

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.

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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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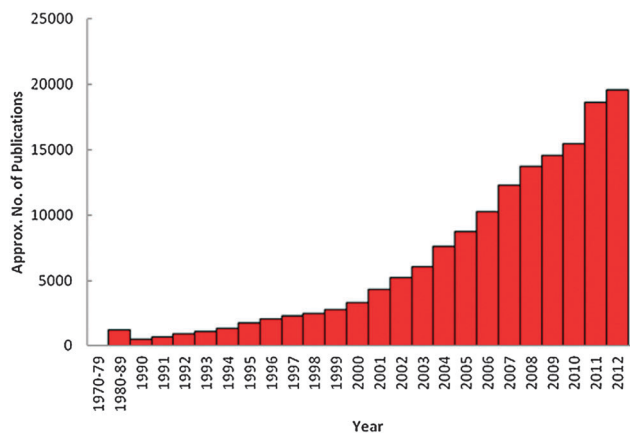
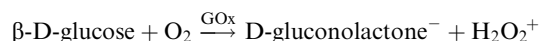


Fig. 1 Trends in biosensing research.

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.^{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.⁸ Historically, these biosensors were based on an enzyme glucose oxidase (GOx) catalyzing the oxidation of glucose to hydrogen peroxide and D-glucono-lactone:



Jeffrey Kirsch

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

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 micro-fabricated platforms for sensing and sorting cells.



Qing Zhou

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

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.

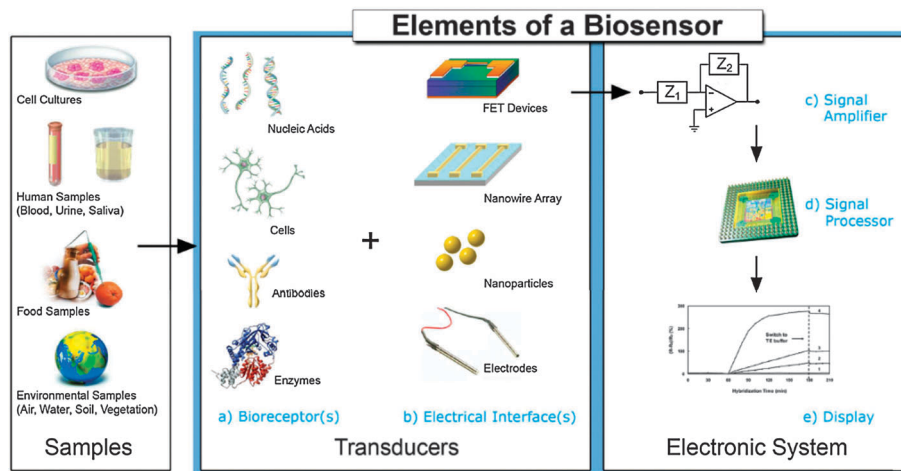


Fig. 2 Elements and selected components of a typical biosensor. Reprinted from an Open Access Journal.⁷

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.⁹ Later, Guilbault and Lubrano suggested the use of H_2O_2 monitoring in reaction (1) for glucose detection.¹⁰ 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.¹¹

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.¹² 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' biosensors²¹³ 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).¹⁴

Significant progress in the development of three generations of biosensors was achieved because of significant efforts of F. W. Scheller's group^{15–17} and Adam Heller's elegant work on the use of flexible polymers with osmium redox sites.¹⁸ Substantial efforts to develop third generation biosensors were put forth by Kulis and Cenas, Palmisano *et al.*, and Aizawa and Koopal.¹⁴

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



Aleksandr Simonian

Aleksandr (Alex) Simonian is currently a Professor of Mechanical Engineering at Auburn University and the founding Program Director of the Biosensing Program in the Division of CBET at the National Science Foundation. He received his MS in Physics from the Yerevan State University (Armenia, USSR), PhD in Biophysics from the USSR Academy of Sciences and a Doctor of Science (DSc) degree in Bioengineering from Moscow Institute of Applied Biochemistry.

His current research interests are primarily in the areas of bio-analytical sensors, nanobiomaterials and functional interfaces. Simonian was elected as a Foreign Member of Armenian National Academy in 2011.

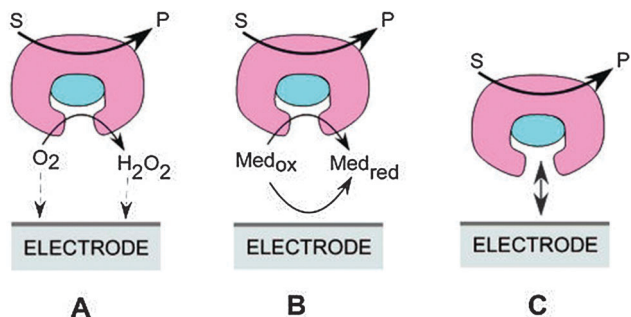


Fig. 3 Three generations of amperometric enzyme electrodes for glucose based on the use of natural oxygen cofactor (A), artificial redox mediators (B), or direct electron transfer between GOx and the electrode (C). Reprinted with permission from Wang, *Chem. Rev.*, 2008, **108**, 814–825. Copyright © 2008, American Chemical Society.¹⁴

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.^{19–21} 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.²² 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

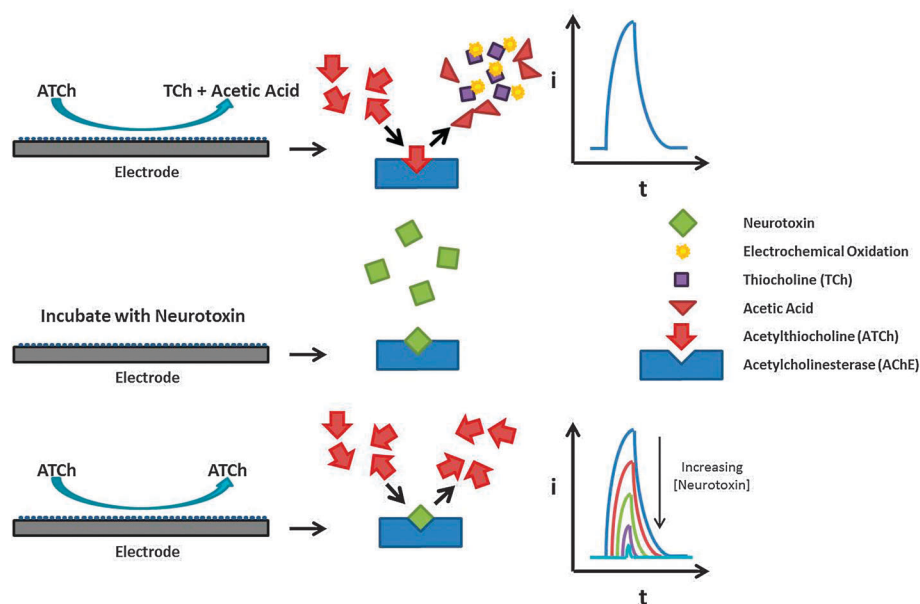


Fig. 4 This scheme shows how AChE can be used in an electrochemical sensing system to determine the quantity of neurotoxin present in a sample. A baseline is first gathered from functioning enzyme. The sensor is then incubated with a concentration of neurotoxin, which binds irreversibly to the active site of the enzyme and destroys its activity. Finally, the response to the substrate is measured again to determine how much of the enzyme has been destroyed, which corresponds to the amount of neurotoxin present. Signal deterioration is proportional to the target analyte concentration in some concentration range.

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.²³ Immunosensors 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”.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.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.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 km² 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.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.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.^{29–32} There is no antidote for ricin, and no widely available, reliable medical test is available to confirm exposure to ricin.^{29,30} The minimum lethal dose is $5 \mu\text{g kg}^{-1}$ of body weight.³⁰

Biosensors have been developed for detection of ricin in several different formats including the fluorescence array biosensor,^{25,33–36} surface plasmon resonance,^{30,32} and magnetoelastic sensors.³¹ Antibodies^{25,30,31,33–36} or oligosaccharides³² 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.^{25,33–36} Uzawa *et al.* used synthetic analogues of β -lactosyl and β -D-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.³²

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 $100 \mu\text{g mL}^{-1}$ 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.³⁷ Botulinum toxin is the most poisonous substance known, and acts on the fusion proteins which release acetylcholine into a neuromuscular junction. It has been developed by several countries as a biological weapon, including four countries classified by the United States government as “state sponsors of terrorism”. Botulism diagnosis must be confirmed through a specialized laboratory, which can require several days to complete, and is frequently misdiagnosed.³⁸

Botulinum toxins have been detected mainly using the sandwich assay format utilizing antibodies with fluorescent tracers,^{25,35,39–42} however, it has also been detected using antimicrobial peptides,⁴³ and aptamers.⁴⁴ The antibody bioassay has the advantages of simplicity and sensitivity, but it is non-selective

