Development of Modern Tools for Environmental Monitoring of Pathogens and Toxicant

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Abstract

Environmental monitoring is required to protect our surrounding from contamination, especially bacteria, virus, and parasitic pathogens & their toxins as well as chemical substances that can be released into a air, soil, and water create serious public health concerns. Presently, traditional methods more popular for the detection of pathogens and its toxins, but they have several limitations due to low concentrations and interference with various enzymatic inhibitors in the environmental samples. This chapter describes the current state of modern tools, the advantages over conventional detection methods, and the challenges due to testing of environmental samples. Future trends in the development of novel detection devices and their importance, use over other environmental monitoring methodologies are also discussed.

9.1 Introduction

For the past few decades, environmental security has become one of the global challenges. Several emerging pollutants (both biological and chemical) from various sources are distributed over environmental matrices. Globally, the problems caused by biological pollutants especially bacterial, viral, and parasitic pathogens and their toxins are likely to be aggravated and pose serious public health concerns. Conventional culture methods of detecting microorganisms in pollutants are based on the integration of the sample into a suitable enriched medium on which the microorganisms can grow multiply and render visual confirmation. These

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A. Singh et al. (eds.), *Environmental Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-15-7493-1_9

conventional test methods are simple, easily resilient, and usually inexpensive. Mostly, conventional methods are very sensitive and laborious and may require several days. Products that are minimally processed have a naturally short shelf life, which prevents the use of many of these conventional methods. Therefore, extensive research work has been executed to reduce assay time and reduce the amount of manual labor by automating methods through the use of interdisciplinary approaches to detect microorganisms and their toxins. It is against this background the detection and monitoring of environmental pollutants are classified into the following groups: Molecular and various sensor-based methods. These methods of detection can be used to effectively combat environmental components and biological tissues. This chapter presents the monitoring technologies for pathogen agents and their toxins and to discuss the advantages, disadvantages, and various characteristics of those methods. It gives an overview of environmental analytical methodologies reliable for public safety and environmental surveillance.

9.1.1 Pathogen and Its Toxins

A pathogen and its toxins that are biological agents cause disease, disability, or seizure to its host. The term is most often used for describing an infectious agent such as a virus, bacterium, protozoa, prion, fungus, or other microorganisms that disrupt the normal physiology of multicellular animals or plants.

9.1.2 Bacteria

Bacteria are microscopic single-celled organisms that flourish in different type of environments. Most catastrophic diseases such as pneumonia, food-borne illness, blood stream infection (sepsis), and sexually transmitted diseases like gonorrhea are caused by bacteria. Pathogenic bacteria, like *Streptococcus* and *Pseudomonas*, cause globally important diseases, such as pneumonia, and *Shigella*, *Campylobacter*, and *Salmonella* cause food-borne illnesses. Hans Christian Gram, a microbiologist, categorizes bacteria into two groups: (a) Gram-positive and (b) Gram-negative bacteria, and the difference between them is in the cell structure of their wall. Nearly 95% of pathogenic bacteria are Gram-negative bacteria, and the rest are Gram-positive bacteria.

9.1.3 Virus

A virus is a small infectious agent that cannot replicate itself. Viruses have either DNA or RNA as genetic material. It can infect all types of life forms, from animals and plants to microorganisms, including bacteria and archaea and directly instruct the cell machinery to reproduce more and more virus cells.

9.1.4 Fungus

A fungus is a eukaryotic organism that admits microorganisms such unicellular yeast and multicellular molds. These are classified under a separate kingdom "fungus," which includes approximately 100,000 described species. Fungi have both useful and harmful properties. In environmental fungi along with its mycelium, various propagules and the metabolites, it produces mycotoxin.

9.1.5 Significance of the Problem

The monitoring of pathogens and their toxins is one of the core issues in understanding and controlling risk to human health. Problem of pathogen and its toxin contaminants has mild-to-severe and short-term or long-term effects and at some circumstances it causes fetal effect and can become a pandemic. Very strict legislation is implemented in areas such as the environment hazards, in order to prevent the terrible consequences of pathogen and its toxins. Thus, there has been a keen interest in designing and developing tool and techniques for the monitoring of pathogens, that is portable and highly robust assays. From this perspective, different type of monitoring tools has achieved intense significance because of their capability to resolve a potentially large number of problems and challenges in pathogen contamination. This chapter aims to provide new trends in the area of pathogen and its toxins detection.

