

Chapter 4

Fundamental Principles for Luminescence Sensing Measuring Devices Used for the Detection of Biological Warfare Agents

Joseph H. Banoub and Farid Jahouh

Abstract This chapter surveys the current detection technologies used in commercially available luminescence biosensor detection equipments currently employed for identifying warfare biological agents (BAs). Brief technical descriptions of these technologies are presented with emphasis placed on the principles of detection. Much of the content presented was obtained from the open-source literature and is an introduction to biosensor fundamentals

4.1 Introduction

Biological agents (BAs) are widely found in the natural environment (work place, hospitals, HVAC, etc.) and as a result of voluntary release of biological warfare or terrorism agents [1, 2]. Biological agents include series of different virulent bacteria, viruses, fungi (yeasts and moulds) and parasites. All of these agents possess irreversible threat to potentially cause ill health and death to soldiers/humans. The incidents of anthrax-laced letters, the emergence of Severe Acute Respiratory Syndrome (SARS), and repeated occurrences of illnesses caused by food-borne pathogens highlight the need for rapid and sensitive identification of the responsible biological agents [3, 4].

Biological agents are usually invisible and possess the ability to infect in very small doses [1, 2]. They also have the cunning ability to replicate rapidly and require minimal resources to survive. For these reasons, it is impossible to initially feel their

J.H. Banoub (✉)

Fisheries and Oceans Canada, Science Branch, Special Projects, Chemistry Department,
Memorial University, St John's, NL, Canada
e-mail: banoubjo@dfo-mpo.gc.ca

F. Jahouh

Department of Chemistry, Memorial University of Newfoundland,
St. John's, NF, 232 Elizabeth Avenue, A1B 3X7, Canada

© Springer Science+Business Media B.V. 2017

J.H. Banoub, R.M. Caprioli (eds.), *Molecular Technologies for Detection of Chemical and Biological Agents*, NATO Science for Peace and Security Series A: Chemistry and Biology, DOI 10.1007/978-94-024-1113-3_4

presence or to predict the risks they present. Recently, it has been demonstrated that bioterrorism raises the specter of exposure to toxins by devising new deliveries by which BAs can be weaponized [5–7]. It has become apparent that BAs weapons pose a real and potentially immediate threat as they are relatively cheap to manufacture and employ, and they have tremendous potential impact as terror weapons [2]. These features make biological weapons attractive to rogue states and terrorist organizations. In this article we briefly describe the threat of biological weapons [5–7].

4.1.1 Classification of Biological Agents

Biological Agents are categorized according to the Code of Practice to the Safety, Health and Welfare at Work (Biological Agents) Regulations, 2013 [8]. This classification system is based on whether: the biological agent is pathogenic to humans, represents a hazard to soldiers, it is transmissible to nearby community and if there is a possible effective treatment available [8–11]. Consequently, BAs are classified into four risk groups (RG), namely, RG1, RG2, RG3 and RG4, as follows:

- RG1 that includes BAs not linked with diseases in healthy adult humans. Examples of RG1 agents include *Bacillus subtilis* or *Bacillus licheniformis*, *Escherichia coli*-K12, and adeno-associated virus (AAV) types 1 through 4 [9, 10].
- RG2 that includes BAs connected with human diseases which is rarely serious and for which preventive or therapeutic interventions are often available. Examples of RG2 agents include *Salmonella* sp., *Chlamydia psittaci*, measles virus, and hepatitis A, B, C, D, and E viruses [9, 10].
- RG3 that contains BAs associated with serious or lethal human diseases for which preventive or therapeutic interventions may be available (high individual risk but low community risk). Examples of RG3 agents include *Brucella*, *Mycobacterium tuberculosis*, *Coccidioides immitis*, yellow fever virus and human immunodeficiency virus (HIV) types 1 and 2 [9, 10].
- RG4 includes BAs that are likely to cause serious or lethal human disease for which preventive or therapeutic interventions are *not usually* available (high individual risk and high community risk). RG4 agents only include viruses. Examples of RG4 agents consist of Crimean-Congo hemorrhagic fever virus, Ebola virus and herpesvirus simiae (B-virus). Under the classification system, These Risk Group 1 agents are the least hazardous whilst Group 4 are the most hazardous [9, 10].

4.2 Analytical Measurement of Biological Agents

Field BAs and urban public health surveillance systems usually provide rapid determination of the presence of BAs in the atmosphere and may in time provide an indication of when and where the biological agent was released [11–15]. Once, the

point source is revealed and the BA identified, rapid clean-up effort could be initiated, after the release of the compound. Moreover, it is essential to monitor the presence of BAs in the environment, to provide people at risk with the means of rapidly identifying contaminated air, water, food and equipment [11–15]. It is well known that antibodies specifically targeting proteins or pathogens, can be generated to a wide variety of target cell bacterial analytes and are the most popular choices for the recognition element in many biosensors. Typical immunoassay formats include competitive, displacement, sandwich, and enzyme-linked immunosorbent assays (ELISA) [14]. ELISA, for example, uses colorimetric or chemiluminescent enzyme substrates for signal transduction and is more suited to automated instruments because of the multiple incubations and washes required.

The classical approach to detect the bacteria species type or microbe involves the use of differential metabolic assays, monitored colorimetrically and on immunochemical routine tests. In addition, the use of cell culture and electron microscopy are vital for the diagnosis of viruses, bacteria and intracellular parasites. Consequently, all samples retrieved from the affected environment must be cultured in order to obtain sufficient numbers of various cell types for reliable identification. Unfortunately, one of the major drawbacks is that the time required for the microbial outgrowth is long and some bacteria obtained are not culturable, as a result of genetic mutation. This chapter has been drafted as a “Comptes Rendus” and general *modi operandi* of novel biodetection approaches using luminescence biosensing approaches, published recently, for the detection of warfare potential BA weapons; it will exclude all the conventional approaches. The readers are encouraged to peruse the references included within the text, to search for the applied commercial used biosensors, that for simplification reasons, we did not discuss herewith.

4.2.1 What Are Biosensors?

According to a recently proposed IUPAC definition 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 (biochemical receptor) which is in direct spatial contact with a transducer element [15]. A biosensor should be clearly distinguished from a bioanalytical system, which requires additional processing steps, such as reagent addition. The term “biosensor” is short for “biological sensor.” The device is made up of a transducer and a biological element that can be an enzyme, an antibody or a nucleic acid. The bioelement interacts with the analyte being tested and the biological response is converted into an electrical signal by the transducer (Fig. 4.1) [16, 17].



Fig. 4.1 Schematic representation of the detection of a bioelement using a biosensor

4.2.2 Overview of Predominant Sensing Techniques

Sensing can be explained as the use of recognition elements (biological in origin) for binding to the biothreat molecule of interest. The binding event must be transduced in a manner that signals the presence of the targeted analyte. Biosensor probes are becoming increasingly sophisticated, mainly owing to combination of advances in two technological fields: microelectronics and biotechnology. Biosensors are highly valuable devices in measuring a wide spectrum of BA analytes [18, 19].

Ideally each sensing detection technology should contain the following characteristics:

- Specific and able to discriminate between closely related pathogenic and non-pathogenic organisms or toxins.
- Sensitive and able to detect small amounts of target within a high background matrix.
- Possess high affinity and being able to maintain binding even through repeated washing steps.
- Stable enough to allow long-term use.

It should be understood that luminescence biosensing technology is totally distinct from other physiochemical methods, such as mass spectrometry (MS) or Fourier transform infrared spectroscopy (FTIR) and Raman based analysis. These methods of course, have their merits and are very sensitive and specific. Therefore, in the following sections, we will provide an overview of the state-of-the-art prime molecular sensing technologies for the detection of BAs.

4.3 Electrical Detection of Pathogenic Bacteria via Immobilized Antimicrobial Peptides

It should be noticed that the current methods for detecting pathogenic bacteria, which include ELISA and PCR [20, 21] are assays that exploit antibodies as molecular recognition elements due to their highly specific targeting of antigenic sites. Nonetheless, these antibodies lack the stability needed to detect pathogenic species under harsh environments, and requires a one-to-one pairing of antibody-based sensors for each target to be detected. Whereas, nucleic acid probe-based techniques such as PCR can reach single-cell detection limits, they still require the extraction of nucleic acids and are limited in portability [21, 22].

McAlpine and coworkers developed a robust and portable biosensor for the detection of pathogenic bacteria that could impact water quality monitoring for bacterial contamination [23–25]. The particular interest of the developed biosensor was that it combined the natural specificity of biological recognition to label-free sensors providing sensitive electronic readout. Thus, McAlpine et al. reported the selective and sensitive detection of infectious agents via electronic detection based on antimicrobial peptide-functionalized microcapacitive electrode arrays [25].

The semi-selective antimicrobial peptide magainin I, which occurs naturally on the skin of African clawed frogs, was immobilized on gold microelectrodes via a C-terminal cysteine residue. Significantly, exposing the sensor to various concentrations of pathogenic *Escherichia coli* revealed detection limits of approximately 1 bacterium/ μL , a clinically useful detection range. The peptide-microcapacitive hybrid device was further able to demonstrate both Gram-selective detection as well as interbacterial strain differentiation, while maintaining recognition capabilities toward pathogenic strains of *E. coli* and *Salmonella* [25].

It is well known that the synthesis of antimicrobial peptides (AMPs) and their resulting intrinsic stabilities render them particularly interesting candidates for the use as molecular recognition elements in electronic biosensing platforms [23–25]. AMPs do exist in nature and are located either in the skin of higher organisms and/or in the extracellular milieu of bacteria [25]. The replacements of current antibody-based affinity probes, with more stable and durable AMPs in biological sensors, have a major advantage as recognition elements. This advantage stems from the AMPs semi-selective binding nature to target cells of a variety of pathogens.

The bioactivity of AMPs toward microbial cells can be classified into groups according to their secondary structures [24, 25]. Many AMPs adopt amphipathic conformations that spatially shield the hydrophobic group of the cationic amino acids, thereby targeting the negatively charged head groups of lipids in the bacterial membrane. In contrast, the membranes of plants and animals separate negative charges to the inner leaflet and contain cholesterol that reduce AMP activity [25]. The AMPs, linear cationic peptides such as magainins, are particularly attractive for microbial sensing applications because of their small molecular size and intrinsic stability [26, 27]. In particular, the positively charged AMP magainin I (GIGKFLHSAGKFGKAFVGEIMKS) binds most selectively to the bacterial cell *E. coli* O157:H7 as a precursor to bactericidal activity [25–27].

4.3.1 Development of an AMP-Based, Label-Free Electronic Biosensor

Magainin I displays a broad-spectrum activity toward other Gram-negative bacteria, which comprise the majority of pathogenic infection in humans [25–28]. The first step toward the development of an AMP-based, label-free electronic biosensor consisted of targeting the microbial cells by magainin I using impedance spectroscopy.

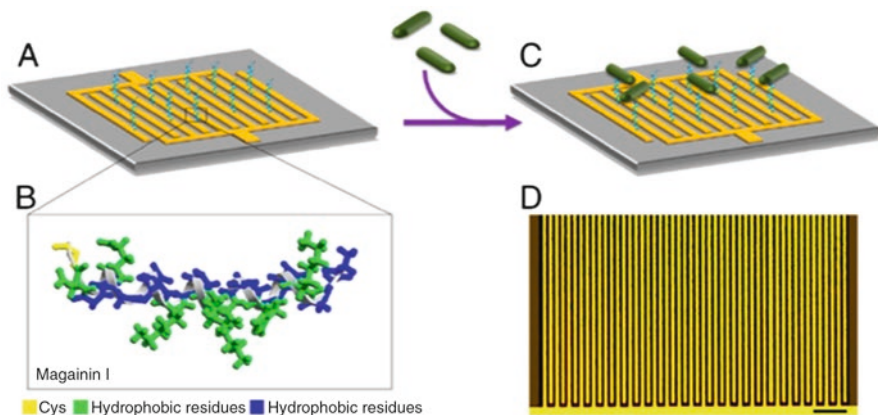


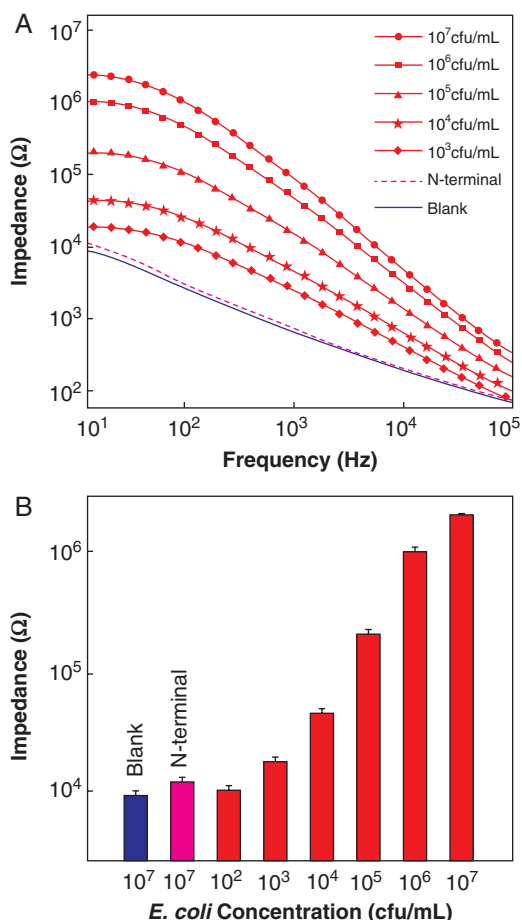
Fig. 4.2 AMP-based electrical detection of bacteria (Adapted from Ref. 25).

