Recent advances in peptide probe–based biosensors for detection of infectious agents

Jason E. Dover[1#], Grace M. Hwang[2#], Elaine H. Mullen[2], Barton Prorok[3], and Sang-Jin Suh[1*]

[1] Department of Biological Sciences, Auburn University, Auburn, AL
[2] The MITRE Corporation, Bedford, MA and McLean, VA
[3] Department of Materials Engineering, Auburn University, Auburn, AL

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[#] Contributed equally to this article.

* Corresponding author: Department of Biological Sciences, 101 Life Sciences Building, Auburn University, AL 36849. Ph: 334-844-1666; FAX: 334-844-1645; e-mail: suhsang@auburn.edu

Sponsor: Laura Silo-Suh, Department of Biological Sciences, 101 Life Sciences Building, Auburn University, AL 36849. Ph: 334-844+1338; email: suhlaur@auburn.edu

ABSTRACT

Recent biological terrorism threats and outbreaks of microbial pathogens clearly emphasize the need for biosensors that can quickly and accurately identify infectious agents. The majority of rapid biosensors generate detectable signals when a molecular probe in the detector interacts with an analyte of interest. Analytes may be whole bacterial or fungal cells, virus particles, or specific molecules, such as chemicals or protein toxins, produced by the infectious agent. Peptides and nucleic acids are most commonly used as probes in biosensors because of their versatility in forming various tertiary structures. The interaction between the probe and the analyte can be detected by various sensor platforms, including quartz crystal microbalances, surface acoustical waves, surface plasmon resonance, amperometrics, and magnetoelastics. The field of biosensors is constantly evolving to develop devices that have higher sensitivity and specificity, and are smaller, portable, and cost-effective. This mini review discusses recent advances in peptide-dependent rapid biosensors and their applications as well as relative advantages and disadvantages of each technology.

INTRODUCTION

Throughout history, pathogenic microorganisms have significantly impacted human activities, whether by causing disease or by being used deliberately in biological warfare (Crawford, 2007; Lim et al., 2005). The anthrax attacks that occurred post-9/11 in the United States highlight the potentially deadly threat posed by the intentional use of biological threat agents (BTA) against both civilians and the military (Fennelly et al., 2004). In addition, recent outbreaks of *Escherichia* and *Salmonella* in the United States make clear the danger of microbial pathogens disseminated through contaminated food (http://www.cdc.gov/; http://www.fda.gov/oc/opacom/hottopics/Salmonellatyph.html; (Goldschmidt, 2006)). Infectious agents also have an indirect effect on agricultural and other related commodities. For example, the recent discoveries in the United States of Huanglongbing disease (citrus greening) in citrus crops and Pierce's disease in grapes threaten to have a severe economic impact on the specialty crop industry at the state, national and international levels (Bové, 2006; Hopkins and Purcell, 2002). To minimize the effects of natural outbreaks or deliberate attacks, near real-time detection of infectious agents is an essential first step in mounting an appropriate response.

Traditionally, infectious agents were detected and identified using standard microbiological and biochemical assays that were accurate but time-consuming. Traditional methods required isolation and/or culturing of large quantities of the infectious agents, and therefore needed several days to complete the analysis. More recently, molecular approaches to identify infectious agents have supplanted traditional microbiological methods because they are more sensitive and take less time. Molecular approaches such as the polymerase chain reaction

(PCR) amplification and analyses of unique DNA sequences and/or 16S rDNA are highly accurate and sensitive (Deisingh and Thompson, 2002). However, these assays require specialized instruments and still take several hours to perform. In addition, DNA-based molecular techniques are limited to the detection of whole organisms and cannot detect toxins and other extracellular products of infectious agents. New techniques are needed that combine the accuracy and breadth of traditional microbiological approaches with the enhanced accuracy and sensitivity of molecular approaches.

Biosensor technology is one such technique that brings together the accuracy and sensitivity of standard approaches with improvement in rapidity of detection. Biosensors also offer the possibility of continuous and real-time monitoring of the environment for the presence of infectious agents to allow timely implementation of preventive and protective measures. The majority of biosensors take advantage of the affinity between a probe molecule and an analyte. Hence, specificity of the probe:analyte interaction is critical for designing an effective biosensor. The sensor platform that detects the probe:analyte interaction and generates a measurable signal needs to be sensitive enough to discern infectious agents even at low concentrations.

An ideal field-ready biosensor should differentiate between pathogenic and non-pathogenic organisms with high sensitivity and accuracy (Ivnitski et al., 2003). Although great technological improvements have been made in continuous collection of environmental samples and increased sensitivity in detection of infectious agents (Chase et al., 2005; Christensen et al., 2006; Jones et al., 2005; Keer and Birch, 2003; Makino and Cheun, 2003), field-ready biosensors continue to be plagued by background interference during collection, duration of detection time

and portability (Petrenko and Sorokulova, 2004). Furthermore, an ideal early warning biosensor system should be designed as an array that can simultaneously detect a multitude of infectious agents while minimizing the probability of false alarms. In this mini review, we discuss recent advances in near real-time peptide-based biosensors for the capture and detection of various infectious agents with an emphasis on label-free detectors suitable for field deployment. Due to the time-consuming nature of the polymerase chain reaction (PCR), we have intentionally minimized discussions of biosensor platforms that contain a PCR step.

2.0 LABEL-FREE BIOSENSORS

A label-free biosensor consists of a sensing element or probe/receptor molecule tethered to a stable sensing surface. A sensing transducer detects the probe:analyte interaction and provides a measurable signal for the binding reaction (Goldschmidt, 2006; Petrenko and Sorokulova, 2004). Ideal biosensor characteristics have been described by Ivnitski et al. (Ivnitski et al., 1999) and these are summarized in Table 1. Specificity is achieved by using a probe that interacts only with the target analyte. This probe is absolutely critical in the overall design and success of a biosensor because it reduces the incidence of false positives. In addition, an effective probe must have high affinity and avidity for the target analyte in order for the sensor to detect the analyte in a complex sample. Without a strong and highly specific probe:analyte interaction, the biosensor loses its efficacy and advantages over the traditional microbiological and molecular biological detection methods. Other desirable features include a long shelf life, reproducibility, capability for continuous monitoring, and portability.

The sensitivity of biosensors is determined in part by the ability of the sensor platforms to generate detectable signals even with a low concentration or frequency of probe:analyte interactions. The majority of sensor platforms detect a perturbance that is created when the unbound probe binds the target analyte, and then translate that interaction into a measurable signal. Label-free biosensors do not require secondary or tertiary reactions to generate measurable signals (i.e., ELISA or DNA sequencing), and are thus ideal for continuous and near real-time monitoring of infectious agents. Sensor platforms with low detection limits can detect the presence of even a minute quantity of infectious agents. (Specifics of each sensor platform are described below in section 4.0.)

Biosensors are classified as single-use sensors, intermittent-use sensors, and continuous-use sensors (Kissinger, 2005). Intermittent-use sensors, typically found in laboratory settings, are accurate and have the capability for data storage. In comparison, single-use and continuous-use sensors currently have relatively poor accuracy and sensitivity (Kissinger, 2005). Further research and development are needed to produce continuous real-time monitoring biosensors that are small, affordable, accurate and sensitive.

