 50-500 nm thick.

46. The fluidic biosensor device of any of paragraphs 40- 44, wherein the metal film is between 75-200 nm thick.

47. The fluidic biosensor device of any of paragraphs 40- 46, wherein at least one dimension of the nanohole is between 10-1000 nm.

48. The fluidic biosensor device of any of paragraphs 40- 46, wherein at least one dimension of the nanohole is between 50-300 nm.

49. The fluidic biosensor device of any of paragraphs 40- 48, wherein the predefined pattern is a non-periodic pattern.

50. The fluidic biosensor device of any of paragraphs 40- 48, wherein the predefined pattern is a periodic pattern.

51. The fluidic biosensor device of paragraph 50, wherein the plurality of nanoholes are separated by a periodicity of between 100-1000 nm.

52. The fluidic biosensor device of paragraph 50, wherein the plurality of nanoholes are separated by a periodicity of between 400-800 nm.

53. The fluidic biosensor device of any of paragraphs 40- 52, wherein the activating agent is a piranha solution.

54. The fluidic biosensor device of any of paragraphs 40- 53, further comprising an adhesion layer, wherein the adhesion layer is between the metal film and the substrate.

55. The fluidic biosensor device of paragraph 54, wherein the adhesion layer comprises titanium, chromium, or a combination thereof.

56. The fluidic biosensor device of paragraphs 54 or 55, wherein the adhesion layer is less than 50 nm thick.

57. The fluidic biosensor device of paragraphs 54 or 55, wherein the adhesion layer is less than 25 nm thick.

58. The fluidic biosensor device of paragraphs 54 or 55, wherein the adhesion layer is less than 15 nm thick.

59. The fluidic biosensor device of any of paragraphs 40- 58, wherein the activated metal film is further functionalized with one or more capture agents.

60. The fluidic biosensor device of paragraph 59, wherein the capture agent is an antibody or antibody fragment thereof, a receptor, a recombinant fusion protein, a nucleic acid molecule, or any combination thereof.

61. The fluidic biosensor device of paragraph 60, wherein the one or more capture agents comprise a first capture agent and second capture agent, wherein the first capture agent is specific for the second capture agent, and the second capture agent is specific for one or more biomolecular targets.

62. The fluidic biosensor device of paragraph 61, wherein the first capture agent is protein A/G.

63. The fluidic biosensor device of paragraphs 61- 62, wherein the second capture agent comprises one or more antibodies or antibody fragments thereof.

64. A method of making a suspended plasmonic nanostructure sensor comprising:

depositing a silicon nitride film on a wafer;

removing at least a portion of the silicon nitride film to form silicon nitride membranes;

depositing a positive e-beam resist over the wafer;

performing e-beam lithography to transfer a nanohole pattern to the silicon nitride film through a dry etching process; and

depositing at least one metal layer over the wafer.

65. The method of paragraph 64, wherein the wafer is silicon.

66. The method of any of paragraphs 64- 65, wherein the silicon nitride film is deposited using Low Pressure Chemical Vapor Deposition (LPCVD).

67. The method of any of paragraphs 64- 66, wherein the at least a portion of the silicon nitride film is removed using optical lithography and one or more of dry and wet etching.

68. The method of any of paragraphs 64- 67, wherein the positive e-beam resist comprises poly(methyl methacrylate) (PMMA).

69. The method of any of paragraphs 64- 68, wherein the positive e-beam resist is removed using an oxygen plasma cleaning process.

70. The method of any of paragraphs 64- 69, wherein the at least one metal layer is a selected from a noble metal, a transition metal, or an alkali metal.

71. The method of any of paragraphs 64- 70, wherein the depositing the at least one metal layer comprises depositing a titanium metal layer and a gold metal layer.

72. The method of any of paragraphs 64- 71, wherein the at least one metal layer defines the nanohole pattern openings of the suspended plasmonic nanostructure sensor.

73. A method of making a suspended nanostructure sensor comprising:

depositing a positive e-beam resist over a substrate; and

performing e-beam lithography to form an array of nanoholes in the substrate.

74. The method of paragraph 73, further comprising depositing at least one metal layer over the substrate.

75. The method of any of paragraphs 73- 75, wherein the at least one metal layer is a selected from a noble metal, a transition metal, or an alkali metal.

76. A biosensor device comprising:

a light source to generate light;

a sensing structure comprising:

a first chamber, the first chamber comprising a fluid inlet,

a second chamber, the second chamber comprising a fluid outlet, and

a suspended nanostructure sensor between the first chamber and the second chamber, the suspended nanostructure sensor comprising a plurality of nanoholes, wherein an analyte is configured to flow from the fluid inlet, through the nanoholes in the suspended nanostructure sensor and to the fluid outlet, wherein the nanostructure sensor changes the refractive index of light when the analyte flows through the nanoholes;

and

a detector to detect the changes to the refractive index of light.

77. The biosensor device of paragraph 76, wherein the upper chamber comprises a glass surface and the lower chamber comprises a glass surface.

78. The biosensor device of paragraphs 76 or 77, further comprising a housing, wherein the upper chamber, lower chamber, and suspended plasmonic nanostructure sensor is enclosed in the housing.

79. The biosensor device of paragraph 78, wherein the housing comprises polydimethylsiloxane (PDMS).

80. Any of the above-described paragraphs wherein the plasmonic nanostructure biosensors, devices, fluidic devices, systems, and methods of use thereof consist essentially of the specified components.

EXAMPLES

Introduction

[00200] Demonstrated herein are plasmonic nanostructure sensors that can combine nanooptics and nanofluidic and methods of use thereof for direct detection of biomolecular targets, such as intact viruses, from analytes, such as biologically relevant media, in a label free fashion with little to no sample preparation. As a group, viruses that utilize RNA as their genetic material make up almost all of the alarming new infectious diseases (Category A, B, and C biothreats) and are a large component of the existing viral threats (influenza, rhinovirus, etc). Some of these viruses, *e.g.* the Ebola hemorrhagic fever virus are both emerging infectious and biological threat agent[41,42.] Patients presenting with RNA virus infections often show symptoms that are not virus specific[43]. Thus, there is great interest in developing sensitive, rapid diagnostics for such viruses to help direct proper treatment. The plasmonic nanostructure sensing platforms described herein use capture agents, such as antiviral immunoglobulins, immobilized at the sensor surface for specific capturing of biomolecular targets, such as virions. Unlike PCR, the plasmonic nanostructure biosensors and methods described herein allow us to take advantage of group specific antibodies, which have historically been able to identify a broad range of known and even previously unknown pathogens (*i.e.* novel mutant strains)[11,44]. In addition, the plasmonic nanostructure detection platforms and systems described herein are capable of quantifying concentrations, such as viral concentrations. Such quantitative detection makes it uniquely possible to detect not only the presence of the intact viruses in the analyzed samples, but also the intensity of the infection process. A dynamic range spanning three orders of magnitude from 10^6 PFU/ml to 10^9 PFU/ml is shown in experimental measurements proving that the detection platforms and systems described herein enable label-free virus detection within a concentration window relevant to clinical testing to drug screening. These studies also show the suitability of this technology for other viral types, including enveloped DNA viruses (vaccinia virus)[45]. Another advantage of these platforms is that due to the non-destructive nature of detection scheme, captured virions and their nucleic acid load (genome) can be exploited in further studies[46]. In these studies described herein, experiments were performed in ordinary biosafety level 1 and 2

laboratory settings without any need for mechanical or light isolation. This technology, enabling fast and compact sensing of biomolecular targets, such as intact viruses, can play an important role in early and point-of-care detection of viruses in clinical settings as well as in biodefense contexts.

[00201] *Device Operation Principle.* The detection scheme based on our nanooptic and nanofluidic plasmonic nanostructure sensors is illustrated in FIGURES 10A-10B. The device consists of a suspended nanohole array grating that couples the normally incident light to surface plasmons, electromagnetic waves trapped at metal/dielectric interface in coherence with collective electron oscillations[35,47-49]. The extraordinary light transmission resonances are observed at specific wavelengths, λ_{res} approximated by [50-53]:

$$\lambda_{res} \approx \frac{a_0}{\sqrt{i^2 + j^2}} \sqrt{\frac{\varepsilon_m \varepsilon_d}{\varepsilon_m + \varepsilon_d}} \qquad (1)$$

where the grating coupling enables the excitation of the surface plasmons (FIGURES 10C-10D). Here, a_0 is the periodicity of the array and i,j are the grating orders. This resonance wavelength is strongly correlated with the effective dielectric constant of the adjacent medium around the plasmonic sensor (Eq. 1)) [51,52]. As biomolecules/pathogens bind to the metal surface or to the ligands immobilized on the metal surface, the effective refractive index of the medium increases, and red-shifting of the plasmonic resonance occurs[54]. Unlike techniques based on external labeling, such resonance shifting operate as a reporter of the molecular binding,phenomena in a label free fashion and enables transduction of the capturing event directly to the far field optical signal[55-57]. Exponential decay of the extent of the plasmonic excitation results in subwavelength confinement of the electromagnetic field to the metal/dielectric interface[58]. As a result, the sensitivity of the biosensor to the refractive index changes decreases drastically with the increasing distance from the surface, thereby minimizing the effects of refractive index variations due to the temperature fluctuations in the bulk medium[58].

[00202] FIGURE 10D demonstrates a representative set of experimental end-point measurements for selective detection of vesicular stomatitis virus (VSV) at a concentration of 10^9 PFU/ml. Here, the transmission light spectra are acquired from a plasmonic nanohole array comprising fluidics of 90μm×90μm with a periodicity of 600 nm and an aperture radius of 200 nm. Spectra are given for both before (thick curve) and after (thin curve) the incubation of the virus containing samples. The sharp resonance feature observed at 690nm (thick curve) with 25nm full width at half maximum (FWHM) is due to the extraordinary light transmission phenomena through the optically thick gold film. This transmission resonance (thick curve) corresponds to the excitation of the (1,0) grating order SPP mode at the metal/dielectric interface of the antibody immobilized detection sensor50. After the incubation process with the virus containing sample, a strong red-shifting (~100nm) of the plasmonic resonance peak is observed (thin curve), due to the accumulated

biomass on the functionalized sensing surface. Such a strong resonance shift results in a color change of the transmitted light, which is, remarkably, large enough to discern visually without a spectrometer. For the un-functionalized control sensors (FIGURE 10C), a negligible red-shifting (~1 nm) of the resonances is observed (thick vs thin curves), possibly due to the non-specific binding events. This measurement clearly demonstrates that nanofluidic plasmonic nanostructure biosensors described herein provide novel platforms that can be used for specific detection of viruses. At lower concentrations of viruses ($<10^8$ PFU/ml) spectral shifts are more modest and require spectral measurements. However, considering that concentrations of certain types of viruses in infected samples reaches to the concentrations comparable to our visual detection limit, our platform offers unique opportunities for the development of rapid point-of-care diagnostics[59].

