Biosensor technology: recent advances in threat agent detection and medicine

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Biosensors are of great significance because of their capability to resolve a potentially large number of analytical problems and challenges in very diverse areas such as defense, homeland security, agriculture and food safety, environmental monitoring, medicine, pharmacology, industry, etc. The expanding role of biosensing in society and a real-world environment has led to an exponential growth of the R&D efforts around the world. The world market for biosensor devices, according to Global Industry Analysts, Inc., is expected to reach $12 billion by 2015. Such expedient growth is driven by several factors including medical and health problems, such as a growing population with a high risk of diabetes and obesity, and the rising incidence of chronic diseases such as heart disease, stroke, cancer, chronic respiratory diseases, tuberculosis, etc.; significant problems with environmental monitoring; and of course serious challenges in security and military applications and agriculture/food safety. A review paper in the biosensor technology area may be structured based on (i) the principles of detection, such as the type of transducer platform, bioanalytical principles (affinity or kinetic), and biorecognition elements origin/properties (i.e. antibodies, enzymes, cells, aptamers, etc.), and (ii) the application area. This review follows the latter strategy and focuses on the applications. This allows discussion on how different sensing strategies are brought to bear on the same problem and highlights advantages/disadvantages of these sensing strategies. Given the broad range of biosensor related applications, several particularly relevant areas of application were selected for review: biological threat agents, chemical threat agents, and medicine.

Introduction

The history of biosensors R&D is an excellent example of how a very simple and elegant idea can foment a revolutionary breakthrough in science and technology which is currently influencing all areas of our life.1,2 The foundation of this field was built by the work of Clark and Lyons in 1962, who used a combination of an electrochemical oxygen sensor (Clark oxygen electrode) and the enzyme glucose oxidase incorporated into a dialysis membrane to demonstrate the capability to quantitatively measure glucose in aqueous media.3 Then Updike and Hicks covered the Clark oxygen electrode with a polyacrylamide gel membrane containing glucose oxidase and demonstrated the first “enzyme electrode” for the rapid and quantitative determination of glucose, while Guilbault and Montalvo used a glass pH electrode with immobilized urease to measure urea concentration.4 These early studies spurred on the integration of biorecognition elements onto physicochemical transducers, creating sensing devices. Early on, the field was largely focused on immobilizing enzymes onto electrodes. Gradually, the field expanded to include other biorecognition elements such as antibodies, cells, and nucleic acids, and the term “enzyme electrode” was transformed to “biosensor”.

A Google Scholar search of the terms “biosensor” and “immunosensor” returned a number of articles for each year as seen in Fig. 1. The first recognized period of research conducted in biosensing was in 1970–1979, and over 50 papers in that decade were published on the subject. The subsequent decade returned close to 1200 articles published on the subject of biosensors. The numbers steadily increased every year in the 1990s. A sharp increase in the rate of publishing can be seen following 2001, which may be attributed to infusion of funds for development of sensors related to homeland security. As the field has widened, the amount of funding available and the amount of research conducted have grown significantly.

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A biosensor (Fig. 2 schematically depicts the concept of a biosensor) is “a self-contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element which is in direct spatial contact with a transducer”, according to the IUPAC definition.\textsuperscript{5,6}

The progress in the biosensors area was catalyzed by the tremendous success of glucose biosensors used for diagnosis and management of diabetes mellitus.\textsuperscript{8} Historically, these biosensors were based on an enzyme glucose oxidase (GOx) catalyzing the oxidation of glucose to hydrogen peroxide and D-glucono-lactone:

\[
\beta\text{-}D\text{-}glucose + O_2 \xrightarrow{\text{GOx}} D\text{-}gluconolactone^- + H_2O_2^+ 
\]

Jeffrey Kirsch received his Masters of Science degree in Materials Science and Engineering from Auburn University in 2013. His work involved the development of electrochemical biosensors utilizing multiple enzymes immobilized onto carbon nanotubes to detect and discriminate between organophosphorus and non-organophosphorus neurotoxins. He received his Bachelors of Science degree in Materials Engineering from Auburn University in 2011 and worked for one year as an undergraduate research assistant under the tutelage of Professor Aleksandr Simonian. His work supported the graduate research efforts on electrochemical sensors for tricresyl phosphate detection. He is currently exploring employment opportunities in North America.

Christian Siltanen earned his BS in Mechanical Engineering from Oregon State University in 2010. His undergraduate work involved fluidic modeling in synthetic capillaries. In 2011, he enrolled at UC Davis where he is currently a PhD student in Prof. Alex Revzin’s group. His research is focused on the design of microfabricated platforms for sensing and sorting cells.

Qing Zhou obtained her BS in Medical Sciences from Peking University, China, in 2011 where she worked on nanofluidics-based biosensing in Dr Wang’s lab in the MEMS center of Peking University. After a half-year training in aptamer selection at the University of Roma La Sapienza, Italy, she joined Prof. Alex Revzin’s group at UC Davis in 2011 where she is currently a PhD candidate. Her project focuses on developing aptamer-based optical and electrochemical biosensors for multiplexed cytokine detection.

Alexander Revzin received his BS (1998) and PhD (2002) in chemical engineering from Wayne State University and Texas A&M University respectively. He did a two year postdoctoral fellowship at Massachusetts General Hospital with Mehmet Toner and then joined Department of Biomedical Engineering at UC Davis in 2004 where he currently holds a rank of professor. Revzin’s laboratory is developing biointerfaces for cell cultivation and sensing.
Since the stoichiometry of this reaction is well known, it is possible to calculate the glucose concentration by monitoring consumption of oxygen or production of hydrogen peroxide. Clark’s original technology was adopted by the Yellow Spring Instrument Company that launched in 1974 the first ever glucose biosensor (the Model 23 YSI analyzer) for the direct measurement of glucose in whole blood; this instrument is still used for clinical glucose measurements. To minimize dependence on oxygen, concentrations of which may vary, Updike and Hicks offered a differential measurement scheme using two oxygen electrodes, where one of them (without GOx) was used as a reference electrode and the working electrode was covered with GOx. Later, Guilbault and Lubrano suggested the use of H₂O₂ monitoring in reaction (1) for glucose detection. An interesting electrochemical system for glucose monitoring, the Biostator, was developed by Clemens et al., where they used a closed-loop approach for continuous glucose monitoring through an extracorporeal shunt and insulin delivery.

Further progress in the electrochemical biosensors was realized in several systems for in vivo glucose monitoring, one of which, a single-use glucose strip, is currently in widespread use, and several other systems for continuous monitoring are currently approved for use. The comprehensive discussion on in vivo glucose sensing issues is provided in a recently published book by Cunningham and Stenken. During the last two decades of the 20th century, innovative approaches in glucose biosensors development predetermined further progress in the field. As discussed in Wang’s excellent review on glucose biosensors history, considerable efforts during those decades focused on the development of different mediator-based ‘second-generation’ biosensors and toward the establishment of electrical communication between the redox center of GOx and the electrode surface. Three generations of glucose biosensors can be distinguished: (i) first-generation devices based on the natural oxygen co-substrate, and the production and detection of hydrogen peroxide; (ii) second generation, based on the synthetic electron acceptor, capable of shuttling electrons from the redox center of the enzyme to the surface of the electrode; and (iii) third generation based on charge-transfer complexes such as tetrathiafulvalene–tetracyanoquinodimethane (TTF–TCNQ) for direct electron transfer between GOx and the electrode (Fig. 3).

Significant progress in the development of three generations of biosensors was achieved because of significant efforts of F. W. Scheller’s group and Adam Heller’s elegant work on the use of flexible polymers with osmium redox sites. Substantial efforts to develop third generation biosensors were put forth by Kulis and Cenas, Palmisano et al., and Aizawa and Koopal.

Further progress in enzyme-based biosensors, which started with a single-enzyme biorecognition element, was extended to the coupled multi-enzyme systems where several enzymes were incorporated into the same platform; such an approach demonstrated strong expansion of the biosensor concept to the detection of new analytes and to multi-parameter assays. Mostly it was based on the unique properties of enzymes to recognize...
one particular substrate (such as glucose oxidase, aspartase, catalase, uricase, etc.) or a selective class of substrates (such as D,L amino oxidase, peroxidase, laccase, alcohol dehydrogenase, etc.). Based on the type and level of integration of biorecognition elements, two main classes of biosensors are distinguished according to the type of interaction leading to the biorecognition signal generation: metabolism sensors and affinity sensors. In metabolism or kinetic sensors (e.g. glucose biosensor) the molecular recognition by enzymes is accompanied by chemical conversion of the analyte to the corresponding products and changes in the parameters throughout the course of the reaction; those changes which correlate with target analyte content are registered by the transducer. In this case, the interaction of the analyte with the biorecognition element is reversible and the biosensor becomes reusable as long as the enzyme is active and is providing an appropriate slope for the calibration curve.

Conversely, in affinity sensors, the molecular recognition results from the formation of an analyte–biorecognition element complex (for example, DNA hybridization) and the physicochemical changes caused by the complex formation are detected by the transducer. In this case, to make the biosensor reusable, the initial state must be regenerated by the splitting of the complex, which is not trivial and in most cases results in poor recovery of the biosensor’s initial parameters. In affinity biosensors, the biorecognition elements are represented by multiple structures, such as antibodies, enzymes, receptors, etc. A classic example of enzyme-based affinity biosensors is a system developed by Guilbault et al. for detection of nerve agents. As seen in Fig. 4, the basic principle behind an acetylcholinesterase (AChE) affinity biosensor relies on the interaction between the target nerve agent and the enzyme active site. Nerve agents inhibit the enzyme by irreversibly binding to the active site of AChE, which prevents it from catalyzing the hydrolysis of acetylcholine and its derivatives. A correlation between the loss in activity (inhibition) and the concentration of the nerve agent can be found due to the large number of enzymes present on the surface of the electrode, and that statistically not all of them will be inhibited. While very sensitive (up to $10^{-11}$ M), this biosensor suffers from the limited specificity, since a large number of different compounds such as heavy metals, detergents, etc. are capable of inhibiting AChE.

Another type of affinity biosensors, where interactions take place without analyte conversion, is a biosensor for biotin detection, where an excellent sensitivity and selectivity was...
achieved due to the extremely high affinity of interaction (the dissociation constant of avidin is $10^{-15}$ M); an electrochemical detection avenue was possible because of catalase-labeled avidin immobilized to the oxygen electrode interface. In the past two decades, the use of avidin–biotin reaction has been ubiquitous in both biosensing and bioconjugation. A variety of pre-labeled biotinylated molecules are available commercially along with reagents for biotinylation of virtually any biomolecule.\textsuperscript{23} Imunosensors are another example of affinity based biosensors. These biosensors are based on detection of antibody–antigen interactions and may be further categorized by direct or indirect methods of detection. In the case of direct sensors, the immunochemical complex formation results in physico-chemical changes at the transducer interface, while indirect methods are based on the specific labels attached to one of the immunochemical reaction partners. A large number of immunosensors are described in the sections below.

Aptamers are another class of affinity biorecognition elements. These molecules were first described approximately two decades ago and have emerged as viable alternatives to antibodies. There are several reasons for the emergence of aptamers including: (1) aptamers are nucleic acids with predictable and easy to engineer molecular structure; therefore, they can be designed into a variety of beacon types. Therefore, aptamers lend themselves to the development of reagent-less biorecognition elements that emit signal directly upon analyte binding. (2) Aptamers are chemically stable, which not only eliminates the need for low temperature storage but also allows for sensor regeneration and reuse. Similar chemical regeneration protocols may cause denaturation of antibodies, rendering traditional immunoassays unreliable. Aptamer based biosensors appear frequently in the discussion of specific applications presented below.

