Biosensor Systems for Homeland Security

by Cynthia J. Bruckner-Lea

The detection of biological agents is important for minimizing the effects of pathogens that can harm people, livestock, or plants. In addition to pathogens distributed by man, there is a need to detect natural outbreaks. Recent outbreaks of *severe acute respiratory syndrome* (SARS), mad cow disease, pathogenic *E. coli* and *Salmonella*, as well as the discovery of letters filled with anthrax spores have highlighted the need for biosensor systems to aid in prevention, early warning, response, and recovery.

The types of biological agents of concern include bacteria, spores, protozoa, and viruses (Fig. 1). From a chemist's viewpoint, pathogens are essentially complex packages of chemicals that are assembled into organized packages with somewhat predictable physical characteristics such as size and shape. Pathogen detection methods can be divided into three general approaches: selective detection methods for specific identification such as nucleic acid analysis and structural recognition, semiselective methods for broad-spectrum detection (*e.g.*, physical properties, metabolites, lipids), and function-based methods (*e.g.*, effect of the pathogen on organisms, tissues, or cells).

The requirements for biodetection systems depend upon the application. While detection-to-warn sensors may require rapid detection on the order of 1 min, detection times of many minutes or hours may be suitable for determining appropriate treatments or for forensic analysis. Ideal sensor systems meet the needs of many applications, and are sensitive, selective, rapid, and simultaneously detect all agents of concern. They are also reliable with essentially no false negatives or false positives, small, easy to use, and low cost with minimal consumables.

Considerations for Sample Collection and Preparation

Pathogen detection in the environment is especially challenging due to the complex nature of the sample (*e.g.*, aerosol, water, food, soil), and the need for trace detection. In all cases, one must consider sample preparation as well as detection to achieve reliable detection. Complex environmental samples may lead to problems with sensor accuracy due to cross reactivity, an increase in detection limit due to inhibition of the detection method, and a decrease in the lifetime of the sensor due to the adsorption of components of the background on the sensor surface.

Trace detection is often needed because small amounts of pathogens can cause illness and releases can be diluted rapidly in the environment. For example, in the food industry, a detection limit of 1 pathogen per gram or 1 pathogen per milliliter is desired. If one assumes only one analyte for detection per pathogen, this translates to a detection limit of only 1.7 zM (1.7×10^{-21} M!). In some cases, one can find thousands of analytes per pathogen, relaxing the required detection limit to 1.7 aM (1.7×10^{-18} M), which is still quite a challenge. It is clear that sample preparation is critical for both purifying and concentrating the pathogen [or components of the pathogen such as deoxyribonucleic acid (DNA) or proteins] of interest prior to detection. Therefore, one must ultimately develop biosensor systems that include sample collection and sample preparation, biodetection (often using multiple biosensors), data integration and analysis, and finally reporting of the results.