[00203] *Device Fabrication*: A lift-off free nanofabrication technique, based on positive resist e-beam lithography and direct deposition of metallic layers, was developed to fabricate the plasmonic nanostructure biosensors[35]. This scheme eliminates the need for lift-off processes, as well as operationally slow focused, ion-beam lithography, which introduces optically active ions. As a result, high quality plasmonic resonances (15-20nm FWHM), and high figure of merits (FOM~40) for refractive index sensitivities, defined as shift per refractive index unit (RUI) divided by the width of the surface plasmon resonances in energy units, are achieved[35]. The fabrication scheme is summarized in FIGURES 11A-11F. Initially, free standing SiNx membranes are created using a series of photolithographic and chemical wet etching (KOH) processes [60]. The membranes are then covered with positive e-beam resist poly(methyl methacrylate) (PMMA) and e-beam lithography is performed to define the nanohole pattern in the resist (FIGURE 11A). This pattern is transferred to the SiNx membrane through a reactive ion etching process (FIGURE 11B). After the removal of the resist with an oxygen plasma etching process (FIGURE 11C),a photonic crystal-like free standing SiNx membrane is defined. Sequential deposition of the metal layers (5nm Ti, 100nm Au) results in free standing plasmonic nanoholes transmitting light at resonance (FIGURE 11D)[35]. As demonstrated repeatedly in the experiments, this scheme allows fabrication of metallic nanohole arrays, without clogging the openings, and with extremely high yield/reproducibility and with minimal surface roughness (FIGURES 11E-11F)[35].

[00204] *Virus preparation.* VSV and virus pseudotypes. Baby hamster kidney (BHK) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% fetal bovine serum and 2 mM glutamine. Cells were grown to 85–95% confluence and then infected with VSV (Indiana serotype, Orsay strain) in DMEM at a low multiplicity of infection (MOI=0.01). 24 hours postinfection (hpi), media was harvested and virus titer was determined by plaque assay. VSV pseudotyped to express the glycoprotein from Ebola Zaire was grown in a similar fashion, but media was harvested at 48hpi. Purified virus was obtained through sedimentation of virus at 100,000XG for 1 hour, followed by resuspension in PBS or 10mM Tris pH 8.0. Resuspended virus was checked for purity by SDS-PAGE and Coomassie Blue staining, aliquoted and stored at -80°C. Vaccinia virus.

A549 cells were cultured in medium described above. Cells were infected with Vaccinia (WR strain) in DMEM at an MOI=0.01. 24hpi media was harvested and virus titers were determined via plaque assay. Aliquots were stored at - 80°C.

[00205] *Antibodies.* Antibodies targeting the single external VSV glycoprotein (called 8G5) were a kind gift of Douglas S. Lyles (Wake Forest). Antibodies were obtained from hybridoma supernatants. Purification of 8G5 from hybridoma supernatants was accomplished by protein A purification. Antibody targeting the Ebola glycoprotein (M-DA01-A5) was kind gift of Lisa Hensley (The United States Army Medical Research Institute of Infectious Diseases-USAMRIID). Antibody against Vaccinia virus (A33L) was the kind gift of Jay Hooper (USAMRIID).

[00206] *Surface Functionalization.* An exemplary surface functionalization scheme is summarized in FIGURES 12A-12B. In accordance with an earlier procedure for immobilization of antiviral immunoglobulins, plasmonic sensors are initially activated, after cleaning in a piranha solution (1:3 hydrogen peroxide in % 45 sulfuric acid solution for 5 min at room temperature)[61]. Activated surfaces are immobilized with protein A/G (Pierce, IL) at a concentration of 1mg/ml in PBS (10 mM phosphate buffer, 137 mM NaCl and 2.7 ml KCl) and incubated for 90 min at room temperature. Weakly bound and unbound molecules are eliminated by washing the chips in a direct stream of deionized, 0.1 μm filtered water. Unless otherwise stated in the following, all post-incubation washing processes were performed in three steps consisting of 5 minutes each PBST, PBS, filtered DI water washing and blow drying with nitrogen. Protein A/G was chosen as a template for the immobilization of the virus specific anti-bodies due to its high affinity to the Fc region of the IgG molecules[62,63]. Protein-AG is a recombinant fusion protein that contains the four Fc binding domains of protein A and two of the Protein G. Unlike protein A, the binding of chimeric protein A/G is less dependent upon the pH. The elimination of the non-specific binding regions to the serum proteins (including albumin) makes it an excellent choice for immobilization of the immunoglobulins. Proper orientation of the antibodies is imposed by this template (FIGURE 12A)[63].

[00207] *Antibody Immobilization.* Specific detection of viruses in a label free fashion requires an effective method to distinguish non-specific binding of the viruses to the plasmonic nanostructure sensor surface. Selectivity is achieved by surface immobilized highly specific antiviral immunoglobulins showing strong affinity to the viral membrane proteins, called glycoproteins (GP)[64]. GPs are presented on the outside of the assembled virus membrane and bind to receptors on the host cell membrane in order to enter into the cell (FIGURE 12A). Complementary antibodies (8G5 to recognize VSV[65-66], M-DA01-A5 to recognize Ebola (kind gifts from Lisa Hensley at USAMRIID) and A33L (a kind gift from Jay Hooper at USAMRIID[67]) having strong affinity to the GPs of the relevant viruses (VSV, pseudotyped Ebola, Vaccinia) were spotted on an array of sensors fabricated on a single chip at a concentration of 0.5 mg/ml in PBS (FIGURE 12A). The sensitivity of any immunoassay is highly dependent on the spotting of the antibodies. Higher concentrations of antiviral antibodies with respect to the virion concentrations are needed [virion]< [IgG], so that the spectral

shift is proportional to the concentration of the virions instead of being limited by the antiviral immunoglobulin concentration[68]. After a 60 min of incubation, unbound antibody was removed by a three step post-incubation washing process. No blocking agent was needed to block the antibody-free protein A/G surface, since the viruses do not directly bind to the protein A/G functionalized surface[61].

[00208] The successful functionalization of the sensing surface is monitored with end-point measurements after each incubation and washing processes. As shown in FIGURE 12B, the accumulated biomass on the sensing surface results in red-shifting of the air (1,0) resonance (black curve) due to the increasing local refractive index at the metal/dielectric of interface of the plasmonic nanostructure biosensor. Initially, a red shifting for about 4 nm was observed (dashed curve), after the protein A/G funtionalization in accordance with the procedure outline above. Protein A/G template is later used to immobilize (in this case) the 8G5-VSV specific antibodies at a concentration of 0.5 mg/ml. A spectral shift of 14 nm (thick curve) is observed after the antibody immobilization, confirming the successful functionalization of the surface.

[00209] *Reference sensors.* Reference sensors were incorporated into the chip design to correct for any drift and noise signal due to the unexpected changes in the measurement conditions or nonspecific binding events. Two different types of control spots, one functionalized with protein A/G only and one without any functionalized biomolecules, were used to determine the optimum configuration for the reference sensors. For the reference sensors functionalized with protein-A/G, it was observed that after the introduction of the antibodies to the detection spots, a red-shifting of the resonance was observed. This observation is associated to the relocation of the anti-viral immunoglobulins during the washing processes from antibody immobilized spots to the protein A/G immobilized reference sensors as a result of the high affinity of the protein A/G to the IgG antibodies. For the reference spots with no protein A/G layer, red shifting of the resonance after the introduction of the viruses was minimal. Accordingly, unfunctionalized nanohole sensors were used for reference measurements.

[00210] *PT-Ebola and Vaccinia Virus Detection.* To determine the broad adaptability of our platform to different types of viruses, we tested the sensors with hemorrhagic fever viruses (like Ebola virus) and poxviruses (like monkeypox or variola, the causative agent of smallpox). These viruses are of particular interest to public health and national security. Though we were not able to directly test these viruses because of biosafety considerations, we use a pseudotyped-VSV, where the Ebola glycoproteins are expressed on the virus membrane instead of the VSV's own glycoprotein[70]. Pseudotyped-Ebola virus (PT-Ebola) is a viable surrogate to analyze the behavior of Ebola, since the expressed glycoprotein folds properly and is fusion competent. The pseudotyped viruses have been successfully used as vaccine against Ebola in nonhuman primate models and can be used at lower biosafety levels (BSL2 versus BSL4). For these experiments, antibody against the Ebola glycoprotein was immobilized on the 9 of 12 sensors on a single chip, while 3 sensors were reserved for reference measurements. Successful functionalization of the protein-A/G and the antibodies were confirmed by

spectral measurements (FIGURE 13A). Following the immobilization of the antibodies, PT-Ebola (at a concentration of 10 PFU U/ml) in a PBS buffer solution) was added onto the chips and incubated for 90 min. After the washing process as summarized above, transmission spectra were collected (FIGURE 13A). Consistent red-shifting of the plasmonic resonances were observed on antibody-coated spots indicating PT-Ebola detection (>= 14nm red shift), while control sensors showed no spectral shift (hatched bars, FIGURE 13B). This occurred with high repeatability (9 of 9 sensors) and excellent signal to-noise ratios. Similarly, the platform was tested for the detection of enveloped DNA poxviruses. To do this, Vaccinia virus was utilized, a poxvirus that is commonly used as a prototype for more pathogenic viruses such as smallpox and monkeypox[71]. A similar approach (Vaccinia antibody to the A33L external protein immobilized on 9 of 12 sensors, incubation with intact vaccinia virus at the same concentration of 108PFU/ml) yielded similar positive results to those seen with PT-Ebola virus (FIGURE 13C). All of the 9 sensors detected the virus, while none of the control sensors indicated more than minimal binding (FIGURE 13D). For sensors close to the spotted sample edges, both weaker (8 nm in the case of Vaccinia virus) and stronger (20-21nm in the case of pseudo-Ebola virus) spectral shifts were observed. This is related to the varying concentrations of viruses due to the edge effects created when the virus sample is spotted. Measurements obtained from multiple sensors improved the robustness of the assay. Repeatability of the measurements was readily observed; all functionalized nanohole sensors showed a consistent shift ranging from 14-2 1 nm (FIGURES 13B, 13D). This observation shows a clear quantitative relation between the spectral shifts and virus concentrations. Such quantification is not possible with techniques based on fluorescent labeling (ELISA). Although Vaccina virus is relatively larger than the pseudo-Ebola viruses, comparable spectral shifts are observed for the pseudo-Ebola viruses. This observation clearly indicates that the capturing efficiency of the viruses, thus the accumulated biomass, is not only controlled by the concentrations of the virions but also controlled by the affinity of the virus-IgG interactions[72]. Without doubt, strength of such interactions is strongly affected by the complex mixture of the envelope proteins and the surroundings of the viral subunits[72,73]. In fact, the structure and the conformation state of the membrane incorporated glycoproteins may strongly differ from those of the purified ones[72]. Accordingly, techniques based on detection of recombinant and refined virus specific proteins or viral peptides are not suitable for medical studies of *in vivo* behavior of live viruses. Instead, techniques enabling direct detection of entire viral particles in medically relevant biological media are needed. While most studies in this field are confined to detection of individual viral components such as glycoproteins and nucleic acids, we demonstrate that our detection platform enables direct detection of entire viruse[73,74].