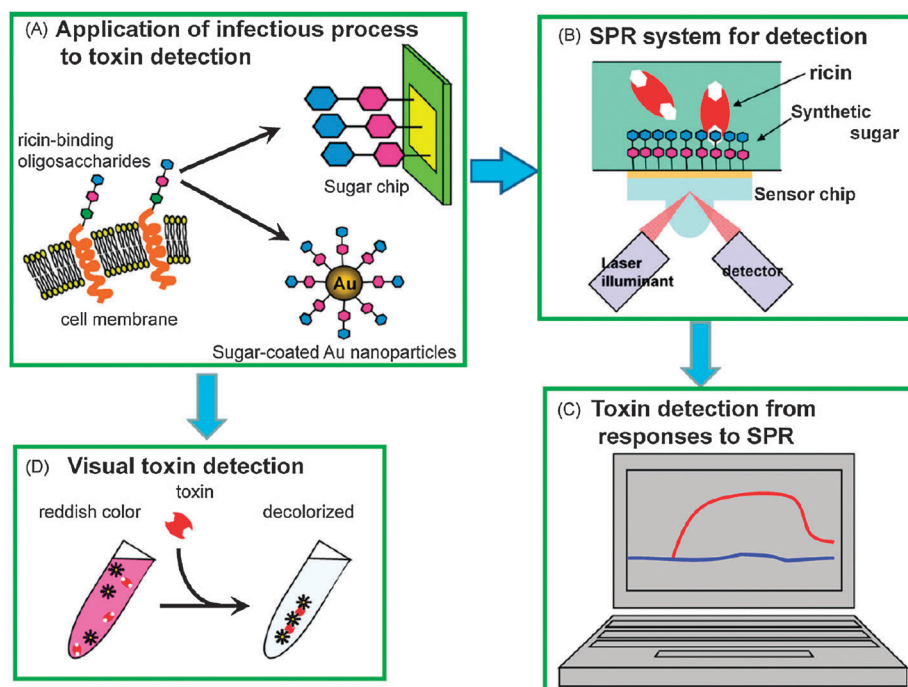


Fig. 5 This scheme demonstrates the application of the infection process to ricin detection, since ricin specifically recognizes cell surface oligosaccharides, and the sugar based nanomaterials (A) were designed for use in surface plasmon resonance (B) and (C), and in a colloidal gold colorimetric assay for the qualitative detection of the toxin (D). Reprinted with permission from Uzawa *et al.*, *Biosens. Bioelectron.*, 2008, **24**, 923–927. Copyright © 2008, Elsevier.³²

among the serotypes, and therefore requires additional assays to determine the serotype.⁴⁵ While fluorescence has been mainly used as the transduction event for botulinum toxins, surface plasmon resonance^{46,47} has also been used with the sandwich assay. Grate *et al.* have developed a sensor with a renewable surface for recognition of botulinum toxin by using Sepharose beads with capture antibodies that can be easily replaced in their system with new beads. Kulagina *et al.* used antimicrobial peptides to detect botulinum toxins, which are similar in some aspects to antibodies, however they could potentially be used to differentiate between the different serotypes as well as have superior detection sensitivity.⁴³

Bruno *et al.* developed a molecular aptamer beacon responsive to botulinum toxins. They identified a series of sequences that were able to form the loop structure necessary to form the quenching action of the molecular beacon, and three of the sequences exhibited fluorescence when exposed to increasing levels of botulinum toxin A. The best sequence they obtained had a detection limit of 1 ng mL⁻¹ in buffer, but it was only responsive to environmental media samples and not to botulism in human serum. This beacon would be suitable for real-time analysis of environmental samples containing several types of biotoxins including staphylococcal enterotoxin B, and other aptamer beacons developed by their group would be more suitable for clinical use biosensors.⁴⁴

Ferracci *et al.* achieved label-free, real-time detection of botulinum neurotoxin B in food and human serum. They immobilized VAMP2, a membrane protein associated with synaptic vesicles, onto surface plasmon resonance chips and monitored the cleavage of this protein by botulinum neurotoxin B. They measured vesicle capture by specific antibodies coupled to the chips and the proteolysis of VAMP2 by botulinum neurotoxin B was attributed to a decrease of the binding of the synaptic vesicles to anti-VAMP2 antibodies. Their sensor provided detection within 10 minutes with a detection limit of 2 pM of the toxin.⁴⁷

Saxitoxin

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.⁴⁸

The standard for detection and screening of saxitoxins is the use of a mouse mortality assay, which is considered ethically problematic.^{49–54} Alternative methods could be provided by developed biosensors such as a surface plasmon resonance (SPR) inhibition assay^{49–52} and electrophysiological assays.^{49–52,54} 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.^{49–51} 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.⁵⁴

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.⁵⁵

Several methods have been developed for detection of SEB including using the antibody sandwich assay,^{25,33–36,39,40,42,56–60} direct detection through surface plasmon resonance (SPR),⁵⁶ magnetoelastic sensors,⁶¹ piezoelectric crystal sensors,^{62,63} and capacitive sensors.⁶⁴ 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.⁵⁶

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.⁶¹

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} .⁶² 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.⁶⁴

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.⁶⁵

Typical detection methods involve optical techniques such as surface plasmon resonance (SPR)^{66–68} or fluorescence based detection, both utilizing antibodies against mycotoxins.^{25,66–71} There are some concerns that antibodies for trichothecene mycotoxins are cross-reactive, increasing uncertainty in measurements.⁶⁸ 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.^{66,67}

Gosselin *et al.* reported a label-free, spectroscopic biosensor based on antibodies for the detection of verrucar-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) and 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.⁷²

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.^{73,74} 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).⁷³ *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.⁷⁴

Pathogenic *Bacillus* species have been detected using antibodies,^{35,36,59,75–80} DNA probes,^{81,82} bacteriophages,^{83–85} and mammalian cells^{86,87} as the recognition element with several different types of transducers including fluorescent array,^{33,35,36,59} evanescent wave fiber-optic,⁷⁷ laser cytometry,^{86,87} electrochemical,^{75,76,78–82} and mass sensitive sensors.^{83–85,88,89}

Tims and Lim developed a fiber-optic biosensor capable of detecting *B. anthracis* spores in common powders at concentrations of 3.2×10^5 spores per mg or higher within an hour.⁷⁷ 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.^{86,87}

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.^{79,80} 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.^{75,78,82} 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.^{83–85}

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

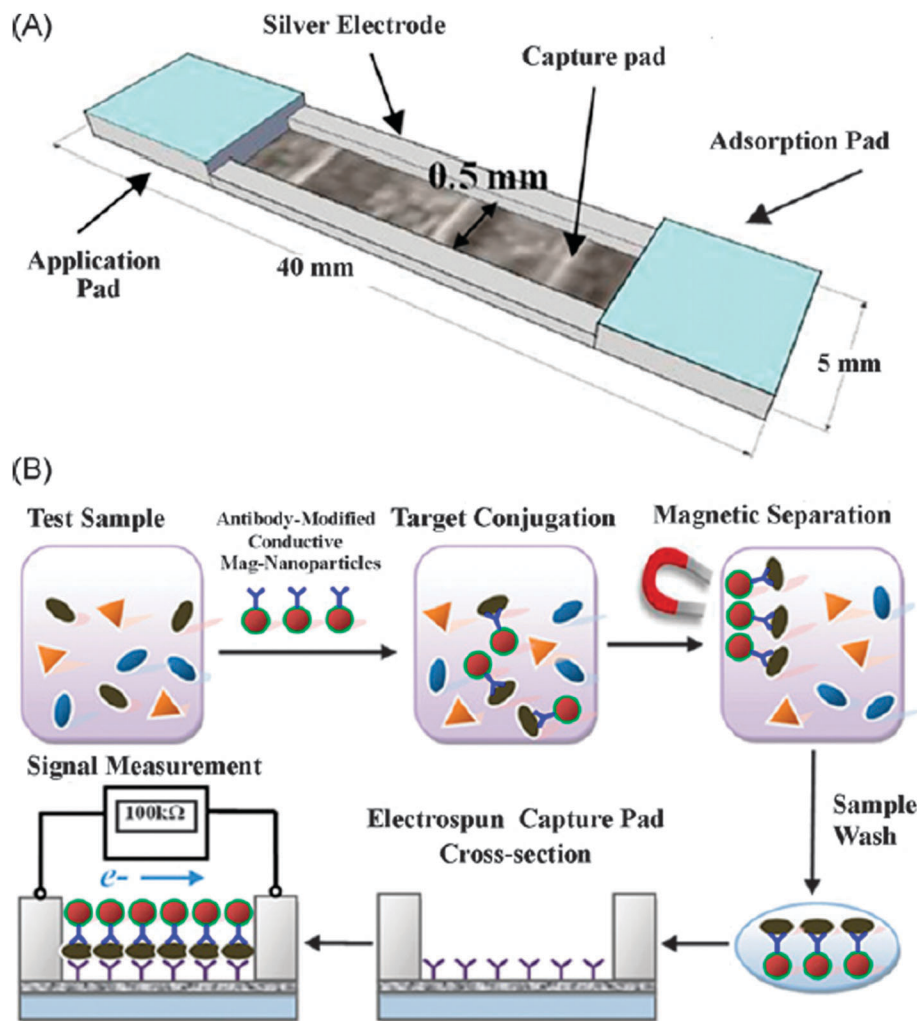


Fig. 6 (A) Schematic showing the biosensor structure and membrane assembly consisting of the application pad, adsorption pad, and the capture pad. (B) Detection scheme of the biosensor based on antibodies and magnetic separation of the analyte from mixed samples. Reprinted with permission from Luo *et al.*, *Biosens. Bioelectron.*, 2010, **26**, 1612–1617. Copyright © 2010, Elsevier.⁹⁰