In the following are descriptions of some primary components of biosensor

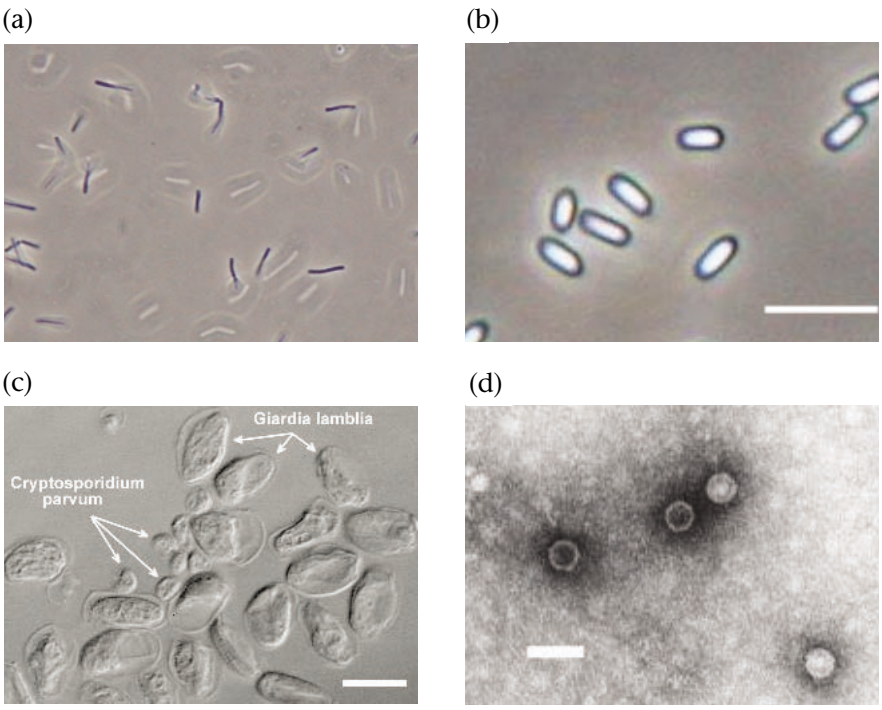

Fig. 1. Images of different types of microorganisms of interest for biodetection. (a) and (b) are optical micrographs of Bacillus subtilis vegetative cells and Bacillus subtilis spores, respectively, which are often used as simulants for anthrax. Scale bar in (b) is 5 µm; image in (a) is approximately 10X lower magnification; images courtesy of N. Valentine, Pacific Northwest National Laboratory (PNNL); (c) images of two protozoa: Cryptosporidium parvum oocysts and Giardia lamblia cysts. The scale bar is 10 µm; image courtesy of H. D. A. Lindquist, U.S. Environmental Protection Agency (EPA); and (d) transmission electron microscope image of poliovirus. The scale bar is 10 nm; image courtesy of F. P. Williams, U.S. EPA.

systems and several examples of biosensor system components that have been developed or are under development for pathogen detection.

Biosensors: Transduction Principles

Biosensors, like chemical sensors, consist of two essential components: a chemically selective layer that binds the target molecule, and a transducer that converts the binding event into a measurable signal that can be monitored, displayed, and used for process control. More detailed information about chemical sensors in general can be obtained from review articles.[1-5] Three of the most common transduction principles for chemical sensing include mass, electrochemical, and optical detection. Mass sensors can produce a signal based on the mass of chemicals that interact with the sensing film. Acoustic wave devices are the most common sensors in this class. They are made of piezoelectric materials, which bend when a voltage is applied to the crystal. Acoustic wave sensors are typically operated by applying an oscillating voltage at the resonant frequency of the crystal, and then measuring the change in resonant frequency when chemicals interact with the sensing surface.

Electrochemical sensors involve using electrodes to measure the electrochemical changes that occur when chemicals interact with a sensing surface. The electrical changes can be based on a change in the measured voltage between the electrodes (potentiometric), a change in the measured current at a given applied voltage (amperometric), or a change in the ability of the sensing material to transport charge (conductometric). Some of the most common types of electrochemical sensors are ion selective electrodes, such as pH electrodes (see tutorial in this issue of *Interface*). These potentiometric sensors include a membrane that selectively passes certain ions, so that the potential drop across the membrane (and therefore the measured electrochemical potential) is dependent on the concentration of ions in the sample.

Optical sensors often employ optical fibers or planar waveguides to direct light to the sensing film. Evanescent waves propagating from waveguides may be used to optically probe only the sensing film, and not the bulk of the sample solution, to decrease the optical background signal from the sample. The measured optical signals may include absorbance, fluorescence, chemiluminescence, surface plasmon resonance (to probe refractive index), or changes in light reflectivity. The most sensitive optical sensors are based upon fluorescence measurements, and with sophisticated optical equipment (not field-portable), single molecule fluorescence measurements are even possible.[6] However, one practical limitation is the limited lifetime of fluorescent molecules due to photobleaching over time. In recent years, fluorescent nanocrystals (quantum dots) have emerged as alternative fluorescent reporters that are typically brighter than molecular dyes, resistant to photobleaching, and amenable to multiplexed detection by controlling the size of the fluorescent nanocrystals to tune the fluorescence wavelength.[7]

Many biosensors that have been developed for pathogen detection utilize sequence-based recognition of nucleic acids or the structural recognition of pathogens or pathogen biomarkers. While optical detection methods are the most widely used in biosensors today, other transduction methods also may be used. Less developed approaches for pathogen detection include the broad spectrum detection of pathogens by monitoring general physical and chemical signatures, and biosensors based on cell function. Below is a summary of these pathogen detection approaches with examples and discussion to hopefully inspire the development of next generation biosensors.