9.2 Detection of Pathogen and Its Toxins

Environmental pollution adversely affects human health and socioeconomic development. Therefore, it is necessary to develop specific and sensitive monitoring protocols in order to avoid false-positive and false-negative results.

9.2.1 Molecular-Based Detection Methods

During the past decade, the use of molecular methods has supplied the means for examining microbial diversity and detecting specific organisms without the need for cultivation. Several molecular techniques have been developed and extensively used for detecting and typing pathogens. These are evaluated in terms of their performance like discriminatory power, reproducibility, and agreement between typing techniques. The application of molecular techniques to the study of natural and engineered environmental systems has enhanced our insight into the interactions of microorganisms in large and complex environments (Table 9.2). Molecular techniques have also been widely used in surveillance, mutation, and other genetic studies of pathogens to increase our understanding about the primary source of pathogens, source of infection, and genetic diversity. Molecular techniques have

Table 9.1 Advantages and disadvantages of some commonly available molecular techniques for identifying food-borne pathogens

Identification method	Advantages	Disadvantages	Reference
Single PCR	Provides a more accurate, sensitive, and rapid detection of single bacteria or genes	Does not produce isolates that can further be characterized, components in foods can interfere with PCR performance and give misleading results, and PCR conditions must be optimized for better performance	Sails et al. (1998), Wang et al. (2000), Abulreesh et al. (2006)
Multiplex PCRa	Reduces cost, limits sample volumes, and allows rapid detection of multiple bacteria	Primer design is critical, as primers may interfere with each other leaving some genes and bacteria undetected	Elnifro et al. 2000, Shi et al. (2010)
Real-time PCRb	Shortens detection time, detects and quantifies bacteria in real time, and possesses high sensitivity, specificity, and reproducibility	Requires expensive equipment and reagents and setting up requires high technical skills	Heid et al. (1996), Wong and Medrano (2005), Shi et al. (2010)
Reverse transcription PCR b	Can detect only viable cells of pathogens	Much skill is required to handle unstable RNA for pathogen detection	Sails et al. (1998), Sharma (2006), Shi et al. (2010)
Nested PCR	Has improved sensitivity and specificity than the conventional PCR method	Contamination level can be high probably from the laboratory environment	Picken et al. (1997)

Source of Table: Adzitey et al. (2013)

the advantage that they are rapid, less laborious, and more sensitive, specific, and efficient (Table 9.1).

9.2.1.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is an in situ DNA replication process that allows for the exponential amplification of target DNA in the presence of synthetic oligonucleotide primers and a thermostable DNA polymerase (Farber 1996; Wang et al. 2000). A wide range of different concentrations or units of DNA templates (5–25 ng), Taq DNA polymerase (0.6–1.25 U), primers (100 μM), and temperature cycles (45–95.8 °C and 30–40 cycles) have been employed to detect or confirm bacteria isolated from environmental pollution (Boonmar et al. 2007; Rahimi et al. 2011; Su et al. 2011). Other components of a PCR reaction such as deoxyribonucleotide triphosphates (dNTPs), magnesium (Mg^{2+}), and buffer solutions have been used in different concentrations to increase detection limits. A PCR process may involve the use of one primer (single PCR) or multiple primers (multiplex PCR) to

Table 9.2 Molecular methods applied to type or characterize bacteria

Typing method	Advantages	Disadvantages	References
PFGE	Has high discriminatory power, reproducibility, and typeability	Requires 3–5 days to complete a test, the cost is relatively high compared to other methods, and this technique has limited availability	Wassenaar and Newell (2000), Trindade et al. (2003)
MLST	Typing data are readily available via the internet, and it is easy to compare results among laboratories and countries, and has good discriminatory ability	This method is expensive and will require skilled researcher to perform	Enright and Spratt (1999), Urwin and Maiden (2003), Dingle et al. (2005)
RAPD	Cheap, rapid, readily available, and easy to perform	Has average reproducibility, discriminatory power, and approximately 80% typeability	Wassenaar and Newell (2000), Shi et al. (2010)
DNA sequencing	Has high discriminatory power, typeability, and reproducibility	Requires more days to complete a test, and this method is complex and relatively expensive	Newell et al. (2000), Wassenaar and Newell (2000)
Denaturing gradient gel electrophoresis (DGGE)			
REP	Cheap, easy to perform, and applicable to small or large number of isolates	Discriminatory power, reproducibility, and typeability are lower compared to PFGE, MLST, and DNA sequencing	Versalovic et al. (1991), Trindade et al. (2003)
ERIC	Quick, cost effective, and does not require much skills to perform	Discriminatory power, reproducibility, and typeability are lower compared to PFGE, MLST, and DNA sequencing	Wassenaar and Newell (2000), Tobes and Ramos (2005)
Ribotyping	Has 100% typeability, good reproducibility, and discriminatory power	It is a complex method and requires 3–4 days to complete a test	Denes et al. (1997), Wassenaar and Newell (2000), Shi et al. (2010)
AFLP	Has good discriminatory power, good reproducibility, and 100% typeability	Requires 3–4 days to complete a test and major capital investment	Wassenaar and Newell (2000), Meudt and Clarke (2007)
RFLP	Inexpensive and very sensitive for strain identification or differentiation	Slow, difficult, and could take up to a month to complete	Mohran et al. (1996), Nachamkin et al. (1996), Babalola (2003)