It should be pointed out that in the last decade, the biosensors field has been significantly advanced by nanotechnology-enabled sensors (nanosensors) due to the powerful combination of recent breakthroughs in nanomaterials with novel biorecognition approaches; this was addressed and recently approved by NSTC Committee on Technology Nanotechnology Signature Initiative: “Nanotechnology for Sensors and Sensors for Nanotechnology”.\textsuperscript{24} It was mentioned that “nanosensors... are providing new solutions in physical, chemical, and biological sensing that enable increased detection sensitivity, specificity, and multiplexing capability in portable devices for a wide variety of health, safety, and environmental assessments”. As seen throughout this review, there have been many biosensors developed using novel nanomaterials in their approaches to both the transduction and biorecognition events.

The field of biosensors is expanding exponentially as is evidenced by citation report shown in Fig. 1. This expansion is fuelled by physical sciences and engineering providing new materials and devices as well as by government and various industries requiring rapid, sensitive and specific sensing technologies. Encompassing the field of biosensors in any one article is not possible, no matter how comprehensive the review is. Therefore, we chose to shine spotlight on certain applications and to discuss sensing technologies in the context of each application.

**Biosensors for biotoxins and biological pathogens**

This section of the review covers biosensing technologies for several different types of toxins and pathogenic organisms, which are the most threat agents in food safety and agriculture and it also discusses the actions of those pathogens because understanding of the mechanism of invasion and interference with normal biochemical processes can lead to the development of new strategies and means to detect, discriminate, and destroy these threats to human safety and well-being.

Pathogenic bacteria and viruses represent a great threat to safety through contamination of food, bioterrorism, and bio-warfare. Many of them, such as ricin, botulinum toxins, saxitoxin, staphylococcal enterotoxin B, and trichothecene mycotoxins, are capable of being weaponized, and therefore early warning of possible exposure to these toxins is of great importance.\textsuperscript{25} Many food-borne illnesses are a result of similar toxins through either contraction of a pathogenic bacterium or virus, or through contamination of the food through toxins excreted while the bacteria grow. Bacillus cereus, Bacillus anthracis, Campylobacter jejuni, cholera toxin, Escherichia coli, Listeria monocytogenes, Salmonella typhimurium, Shigella spp., Staphylococcus aureus, and Staphylococcal enteritis are just a few of the many types of pathogenic bacteria which are capable of infecting and harming humans.\textsuperscript{26} For bioterrorism/biological weapons, examination of the relationship between aerosol infectivity and toxicity versus quantity of the agent reduces the number of effective agents able to produce the highest number of casualties. As an example, the necessary amount of agent needed to cover a 100 km$^2$ area with 50% lethality is 8 metric tons for agents such as ricin classified as “highly toxic” versus only kilogram quantities of anthrax with the same coverage and results.\textsuperscript{27} In the last 10 years, the focus of biosensors for detection of pathogenic bacteria has been on not only utilizing new transduction methods, but also developing more robust and flexible bio-recognition elements. The most common type of recognition element for biosensors are antibodies/lectins and nucleic acid probes.\textsuperscript{28} Other types of recognition elements include aptamers, whole cells, and bacteriophages, each with their own distinct advantages and challenges.

**Ricin**

Ricin is a natural toxin found in castor beans, and can be produced from the waste material left over after processing the beans. Ricin poisoning is not contagious, and must be deliberately produced and used in order to poison people. The effects vary based on how ricin was introduced into the body; however, it works by shutting down protein production inside the cells, and without the ability to produce new proteins those cells will eventually die. The high toxicity of this poison has led several countries to study and develop weapons from this agent,
as well as terrorist organizations due to the relative ease to obtain ricin. There is no antidote for ricin, and no widely available, reliable medical test is available to confirm exposure to ricin. The minimum lethal dose is $5 \mu g \text{kg}^{-1}$ of body weight.

Biosensors have been developed for detection of ricin in several different formats including the fluorescence array biosensor, surface plasmon resonance, and magnetoelastic sensors. Antibodies or oligosaccharides are used as the recognition element. The fluorescence array biosensor, pioneered by Rowe-Taitt et al., includes an array of capture antibodies, each specific to their target antigen, that are immobilized onto the sensor surface. The analyte is passed over the antibodies and bound to its specific antibody, and then a fluorescent tracer coupled with the antibody is passed over the sensor to label the bound analyte. The sensor is then imaged with a charged coupled device (CCD) camera and the amount of analyte bound is calculated from the amount of fluorescent signal obtained. Uzawa et al. used synthetic analogues of $\beta$-lactosyl and $\beta$-$\delta$-galactosyl ceramides as ligands for ricin, since it binds to cell-surface oligosaccharides (Fig. 5). These sugar-probes were bound to the surface plasmon resonance (SPR) sensor and allowed them to detect ricin at 10 pg mL$^{-1}$ within 5 minutes. Additionally they coated Au nanoparticles with the sugar-probes to discriminate ricin from other proteins within 30 minutes, by the agglomeration/cross-linking of the nanoparticles in solution, producing a color change.

Shankar et al. developed a disposable magnetoelastic sensor utilizing a sandwich complex of the antibody–antigen interaction and measured the concentration of ricin through shifts in both resonant frequency and resonance quality factor of the sensor. These are due to mass change in the sensor from the binding of ricin to the sensor surface, and the sensor shows a linear response in concentrations ranging from 10 ng mL$^{-1}$ to 100 ng mL$^{-1}$ and a LOD of 5 ng mL$^{-1}$.31

**Botulinum toxins**

Botulinum toxin is made by a bacterium called *Clostridium botulinum*, and causes the muscle-paralyzing disease Botulism. Food-borne botulism occurs when the toxin is ingested from contaminated food, which will lead to the illness within a few hours to several days. It is especially dangerous because the contaminated food can be easily available to other people besides the person first exposed. Botulism is not contagious, but the effects cause a variety of health issues and eventually death if left untreated. Botulism diagnosis must be confirmed through a specialized laboratory, which can require several days to complete, and is frequently misdiagnosed.

Botulinum toxins have been detected mainly using the sandwich assay format utilizing antibodies with fluorescent tracers, however, it has also been detected using antimicrobial peptides, and aptamers. The antibody bioassay has the advantages of simplicity and sensitivity, but it is non-selective.

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**Fig. 5** This scheme demonstrates the application of the infection process to ricin detection, since ricin specifically recognizes cell surface oligosaccharides, and the sugar based nanomaterials (A) were designed for use in surface plasmon resonance (B) and (C), and in a colloidal gold colorimetric assay for the qualitative detection of the toxin (D). Reprinted with permission from Uzawa et al., *Biosens. Bioelectron.*, 2008, 24, 923–927. Copyright © 2008, Elsevier.
Saxitoxins are produced by the dinoflagellate planktons commonly associated with the “red tide”, and accumulate in shellfish which consume the microalgae. This toxin acts on sodium channels in nerve cells by binding to the voltage gates, which causes neurotoxic effects. Saxitoxin can be ingested, inhaled through an aerosol, or enter open wounds, and is 1000 times more toxic than Sarin nerve gas. It is similar to the botulinum toxin as a cholinergic agonist which inhibits the release of acetylcholine at the synapses. Symptoms occur between 10 minutes and four hours depending on the dose and exposure method, with intravenous injection causing death in less than 15 minutes. There is no antidote therapy, and is extremely difficult to detect and identify without expensive and complicated analytical laboratory equipment. Faulty identification of saxitoxin as a nerve agent could be fatal in military medicine and treatment with atropine would increase fatalities. The compounds are extremely toxic and easy to produce in large quantities due to high yield harvesting from cultures of the species Protogonyaulax, making them ideal for bioterrorism.

The standard for detection and screening of saxitoxins is the use of a mouse mortality assay, which is considered ethically problematic. Alternative methods could be provided by developed biosensors such as a surface plasmon resonance (SPR) inhibition assay and electrophysiological assays. Campbell et al. used several different recognition elements such as the sodium channel receptor and different antibodies in their SPR biosensor. They immobilized the toxin to the sensor surface and passed the recognition elements across the surface with different concentrations of different types of saxitoxin, and the amount of binding was measured, with higher concentrations inhibiting the ability of the binder to stick to the surface. Velez et al. measured sodium currents in the sodium channels of HEK 293 cells and their inhibition by saxitoxins, although it requires some advanced training of personnel. The recombinant sodium channel could be tailored to specific toxins which affect sodium channels and therefore could provide the biorecognition event for those other toxins.

**Staphylococcal enterotoxin B**

Staphylococcal enterotoxin B (SEB) is a toxic protein produced by several species of bacteria including *Staphylococcus*, *Streptococcus*, and *Mycoplasma*. The toxin is a “super-antigen” capable of nonspecific activation of the immune system which includes the release of large quantities of cytokines. This toxin is incapacitating in nature, and is generally non-lethal, but high levels of exposure can lead to death, and depending on the method of exposure, can have serious health implications. SEB can be used to contaminate food or water, but is non-contagious. The relative ease of production and low quantities necessary for intoxication, ease of dispersion, delayed onset of symptoms, high morbidity, and difficulty in discernment as a weapon make this toxin desirable as a bioweapon.

Several methods have been developed for detection of SEB including using the antibody sandwich assay, direct detection through surface plasmon resonance (SPR), magnetoelastic sensors, piezoelectric crystal sensors, and capacitive sensors. Homola et al. were able to directly detect SEB (LOD of 5 ng mL\(^{-1}\)) on an antibody functionalized SPR chip, and then were able to amplify their signal using a secondary antibody binding interaction (sandwich assay) after direct detection with a limit of detection of 0.5 ng mL\(^{-1}\) in both buffer and milk.

Ruan et al. used magnetoelastic sensors functionalized with antibodies against SEB (anti-SEB) to detect it at concentrations as low as 0.5 ng mL\(^{-1}\) by measuring the change in resonance frequency. They were able to amplify their signal through enzymatic catalytic precipitation and biotin–avidin system. After binding SEB to the antibody-coated sensor, they exposed the SEB loaded sensor to biotin-labeled anti-SEB, then to avidin loaded with alkaline phosphatase, which hydrolyzed and polymerized 5-bromo-4-chloro-3-indolyl phosphate, leading to further change in resonance frequency.
Campbell et al. developed a piezoelectric-excited millimeter-sized cantilever (PEMC) sensor functionalized with anti-SEB and measured the resonant frequency shift as SEB binds to the antibodies. They reported a detection limit between 12.5 pg mL\(^{-1}\) and 50 pg mL\(^{-1}\). Labib et al. developed a capacitive biosensor using horseradish peroxidase labeled anti-SEB to detect SEB at concentrations as low as 0.3 pg mL\(^{-1}\) within 10 minutes.

**Trichothecene mycotoxins**

Trichothecene mycotoxins are toxins created by fungal metabolism of species such as *Fusarium*, and they cause multi-organ effects such as emesis, diarrhea, nervous disorders, cardiovascular alterations, skin toxicity, bone marrow damage, etc. Trichothecene mycotoxins can be delivered as dusts, droplets, aerosols, or smoke through a multitude of aerial delivery systems, and due to the ease of production on a large scale, they are highly regarded as a biological weapon. Aerosolized trichothecenes in large doses can produce death within minutes, and at low doses produce a host of symptoms that affect the eyes, skin and gastrointestinal tract. Traditional detection and identification requires expensive, highly analytical instrumentation such as chromatography and mass spectrometry, and battlefield screening is conducted by observation of symptoms.