3.0 PEPTIDE-BASED RECEPTOR MOLECULES (PROBES)

Peptides are remarkable in their ability to form various tertiary structures that interact with numerous molecules. This ability is clearly demonstrated in antibodies, which contain

highly specific complementarity determining regions (CDR) for recognition of various antigens. As a result, peptides have been explored as ideal probe molecules for biosensors. In this section, relative advantages, disadvantages, and experimental application of various peptide probes in biosensors will be discussed.

3.1 ANTIBODIES, ANTIBODY FRAGMENTS, LLAMABODIES

Due to their specificity and affinity for diverse analytes, antibodies have been a natural choice for molecular receptors and probes in biosensors (Luppa et al., 2001; Ziegler and Gopel, 1998). The biggest advantage of antibody-based probes is the specificity and affinity of these polypeptides to target analytes. Antibodies form tight non-covalent bonds with specific target molecules with apparent K_d values of 10^{-7}–10^{-11} M (Abbas and Lichtman, 2005). Thus, antibodies can interact strongly with the target analyte even in a complex mixture, resulting in a biosensor that is highly specific.

Unfortunately, despite their high affinity and specificity for infectious agents, antibodies have limitations as biosensor probes that affect their applicability in the field. One of the major disadvantages of antibodies is their relative instability to environmental fluctuations, especially high temperature, compared to other peptide-based probes (see below). This limitation may require antibody-based biosensors to be stored in refrigerated containers, and can reduce long-term storage and field applicability. In addition, production of polyclonal antibodies in animals is time-consuming and costly. Furthermore, polyclonal antibodies frequently lack selectivity

because they recognize different epitopes on the same pathogen. Some of these antigens may be present in other closely related but non-pathogenic organisms and may lead to false positive results. This lack of selectivity also leads to a decrease in affinity and specificity to the desired target molecule. In contrast, monoclonal antibodies are more selective and can be produced rapidly in vitro. A recently developed antibody against the spore coat of *Bacillus anthracis* that can differentiate *B. anthracis* spores from vegetative cells or from spores of other *Bacillus spp.* demonstrates the enhanced specificity of monoclonal antibodies (Swiecki et al., 2006). However, higher production costs and susceptibility to unfavorable environmental conditions limit the broad use of monoclonal antibodies in field-ready sensors (Goldman et al., 2006; Pancrazio et al., 1999; Shone et al., 1985).

The discovery in the 1990s of camelid and shark antibodies composed only of single heavy chains with very small antigen-binding domains (Goldman, et al., 2006; Greenberg et al., 1995; Hamers-Casterman et al., 1993) facilitated the development of thermostable antibodies that retain specificity. The hypervariable regions from these antibodies have been cloned and expressed as 12–15 kDa single-domain antibodies (sdAbs or nanobodies) that are stable to temperatures as high as 90ºC. Using this technology, Goldman et al. (Goldman, et al., 2006) successfully developed heat-stable llama sdAbs for a broad range of Marburg hemorrhagic fever virus antigens . In 2007, Sherwood et al. (Sherwood et al., 2007) developed a sensitive assay for Marburg virus variants based on llama sdAbs that did not cross-react with Ebola virus antigens. In an unoptimized chemiluminescent assay, the most specific clone could detect 0.1–1 pfu/well within 30 minutes. In the presence of a detergent, the signal-to-noise ratio exceeded 1000 at best.

These highly sensitive and selective sdAb probes could be used in any antibody-based biosensor designed to detect infectious agents (Goldman, et al., 2006).

Phage-antibody technology has also been developed (Marks et al., 1991; McCafferty et al., 1990) to overcome some of the disadvantages associated with antibodies. With this technology, a particular fragment of the antibody, including antigen binding sites, is produced and displayed on the surface of a bacteriophage. The advantages of displaying antibody fragments on the surface of a bacteriophage include smaller size and increased stability of the peptide probe (Brigati and Petrenko, 2005). For display of antibodies and peptides, the Ff class of filamentous phages (M13 and Fd) has been most commonly used. In this technique, cDNA encoding antibody heavy and light chains (V_H, V_L) is amplified from human B-cells, combined, and cloned into the bacteriophage pIII gene to generate a random library with potentially billions of clones with differing antigen specificities (Hoogenboom, 2002). Phages that display antibody fragments against a particular analyte are then selected by biopanning and are further characterized (Iqbal et al., 2000; Petrenko and Vodyanoy, 2003).

Three different types of libraries can be generated through phage-antibody technology: naïve, synthetic, and classical. A naïve library is constructed by amplifying the V (variable) genes from B-cell lymphocytes of a naïve animal host. A synthetic library is constructed by incorporating human V genes into any germ line. The V genes can be further mutated to increase the CDR diversity of the library. A classic library contains clones of developed V_H and V_L chains from an immunized host that have been recombined into scFv (single chain fragment variable) antibodies (Conrad and Scheller, 2005). These antibodies can be displayed as the Fab (fragment

antigen binding), which consists of a fragment of the heavy chain connected by a disulfide bond to the light chain with the Fv (fragment variable) (Skerra and Pluckthun, 1988) antigen binding domain, which is stabilized with a second disulfide bond (Hoogenboom, 2002). These antibodies can also be displayed as an scFv fragment that is a contiguous linear polypeptide of V_H and V_L domains held together by a flexible amino acid linker (Huston et al., 1991).

As expected, antibody fragments displayed on phages offer an advantage over antibodies in terms of specificity, sensitivity, and robustness (Crowther, 1995; Petrenko and Sorokulova, 2004). The smaller size of the Fv or scFv fragments is also an advantage. The typical IgG antibody is approximately 150 kDa, while the antibody fragments, F_V and scFv, are approximately 30 kDa. The Fab is less stable compared to the scF_V, but is less prone to some of the problems experienced with the scFv, which include the formation of aggregate bodies (Marks et al., 1992). However, the Fab can be stabilized in a similar manner to the scFv via a polypeptide linker between the Fd (fragment difficult) and the light chain to form a single-chain Fab (scFv). In addition, thermodynamic stability of the Fab can be augmented through point mutations in the constant domains of heavy and light chains (Teerinen et al., 2006). (For a more detailed review of antibody phage display, see Conrad and Scheller (Conrad and Scheller, 2005).)

Biosensors equipped with antibody/antibody fragments have been used to detect an assortment of targets including *Brucella melitensis* (Hayhurst et al., 2003); *B. anthracis* (Campbell and Mutharasan, 2006b; Wang et al., 2006); *Francisella tularensis* and *B. subtilis* spores (Chinowski et al., 2007); *E. coli* O157:H7, *Salmonella typhimurium*, *Legionella*

pneumophila, and Yersinia enterocolitica (Oh et al., 2005); Sin Nombre hantavirus (Bisoffi et al., 2008; Velappan et al., 2007); Avian influenza virus (Zhang et al., 2006) ; Venezuelan equine encephalitis (VEE) virus (Hu et al., 2004); and toxins (Emanuel et al., 2000).