It should be repeated that electrical impedance measures the total opposition to a circuit or a part of the circuit presented to an electric current. Usually the impedance is the results for both resistance and reactance. It is important to remember that the resistance component arises from collisions of the current-carrying charged particles with the internal structure of the conductor. Furthermore, the reactance component is an additional opposition to the movement of the electric charge that result from the changing magnetic and electric fields in circuits carrying alternating current.

Figure 4.2 outlines the sensing platform. First, the AMPs are immobilized on the micro-fabricated interdigitated gold electrodes (Fig. 4.2a). It should be specified that magainin I contains a cysteine residue on the C terminus (Fig. 4.2b), which allows the facile site-specific covalent attachment to the gold electrodes. Next, the heat-killed bacterial cells are injected and incubated on the AMP-modified electrodes [25]. When, the bacteria are recognized by the AMPs, binding will ensue (Fig. 4.2c), causing the dielectric property to changes that can be monitored by a spectrum analyzer. Usually, the impedance is measured over a frequency range of 10 Hz to 100 kHz. Figure 4.2d shows an optical micrograph of the device, which is made using standard microfabrication techniques.

The results of measurements performed after incubation of the immobilized AMPs with pathogenic *E. coli* O157:H7 cells concentrations ranging from 10^3 to 10^7 cfu/mL are shown in Fig. 4.3. When, a blank device with no immobilized AMPs was also tested for comparison, it was found that there was no change in the impedance of the blank device without immobilized AMPs, upon exposure to various bacterial concentrations. Figure 4.3a shows that at low frequencies, the different concentrations of bacterial cells have the effect of increasing the impedance in proportion to the number of cells present in the sample. As the frequency increases, the contribution to the impedance from the bacterial cells decreases, leaving only the dielectric relaxation of small dipoles including water molecules in the buffer solution to affect the measured impedance. Figure 4.3b depicts the impedance change at a fixed frequency of 10 Hz. The variation in the impedance is directly proportional

Fig. 4.3 Impedance spectra and sensitivity of the AMP electronic biosensor (Adapted from Ref. 25)



to the number of bacterial cells bound to the immobilized AMPs and manifested in a logarithmic increase with respect to serially diluted bacterial concentrations. Significantly, the detection limit of response of the hybrid AMP-microelectrode device to *E. coli* was found to be 10^3 cfu/mL (1 bacterium/ μ L). This lowest limit of detection appears to be limited by the presence of impedance due to the electrical double layer resulting from the electrode polarization effect at low frequencies. Notably, these sensitivity limits is clinically relevant [30] and compares favorably to AMP-based fluorescent assays [26], antibody-based impedance sensors [27], and to the LAL test [25].

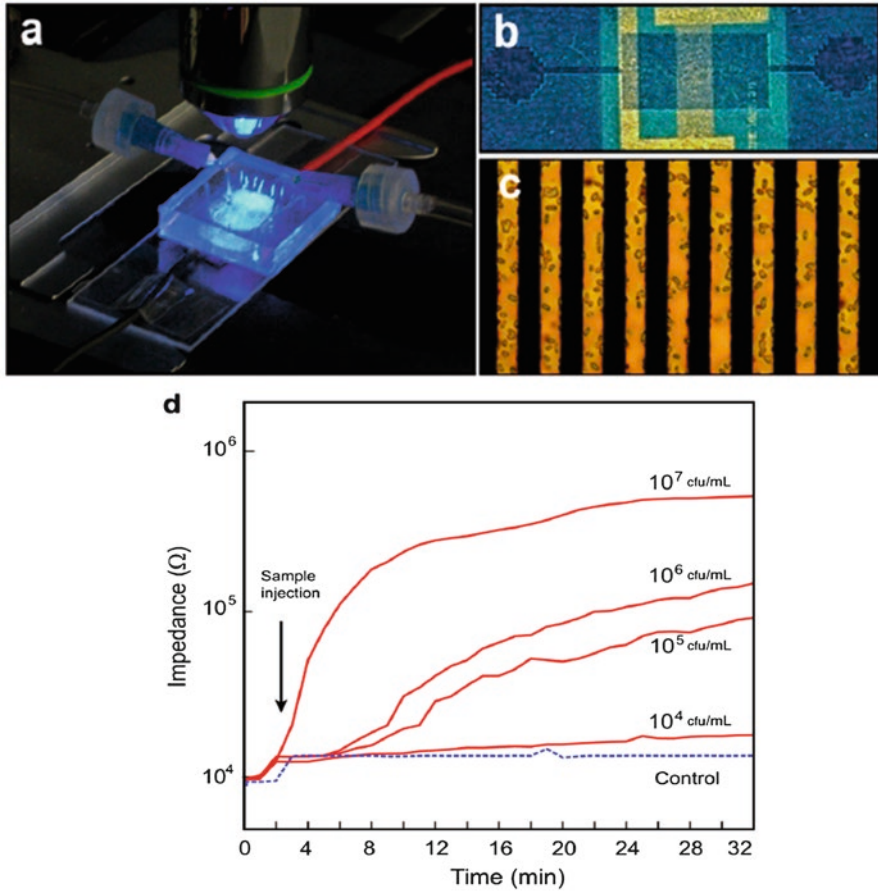


Fig. 4.4 Real-time binding of bacteria to AMP biosensors (Adapted from Ref. 25)

4.3.2 Pathogenic Bacteria Real-Time Detection

To simulate the use of the AMP microelectrodes in everyday applications, such as direct water sampling, the biosensor response was investigated in real time, as shown in Fig. 4.4. First, a microfluidic cell was bonded to the interdigitated biosensor chip (Fig. 4.4a), such that the electrodes were perpendicular to the direction of the sample flow (Fig. 4.4b) [25].

Next, the fluid was injected using a syringe pump connected to the inlet port and allowed to flow through to the outlet port at a flow rate of $100 \mu\text{L}/\text{min}$. The flow cell was first flushed with buffer to establish a baseline. This was followed by injection of various dilutions (10^4 – 10^7 cfu/mL) of the pathogenic *E. coli* cells in PBS to the channel at a reduced flow rate of $5 \mu\text{L}/\text{min}$ for 30 min. For example, Fig. 4.4c shows the microelectrode array after exposure to 10^7 cfu/mL bacterial cells.

Simultaneously, the impedance response was continuously monitored during the sample flow-through process (Fig. 4.4d).

All samples produced a measurable response relative to the control sample within 5 min, with the highest concentration sample yielding a response within 30 s; the responses saturated after after 20 min. These results augured well for the implementation of this sensor in continuous monitoring of flowing water supplies.

4.3.3 Selectivity Measurements

McAlpine also examined the selectivity of the AMP-functionalized biosensors toward the following various bacterial species (i) Gram-negative pathogenic *E. coli* O157:H7, (ii) the nonpathogenic *E. coli* strain American Type Cell Culture (ATCC) 35,218, (iii) Gram-negative pathogenic *Salmonella typhimurium*, and (iv) the Gram positive *Listeria monocytogenes*. The selectivity was first investigated using fluorescent microscopy methods, by staining bacterial cells and optically mapping their binding density to gold films hybridized with AMPs. The discriminative binding patterns of immobilized magainin I to and the surface density of the various bacterial cells (all 10^7 cfu/mL) stained with propidium iodide (PI) nucleic acid stain are showed in Fig. 4.5. In summary, coupling of AMPs with microcapacitive biosensors has resulted in the implementation of a portable, label-free sensing platform for the detection of infectious agents. The achievable sensitivity approached 1 bacterium/ μL -a clinically relevant limit-and the AMPs allowed for sufficient selectivity to distinguish pathogenic and Gram-negative bacteria, while retaining broad-band detection capabilities [25–28]. In addition, the simulated water sampling chip which consisted of a microfluidic flow cell integrated onto the hybrid sensor, demonstrated the potential of real-time on-chip monitoring of the interaction of *E. coli* cells with the antimicrobial peptides [25].

4.4 NRL Array Biosensor for Toxin Detection

This following part discusses the progress made with the NRL Array Biosensor, which is a portable instrument for rapid and simultaneous detection of multiple targets which was developed, automated and miniaturized for operation at the point-of-use by the US Navy Research Laboratories. This Array Biosensor has been used for the quantitative immunoassays against an expanded number of toxins and toxin indicators in food and clinical fluids, and for its usefulness as semi-selective molecules which can be used as alternative recognition moieties [29–31]. In this sensor, the antibodies or other capture molecules are immobilized in a two-dimensional array on an optical waveguide (as either stripes or spots) and standard fluoroimmunoassays are performed within the channels of a multi-channel flow cell, which is placed on the waveguide surface (Fig. 4.6, left). The spots are interrogated using

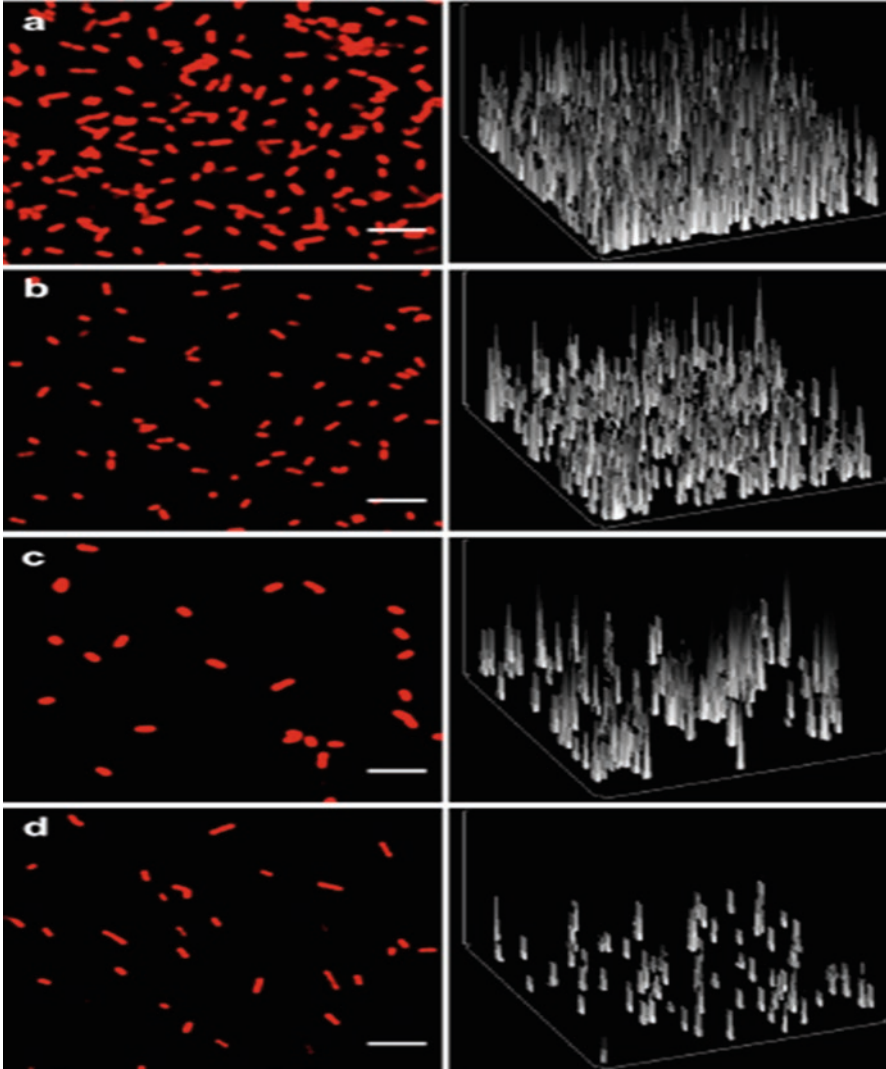


Fig. 4.5 Optical microscopy of the selectivity of AMPs (Adapted from Ref. 25)

evanescent wave technology: light from a 635 nm diode laser is focused into the edge of the patterned slide/waveguide and after propagation and mixing within the waveguide; the confined beam produces an evanescent field within the sensing portion of the waveguide [29]. In the NRL array biosensor the spots are interrogated using evanescent wave technology that is specifically: a light issued from a 635 nm diode laser that is focused into the edge of the patterned slide/waveguide and after propagation and mixing within the waveguide, the confined beam produces an evanescent field within the sensing portion of the waveguide [29, 32]. The definition of

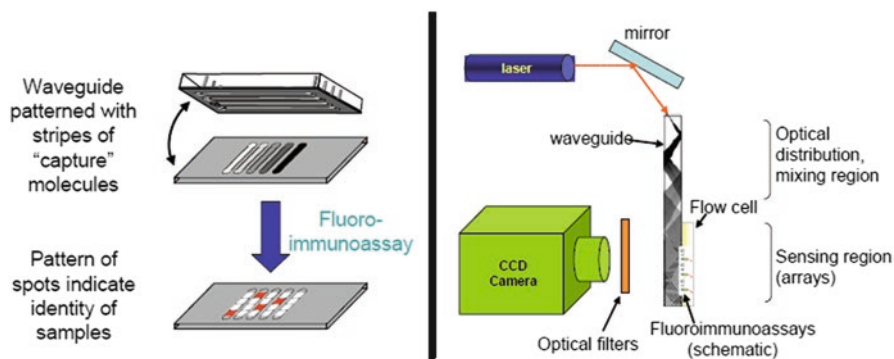


Fig. 4.6 NRL array biosensor (Adapted from Ref. 29)

an evanescent field or wave is: an oscillating electric and/or magnetic field, which does not propagate as an electromagnetic wave but whose energy is spatially concentrated in the vicinity of the source (oscillating charges and currents) [32].