Typical detection methods involve optical techniques such as surface plasmon resonance (SPR) or fluorescence based detection, both utilizing antibodies against mycotoxins. There are some concerns that antibodies for trichothecene mycotoxins are cross-reactive, increasing uncertainty in measurements. In-field test strips involving a lateral flow device have been developed for visual confirmation of trichothecene deoxynivalenol exposure in wheat samples. Lateral flow devices utilize capillary forces to move samples across an immune-analytical membrane containing antibodies or other immunoreagents. However they only provide a positive/negative result, verifying the presence or absence of the analyte. Attempts have been made to remedy this by adding zones to a test strip which only react in certain ranges of the analyte, thereby giving confirmation of the presence and a concentration range.

Gosselin et al. reported a label-free, spectroscopic biosensor based on antibodies for the detection of verrucarin-A at concentrations as low as 2 pg mL\(^{-1}\) in buffer and 6 pg mL\(^{-1}\) in dust samples. Their sensor is based on the sensitivity of Fourier transform infrared-attenuated reflection (FTIR-ATR) antibodies that are directly placed at the surface of the infrared sensing element. FTIR-ATR allows for spectrometric analysis of compounds in solution when they come in contact with the ATR element, while the FTIR provides quantitative information in addition to spectroscopic signatures for precise determination of the interacting molecules.

**Bacillus anthracis and Bacillus cereus**

*Bacillus anthracis* and *Bacillus cereus* are two pathogenic species of the bacterial genus *Bacillus*. *B. anthracis* is the pathogen responsible for what is known as “Anthrax,” which is a disease affecting livestock that consume the spores from the soil. The symptoms are severe and vary based on the form, but can include hemorrhage, necrosis, edema, and eventually death. Both *B. anthracis* and *B. cereus* are spore forming, which allows them to survive in harsh conditions outside of a host for long periods of time. Anthrax can be weaponized by aerosolizing the spores, and is considered as a potential tool for bioterrorism, but is not transmitted between persons. Traditional methods for detection involve the standard blood culture, which can give results within a day, and definitive diagnosis would require up to two days, if they are looking for anthrax (most laboratories do not further identify the *Bacillus* species unless it is specifically requested).

*B. cereus* is a food-borne pathogen causing two different types of gastrointestinal disease, either diarrheal or emetic. Food safety and clinical methods for identification of *B. cereus* group strains do not distinguish between *B. cereus* and *B. thuringiensis*, another member of the *Bacillus* genus.

Pathogenic *Bacillus* species have been detected using antibodies, DNA probes, bacteriophages, and mammalian cells as the recognition element with several different types of transducers including fluorescent array, evanescent wave fiber-optic, laser cytometry, electrochemical, and mass sensitive sensors.

Tims and Lim developed a fiber-optic biosensor capable of detecting *B. anthracis* spores in common powders at concentrations of 3.2 \(\times\) 10\(^5\) spores per mg or higher within an hour. Banerjee et al. used multi-well plates containing Ped-2E9 cells encapsulated in a collagen matrix and colorimetrically measured the alkaline phosphatase released from infected cells and the live or dead status of the cells were confirmed by laser cytometry. Their device was capable of measuring *B. cereus* at initial concentrations of 10\(^2\)–10\(^4\) CFU per g in meat and rice samples.

Pal et al. used polyaniline labeled with *Bacillus* antibodies to build resistance based biosensors capable of detecting 35–88 CFU per mL in food samples within 6 minutes. Additionally, they built magnetic polymer nanostructures as pre-concentrators for DNA targets looking for the *Bacillus anthracis* protective antigen A gene, using a biotinylated capture probe and a detector probe labeled with the magnetic nanoparticles. They detected the nanoparticles using electrochemical oxidation–reduction. This is schematically represented in Fig. 6. Johnson et al. developed a wireless biosensor utilizing magnetoelastic sensors coated with bacteriophage engineered to bind *B. anthracis* spores and measured the shift in resonant frequency.

**Campylobacter spp.**

Campylobacteriosis is an infectious disease of the digestive system and is caused by bacteria of the *Campylobacter* genus. Symptoms of this disease include diarrhea, cramping, abdominal pain, and fever, and *Campylobacter* is considered one of the most common causes of diarrheal illness in the United States. *C. jejuni* is the most common cause of human illness; however, all species
can be dangerous and carried by birds. Transmission occurs through consuming contaminated food (especially poultry) and water, or when one comes in contact with an infected animal, and the minimum amount of cells necessary to cause infection is 500. The challenges presented by these bacteria include early detection and decreasing the amount of contamination of food, prevention and reduction of the development of resistant strains, and detection of outbreaks.\(^9^1,9^2\)

*Campylobacter* species have been detected using DNA,\(^9^3–9^5\) aptamers,\(^9^6,9^7\) antibodies,\(^4^0,7^1,9^2,9^8–1^0^0\) or bacteriophages\(^9^6,1^0^1\) as the recognition element and using optical,\(^3^9,5^7,7^1,9^2–9^4,9^9,1^0^1–1^0^4\) electrochemical,\(^1^0^5–1^0^7\) or mass sensitive transducers.\(^9^8,1^0^0,1^0^8–1^1^0\) Gnanaprakasa *et al.* used the dotLab system (a commercial diffractive optics sensor) to detect DNA segments unique to *C. jejuni* and compared the results to those obtained using the surface plasmon resonance (SPR) biosensor (Fig. 7). The *hippuricase* gene is expressed only in *C. jejuni*, which provides specificity for the biosensor, so corresponding DNA was immobilized onto the sensors and this gene was detected with a calculated limit of detection (LOD) of 2.5 nM for SPR and 5 nM for dotLab.\(^9^4\)

Singh *et al.* exploited the receptor binding proteins of the *Campylobacter* bacteriophage NCTC 12673 to detect *C. jejuni* using SPR. The authors found that they were able to orient the binding proteins to optimize the capture of the bacteria by modifying the self-assembled monolayer chemistry.\(^1^0^1\) Sapsford *et al.* developed 25 minute sandwich assays on the array biosensor to detect *C. jejuni* at concentrations as low as \(9.7 \times 10^2\) CFU per mL.\(^9^2\) Viswanathan *et al.* used screen printed electrodes modified with antibody functionalized carbon nanotubes to detect *Campylobacter* species in bovine milk. The sensor was immersed in an antibody-PbS nanocrystal conjugate solution to build a sandwich complex which was then stripped away electrochemically, and the dissolution of the metal ions correlated to the amount of bacteria present.\(^1^0^7\) Safina *et al.* utilized several different lectins to develop a quartz crystal microbridge biosensor for use in flow injection analysis. The lectins are selective to carbohydrates on the surface of the
cell wall, and by using several different types they could detect different strains of *Campylobacter*. Additionally, they could use a glycine solution (pH 2.5) to wash the surface allowing them to reuse the sensor, capable of detecting $10^3$ cells within 30 minutes.98,109

**Cholera toxin**

Cholera toxin (CT) is produced by the bacterium *Vibrio cholera*, which is usually found in water or food sources which have been contaminated by feces from a person infected with cholera. Cholera causes severe diarrhea, and in serious cases can lead to dehydration and shock, and is diagnosed by standard culture methods.111 Cholera toxin has a ring of five identical protein chains which attaches to carbohydrates on the surface of the cell, and delivers the toxic portion of its structure into the cell. Once inside, the toxic portion uses the cell to convert itself into a toxic enzyme, which then starts to destroy the cell molecule by molecule. The function of the toxic enzyme is to convert signal proteins (the G-protein) into a permanently active state, sending a never ending signal, which causes the cell to begin to transport water and sodium out of the cell, flooding the intestines.112

Cholera toxin has been detected using antibodies/lectins25,33–36,113,114 and/or gangliosides115–117 in fluorescence,25,33–36,113,114,116 electrochemical,115,117 and piezoelectric118 biosensors. To detect pathogenic cholera, Rowe-Taitt *et al.* immobilized ganglioside GM1, the receptor for the cholera toxin, onto their array biosensor, and were able to detect as low as 40 ng mL$^{-1}$ of the toxin, and with the addition of CT-specific antibodies in their array system, they were able to obtain specificity as well.116 Viswanathan *et al.* used potassium ferrocyanide encapsulated and ganglioside functionalized liposomes attached to a surface-assembled monolayer modified electrode, and when exposed to cholera toxin, the change in mass of the piezoelectric crystal results in a frequency shift, with a detection limit of 25 ng mL$^{-1}$.118

**Escherichia coli**

*Escherichia coli* bacteria are representative of a large class of bacteria, of which most are harmless and important to a healthy human intestinal tract. However, there are several pathogenic types of *E. coli* which can cause diarrhea or illness outside of the intestinal tract. The most common types of pathogenic *E. coli* are the six types that produce the Shiga toxin, of which *E. coli* O157:H7 is the most commonly identified Shiga toxin producing bacteria found in North America. Although the symptoms are rarely life-threatening, 5–10% of those infected develop a complication known as hemolytic uremic syndrome, which can result in kidney shutdown and other serious problems. Pathogenic *E. coli* can easily contaminate food through unsanitary preparation conditions or through exposure to fecal matter.119

*E. coli* has been detected using many different means including antibodies,90,120–126 antimicrobial peptides,127,128 aptamers,28 bacteriophages,121 β-galactosidase detection,129,130 and DNA probes31,131 using optical,123–125,127–131 electrochemical,90,120–122,126 and mass sensitive biosensors.132 Mejri *et al.* compared the use of antibodies and phages as the recognition element in an impedance biosensor. Both recognition elements were immobilized onto interdigitated gold microelectrodes (schematically depicted in Fig. 8) and exposed to *E. coli*. The phage produced two successive signals with an initial increase in impedance as the bacteria are captured, and then a decrease over time attributed to phage-induced lysis. This allows for
confirmation of capture of the target analyte, eliminating concern for non-specific adsorption or cross-binding. This biosensor was capable of detecting *E. coli* in the range of $10^4$ to $10^7$ CFU per mL.\(^{121}\)

Mathew and Alocilja developed a porous silicon-based chemiluminescence biosensor which detected the emission of light when the β-galactosidase enzyme from *E. coli* catalyzes its substrate dioxetane, with a sensitivity of 10–100 CFU of *E. coli*.\(^{129}\) Simpson and Lim described possible methodologies to reduce PCR confirmation time by a factor of 5 by direct PCR of bacteria using fiber optic waveguides, eliminating the need for culture or enrichment steps.\(^{131}\) Fu *et al.* developed a magnetostrictive microcantilever biosensor using antibodies against *E. coli* to detect the bacteria in water by measuring changes in frequency shift. They found that reducing the size of their sensor increased the limit of detection, with a sensor at 1.5 mm × 0.8 mm × 35 μm having a detection limit of $10^5$ CFU per mL.\(^{132}\)

**Listeria monocytogenes**

*Listeria monocytogenes* is a food-borne pathogen which causes listeriosis, and is the leading fatal food-borne infection in the United States. Listeriosis affects the central nervous system, causing meningitis or encephalitis, and has also been found to cause newborn meningitis, when pregnant mothers become infected. Traditionally the bacterium is detected in food samples through conventional culture methods.\(^{133}\)

*L. monocytogenes* has been detected using antibodies,\(^{57,76,124}\) aptamers,\(^{134}\) mammalian cells,\(^{86,87,135}\) and bacteriophages\(^{136}\) as the recognition element in optical\(^{57,86,87,134,136}\) and electrochemical\(^{76}\) biosensors. Susmel *et al.* immobilized antibodies against *L. monocytogenes* onto screen printed gold electrodes using self-assembled monolayers (SAMs). After immobilization, the redox behavior and diffusion coefficient of a potassium hexacyanoferrate(II) probe was monitored in the presence and absence of the bacteria, with the former causing a shift in the diffusion coefficient of the redox probe. This was attributed to the impedance of redox electrons during the binding of the bacteria to the antibodies, with no change occurring when just the antigen was immobilized.\(^{76}\) Ohk *et al.* used an antibody–aptamer fiber optic biosensor to detect *L. monocytogenes* in food products, and was able to selectively detect pathogenic *Listeria* in mixed bacteria samples at a concentration of $10^3$ CFU per mL. The fiber-optic waveguide was coated with antibodies to capture the bacteria. Aptamer-A8, a single-stranded oligonucleotide ligand which is specific to internalin A, an invasive protein from *L. monocytogenes*, was used as the reporter.\(^{134}\) A schematic depicting aptamer-based detection schemes is shown in Fig. 9.