3.2 BACTERIOPHAGE AND BACTERIOPHAGE-DISPLAYED PEPTIDES

For biosensor technology, lysogenic filamentous phages of the Ff class have been used extensively. The Ff class of filamentous phages contains single-stranded plus-sense DNA that is encapsulated within a capsid composed of several different proteins (Figure 1). To display oligopeptides, a DNA sequence that encodes a peptide is cloned in frame to either a pIII or pVIII gene without disrupting its function. An oligopeptide fused to pIII is then displayed in five copies at the polar end of the phage. In contrast, an oligopeptide fused to pVIII is displayed in approximately 2800 copies and forms a unique “landscape” on the surface of the phage due to interaction among the displayed oligopeptides. The major advantage of a landscape phage is the greater avidity compared to pIII-displayed probes. Addition of random nucleotides in frame to pIII or pVIII genes results in construction of a random phage display library. Phage-displayed oligopeptide probes have been described in detail in several excellent articles by Petrenko and colleagues (Petrenko and Smith, 2000; Petrenko and Sorokulova, 2004; Petrenko and Vodyanoy, 2003; Smith and Petrenko, 1997). The major disadvantage of displaying an oligopeptide on pVIII is the relatively short peptide (maximum of 8–9 amino acids) that can be fused to the pVIII protein without affecting its function.

Phage-displayed peptides are attractive alternatives to antibody probes for several reasons. First, phage display is more cost-effective than monoclonal antibodies. Second, oligopeptides displayed on phages are more stable and resistant to environmental stressors than typical non-camelid antibodies (Brigati and Petrenko, 2005). Finally, phage-displayed peptide probes are more amenable than antibodies to manipulation at the molecular level to improve their interaction with the analyte.

Of interest in this review are applications of phage-displayed peptide probes to detect BTA such as *B. anthracis* spores. Recently, several classes of pVIII- or pIII-displayed oligopeptides that recognize *B. anthracis* spores were identified (Brigati et al., 2004; Turnbough, 2003; Uithoven et al., 2000; Williams et al., 2003). Unfortunately, none of the peptides demonstrated 100% specificity for the target spores, which is most likely due to a strong structural similarity of glycoproteins surrounding *Bacillus spp.* spores. A recent experiment using ruthenium red to stain *Bacillus spp.* spores demonstrated a glycoprotein layer surrounding both *B. subtilis* and *B. anthracis,* and suggested a higher ultrastructural similarity among various *Bacillus* species than had been recognized previously (Waller et al., 2004).

Despite the limited specificity for *B. anthracis* spores, pVIII landscape phage-displayed oligopeptides have been successfully used to identify other infectious agents, such as *S. enterica* serovar Typhimurium (Olsen et al., 2003; Olsen et al., 2006; Sorokulova et al., 2005), an important foodborne pathogen. Other interesting developments include genetically engineered T7 phage that can be biotinylated in vivo for highly sensitive and specific detection of *E. coli* using streptavidin conjugated quantum dots (Edgar et al., 2006); use of a lytic phage for highly

specific capture and detection of *Staphylococcus aureus* (Balasubramanian et al., 2007); and enhancing expression of peptides on the major coat protein, pVIII, via the twin arginine translocation (TAT) system to improve secretion of the fusion protein to the periplasm. The TAT pathway is one of many secretion pathways in prokaryotes. It differs from the more common Sec pathway in its ability to directly transport proteins in their native conformation across the membrane. This pathway has been exploited to improve the expression of probe peptide:pVIII fusion proteins on filamentous phages (Paschke and Hohne, 2005; Thammawong et al., 2006).

In summary, recent improvements in peptide probes have focused on enhancing stability and lowering production costs. The discovery of thermostable antibodies, the use of phages to display antibodies and peptides, and the use of phages as probes, have resolved these issues and promise a bright future for peptide probes in rapid biosensors.

4.0 SENSOR PLATFORMS

In this section we discuss various types of biosensor platforms, with an emphasis on receptor probes, detection limits, and potential for field deployment. In general, probe:analyte interactions are measured by monitoring perturbation in optical characteristics, mass-induced resonance changes, or electrical properties.

4.1. MASS PERTURBANCE BIOSENSORS

Biosensors that monitor mass-induced perturbations include piezoelectric cantilever arrays, quartz crystal microbalance (QCM) sensors, surface acoustic wave (SAW) devices, and magnetoelastic transducers.

4.1.1. Piezoelectric sensors, also referred to as “acoustic wave” or “microbalance” sensors, measure changes in resonant frequency that result from an alteration in mass when the analyte binds to the probe. Bulk acoustic wave devices use the entire piezoelectric substrate for wave propagation. The maximum amplitude of vibration occurs at the top and bottom faces of the crystal, thus allowing bulk acoustic wave devices to function as surface detectors. The typical bulk wave device operates at between 10 and 50 MHz. Unlike bulk acoustic wave devices, surface acoustic wave sensors operate on the principle of acoustic energy confined near a thin surface region of the substrate (Figure 2). Most SAW devices oscillate in the 50 MHz to low GHz range.

Typically, acoustic energy confinement is generated and detected with interdigital transducers located on the surface of a piezoelectric crystal. The basic SAW device wave speed is determined by the mass and material properties of the probe coating. The relation between change in resonant frequency and mass adsorbed is defined by the Sauerbrey equation, $\Delta f = (-2.3 \times 10^6 F^2 \Delta m)/A$, where F is the resonant frequency in MHz, Δm is the mass change in grams, and A is the coated sensing area in cm^2 (Lazcka et al., 2007). Therefore, the amount of analyte mass adsorbed by the probe determines the magnitude of change in the oscillator circuit frequency. For acoustic wave sensors of equal cross-sectional area, the sensitivity is proportional to the change in mass and the square of the resonant frequency. Therefore, devices with higher

resonant frequencies are more sensitive. Thus the primary advantage of SAWs over QCM devices is higher sensitivity. However, the most common type of bulk acoustic wave device is the QCM, due in part to quartz's tolerance to high temperatures.

Some SAW designs are intended to work in liquid media (e.g horizontally polarized shear waves), whereas QCMs are damped by liquid. To overcome the high dielectric constant of water, SAW devices have been fabricated since the 1990s with lithium niobate and lithium tantalite instead of quartz (Lange et al., 2008). Newer materials such as piezoelectric aluminum nitride thin film have been used to produce SAW devices in the 2 GHz resonant frequency range, but these materials have not yet been utilized in SAW-based biosensors (Gronewold, 2007). (Comprehensive reviews of the last 20 years of acoustic wave research can be found in Lange et al., 2008 and Gronewold, 2007.)

Oligopeptides have been used successfully as probes on SAW sensors. To detect *S. enterica* serovar Typhimurium, Olsen et al. (Olsen, et al., 2006) immobilized a pathogen-specific landscape phage and demonstrated the usefulness of a phage-displayed probe bound to an acoustic wave transducer. The phage was attached to the transducer via physical adsorption and tested against logarithmic concentrations of *S. enterica*. The sensor demonstrated a strong dose-response and detected the interaction between phages and bacteria in approximately 200 seconds with a lower detection limit of 100 cells/ml. These results correlated with a previous study performed with polyclonal antibodies bound to a Langmuir-Blodgett (LB) layer on an acoustic wave transducer (Olsen, et al., 2003; Olsen, et al., 2006) and suggested that physical adsorption of phage to QCM was comparable to deposition of antibodies on an LB layer. Nanduri et al.

(Nanduri et al., 2007) verified that apparent K_d values of phage bound to a target molecule, β-galactosidase in solution, were similar to physical adsorption (1.7 ± 0.5 nM) and LB monolayer deposition (0.6 ± 0.4 nM).