Nucleic Acid-based Methods for Pathogen Detection

Selective identification of biological agents can be done by analyzing the genetic components of the cells, DNA and RNA (ribonucleic acid). While typical microorganisms contain 0.6 to 5 million DNA base pairs,[8] the presence of several specific DNA sequences only about 25 bases in length can often be used for the specific identification of microorganisms. Because DNA is a double helix containing two strands of a sequence of four bases that form specific hydrogen bonded pairs between the helices (adenine binding to thymine and guanine binding to cytosine), one short single strand of DNA bases (the probe) can be used within a biosensor to encode for the matching strand of DNA (the target).

One can imagine that specific hybridization of intact genomic DNA (many tens of thousands of bases) onto a sensor surface containing a specific probe sequence for detection (typically only 25 bases), is not often successful without some sample preparation to shear the DNA, or DNA amplification reactions such as polymerase chain reaction (PCR) to produce millions of copies of smaller DNA oligonucleotides (about 100 bases in length) that can then hybridize to the sensor surface. So again, a DNA-based biosensor by itself has little value for pathogen detection unless it is coupled with a sample preparation system to perform functions such as cell concentration, cell lysis (breaking open the organisms to release DNA from the cell interior), DNA amplification, and finally detection using the biosensor.

One function of RNA within cells is to translate the DNA sequence into many individual proteins. One prevalent type of RNA, messenger RNA (mRNA), is used as a template for protein synthesis within cells. This type of RNA is rapidly synthesized and degraded within cells, with a turnover rate of only 2-3 min in microorganisms.[8] The presence of high levels of mRNA can therefore be used as an indicator of cell activity and viability. Dormant cells, such as spores, do not have high levels of mRNA, but still contain other types of nucleic acids such as ribosomal RNA (rRNA) and DNA. Since both rRNA and highly expressed mRNA can be present at thousands of copies per cell, they are potential targets for rapid and sensitive detection without the need for PCR to amplify DNA (which is present at only one copy per cell). Viruses are made primarily of RNA and protein, and also may be detected using nucleic acid-based methods that target RNA. Following is a description of the most common nucleic acid-based methods for pathogen detection: DNA amplification methods such as PCR, and the use of DNA microarrays for direct detection of RNA or detection of DNA amplification products.

DNA amplification.—DNA amplification methods require the use of a reaction mixture containing enzymes and individual DNA bases to generate many copies of a specific region of DNA for detection. While there are many methods under development for DNA amplification [*e.g.*, exponential amplification reaction (EXPAR), strand displacement amplification (SDA), transcription mediated amplification (TMA), and nucleic acid sequence-based amplification (NASBA)], by far the most widely used method for DNA amplification is PCR[9] which involves using two primers of about 20 bases each to define the region on the DNA for amplification (typically 100 to 500 bases in length). This reaction requires thermal cycling to accomplish the following tasks: hybridizing the primers to the target (typically 50°C), extension of the primers (typically 70°C),

and finally separation of the product from the target (typically 95°C). Ideally, the quantity of PCR product doubles with each complete thermal cycle, producing more than a billion copies of PCR product from one double stranded DNA target in 30 cycles. Traditionally, PCR product was detected by gel electrophoresis, but newer methods include PCR product analysis in real-time using methods such as Taqman PCR,[10] and detection of PCR product by hybridization onto various DNA microarrays.[11-18]

TaqMan PCR requires optical monitoring of the reaction mix for detection in real-time during PCR. An additional probe sequence (also about 20 bases) is added to the reaction mix, and binds to the target DNA between the primers. The probe contains a fluorescent dye and a quencher, so that the fluorescence is quenched prior to DNA amplification. During the primer extension process, the probe sequence is cleaved, separating the dye and the quencher, and increasing the fluorescence in the reaction. The fluorescence is typically monitored in real-time during the reaction, and the number of thermal cycles required to achieve a fluorescent threshold is correlated to the target concentration.