Koo *et al.* used heat shock protein 60 (Hsp60), a receptor for the *Listeria* adhesion protein during *L. monocytogenes* infection, as a recognition element in their microfluidic fluorescence biosensor. It was shown to have a much higher (83×) capture efficiency than the monoclonal antibody mAb-C11E9, and therefore...
could be used as an alternative recognition element to antibodies for specific detection of *L. monocytogenes*. Nanduri *et al.* detected *L. monocytogenes* with a surface plasmon resonance (SPR) sensor modified by physical adsorption with phage-displayed antibodies. The sensor showed specificity towards *L. monocytogenes* whole cells with a detection limit of $2 \times 10^6$ CFU per mL.

**Salmonella**

Pathogenic *Salmonella* are bacteria which cause salmonellosis, an infection in which the patient develops diarrhea, fever, and abdominal cramps. In rare cases, the infection may spread to the bloodstream and can cause death if left untreated. *Salmonella* are typically found in the intestinal tracts of humans and other animals, and infection occurs through consumption of food contaminated with fecal matter or through unsafe food preparation methods.

Pathogenic *Salmonella* has been detected using antibodies, antimicrobial peptides, bacteriophages, and DNA probes coupled with optical, electrochemical, and mass sensitive transduction techniques. Nyquist-Battie *et al.* examined the effects of low-shear modeled microgravity (LSMMG) on antibodies and the growth of *Salmonella*, for monitoring food and water during spaceflight. They found that LSMMG does not have any significant effects on either the binding of antibodies to bacteria or surface antigens of those grown in LSMMG.

Zhao *et al.* developed a sensor (Fig. 12) based on the insertion element gene of *Salmonella*. They used two different types of nanoparticles, Au-NP and magnetic nanoparticles, coated with the target specific DNA probe (Fig. 10). A sandwich complex is formed between the nanoparticles and the target DNA in a mixed medium, and the captured DNA is removed from the medium using a magnet. The bio-barcode DNA is released from the Au-NP and is detected by fluorescence with a detection limit as low as 1 ng mL$^{-1}$. They further developed this to detect multiple pathogens, but added nanoparticle tracers which when dissolved after capture allowed them to detect the DNA electrochemically.

Kim *et al.* developed an impedance biosensor for detection of *Salmonella* using antibodies to capture the bacteria and detect $10^6$ CFU per mL in pork meat extract within 5 minutes. Li *et al.* directly detected *Salmonella* on fresh produce using phage-based magnetoelastic biosensors (Fig. 11). The surface of a tomato was spiked with *Salmonella* and the biosensors and control sensors (magnetoelastic sensors with no phage) were placed onto the tomato for 30 minutes. Shifts in resonant frequency were measured before and after exposure to the surface of the spiked tomato, and the limit of detection was $5 \times 10^2$ CFU per mL.

**Shigella spp.**

*Shigella* is a family of pathogenic bacteria that infect humans and cause shigellosis, characterized by diarrhea, fever, and stomach cramps. *Shigella* is currently a major disease-causing pathogen in the developing world, and *Shigella dysenteriae* type 1 can cause deadly epidemics. The main route of transmission is through contact with the feces of infected persons, which happens when basic hygiene is inadequate, and can also contaminate food and water.

*Shigella* species are typically detected using antibodies against the specific strain as the recognition element, and fluorescence array or electrochemistry as the method of transduction. Zhao *et al.* developed a sensor (Fig. 12) based on
carbon nanotubes and chitosan/sodium alginate wherein they trapped horseradish peroxidase (HRP) labeled antibodies against *Shigella flexneri*. They used cyclic voltammetry to detect changes in the reduction peak for H$_2$O$_2$ (the substrate for HRP), which would decrease after exposure to the *Shigella* bacterium with a detection limit of 2.3 × 10$^3$ CFU per mL for chitosan and 3.1 × 10$^3$ CFU per mL for sodium alginate. They attributed the decrease in current to the immobilized bacteria...
Review Article


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of multiple analytes in each sample. Further development yielded an automated system which was able to increase the limit of detection, since the operator was only required to load the samples. They were also able to miniaturize the array biosensor, which then allowed them to build a portable biosensing platform.

**Novel methods for biosensing of biotoxins and biological species**

There have been several proposed improvements for increasing selectivity and sensitivity of biosensors (many have been mentioned previously). Setterington *et al.* proposed using immunomagnetic nanoparticles to rapidly screen food samples and concentrate food-borne pathogens. They attached antibodies to magnetic nanoparticles and exposed them to a mixed sample of bacteria, and then used a magnet to separate the target species and concentrate them for detection. Similarly, Torres-Chavolla and Alocilja developed a biosensor to detect tuberculosis based on nanoparticle-DNA capture probes for both recognition and sensing of tuberculosis-specific DNA fragments (Fig. 14).

Justin *et al.* proposed using hydrodynamic focusing in microfluidic sensors, which provides a highly controllable sensitivity to the detection.
in impedance-based biosensors by creating an adjustable, soft-walled “virtual” microchannel.\textsuperscript{166,167} McGraw \textit{et al.} proposed using electrotextiles such as a polypropylene microfiber membrane coated with polypyrrole and antibodies for highly sensitive detection of pathogens, allowing for the creation of light-weight and flexible sensors.\textsuperscript{126,168} Wojciechowski \textit{et al.} proposed using organic photodiodes to provide cheap and miniaturized optical transduction, allowing for the possibility of portable and disposable optical biosensors.\textsuperscript{169}

One type of transducer that was developed in the late 1980s but has seen very limited use recently is the light-addressable potentiometric sensor or LAPS. LAPS incorporates lightly doped silicon with a thin silicon nitride insulator that is in contact with an aqueous solution, and the electronic equipment used with it controls the potential and measures the photocurrent generated by rapidly flashing light-emitting diodes (LEDs), and is very similar to other types of pH field effect transistors. Minute changes in pH are transduced into voltage per time differentials by the instrument.\textsuperscript{170,171} Gehring \textit{et al.} used LAPS in conjunction with an immunoligand assay for the rapid detection of \textit{E. coli} with a limit of detection of $7.1 \times 10^2$ cells per mL of heat-killed or $2.5 \times 10^4$ cells per mL of live cells within 45 minutes.\textsuperscript{170} It can be applied to multi-analyte sensing and imaging, and is capable of measuring various ions and molecules.\textsuperscript{172} Xu \textit{et al.} described its potential to be used in a cell-based biosensor able to monitor extracellular action of a single living cell under different stimuli.\textsuperscript{173}

Another area that has yet to be fully exploited for biosensors development includes the use of “molecular beacons” as a biosensing recognition element. Molecular beacons (MBs) are single stranded nucleic acid probes composed of the functional areas including a stem, loop and fluorophore–quencher pair. Depending on the conformation state of the molecular beacon the fluorophore–quencher pair provides an on/off logic control, \textit{i.e.} when they are in close proximity, the energy is transmitted from the fluorophore to the quencher, and when they are far apart there is no energy transfer. The stems provide a locking mechanism to keep the beacon in a “hairpin” structure, and the loop acts as the recognition element for the target DNA. When the target DNA hybridizes the MB, there is a change in conformation of the MB and the fluorophore–quencher distance increases, giving off fluorescence (Fig. 15).

A molecular beacon array has several distinct advantages over conventional arrays. The first is that no target labeling is necessary to achieve detection. MBs also allow for real time detection/monitoring of target nucleic acids with very high sensitivity, and do not require the probe–target hybrids to be extracted from the unbound probes.\textsuperscript{174} Goel \textit{et al.} reviewed several papers which discussed using molecular beacons to detect pathogens such as \textit{E. coli}, HIV, \textit{M. tuberculosis}, Papillomavirus, \textit{Salmonella} and many more.\textsuperscript{175} Du \textit{et al.} reported using a molecular beacon biosensor to detect DNA from methicillin resistant \textit{S. aureus}.\textsuperscript{176} This has potential to be a very powerful analytical tool for real-time analysis of any pathogen, so long as there is a DNA/RNA sequence.

**Challenges and future direction**

Although there are many solutions to detecting pathogenic biological species, there is still much room for improvement. Many of the species mentioned currently require antibodies to detect, which have several disadvantages, including extensive use of expensive reagents,\textsuperscript{177} dependency on assumed antigenic motifs,\textsuperscript{156,177} limited stability under extreme environmental conditions,\textsuperscript{128,134} lack of batch consistency, high costs of development, and possible non-specificity in multiplex formats.\textsuperscript{128} Therefore one direction that biosensors should go is into development of new recognition elements to provide alternatives to detecting pathogenic species and toxins, for applications which have more stringent requirements, such as battlefield deployment. Aptamers may provide an express avenue towards an alternative to antibodies, since they function similarly, yet can be produced at a low cost and are a little more robust.\textsuperscript{134}

Another huge challenge is there are currently many diagnostic biosensors available, which have no capability for real-time monitoring. Understanding the mechanisms of how these toxins and pathogens (and similar ones) invade and destroy the human body is essential to creating new strategies to detect, discriminate, and destroy these pathogenic materials and organisms. Development of label-free biosensors for many of

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**Fig. 15** Schematic of a molecular beacon detection system. The optical imaging setup excites and detects the fluorescence (or lack thereof) from the fluorophore attached to the molecular beacon. Without the target DNA, the molecular beacon forms a hairpin structure, with the fluorophore emission being quenched. However when the target DNA hybridizes with the beacon, the change in conformation frees the fluorophore from the quencher and the emitted light can be measured. Reprinted from an Open Access Journal.\textsuperscript{174}
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Review Article

Biosensors for detection of neurotoxic compounds

Neurotoxic compounds can be separated into one of two types: organophosphorus (OP) and non-organophosphorus. Organophosphorus compounds compose the largest class of urban and rural pesticides, with 100 different compounds currently commercially used, and over 1500 different OP neurotoxins have been synthesized in the last century. They also include many chemical warfare agents, notably Tabun (GA), Sarin (GB), Soman (GD), Cyclosarin (GF), and VX, all of which are or were a part of the United States domestic inventory. While the use of these agents was banned in 1997 by Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on their Destruction, the deliberate release of Sarin has occurred in Japanese cities in 1994 and 1995 by terrorists, demonstrating the concern that these weapons could be used by rogue nations or terrorist groups. The distribution of these agents is typically considered to occur by single-source airborne release from an intentional terrorist attack, and would result in an acute exposure to these agents, through inhalation or dermal sorption. However, there is also concern for environmental monitoring due to the means of storage of these agents, and also for non-agent neurotoxins (pesticides/insecticides) of food and water, leading to exposure through ingestion. Fig. 16 depicts the chemical structures of several different types of organophosphate neurotoxins. The G-agents are all viscous liquids with varying volatility and faint odors, while VX is an amber-colored liquid with a high vapor density and is odorless. Of the G-series, Sarin is considered to be the greatest vapor threat, while VX was formulated to have a low volatility, thereby increasing the persistence of the toxin wherever it is spread. However VX vapors, if allowed to accumulate, are more potent than any of the G-series agents.