Monoclonal antibodies have also been successfully used as probes on a SAW sensor to monitor real-time binding of viruses. Bisoffi et al. (Bisoffi, et al., 2008) demonstrated detection of *Coxsackie* virus B4 and *Sin Nombre* virus (SNV) spiked into real world liquid samples (river, sewer effluent) using a hand-portable 325 MHz SAW biosensor. The SAW sensor showed a log-linear dose-response that spanned up to five orders of magnitude in viral concentrations (SNV [ul^{-1}]: 1.8 x 10^1–1.8 x 10^4, R^2=0.95; B4 [ul^{-1}]: 9 x 10^5–3.6 x 10^6, R^2=0.99), and detected these probe:virus responses within 15 seconds of virus injection for both *Coxsackie* virus B4 and SNV. The investigators also verified the specificity of their probes by introducing high concentrations of HSV-1 virus (~3.6 x 10^6 ul^{-1}) as a confounding agent, and they observed no significant effect. Furthermore, the SAW device was able to detect analytes after surface regeneration using organic solvents, ultrasound, and ultraviolet-ozone.

The piezoelectric-excited millimeter-sized cantilever (PEMC) sensor developed by Campbell and Mutharasan (Campbell and Mutharasan, 2006b) has been successfully used for detection of *B. anthracis* spores. Similarly to QCM, when the analyte binds to the sensing surface of the cantilever that is covered with the probe molecule, a change in mass results in a change in the cantilever's resonant frequency. The cantilevers were constructed of a layer of zircon titanite (PZT) bonded to a glass cover slip (Campbell and Mutharasan, 2005). The surface was cleaned and treated to bond with the *B. anthracis* spore–specific antibody probe. Different

mixtures of *B. anthracis* and *B. thuringiensis* spores were used to test for specificity of the PEMC sensor. Although the PEMC sensor could selectively identify the correct target, the sensitivity decreased with increasing amounts of *B. thuringiensis* spores. The investigators hypothesized that *B. thuringiensis* spores crowded onto the surface of the cantilever and hindered transport of the *B. anthracis* spores to the cantilever. Campbell and colleagues also analyzed the PEMC sensor for detection of *B. anthracis* spores in a flow cell (Campbell and Mutharasan, 2006a). Investigators determined that the binding rate of spores to antibodies increased five-fold in the flow cell compared to static conditions. One reason for this may be the increased deposition of antibody on the PEMC under flow (which was reflected in the differential resonant frequency). When exposed to spores, the PEMC reached a steady-state resonant frequency (i.e., indicated by signal saturation) within two minutes. The detection limit was 300 spores/ml.

Recently, Campbell and Mutharasan (Campbell and Mutharasan, 2008) utilized PEMC to detect a potent waterborne pathogen, the *Cryptosporidium parvum* oocyst. To capture the oocysts, they functionalized PEMC with antibody probe IgM and successfully detected the pathogen at various concentrations in a dose-dependent response in less than 30 minutes. The limit of detection was extrapolated based on the dose-response of the sensor, and the investigators determined that 1–10 oocysts could be measured in 15 minutes.

4.1.2. Magnetoelastic sensors (Figure 3) are constructed of amorphous ferromagnetic ribbons or wires. They have high tensile strength and are cost effective (Barandiaran and Gutierrez, 1997; Grimes et al., 2002),which makes them an attractive biosensor platform. The magnetoelastic sensor can be coated with various probe molecules to capture analytes. Binding of analyte to

magnetoelastic sensor results in a resonant frequency change that can be measured rapidly and accurately. Ruan and colleagues have used magnetoelastic sensors to detect *E. coli* O157:H7, avidin, and *staphylococcal enterotoxin* Type B (SEB) (Ruan et al., 2003; Ruan et al., 2004; Ruan et al., 2004). To detect *E. coli* O157:H7, antibody was covalently attached to the sensor, the sensor was exposed to the pathogen in varying concentrations, and the resulting resonant frequency was measured. To detect lower concentrations of the bacterium, a secondary alkaline phosphate (AP) labeled anti-*E. coli* O157:H7 antibody was added to amplify the frequency above background. Using this system the investigators were able to detect 100 *E. coli* O157:H7 cells/ml with a linear range of 10^2–10^6 bacterial cells/ml. For detecting SEB, the sensor was coated with covalently bound anti-SEB antibody and exposed to the toxin, biotin-labeled anti-SEB antibody, and AP-labeled avidin (Ruan, et al., 2004). From this study, the investigators determined a linear dose-response in the range of 0.5 to 5 ng/ml of SEB. These studies demonstrate the sensitivity of magnetoelastic sensors. Another advantage of the magnetoelastic sensor is its ability to detect analyte in near real-time measurement intervals of 10 ms.

As recently demonstrated by Guntupalli et al. (Guntupalli et al., 2007), size of the magnetoelastic sensor greatly affects its sensitivity. Using sensors covered with polyclonal anti-*S. enterica* serovar Typhimurium antibody, they determined that smaller the sensor, higher the sensitivity. This inverse relationship of sensor size to sensitivity was described by the equation $\Delta f = -f\,(\Delta m/2M)$, in which M is the initial mass, Δm is the mass change, and Δf is the change in resonance frequency. Thus a smaller M results in a larger Δf. The detection limit for the smallest sensor tested was 5×10^3 cfu/ml, while the largest sensor's lower detection limit was 10^7 cfu/ml.

The efficacy of magnetoelastic sensors was also demonstrated by Wan and colleagues who detected 10^3 *B. anthracis* spores/ml (Wan et al., 2007) with a magnetoelastic platform covered with landscape phage probe. This study also verified the previous results of Brigati et al. (Brigati and Petrenko, 2005) that phage-displayed oligopeptide probes were much more robust than polyclonal antibody probes for retaining relative stability and binding characteristics.

4.2. OPTICAL

4.2.1 Surface plasmon resonance (SPR) was conceptualized as an optical biosensor in 1983 (Liedberg et al., 1983). Surface plasmons (SPs) are electron density waves formed at the interface of a metal (e.g., gold) and a dielectric (e.g., glass). Typically, SPs are characterized by optically monitoring changes in the refractive index that arise from an analyte binding to a metal-coated surface of a prism (Homola, 2003; Mullett et al., 2000). The two most common methods for monitoring SPs in biosensing are 1) wavelength interrogation, in which the angle of incidence is fixed and the reflected photons are monitored as a function of wavelength, and 2) angle interrogation, in which the illumination wavelength is fixed and the reflected photons are measured as a function of the reflectance angle. The most common SPR method uses the prism-based Kretschmann-Raether geometry (Figure 4) (Biacore, 2006). Fluidics channels can be constructed to abut the thin gold layer where receptor molecules (illustrated as antibodies in Figure 4 inset) for target analytes are anchored. When the analytes are introduced into the fluidics channel, binding interactions between target analytes and the fixed layer of receptors generate perturbations that result in refractive index changes at the metal and dielectric interface.

The magnitude of SPR shift in the monitored parameter (e.g., wavelength or angle) corresponds to the number of bound analytes.