While PCR can be exquisitely sensitive, drawbacks include the time required for PCR (typically 30 min to 1 h or more), difficulty in using this approach to simultaneously detect large numbers of pathogens in a single reaction, and the sensitivity of PCR to inhibition by sample matrix components, such as humic acids.[16] The time for PCR has been decreased to only 5-7 min in control samples with no PCR inhibitors.[10] However, a typical sample preparation process requires 1 h or more in a laboratory. Work is ongoing in several laboratories to automate the sample preparation steps (cell concentration, cell lysis, nucleic acid purification) required for reliable PCR analysis, and bridge the gap between environmental samples of milliliters or more in volume, and detection methods such as PCR, which typically require 10 to 50 µL total volume. Figure 2 shows an automated DNA sample preparation system that is under development at Pacific Northwest National Laboratory, and includes immunomagnetic concentration of organisms, cell lysis, and flow-through PCR to process up to 100 mL and detect as few as 10 cells in 1 mL starting material.[19-23]

DNA microarrays.—One alternative to detection during the PCR reaction is hybridization of the PCR product onto a microarray for detection. A microarray is a planar substrate such as a glass slide, silicon wafer, or polymer surface that is patterned with specific probe sequences to bind within the sequence of the PCR product. Optically or electrochemically labeled PCR primers may be used to generate PCR product that is optically or electrochemically active, and detection methods without labeling, such as acoustic wave and surface plasmon resonance methods, also may be used to detect hybridized PCR product. One advantage of using a microarray readout, is that one may use a collection of probes on the microarray to detect small differences in the generated PCR product, such as single base changes that may be used occasionally for strain identification or forensic analysis.[24] The rate-limiting step in the hybridization process is the mass transport of the DNA to the microarray surface. Electrophoretic transport of the nucleic acid in solution to electrically addressed sites on the planar array dramatically decreases hybridization times[25] (Fig. 3), and additional methods for rapid hybridization assays are needed.

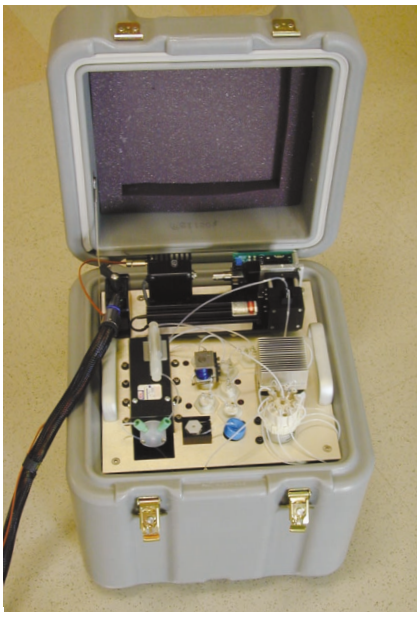

FIG. 2. The BEADS automated sample preparation and detection system under development at PNNL is about 1 ft³ in size and uses microbeads to concentrate and purify the analytes of interest. The system shown here also includes a flow-through PCR module and a fluorescence detector.

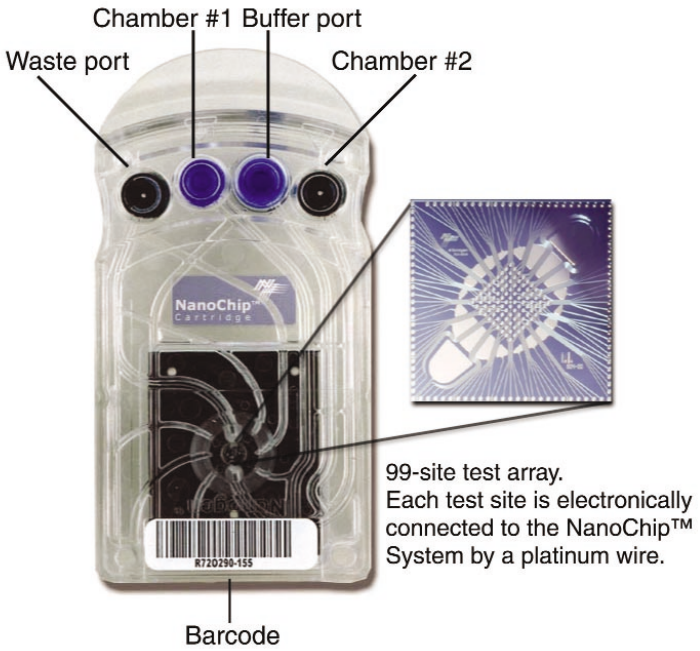

FIG. 3. Photo of an electronically addressable microarray for accelerating nucleic acid hybridization. Image courtesy of Nanogen, Inc.; www.nanogen.com

Because RNA also hybridizes to DNA oligonucleotides, DNA microarrays may be used for the direct detection of rRNA[26,27] or mRNA. Direct RNA analysis may enable highly multiplexed and rapid detection which is difficult with PCR. Figure 4 shows data obtained using a gel pad microarray for rRNA analysis.[27] Figure 5 shows an mRNA array readout for the simultaneous detection of multiple organisms, including pathogenic *E. coli, Salmonella,* and *Shigella*.