[00211] *Virus Detection in Biological Media.* To demonstrate the applicability of the plasmonic nanostructure detection platforms described herein in biologically relevant systems, experiments were extended to detection of intact viruses directly from biological media (cell growth medium + 7% fetal calf serum). These conditions provide a number of potentially confounding factors

(high serum albumin levels, immunoglobulins and growth factors) that could add unwanted background signal, thus this was an important test for the robustness of the detection systems described herein. In FIGURES 14A-14B, it is shown that the initial Pr-AG functionalization (1 mg/ml) resulted in 4 nm red shifting of the resonances. Subsequently, anti-VSV (0.5 mg/ml) immobilization was confirmed with the ~15 nm red shifting of the resonances. Finally, VSV was applied to the chips at a concentration of 10^6 PFU/ml in a DMEM/FBS medium. Measurements, following an incubation period of 90 min and post washing processes, showed a 4 nm resonance shift for the anti-viral immunoglobin functionalized spots. In control sensors, red-shifting of the resonances was seen, but was limited to only 1.3 nm due to the non-specific binding of the serum proteins. The specific capturing of the intact viruses at a low concentration of 10^6 PFU/ml is clearly distinguishable at the antibody functionalized sensors. This observation demonstrates the potential of this platform for clinical applications. Due to the ability to quantify non-specific binding on an individual chip, the presence of a small amount of background does not pose a fundamental bottleneck for the viability of this technology. In fact, this technology is sufficient for microbiology laboratories involving culturing of the viruses. In addition, it is likely that the technology can be adapted "as is" for successful diagnosis of herpesvirus, poxvirus and some gastroenteric infections, since a detection limit of 10^7-10^8 PFU/ml is usually sufficient for clinical applications[59]. Given that the resolution limit of detection system is 0.05 nm, it is likely that much lower concentrations can be detected with the current platform. Background shifting due to the non-specific binding could be a problem at lower concentrations of analytes ($<10^5$ PFU/ml), however this limitation can be considerably reduced and significant improvements in detection limits of the devices can be achieved by optimizing the surface chemistry.

[00212] *Conclusion.* The studies described herein provide biosensing platforms and methods of use thereof for fast, compact, quantitative and label free sensing of biomolecular targets, such as viral particles ,with minimal sample processing. Demonstrated herein is the extraordinary light transmission phenomena using plasmonic nanohole arrays that can be adapted for pathogen detection without being confounded by surrounding biological media. In some embodiments, the plasmonic nanostructure sensing platform uses antiviral immunoglobulins immobilized at the sensor surface for specific capturing of the intact virions and is capable of quantifying their concentrations. Direct detection of different types of viruses (VSV, pseudo-Ebola and Vaccinia) are shown. A dynamic range spanning three orders of magnitude from 10^6 PFU/ml to 10^9 PFU/ml is shown in experimental measurements corresponding to virion concentration within a window relevant to clinical testing to drug screening. Moreover, detection of the viruses at low concentrations in biologically relevant media at detection limits $<10^5$ PFU/ml clearly demonstrates the feasibility of the technology for earlier diagnosis of viruses directly from the human blood. It is important to note that the ease of multiplexing afforded by this approach is a crucial aspect of the biosensor designs described herein. The nanofluidic plasmonic nanostructure sensors can be readily expanded into a multiplexed format,

where various viral antibodies are immobilized at different locations to selectively detect the pathogens in an unknown sample. The advantage of the plasmonic nanostructure sensors combining plasmonic optics and nanofluidics is the ability to detect intact virus particles and identify them without damaging the virus structure or the nucleic acid load (genome), so that the samples can be further studied [46]. The approaches described herein open up biosensing applications of extra-ordinary light transmission phenomena for a broad range of pathogens, and can be directly utilized in any biology lab.

[00213] It should be understood that processes and techniques described herein are not inherently related to any particular apparatus and may be implemented by any suitable combination of components. The present invention has been described in relation to particular examples, which are intended in all respects to be illustrative rather than restrictive. Those skilled in the art will appreciate that many different combinations will be suitable for practicing the present invention. Moreover, other implementations of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. Various aspects and/or components of the described embodiments may be used singly or in any combination. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

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CLAIMS

What is claimed is:

1. A plasmonic nanostructure biosensor comprising a substrate and a metal film disposed on the substrate, wherein said metal film comprises one or more surfaces comprising a plurality of nanoelements arranged in a predefined pattern, wherein each said nanoelement has a dimension less than one wavelength of an incident optical source to which said metal film produces surface plasmons, and wherein said metal film is activated with an activating agent.

2. The plasmonic nanostructure biosensor of claim 1, wherein the substrate comprises silicon, silicon dioxide, silicon nitride, glass, diamond, quartz, magnesium fluoride (MgF_2), calcium fluoride (CaF_2), ZnSe, germanium, or a polymer.

3. The plasmonic nanostructure biosensor of claims 1 or 2, wherein the metal film produces surface plasmons to incident light in the UV-VIS-IR spectral range.

4. The plasmonic nanostructure biosensor of any of claims 1-3, wherein the metal is a Noble metal, a transition metal, or an alkali metal.

5. The plasmonic nanostructure biosensor of any of claims 1-4, wherein the metal is selected from the group consisting of gold, rhodium, palladium, silver, osmium, iridium, platinum, titanium, aluminum, or any combination thereof.

6. The plasmonic nanostructure biosensor of any of claims 1-5, wherein the metal film is between 50-500 nm thick.

7. The plasmonic nanostructure biosensor of any of claims 1-5, wherein the metal film is between 75-200 nm thick.

8. The plasmonic nanostructure biosensor of any of claims 1-7, wherein the nanoelement is a nanohole.

9. The plasmonic nanostructure biosensor of claim 8, wherein at least one dimension of the nanohole is between 10-1000 nm.

10. The plasmonic nanostructure biosensor of claim 8, wherein at least one dimension of the nanohole is between 50-300 nm.

11. The plasmonic nanostructure biosensor of any of claims 1-10, wherein the predefined pattern is a non-periodic pattern.

12. The plasmonic nanostructure biosensor of any of claims 1-10, wherein the predefined pattern is a periodic pattern.

13. The plasmonic nanostructure biosensor of claim 12, wherein the plurality of nanoelements are separated by a periodicity of between 100-1000 nm.

14. The plasmonic nanostructure biosensor of claim 12, wherein the plurality of nanoelements are separated by a periodicity of between 400-800 nm.

15. The plasmonic nanostructure biosensor of any of claims 1-14, wherein the activating agent is a piranha solution.

16. The plasmonic nanostructure biosensor of any of claims 1-15, further comprising an adhesion layer, wherein the adhesion layer is between the metal film and the substrate.

17. The plasmonic nanostructure biosensor of claim 16, wherein the adhesion layer comprises titanium, chromium, or a combination thereof.

18. The plasmonic nanostructure biosensor of claims 16 or 17, wherein the adhesion layer is less than 50 nm thick.

19. The plasmonic nanostructure biosensor of claims 16 or 17, wherein the adhesion layer is less than 25 nm thick.

20. The plasmonic nanostructure biosensor of claims 16 or 17, wherein the adhesion layer is less than 15 nm thick.

21. The plasmonic nanostructure biosensor of any of claims 1- 20, wherein the activated metal film is further functionalized with one or more capture agents.

22. The plasmonic nanostructure biosensor of claim 21, wherein the capture agent is an antibody or antibody fragment thereof, a receptor, a recombinant fusion protein, a nucleic acid molecule, or any combination thereof.

23. The plasmonic nanostructure biosensor of claims 1-22, wherein the one or more capture agents comprise a first capture agent and second capture agent, wherein the first capture agent is specific for the second capture agent, and the second capture agent is specific for one or more biomolecular targets.

24. The plasmonic nanostructure biosensor of claim 23, wherein the first capture agent is protein A/G.

25. The plasmonic nanostructure biosensor of claims 23- 24, wherein the second capture agent comprises one or more antibodies or antibody fragments thereof.

26. A plasmonic nanostructure biosensor system for detecting one or more biomolecular targets comprising:

(i) a plasmonic nanostructure biosensor of any of claims 1-25;

(ii) a device for contacting one or more samples comprising one or more biomolecular targets to the metal film surface(s) of the plasmonic nanostructure biosensor;

(iii) an incident light source for illuminating a surface of said metal film to produce said surface plasmons; and

(iv) an optical detection system for collecting and measuring light displaced from said illuminated metal film, wherein said displaced light is indicative of surface plasmon resonance on one or more surfaces of said metal film.

27. The plasmonic nanostructure biosensor system of claim 26, wherein the device for contacting one or more samples comprises a fluidic system.

28. A method for detecting one or more biomolecular targets comprising:

(i) providing a plasmonic nanostructure biosensor system of claims 26 or 27;

(ii) contacting one or more samples comprising one or more biomolecular targets to the metal film surface of the plasmonic nanostructure biosensor;

(iii) illuminating one or more surfaces of the metal film of the plasmonic nanostructure biosensor with the incident light source to produce surface plasmons, before and after the contacting with the one or more samples;

(iv) collecting and measuring light displaced from the illuminated film with the optical detection system, before and after the contacting with the one or more samples; and

(v) detecting the one or more biomolecular targets based on a change or difference in the measurement of the light displaced from the illuminated film before and after the contacting with the one or more samples.

29. The method of claim 28, wherein the biomolecular target is a eukaryotic cell, a eukaryotic cellular component, a prokaryotic cell, a prokaryotic cellular component, a viral particle, a protein, an oligonucleotide, a prion, a toxin, or any combination thereof.

30. The method of claims 27- 29, wherein said collected light comprises light in a transmission mode, in a reflection mode, or a combination thereof.

31. The method of any of claims 27- 30, wherein the step of measuring displaced light comprises measuring light over a spectral range selected to comprise at least one plasmon band.

32. The method of any of claims 27- 31, wherein the change in the measurement of the displaced light before and after the contacting is a resonance peak shift, a change in a resonance peak intensity, a broadening of a resonance peak, a distortion in resonance of peak, or a change in refractive index.

33. A fluidic biosensor device comprising:

an upper chamber, the upper chamber comprising a fluid inlet;

a lower chamber, the lower chamber comprising a fluid outlet; and

a suspended nanostructure sensor placed between the upper chamber and the lower chamber, the nanostructure sensor comprising a plurality of nanoholes, wherein an analyte is configured to flow from the fluid inlet, through the nanoholes in the suspended nanostructure sensor and to the fluid outlet.

34. The fluidic biosensor device of claim 33, wherein the upper chamber comprises a glass surface.

35. The fluidic biosensor device of claims 33- 34, wherein the lower chamber comprises a glass surface.

36. The fluidic biosensor device of any of claims 33- 35, further comprising a light source to direct light through one chamber, and a light detector to detect the light through the other chamber.

37. The fluidic biosensor device of any of claims 33- 36, further comprising a housing, wherein the housing encloses the upper chamber, the lower chamber, and the suspended nanostructure sensor.

38. The fluidic biosensor device of claim 37, wherein the housing comprises polydimethylsiloxane (PDMS).

39. The fluidic biosensor device of any of claims 33- 38, wherein the suspended nanostructure sensor comprises a photonic crystal sensor.

40. The fluidic biosensor device of any of claims 33- 38, wherein the suspended nanostructure sensor comprises a plasmonic nanohole biosensor comprising a substrate and a metal film disposed on the substrate, wherein said metal film comprises one or more surfaces comprising a plurality of nanoholes arranged in a predefined pattern, wherein each said nanohole has a dimension less than one wavelength of an incident optical source to which said metal film produces surface plasmons, and wherein said metal film is activated with an activating agent.

41. The fluidic biosensor device of claim 40, wherein the substrate comprises silicon, silicon dioxide, silicon nitride, glass, diamond, quartz, magnesium fluoride (MgF_2), calcium fluoride (CaF_2), ZnSe, germanium, or a polymer.

42. The fluidic biosensor device of claims 40 or 41, wherein the metal film produces surface plasmons to incident light in the UV-VIS-IR spectral range.