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.^{91,92}

Campylobacter species have been detected using DNA,^{93–95} aptamers,^{96,97} antibodies,^{40,71,92,98–100} or bacteriophages^{96,101} as the recognition element and using optical,^{39,57,71,92–94,99,101–104} electrochemical,^{105–107} or mass sensitive transducers.^{98,100,108–110} 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.⁹⁴

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.¹⁰¹ Sapsford *et al.* developed 25 minute sandwich assays on the array biosensor to detect *C. jejuni* at concentrations as low as 9.7×10^2 CFU per mL.⁹² 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.¹⁰⁷ 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

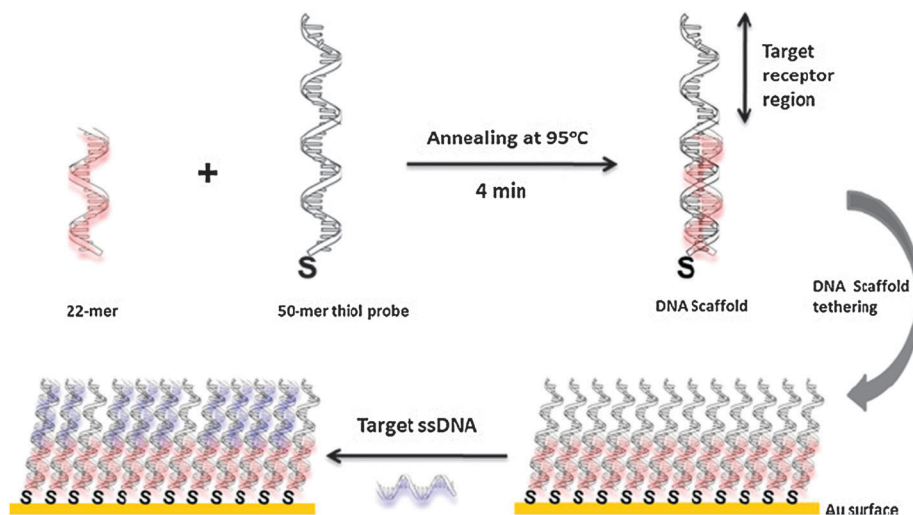


Fig. 7 Schematic showing the formation of DNA scaffolds using a 50-mer long probe and a 22-mer short probe. Annealing at 95 °C binds the short and long strands to form the DNA scaffold, which is used to form a self-assembled monolayer on the gold surface of an SPR biosensor to detect complementary single stranded DNA. Reprinted with permission from Gnanaprakasa *et al.*, *Sens. Actuators, B*, 2011, **156**, 304–311. Copyright © 2011, Elsevier.⁹⁴

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/lectins^{25,33–36,113,114} and/or gangliosides^{115–117} 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 as labels for cholera toxin. An electrode modified with carbon nanotubes which are coated with poly(3,4-ethylenedioxythiophene) was functionalized with antibodies against cholera toxin. When a sandwich complex (described in the previous section)

is built, stripping voltammetry releases the potassium ferrocyanide which is measured and correlated to the amount of bound cholera toxin.¹¹⁷ Chen *et al.* utilized 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 probes^{93,131} 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

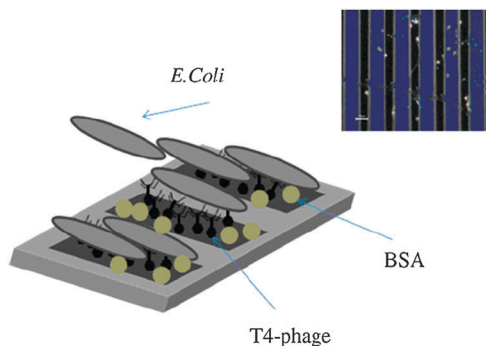


Fig. 8 Schematic showing the structure of a T4-phage biosensor with an amplified photograph of a section of the interdigitated microelectrodes. Reprinted with permission from Mejri *et al.*, *Biosens. Bioelectron.*, 2010, **26**, 1261–1267. Copyright © 2010, Elsevier.¹²¹

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 $1.5\text{ mm} \times 0.8\text{ mm} \times 35\text{ }\mu\text{m}$ 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 bacteriophages¹³⁶ as the recognition element in optical^{57,86,87,124,134,136} and electrochemical⁷⁶ 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 $\times$) capture efficiency than the monoclonal antibody mAb-C11E9, and therefore

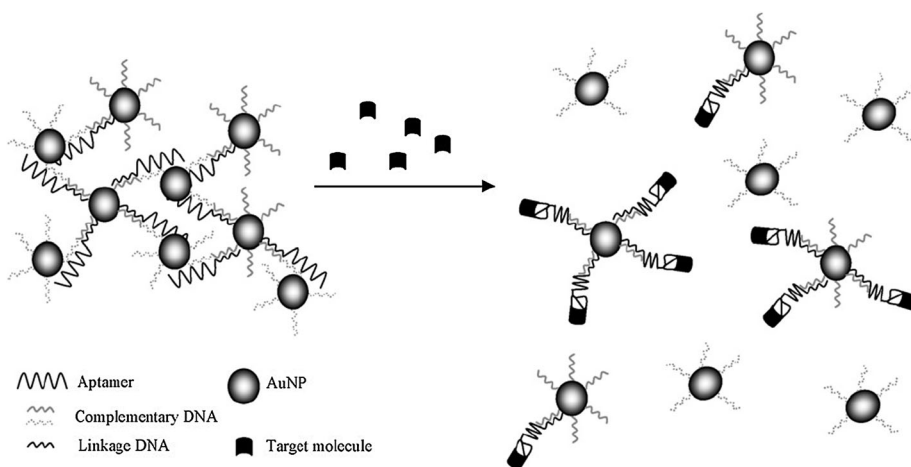


Fig. 9 Schematic showing a gold nanoparticle aptasensor for colorimetric-based detection. Aptamer-functionalized gold nanoparticles were aggregated using aptamer complementary oligonucleotide sequences, and in the presence of the target molecule the aptamers change their conformation, resulting in disassembly of the aggregates to produce a change in color. Reprinted with permission from Torres-Chavolla and Alocilja, *Biosens. Bioelectron.*, 2009, **24**, 3175–3182. Copyright © 2009, Elsevier.²⁸

could be used as an alternative recognition element to antibodies for specific detection of *L. monocytogenes*.¹³⁵ 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 2×10^6 CFU per mL.¹³⁶

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.¹³⁷

Pathogenic *Salmonella* has been detected using antibodies,^{124,138–141} antimicrobial peptides,^{127,128} bacteriophages,^{85,142} and DNA probes^{81,143–145} coupled with optical,^{124,127,128,138,145} electrochemical,^{81,141,144} and mass sensitive^{85,139,142} 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.¹⁴⁰ Zhang *et al.* developed a highly amplified bio-barcode DNA assay for 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} .¹⁴⁵ They further developed this to detect multiple pathogens, but added nanoparticle tracers which when dissolved after capture allowed them to detect the DNA electrochemically.⁸¹

Kim *et al.* developed an impedance biosensor for detection of *Salmonella* using antibodies to capture the bacteria and detect 10^3 CFU per mL in pork meat extract within 5 minutes.¹⁴¹ 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 5×10^2 CFU per mL.¹⁴²

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.¹⁴⁶

Shigella species are typically detected using antibodies against the specific strain as the recognition element,^{92,147,148} and fluorescence array⁹² or electrochemistry^{147,148} as the method of transduction. Zhao *et al.* developed a sensor (Fig. 12) based on

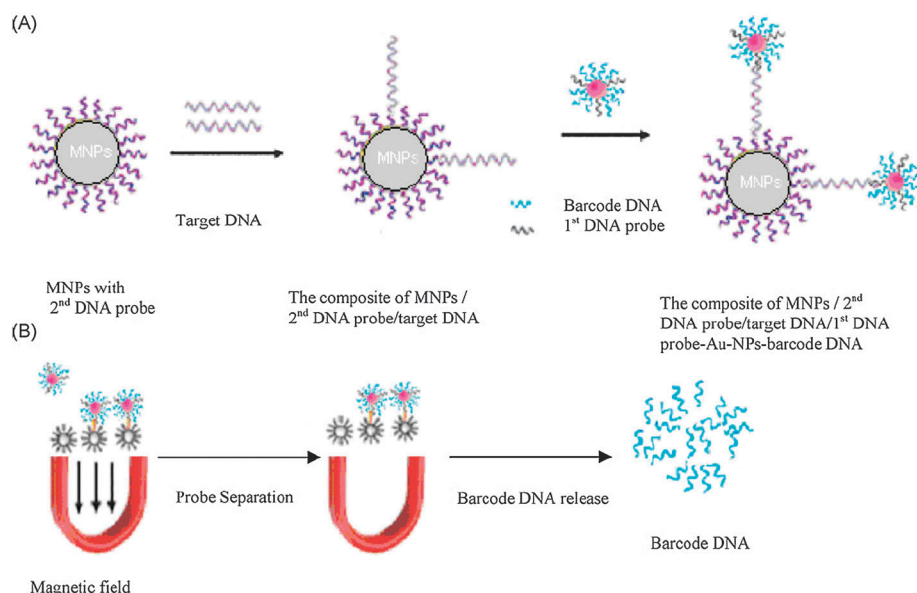


Fig. 10 Schematic of the bio-barcode assay with (A) formation of the magnetic nanoparticles (MNPs) with the DNA probe complex including the target DNA and the barcode DNA on gold nanoparticles (Au-NPs) and (B) the separation of the complex and release of the barcode DNA. Reprinted with permission from Zhang *et al.*, *Biosens. Bioelectron.*, 2009, **24**, 1377–1381. Copyright © 2009, Elsevier.¹⁴⁵