While OP and non-OP neurotoxins are different, their mechanism of action is the same. Acetylcholine (ACh) is a chemical neurotransmitter found at neural synapses and neuromuscular junctions. Acetylcholinesterase (AChE) is a kinetic enzyme which hydrolyzes and regulates the amount acetylcholine present. Neurotoxic compounds bind irreversibly to the active site of AChE, thereby inhibiting its ability to hydrolyze acetylcholine, which accumulates in the synapses. Depending on the route of exposure and the degree of exposure, the symptoms of poisoning include excessive bronchial, salivary, ocular, and intestinal secretions, sweating, miosis, bronchospasm, intestinal hypermotility, bradycardia, muscle fasciculations, twitching weakness, paralysis, loss of consciousness, convulsions, depression of the central respiratory drive, and death through asphyxiation. Treatment of neurotoxin poisoning has varying success depending on the amount of neurotoxin and the amount of time between exposure and treatment. AChE can be reactivated within a certain time period depending on the agent, using pralidoximes and atropine, allowing the victim to recover. Discovery and development of the phosphotriesterase enzymes have led to their use in biosensing technologies, including organophosphate hydrolase, organophosphate acid anhydrase, human paraoxonase, and squid diisopropyl fluorophosphatase. This section of the review will cover the various biosensing strategies developed over the past 15 years for the detection and decontamination of organophosphorus and non-organophosphorus compounds, focusing mainly on the most common enzymatic means.

Acetylcholinesterase and its variants

Acetylcholinesterase (AChE) and other cholinesterase enzymes traditionally have been used as the bio-recognition element for detection of neurotoxic compounds through inhibition assays based on potentiometric, amperometric and piezoelectric transducers. This provides very sensitive detection of pesticides even in the nanomolar concentration range. The most common method for detecting pesticides is through the use of electrochemical amperometric sensors, with recent developments being focused on incorporating the enzyme with novel nanomaterials to increase its sensitivity. Acetylthiocholine (ATCh) is a synthesized derivative of acetylcholine, and is susceptible to hydrolysis by acetylcholine esterase (AChE). The product thiocholine (TCh) is electroactive, which allows direct amperometric study of AChE without using a mediator and choline oxidase (ChOx). This simplifies the system and allows for rapid determination of enzyme activity. Below is the reaction scheme for the hydrolysis of ATCh by AChE.

\[
\text{ATCh} + \text{H}_2\text{O} \xrightarrow{\text{AChE}} \text{TCh} + \text{acetic acid}
\]

The main drawback of these methods is that the enzyme activity is destroyed and either the sensor has to be discarded or the enzyme replaced. Additionally, different pesticides (both organophosphates and carbamates) deactivate the enzyme to...
different extents, and calibration of the sensor to an unknown sample would be almost impossible. Therefore, Bachmann et al. have developed and used different variants of AChE to detect pesticides using artificial neural networks and discriminate between paraoxon and carbofuran. Using four different types of AChE, they built a disposable multisensor and processed their data using an artificial neural network algorithm, with the ability to discriminate between binary samples of pesticides.

Many different groups have attempted over the years to genetically engineer AChE to become more sensitive to low concentrations of pesticide, as well as improve the selectivity of AChE by increasing its sensitivity to specific organophosphates and carbamates. Standard electric eel AChE is not sensitive enough for trace analysis of pesticides. Schulze et al. engineered an AChE enzyme from Nippostrongylus brasiliensis AChE B through introducing 10 single and 4 double mutations into the AChE peptide chain. This led to an increase in the sensitivity of the enzyme to 10 of the 11 pesticides they tested, and they were able to detect concentrations of 11 of the 14 most important organophosphates and carbamates below 10 μg kg⁻¹. The three mutants they created also significantly increased the storage stability at high temperatures. There was no loss of sensitivity of the mutant enzyme immobilized onto screen printed electrodes after 17 months in storage at room temperature.

Some pesticides, such as organophosphorothionates, have very minimal inhibitory effect on AChE while in their native form. However, the AChE inhibition is significantly increased when they are oxidized by cytochrome P-450 monooxygenase (found in living organisms). They are not detectable by AChE inhibition in commonly found concentrations. Therefore Roepcke et al. described a new enzymatic method for the direct detection of phosphorothionates in food samples without extraction by using chloroperoxidase in conjunction with tert-buty1 hydroperoxide and two halides as a pretreatment step. This enzyme converts the pesticides into their oxon form, which they were able to detect with a standard AChE inhibition assay.

Liao et al. developed a liquid crystal biosensor based on enzymatic growth of gold nanoparticles (Au NPs) in the presence of acetylcholine (depicted schematically in Fig. 17). AChE was used to hydrolyze acetylthiocholine (ATCh), forming thiocarboxylic acid which reduces AuCl₄⁻ to Au NPs without the need for Au nanoseeds. The large Au NPs attenuate the optical signal of the liquid crystal biosensor, disrupting the orientation of the liquid crystals. However, when hydrolysis is mediated by AChE in the presence of acetylcholine or organophosphate pesticides (any AChE inhibitor would work), the ATCh hydrolysis would be inhibited, reducing the catalytic growth of the Au NPs and reducing the response of the liquid crystal orientation. They could detect OP neurotoxins at 0.3 nmol L⁻¹ and acetylcholine at 15 μmol L⁻¹.

Other inhibition-based enzymes

In addition to acetylcholinesterase and its derivatives, there are many other enzymes that have been used in inhibition based biosensing systems. There have been several reviews covering these different enzymes and the strategies in which they are being used. Luque de Castro and Herrera highlighted in their review the challenges of non-selectivity of inhibition-based enzyme sensors, and also the use of continuous and discontinuous (flow injection analysis vs. batch analysis) approaches in inhibition-based biosensing. Patel reviewed the availability of inhibition based biosensors for the detection of a range of chemical compounds and food-borne contaminants. Amine also reviewed a number of biosensors based on enzyme inhibition to determine pollutants and toxic compounds.

![Fig. 17](http://www.chemsocrev.org/content/11/1/45/F2.large.jpg)

The left schematic shows the assembly of the liquid crystal biosensor for detection of pesticides: (a) plain glass, (b) self-assembled TEA/DMOAP film, (c) immobilization of AChE, and (d) gold nanoparticles obtained through biocatalytic growth. The right figure shows a series of optical images under the cross polarizer of the liquid crystal biosensor when exposed to (a) 0, (b) 0.3, (c) 3, (d) 30, (e) 300, and (f) 3000 nM pesticide. Reprinted with permission from Liao et al., Anal. Chem., 2011, 84, 45–49. Copyright © 2011, American Chemical Society.
Cholinesterases are clearly the most widely used recognition elements in inhibition-based neurotoxin biosensing, however there are many other enzymes that have been used in similar approaches including: horseradish peroxidase, tyrosinase, urease, glucose oxidase, invertase, ascorbic acid oxidase, carboxyl esterase, catalase, etc.\textsuperscript{198}

Organophosphate hydrolase

Traditionally, pesticides and chemical warfare agents have been detected using a variety of methods including immunoassays, which offer advantages such as decreased sample processing, high specificity, and the ability to analyze a large number of samples. However, these are plagued by a number of limitations including the use of expensive, non-renewable materials, complicated ligand interactions, and advanced knowledge of the expected analytes. The most commonly used method involves the use of cholinesterase enzymes utilizing thiocholine and the inhibition of the cholinesterase by the analyte. This technique has the advantage of high sensitivity (down to parts per billion of pesticide), but lacks specificity, as there are many non-pesticide materials that can affect and even inhibit these enzymes, leading to false positive readings.\textsuperscript{178,199}

Rainina et al., from Wild’s group at Texas A&M, described in 1996 a special enzyme developed from recombinant E. coli for the direct detection of organophosphorous neurotoxins. This enzyme belongs to a group of enzymes known as the phosphotriesterases, and was the first as such described for use as a biosensing recognition element. This enzyme, known as organophosphate hydrolase (OPH), cleaves the P–O, P–F, P–S, or P–CN bonds and releases two protons, and the protons release corresponds to the quantity of organophosphate hydrolyzed. They developed a biosensor based on potentiometry and pH with a linear detection range for paraoxon of 0.25–250 ppm (0.001–1.0 mM).\textsuperscript{178} Richins et al. developed a similar biocatalyst based on the work pioneered by James Wild, in which they anchored OPH to the surface of E. coli through manipulating the cell to express the proteins on the surface to degrade OP neurotoxins.\textsuperscript{200} Genetic modification of the enzyme by Wild’s group led to multiple mutants which improved the ability of the enzyme to hydrolyze specific agents, such as Soman, acephate, or VX adding the possibility to selectively destroy (and detect) pesticides and chemical warfare agents.\textsuperscript{201} Russell et al. developed poly(ethylene glycol) hydrogel encapsulated seminaphthofluorescein (SNAFL)–OPH conjugates for use as a self-reporting biorecognition element. SNAFL is a pH-sensitive dye in which the emission spectrum changes at a wavelength of 550 nm in response to pH. With spectrofluorimetry, they were able to readily detect paraoxon at concentrations as low as 8 \times 10^{-7} \text{M}, and the hydrogel encapsulated enzyme conjugates remained stable against denaturing, leaching, and photobleaching when stored under ambient conditions.\textsuperscript{201}

Several different transduction strategies have been incorporated using organophosphate hydrolase (OPH) as the biorecognition element including pH electrodes,\textsuperscript{178} fluoride ion-sensitive electrodes,\textsuperscript{202} pH responsive dyes,\textsuperscript{201} pH sensitive field effect transistors (pHFETs),\textsuperscript{203,204} optical techniques,\textsuperscript{205–212} and electrochemical techniques.\textsuperscript{213–215} Simonian et al. immobilized OPH to the gate of one pHFET while a second unmodified pHFET was kept as a reference (Fig. 18). Changes in bulk pH were measured as changes in the common source voltage, while local pH changes at the modified FET gate were amplified and the voltage was measured.\textsuperscript{204}

Simonian et al. also demonstrated the use of Au nanoparticles with fluorophore decoys, exploiting the changes in fluorescent intensity based on the distance between the two (Fig. 19). OPH was immobilized onto Au nanoparticles and a decoy fluorophore was incubated with the nanoparticle–enzyme conjugate. When exposed to the substrate, the affinity of the enzyme for the substrate caused it to release the decoy, thereby reducing the intensity of fluorescence, which was correlated to the concentration of the substrate.\textsuperscript{205}

Ji et al. immobilized OPH onto quantum dots and used circular dichroism spectroscopy to observe changes in the secondary
structure of the enzyme when exposed to organophosphorus compounds. Then they used photoluminescence (PL) spectroscopy to show that the PL intensity of the bioconjugates was quenched when in the presence of paraaxon, and correlated the overall quenching percentage to the amount of paraaxon, with a detection limit around $10^{-8}$ M. Rogers et al. developed a fluorescence based assay for detecting a common product from all substrates to OPH, which provides the advantage of being able to detect any substrate using the same detection mechanism. They immobilized fluorescein isothiocyanate to the enzyme, a pH dependent fluorescent reporter, which monitored the change in pH when exposed to the substrate. Simonian’s group has demonstrated their ability to immobilize OPH onto carbon nanotubes and directly detect the product of the hydrolysis reaction using amperometric electrochemical sensors.