43. The fluidic biosensor device of any of claims 40- 42, wherein the metal is a Noble metal, a transition metal, or an alkali metal.

44. The fluidic biosensor device of any of claims 40- 43, wherein the metal is selected from the group consisting of gold, rhodium, palladium, silver, osmium, iridium, platinum, titanium, aluminum, or any combination thereof.

45. The fluidic biosensor device of any of claims 40- 44, wherein the metal film is between 50-500 nm thick.

46. The fluidic biosensor device of any of claims 40- 44, wherein the metal film is between 75-200 nm thick.

47. The fluidic biosensor device of any of claims 40- 46, wherein at least one dimension of the nanohole is between 10-1000 nm.

48. The fluidic biosensor device of any of claims 40- 46, wherein at least one dimension of the nanohole is between 50-300 nm.

49. The fluidic biosensor device of any of claims 40- 48, wherein the predefined pattern is a non-periodic pattern.

50. The fluidic biosensor device of any of claims 40- 48, wherein the predefined pattern is a periodic pattern.

51. The fluidic biosensor device of claim 50, wherein the plurality of nanoholes are separated by a periodicity of between 100-1000 nm.

52. The fluidic biosensor device of claim 50, wherein the plurality of nanoholes are separated by a periodicity of between 400-800 nm.

53. The fluidic biosensor device of any of claims 40- 52, wherein the activating agent is a piranha solution.

54. The fluidic biosensor device of any of claims 40- 53, further comprising an adhesion layer, wherein the adhesion layer is between the metal film and the substrate.

55. The fluidic biosensor device of claim 54, wherein the adhesion layer comprises titanium, chromium, or a combination thereof.

56. The fluidic biosensor device of claims 54 or 55, wherein the adhesion layer is less than 50 nm thick.

57. The fluidic biosensor device of claims 54 or 55, wherein the adhesion layer is less than 25 nm thick.

58. The fluidic biosensor device of claims 54 or 55, wherein the adhesion layer is less than 15 nm thick.

59. The fluidic biosensor device of any of claims 40- 58, wherein the activated metal film is further functionalized with one or more capture agents.

60. The fluidic biosensor device of claim 59, wherein the capture agent is an antibody or antibody fragment thereof, a receptor, a recombinant fusion protein, a nucleic acid molecule, or any combination thereof.

61. The fluidic biosensor device of claim 60, wherein the one or more capture agents comprise a first capture agent and second capture agent, wherein the first capture agent is specific for the second capture agent, and the second capture agent is specific for one or more biomolecular targets.

62. The fluidic biosensor device of claim 61, wherein the first capture agent is protein A/G.

63. The fluidic biosensor device of claims 61- 62, wherein the second capture agent comprises one or more antibodies or antibody fragments thereof.

64. A method of making a suspended plasmonic nanostructure sensor comprising:
depositing a silicon nitride film on a wafer;
removing at least a portion of the silicon nitride film to form silicon nitride membranes;
depositing a positive e-beam resist over the wafer;
performing e-beam lithography to transfer a nanohole pattern to the silicon nitride film through a dry etching process; and
depositing at least one metal layer over the wafer.

65. The method of claim 64, wherein the wafer is silicon.

66. The method of any of claims 64- 65, wherein the silicon nitride film is deposited using Low Pressure Chemical Vapor Deposition (LPCVD).

67. The method of any of claims 64- 66, wherein the at least a portion of the silicon nitride film is removed using optical lithography and one or more of dry and wet etching.

68. The method of any of claims 64- 67, wherein the positive e-beam resist comprises poly(methyl methacrylate) (PMMA).

69. The method of any of claims 64- 68, wherein the positive e-beam resist is removed using an oxygen plasma cleaning process.

70. The method of any of claims 64- 69, wherein the at least one metal layer is a selected from a noble metal, a transition metal, or an alkali metal.

71. The method of any of claims 64- 70, wherein the depositing the at least one metal layer comprises depositing a titanium metal layer and a gold metal layer.

72. The method of any of claims 64- 71, wherein the at least one metal layer defines the nanohole pattern openings of the suspended plasmonic nanostructure sensor.

73. A method of making a suspended nanostructure sensor comprising:
depositing a positive e-beam resist over a substrate; and
performing e-beam lithography to form an array of nanoholes in the substrate.

74. The method of claim 73, further comprising depositing at least one metal layer over the substrate.

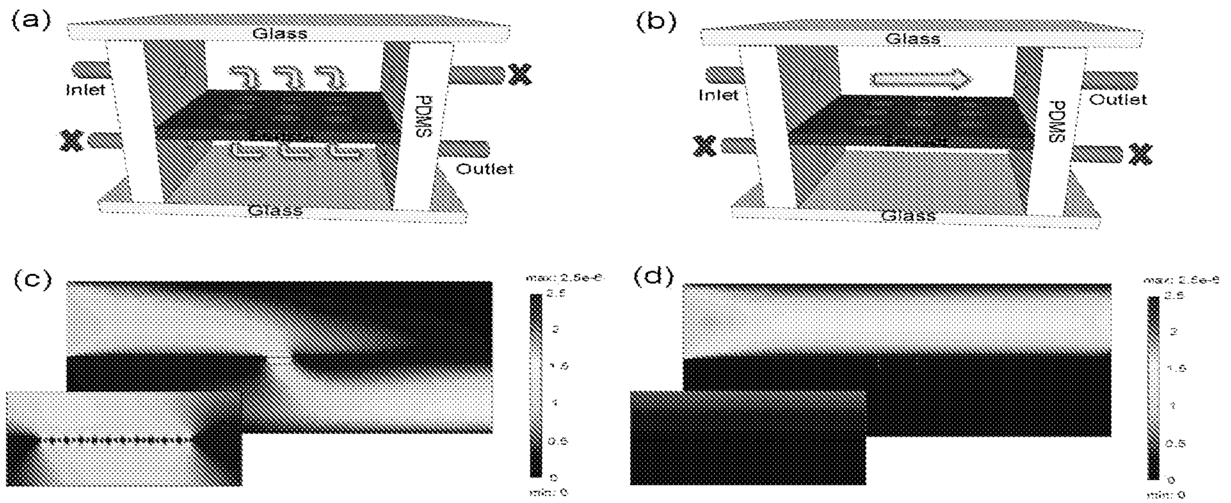
75. The method of any of claims 73- 75, wherein the at least one metal layer is a selected from a noble metal, a transition metal, or an alkali metal.

76. A biosensor device comprising:
a light source to generate light;
a sensing structure comprising:
a first chamber, the first chamber comprising a fluid inlet,
a second chamber, the second chamber comprising a fluid outlet, and
a suspended nanostructure sensor between the first chamber and the second chamber, the suspended nanostructure sensor comprising a plurality of nanoholes, wherein an analyte is configured to flow from the fluid inlet, through the nanoholes in the suspended nanostructure sensor and to the fluid outlet, wherein the nanostructure sensor changes the refractive index of light when the analyte flows through the nanoholes;
and
a detector to detect the changes to the refractive index of light.

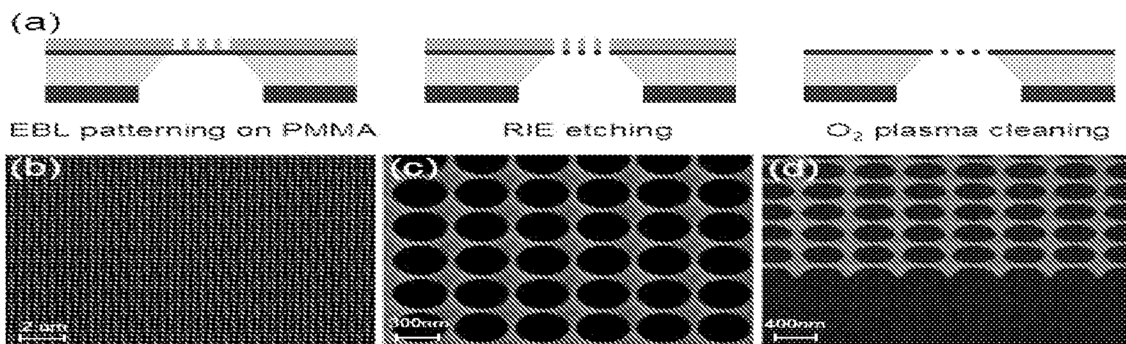
77. The biosensor device of claim 76, wherein the upper chamber comprises a glass surface and the lower chamber comprises a glass surface.

78. The biosensor device of claims 76 or 77, further comprising a housing, wherein the upper chamber, lower chamber, and suspended plasmonic nanostructure sensor is enclosed in the housing.

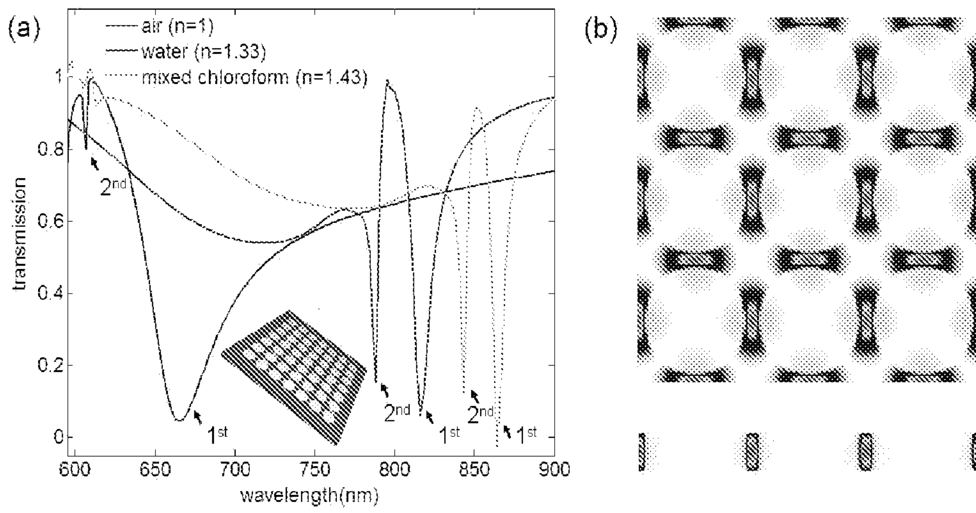
79. The biosensor device of claim 78, wherein the housing comprises polydimethylsiloxane (PDMS).