detect bacterial isolates (Table 9.1). Microorganisms contain a number of well-conserved genes, such as the ribosomal 16S gene and the heat-shock protein/chaperonin Hsp60/65 (or GRAEL), which are excellent targets for PCR. Analysis of the16S ribosomal RNA gene in bacteria in PCR and subsequent sequencing is particularly informative, as there are well-conserved sequences that can be used as binding sites for universal PCR primers adjacent to variable sequences and then a database of known sequences can be compared and analyzed. Other forms of PCR are real-time PCR, nested PCR, reverse-transcription PCR, and many more. Polymerase chain reaction assays have been routinely used for rapid detection, identification, and differentiation of pathogens. They have been used in areas such as DNA cloning, diagnosis of hereditary and infectious diseases, identification of genetic fingerprints, and detection and diagnosis of infectious diseases. Polymerase chain reaction technique plays an important role in the identification of typical bacterial strains that exist in viable but nonculturable coccoid forms (e.g., *Campylobacter* spp.), which are often missed by the conventional method (Magistrado et al. 2001). The use of PCR also avoids situations where phenotypic characteristics are ambiguous and wrongly interpreted, for instance, the occurrence of hippurate negative *C. jejuni* strains (Adzitey and Corry 2011). However, some PCRs may not be suitable for processed and certain foods because amplification can be obtained from DNA originating from both viable and nonviable cells (Sails et al. 1998; Wang et al. 2000). The technique can be expensive and its sensitivity and performance can be inhibited by components of enrichment broth and DNA extraction solution, concentration of the PCR mixtures (primers, DNA templates, dNTPs, and Mg^{2+}), and temperature and cycling conditions (Rossen et al. 1992; Wilson 1997; Wassenaar and Newell 2000). Table 9.1 shows commonly available molecular techniques that have been applied to identify bacteria isolated from environmental samples, while Table 9.2 summarizes the advantages and disadvantages of some commonly available molecular techniques for identifying pathogens. After popularization of polymerase chain reaction (PCR), nucleic acid-based assays for the detection and identification of environmental pathogens have been successfully developed. There are several DNA-based assay formats here, but only nucleic acid amplification techniques have been developed commercially to detect pathogens. PCRs, involving amplification step, are becoming more popular due to their higher sensitivity and fast identification of the pathogens and their toxins. Naravaneni and Jamil (2005) had standardized PCR-based technique for detection of *Salmonella* and *Escherichia coli*. They designed specific genes for examples for *Salmonella* used fimA, pathogenic *E. coli* used afa gene primers for amplification. Adleyb et al.'s (2009) studies have established that BCFomp1/BCRomp1, the DNA sequences, can be used for the specific detection of the *B. cereus* group spp. Analysis of these primers using standard PCR analysis showed that the minimum level of detection was 10^3 CFU/ml and the lowest number of bacterial cell per reaction tube amplified was 5 CFU with initial need of DNA found to be 1 pg. Malorny et al. (2004) developed robust real-time PCR for the specific detection of *Salmonella*. The assay used specifically designed primers and a probe targeted within the *ttrRSBCA* locus, which is located near the *Salmonella* pathogenicity island 2 at centisome 30.5. The