The Surface bound molecules labeled with fluorophore are excited by this evanescent field, producing a fluorescence signal; this fluorescence is then detected using a CCD camera fitted with appropriate bandpass and longpass filters (Fig. 4.6, right). Since the penetration depth of the evanescent field is limited, only surface-bound fluorophores are excited, enabling analysis of non-homogeneous or turbid samples. The locations and intensities of the fluorescent spots indicate the identity and concentration of the target sample in each lane [29, 32].

The NRL Array Biosensor was used successfully for the detection of toxins and for multiple toxins simultaneously in multiple samples, it could also detect toxin levels as low as 500 pg/mL and quantify the toxin concentration, and finally it could perform toxin assays in clinical, food, and environmental samples [29]. Furthermore, both sandwich immunoassays for protein toxins (e.g., staphylococcal enterotoxin B [SEB] and ricin) and competitive immunoassays for low molecular weight toxins (e.g., trinitrotoluene and fumonisin B1) were reported (Fig. 4.6) [29].

4.4.1 Toxins Environmental Testing in Food and in Air

Determination of bacteria and large toxins in foods and air by the NRA Array Biosensor Assays normally employ a sandwich immunoassay format. However, mycotoxins are smaller in size and are therefore better assayed using an indirect competitive immunoassay [32–35].

The validity of the NRL Array Biosensor was demonstrated for the detection of mycotoxins (ochratoxin A, deoxynivalenol and aflatoxin B1) in various food matrices and in air [32–35]. This competitive assay protocol involved attaching the biotinylated mycotoxin derivatives onto the waveguide, this was followed by incubating

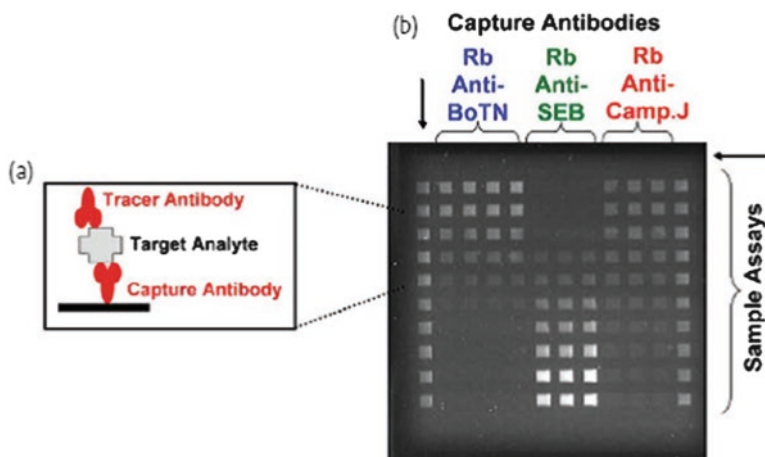


Fig. 4.7 Immunoassay with the NRL array biosensor using the sandwich immunoassay format (Adapted from Ref. 29)

the test sample with cyanine 5 (Cy5)-labeled anti-toxin antibodies and then passing the pre-incubated mix over the immobilized mycotoxin derivatives (Fig. 4.7). Since, the immobilized mycotoxin derivatives competed with the toxin in the test sample for binding to the fluorescent antibodies; it was found that the resulting fluorescent signal of the immunocomplex on the waveguide surface was inversely proportional to the concentration of toxin in the sample (decrease in signal with increasing concentration [32–35].

This type of Array Biosensor has been automated and miniaturized for operation as point-of-use quantitative immunoassay arrays. This methodology was utilized to measure an expanded number of toxins and toxin indicators in food and clinical fluids. In addition, semi-selective recognition molecules were also used to expand the repertoire of toxins that can be detected on a single array. In the automated system, up to 6 samples can be analyzed simultaneously while the non-automated system can test up to 12 samples [30B].

4.4.2 Use of Antimicrobial Peptides for Toxin Detection

Taitt and coworkers investigated the possibility to use antimicrobial peptides (AMPs) for the detection of inactivated botulinum toxins A, B, and E as well as other toxins in assays analogous to the AMP-based bacterial assays [36]. They observed clear differences in the patterns of binding between botulinum neurotoxoids A, B, and E. It was found that the detection limits were improved when immobilized AMPs were used for target capture [36].

4.5 Surface Acoustic Wave (SAW) Sensors

The Love wave (LW) physical effect was originally discovered by the mathematician Augustus Edward Hough Love [37, 38]. Typically, LW sensors consist of a transducing area and a sensing area. The transducing area consists of the interdigital transducers (IDTs), which are metal electrodes, sandwiched between the piezoelectric substrate and the guiding layer [39–41]. The input IDT is excited electrically (applying an rf signal) and launches a mechanical acoustic wave into the piezoelectric material which is guided through the guiding layer up to the output IDT, where it gets transformed back to a measurable electrical signal (Fig. 4.8).

It should be understood that the sensing area, is the area of the sensor surface, located between the input and output IDT, which is exposed to the analyte. Consequently, to permit the use of a SAW device as a biosensor, the device has to be coated with a biospecific layer corresponding to the analyte. The immobilization chemistry strongly depends upon the underlying SAW substrate with or without a guiding layer and hence on the chemical environment available. Gold surfaces, for example, allow the use of functionalized thiols, whereas quartz or SiO₂ surfaces enable the use of various silanes [42]. Analyte-specific molecules (e.g., antibodies) are immobilized on the SAW device to catch analyte molecules (e.g., antigens) from the sample stream. Analytes binding to the immobilized capture molecules will influence the velocity of the SAW and hence the output signal generated by the driving electronics [39].

SAW detectors have the ability to identify and measure many BAs simultaneously and are relatively inexpensive, making them a popular choice amongst civilian response units [39–42]. SAW detect changes in the properties of acoustic waves as they travel at ultrasonic frequencies in piezoelectric materials. The basic transduction mechanism involves interaction of these waves with surface-attached matter. Multiple sensor arrays with multiple coatings and pattern recognition algorithms provide the means to identify agent classes and reject interferant responses that could cause false alarms [39–43].

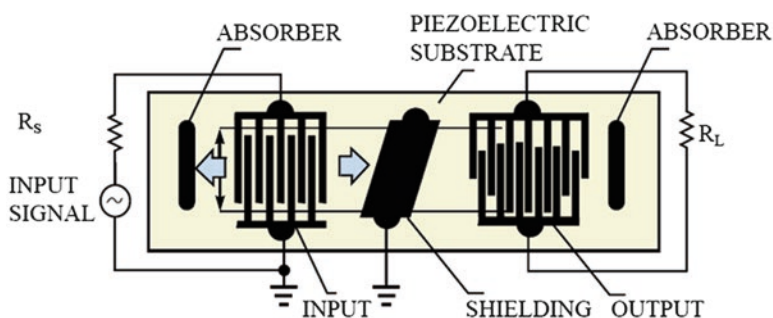


Fig. 4.8 Surface Acoustic Wave Sensor (SAW) (Adapted from Ref. [40])

Recently, SAW immunosensors were successfully applied to detect *E. coli*, *Legionella*, the anthracis simulant B8 *Bacillus thuringiensis* (B8) and M13 bacteriophage (M13) acting as model analyte for bacteria or viruses. Tamarin et al. used Love wave sensors based on quartz substrate with a SiO₂ wave-guiding layer. Antibodies against M13 were immobilized on the sensor surface to detect the bacteriophage directly [43].

Stubbs et al. developed a SAW immunoassay for the detection of analytes in the gas phase, e.g., cocaine plumes [44]. For this purpose, SAW devices based on quartz were used. Antibodies were coupled to the SAW device via adsorbed protein A and coated with a hydrogel layer to overcome the problem of hydration of the biomolecule [44]. Benzoylcegonine, the major metabolite of cocaine, could be detected in vapor [45]. In general, it can be specified that SAW based biosensors offer the possibility of observing real-time binding events of proteins at relevant sensitivity levels [45, 46].

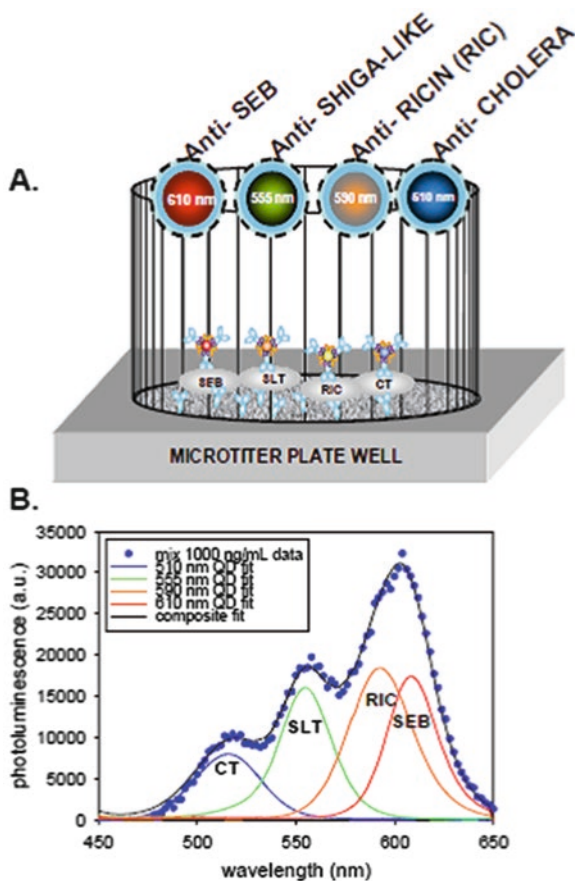
4.6 Biosensing with Luminescent Semiconductor Quantum Dots

Recently, luminescent semiconductor nanocrystals or quantum dots (QDs) have become a successful novel nanomaterial possessing unique photophysical fluorescent properties, which have helped create a new generation of robust fluorescent biosensors [47–53]. It should be stated that the fluorescent properties of QDs have overcome most of the liabilities of conventional organic and protein-based fluorophores. The biosensing QD properties of interest include high quantum yields, broad absorption spectra coupled to narrow size tunable photo-luminescent emissions and exceptional resistance to both photo-bleaching and chemical degradation. In this section, we will examine the progress in adapting QDs for several predominantly in vitro biosensing applications including use in immunoassays, as generalized probes [47–53].

4.6.1 Immunoassays Using Quantum Dots

The unique advantages of using QDs are owed to their inherent photostability, their improved sensitivity, and size tunable photoluminescence coupled to their broad absorption spectra. These unique advantages have allowed them to serve as multi-color or multiplexed immunoassays. It should be mentioned that in terms of coupling QDs to antibodies, the most common method reported in the literature utilizes biotin-avidin interactions [48]. The avidin/streptavidin coated QDs are commercially available, while biotin-labeling of antibodies are usually prepared in-house. QDs presenting available carboxylic acids from their capping agents may also be covalently attached

Fig. 4.9 QD multiplexed biosensing (Adapted from Ref. 54)



to the epsilon amine of an antibody's lysine residues by using EDC/NHS coupling chemistry [47–53]. Alternatively, simple electrostatic interactions can be used depending on the overall protein charge at the pH of conjugation [48].

Goldman et al. used sandwich immunoassays for the simultaneous detection of four toxins: cholera toxin, ricin, shiga-like toxin 1 and staphylococcal enterotoxin B (SEB), in a single microtiter well, see Fig. 4.9 [47, 48, 54].

In this assay capture antibodies immobilized in a microtiter well plate were first exposed to the mixed toxin sample. Antibodies specific for each of the toxins coupled to a different color QD were then added to the microtiter well plate. The resulting signal from the mixed toxin samples was then deconvoluted using a simple algorithm. Similarly, QD-antibody bioconjugates were used to identify and differentiate between diphtheria toxin and tetanus toxin proteins which were non-specifically immobilized onto poly-L-lysine coated cover slips and for the simultaneous detection of *Escherichia coli* O157:H7 and *Salmonella typhimurium* bacteria using different colored QDs as immunoassay labels [55].