Although the advantages of direct measurements are substantial, in practice it has been necessary to use secondary antibodies tethered to latex spheres or gold beads to enhance the SPR signal (Anderson et al., 2005; Homola, 2003). Anderson et al. (Anderson, et al., 2005) employed a wavelength-interrogation, prism-based SPR system to measure concentrations of SEB, ricin, and *B. globigii* spores in conjunction with antibodies as probes and secondary antibodies. Detection limits for SEB, ricin, and *B. globigii* were 25 ng/ml, 100 ng/ml, and 10^6 spores/ml, respectively. Although adding secondary antibodies enlarged the amplitude of the SPR signal, it only enhanced the detection limits for SEB and ricin by a factor of 3 and 10, respectively. It is worthy to note that the investigators were able to obtain up to five regenerations for each gold surface without a loss of function.

Two recent developments have improved portability for field applications by decreasing the size of prism-based SPR. The first is a portable 24-analyte SPR sensor based on the SPREETA^{TM} technology, which was developed by Texas Instruments but is now part of ICx Nomadics (Chinowski, et al., 2007). SPREETA^{TM} is unique in its use of separate reference and sensing channels, which allows the background (reference signal) to be subtracted from the sensing signal and thus increases the sensitivity of detection. However, even with SPREETA^{TM}, detection of *B. subtilis* spores required a signal amplification step using secondary antibodies. The second improvement is a hand-held single analyte SPR developed by Feltis et al. (Feltis et al., 2008) that offers a portable, wireless alternative to current SPR systems. Using this system

along with anti-ricin antibody, the investigators detected 200 ng/ml of ricin in 10 minutes (Feltis, et al., 2008). Although this system has relatively poor sensitivity and requires a large sample size, its portability makes it an attractive alternative to other SPR-based biosensor platforms.

To increase sensitivity while obviating the need for secondary antibodies, Endo et al. (Endo et al., 2006) developed a localized SPR (LSPR) that can analyze a nanoscale microarray chip containing 300 spots. This nanochip can be spotted with an assortment of antibodies for multiplex capability, and has a lower detection limit of 100 pg/ml of analyte. This platform is inexpensive and small, is capable of multiplex analysis, requires only small amounts of fluids, and is highly sensitive. Once LSPR substrates can be mass-produced, these direct label-free optical immunoassays will likely revolutionize hand-portable biosensors for biosecurity applications and point-of-care clinical diagnostics.

Oh et al. (Oh, et al., 2005) used a prism-based SPR system to develop a multiplex biosensor chip for simultaneous detection of multiple infectious agents. Using specific monoclonal antibody probes, they simultaneously detected *E. coli* O157:H7, *S. enterica* serovar Typhimurium, *Legionella pneumophila, and Yersinia enterocolitica.* The chips were exposed to approximately 10^5/ml of various pathogens and the refractive SPR angles were measured. Based on the shift in the SPR angle that resulted from binding of a particular pathogen to its cognate antibody, the investigators were able to differentiate the interaction of specific probe:analyte pairs.

In a separate study, Balasubramanian et al. (Balasubramanian, et al., 2007) used a lytic phage (bacteriophage ATCC 12600) and SPREETA^{TM} to detect *S. aureus*. In this study, the phage was immobilized on the gold surface of the SPR sensor and allowed to capture various concentrations of bacteria. A dose-response curve indicated a detection limit of 10^4/ml. Because the probe used was a natural *S. aureus* phage, the interactions were very specific and suggested the potential of this biosensor for real-world applications.

Finally, advances in nanofabrication techniques have made it possible to excite surface plasmons using metallic subwavelength structures (e.g., grating-coupled) instead of prism coupling (Genet and Ebbesen, 2007; Homola, 2003). Enhanced transmission through metallic subwavelength structures already shows potential for high throughput biosensing (Brolo et al., 2004; Ebbesen et al., 1998; Hwang et al., in press; Leebeeck et al., 2007; Pang et al., 2007; Steiner, 2004; Stewart et al., 2006). We anticipate that further biosensor research in two-dimensional grating coupler SPR and LSPR systems will permit massively parallel processing to reduce sampling time and decrease the probability of false alarms in field applications.

4.2.2 Non “label-free” optical assays. Although the emphasis of this review is on fieldable label-free sensors, there are a few non label-free sensors that show promise.

4.2.2.1 Proximity ligation assay (PLA) via multivalent burr is a technique that combines the power of PLA and real-time PCR for highly sensitive detection of pathogens (Fredriksson et al., 2002; Gullberg et al., 2004). As illustrated in Figure 5 and described in Pai et al. (Pai et al., 2005), the multivalent burrs are peptide/antibody probes and oligonucleotide tags conjugated to a

protein base. Binding of multivalent burrs to an analyte (a cell or a spore) results in oligonucleotide tags being brought together in close proximity to generate a unique amplicon via ligation. Presence of this amplicon is then detected via real-time PCR with high sensitivity. Pai et al. (Pai, et al., 2005) successfully detected as few as 10 spores for *B. subtilis* and *B. cereus,* and 100 spores for *B. anthracis*.

Gustafsdottir et al. (Gustafsdottir et al., 2006) developed a slight variation of the PLA to detect porcine parvovirus (PPV) and the bacterium *Lawsonia intracellularis*. In this study, oligonucleotides were conjugated directly to antibody to generate antibody-oligonucleotide probes that captured even a few copies of the analyte. Although PLA and related techniques show promise because of extreme sensitivity, reliance on real-time PCR limit their utility due to the cost.

4.2.2.2 Common fluorophores can suffer from low signal-to-noise ratio because of auto-fluorescence and variable photostability. Quantum dots, which are fluorescent semiconductor nanocrystals, have an advantage over these fluorophores because they have a broad range absorption spectrum and narrow range emission based on the size of the crystal. In addition, quantum dots are photostable due to the presence of an outer shell, which also increases the quantum yield (Michalet et al., 2005). Furthermore, by changing the size and the makeup of the crystal core, dots emitting different colors can be created and used in multiplex assays (Deng et al., 2007; Lim, et al., 2005). Quantum dots have been used to label antibodies, phages, and magnetic beads for the detection of various analytes.

Edgar et al. recently used a biotin-tagged lytic T7 phage to form quantum dot complexes for detection of *E. coli* (Edgar, et al., 2006). To label the phage with quantum dots, the phage was genetically modified to incorporate biotin on its capsid protein, and then streptavidin-coated quantum dots were conjugated to the capsid. This quantum dot–labeled phage was then successfully used to capture *E. coli* from an environmental sample (river water) as detected by flow cytometry and microscopy. The sensitivity of this assay is demonstrated by its ability to detect 20 *E coli* cells in 1 ml of river water sample.

4.3 ELECTRICAL PERTURBANCE BIOSENSORS

Biosensors that detect electrical perturbances include amperometric and potentiometric platforms which measure changes in current and voltage, respectively. The probe:analyte interaction causes perturbances at the sensing interface that are measured by the transducer.