detection probabilities were 70% when a *Salmonella* cell suspension containing 10^3 CFU/ml was used as a template in the PCR (5 CFU per reaction) and 100% when a suspension of 10^4 CFU/ml was used. Sharma (2006) developed a method for detection of mRNA encoded by rfbE and eae genes of enterohemorrhagic *Escherichia coli (EHEC) O157:H7*. A 129-bp and a 106-bp sequence specific to rfbE and eae, respectively, were targeted for real-time detection. This method may contribute to meet the enhancing demand for quality assurance laboratories as standard diagnostic methods. Obeid et al. (2003) characterized and developed reusable glass chip-based microfibricated monolithic microdevices using reverse transcription (RT) and functional integration of PCR in a continuous flow mode. This allows the selection of the number of chip amplification cycles. Samples and reagents for PCR were pumped continuously through appropriate entry holes. After cycles 20, 25, 30, 35, and 40, products were collected from outlet channels. Products were collected in 0.2 ml tubes and analyzed by agarose gel electrophoresis and ethidium bromide staining after 30 cycles in only 6 min. The requirement of the initial DNA and RNA input molecules was used during these studies in the range of $2.5 \times 10^6 - 1.6 \times 10^8$, respectively.

Emerging molecular techniques, such as pulsed field gel electrophoresis (PFGE), denaturing gradient gel electrophoresis (DGGE), multilocus sequence typing (MLST), random amplified polymorphism deoxyribonucleic acid (RAPD), plasmid profile analysis, and deoxyribonucleic acid (DNA) sequencing are among most often used typing techniques and have been applied to pathogens isolated from environmental samples (Table 9.2). Others such as repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), ribotyping, amplified fragment length polymorphism (AFLP), and restriction fragment length polymorphism (RFLP) and so on are yet to be reported in terms of their application. Table 9.1 summarizes the advantages and disadvantages of some commonly available molecular techniques for typing or characterizing pathogens.

9.2.1.2 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is often used to examine microbial diversity of environmental samples and to monitor changes in microbial communities. The number, exact position, and intensity of bands in a gel track in DGGE gel numerically approximate the number and relative abundance of dominant ribotypes in the sample. This approach allows comparison of different microbial communities. Banding patterns of highly diverse microbial communities, present in soils, activated sludge and sediment, are usually very complex when bacterial primer is used. Furthermore, only the major populations of the analyzed community are represented on these DGGE patterns and thus are relatively less abundant but possibly very important species that cannot be detected by this molecular method. The DGGE gel provides a valuable tool for monitoring the structure and dynamics of microbial populations over time or under the influence of environmental changes. This approach has already been used in a few studies, which investigated specific microbial groups such as methanotrophic members of the Proteobacteria, actinomycetes, ammonia-oxidizing bacteria, Archaea, and fungi. Lee et al. (2009)

study Yellow Sand dust, a seasonal meteorological phenomenon affecting East Asia. These storms often provide long-range transport to various microorganisms. Microbiological air samples were collected using a PM2.5 cyclones, Yellow Sand events, and non-Yellow Sand events. Total nucleic acids were also extracted, and the 16S rDNA was amplified by PCR and analyzed by denaturing gradient gel electrophoresis (DGGE). Dendrogram analysis, based on DGGE, indicated that the microbial profiles from the Yellow Sand were distinctive from those of the non-Yellow Sand samples. These results suggest that, as a result of Yellow Sand events, humans in the affected regions are exposed to communities of microorganisms that might cause various adverse health effects. In DGGE, group-specific 16S rRNA primers are useful to compare different microbial communities, as well as to monitor microbial communities in function of time.

9.2.1.3 Pulsed Field Gel Electrophoresis (PFGE)

Pulsed field gel electrophoresis (PFGE) is an agarose gel electrophoresis technique used for separating larger pieces of DNA by applying electrical current that periodically changes direction (three directions) in a gel matrix unlike the conventional gel electrophoresis where the current flows only in one direction (Schwartz and Cantor 1984; Arbeit 1999; Trindade et al. 2003). In PFGE, intact chromosomes are digested using restriction endonucleases to generate a series of DNA fragments of different sizes and patterns specific for a particular species or strain (Shi et al. 2010). This method has good reproducibility, discriminatory power, and typeability, but PFGE is sensitive to genetic instability, has limited availability, and requires at least 3–4 days to complete a test (Wassenaar and Newell 2000).