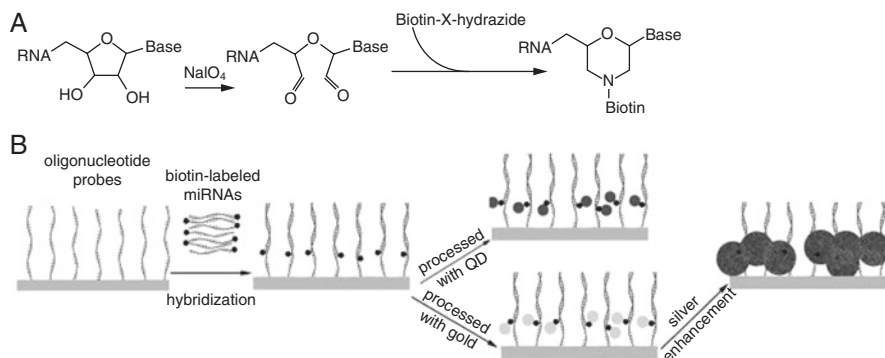


Fig. 4.10 Schematic principles of the miRNA profiling microarray (Adapted from Ref. 53)

4.6.2 Nucleic Acid Detection

Liang et al. applied QDs to miRNA microarray assays by using streptavidin QDs probes to label biotinylated miRNA targets derived from rice, see Fig. 4.10 [53]. Initially, miRNAs were oxidized with sodium periodate to oxidize the ribose 2'- and 3'- hydroxyl groups into aldehydes. The resulting dialdehyde was then reacted with biotin-X-hydrazide resulting in biotinylated miRNA. This was followed by immobilizing the 5' amine-modified oligonucleotide probes antisense to miRNAs on the amine-reactive glass slides. The biotinylated miRNAs were captured on the microarray by oligonucleotide probes in hybridization. Quantum dots were labeled on the captured miRNAs through the strong specific interaction of streptavidin and biotin. As QDs have a high extinction coefficient and a high quantum yield, so trace amounts of miRNAs are easily detected with a laser confocal scanner. In addition as alternative, the colorimetric gold–silver detection method was used in which captured miRNAs were labeled with streptavidin-conjugated gold followed by silver enhancement. During silver enhancement, the gold nanoparticles bound to miRNAs catalyzed the reduction of silver ions to metallic silver, which further autocatalyzed the reduction of silver ions to form metallic silver precipitation on gold, resulting in a signal enhancement [53]. This process allowed straightforward detection of the microarray with an ordinary charge-coupled device (CCD) camera mounted on a microscope.

They found that QD probes provided good sensitivity down to sub-femtomolar concentrations and dynamic range over several orders of magnitude. This was far better than other dye-based methods and further obviated the use of amplification while allowing a semi-quantitative comparison of the amount of miRNA in different samples [53].

Figure 4.11 displays a set of images for various concentrations of miRNA (21 nt siRNA) detected by QD [53]. It should be noted that the signals become gradually weaker with the decrease in miRNA concentration (Fig. 4.11a). When the miRNA concentration was as low as 39 pM, the fluorescence signal could be detected, indi-

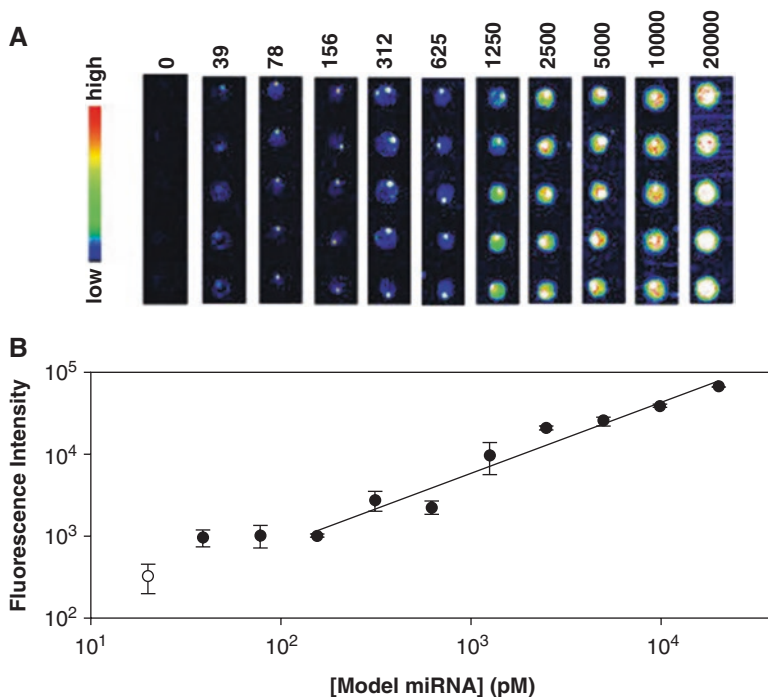


Fig. 4.11 Detection limit and dynamic range of the model miRNA detection microarray. (a) Image sets of microarrays hybridize with various concentrations of miRNAs from 20 nM to 39 pM and the background. The 50 μ M concentration of oligonucleotide probes printed on slides pentaplicately. The volume of model miRNA needed to hybridize with microarray was 10 μ l. (b) Correlation between fluorescence intensity of spots and concentrations of model miRNA. The values were calculated from image in (a). Open circle represents the background (Adapted from Ref. 53).

cating that the lower detection limit of miRNA microarrays is at least 0.4 fmol. As shown in Fig. 4.11b, the fluorescence intensity of the spots is linear to the model miRNA in a logarithmic fashion from 156 to 20,000 pM, and the dynamic range is about 2 orders of magnitude. This implies that this method can be used to quantify miRNAs with broad concentration range [53].

4.6.3 Sensing Based on FRET with Quantum Dot Bioconjugates

Fluorescence Resonance Energy Transfer (FRET) is a physical radiationless transmission of energy phenomenon which relies on the distance-dependent transfer of energy from a donor molecule to an acceptor molecule. FRET has been extensively used in biophysical and biochemical studies to probe ligand-receptor binding and molecular structural changes [56–58]. In the following example, the authors

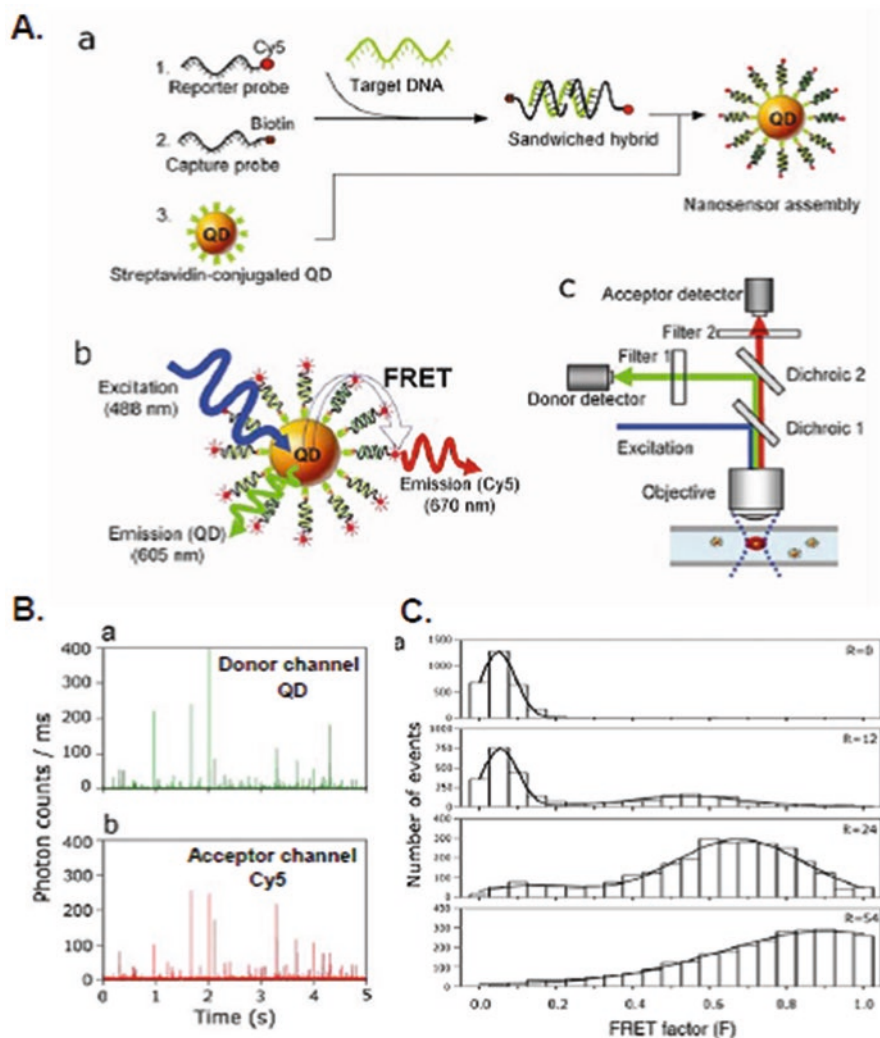


Fig. 4.12 Single QD-based DNA nanosensor (Adapted from Ref. 54)

incubated the dye-labeled DNA targets with biotinylated capture DNA probes, which were allowed to be conjugated to streptavidin QDs, only when the two DNA sequences hybridize. The resulting hybridization was then detected via FRET hybridization between the QD and the dye acceptor (Fig. 4.12) [54]. It should be noted that the additional background caused by the acceptor direct excitation is virtually eliminated through the choice of an appropriate excitation wavelength; this led to a 100-fold improvement in sensitivity compared to single organic dye molecular beacon-based detection. This type of sensing schemes can also be amenable to use in a multiplex format. The narrow and symmetric QD emissions allow easy spectral

deconvolution and the most straightforward configuration relies on several QD populations interacting with the same dye acceptor, rather than the opposite [54].

4.7 Engineered Cell-Based Sensors: The CANARY System

Petrovick et al. have developed a novel inexpensive genetically engineered white-blood cells biosensor for the rapid identification of warfare BA pathogens and toxins [59]. This new sensor was named and abridged as CANARY for “Cellular Analysis and Notification of Antigen Risks and Yields”. CANARY sensors are capable to detect soluble protein toxins, which are an important class of potential bioweapon, and can also be used for sequencing DNA and RNA [60].

4.7.1 CANARY Bioelectronic Sensor

The CANARY technology is based on genetically engineered white blood B cells, which has the ability to bind to and to recognize pathogens quickly and assists other parts of the immune system to fight the infection. It is well known that B cells are the fastest known pathogen identifiers (intrinsic response less than 1 sec). The B-lymphocytes recombinantly express cytosolic aequorin, a Ca-sensitive bioluminescent protein from the jellyfish *Aequoria victoria*, which emits light in response to elevations of intracellular Ca, along when membrane-bound by antibodies. Binding of the pathogen to the cell surface antibodies causes an increase in intracellular Ca levels, resulting in the emission of light from the cytosolic aequorin [60–63].

Two routine genetic modifications enable engineered B-cell lines to express cytosolic aequorin, a calcium sensitive bioluminescent protein, as well as membrane bound antibodies specific for pathogens of interest [62, 63]. This was achieved by crosslinking the membrane-bound antibodies to a polyvalent antigen that induces a signal-transduction cascade. This latter, sequentially involves tyrosine kinases, phospholipase C and inositol triphosphate (IP3). The IP3 activates calcium channels, thereby increasing cytosolic calcium from both internal stores and the extracellular medium, which activates the aequorin, causing it to emit light (Fig. 4.13) [59, 62–64].

The CANARY sensor can detect less than 50 colony-forming units (cfu) of pathogen in less than 3 min, which include the time required to concentrate the sample [65]. It should be mentioned that state-of-the-art immunoassays take at least 15 min, whereas polymerase chain reaction (PCR) takes longer than 30 min. The novel genetic-engineering system developed by Petrovick et al. have consisted of the efficient production of B-cell lines that can react specifically and rapidly to a variety of pathogens [59]. The antibody genes were cloned from hybridomas and inserted into expression vectors. These were transfected into a parental B cell line that expresses active aequorin, and the cells are screened for their response to patho-

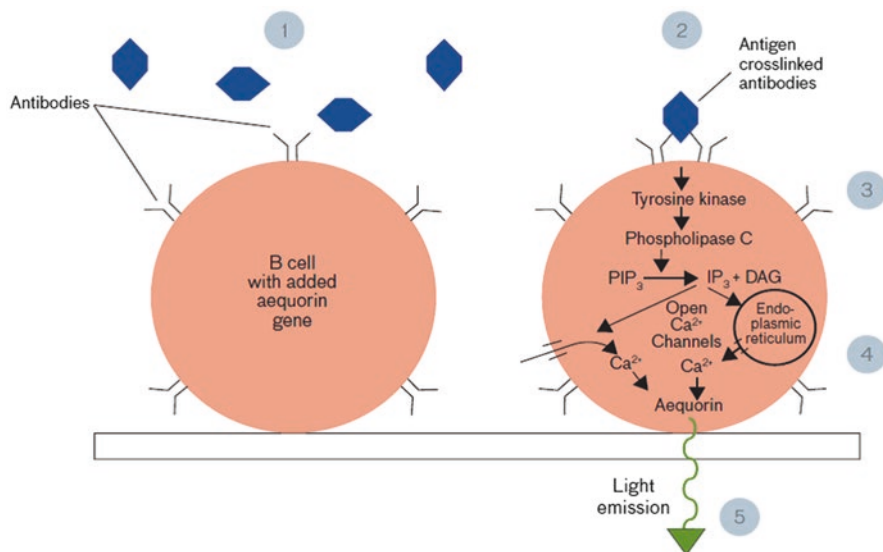


Fig. 4.13 The CANARY bioelectronic sensor (Adapted from Ref. 59)

gen. The genetically engineered CANARY cells can be used separately in a single identification assay, or as many as three can be combined to achieve a multiplexed assay. Alternatively, several antibodies can be expressed in a single cell line to provide a classification assay [59]. It is also feasible to create B cells that emit at different wavelengths of light, enabling multiplexed assays that simultaneously distinguish among several targets [59].