For amperometric devices, the potential is kept constant and set at a value that produces a change in current only when the analyte comes into contact with the probe (Goldschmidt, 2006; Lazcka, et al., 2007). This type of sensor is commonly used to detect diagnostically significant measurements such as blood sugar levels (Aubree-Lecat et al., 1989; Wolfson et al., 1989) but it has also been used to detect infectious agents (Abdel-Hamid et al., 1999; Boyaci et al., 2005; Mittelmann et al., 2002; Sippy et al., 2003; Thomas et al., 2004). For example, an amperometric immunofiltration sensor was used to detect *E. coli* O157:H7 (Abdel-Hamid, et al., 1999) captured on a disposable filter membrane coated with a primary antibody. As the bacterial

sample passed through the filter, cells were captured on the primary antibody and labeled with an enzyme-conjugated secondary antibody. The addition of the enzyme substrate caused a chemical reaction that produced a shift in current which increased with increasing conjugate concentration. This amperometric immunofiltration sensor is simple and rapid (detection within 30 minutes). The technology is potentially portable and could be used to identify other important infectious agents in the field. The limiting factor for this type of biosensor platform is the availability of primary antibodies for the target agents.

Sippy et al. (Sippy, et al., 2003) also used an amperometric immunofiltration sensor to capture and measure *E. coli* O55 at a detection limit of 100 cells/ml, which was comparable to that achieved by Abdel-Hamid et al. (Abdel-Hamid, et al., 1999). However, Sippy and colleagues (Sippy, et al., 2003) decreased sample detection time to 10 minutes by using a lateral flow immunoassay rather than a filter membrane during the capture step.

An amperometry biosensor was also successfully used to detect waterborne infectious agents. Boyaci et al. (Boyaci, et al., 2005) combined amperometry and paramagnetic bead approaches to detect *E. coli* in contaminated water sources. Paramagnetic beads can be easily isolated following an initial reaction through application of a magnetic field. For this application, β-galactosidase, an enzyme produced by *E. coli*, was used to detect the presence of the bacterium. The beads were first functionalized with streptavidin and then reacted with biotinylated anti-*E. coli* polyclonal antibodies. The antibody-coated beads were then mixed with *E. coli* cultures and the antigen-antibody complexes were isolated by a magnetic field. An inducer (isopropyl β-D-thiogalactopyranoside, or IPTG) of the *lac* operon, which encodes for β-

galactosidase, was added to the complex and the enzymatic activity was then measured amperometrically by detecting oxidation of *p*-aminophenyl β-D-thiogalactopyranoside (PAPG) to *p*-aminophenol (PAP). Total analysis time for this system was approximately 1 hour, and the sensitivity was $2x10^6$ cfu/ml. Under optimal conditions it was possible to detect 20 cfu/ml within 7 hours. The limiting factor for this assay was the time it took for the captured *E. coli* to produce a measurable quantity of β-galactosidase upon induction.

Potentiometric sensors are not commonly used as biosensor platforms. However, these sensors can detect very small concentrations of a particular analyte due to their logarithmic response (Lazcka, et al., 2007). Thus, they should be further exploited for detection of minute quantities of infectious agents. One particular application of the potentiometric sensor, the light addressable potentiometric sensor (LAPS), has been described by Hafeman et al. (Hafeman et al., 1988). LAPS has high sensitivity due to the relative uniformity of its surface potential which results in stability of the signal and the ability of the sensor to detect a small concentration of the analyte. LAPS has successfully detected *Y. pestis* and *B. globigii* that first had been captured by an antibody-mediated capture filtration method (Dill et al., 1997). In this study, biotinylated primary antibodies, the analytes, and secondary antibodies formed immunocomplexes on the nitrocellulose capture filter in which changes in pH were measured as a function of time that registered as a rate change (μV/s). The sensor had a very low detection limit of 10 cells for *Y. pestis*, and 15 spores for *B. globigii*. Using a similar approach, Uithoven et al. (Uithoven, et al., 2000) detected 3×10^3 spores/ml of *B. subtilis* in less than 15 minutes. While antibodies are the traditional probes used with this sensor platform, Hu et al. (Hu, et al., 2004) successfully used

biotinylated scFv to detect Venezuelan equine encephalitis (VEE) virus in 90 minutes with a detection limit of 30 ng/ml.

4.4. MAGNETIC BEADS

The use of immunomagnetic beads to separate and detect infectious agents began in the late 1980s when Lund et al. (Lund et al., 1988) selectively captured pathogenic *E. coli* cells on magnetic beads coated with monoclonal antibodies. Cells were stained with acridine orange and detected through fluorescence microscopy. In 1992, Fratamico et al. (Fratamico et al., 1992) reported the capture on antibody-coated magnetic beads of *E. coli 0157:H7* with a sensitivity of 10 cells per ml. During the 1990s, several research groups developed immunomagnetic technologies that could detect 10 to 100 cells per gram of ground beef. All methods required overnight enrichment of cells prior to capture and concentration, followed by an 8-hr ELISA (Weimer et al., 2001).

In 1996, Bruno and Yu (Bruno and Yu, 1996) reported strain-dependent detection limits of 10^2 to 10^5 *Bacillus anthracis* spores per mg of soil suspension using immunomagnetic separation combined with an electrochemiluminescence sensor .

In 2006, Aytur et al. (Aytur et al., 2006) described a gold-plated semiconductor chip coated with antigen that quantified the number of bound antibody-coated magnetic beads through their production of local magnetic fields upon excitation. The analyte concentration was directly proportional to the number of beads detected after magnetic washing of the capture surface. The

signal to background ratio was 16 compared to 7 in a comparable ELISA. Although this disposable format has not been employed yet for whole organisms, its low cost, ease of use, and portability show promise for the future development of direct sensing technologies. The complementary metal oxide semi-conductor (CMOS) chip would allow integration of signal processing electronics in a fieldable biosensor (Aytur, et al., 2006) .

In 2007, Meyer et al. (Meyer et al., 2007) reported a highly specific and sensitive immunomagnetic bead sensor designed for batch processing of complex samples. The sensor employed small plastic columns containing sintered polyethylene filters on which monoclonal antibodies were adsorbed. Paramagnetic beads conjugated to the same antibodies accumulated in the column and were detected by a measurement head that applied magnetic fields of different frequencies to the beads when they bound to immobilized antigens. A detection limit of 2.5 ng/ml was demonstrated in samples containing human blood serum. This transportable system can also be used to measure living cells extracted from tissue samples, as well as aerosols suspended in phosphate buffered saline (PBS) solution (Meyer, et al., 2007).

5.0 CONCLUDING REMARKS

To effectively combat the rapid dissemination and outbreak of infectious agents that occur either deliberately or naturally, it is imperative to develop near real-time biosensors that can specifically and accurately detect various biological and infectious threat agents. There has been tremendous advancement in BTA detection technology in the past few years, but a

dependable near real-time biosensor has not yet been developed. Accurate biosensor development requires a highly specific probe for the infectious agent of interest. Currently, peptide-based probes, including oligopeptides and antibodies, are most commonly used to capture infectious agents. Recent improvements in this area of research have focused on development of capture platforms, such as micro- or nanochips in which multiple agents can be differentially captured. Developments in detection/sensing technologies are focused on improving sensitivity, cost effectiveness, and portability. Ideally, to evoke the most efficient defensive response, a network of "alarm-type" biosensors is needed to serve as an initial warning for the presence and spread of an infectious agent. Short of this goal, a portable biosensor with high sensitivity and accuracy that can detect infectious agents in near real-time is desirable. Continued research to improve probes and platforms should result in effective biosensors that can be used in real-world situations in the near future. Using a combination of different probe types (e.g., llamabodies, peptides, and antibodies) that target different epitopes on the analyte pathogen will most likely achieve a low probability of false alarm when the collective responses from several simultaneous probe:analyte interactions are considered.