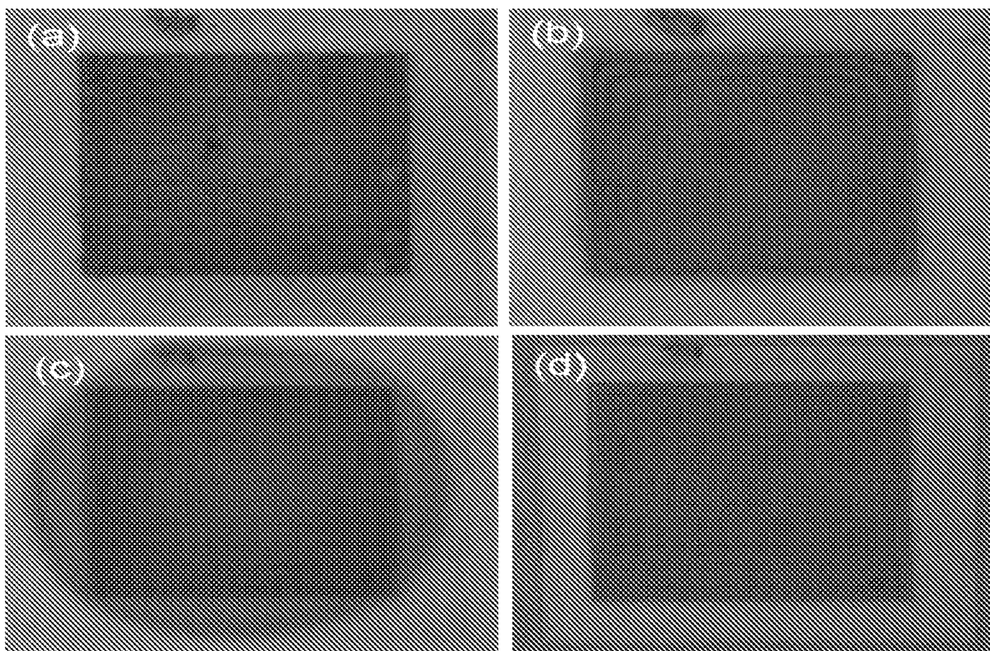
FIGURES 1A-1D



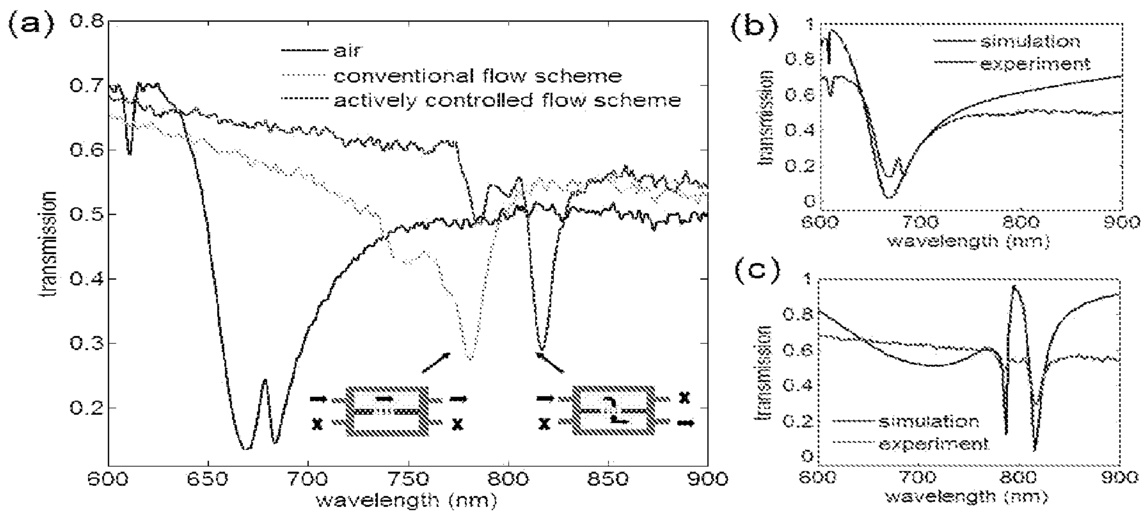
FIGURES 2A-2D



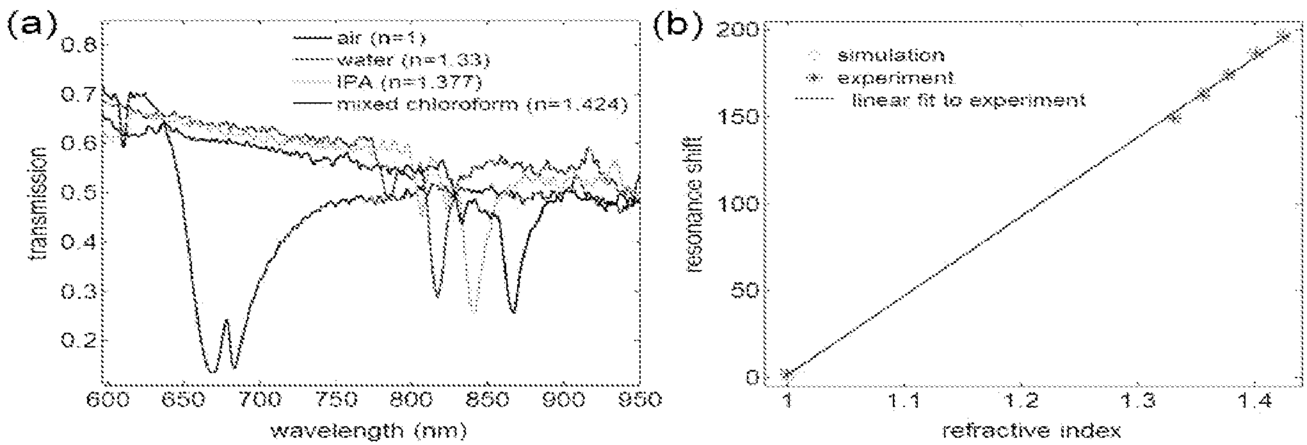
FIGURES 3A-3B



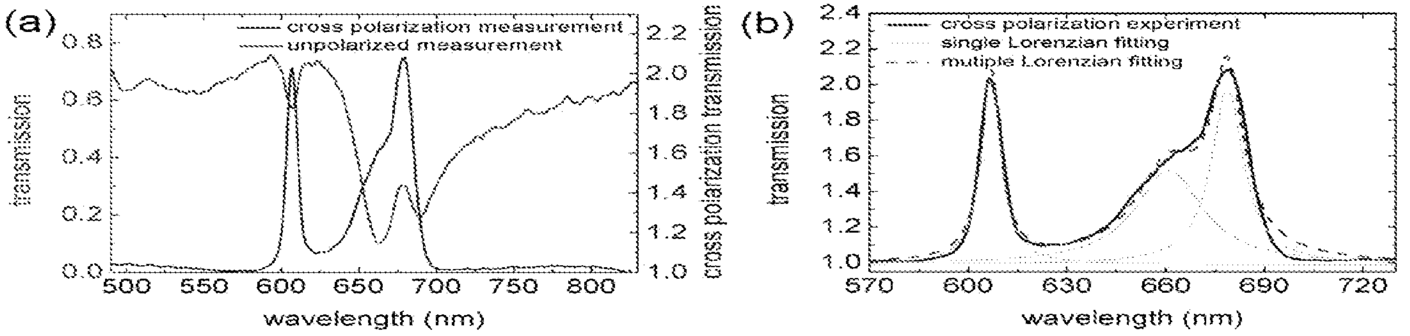
FIGURES 4A-4B



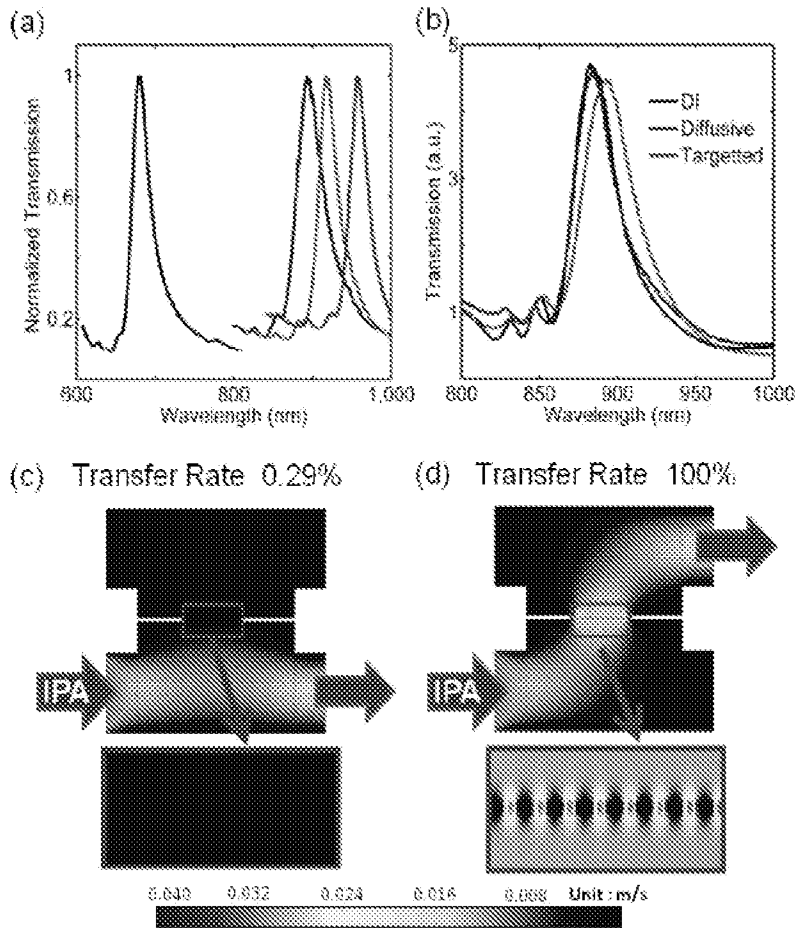
FIGURES 5A-5C



FIGURES 6A-6B



FIGURES 7A-7B



FIGURES 8A-8D

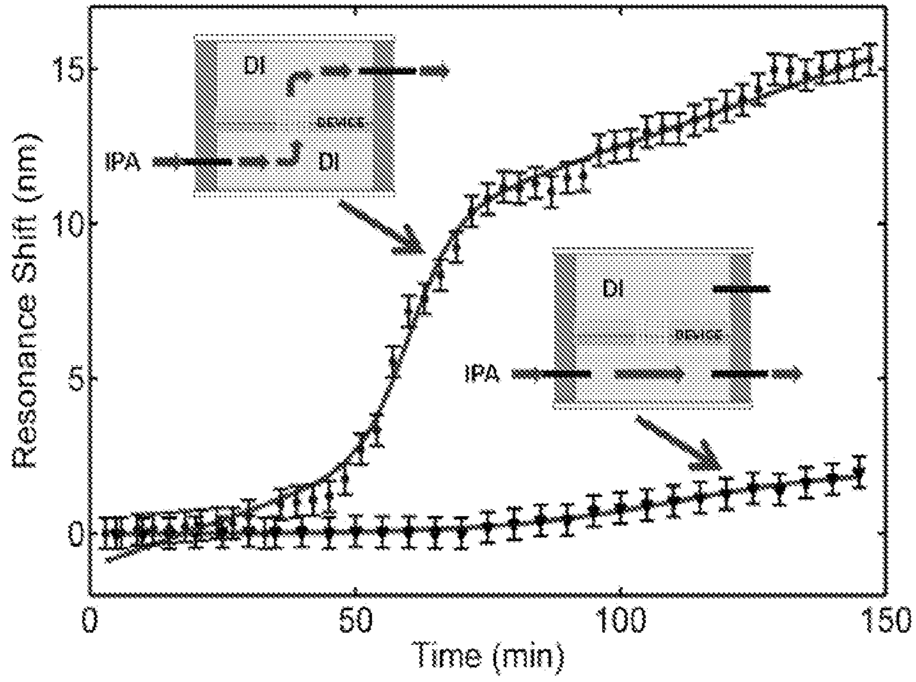
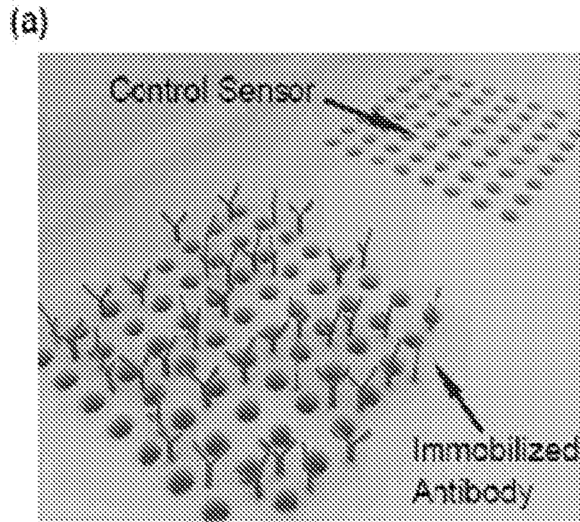
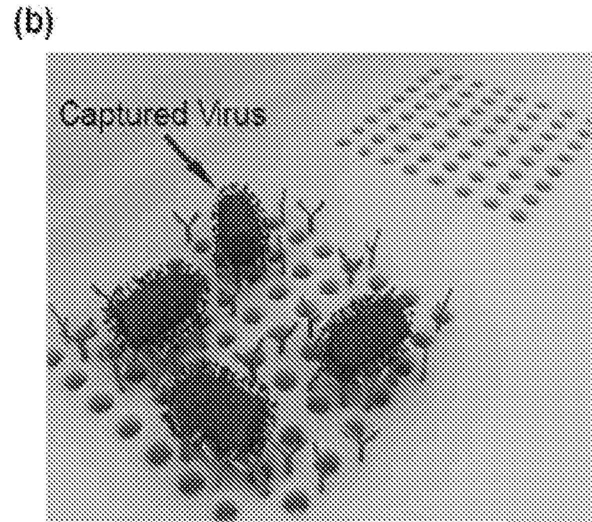