4.7.2 Toxin Detection

A novel effective method described by Petrovick et al. for the immobilization of a toxin was to capture it on beads coated with antibodies against that specific toxin [59]. The antibody-coated beads were then incubated in a solution containing the suspected toxin, and washed to remove the contaminating proteins and other materials. The toxin-obtained decorated beads are excellent candidates for the purification and immobilization of toxin analytes by the CANARY cells, as illustrated in Fig. 4.14 [59].

It should be mentioned, that the CANARY cells should express an antibody that binds to the toxin at a different site from that of the capture antibody. Because the toxin is immobilized on the bead, the antibodies on the CANARY cell that bind to the toxin are also immobilized, and therefore light emission is stimulated [59]. This approach has been used to develop a very effective CANARY assay for botulinum neurotoxin type A (BoNT/A) which is the most poisonous toxin known to man, with

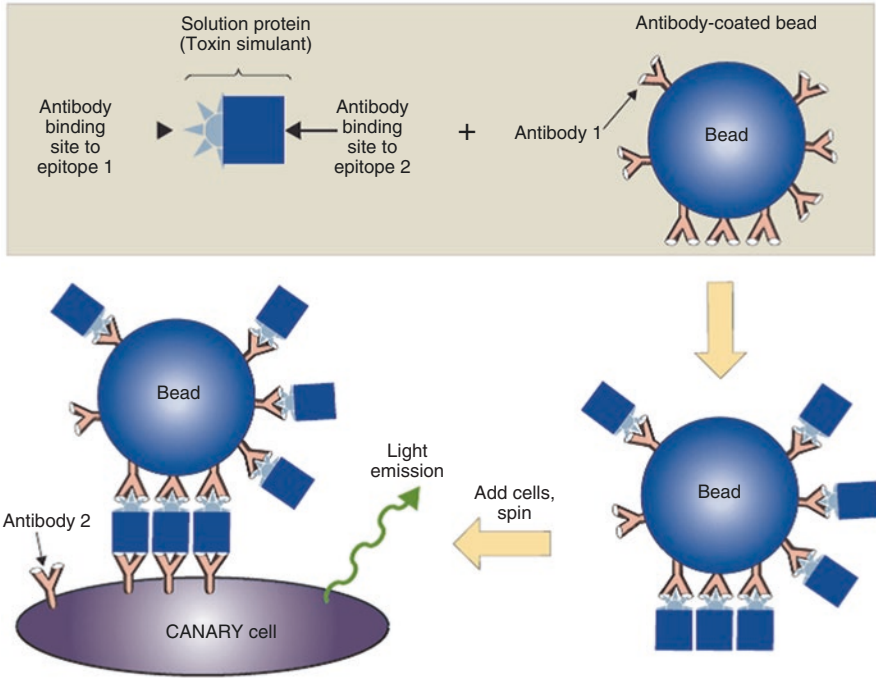


Fig. 4.14 Two antibodies assay for toxins detection. (Adapted from Ref. 59)

an LD50 for a 55 kg adult of about 550 ng by inhalation. Under ideal conditions, the assay sensitivity is currently 16 pg (1.6 ng/mL) [59].

4.7.3 DNA Sequence Detection

The detection of soluble macromolecules has a second interesting application: identification of DNA and RNA sequences. The ability to identify nucleic acid (NA: DNA or RNA) sequences is central in the deduction of the first hard NA sequence information, concerning a new or genetically modified pathogen [59]. It is important to develop assays that have the flexibility to respond quickly to new threats. For this reason, developing NA probes allow for the rapid and quick examination of the actual genetics of the target organism [59]. Once the NA sequence of a pathogen is determined, multiple short probes are synthesized that bind adjacent to each other along a specific sequence on the target NA [59]. Consequently, Petrovick et al. developed a novel assay that uses a single CANARY cell line that expresses an antibody against digoxigenin. Each of these probes is labeled with a single digoxigenin molecule. If these probes are added to solution containing the target NA sequence, the binding of multiple digoxigenin-containing probes produces a tight

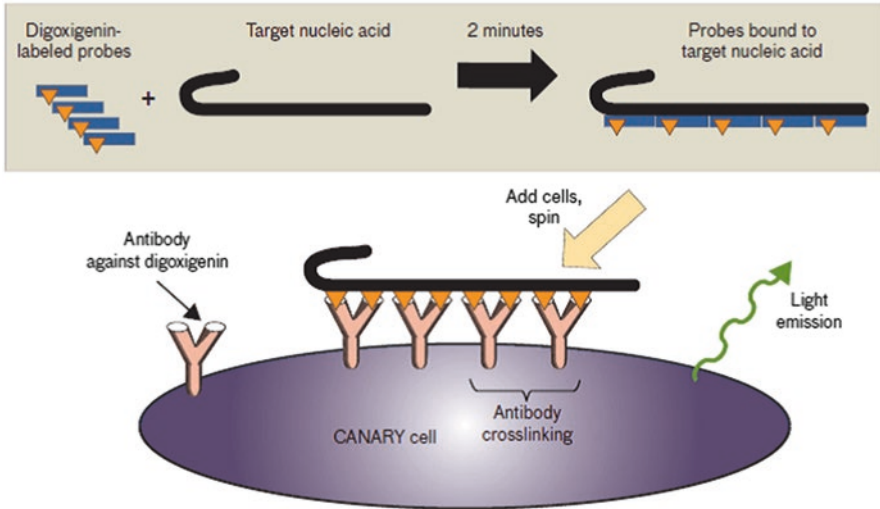


Fig. 4.15 DNA probes designed that bind to a specific region on a single-target nucleic acid (NA) (Adapted from Ref. 60)

cluster of immobilized digoxigenin molecules, which will stimulate light production from the CANARY cell (see Fig. 4.15) [59].

In the absence of target NA, each digoxigenin-labeled probe remains monomeric, and therefore cannot crosslink antibodies on the surface of CANARY cells. There are advantages to detecting RNA as compared to DNA. Primarily, while there is only one copy of genomic DNA per bacterium, there can be thousands of copies of a single RNA; so the number of target molecules per bacterium is much higher. Furthermore, since probe binding requires that the target NA be single stranded, a denaturing step must be performed to separate the two constituent strands of DNA. RNA, however, is normally single stranded [59].

4.7.4 CANARY Detection of *Francisella tularensis* and *Yersinia pestis*

The CANARY approach takes advantage of a receptor that binds to the constant region of antibodies, leaving the antigen-binding region of the antibody free. After binding bacteria with the captured antibodies, the receptor initiates a signal cascade, similar to the one induced by the crosslinking of membrane-bound antibodies on B cells, which activates aequorin [59].

The excellent combination of speed and sensitivity of the CANARY system was demonstrated with cell lines expressing an antibody specific for the F1 antigen of *Yersinia pestis* (*Yp*), shown in Fig. 4.16 [59]. When concentrated in the centrifuge

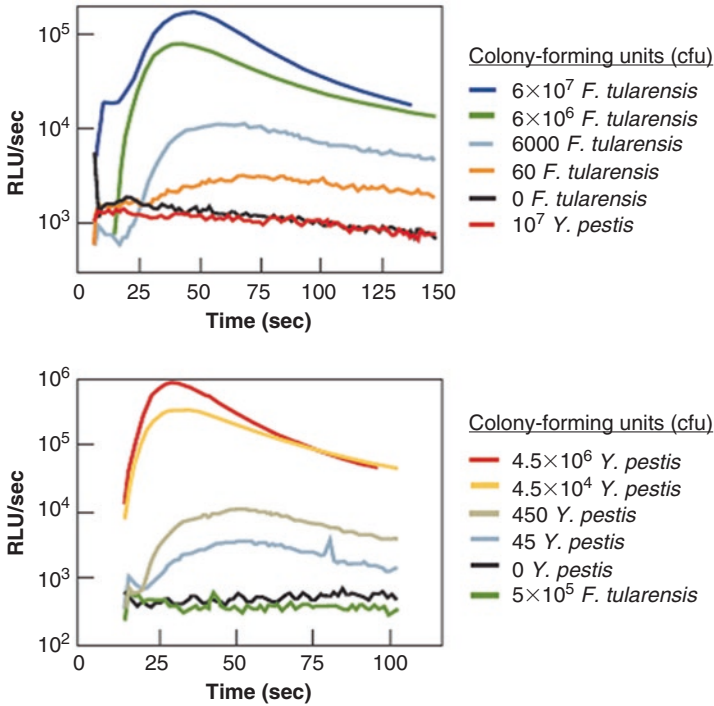


Fig. 4.16 The dose-response curves for inactivated *Francisella tularensis* (top) and *Yersinia pestis* (bottom) (Adapted from Ref. 60)

luminometer, as little as 45 cfu of formalin-inactivated Yp are detected. However, there was no response to relatively large numbers of *F. tularensis* [59].

For biological defense applications, the CANARY technology was incorporated into a flexible biological-aerosol sensor platform called PANTHER that can form the core of a family of mission-specific bio-aerosol identification sensors useful as standalone sensors for site/building protection, emergency response, rapid screening, and environmental monitoring [59].

To summarize, the CANARY's capabilities open possible applications in pathogen genotyping, virulence testing, antibiotic resistance screening, and viability assessment. Moreover, using these cells demonstrate the best known combination of speed and sensitivity. Other applications of CANARY technology include biological aerosol sampling, point-of-care diagnostics, pre-symptomatic diagnosis in the aftermath of a biowarfare attack, detection of agricultural pathogens at ports of entry, or screening of perishable food supplies medium [62], which activates the aequorin, causing it to emit light [59, 60].

4.8 High-Density Microsphere-Based Fiber Optic DNA Microarrays

It is well known that an optical fiber or optical fibre is a flexible, transparent fiber made by drawing glass (silica) or plastic to a diameter slightly thicker than that of a human hair [66, 67]. Optical fibers consist of an inner core which is surrounded by a clad material of lower refractive index. Because of the differences in refractive index light is totally reflected. A fiber optic bundle consists of thousands of individual fibers fused together such that each fiber retains its ability to transmit light independently of its neighbors (Fig. 4.17) [68].

Pantano and Walt showed that by selectively etching the fiber core, an array of microwells can be formed [69]. These microwells can be filled with oligonucleotide-functionalized microspheres. The array dimensions can be tailored to suit any size of oligonucleotide-functionalized microsphere. The well diameters are equal to those of the fiber cores, and the depths are dependent on the etchant concentration, the exposure time, and the fiber composition. Because each microsphere is optically wired to a fiber, the specific interactions on each microsphere surface can be independently monitored. Walt and coworkers developed a high-density fiber optic DNA microarray consisting of oligonucleotide-functionalized, 3.1- μm -diameter microspheres randomly distributed on the etched face of an imaging fiber bundle [69].

Usually these fiber bundles are composed of around 6000/50000 fused optical fibers, in which each fiber –contains an etched well [70]. The desired oligonucleotide sequences are attached to individual microspheres, then added to each etched wells on the fiber optic bundle face. The produced microwell arrays are capable of casing complementary-sized microspheres, each containing thousands of copies of a unique oligonucleotide probe sequence. Walt and coworkers showed that the array fabrication process resulted in random microsphere placement. It should be understood that the determination of the position of microspheres in the random array, essentially required an optical encoding scheme. The detection schemes, which are combined the intrinsic recognition abilities of nucleic acids, are usually measured with fluorescence-based detection methods. This “optical bar code” obtained is simply a combination of fluorescent dyes with different excitation and emission wavelengths and intensities that allows each bead to be independently identified [70].

Nonetheless, additional degrees of freedom are available in the fiber-optic format, that allows additional number of excitation and emission wavelengths that can be used. It should be mentioned that the optically bar-coded arrays, can be decoded in a matter of seconds. This occurs byh conventional image processing software that collect series of fluorescence images at different excitation and emission wavelengths and then analyze the relative intensities of each bead. Alternatively, preformed oligonucleotides may be added directly to surface-activated microspheres [70].