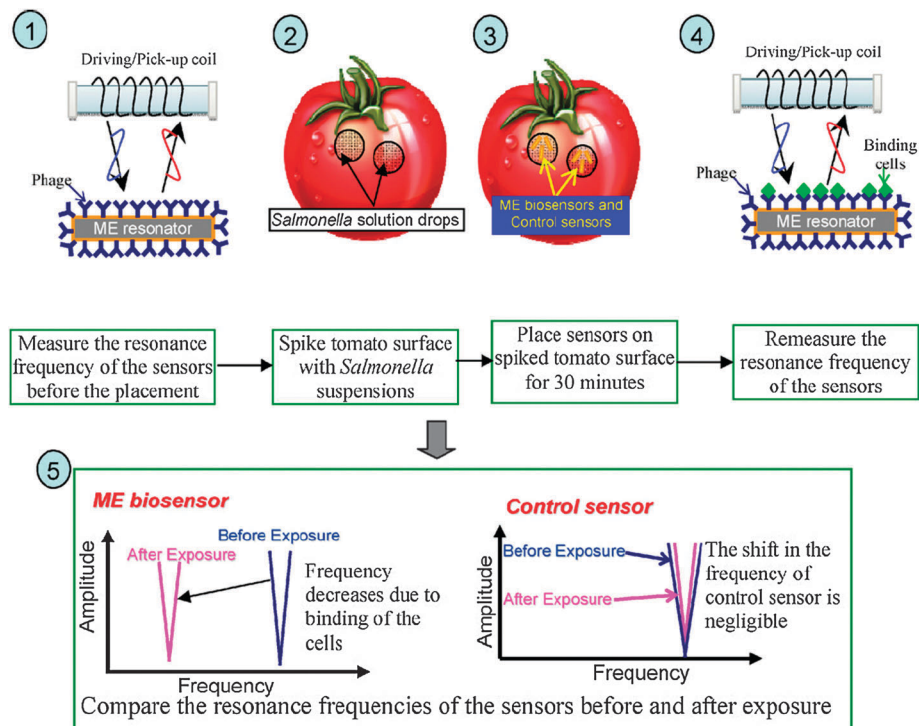


Fig. 11 Scheme of the process used to directly detect *Salmonella* on tomato surfaces using magnetoelastic biosensors. Reprinted with permission from Li *et al.*, *Biosens. Bioelectron.*, 2010, **26**, 1313–1319. Copyright © 2010, Elsevier.¹⁴²

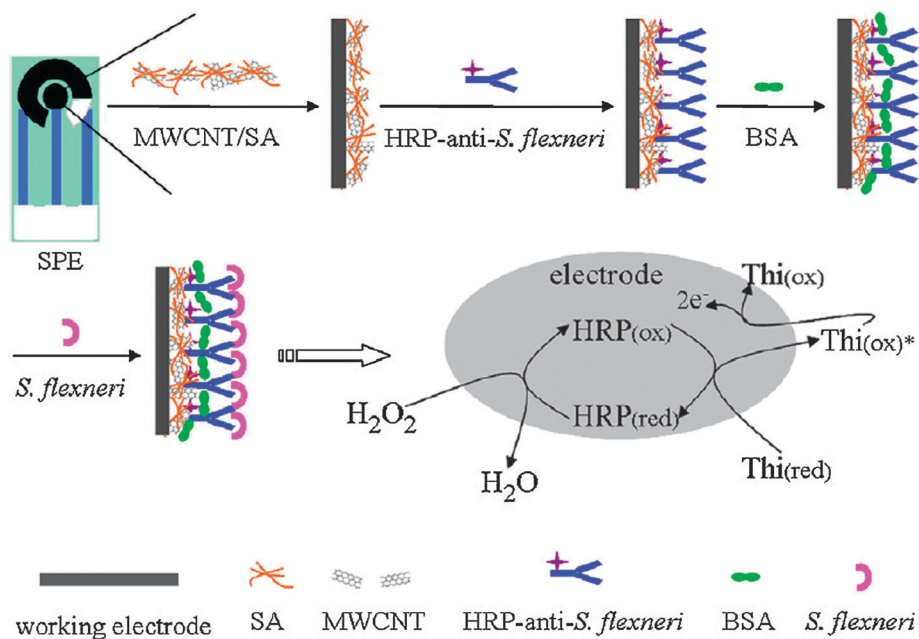


Fig. 12 Scheme showing the fabrication and electrochemical process for the immunoelectrode used in the determination of *Shigella flexneri*. Thi(ox) and Thi(red) are the oxidized and reduced forms of thionine; * represents transition state. MWCNT is multi-walled carbon nanotube; HRP is horseradish peroxidase; BSA is bovine serum albumin. Reprinted with permission from Zhao *et al.*, *Anal. Biochem.*, 2011, **408**, 53–58. Copyright © 2011, Elsevier.¹⁴⁸

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 H₂O₂ (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

shielding the active site of HRP, reducing its catalytic activity towards H_2O_2 .^{147,148}

Staphylococcus aureus

Staphylococcus aureus is responsible for staphylococcal food poisoning due to its ability to produce seven different toxins that can contaminate food. This bacterium is relatively common and found in the noses and on the skin of 25% of healthy people and animals. *Staphylococcus* can contaminate food through unsafe food preparation methods by people who carry the bacteria, and as it grows it releases toxins which are heat resistant and not destroyed by cooking. Staphylococcal food poisoning is usually fast acting and has mild gastrointestinal symptoms. Staphylococcal enterotoxin B (mentioned previously) in relatively high doses can have serious health implications.¹⁴⁹

S. aureus was detected using antibodies^{150,151} and phages,¹⁵² and optical^{150–152} and mass sensitive techniques.¹⁵⁰ Subramanian *et al.* built a surface plasmon resonance (SPR) biosensor using self-assembled monolayers (SAMs) and antibodies against *S. aureus* in both direct and sandwich assays. Their primary investigation was to determine the capability of mono and dithiol tether based SAMs in biosensing, and they were both capable of specifically detecting *S. aureus* in a mixture of *E. coli* at 10^5 CFU per mL.¹⁵¹ Balasubramanian *et al.* used a lytic phage as a highly specific biorecognition element in an SPR for label-free detection of *S. aureus*. The phage was immobilized onto the gold surface through direct physical adsorption and was able to detect the bacterium with a detection limit of 10^4 CFU per mL.¹⁵²

Boujday *et al.* used several transduction techniques including quartz crystal microbalance (QCM), polarization modulation reflection absorption infrared spectroscopy (PM-RAIRS), and fluorescence to detect *S. aureus* on antibody-conjugated gold. Fluorescence was able to verify the specificity and quantify the detection of the pathogenic bacteria, and QCM and PM-RAIRS were used to examine the homogeneity and accessibility of the receptors on the gold surface. With PM-RAIRS, they were able to enhance the sensitivity of their device and reach a detection level of 10^5 CFU per mL.¹⁵⁰

Viral threats

Smallpox, viral hemorrhagic fevers, viral encephalitis, and several other viral pathogens have been classified by the CDC as potential weapons of mass destruction or candidates for bioterrorism. Dispersion of these agents may be difficult; however the risk is greatly magnified by the infectious capabilities if these agents were introduced into large populations due to limited available treatment and vaccination strategies.¹⁵³ Along with anthrax, smallpox is one of the two potential agents that exhibit the greatest potential for weaponization, due to its high lethality, stability in aerosol transmission, and capability for large scale production. Availability of the vaccine for smallpox is limited, as the viability of stored vaccine is no longer guaranteed. Smallpox virus is difficult to obtain, however its effects could be devastating due to the communicability,

and less than 20% of the population has any immunity due to prior vaccination. There are several different viruses that can cause hemorrhagic fever including Lassa fever, Rift Valley fever, Ebola hemorrhagic fever, and Marburg disease. These viral organisms are potential agents due to their high infectivity by aerosol, lethality, and possibility for replication in tissue cultures.²⁷

Existing techniques for detecting viruses include cell culturing, enzyme-linked immunosorbent assays (ELISA), and PCR which are not readily compatible for point of care use without expensive infrastructure, and these techniques are time consuming and labor intensive.¹⁵⁴ Antibodies^{154,155} and antimicrobial peptides¹⁵⁶ have provided selectivity as biorecognition elements in biosensors, and optical,^{154–156} electrochemical,¹⁵⁷ and mass sensitive¹⁵⁸ techniques have been employed as transducers. Donaldson *et al.* proposed using antibodies against the Vaccinia virus (a surrogate for the smallpox virus) labeled with fluorescent tracers to detect the Vaccinia virus at 2.5×10^5 PFU per mL in throat culture swabs.¹⁵⁵ Kulagina *et al.* used antimicrobial peptides to directly capture multiple pathogens including the Vaccinia virus in a sandwich fluorescent assay, which showed higher sensitivity *versus* the antibodies (5×10^5 PFU per mL *vs.* $>1 \times 10^8$).¹⁵⁶