Structure-based Methods for Pathogen Detection

Structure-based detection involves affinity recognition in a lock-and-key fashion of proteins on or within the pathogen, or other molecules such as lipopolysaccharides on the cell surface. Just as DNA is the blueprint for all biological activity in a biological cell, proteins are the molecules that carry out much of that activity. Many studies have evaluated the feasibility of identifying microorganisms by detecting the proteins present within the organisms. The key is identifying the biomarkers that can be used for pathogen identification.

The most common biosensors that use structure-based molecular recognition are immunoassays, which utilize antibodies for molecular recognition. Vertebrates produce in excess of 10^{11} different antibodies to bind to molecules (antigens) that are recognized as foreign, such as potentially dangerous viruses, cells, or nonbiological chemicals. Antibodies have historically been produced by inoculating animals with the target analyte of interest, and isolating the antibodies from the serum or the specific cells that generate the antibodies. This is a relatively costly and laborious process. Recently, methods have been developed for generating antibodies *in vitro* without the need for inoculation of vertebrates. For example, methods have been developed for generating antibodies on the surface of bacteriophage,[28] and libraries of more than 10^9 antibody fragments have been generated on the surface of yeast.[29] These high throughput *in vitro* methods used for

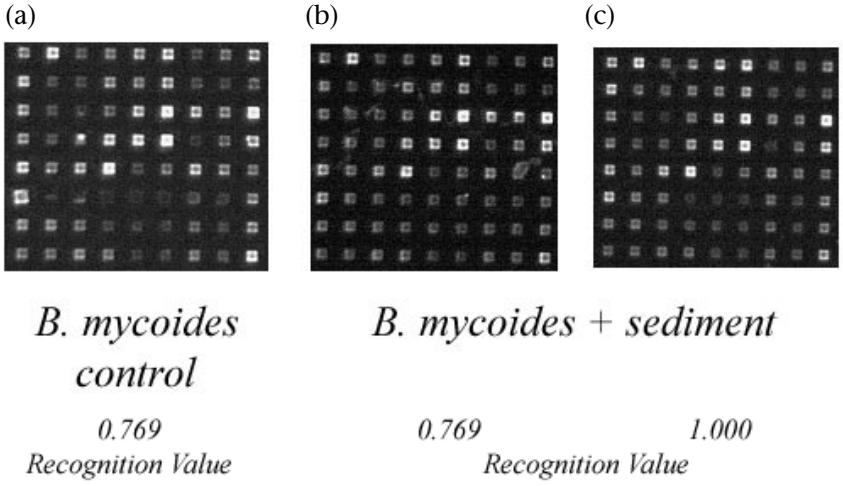

FIG. 4. Experiments at Argonne National Laboratory (ANL) show that environmental admixtures do not affect the sample preparation and detection process that includes nucleic acid purification, labeling, and fragmentation, followed by hybridization of labeled targets with three-dimensional gel microarrays. Gel pad microarray images of pure cultures (a) and cells added to ground water sediments (b and c) show statistically identical patterns after 1 h of hybridization. Figure courtesy of D. P. Chandler, ANL.