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FIGURE LEGENDS

Figure 1. Filamentous bacteriophage. Lysogenic filamentous phages of the Ff class contain single-stranded DNA genome that is encapsulated by approximately 2800 copies of the major coat protein (pVIII). In addition, there are approximately five copies each of minor coat proteins, pIII and pVI, at the “head” of the phage. The “tail” of the phage contains approximately five copies of the minor proteins pVII and pIX (Petrenko and Sorokulova, 2004; Smith and Petrenko, 1997). Of the capsid proteins, pIII and pVIII have been utilized the most to display peptides or proteins.

Figure 2. Schematic of surface acoustical wave sensor principle. A typical acoustic wave device consists of a piezoelectric quartz substrate with input/output transducers and a probe coating. The transducers are part of an oscillator circuit whose frequency is determined by acoustic wave speed in the quartz substrate. An input transducer generates a SAW that propagates to the output transducer and is fed back through an amplifier. Perturbations at the surface of the piezoelectric crystal induce a detectable change in the resonant frequency of the device.

Figure 3. Schematic of magnetoelastic sensor principle (adapted from (Ruan, et al., 2003)). A magnetic pulse is generated when an analyte makes contact with the probe. This impulse is converted by the sensor transducer to a frequency and provides information for analysis of the interaction between the probe:analyte.

Figure 4. Schematic of a prism-based surface plasmon resonance sensor (adapted from (Oh, et al., 2005)). A typical SPR schematic consists of a prism with one side coated with a thin layer of gold (<100 nm), to which receptor molecules such as antibodies are adhered. The prism is illuminated at an oblique angle through one of the uncoated sides. Reflected photons are monitored by a photo detector that collects the photons emitted from the uncoated side of the prism.

Figure 5. Enhanced Proximal ligation assay for detection of whole cells (adapted from (Pai, et al., 2005)). (A) Multivalent burr is constructed as a specific peptide probe–oligonucleotide–protein (P) conjugate. (B) When multivalent burrs bind to the analyte (whole cells or spores) close together, the proximity of the oligonucleotide tags allow ligation of DNA to create a unique amplicon that can be detected via real-time qPCR with high sensitivity.

Figure 1.

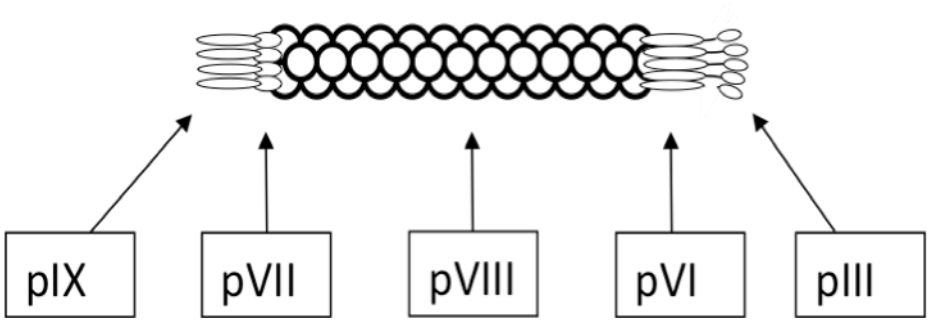


Figure 2.

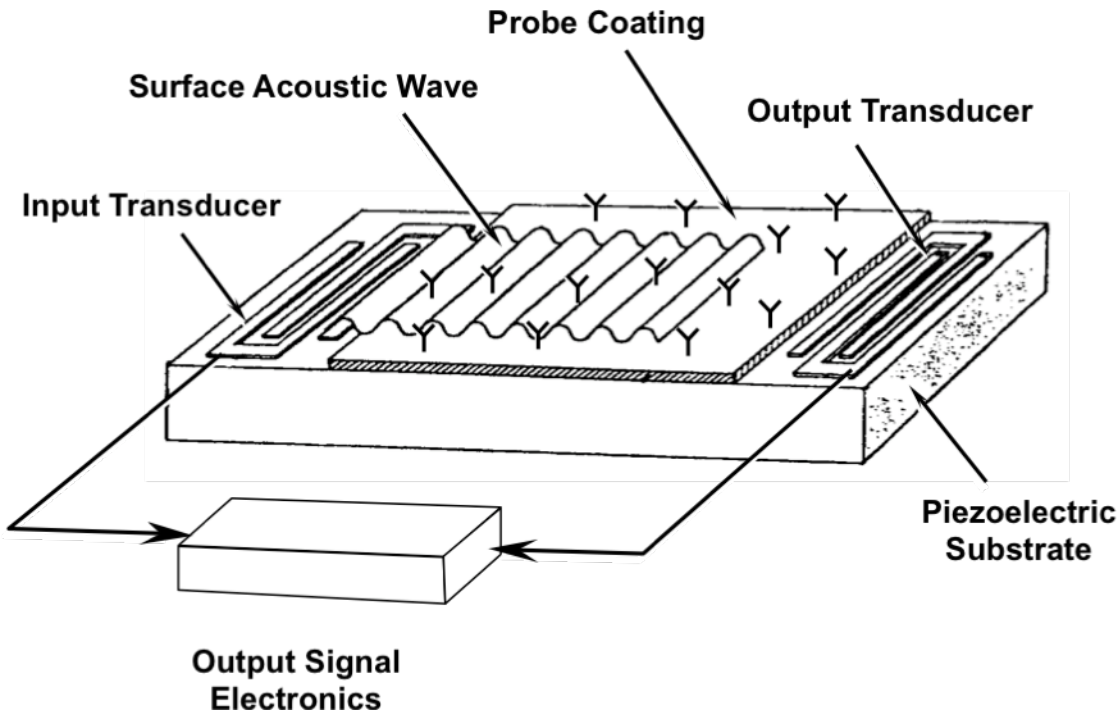

Probe Coating
Surface Acoustic Wave
Output Transducer
Input Transducer
Piezoelectric
Substrate
Output Signal
Electronics

Figure 3.