Needless to say those fluorescence-based assays are more desirable than traditional radiolabeled methods due to their increased safety and experimental versatil-

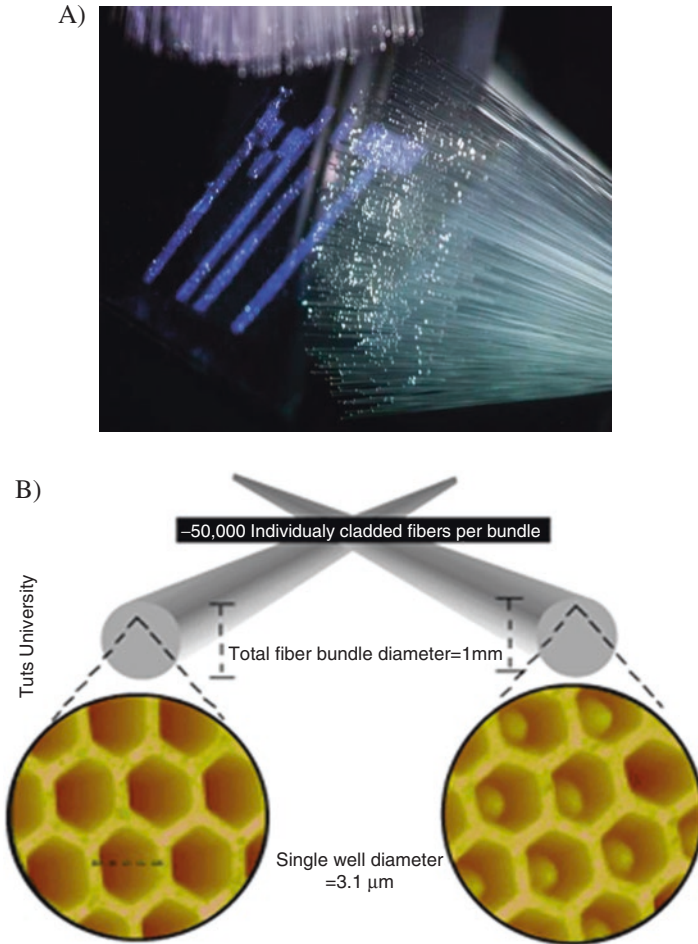


Fig. 4.17 (a) Individual optical fibre is a flexible, transparent fiber and a fiber optic bundle consists of thousands of individual fibers fused together bundle. (b) Scanning force images of the etched face of an optical fiber imaging bundle. The first (*left*) is empty, and the second (*right*) contains complementary-sized microspheres. Each well diameter is approximately 3.1 μm [68]

ity. Fluorescence can be incorporated into microarray assays by fluorescent intercalating dyes, fluorescently labeled targets, or label-less methods employing fluorescence resonance energy transfer (FRET). Fluorescence-based assays enable the measurement of multiple wavelengths independently and simultaneously. The use of multiple fluorophores enables parallel interrogation schemes [70–73].

4.8.1 *Fiber Optic DNA Biosensors*

A general array protocol entails immobilizing a probe sequence (primer) that can hybridize to its fluorescently-labeled complementary target. The fluorescent tag is commonly incorporated into the target molecules, via polymerase chain reaction (PCR) [74]. This primer labeling method is convenient when amplification is needed for detection, or when it is used to construct a cDNA library from a genomic RNA pool via reverse transcription. In addition, the derivatized fiber core experiments are able to detect unlabeled (non-fluorescent) target solutions [74]. This is achieved by competitive hybridization with fluorescent target samples. In this method, the fluorescent synthetic target complements are synthesized and initially hybridized to the array to saturate the array probe elements [68]. The unlabeled target solution is then hybridized to the same array, competing with the prehybridized synthetic targets. The presence of unlabeled target is determined by a fluorescence decrease caused by displacement of the fluorescent synthetic target by the unlabeled species. This procedure eliminates the need to incorporate fluorescence into the target and allows quantitative measurements to be performed. These fiber optic platforms are the basis for microsphere array designs that improved array fabrication and allowed extremely high-density sensor placement [68].

4.8.2 *Analysis Setup and Protocol*

The imaging system consists of a light source, an inverted microscope, and a modified Olympus epifluorescence microscope/charge-coupled device camera (Photometrics PXL). A fiber chuck held the imaging fiber in a fixed position while electronically controlled filter wheels switched between the analytical wavelength and the encoding wavelengths, enabling complete analysis and identification of the microspheres within minutes. Excitation light is sent into the proximal tip of the imaging fiber, and emission from the fluorescing molecules captured and directed onto the CCD camera detector (Fig. 4.18) [72].

The multiplex analysis images were acquired for 1 and 0.5 s at wavelengths specific to each encoding dye. A 365-nm excitation filter and a 600-nm long-pass emission filter were used for the Eu-dye. A 620-nm excitation filter and a 670-nm emission filter were used for the Cy5 dye. A 530-nm excitation filter and a 580-nm emission filter were used for TAMRA. That is detection of amplified DNA fragments which were incubated with a fluorescein-labeled sequencing primer in the presence of the two allelic ROX-ddNTP or TAMRA-ddNTP terminators. All targets were labeled with fluorescein. It was shown that the fluorescence intensity was proportional to the extent of hybridization at each probe position [72A]. In addition, the camera was equipped with an internal chip that provides megapixel resolution (1280/1024). This megapixel chip was able to resolve the arrays miniaturized feature sizes (3 μm) and provided multiple pixels for each optical channel in the fiber bundle (Fig. 4.19).

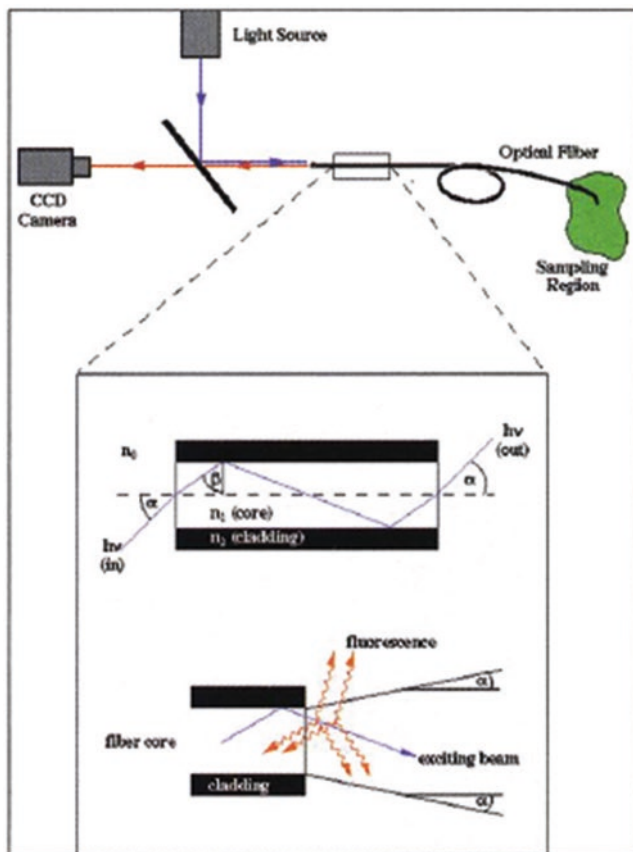


Fig. 4.18 Schematic diagram of the fiber optic imaging system (Adapted from Ref. 73)

Finally, it can be surmised that the described fiber optic microsphere-based biosensor is a versatile platform that possesses micro-scale features and an overall array size that enables rapid analysis and extremely low detection limits. Redundant detection elements in the array increase the signal-to-noise ratio and avoid the potential for false positive and false negative results. Microsphere-based arrays are reusable, and are easy to fabricate. The fiber optic platform has also been applied to other applications including artificial olfaction and cell-based array sensing [68, 73].

4.9 Surface Plasmon Resonance

Recently, numerous strategies for protein labeling were developed and they allowed the characterization of proteins regarding their structure, folding, or interaction with other proteins [75]. Surface plasmon resonance (SPR) is a label-free detection

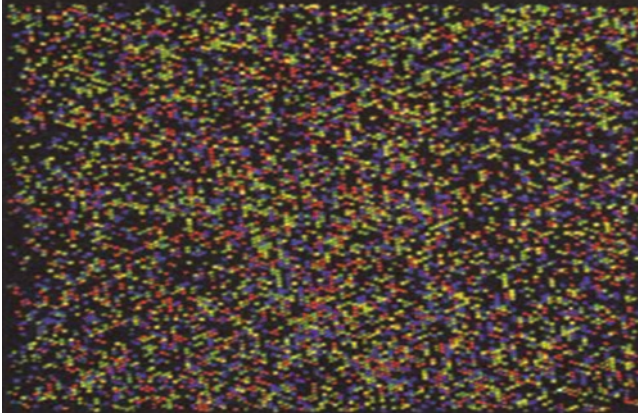


Fig. 4.19 An image of a 13,000-well square fiber bundle microarray, with a diameter of ~ 1.2 mm. This entire array is about the size of a single spot on a traditional spotted array (Adapted from Ref. 73).

method which is a suitable and reliable platform in clinical analysis for biomolecular interactions. Undeniably, SPR has been proven to be one of the most powerful technologies to determine the specificity, affinity and kinetic parameters displayed during the binding of macromolecules. These binding of macromolecules include, but are not limited to, protein-protein, protein-DNA, enzyme-substrate or inhibitor, receptor-drug, lipid membrane-protein, protein-polysaccharide and cell or virus-protein [76–85]. SPR is an optical technique which measures the refractive index changes in the vicinity of thin metal layers (i.e., gold, silver, or aluminum films) in response to biomolecular interactions.

4.9.1 SPR Principle

When the photon of an incident light strikes a metal surface, typically a gold surface, surface plasmon resonance occurs. When a portion of the light energy excites the electrons of the metal surface layer, at a certain angle of incidence, it creates electron movements which propagate parallel to the metal surface. This electric oscillation is termed as a “plasmon”. This oscillation, in turns, generates an electric field whose range is around 300 nm from the boundary between the metal surface and sample solution [76, 79]. In a commercial SPR biosensor configuration, the incident light employed is generated by a high-reflective index glass prism of the Kretschmann geometry of the attenuated total reflection (ATR) method (Fig. 4.20) [79].

The defined SPR angle, at which resonance occurs, relies on the refractive index of the material coating the metal surface, and the constant light source wavelength. When there is no change in the reflective index of the sensing medium, the plasmon

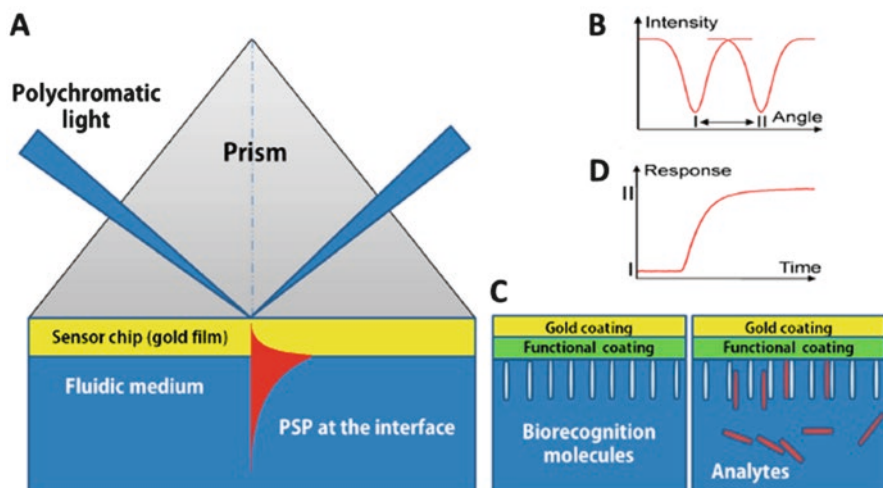


Fig. 4.20 Concept of a surface plasmon resonance (SPR) biosensor: (a) Kretschmann geometry of the ATR method; (b) spectrum of reflected light before and after refractive index change; (c) analyte-biorecognition elements binding on SPR sensor surface and (d) refractive index changes caused by the molecular interactions in the reaction medium (Adapted from Ref. [79]).

oscillation cannot be formed (Fig. 4.20a). In addition, it should be noted that when the surface of the sensing material has been coated through biomolecule attachment only, there will be perhaps an unnoticed small change in the reflective index of the sensing medium, and as a result, the plasmon oscillation cannot be formed (Fig. 4.20c, left side) [79]. However, when the metal surface has been coated with an analyte-biorecognition couple of biomolecules, detection is accomplished by measuring the changes in the reflected light obtained on a detector (Fig. 4.20c, right side). In addition, the amount of surface concentration can be quantified by monitoring the reflected light intensity or tracking the resonance angle shifts. Typically, an SPR biosensor has a detection limit of 10 pg/mL [76–79].

In all commercial SPR biosensors, probe molecules are firstly immobilized on to the sensor surface. When the solution of target molecules is flown into contact with the surface, a probe-target binding via affinity interaction occurs, which consequently induces an increase in the refractive index at the SPR sensor surface (Fig. 4.20d) [79]. In SPR experiments, resonance or response units (RU) are used to describe the signal change, where 1 RU is equivalent to a critical angle shift of 10^{-4} degree [80–84]. At the start of the experiment whereas probe target interactions have not occurred, the initial RU value corresponds to the starting critical angle. The change in refractive index Δn_d arisen within a layer of thickness h can be calculated as:

$$\Delta n_d = \left(\frac{dn}{dc} \right)_{\text{vol}} \Delta \Gamma / h \quad (4.1)$$

where $(dn/dc)_{vol}$ is the increase of refractive index n with the volume concentration of analyte c , and $\Delta\Gamma$ is the concentration of the bound target on the surface [76].

4.9.2 High-Throughput Screening (HTS)

There are several different formats of SPR biosensors. These comprise the array format, multi-channel unit format, and SPR imaging format, which allow simultaneous and continuous detection to analyze the performance of hundreds to thousands of affinity binding events on a chip surface [81–83]. In SPR imaging, the incidence angle remains fixed, and the binding of biomolecules on a gold surface is measured as the change in reflectivity (or reflectance) in relation to the incident ray intensity, unlike SPR sensors that depend on the measurement of the absorption dip in the SPR angle or SPR wavelength [80–84]. Recently, it was shown that SPR imaging technology using a multi-analyte biosensor allows the measurement of a high-throughput approach, and it achieves a similar degree of sensitivity of conventional SPR biosensors (Fig. 4.21) [83].