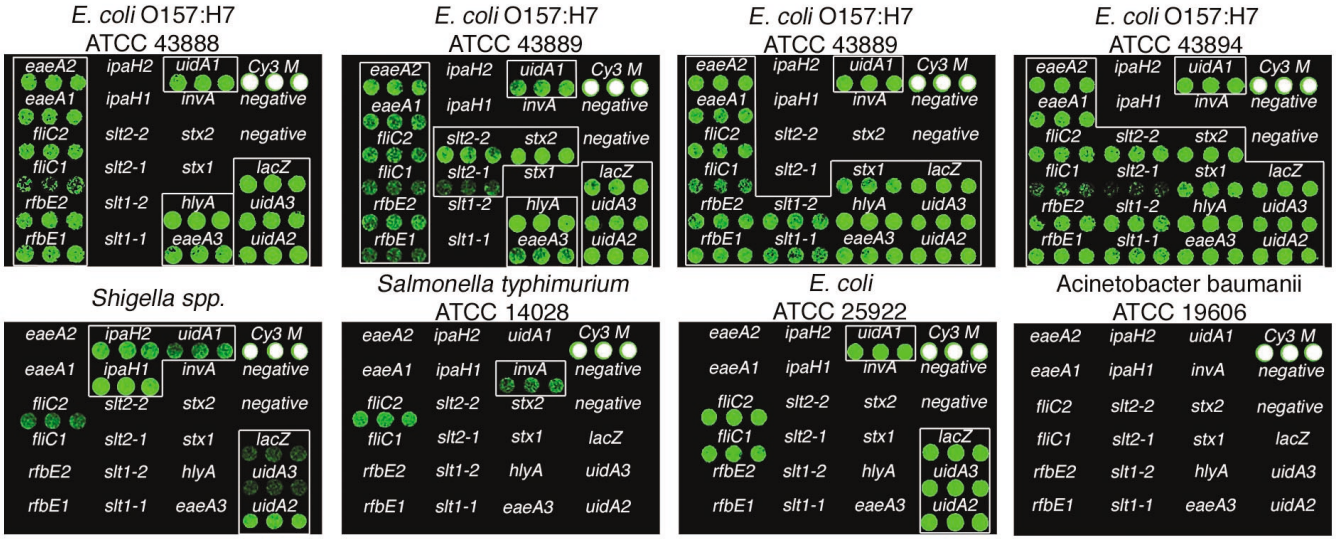

FIG. 5. PNNL experiments show that direct mRNA analysis without PCR may be used for pathogen identification. Hybridization of fluorescently labeled mRNA onto a DNA microarray printed on a glass slide (~100 μm spots) illustrates how the unique mRNA content of each organism may be used to identify various strains of pathogenic and non-pathogenic E. coli as well as Salmonella typhimurium and Shigella spp. Acinetobacter baumanii, with no mRNA probes on the array, shows a positive response only at the location of the positive control probes. Figure courtesy of T. M. Straub, PNNL.

antibody selection and molecular evolution that are used to increase binding affinity have the potential to generate low cost, high affinity reagents.

In addition to antibodies, a wide variety of other molecular recognition elements may be used for biosensing.[30] For example, one may use other proteins (*e.g.*, receptors, lectins, and enzymes), aptamers (RNA or DNA oligomers), peptides, small molecules, and imprinted polymers. Many of these approaches involve selection from large combinatorial libraries, analogous to the approach used for antibody selection. Key challenges in the development of molecular recognition elements for structure-based pathogen detection are the development of high affinity, lowcost, stable reagents.

The most common structure-based sensors are immunoassay tickets. Several different types are under development and commercially available for the detection of a wide range of bioagents. These handheld, easy to use disposable sensors are analogous to widely used pregnancy test kits. A liquid sample is added manually to the test strip (ticket), and other reagents are added as required. As target molecules in the sample wick through the ticket, they bind to immobilized antibodies and detection molecules in a sandwich format. The appearance of a colored pattern on the ticket indicates a positive result. Some examples include BTA Test Strips, and the Sensitive Membrane Antigen Rapid Test (SMART) system. While these devices are easy to use, and suitable for some applications, the cost per assay is on the order of $1 or more, making them unsuitable for many continuous monitoring situations. In addition, at least 10,000 targets bound to the sensor surface are required for detection, and analysis requires 15 min or longer. Therefore, alternative sensor platforms are needed for rapid, sensitive, low-cost detection of multiple pathogens.

One platform that is under development for the simultaneous detection of multiple pathogens is the Autonomous Pathogen Detection System (APDS) developed at Lawrence Livermore National Laboratory (Fig. 6). This instrument includes both aerosol sample collection and automated pathogen detection by a sandwich immunoassay on a bead suspension array.[31] Bead suspension arrays are analogous to planar arrays; the optical properties of beads (rather than locations on a planar array) are used to code up to 100 different antibody surfaces for binding specific bioagents. The sample and a secondary antibody with a fluorescent tag are added to form a sandwich assay. The beads are optically interrogated one at a time using a flow cytometer (*e.g.*, Luminex, Austin, TX). Recent published results showed that the APDS system can simultaneously analyze samples in 60 min for four bioagent simulants at concentrations of about 1 to 5 colony forming units per milliliter for bacteria and spores, which is comparable to enzyme-linked immunoassays.[31] Currently, the system is designed to detect 11 agents, with four internal controls and automatic PCR confirmation of agent DNA.