FIGURE 9

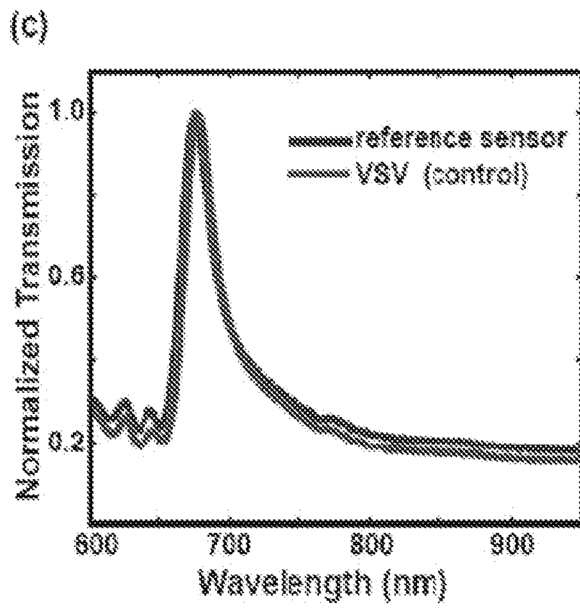
(a)



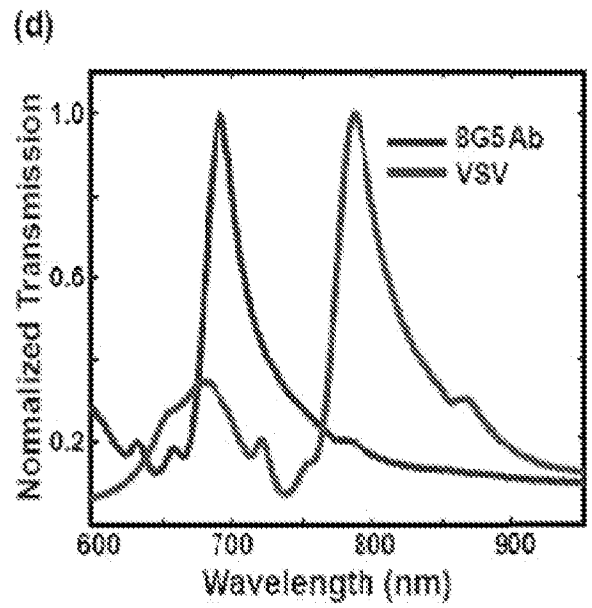
(b)



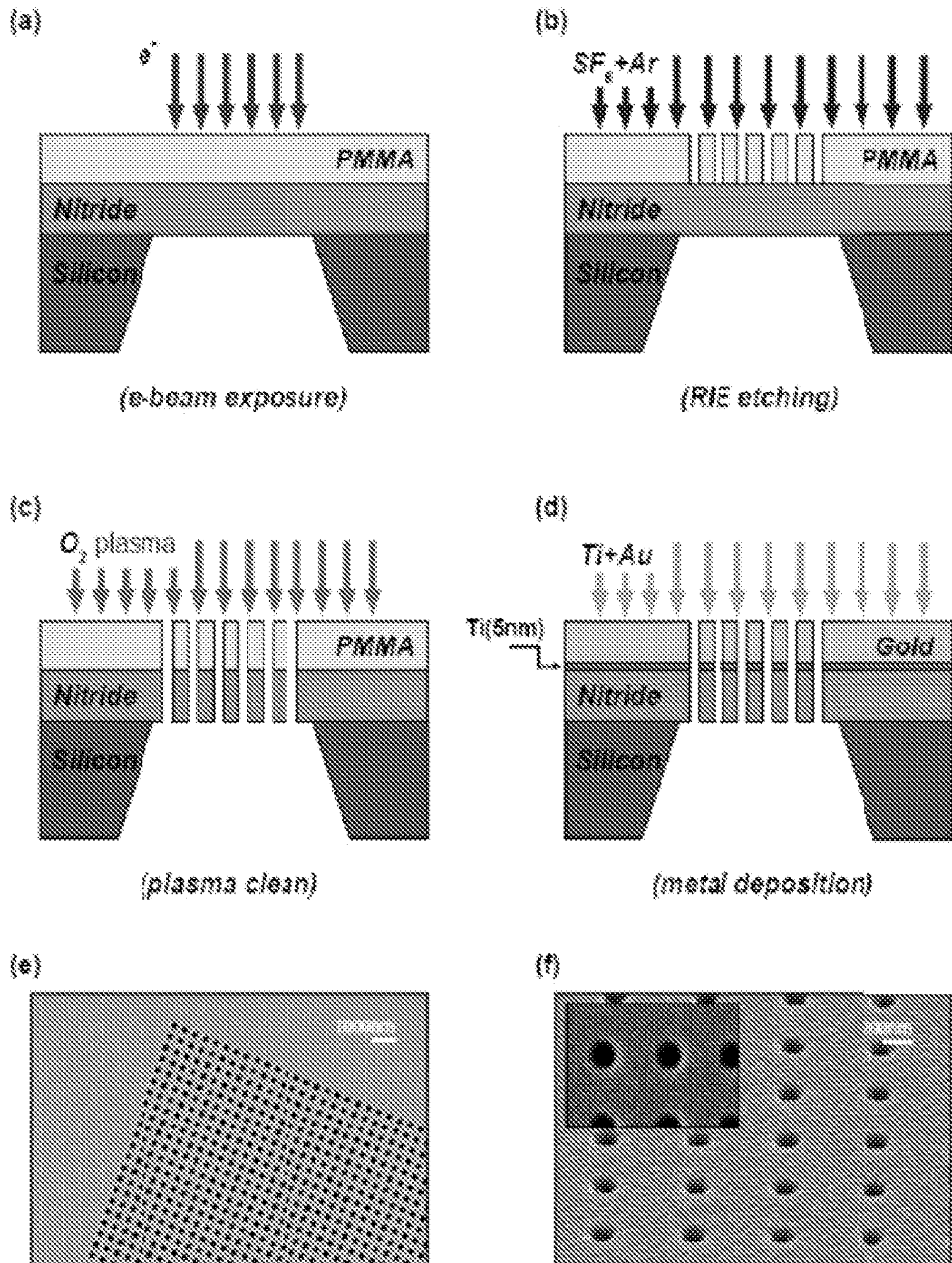
(c)



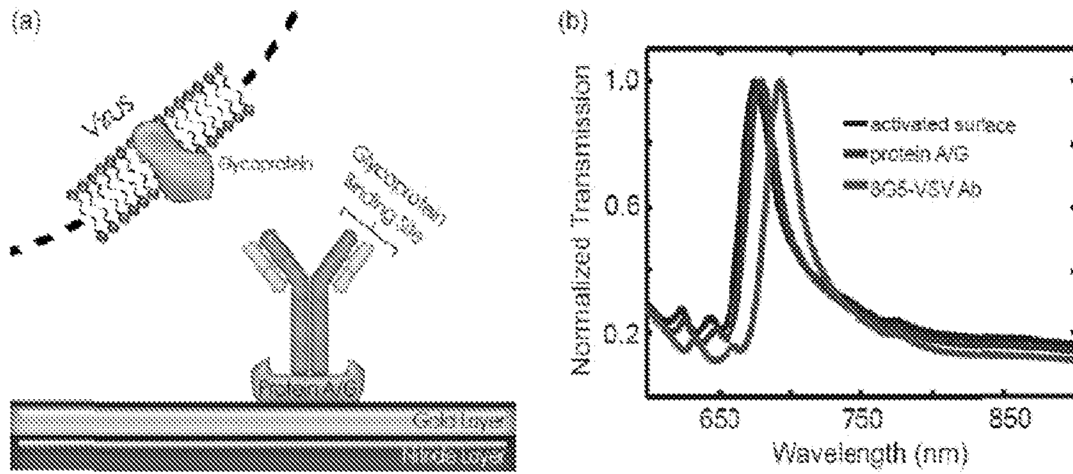
(d)