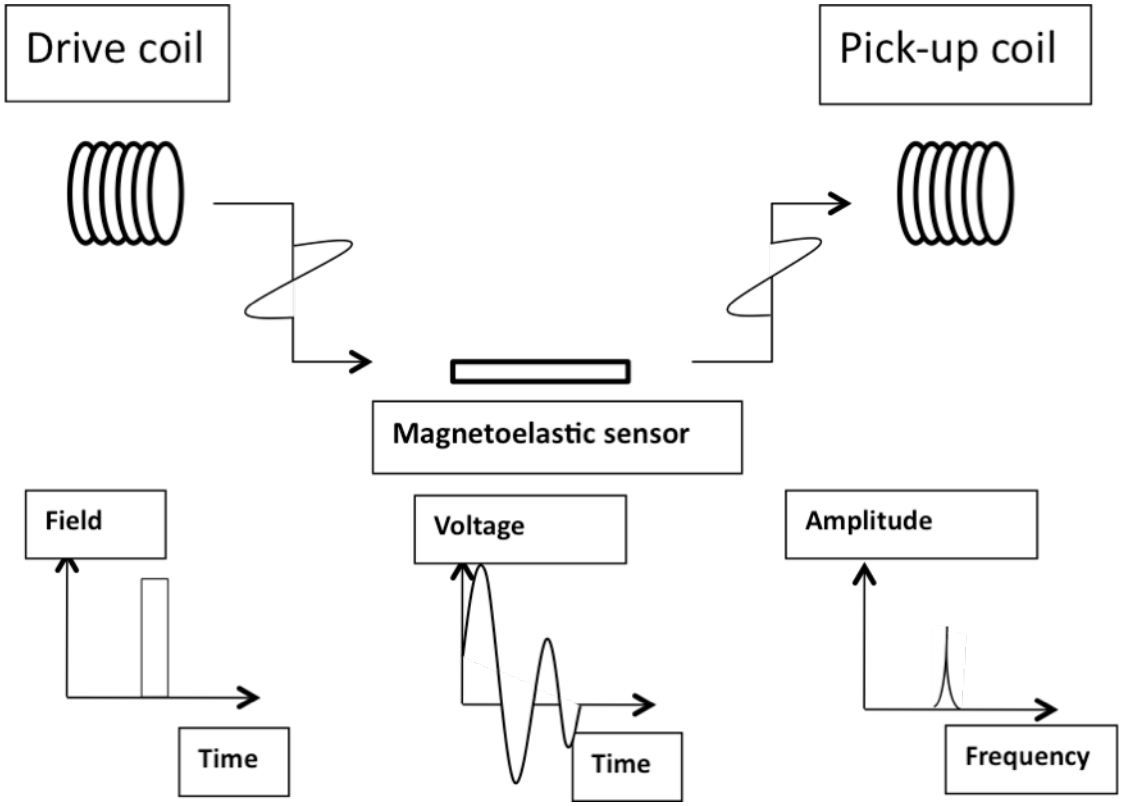

Drive coil
Pick-up coil
Magnetoelastic sensor
Field
Time
Voltage
Time
Amplitude
Frequency

Figure 4.

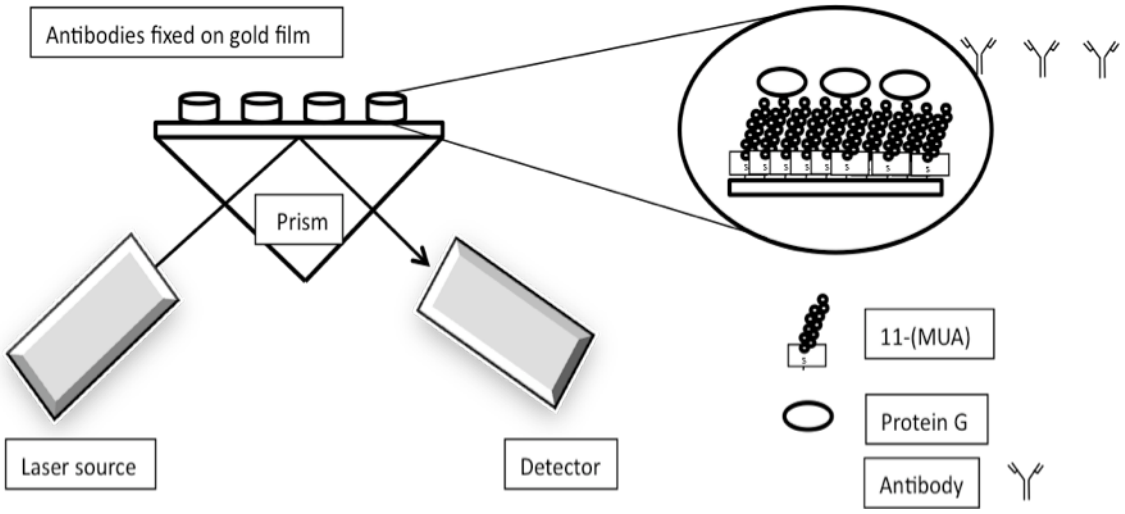
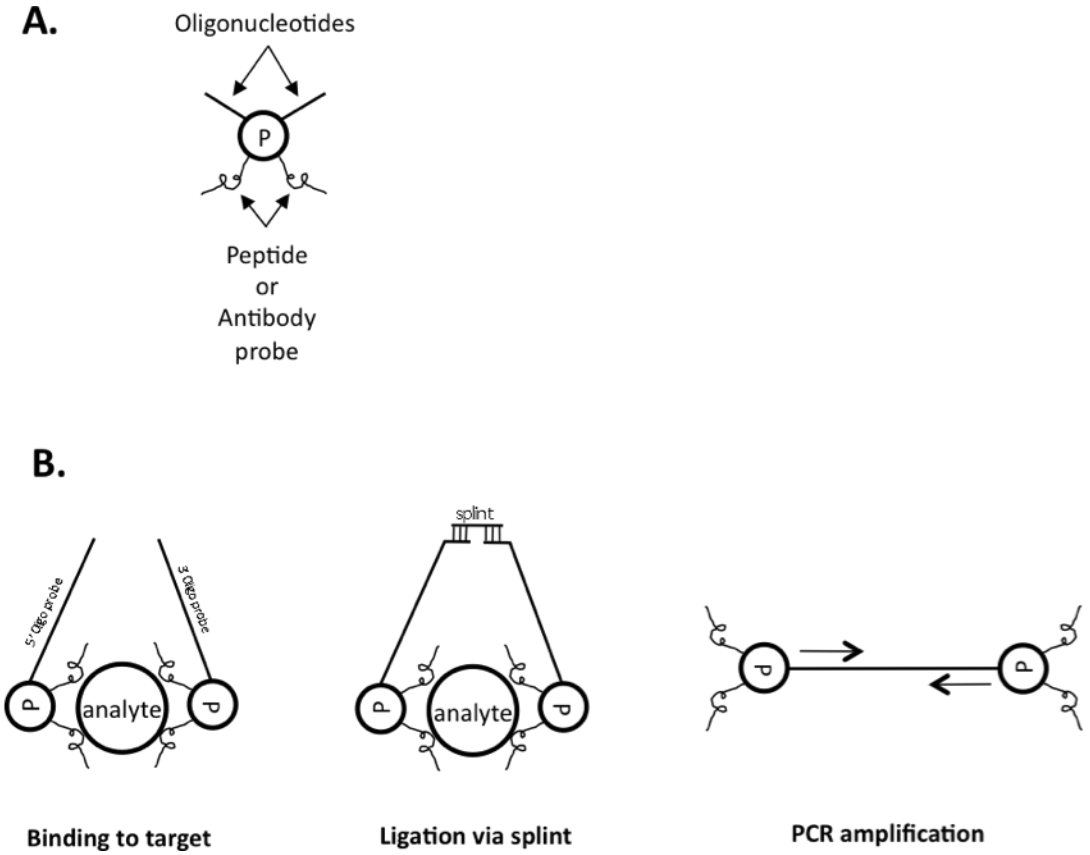

Antibodies fixed on gold film
Prism
Laser source
Detector
11-(MUA)
Protein G
Antibody

Figure 5.


A.
Oligonucleotides
P
Peptide
or
Antibody
probe
B.
5' oligo probe
3' oligo probe
P
analyte
P
Binding to target
splint
P
analyte
P
Ligation via splint
P
P
PCR amplification