Organophosphate acid anhydrase

Organophosphate acid anhydrolase (OPAA) is a prolidase enzyme and genes encoding this enzyme were cloned and sequenced from two species of Alteromonas. It has the capability, similarly to OPH, to hydrolyze a wide range of organophosphates (OPs) with P–F, P–O, P–CN, and P–S bonds. It has preferential activity toward P–F bonds and minimal activity towards P–O or P–S bonds, and as such is the enzyme of choice for destroying fluorine-containing organophosphates, such as Sarin and Soman. Diisopropylfluorophosphatase (DFP) is a phosphonofluoridate neurotoxin similar in structure to Sarin and Soman with a significantly reduced toxicity, which makes it an ideal analogue for chemical warfare agents. Simonian et al. used OPAA in conjunction with a pH electrode and a pH sensitive field effect transistor (in a similar configuration as above) to build a biosensor to detect DFP at concentrations as low as 25 µM and 20 µM, respectively, with no response to P–O and P–S bond containing OP pesticides paraaxon and demeton-S.

Zheng et al. used fluorescence and other spectroscopic techniques to analyze the hydrolysis of DFP catalyzed by OPAA at the air–water interface, demonstrating the ability of OPAA to be used as a recognition element in biosensors.

Multi-enzyme strategies

The use of multiple enzymes in any sensor has a great deal of attractiveness. It can provide versatility for detection of many analytes or for complex processes where more than one type of enzyme may be needed to “cascade” a chemical into a detectable analyte. A classic example of this would be glucose oxidase paired with horseradish peroxidase, eliminating the need for a redox mediator to detect glucose. It can open up the possibility for multiplexed assays of different chemical analytes. Simonian et al. described the use of acetylcholinesterase (AChE) in conjunction with organophosphate hydrolase (OPH) to screen samples for organophosphate (OP) neurotoxins and carbamates. They found that mutual interactions of various neurotoxins with AChE were not additive, but dependent on the type of chemicals, and they separated the effects of the inhibitors using a joint action of AChE and OPH. They demonstrated that although it was impossible to have an adequate understanding of the composition of an unknown sample containing multiple inhibitors, they were able to eliminate OP neurotoxins from the samples, and detect the non-OP neurotoxins to their true concentration with AChE inhibition. They also demonstrated the ability of OPH with organophosphate acid anhydrase (OPAA) to discriminate between different OP neurotoxins based on their unique ability to catalyze different OPs with varying kinetic rates (discriminating between P–S and P–O versus P–F bonds). With various other mutant enzymes having different kinetic abilities toward the different types of bonds, it may be possible to create a multiplex array, wherein each neurotoxin could be “fingerprinted” with respect to its catalytic response to different enzymes.
Mantha et al. demonstrated the capability of carbon nanotubes as a scaffolding support for OPH and DNA to build a layer-by-layer (LbL) assembled OP biosensor. The concept of LbL assembly involves oppositely charged species used to build a nanostructure of alternating monolayers of the charged species through electrostatic interactions. Not only does the catalytic response of the sensor increase with each additional layer of enzyme, but it is also easily renewed by adsorption to replace the lost layer. This novel technique has been developed for use in many different biosensors and could be exploited to build multi-enzyme structures. Kirsch et al. demonstrated this using OPH and glucose oxidase to detect paraoxon and glucose with the same sensor (Fig. 20).

Challenges and future outlook

Currently there are many biosensors able to detect organophosphates and carbamate pesticides, most of them based on acetylcholinesterase inhibition. However, there are not many biosensors able to detect and discriminate between different types (OP and non-OP) with any sort of accuracy, or that are able to discriminate between different OPs in multicomponent samples. The multi-enzyme capability will go a long way to solving these problems. Another challenge for enzymatic catalysis of pesticides is dealing with long term stability without compromising activity. There are currently no ways of controlling, manipulating, or definitively verifying the conformation, orientation, or viability of an enzyme immobilized to a surface. The current way to verify activity is through response to a substrate, but this cannot describe what is going on at the interface between the solid support and the enzyme, or how the enzyme has changed in its transition from solution to immobilization.

Medical biosensors

Immunology and infectious disease diagnosis

Human immunodeficiency virus (HIV). There are 1.2 million HIV infected people in the US, 20% of whom are unaware of their infection status. The diagnosis of HIV-1 infection typically relies on the detection of HIV-1 RNA, capsid antigen p24, or antibodies. Current HIV-1 diagnostic technologies such as dipsticks, enzyme immunoassays (ELISA) and OraQuick HIV test kits rely on the detection of HIV antibodies in serum. Given that seroconversion does not occur until 3–8 weeks after HIV-1 infection, alternative strategies for early stage HIV diagnosis are being developed. These strategies rely on the detection of antigens/proteins and nucleic acids and represent the two major thrusts in the development of biosensors for HIV detection.

One approach for early detection is to target p24, an HIV capsid protein that appears before detectable antibodies, due to a burst of virus replication upon infection. Although genetic variability is a notable hurdle for direct HIV-1 detection, HIV-1 p24 is one of the most conserved proteins among HIV-1 variants, making it a consistent and universal marker for detection. It has been demonstrated that p24 antigen testing assays have great value in early HIV infection diagnosis, blood screening, and monitoring of antiviral therapy. Furthermore, specificity of the p24 antigen test for HIV detection is approximately 99.9%. The standard sensing method for p24 antigen has traditionally been ELISA based. However, despite the ELISA test’s specificity for p24, it lacks sufficient sensitivity for HIV diagnosis. To overcome this problem, several strategies have attempted to amplify the signal, most of which involve the use of nanoparticles. Kim et al. labeled anti-p24 antibody immobilized gold nanoparticles with barcode DNA and detected p24 in a sandwich format. Following target binding, barcode DNAs were released from nanoparticles and quantified. By labeling single gold nanoparticles with multiple barcode DNAs, the binding event signal was amplified, resulting in 99% sensitivity, far superior to the conventional ELISA test for detection (20.5%). Similarly, Tang and Hewlett developed an ultrasensitive assay using this bio-barcode amplification (BCA) concept. Instead of gold nanoparticles carrying the DNA barcode for subsequent microarray or silver staining, highly fluorescent europium (Eu) nanoparticles were utilized for direct fluorescence measurements. This alternative assay had an approximate 100–150-fold detection limit enhancement compared to ELISA (10–15 pg mL⁻¹). In addition to optical detection, nanoparticles have also been incorporated into electrochemical sensors to improve sensitivity. Teeparuksapun et al. designed a capacitive sensor by chemically adsorbing mAb-coupled gold nanoparticles onto electrode surfaces. High sensitivity in this assay was attributed to two factors: increased mAb loading density due to the large surface area of nanoparticles, as well as a significant decrease in interfacial resistance owing to assembly of nanoparticles on a polytyramine.

In a similar manner, Gan et al. constructed an amperometric sensor by labeling gold nanoparticles with high density HRP to induce enhanced reductive current, achieving a detection limit of 0.5 pg mL⁻¹.

Another category of sensors for early diagnosis detects the genetic material of HIV. The most conventional approach is to lyse the virus, extract the RNA, amplify via PCR and read the copy number. Due to both sensitivity and specificity, this method serves as the gold standard for HIV diagnosis, though it requires professional operation by skilled personnel. In a more automated approach, Lee and colleagues have integrated these protocols into a portable device for point-of-care purposes. Real-time detection could be achieved in 95 minutes, far faster than the conventional method of gel electrophoresis (3–4 hours). With the successful integration of PCR, detection of a single infected cell could be realized. Demirci and coworkers took an alternative approach to virus lysate sensing, in which the presence of HIV was detected by identifying the impedance spectrum change induced by all released ions and charged molecules in virus nano-lyte. In this label-free electrical sensing format, magnetic beads were functionalized with anti-gp120 antibodies to selectively capture HIV-1 virus. The virus was then lysed and the released ions and charged molecules resulted in a detectable change in the impedance spectrum (Fig. 21). By obviating the amplification...
step (typically required for nucleic acid tests (NAT)).

Although the identification of HIV-1 by protein or nucleic acid markers is specific, it cannot provide information regarding the metabolic state of the virus, and these types of sensors are therefore less suitable for drug screening or evaluation. HIV-1 protease (HIV-1 PR) is an essential enzyme that is required for the proper assembly and maturation of the infectious virions, by cleaving viral polyprotein precursors into a mature form. Hence, the HIV-1 PR is a promising target for effective HIV inhibitor development. An optical method utilizing SPR has been applied to the screening of compounds that interact with HIV-1 PR. In this method, probe HIV-1 PR was immobilized onto the sensor surface, and the binding of various potential inhibitors for HIV-1 PR was monitored and distinguished by their interaction kinetics. Similarly, an electrochemical method with ferrocenyl (Fc)-conjugated pepstatin immobilized on gold was applied to sense the binding of HIV-1 PR with the substrate. The more HIV-1 PR bound on pepstatin, the more Fc redox center encapsulated into the complex, making the oxidation of the Fc group more difficult. This process was indicated by a significant increase in formal potential and decrease in current. The presence of HIV-1 PR inhibitors such as cytochalasin A decreased the observed peak potential, with signal suppression proportional to cytochalasin A concentration, demonstrating the sensor’s potential use in drug screening. A low-cost and label-free impedimetric method employing magnetic beads was also used for HIV-1 PR detection. In this approach, a specific HIV-1 protease substrate was conjugated to both magnetic beads and a gold surface. Upon cleavage of the probe peptide by HIV-1 PR, the physical link between the beads and the sensing surface was destroyed, resulting in increased charge transfer resistance due to the dissociation of beads from the gold sensing surface. As low as 10 pg mL\(^{-1}\) HIV-1 protease was detected using this method and the inhibitory activity of the anti-HIV drug saquinavir mesylate was attested.

Another indicator with great clinical significance for HIV/AIDS patients is the CD4 T cell number. CD4 T cells are specifically attacked and killed by the virus leading to compromised immunity and AIDS. In fact, diagnosis of AIDS is based on enumeration of CD4 T-cells, 200 or fewer CD4 cells per μL of blood being the indicator of AIDS. The effectiveness of anti-retroviral therapy is monitored by evaluating CD4 T-cell numbers, which are supposed to increase as the viral load decreases. Flow cytometry is currently the most common technique for quantifying CD4\(^{+}\) cells; however, this approach uses expensive instrumentation and requires a skilled operator. The biosensing community is producing simpler and more robust solutions to the challenge of T-cell quantification. For example, novel and portable imaging systems were established for CD4 T cell counting in resource-poor settings. Compact electrical sensing formats have also been developed, which typically exploit resistance change and generated impedance pulse. Mechanical sensors that detect the mass increase due to the CD4\(^{+}\) cell adhesion by monitoring the variation in resonant frequency offer another simple platform. Recently, a more stable and cost-efficient selective cell capture surface incorporating an anti-CD4 aptamer was created, and high cell capture specificity was established.

**Tuberculosis.** Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis* (MTB) that is a cause of mortality and morbidity, particularly in the developing world. Similar to HIV diagnosis, techniques for sensing TB presence can be classified into two categories: indirect tests based on MTB-specific immune responses, and direct tests based on MTB nucleic acids or bacillus.