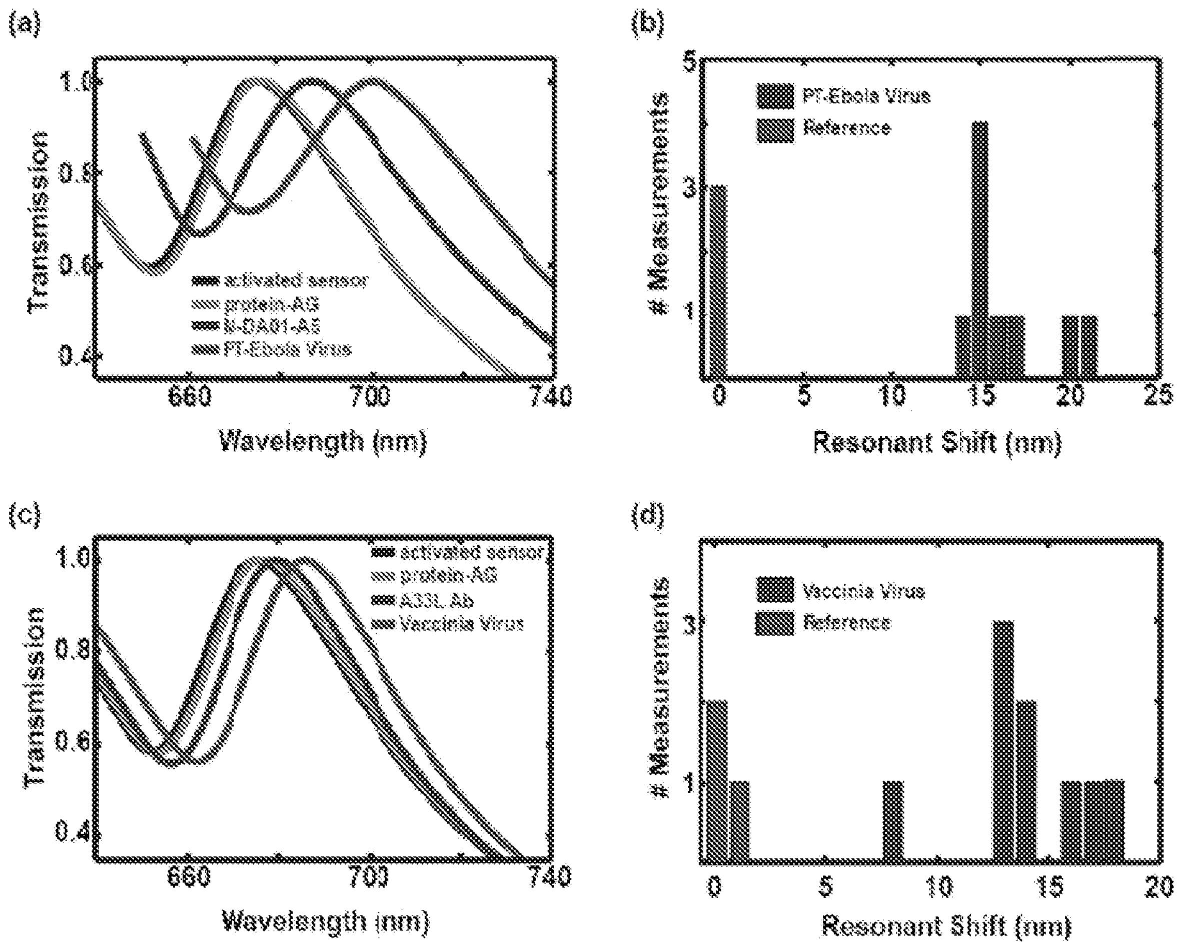
FIGURES 10A-10D



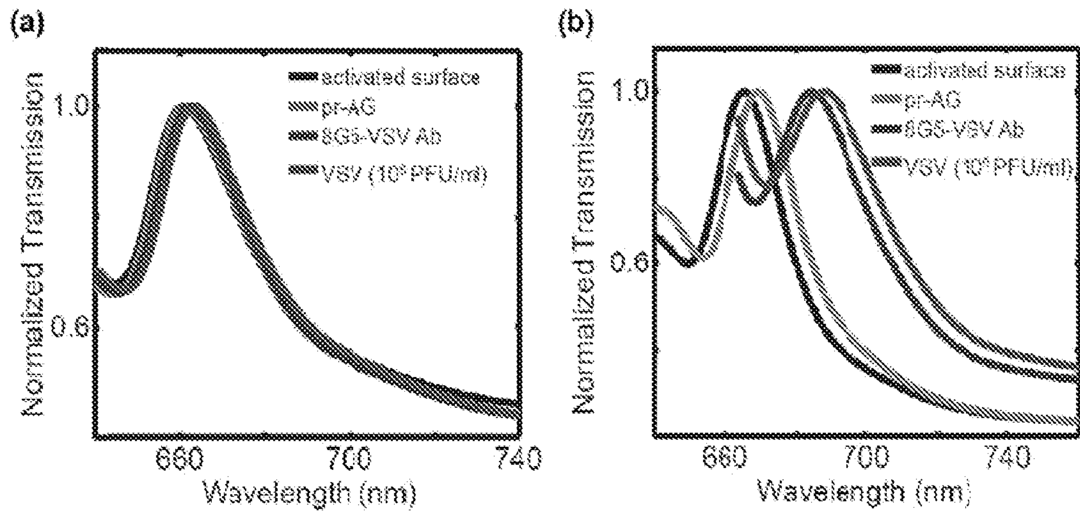
FIGURES 11A-11F



FIGURES 12A-12B



FIGURES 13A-13D



FIGURES 14A-14B

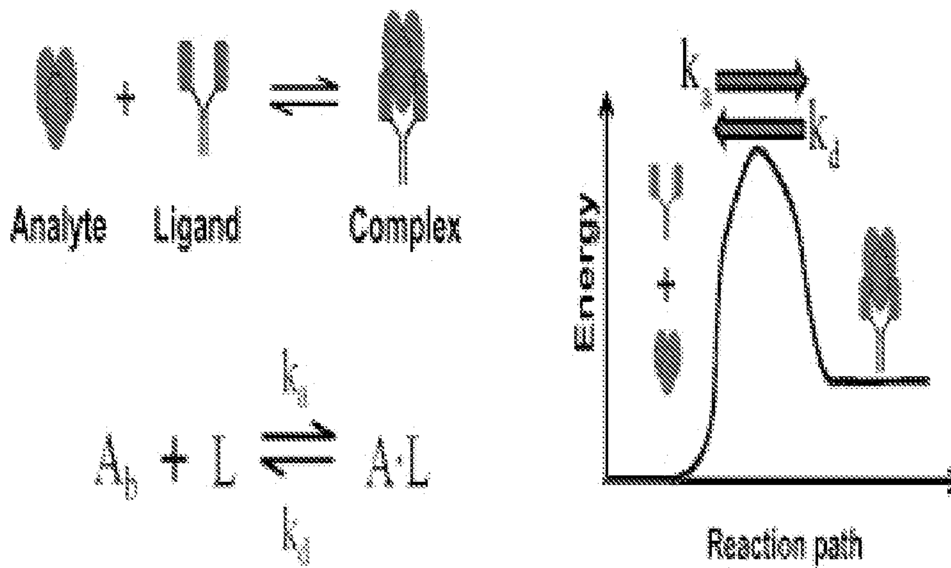


FIGURE 15

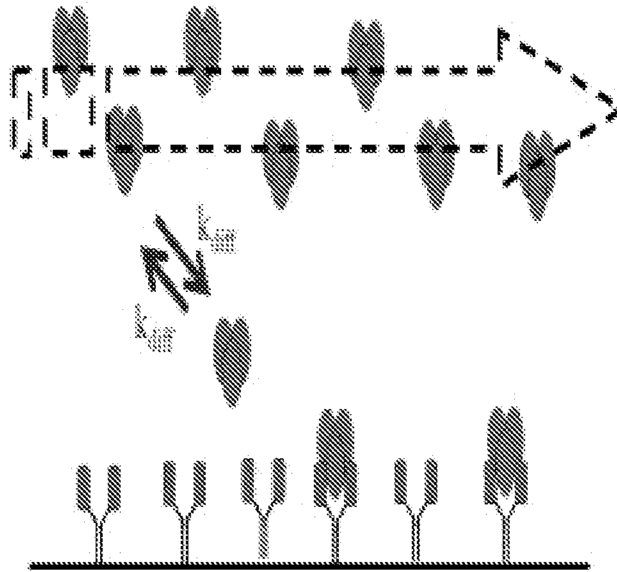


FIGURE 16

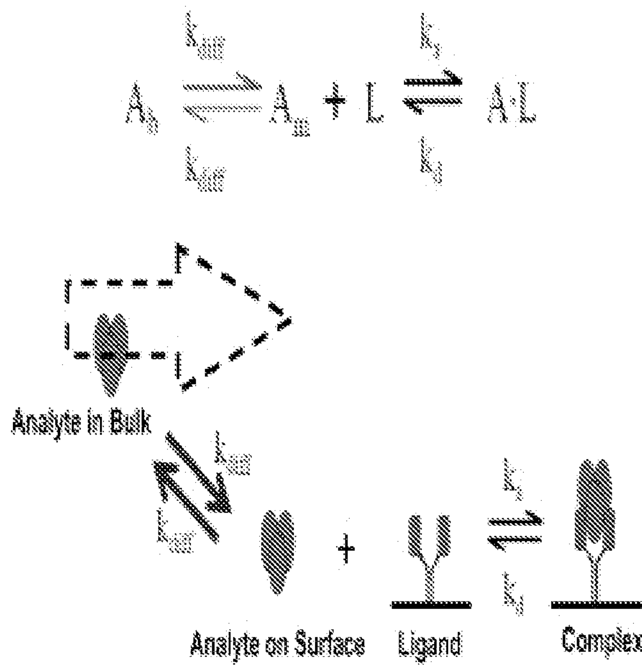


FIGURE 17

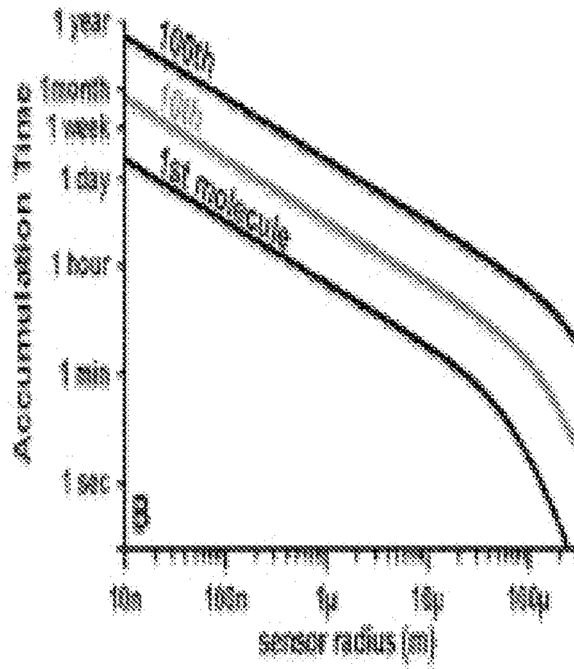


FIGURE 18

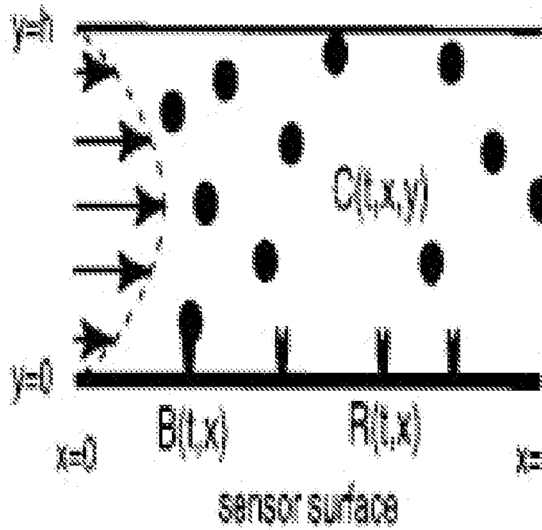
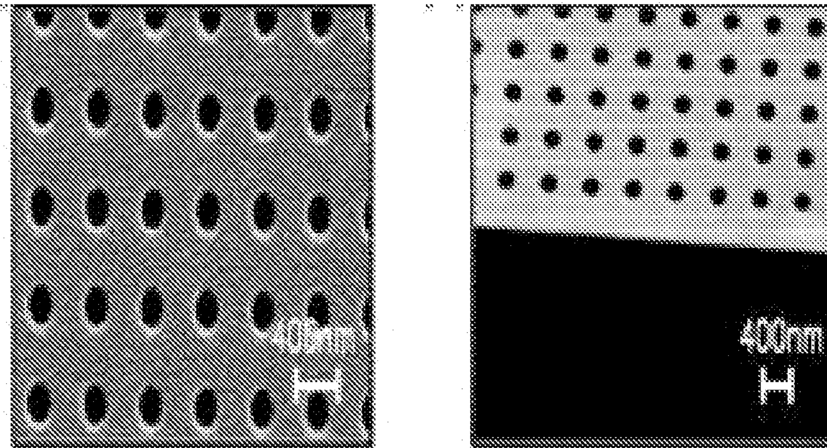