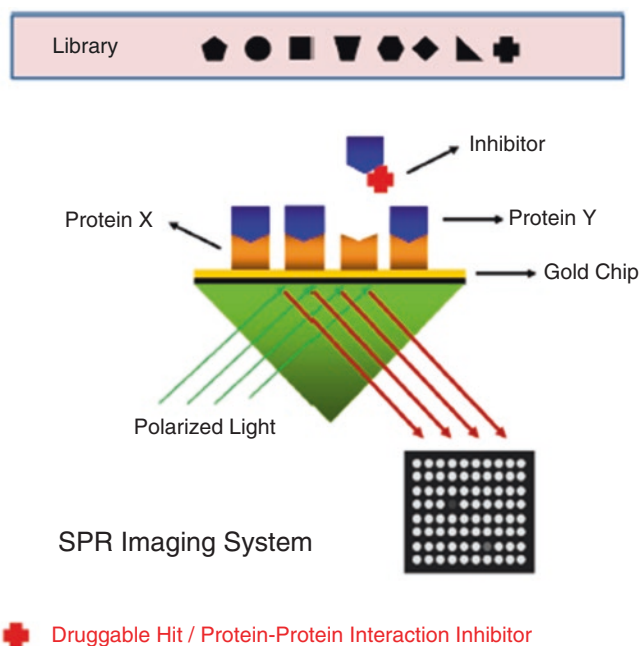


Fig. 4.21 High-throughput drug screening using an SPR imaging protein chip system. The bright image indicates protein-protein interaction on a gold surface. Upon the binding of an inhibitor to the target protein, protein-protein interactions are disrupted, resulting in changes in SPR imaging signal intensity and a darker image (Adapted from Ref. [83])

Consequently, SPR imaging systems without any labeling requirements can be used for high-throughput screening (HTS), particularly in drug discovery, than any other optics-based detection techniques [83, 84].

4.9.3 Surface Plasmon Resonance Sensing of Biological Warfare Agent Botulinum Neurotoxin A

Dhaked and coworkers developed a label free real time method for the detection and quantification of botulinum neurotoxin A (BoNT/A) using surface plasmon resonance (SPR) [85]. The authors used antibody against rBoNT/A-HCC fragment and synaptic vesicles (SV), which were immobilized on a carboxymethyl dextran modified gold chip. The immobilization of BoNT/A antibody and interaction of BoNT/A with immobilized antibody were characterized in-situ by SPR and electrochemical impedance spectroscopy. A sample solution containing BoNT/A antigen in concentrations ranging from 0.225 to 4.5 fM and 0.045 to 5.62 fM was interacted with the immobilized antibody and immobilized SV, respectively [85].

4.9.3.1 Immobilization of BoNT/A Antibody and SV Protein on CM5 SPR Sensor Chip

Dhaked and coworkers described the stepwise immobilization of BoNT/A antibody and SV protein on a CM5 chip, respectively in nine steps. These steps involved the stabilization of the baseline, the activation of carboxyl groups on the CM5 chip, and converting them into activated carboxymethylated groups on the sensor chip for future bounding to the free amino groups of BoNT/A antibody. This was followed by washing with PBS and measuring the SPR angle that has shifted nearly to the baseline [85]. When the BoNT/A antibodies are injected on the CM5 chip an increase in SPR angle was observed. Finally, washing with 1000 mM ethanolamine was performed to prevent non-specific binding and to block the unreacted NHS-ester groups on CM5 chip. From Figs. 4.22 and 4.23, a net angle change of 95.44 m° and 48 m° are observed and this ascribes the attachment of 0.79 ng/ mm² of antibody and 0.4 ng/mm² SV protein on CM5 chip, respectively [87].

4.10 Conclusion

Most of the sensing measuring devices used for the detection of biological warfare agents are based on luminescence immunoassay signal transduction mechanisms which are optical. In this chapter, we have discussed the following subjects: impedance spectroscopy, evanescent wave technology and internal reflection fluorescence

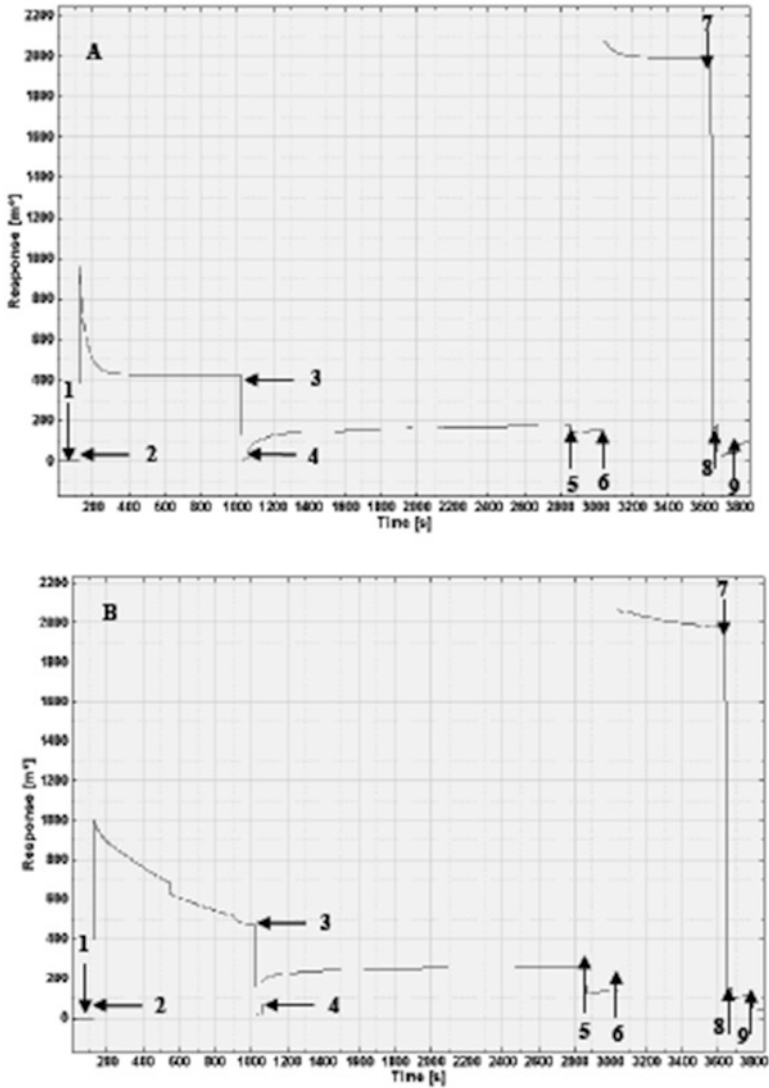


Fig. 4.22 Sensorgram showing different steps [(1) Baseline (2) EDCNHS activation (3) Washing (4) Antibody coupling (5) Washing (6) Deactivation (7) Washing (8) Regeneration and (9) Back to baseline] involved in the (a) immobilization of BoNT/A antibody (1: 500 dilution); (b) immobilization of SV on CM5 chip [87]

(TIRF) excitation, surface acoustic wave sensors, fluorescent biosensors, Fluorescence Resonance Energy Transfer (FRET), light emission (CANARY), microsphere-based fiber optic biosensors, and surface plasmon resonance. It should be noted that although fluorescence detection is the most popular, surface plasmon resonance (SPR)-based immunoassay formats with surface imaging capabilities are growing in popularity

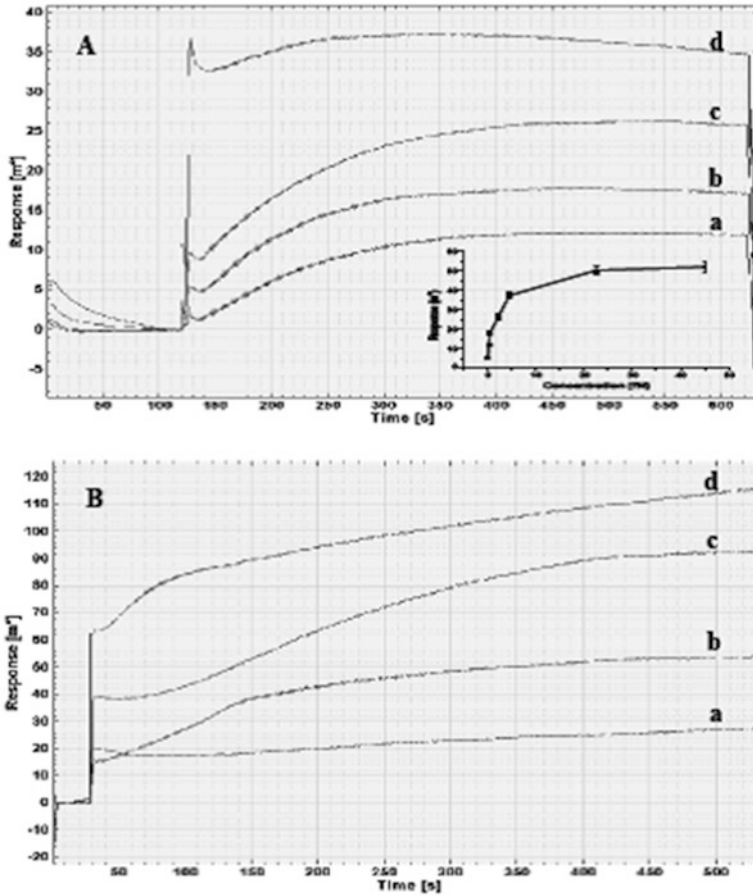


Fig. 4.23 SPR sensor response for the interaction of different concentration of BoNT/A antigen with (a) immobilized BoNT/A antibody (a) 0.225 fM, (b) 0.45 fM, (c) 2.25 fM and (d) 4.5 fM (b) immobilized SV (a) 0.045 fM, (b) 0.225 fM, (c) 1.12 fM and (d) 5.62 fM Temperature: 25o C and pH: 7.5 [87]

[80–86]. Multiplexing, or simultaneous detection of multiple analytes, is one of the most important prerequisites for biothreat agent detection. As mentioned in our introduction, the presented work is more a “Comptes Rendus” and an introduction to this series of sensing measuring devices used for the detection of biological warfare agents that were presented to the participants of this NATO-ASI meeting.

References

1. Eitzen EM Jr, Takafuji ET (1997) Historical overview of biological warfare. In: Sidell FR, Takafuji ET, Franz DR (eds) *Medical aspects of chemical and biological warfare*. Office of the Surgeon General, Borden Institute, Walter Reed Army Medical Center, Washington, DC, pp 415–423
2. Ridell R (2004) Biological warfare and bioterrorism: a historical review. *Proc (Baylor Univ Med Cent)* 17(4):400–406
3. WHO. Severe acute respiratory syndrome (SARS) (2003) *Wkly Epidemiol Rec*, 78: 86–92
4. Gray C (2007) Another bloody century: future warfare, pp 265–266, Phoenix
5. Heymann DL (2001) Strengthening global preparedness for defense against infectious disease threats. Committee on foreign relations, United States Senate. Hearing on the threat of bioterrorism and the spread of infectious diseases
6. Hoffman RE, Norton JE (2000) Lessons learned from a full-scale bioterrorism exercise. *Emerg Infect Dis* 6:652–653
7. US Commission on National Security in the 21st Century. *New world coming: American security in the 21st century, supporting research and analysis*. September 15, 1999
8. Szinicz L (2005) History of chemical and biological warfare agents. *Toxicology* 214(3):167–181
9. CDC. *Biological and Chemical Terrorism: Strategic Plan for Preparedness and Response (2005) Recommendations of the CDC Strategic Planning Workgroup*. *MMWR Recomm Rep* 49(RR-4):1–26
10. Cenciarelli O, Rea S, Carestia M, D’Amico F, Malizia A, Bellecci C, Gaudio P, Gucciardino A, Fiorito R (2013) Bioweapons and bioterrorism: a review of history and biological agents. *Defence S & T Tech Bull* 6(2):111–129
11. Barrett JA, Bowen GW, Golly SM, Hawley C, Jackson WM, Laughlin L, Lynch ME (1998) Assessment of biological agent detection equipment for emergency responders, June 1, 1998. Chemical Biological Information Analysis Center (CBIAC), P.O. Box 196, Gunpowder, MD 21010–0196
12. *Chemical and biological terrorism: research and development to improve civilian medical response to chemical and biological terrorism incidents*, National Academy of Sciences, 1999. National Academy Press, 2101 Constitution Avenue, N.W., Box 285, Washington, DC 20055
13. Barrett JA, Bowen GW, Golly SM, Hawley C, Jackson WM, Laughlin L, Lynch ME (1998) Final report on the assessment of biological agent detection equipment for emergency responders, U.S. Army Chemical and Biological Defense Command (CBDCOM), CBIAC, P.O. Box 196, Gunpowder, MD 21010–0196
14. Marquette CA, Blum LJ (2006) State of the art and recent advances in immunoanalytical systems. *Biosens Bioelectron* 15;21(8):1424–1433
15. IUPAC (1992) 64: 143
16. Sapsforda KE, Bradburne C, Delehanty JB, Medintzb I (2008) Sensors for detecting biological agents (2008). *Mater Today* 11(3):38–49
17. Thevenot R, Toth K, Durst RA, Wilson GS (1999) Electrochemical biosensors: recommended definitions and classification. *Pure Appl Chem* 71:2333–2348
18. Thevenot R, Toth K, Durst RA, Wilson GS (2010) Electrochemical biosensors: recommended definitions and classification. *Biosens Bioelectron* 16:121–131
19. Thevenot R, Toth K, Durst RA, Wilson GS (2001) Electrochemical biosensors: recommended definitions and classification. *Anal Lett* 34:635–659
20. Daly P, Collier T, Doyle S (2002) PCR-ELISA detection of *Escherichia coli* in milk. *Lett Appl Microbiol* 34:222–226
21. Tsai W-L, Miller CE, Richter ER (2000) Determination of the sensitivity of a rapid *Escherichia coli* O157:H7 assay for testing 375-gram composite samples. *Appl Environ Microbiol* 66(9):4149–4151
22. Nicolas P, Mor A (1995) Peptides as weapons against microorganisms in the chemical defense system of vertebrates. *Annu Rev Microbiol* 49:277–304