The most widely used indirect clinical method for TB detection is the tuberculin skin test (TST), which measures the body’s immune response to injected purified protein derivative (PPD) tuberculin. This test is robust, inexpensive and well entrenched, first becoming available in the late 19th century. However, it frequently results in false positive and false negative results, and also requires a return visit for a patient. Interferon gamma (IFN-γ) release assays (IGRAs) are emerging as an alternative to TB skin tests. These assays rely on memory T-cells that are stored in the human immune system after infections and that may be queried through stimulation with disease specific antigens. In the case of TB testing, immune cells are exposed to TB-specific antigens and respond by production of inflammatory cytokine IFN-γ. Hence, if the patient’s immune system has been previously challenged by MTB, T cells will respond to the highly purified and specific MTB antigens (ESAT6, CFP10, TB7.7) by producing higher levels of IFN-γ. To reduce sample volume requirements, microfluidic systems for evaluating IFN-γ secretion have been developed. MTB-specific antibodies present in serum have been applied as an alternative sensing target. Nagel et al. developed three optical sensor models by immobilizing MTB lipoprotein antigen probes onto surfaces and monitored antibody binding using reflectometric interference spectroscopy.
In addition to indirect strategies mentioned above, sensors based on direct detection of MTB nucleic acids or cells have also been established, including a PCR-free MTB genomic DNA sensor developed by Thiruppathiraja et al. In this format, MTB DNA fragments hybridize with capture probes on both electrodes and gold nanoparticles co-modified with enzyme alkaline phosphatase (ALP). The recognition event triggers the generation of an electroactive species of para-nitrophenol and induces a detectable electrical signal.\(^{265}\) However, nucleic acid recognition requires a genome DNA extraction process, making it inappropriate for point-of-care applications. The direct detection of MTB cells is another current trend. Novel and miniaturized imaging techniques have been involved in the recognition of MTB from clinical samples, including an on-chip nuclear magnetic resonance (NMR) biosensor\(^{266}\) and cell phone based fluorescence microscopy sensor (Fig. 22). The miniaturized diagnostic magnetic resonance (DMR) system monitored the change in spin–spin relaxation time due to the presence of targets and could detect as few as 20 bacteria of MTB per mL of sputum samples without processing.\(^{266,267}\) The progress in mobile phone fluorescence microscopy has made the optical detection of TB bacilli from sputum smears more flexible and more effective in serving point-of-care purposes.\(^{268}\)

**Immunology research.** Cytokines are small proteins secreted by immune cells and serve as signals for immune cell migration, differentiation and proliferation. Cytokine signaling is complicated in that the same cytokine may originate from different immune cell types which are frequently defined by the profile of cytokines they release. Thus, it is desirable to resolve the heterogeneity among leukocytes and identify the unique subset most closely associated with a certain immune response. In other words, there is strong interest in connecting cytokine profiles of immune cells to infectious diseases such as HIV and TB.\(^{269-271}\)

Current methods for the detection of cytokines include real-time reverse-transcriptase PCR, intracellular cytokine staining (ICS) coupled with flow cytometry,\(^{272}\) and (ELISA) or immunospot (ELISPOT) assays.\(^{273}\) In recent years, more high-throughput methods aiming at characterizing cytokine profiles for individual groups of cells, or even single cells have been established. A single-cell secretome analysis approach was developed by Love and co-workers, and utilized a microengraving platform in which arrays of microwells fabricated in PDMS by soft lithography housed single cells.\(^{274}\) Glass slides bearing multiple capture antibodies against cytokines were subsequently pressed against the PDMS mold to enclose the cell in a 100 pL volume. After incubation, the slides were removed and interrogated by fluorescence-labeled secondary antibodies to calculate cytokines secreted from each cell. Cytokines including interleukin-6 (IL-6), interleukin-17 (IL-17), IFN-\(\gamma\) and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) secreted from human peripheral blood mononuclear cells (PBMCs) were quantified. In a similar manner, antigen specific antibodies from B cells\(^{275}\) and cytokine/antibody production from hematopoietic cells\(^{276}\) were concurrently identified.

The Heath group has reported a different approach to perform multiplexed cytokine detection from lipopolysaccharide (LPS)-stimulated macrophages by using a single-cell barcode chip (SCBC).\(^{277}\) The chip included microchambers containing a dozen antibody “barcodes” specific for several cytokines (Fig. 23).
To achieve consistent loading, antibodies were incorporated into fluidic channels using a DNA-encoded antibody library (DEAL) method. Cells were then loaded into the microfluidic channels, and pneumatic actuation segmented the channels into 3 nL single cell laden microchambers to restrict cytokine diffusion. Barcodes were then read by a sandwich assay applying labeled secondary antibodies. With this approach, the polycytokine secretion from LPS-stimulated macrophages and antigen stimulated cytotoxic T lymphocytes (CTLs) from metastatic melanoma donors were evaluated. Similarly, Lu et al. developed a single cell analysis platform by combining sub-nanoliter chambers with antibody barcodes. In contrast to the Heath group’s SCBC, this antibody barcode was fabricated by a simpler, microchannel-guided flow pattern technique, and performed protein secretomic analysis for cell lines and single tumor cells. Notably, this platform has correlated the secretion profiles with cellular migration activity, linking the protein secretomic signature with physical behaviors of the cell.

In addition to planar microarrays for cytokine profiling, antibody-carrying permeable hydrogels have also been employed due to improved loading capacity and binding kinetics as well as reduced non-specific binding of proteins. Doyle and colleagues developed hydrogel microparticles that recognize IL-2, IL-4 and TNF-α by cytokine-specific conjugated antibodies, with antibody category information encoded by graph barcode patterns on the carrying gel. Combined with a microfluidic scanning system, this approach achieved a detection limit of 1 pg mL⁻¹. A new trend in immune sensor development is the analysis of dynamic cytokine release. One strategy is to sample extracellular space of a cell-enclosed chamber at regular time points. Alternative approaches include immobilizing the antibody onto surfaces and monitoring binding via surface plasmon resonance (SPR). The application of aptamers in biosensors has provided an alternative platform for real-time cytokine monitoring, exhibited by recent developments of optical and electrochemical aptasensors. In both cases, the cytokine-aptamer binding event was reported via an aptamer structure change, resulting in quencher-carrying complementary strand release and continuous fluorescence recovery, or attenuated electron transfer efficiency and depression of current signal.

Cancer diagnostics and monitoring

Cancer is responsible for 1 in 4 deaths in the United States, with higher fatality than heart disease in people younger than 85 years of age. Survival is highly dependent on early detection (note decreased mortality in breast and colorectal cancer due to scheduled screening), so the development of cancer specific biomarkers and the tools to detect them is a vital area of research. Conventional screening typically involves morphological or histological analysis of cells and individual tumor associated antigens. High levels of individual markers are associated with certain cancers, such as prostate specific antigen (PSA) and CA125 for prostate and ovarian cancers, respectively. Despite their sensitivity, tests for these markers often lack specificity: both PSA and CA125 concentrations

Fig. 23  Layout of the single-cell barcode chip (SCBC) system. (a and b) Optical image of an SCBC system and cells isolated in microchambers; (c) scheme of cytokine detection by antibody barcode array; (d) scanned antibody barcode array; (e) calibration curve for recombinant cytokine proteins. Reprinted with permission from Ma et al., Nat. Med., 2011, 17, 738–743. Copyright © 2011, Nature Publishing Group.
can be strongly affected by benign diseases.\textsuperscript{293} Hence, much of the difficulty in developing tools for the acquisition of meaningful data regarding cancer biomarkers arises from the complexity of clinical diagnosis. There are hundreds of distinct human cancers, with many more associated deregulated genes and signaling pathways. One study compiled a list of 1261 proteins believed to have altered expression levels in human cancers.\textsuperscript{294} Accurate cancer diagnosis, prognosis, and drug response monitoring will therefore rely heavily on multi-target analysis. Biosensors offer a promising platform for rapidly accomplishing this in real time, and have recently served applications from molecular signature profiling, to dynamic functional analysis, to tumor cell enrichment and purification.

**Genomic signature profiling.** Microarray technologies have played an important role in identifying oncogenes and tumor suppressor genes, as well as tumor associated proteins.\textsuperscript{295} DNA microarrays, which hybridize with target DNA and RNA sequences and employ a variety of transduction techniques, can interrogate an entire tumor cell genome concurrently.\textsuperscript{290} These techniques have been utilized to profile molecular signatures for tumor classification. In a prominent example, Golub and colleagues employed Affymetrix Genechip\textsuperscript{44} technology (a DNA microarray assay involving fluorescent labeling and off-chip DNA pre-amplification) to distinguish acute myeloid leukemia from acute lymphoblastic leukemia, without prior knowledge of these classes.\textsuperscript{296} Other groups have narrowed the focus to identify aberrant expression of gene subsets in tissues such as ovarian carcinomas, melanoma, and colorectal, prostate, and oral cancers.\textsuperscript{297} Despite these successes, genomic profiling can become very complex, requiring expensive equipment and experienced personnel. For this reason, significant effort has been directed toward the development of protein and small molecule biomarker analytical tools.\textsuperscript{295}

**Detecting cancer biomarkers.** As molecular profiling continues to reveal new cancer biomarkers, creative new tools are being developed to detect and quantify them from bodily fluids in a minimally invasive fashion. Vascular endothelial growth factor (VEGF) is a signaling protein that promotes angiogenesis by activating VEGF-receptor kinases in endothelial cells, and its overexpression is related to tumor growth and metastasis.\textsuperscript{298} VEGF is therefore a common target for both diagnosis and therapy for multiple cancers. Several VEGF sensing molecules have been developed, including antibodies\textsuperscript{299} and peptides,\textsuperscript{300} however aptasensors have become increasingly popular. Liet al. developed an anti-VEGF aptamer microarray, combined with an antibody sandwich type assay for SPR based detection.\textsuperscript{301} Sensitivity at 1 pM concentration was achieved by enzymatic amplification: after biotinylated anti-VEGF antibody was added to the immobilized aptamer-analyte complex, horseradish peroxidase (HRP) conjugated anti-biotin antibody further enhanced the SPR signal by causing localized precipitation of HRP substrate tetramethylbenzidine (TMB). More recently, another VEGF aptasensor was developed based on the inactivation of surface plasmon enhancement of fluorescent probes attached to gold nanoparticle bound aptamers. This single step sensing method measures target recognition directly at clinically relevant sensitivity and a broad linear range of detection (1.25 pm to 1.25 \(\mu\)M).\textsuperscript{302} Anti-VEGF aptamers have also been incorporated onto transducer surfaces for field effect transistor (FET) biosensors, namely because of their sub-Debye length size in the conducting channels, which is the required distance for electrical detection of mobile carriers.\textsuperscript{303,304} Lee et al. successfully detected VEGF concentrations as low as 1.04 pM with an aptamer-modified Si nanowire FET.\textsuperscript{305} An FET was later developed based on carboxylated polypyrrole nanotubes (CPNTs) to detect VEGF down to 400 fM concentration.\textsuperscript{304} Fig. 24 schematically depicts an aptasensor for detecting VEGF.