Yanik *et al.* developed an optofluidic biosensor to detect viruses based on plasmonic nanoholes, and has the advantages of possibility for multiplexing and detecting intact virus particles without damaging the structure or the nucleic acid load, allowing for further study. Antibodies are conjugated onto a nanohole array, and the resonance transmission of light is measured, similar to the surface plasmon resonance phenomena. They were capable of detecting Vaccinia and Ebola between 10^6 and 10^9 PFU per mL.¹⁵⁴ Gupta *et al.* proposed using microresonators in conjunction with antibodies to detect single virus particles, by measuring the change in resonant frequency of microcantilevers. They were able to measure the average dry mass of a single Vaccinia virus particle to be 9.5 fg, but did not incorporate any kind of recognition element into their system.¹⁵⁸

Multi-detection biosensors

Currently, there are many groups working towards multi-detection of biological species on a single device, which would allow for inexpensive, rapid screening of foodstuffs and water supplies for many different pathogens.^{25,33–36,39,40,42,57–60,71,78,81,85,86,92,124,140,159–162}

The work performed by Rowe *et al.* on the array biosensor in 1999 capable of detecting multiple analytes on a single platform from mixed samples forms the basis of most of the works recently performed in biodefense, and was the first to successfully demonstrate a device that performed multi-detection (Fig. 13).⁵⁹ Not only was it able to detect bacterial samples, but it was also capable of detecting viral and protein analytes as well within 14 minutes with comparable sensitivity to ELISA. Their work also demonstrated several unique advantages at that period of time, including analysis of multiple samples in parallel, quantitative detection of the analytes at physiologically relevant concentrations, and simultaneous detection

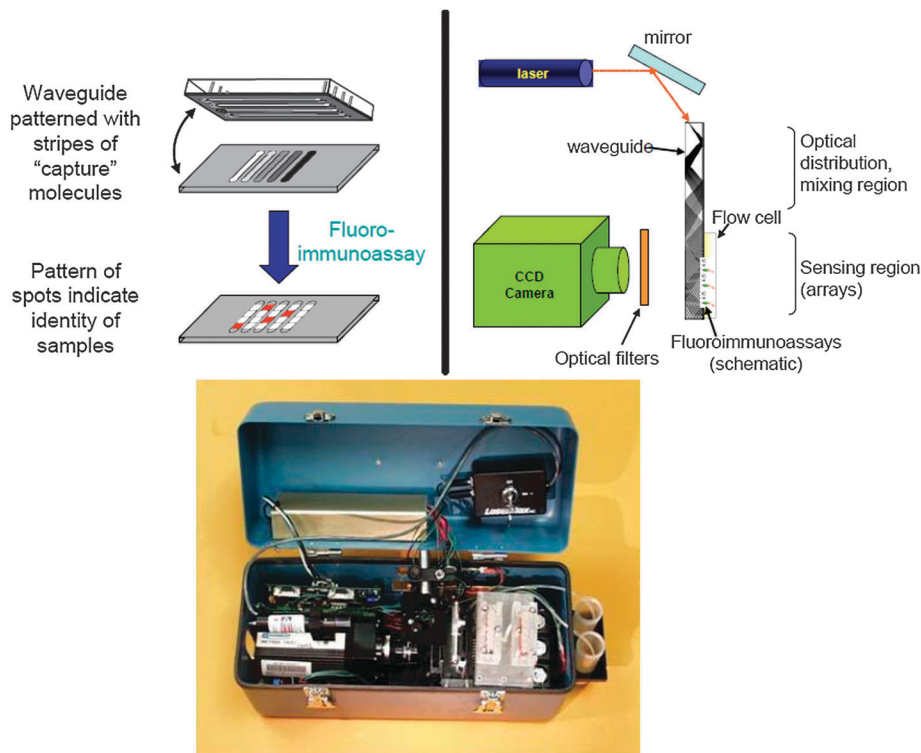


Fig. 13 The NRL Array Biosensor. Upper left: schematic showing the waveguide surface patterned with the capture elements and flow cell. Upper right: schematic of the optical configuration of the sensor. Bottom: prototype of the NRL array biosensor, with all imaging components (left side) separated from the fluidic components (right side) to minimize damage from potential leakage. Reprinted from an Open Access Journal.¹⁶⁴

of multiple analytes in each sample.⁶⁰ 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.³⁵ They were also able to miniaturize the array biosensor, which then allowed them to build a portable bio-sensing platform.^{33,160,162,163}

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.¹⁵⁹ 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 micro-fluidic sensors, which provides a highly controllable sensitivity

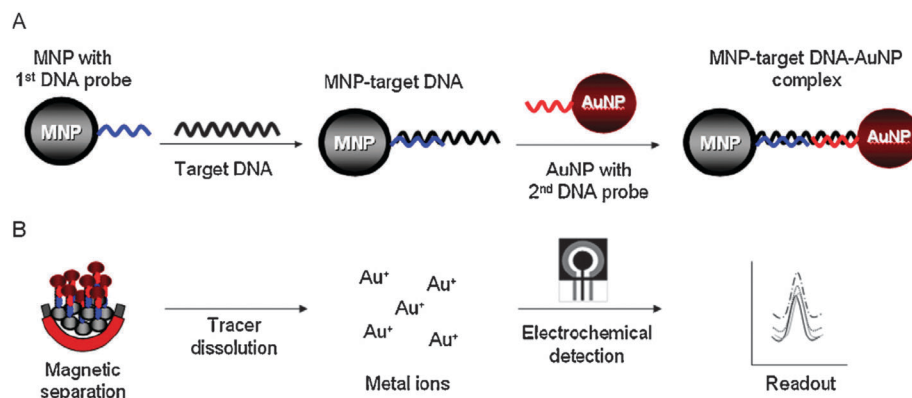


Fig. 14 Schematic of a target DNA detection system using (A) magnetic nanoparticles with DNA probes to form a target complex with gold nanoparticle DNA probes and (B) magnetic separation and dissolution of the tracers to detect the analyte electrochemically. Reprinted with permission from Torres-Chavolla and Alocilja, *Biosens. Bioelectron.*, 2011, **26**, 4614–4618. Copyright © 2011, Elsevier.¹⁶⁵

in impedance-based biosensors by creating an adjustable, soft-walled “virtual” microchannel.^{166,167} McGraw *et al.* proposed using electrotexiles 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.^{126,168} Wojciechowski *et al.* proposed using organic photodiodes to provide cheap and miniaturized optical transduction, allowing for the possibility of portable and disposable optical biosensors.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.^{170,171} Gehring *et al.* used LAPS in conjunction with an immunoligand assay for the rapid detection of *E. coli* with a limit of detection of 7.1×10^2 cells per mL of heat-killed or 2.5×10^4 cells per mL of live cells within 45 minutes.¹⁷⁰ It can be applied to multi-analyte sensing and imaging, and is capable of measuring various ions and molecules.¹⁷² Xu *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.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, *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.¹⁷⁴ Goel *et al.* reviewed several papers which discussed using molecular beacons to detect pathogens such as *E. coli*, HIV, *M. tuberculosis*, Papillomavirus, *Salmonella* and many more.¹⁷⁵ Du *et al.* reported using a molecular beacon biosensor to detect DNA from methicillin resistant *S. aureus*.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,¹⁷⁷ dependency on assumed antigenic motifs,^{156,177} limited stability under extreme environmental conditions,^{128,134} lack of batch consistency, high costs of development, and possible non-specificity in multiplex formats.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.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

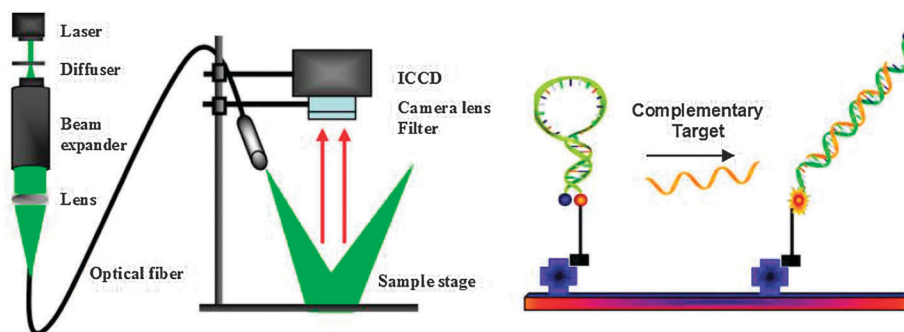


Fig. 15 Schematic of a molecular beacon detection system. The optical imaging setup excites and detects the fluorescence (or lack thereof) from the fluorophore attached to the molecular beacon. Without the target DNA, the molecular beacon forms a hairpin structure, with the fluorophore emission being 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.¹⁷⁴

these pathogens would be a step towards real-time capabilities, such as molecular beacon biosensors. For many of the toxins, engineered enzymes capable of catalytic destruction may provide a means to detect them in real-time, without being consumed or irreversibly altered, as is the case with other recognition elements like antibodies, aptamers, DNA probes, cells, *etc.*