FIGURE 19

PLASMONIC NANOHOLE (METAL BASED)



PHOTONIC CRYSTAL (DIELECTRIC BASED)

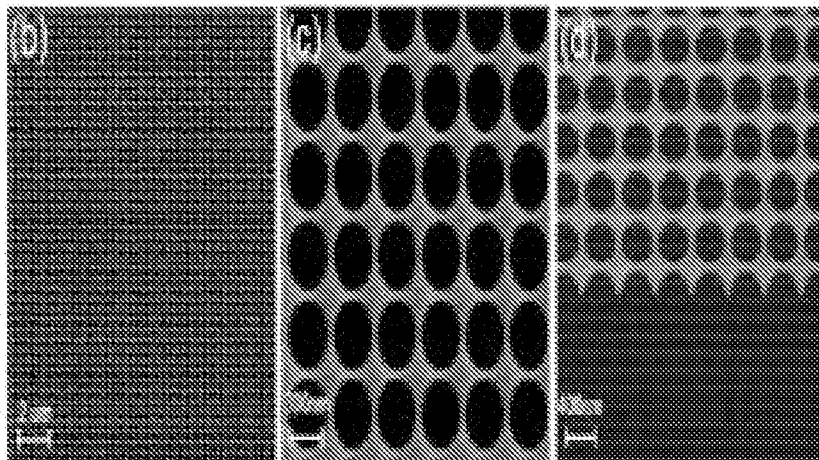


FIGURE 20

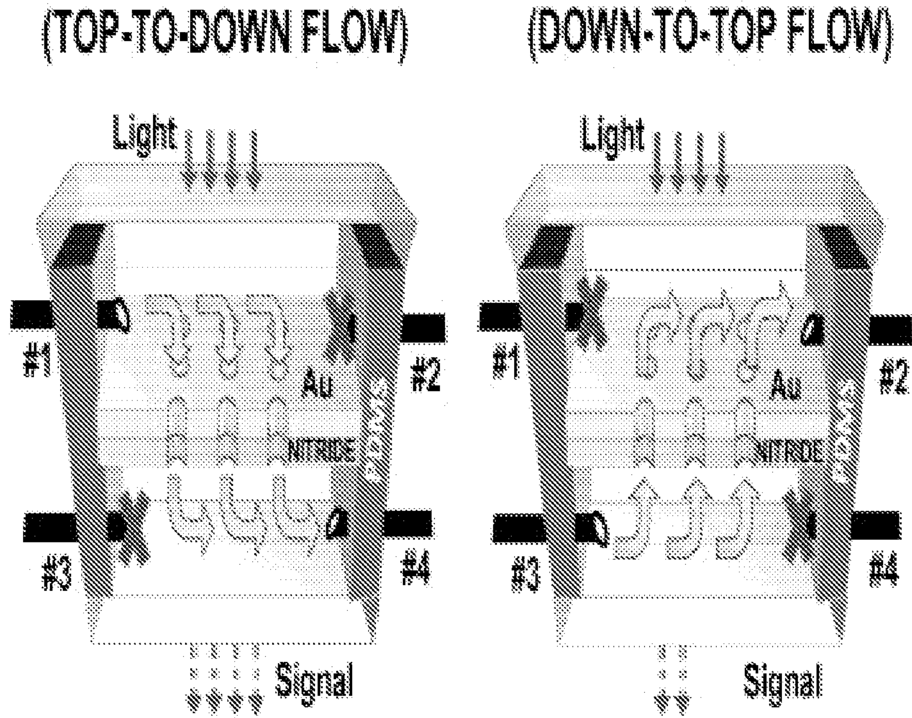


FIGURE 21

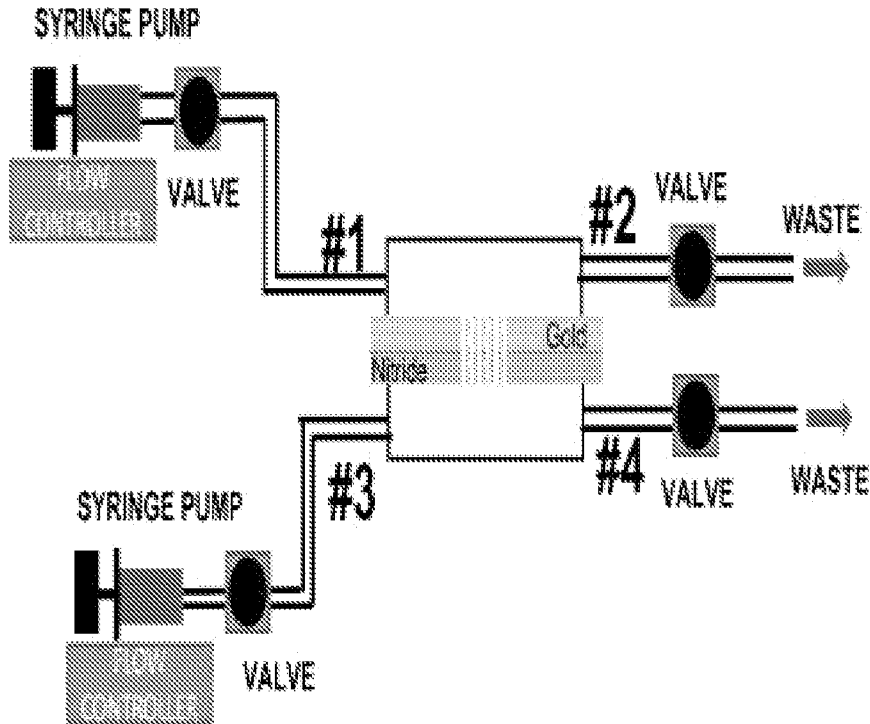


FIGURE 22

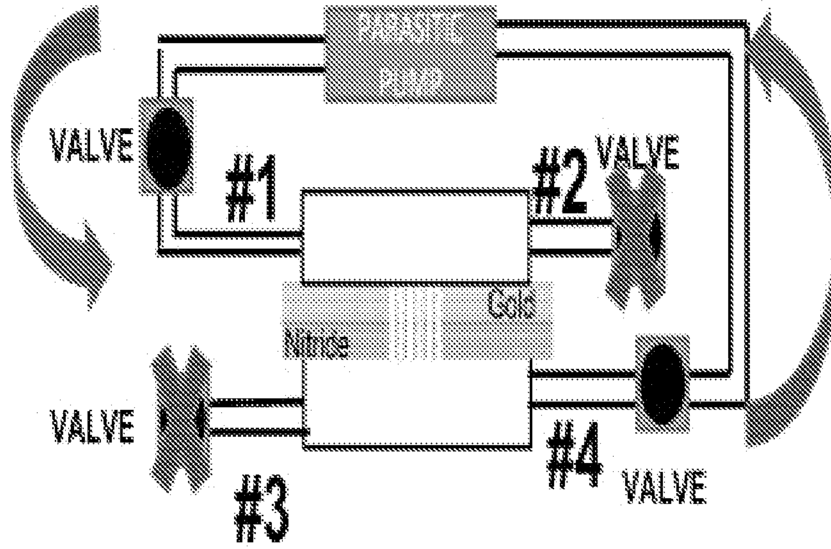
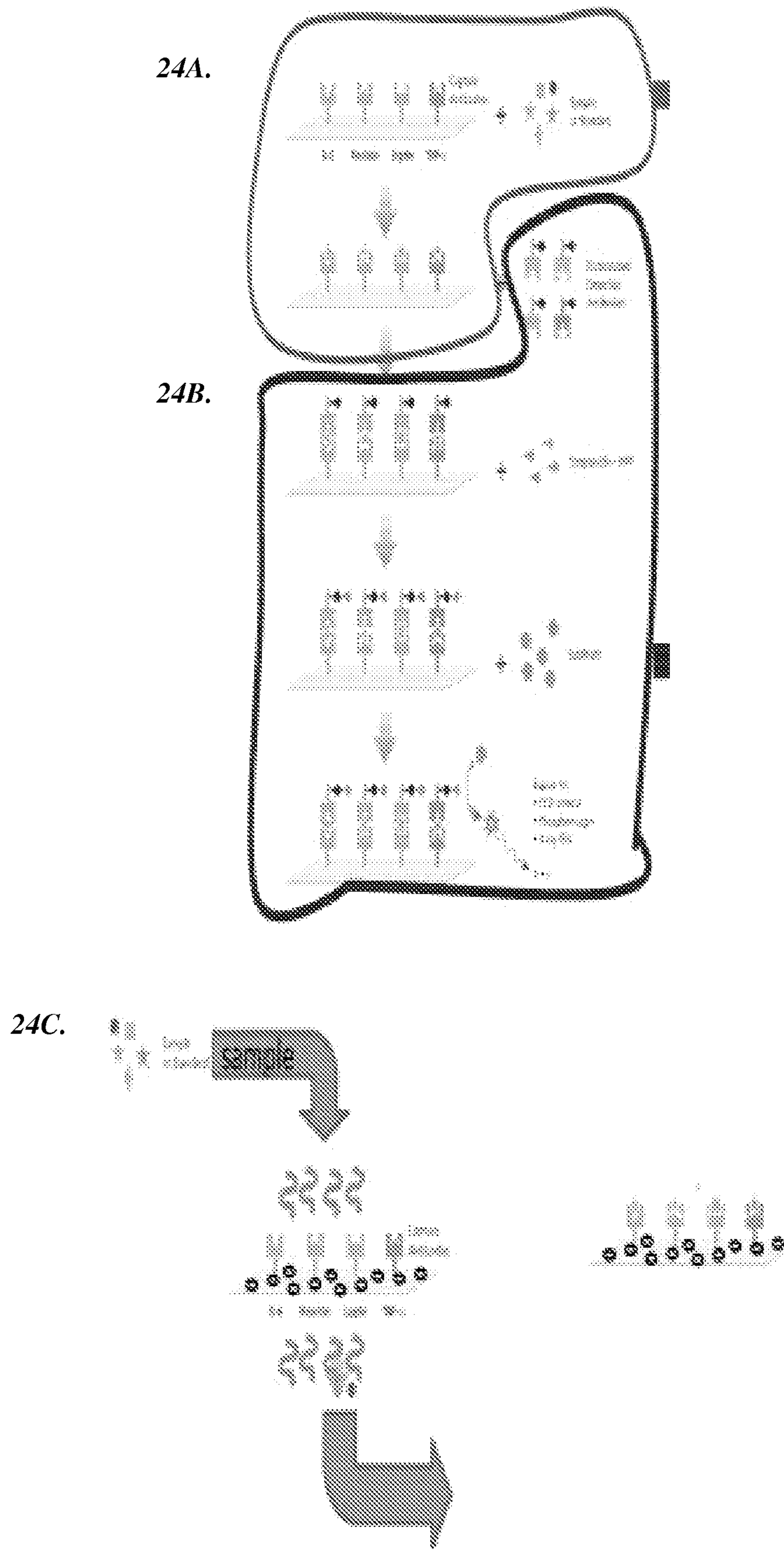


FIGURE 23



FIGURES 24A-24C