HER2 belongs to a family of human epidermal growth factor receptors (HER) responsible for normal cellular growth and differentiation. This transmembrane protein is rarely expressed on healthy cell surfaces, however its overexpression leads to uncontrolled malignant growth.\textsuperscript{306} High levels of HER2 can be found in the serum of breast cancer patients, and accordingly, it is a strong prognostic indicator and a target for FDA licensed breast cancer therapy (Herceptin).\textsuperscript{307} The standard HER2 detection method is ELISA based, however these tests are sensitivity limited and time consuming. Alternatively, researchers have recently developed piezoelectric microcantilever sensors (PEMS) for label-free electrical detection of HER2 at clinically relevant concentrations (ng mL\(^{-1}\)).\textsuperscript{293,308} Fan and co-workers recently adopted a ring resonator technology combined with microfluidics for the label-free optical detection of HER2. This opto-fluidic ring resonator (OFRR) biosensor measures a change in the relative refractive index in a thin mAB coated capillary upon binding with target HER2 molecules in a rapid (30 min) fashion.\textsuperscript{309}

Another interesting trend is the application of biosensors for the dynamic monitoring of protein kinases. This family of enzymes is responsible for the orchestration of numerous signaling cascades in response to external cues. Hyper-activation of protein kinases by abnormal protein expression is a cause of uncontrolled cell growth and proliferation, and as a result, kinases have become a target of interest for inhibitory drug development.\textsuperscript{310,311} While conventional studies involve radioactive or antigenic end point assays, new fluorescence biosensing techniques have facilitated the study of kinase activity in real-time in living cells.\textsuperscript{312} Tunceroglu et al. developed a genetically encoded fluorescence resonance energy transfer (FRET) based assay to monitor inhibition of Bcr-Abl kinase by small molecules in vivo, and correlated its activity with disease state in chronic myelogenous leukemia (CML).\textsuperscript{313} Fluorescent peptide biosensors have also been employed for the monitoring of drug resistant CML,\textsuperscript{314} in addition to probing kinase activity in cell lysates for high throughput drug screening.\textsuperscript{315}

**Circulating tumor cells.** Circulating tumor cells (CTCs) offer an alternative approach for detecting cancer. CTCs are the origin of metastases, as they are shed from primary tumors and can possess the ability to invade distant organs. Although extremely sparse (as few as 1 per 10\(^6\) haematologic cells),\textsuperscript{316} CTC enumeration offers a convenient and non-invasive means for detection and monitoring of several cancers. Early techniques for detection in whole blood included the commercially
available Veridex Cell Search system, which binds antibody coated magnetic nanoparticles to CTCs in whole blood, followed by a sandwich type immunofluorescence assay. More recently, several groups have exploited microfluidics for less time and labor intensive purification of CTCs. One of the simplest methods is based on microfiltration of relatively large CTCs. Zheng et al. used this method for CTC cell enrichment (90% recovery), followed by on-chip cell lysis with embedded electrodes for PCR based genomic analysis. Other groups have employed aptamers and anti-EpCAM mAb coated micropost arrays fabricated in microfluidic chips to isolate CTCs from whole blood in a user-friendly fashion (Fig. 25). Stott et al. incorporated a herringbone chip microfluidic design with this method to maximize interactions between cells and Ab coated surfaces. Finally, stimulated release of captured CTCs was recently achieved with responsive polymer brushes. In this approach, Ab-functionalized poly(N-isopropylacrylamide) (PIPAAm) polymer brushes were immobilized on a silicon nanowire substrate for the CTC capture. Upon temperature decrease, polymer brushes underwent conformational change, internalizing capture antibodies and releasing cells.

Applications in neuroscience

Neurotransmitters. Neurons relay chemical signals across synapses via neurotransmitters. These molecules are grouped into pre-synaptic vesicles, released into the synaptic cleft and subsequently loaded onto the post-synaptic membrane to induce action potentials. Elucidating the role of various neurotransmitters relies heavily on the detection platform, and several sensing devices have accordingly been developed.
One important example is the sensing of dopamine, a neurotransmitter that is linked to multiple neurological disorders including Huntington’s and Parkinson’s diseases. Dopamine serves as a potential marker for molecular Parkinson’s diagnosis, in light of the fact that the disease is characterized by severe depletion of dopamine.322,323 Most early sensors were based on the principle that dopamine can be easily oxidized and detected by conventional electrode324,325 or ion sensors.326 Though simple and effective, some limitations are associated with this method: ascorbic acid, the major competitor of dopamine for oxidation detection, has several orders of magnitude higher concentration than dopamine in the extracellular fluid, and shares a similar oxidation potential window.327 Efforts have been made to modify the electrode surface to exclude interaction with ascorbic acid while allowing access to dopamine.328,329 However, problems with this strategy still exist, in that products of oxidized dopamine are capable of catalyzing the oxidation of ascorbic acid to regenerate dopamine.330 To counter this problem, non-oxidative electrochemical detection strategies have been developed. Arrigan et al. reported a sensing method based on the difference between dopamine and ascorbic acid transfer efficiency across the aqueous-to-organic phase boundary.331 Other strategies use preferential dopamine receptors as bio-recognition elements, including phenylboronic acid332 and poly(amine)boronic acid (PABA).333 Owing to high detection limits in the micromolar range, the integration of novel immobilization substrates like nanotubes has also gained popularity for signal enhancement.334

Acetylcholine (ACh) is another intensely studied neurotransmitter. In fact, it was the first to be isolated almost a century ago, and has since been identified as a major player in cognitive functions such as attention, learning and memory.335 Pathological signaling in the ACh pathway is associated with neurological disorders including Alzheimer’s disease, Parkinson’s disease, and schizophrenia, necessarily driving bio-sensing technologies for its detection and in vivo monitoring.335

Typical enzyme assays for ACh detection involve the conversion of ACh to choline by acetylcholinesterase, followed by oxidation by choline oxidase to produce electrochemically336–338 or electrochemiluminescence339 detectable hydrogen peroxide.335 Similar to the abovementioned dopamine detection example, sensors of this type are susceptible to interference caused by electroactive species, and efforts have therefore been made for non-specific exclusion on electrode surfaces using semi-permeable membranes.340 While reported limits of detection for these ACh enzyme biosensors span a nM to µM range, several groups have applied mass spectrometry (MS) based approaches coupled with various sampling techniques to achieve pM sensitivity.335 For example, liquid chromatography tandem mass spectrometric (LC-MS) methods coupled with microdialysis have been employed to measure ACh in pharmaceutical preparations341 and in vivo in rat and mouse brain.342–344

Sweedler et al. quantified single neuron metabolomics by coupling single cell electrophoresis with electrospray ionization (ESI) time-of-flight MS.345 Furthermore, MS-based technologies have been applied to multianalyte detection346,347 and proteomic/peptidomic based neurodegenerative disease biomarker discovery.348,349 In a recent example, Greco et al. utilized amine derivatization of several neurotransmitters to achieve enhanced sensitivity (30 pM) for several monoamine neurotransmitters simultaneously.347

Although many of these examples involve sampling methods coupled to large instrumentation, the emergence of microfluidics has permitted the miniaturization of analytical devices for neuroscience. Kennedy and coworkers have implemented microfluidics into both sampling350 and sensing351 elements for in vivo capillary electrophoresis based monitoring of neurotransmitters. Microfluidics coupled with soft lithography and surface patterning techniques have also been employed for in vitro neurotransmitter monitoring, as well as guided axon/dendrite growth and synapse formation with precise control of cellular microenvironment.352–356 These devices have demonstrated advantages for disease modeling, electrophysiology, and the culture of neural stem cells.357

**Action potentials.** Action potentials (AP) in neurons play a critical role in cell–cell communication. The conventional patch clamp technique allows for recording of AP with a high signal-to-noise ratio,358 however the extremely low-throughput format makes this system inappropriate for multiplexed measurement. Other methods that utilize field-effect transistors (FET) and multi-electrode arrays (MEA) have been developed to address multiplexing, and in recent years, nanoscale transistors and electrodes have enhanced the signal strength of AP detection. For example, a localized nanoscale FET at the tip of kinked silicon nanowires was used to create a three-dimensional AP sensor.359 For contact with cell cytosol, SiO2 nanotubes were integrated on top of a nano-scale FET to penetrate cell membranes and achieve intracellular AP recording.360 Finally, nanowire FET arrays fabricated on flexible plastic/biopolymer substrates were also developed to apply FET-based AP sensing to three-dimensional soft objects such as whole embryonic heart.361 In MEA-based sensing, recent efforts have focused on the establishment of a tight cell membrane-to-electrode seal to minimize signal loss. In contrast to planar electrode schemes, Hai et al. used mushroom-shaped microspines for “in-cell recording” and detected APs with increased signal-to-noise ratio.362 As a next step, they incorporated a neuron stimulation function into the electrodes to realize cell stimulation without causing electroporation.363 Interestingly, Xie and coworkers exploited the electroporation phenomenon by applying a voltage pulse to platinum nanopillars to create nanopenores on cell membranes adjacent to electrodes. In this way, both extracellular APs pre-electroporation and intracellular APs post-electroporation were recorded, and the application of this device in ion-channel drug screening was demonstrated.364 Fig. 26 shows a working sensor and the principle for detection of action potentials in the neurons.

**Challenges and future outlook**

For widespread clinical application, biosensor development must continue in the direction of point-of-care; that is, sensors must be portable, cost-efficient and user-friendly. One promising
example is the paper-based analytical device (µPAD), a low cost sensing platform that affords accessibility to the general population. Because the driving force for fluid movement in these devices is based on evaporation or capillary forces, the demand for bulky external equipment is largely eliminated. Examples of paper-based, environmentally friendly, and disposable sensors emerged early in the 20th century with simple bio-analysis strips such as pregnancy tests, which have had years of clinical relevance. Whitesides and colleagues have recently expanded application of paper-based devices for a wider range of analytes, including glucose and proteins in urine. In research laboratories, future trends will likely lie in multiplexed, high-throughput single-cell analysis to reveal the complexity and heterogeneity in cell populations. Real-time probing is another focus for sensor development to study cytokine secretion dynamics, cell–cell and cell–environment interaction. Recently, novel approaches in the areas of biosensors have been pioneered based on the latest advances in biomolecular computing, particularly in enzyme-based logic systems. Application of the biocomputing concepts has been successfully demonstrated for systems with logic analysis of biomarkers of different injuries, including soft-tissue injury, traumatic brain injury, liver injury, abdominal trauma, hemorrhagic shock, and oxidative stress. Compared to traditional single-analyte sensing devices, the biocomputing approach enabled high-fidelity multi-analyte biosensing, particularly beneficial for point-of-care and field applications. Further integration of binary operating biosensors with biofuel cells, particularly with implantable biofuel cells, allowed extension of the sensing process to the self-activated actuation, thus resulting in integrated “sense-and-treat” systems. Finally, coupled with data analysis and bioinformatics tools, development of simultaneous multi-analyte sensing platforms may elucidate the genomic and proteomic signature profiles of numerous disease states, including cancers and neurological disorders, advancing pursuits of personalized medicine.

Overall future outlook

A review of the biosensing technology shows that there is enough research into the basic principles of transduction to be able to build a large variety of commercial devices, and solve most of the problems associated with the transduction event. New horizons might be achievable by combining different transduction platforms (electrochemical/optical/mass sensitive, etc.) for enhanced data acquisition in biosensor applications. However, at this point the bottleneck of biosensors is the lack of fundamental understanding of biorecognition macromolecules confinement and orientation at the micro- and nano-interfaces for high-throughput biosensing applications. Thus more studies should be devoted to understanding how a nanoscale biorecognition element (such as an antibody or enzyme) binds to a surface, how it changes when immobilized, how it can be manipulated to have the correct orientation to perform the recognition event, and how it can be made to remain stable and reliable for long periods of time in ambient conditions. For further progress in the biosensors field we need revolutionary ideas in the development of novel target recognition strategies, such as new nanoscale structures with variable selectivity, engineered proteins, signaling aptamers, natural and artificial ion-channels, bio-designed and molecular-imprinted polymers, etc. We also need new paradigms for the identification and detection of existing or emerging pathogenic microorganisms, unknown toxins and viral threat agents. There is a significant need for the development of materials and methods for bio-interfaces that afford general solutions to the problem of nonspecific sensor response. The development of new receptor designs that allow for highly selective and reversible recognition action (i.e. methods for manageable triggered dissociation of analytes from bioreceptors) for extending continuous monitoring technologies will be extremely important. And finally, widespread penetration of biosensing innovations will not be achieved unless: (i) the long-term stability and selectivity can be significantly improved; (ii) environmental susceptibility to deterioration of biorecognition elements will be made insignificant; (iii) highly standardized bio-nano-interfaces will be readily available; and (iv) costs can be significantly reduced.

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