23. Zasloff M (2002) Antimicrobial peptides of multicellular organisms. *Nature* 415:389–339
24. Meng H, Kumar K (2007) Antimicrobial activity and protease stability of peptides containing fluorinated amino acids. *J Am Chem Soc* 129:15615–15622
25. Mannoro MS, Zhang C, Link J, McAlpine MS (2010) Electrical detection of pathogenic bacteria via immobilized antimicrobial peptides. *Proc Natl Acad Sci U S A* 107(45):19207–19212
26. Zasloff M, Martin B, Chen HC (1988) Antimicrobial activity of synthetic magainin peptides and several analogues. *Proc Natl Acad Sci U S A* 85:910–913
27. Zasloff M (1987) Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci U S A* 84:5449–5453
28. Matsuzaki K, Sugishita KI, Harada M, Fujii N, Miyajima K (1997) Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria. *BBA-Biomembranes* 1327:119–130
29. Taitt CR, Shriver-Lake LC, Ngundi MM, Ligler FS (2008) Array biosensor for toxin detection: continued advances. *Sensors* 8:8361–8377
30. Ligler FS, Taitt CR, Shriver-Lake LC, Sapsford KE, Shubin Y, JPB G (2003) Array biosensor for detection of toxins. *Anal Bioanal Chem* 377(3):469–477
31. Feldstein MJ, Golden JP, Ligler FS, Rowe CA (2001) Reflectively coated optical waveguide and fluidics cell integration. *US Pat* 6:192, 168
32. Golden JP, Taitt CR, Shriver-Lake LC, Shubin YS, Ligler FS (2005) A portable automated multianalyte biosensor. *Talanta* 65(5):1078–1085
33. Ngundi MM, Qadri SA, Wallace EV, Moore MH, Lassman ME, Shriver-Lake LC, Ligler FS, Taitt CR (2006) Detection of deoxynivalenol in foods and indoor air using an array biosensor. *Environ Sci Technol* 40(7):2352–2356
34. Sapsford KE, Taitt CR, Fertig S, Moore MH, Lassman ME, Maragos CA, Shriver Lake LC (2006) Indirect competitive immunoassay for detection of aflatoxin B-1 in corn and nut products using the array biosensor. *Biosens Bioelectron* 21(12):2298–2305
35. Ngundi MM, Taitt CR (2006) An array biosensor for detection of bacterial and toxic contaminants of foods. In *Diagnostic Bacteriology Protocols*, 53–68
36. Shriver-Lake LC, Erickson JS, Sapsford KE, Ngundi MM, Shaffer KM, Kulagina NV, Hu JE, Gray SA, Golden JP, Ligler FS, Taitt CR (2007) Blind laboratory trials for multiple pathogens in spiked food matrices. *Anal Lett* 40(16–18):3219–3323
37. Milne EA (1941) Augustus Edward Hough Love. 1863–1940. *Obituary Notices of Fellows of the Royal Society* 3(9):466
38. O'Connor JJ, Robertson EF. “Augustus Edward Hough Love”, [MacTutor History of Mathematics archive, University of St Andrews](#)
39. Länge K, Rapp BE, Rapp M (2008) Surface acoustic wave biosensors: a review. *Anal Bioanal Chem* 391:1509–1519
40. A) Grate JW, Frye GC (1996) Acoustic wave sensors. In: Baltés H, Göpel W, Hesse J (eds) *Sensors update, vol 2*. Wiley-VCH. B) [https://wiki.metropolia.fi/display/sensor/Surface+acoustic+wave+\(SAW\)+sensors](https://wiki.metropolia.fi/display/sensor/Surface+acoustic+wave+(SAW)+sensors)
41. Weinheim E, Wohltjen H, Dessy R (1979) *Anal Chem* 51:1458–1464
42. Collings AF, Caruso F (1997) *Rep Prog Phys* 60:1397–1445
43. Tamarin O, Comeau S, Déjous C, Moynet D, Rebière D, Bebian J, Pistré J (2003) *Biosens Bioelectron* 18:755–763
44. Stubbs DD, Hunt WD, Lee SH, Doyle DF (2002) *Biosens Bioelectron* 17:471–477
45. Stubbs DD, Lee SH, Hunt WD (2003) *Anal Chem* 75:6231–6235
46. Sapsford KE, Berti L, Medintz IL (2005) Fluorescence resonance energy transfer concepts, applications and advances. *Minerva Biotech* 16:253–279
47. Goldman ER, Medintz IL, Mattoussi H (2006) Luminescent quantum dots in immunoassays. *Anal Bioanal Chem* 384:560–563
48. Goldman ER, Balighian ED, Mattoussi H, Kuno MK, Mauro JM, Tran PT, Anderson GP (2002) Avidin: a natural bridge for quantum dot-antibody conjugates. *J Am Chem Soc* 124:6378–6638

49. Wang SP, Mamedova N, Kotov NA, Chen W, Studer J (2002) Antigen/Antibody immunocomplex from CdTe nanoparticle bioconjugates. *Nano Lett* 2:817–822. 70
50. Sun BQ, Xie WZ, Yi GS, Chen DP, Zhou YX, Cheng J (2001) Microminiaturized immunoassays using quantum dots as fluorescent label by laser confocal scanning fluorescence detection. *J Immunol Methods* 249:85–89
51. Hoshino A, Fujioka K, Manabe N, Yamaya S, Goto Y, Yasuhara M, Yamamoto K (2005) Simultaneous multicolor detection system of the single-molecular microbial antigen with total internal reflection fluorescence microscopy. *Microbiol Immunol* 49:461–470
52. Zhu L, Ang S, Liu WT (2004) Quantum dots as a novel immunofluorescent detection system for *Cryptosporidium parvum* and *Giardia lamblia*. *Appl Environ Microbiol* 70: 597–598.
53. Liang RQ, Li W, Li Y, Tan CY, Li JX, Jin YX, Ruan KC (2005) An oligonucleotide microarray for microRNA expression analysis based on labeling RNA with quantum dot and nanogold probe. *Nucleic Acids Res* 33: e17
54. Sapsford KE, Thomas P, Medintz IL, Hedi M (2006) Biosensing with luminescent semiconductor quantum dots. *Sensors* 6:925–953
55. Zhang CY, Ma H, Nie SM, Ding Y, Jin L, Chen DY (2000) Quantum dot-labeled Trichosanthin. *Analyst* 125:1029–1031
56. Jares-Erijman E, T b J (2003) FRET Imaging. *Nat Biotechnol* 21:1387–1395
57. Sapsford KE, Berti L, Medintz IL (2005) Fluorescence resonance energy transfer concepts, applications and advances. *Minerva Biotechnol* 16:253–279
58. Sandros MG, Shete V, Benson DE (2006) Selective, reversible, reagentless maltose biosensing with core-shell semiconducting nanoparticles. *Analyst* 131:229–235
59. Petrovick MS, James D, Harper JD, Frances E, Nargi FE, Eric D, Schwoebel ED, Mark C, Hennessy MC, Todd H, Rider TH, Hollis MA (2007) Rapid sensors for biological-agent identification. *Lincoln Laboratory J* 17:63–84
60. Cormie MJ, Prasher DC, Longiaru M, McCann RO (1989) The enzymology and molecular biology of the Ca²⁺-activated photoprotein, aequorin, photochem. *Photo-Dermatology* 49(4):509–512
61. Shimomura O, Musicki B, Kishi Y (1989) Semi-synthetic aequorins with improved sensitivity to Ca²⁺ ions. *Biochem J* 261:913–920
62. Wilson HA, Greenblatt D, Poeni M, Finkelman FD, Tsien RY (1987) Cross-linkage of B lymphocyte surface immunoglobulin by anti-Ig or antigen induces prolonged oscillation of intracellular ionized calcium. *J Exp Med* 166:601–606
63. Tsuji FJ, Inouye S, Goto T, Sakaki Y (1983) Site-specific mutagenesis of the calcium-binding photoprotein aequorin. *Proc Natl Acad Sci U S A* 83:8107–8111
64. Shimomura O, Johnson FH (1978) Peroxidized coelenterazine, the active group in the photoprotein aequorin. *Proc Natl Acad Sci U S A* 75(6):2611–2615
65. Rider TH, Petrovick MS, Nargi FE A B cell-based sensor for rapid identification of pathogens. *Science* 301:213–215
66. Senior JM, Yousif JM (2009) Optical fiber communications: principles and practice, Pearson Education
67. Epstein JR, Leung APK, Kyong-Hoon L, Walt DR (2003) High-density, microsphere-based fiber optic DNA microarrays. *Biosens Bioelectron* 18:541–546
68. <http://spie.org/newsroom/decoding-dna>
69. Pantano P, Walt DR (1996) Ordered nanowell arrays. *Chem Mater* 8:2832–2835
70. Fodor SPA, Read JL, Pirrung MC, Stryer L, AT L, Solas D (1991) Light-directed, spatially addressable parallel chemical synthesis. *Science* 251:767–773
71. Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270:467–470
72. Epstein JR, Lee M, Walt DR (2002) High-density fiber-optic genosensor microsphere array capable of zeptomole detection limits. *Anal Chem* 74:1836–1840
73. A) Ferguson JA, Steemers FJ, Walt DR (2000) High density fiber optic DNA random microsphere array. *Anal Chem* 72: 5618–5624; B) Olivas SJ, Arianpour A, Stamenov I, Morrison R,

- Stack RA, Johnson AR, Agurok P, Ford JE (2015) Image processing for cameras with fiber bundle image relay. *Applied Optics* 54(5): 1124–1137
73. Mullis KB (1994) Polymerase chain reaction (Nobel Prize). *Angew Chem* 106:1271–1276
74. Nguyen HH, Park J, Sebyung Kang S, Kim M (2013) Surface plasmon resonance: a versatile technique for biosensor applications. *Sensors* 15:10481–10510
75. Stephanopoulos N, Francis MB (2006) Choosing an effective protein bioconjugation strategy. *Nat Chem Biol* 7:876–884
76. Tugarinov V, Kanelis V, Kay LE (2006) Isotope labeling strategies for the study of high-molecular-weight proteins by solution NMR spectroscopy. *Nat Protoc* 1:749–754
77. Phelan ML, Nock S (2003) Generation of bioreagents for protein chips. *Proteomics* 3:2123–2134
78. Šípová H, Homola J (2013) Surface plasmon resonance sensing of nucleic acids: a review. *Anal Chim Acta* 773:9–23
79. De Feijte JA, Benjamins J, Veer FA (1978) Ellipsometry as a tool to study the adsorption behavior of synthetic and biopolymers at the air-water interface. *Biopolymers* 17:1759–1772
80. Smith EA, Corn RM (2003) Surface plasmon resonance imaging as a tool to monitor biomolecular interactions in an array based format. *Appl Spectrosc* 57:320A–332A
81. Steiner G (2004) Surface plasmon resonance imaging. *Anal Bioanal Chem* 379:328–331
82. Jung SO, Ro HS, Kho BH, Shin YB, Kim MG, Chung BH (2005) Surface plasmon resonance imaging-based protein arrays for high-throughput screening of protein-protein interaction inhibitors. *Proteomics* 5:4427–4431
83. Kim M, Han SH, Shin Y (2007) Surface plasmon resonance biosensor chips. *Biochip J* 1:81–89
84. Tomar A, Gupta G, Singh MK, Boopathi M, Singh B, Dhaked RK (2016) Surface plasmon resonance sensing of biological warfare agent botulinum neurotoxin A. *J Bioterror Biodef* 7(2):142–154
85. Tsai WC, Li IC (2009) SPR-based immunosensor for determining staphylococcal enterotoxin A. *Sensors Actuators B Chem* 136:8–12
86. Stenberg E, Persson B, Roos H, Urbaniczky C (1991) Quantitative determination of surface concentration of protein with surface plasmon resonance using radiolabeled proteins. *J Colloid Interface Sci* 143:513–526
87. Tomar A, Gupta G, Boopathi M, Singh B, Dhaked RK (2016) Surface plasmon resonance sensing of biological warfare agents. *J Bioterror Biodef* 7(1), 1–12