9.2.1.4 Multilocus Sequence Typing (MLST)

Multilocus sequence typing (MLST) is an unambiguous, portable, and nucleotide-based technique for typing bacteria using the DNA sequences of internal fragments of multiple housekeeping genes (Maiden et al. 1998; Spratt 1999; Urwin and Maiden 2003). In MLST, approximately 450–500 bp internal fragments of each gene are used and most bacteria have enough variation within the house-keeping genes to provide many alleles per locus, thus allowing billions of distinct allelic profiles to be differentiated utilizing the multiple house-keeping loci (Enright and Spratt 1999; Urwin and Maiden 2003). The advantages of MLST are that it provides typing data that are unambiguous, portable, more accurate, and more discriminatory for most bacteria. These data are readily available, comparable, and accessible via the internet in contrast to most typing procedures involving the comparison of DNA fragment sizes on a gel (Dingle et al. 2005). Furthermore, MLST data can be used to investigate evolutionary relationships among bacteria (Urwin and Maiden 2003).

9.2.1.5 Random Amplified Polymorphism Deoxyribonucleic Acid (RAPD)

Random amplified polymorphism deoxyribonucleic acid (RAPD) is a PCR-based technique in which arbitrary primers (typically 10-mer primers) are used to randomly amplify segments of target DNA under low-stringency PCR condition (Wassenaar and Newell 2000). This process leads to the amplification of one or more DNA

sequences and generates a set of finger printing patterns of different sizes specific to each strain (Farber 1996; Trindade et al. 2003). The advantages of RAPD are that it is relatively cheap, rapid, readily available, and easy to perform (Wassenaar and Newell 2000; Shi et al. 2010; Rezk et al. 2012). In RAPD, the efficiency of amplification, annealing, and the length of the product varies with the primed sites, giving rise to both weak and strong amplicons, which makes interpretation of the results difficult. In addition, RAPD has low reproducibility, average discriminatory power, and approximately 80% typeability (Wassenaar and Newell 2000). The use of two or more primers improves the discriminatory power of RAPD (Trindade et al. 2003).

9.2.1.6 Deoxyribonucleic Acid (DNA) Sequencing Techniques

Deoxyribonucleic acid (DNA) sequencing techniques involve technologies used to determine the order of the nucleotide bases (namely adenine, cytosine, guanine, and thymine) in a DNA molecule. In recent times, DNA sequencing is widely and routinely used in the identification, typing, characterization, and/or taxonomic classification of unknown or novel pathogens isolates by many researchers. DNA sequencing has always been preceded by PCR to amplify the target genes. 16S rRNA is a common gene that is amplified for sequencing and subsequently for the identification, typing, and/or taxonomic classification of the pathogen in question. Sequencing has high discriminatory power, 100% typeability, and good reproducibility (Newell et al. 2000; Wassenaar and Newell 2000). The disadvantage is that it requires 2–3 days to complete a test, has limited availability, and costs are higher than other typing methods (Newell et al. 2000; Wassenaar and Newell 2000).

Other typing methods are enterobacterial repetitive intergenic consensus (ERIC), repetitive extragenic palindromic (REP), ribotyping, amplified fragment length polymorphism (AFLP), and restriction fragment length polymorphism (RFLP). Enterobacterial repetitive intergenic consensus (ERIC) PCR uses primers specific for enterobacterial repetitive intergenic consensus sequences. These primers can be used under high stringency conditions to match the target DNA to produce DNA finger printing that are different in sizes (Wassenaar and Newell 2000; Trindade et al. 2003). Enterobacterial repetitive intergenic consensus (ERIC) PCR is quick, easy to perform, and cost effective. Nonetheless, reproducibility is low compared to pulsed field gel electrophoresis. Repetitive extragenic palindromic sequences (REP) also depend on repetitive DNA elements present in pathogens (Trindade et al. 2003). In repetitive extragenic palindromic sequences, repetitive DNA elements present within bacterial genome are amplified to produce finger printing of different sizes specific to each strain (Versalovic et al. 1991). Trindade et al. (2003) reported that REP is cheaper, easy to perform, and applicable to small or large number of isolates, and the results have a good correlation with those obtained by PFGE but have lower discriminatory power. Ribotyping is a molecular technique that uses unique DNA sequences to differentiate strains of bacteria. In ribotyping, first isolation of genomic DNA then digestion of isolated DNA with selected restriction endonuclease at specific sites and generates pieces of DNA of different lengths, then go for separation of pieces of DNA by gel electrophoresis and at last identified bands of DNA using