Biosensors for detection of neurotoxic compounds

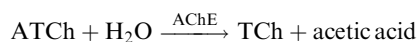
Neurotoxic compounds can be separated into one of two types: organophosphorus (OP) and non-organophosphorus. Organophosphorus compounds compose the largest class of urban and rural pesticides, with 100 different compounds currently commercially used, and over 1500 different OP neurotoxins have been synthesized in the last century.¹⁷⁸ 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.¹⁷⁹ 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,¹⁸⁰ 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.¹⁷⁹

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.¹⁷⁹ Discovery and development of the phosphotriesterase enzymes have led to their use in biosensing technologies, including organophosphate hydrolase, organophosphate acid anhydrolase, human paraoxonase, and squid diisopropyl fluorophosphatase.^{182,183} 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.^{184–186} 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.¹⁸⁷



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

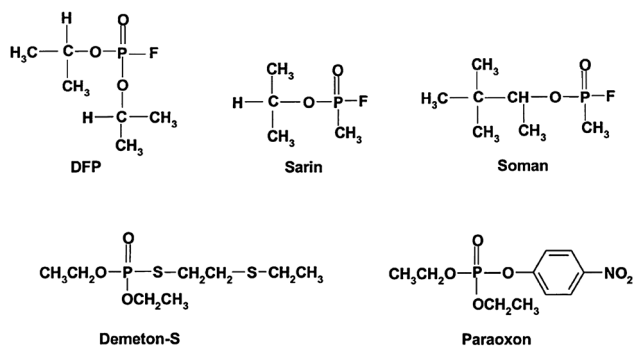


Fig. 16 Chemical structures of some organophosphate neurotoxins. Reprinted with permission from Simonian *et al.*, *Anal. Chim. Acta*, 2001, **442**, 15–23. Copyright © 2001, Elsevier.¹⁸¹

different extents, and calibration of the sensor to an unknown sample would be almost impossible.^{188,189} 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.^{188,189}

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.¹⁹⁰ 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 $10\ \mu\text{g kg}^{-1}$. 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.^{191,192}

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*-butyl 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.¹⁹³

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 thiocholine which reduces AuCl_4^- 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 $0.3\ \text{nmol L}^{-1}$ and acetylcholine at $15\ \mu\text{mol L}^{-1}$.¹⁹⁴

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.¹⁹⁵ Patel reviewed the availability of inhibition based biosensors for the detection of a range of chemical compounds and food-borne contaminants.¹⁹⁶ Amine also reviewed a number of biosensors based on enzyme inhibition to determine pollutants and toxic compounds.¹⁹⁷

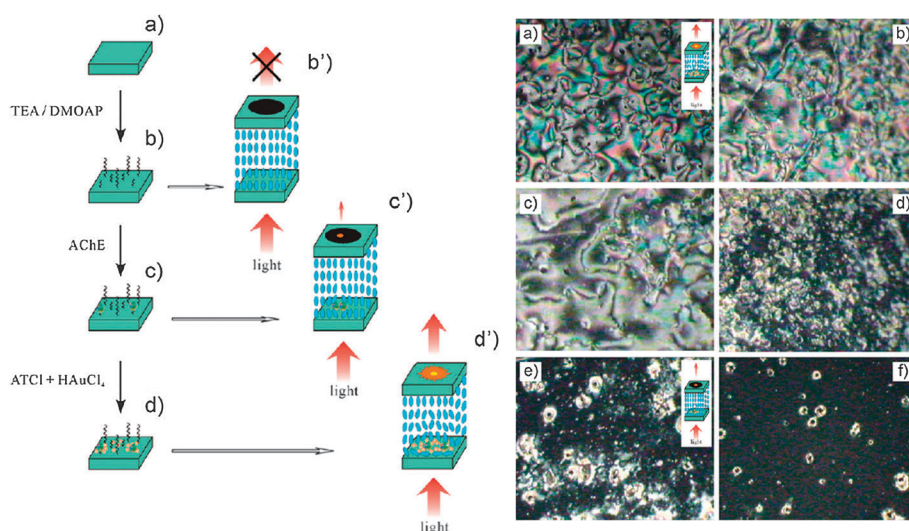


Fig. 17 The left schematic shows the assembly of the liquid crystal biosensor for detection of pesticides: (a) plain glass, (b) self-assembled TEA/DMOAP film, (c) immobilization of AChE, and (d) gold nanoparticles obtained through biocatalytic growth. The right figure shows a series of optical images 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.¹⁹⁴

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.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.^{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 organophosphorus 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).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.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.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 8×10^{-7} M, and the hydrogel encapsulated enzyme conjugates remained stable against denaturing, leaching, and photobleaching when stored under ambient conditions.²⁰¹

Several different transduction strategies have been incorporated using organophosphate hydrolase (OPH) as the biorecognition element including pH electrodes,¹⁷⁸ fluoride ion-sensitive electrodes,²⁰² pH responsive dyes,²⁰¹ pH sensitive field effect transistors (pHFETs),^{203,204} optical techniques,^{205–212}

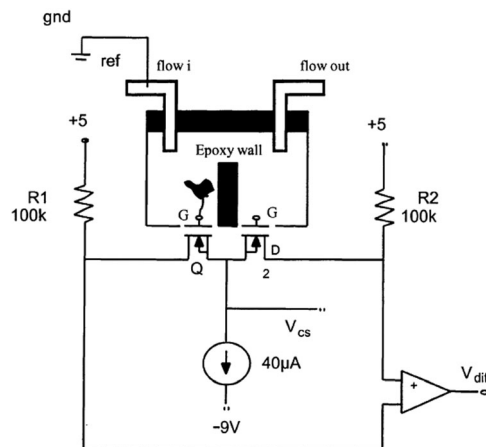


Fig. 18 This scheme shows the circuit diagram for a constant current differential sensor based on two pH sensitive field effect transistors, one with enzyme and the other without. Reprinted with permission from Simonian *et al.*, *Anal. Chim. Acta*, 2001, **442**, 15–23. Copyright © 2001, Elsevier.¹⁸¹

and electrochemical techniques.^{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.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.²⁰⁵

Ji *et al.* immobilized OPH onto quantum dots and used circular dichroism spectroscopy to observe changes in the secondary

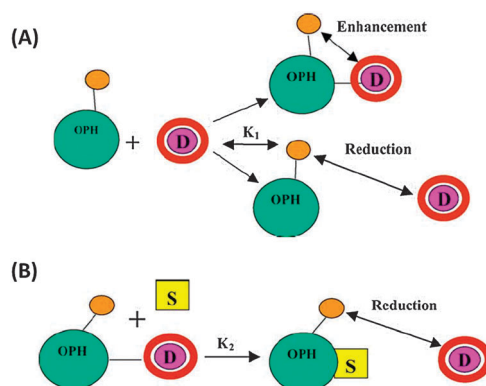


Fig. 19 This scheme shows the decoy–enzyme interaction in the (A) absence and (B) presence of a substrate. (A) The decoy (D) binds to the enzyme–nanogold conjugate (OPH), leading to surface enhanced fluorescence of the decoy. (B) The substrate (S) displaces the decoy (D) from the OPH–gold complex, which causes a decrease in fluorescence signal from the decoy. Reprinted with permission from Simonian *et al.*, *Anal. Chim. Acta*, 2005, **534**, 69–77. Copyright © 2005, Elsevier.²⁰⁵

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 paraoxon, and correlated the overall quenching percentage to the amount of paraoxon, with a detection limit around 10^{-8} M.²¹¹ 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.²¹² 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.^{213–216}

Organophosphate acid anhydrolase

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.^{181,217} 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. Diisopropylfluorophosphate (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.^{181,218} 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 μ M and 20 μ M, respectively, with no response to P-O and P-S bond containing OP pesticides paraoxon and demeton-S.¹⁸¹

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.²¹⁸

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.¹⁹⁹ They also demonstrated the ability of OPH with organophosphate acid anhydrolase (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).¹⁸¹ 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.²⁰⁴

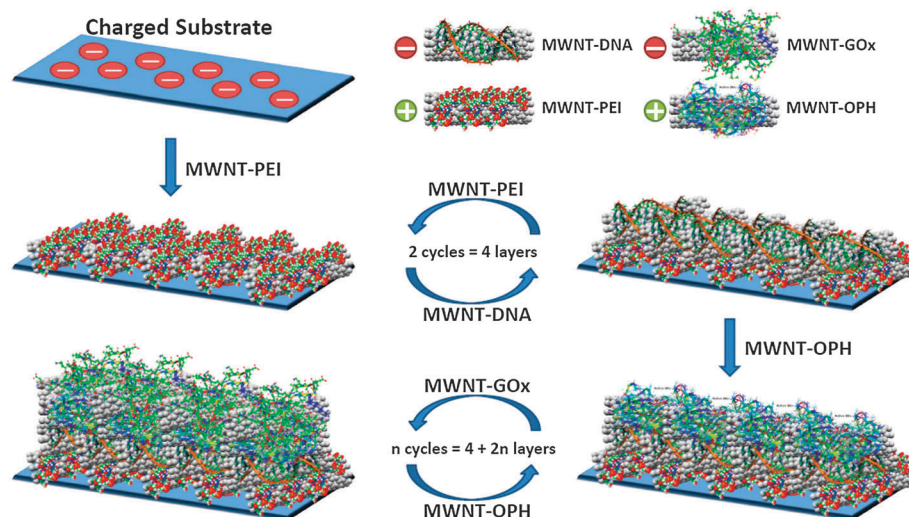


Fig. 20 This schematic illustrates the layer-by-layer assembly process for carbon nanotubes armored with biopolymers and enzymes to build a multi-substrate biosensor. This process can be applied to any system involving oppositely charged species. Reprinted with permission from Kirsch *et al.*, *ECS Trans.*, 2012, **50**, 345–355. Copyright © 2012, The Electrochemical Society.²²⁴