While structure-based assays such as immunoassays are widely used, research is needed to increase sensitivity and decrease false positive rates. In addition, many one-step binding assays (rather than sandwich assays) are promising for decreasing analysis time and minimizing the cost of reagents.

Broad Spectrum Pathogen Detection

While nucleic acid and protein-based recognition may result in selective biosensors for pathogen detection, many other physical and chemical properties of the organisms can be used for broad spectrum (and maybe even selective) detection of the bioagents. For example, size, shape, and optical properties of aerosols can be used to distinguish between biological and nonbiological particles.[32] Mass spectrometry has been investigated as a means for obtaining information about the protein composition of organisms. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) generates protein fragment (peptide) fingerprints to identify organisms,[33,34] however the identification of organisms in complex mixtures remains a challenge. To circumvent the analysis of complex mixtures, mass spectrometry of single bioaerosol particles has also been demonstrated.[35] However, the mass spectrometry detection systems are relatively large, expensive, and complex. Much simpler, inexpensive biosensors that operate on similar principles—the analysis of the chemical composition of organisms—would be valuable.

In addition to peptides and proteins, other chemicals such as lipids and metabolites can be used for the semi-

FIG. 6. Photo of the Autonomous Pathogen Detection System which includes aerosol collection and automated detection of multiple pathogens using sandwich immunoassays on bead suspension arrays. The complete instrument is a few feet tall with a footprint of about 4 ft^2. Image courtesy of J. Dzenitis, Lawrence Livermore National Laboratory (LLNL).

selective detection of organisms. For example, calcium dipicolinate is a unique component of endospores and can be used to detect spores, but cannot distinguish between *Bacillus anthracis* and other nontoxic Bacillus spores. For example, semiselective detection of bacterial spores has been achieved by immobilizing calcein dye on a sensor surface, which binds to the calcium ions associated with the spores, resulting in a fluorescence shift on binding.[36]

Despite the inherent limitations of semiselective pathogen detection, semiselective detection methods are useful in triggering more selective pathogen detection systems, which require more time and expense for each analysis. It also may be possible to increase the accuracy of semiselective pathogen detection systems by developing sensor arrays that monitor multiple semiselective pathogen biomarkers simultaneously. Sensor arrays have been widely used in the chemical sensor field,[1-3] but have not been applied extensively to pathogen detection. In the sensor array approach, each element of an array responds to different properties (chemical or physical) of an organism. Such an approach requires a well-characterized environmental background signal and research to determine sensor array fingerprints that constitute a positive signal.

Function-based Pathogen Detection

Function-based detectors utilize either whole organisms or portions of organisms (organs, tissues, cells, or receptor membranes) for detection. While nucleic acid and structure-based detection methods are highly specific, they are able to recognize only structural elements that have been characterized previously and identified as molecular recognition features of interest for pathogen detection. In contrast, function-based sensors respond to any insults that affect the biological function being monitored. The classic examples of function-based sensors are the caged canaries that were used to provide early warning of toxic levels of methane, an odorless gas, in coal mines. Most examples of function-based detection are for the detection of toxins or chemical agents, rather than biological agents. Compared to responses to chemical insults, the pathways for the action of bioagents are generally more complex, less understood, and take much longer times for a measurable response at the whole organism level. However, it may be possible to use similar concepts to develop synthetic components of biological systems that respond to the insults of bioagents.

One example of a function-based detection system is the use of nerve cells or synthetic nerve cell membranes to detect nerve agents. Neuronal cell networks have been cultured onto microelectrode arrays,[37] and the responses of the cells have been characterized by recording the extracellular action potentials due to changes in the flux of ions through the cell membranes. In addition to response time challenges in developing function-based detection systems for bioagents, there are significant operational challenges such as sensor maintenance, shelf life, and compatibility with environmental samples. However, the hope is that a detection approach based on biological responses will have broad applicability for the detection of both known and unknown bioagents.

Conclusions

Biosensor systems for pathogen detection are complex devices, including sample collection, sample preparation, biodetection, and data analysis functions. Novel approaches are under development to minimize consumables and cost per analysis, decrease analysis time, improve reliability, and provide additional capabilities for the detection of a wide range of known and unknown bioagents. Undoubtedly, the development of the next generation of biosensor systems will improve the biosensor systems available today, and help in achieving a cleaner environment, a safer food supply, and also provide human health benefits, and enhanced homeland security.

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