Southern blot hybridization with specific probe of rRNA genes (Shi et al. 2010). Ribotyping has 100% typeability and good reproducibility, but it is a complex method, is sensitive to genetic instability, and requires 3–4 days to complete a test (Wassenaar and Newell 2000). Ribotyping has higher discriminatory power at the species and subspecies level compared to the strain level (Denes et al. 1997; Shi et al. 2010). Amplified fragment length polymorphism (AFLP) involves the use of two restriction enzymes to digest total genome DNA, one with an average cutting frequency (4-bp recognition site) and the other with a higher cutting frequency (6-bp recognition site) followed by linking of adapters to the sticky ends of the restriction fragments and amplification of a subset of selected restriction fragments (Wassenaar and Newell 2000; Shi et al. 2010). The primers used for amplification are radioactive or fluorescent labeled, and denaturing polyacrylamide gel analysis is used to determine the presence or absence of DNA fragments to identify polymorphisms (Blears et al. 1998; Wassenaar and Newell 2000). Amplified restriction length polymorphism has good discriminatory power, good reproducibility, and 100% typeability, needs no prior sequence information for amplification, and is insensitive to genetic instability, but AFLP is a complex method, requires 3–4 days to complete a test, and requires major capital investment (Wassenaar and Newell 2000; Meudt and Clarke 2007). Restriction fragment length polymorphism (RFLP) involves the use of restriction enzyme to digest DNA and to separate the resulting restriction fragments according to their length on agarose gel electrophoresis. Restriction fragments are then transferred into a membrane through Southern blot procedure and hybridized to a membrane bound labeled DNA probe (Babalola 2003; Foley et al. 2009). This method utilizes the variations in homologous DNA sequences to characterize bacteria. This technique is inexpensive, is very sensitive for strain identification or differentiation, and has widespread application, although it has become obsolete in the present time due to the emergence of relatively inexpensive sequencing technologies (Mohran et al. 1996; Babalola 2003). The technology is also slow and difficult and could take up to a month to complete (Mohran et al. 1996; Nachamkin et al. 1996).

9.2.2 Enzyme-Linked Immunsorbent Assay (ELISA)

Many immunological techniques provide quantitative assessment of the concentration of analytes in pure solutions or complex mixtures. In this area have great potential due to its sensitive and specificity towards diverse range of chemical and biological molecules and the immunoassays can also be used to provide real-time information.

The enzyme-linked immunosorbent assay (ELISA) is a test that identifies the substrate by the interaction of antibodies with antigen. An antibody is "Y"-shaped immunoglobin (Ig) that is made up of two heavy chains (H) and two light chains (L). Each of the chain has a constant and a variable part. The variable part is specific to the antigen that binds with corresponding antigen that is highly specific and selective (Conroy et al. 2009; Donahue and Albitar 2010). An ELISA required at least one

antibody with specificity towards particular antigen. The subsequent reaction produces a detectable signal, most commonly a color change in the substrate. The sample with an unknown amount of antigen is immobilized on a solid support known as a polystyrene microtiter plate either nonspecifically or specifically. After the antigen is immobilized, the primary antibody (detection antibodies) is added, forming a complex with the antigen. The primary antibody can be covalently linked to an enzyme or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation. The main enzymes used are horseradish peroxidase, alkaline phosphatase, and β-galactosidase. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are nonspecifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample.

9.2.2.1 Basic Steps for Developing and Running an Immunoassay (Karen et al. 2012)

1. Establish assay critical success factors (i.e., sensitivity required).
2. Ensure that appropriate antibody and antigen reagents are available.
3. Adsorb antigen or capture antibody to a solid surface.
4. Wash off unbound reagents.
5. Block nonspecific binding sites to reduce background.
6. Incubate the secondary antibody with the sample.
7. Wash off unbound reagents.
8. Incubate secondary antibody conjugate with sample.
9. Wash off unbound reagents.
10. Incubate substrate to generate signal.
11. Calibrate curve fitting, data analysis, and quantitation by nonlinear regression.