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.²¹⁴ This novel technique has been developed for use in many different biosensors^{214,219–223} 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).²²⁴

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.²²⁵ The diagnosis of HIV-1 infection typically relies on the detection of HIV-1 RNA, capsid antigen p24, or antibodies.²²⁶ 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.^{227,228} 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.^{229–232}

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.^{229,233} 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.^{230,234} It has been demonstrated that p24 antigen

testing assays have great value in early HIV infection diagnosis,²³³ blood screening, and monitoring of antiviral therapy.²³⁵ Furthermore, specificity of the p24 antigen test for HIV detection is approximately 99.9%.²³⁰ 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.^{236–238} 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,²³⁰ resulting in 99% sensitivity, far superior to the conventional ELISA test for detection (20.5%).²³⁰ 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 (Eu³⁺) 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).²²⁹ 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.^{231,239} 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⁻¹.²³²

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.^{241–243} Due to both sensitivity and specificity, this method serves as the gold standard for HIV diagnosis, though it requires professional operation by skilled personnel.²³¹ 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.²⁴⁴ 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).²⁴⁰ By obviating the amplification

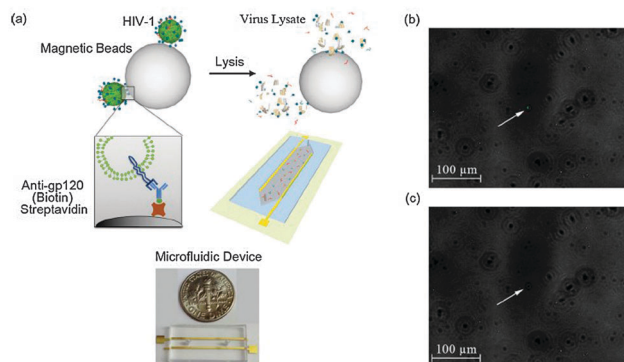


Fig. 21 Scheme of the impedance sensing system and fluorescent/bright-field images of captured HIV-1. (a) 3D schematic illustration of viral capture by anti-gp120 antibody coated magnetic beads and the image of the label-free sensing system containing two rail electrodes; (b and c) fluorescent and bright-field images of captured HIV-1 on magnetic beads, indicated by white arrows. Reprinted with permission from H. Shafiee *et al.*, *Small*, 2013. Copyright © 2013, John Wiley and Sons.²⁴⁰

step (typically required for nucleic acid tests (NAT)),²³¹ this sensing method provides a facile scheme for rapid and low-cost acute stage HIV-1 infection diagnosis.

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.²⁴⁵ 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.²⁴⁶ Similarly, an electrochemical method with ferrocenoyl (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.²⁴⁷ The presence of HIV-1 PR inhibitors such as cytochalasin A²⁴⁸ decreased the observed peak potential, with signal suppression proportional to cytochalasin A concentration, demonstrating the sensor's potential use in drug screening.²⁴⁷ A low-cost and label-free impedimetric method employing magnetic beads was also used for HIV-1 PR detection.²⁴⁹ 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.²⁴⁹

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.^{250–252} 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.²⁵³ 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.^{254,255} Compact electrical sensing formats have also been developed, which typically exploit resistance change and generated impedance pulse.^{256–258} Mechanical sensors that detect the mass increase due to the CD4⁺ cell adhesion by monitoring the variation in resonant frequency offer another simple platform.²⁵⁹ 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.²⁶⁰

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.²⁶¹ 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.²⁶² To reduce sample volume requirements, microfluidic systems for evaluating IFN-γ secretion have been developed.^{251,263} 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.²⁶⁴

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) biosensor²⁶⁶ 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- γ and tumor necrosis factor- α (TNF- α) secreted from human peripheral blood mononuclear cells (PBMCs) were quantified. In a similar manner, antigen specific antibodies from B cells²⁷⁵ and cytokine/antibody production from hematopoietic cells²⁷⁶ 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).

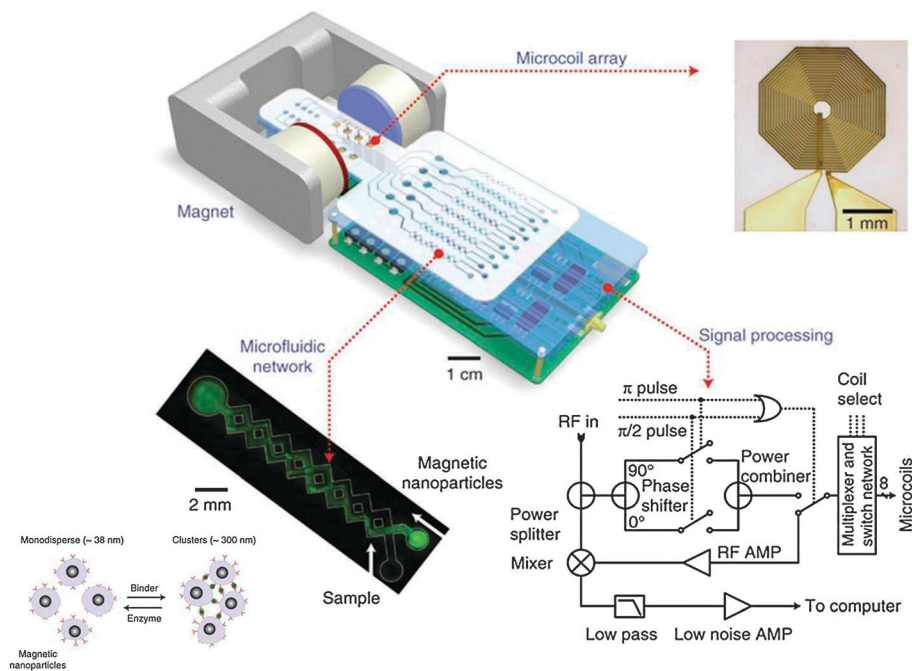


Fig. 22 Schematic diagram of the DMR system structure and working principle. Clustering of nanoparticles mediated by targets results in nuclear spin dephasing efficiency change and decreased spin-spin relaxation time. Reprinted with permission from Lee *et al.*, *Nat. Med.*, 2008, **14**, 869–874. Copyright © 2008, Nature Publishing Group.²⁶⁶

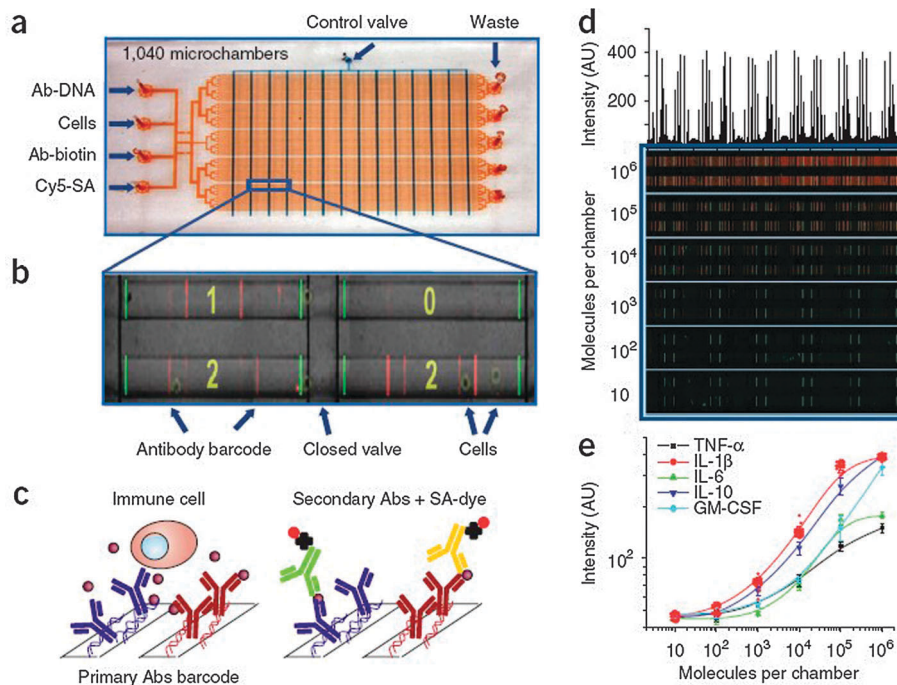


Fig. 23 Layout of the single-cell barcode chip (SCBC) system. (a and b) Optical image of an SCBC system and cells isolated in microchambers; (c) scheme of cytokine detection by antibody barcode array; (d) scanned antibody barcode array; (e) calibration curve for recombinant cytokine proteins. Reprinted with permission from Ma *et al.*, *Nat. Med.*, 2011, **17**, 738–743. Copyright © 2011, Nature Publishing Group.²⁷⁷

To achieve consistent loading, antibodies were incorporated into fluidic channels using a DNA-encoded antibody library (DEAL) method.²⁷⁸ 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.²⁷⁷ 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.²⁷⁹

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.^{280,281} 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} .²⁸²

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.²⁷⁴ Alternative approaches include immobilizing the antibody onto surfaces and monitoring binding *via* surface plasmon resonance (SPR).²⁸³ 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.^{284,285} 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,²⁸⁶ or attenuated electron transfer efficiency and depression of current signal.^{263,287}

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.²⁸⁸ 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.²⁸⁹ Conventional screening typically involves morphological or histological analysis of cells and individual tumor associated antigens.²⁹⁰ High levels of individual markers are associated with certain cancers, such as prostate specific antigen (PSA) and CA125 for prostate and ovarian cancers, respectively.^{291,292} Despite their sensitivity, tests for these markers often lack specificity: both PSA and CA125 concentrations

can be strongly affected by benign diseases.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.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.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.²⁹⁰ These techniques have been utilized to profile molecular signatures for tumor classification. In a prominent example, Golub and colleagues employed Affymetrix Genechip™ 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.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.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.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.²⁹⁸ VEGF is therefore a common target for both diagnosis and therapy for multiple cancers. Several VEGF sensing molecules have been developed, including antibodies²⁹⁹ and peptides,³⁰⁰ however aptasensors have become increasingly popular. Li *et al.* developed an anti-VEGF aptamer microarray, combined with an antibody sandwich type assay for SPR based detection.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 μ M).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.^{303,304} Lee *et al.* successfully detected VEGF concentrations as low as 1.04 pM with an aptamer-modified Si nanowire FET.³⁰³ An FET was later developed based on carboxylated polypyrrole nanotubes (CPNTs) to detect VEGF down to 400 fM concentration.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.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).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).^{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.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.^{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.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).³¹³ Fluorescent peptide biosensors have also been employed for the monitoring of drug resistant CML,³¹⁴ in addition to probing kinase activity in cell lysates for high throughput drug screening.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 10⁹ haematologic cells),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

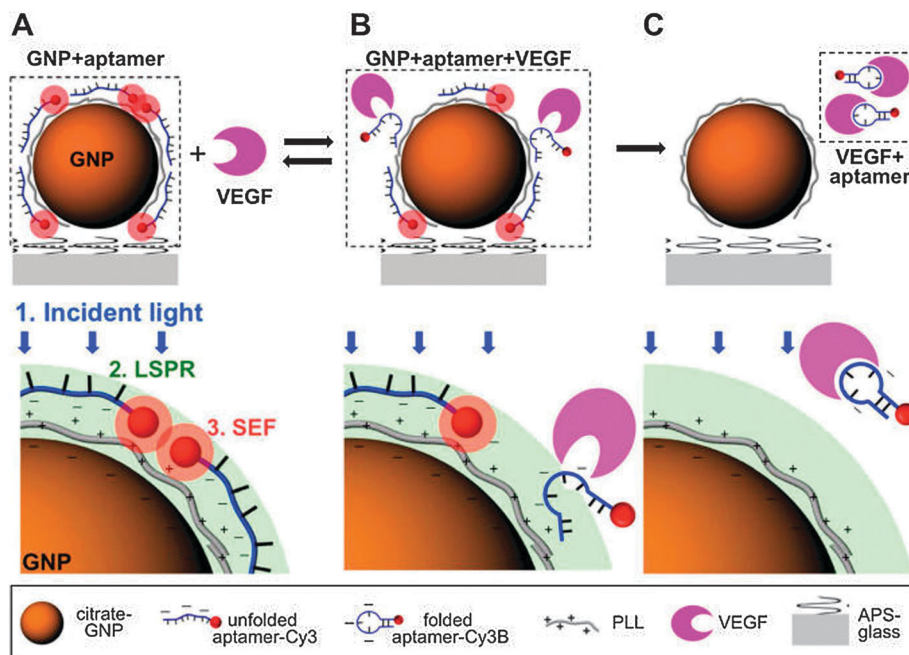


Fig. 24 Schematic representation of the detection mechanism of the aptasensor for VEGF165. (A) In the absence of target molecules, VEGF165, unfolded VEGF165 aptamer is electrostatically bound to a positively charged PLL-coated gold nanoparticle (GNP) surface and surface-enhanced fluorescence (SEF) of Cy3B conjugated with the VEGF165 aptamer is created by both a metal interaction increasing the radiative fluorescent decay rate of Cy3B and the local surface plasmon resonance (LSPR) enhancing the intensity of an incident light. (B) The interaction of the VEGF165 aptamer with its target induces the reversible conformation change of the aptamer and, consequently, the decreased electrostatic binding force. (C) As a result, the target-binding interaction of the aptamer causes the irreversible detachment of the aptamer from the GNP surface and avoids the SEF effect of Cy3B. Reprinted with permission from Cho *et al.*, *ACS Nano*, 2012, **6**, 7607–7614. Copyright © 2012, American Chemical Society.³⁰⁵

available Veridex Cell Search system, which binds antibody coated magnetic nanoparticles to CTCs in whole blood, followed by a sandwich type immunofluorescence assay.³¹⁷ More recently,

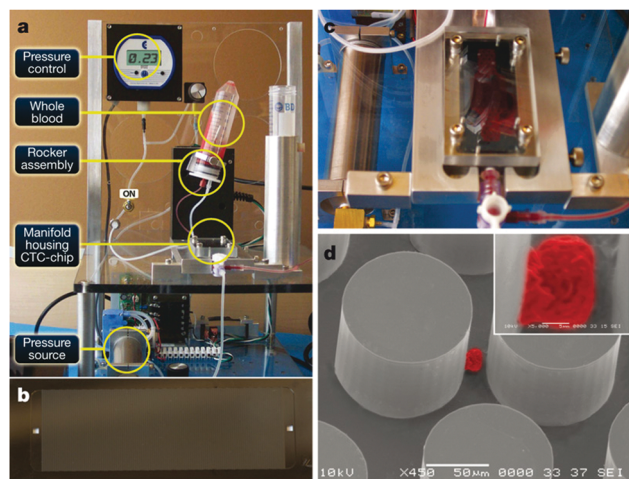


Fig. 25 (a) The workstation setup for CTC separation. The sample is continually mixed on a rocker, and pumped through the chip using a pneumatic-pressure-regulated pump. (b) The CTC-chip with microposts etched in silicon. (c) Whole blood flowing through the microfluidic device. (d) Scanning electron microscope image of a captured NCI-H1650 lung cancer cell spiked into blood (pseudo coloured red). The inset shows a high magnification view of the cell. Reprinted with permission from Nagrath *et al.*, *Nature*, 2007, **450**, 1235–1239. Copyright © 2007, Nature Publishing Group.³¹⁶

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.³¹⁸ Other groups have employed aptamers³¹⁹ and anti-EpCAM mAb³¹⁶ 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.³²⁰ 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.³²¹

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 electrode^{324,325} 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(anilineboronic 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.³²⁷

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.³³⁴ 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 electrochemically^{336–338} 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 μ M 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 detection^{346,347} 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 nanopores 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

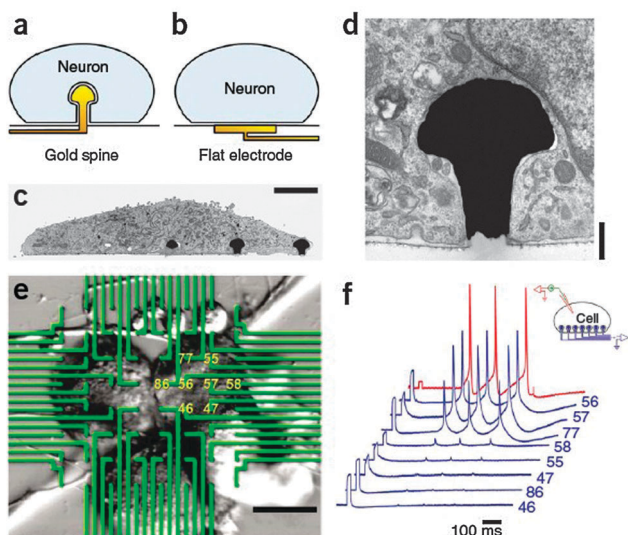


Fig. 26 A mushroom-shaped microelectrode (gMμE) engulfed by a neuron for electrical coupling. (a and b) Schematic illustration of a neuron on a gold-spine electrode (a) and a flat electrode (b); (c) electron micrograph image of the cross section of a cell which engulfs three gold spine electrodes; (d) transmission electron micrograph of a gMμE engulfed by a cell; (e) three neurons cultured on gold spine electrode arrays; (f) action potential signal monitored from 8 spine electrodes. Reprinted with permission from Hai et al., *Nat. Methods*, 2010, 7, 200–202. Copyright © 2010, Nature Publishing Group.³⁶⁵

example is the paper-based analytical device (μPAD),³⁶⁶ 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,³⁶⁷ 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.³⁶⁸ Whitesides and colleagues have recently expanded application of paper-based devices for a wider range of analytes, including glucose and proteins in urine.^{369,370} 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^{371,372} based on the latest advances in biomolecular computing,³⁷³ particularly in enzyme-based logic systems.³⁷⁴ 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.^{375,376} 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,³⁷⁷ particularly with implantable biofuel cells,³⁷⁸ 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 viable 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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