Enzyme-linked immunosorbent assay (ELISA) is a widely used immunoassay for environmental purposes. Three different types of ELISA are carried out according to different formats: direct competitive, indirect competitive, or sandwich type. Competitive assays are most common and can be performed in different ways. Analyte and the tracer (direct competitive ELISA) or analyte and the immobilized ligand (indirect ELISA) may compete for a limited number of binding sites. Sandwich-type ELISA is a noncompetitive assay, in which the analyte is recognized by two different antibodies, immobilized Ab and marker Ab (Harris 1999; Farre et al. 2005). Flow-injection immunoassay (FIIA) is a technique, based on the introduction of the sample into carrier stream, which enters the reaction chamber where the immunoreaction takes place. FIIA has been successfully used for detection of different pollutants, e.g., triazines. At present, this method is integrated into different immunosensors. Immunoassay and other immune techniques are the powerful and elegant techniques for rapid detection of environmental pathogen and its toxins. They also provide accurate and convenient means of detection of adulteration and authentic assay. These assays are fast and relatively inexpensive. Immunoassays are not as susceptible to matrix effects as PCR assays. Meng and Doyle (2002) and Taitt et al. (2004)

developed single-analyte sandwich immunoassays for the detection of *Salmonella enterica* serovar Typhimurium, with a detection limit of 10^4 CFU/ml; the limit of detection was improved ten-fold by lengthening the assay protocol to 1 h. *S. enterica* serovar Typhimurium was also detected in the following spiked foodstuffs, with minimal sample preparation: sausage, cantaloupe, whole liquid egg, alfalfa sprouts, and chicken carcass rinse. To determine its efficacy as a screening tool for the diagnosis of asymptomatic *Salmonella* infection of poultry, chicken samples were tested and the limit of detection of pathogen was 10^3 CFU/g. The most commonly used immunoassays for the detection of the pathogen are based on the use of whole cells, or heat killed (Silbernagel et al. 2005), or formalin fixed (Solve et al. 2000), and then detected by an ELISA. Feldstine et al. (1997) developed an immunoprecipitation method that made use of heat killed *L. monocytogenes* cells to detect contamination between 0.003 and 11 cfu/ml of food samples. An enzyme-linked immunosorbent assay (ELISA) for *Clostridium botulinum* type A and type B toxins was assessed for diagnostic accuracy in cases of infant botulism. Botulism is a serious food-borne neuroparalytic disease, caused by botulinum neurotoxin (BoNT), produced by the anaerobic bacterium *Clostridium botulinum*. Stanker et al. (2013) developed serotype B-specific monoclonal antibodies for sandwich (capture) ELISA antibodies ranging from 10 to 48 $\times$ 10^{-11} M. Assay performance for all possible combinations of capture–detector antibody pairs was evaluated, and the antibody pair resulting in the lowest level of detection (L.O.D.) ~20 pg/mL was determined.

9.2.3 Laser-Induced Breakdown Spectroscopy

Laser-induced breakdown spectroscopy (LIBS) has a flexible and convenient technique for rapidly determining the elemental composition of samples with minimal or no sample preparation. This technique is used to analyze the spectral emission from laser-induced plasmas, the plasma emission intensity being proportional to the abundance of an element in the sample. The relative simplicity and capability of fast multielemental analyses of solid, liquid, or gaseous samples make LIBS an ideal tool to study a wide range of samples. Although the use of LIBS has been most popular in metallurgical and biological samples, in recent years, it has been used to study environmental and biological samples. Yu et al. (2010) separated a variety of bacteria by detecting the trace mineral elements contained in five different types of bacteria that were grown in the same nutrient liquid, among them are four Gram-negative species (*Acinetobacter baylyi*, *Erwinia chrysanthemi*, *Escherichia coli*, and *Shewanella oneidensis*) and one Gram-positive bacterium (*Bacillus subtilis*). In the next round of this work, they evaluated the performance of LIBS for both sensitive detection of mineral trace elements in fresh vegetables and highly spatially resolved measurements of the amounts. Rosalie et al. (2010) described the use of LIBS to differentiate live pathogens and killed viruses on substrates. They differentiat the live pathogens *B. anthracis* Stern strain and *F. tularensis* live vaccine strain colonies on agar and demonstrate that it was found possible to differentiate between a

samples. UV-killed *hantavirus* strains were studied as dilutions on slides, and it was also found possible to differentiate between strains. Jonathan and Pourmand (2007) also identified and compared a pathogenic with nonpathogenic strain by LIBS. In their experiment they identified a pathogenic strain of bacteria, *Escherichia coli* O157:H7 enterohemorrhagic *E. coli* or EHEC, and compared three nonpathogenic *E. coli* strains (a laboratory strain of K-12 AB), a derivative of the same strain termed HF4714, by LIBS with nanosecond pulses in environmental samples. Multari et al. (2012) described the rapid detection of biological contaminants, such as *Escherichia coli O157:H7* and *Salmonella enterica*, on perishable foods items present in environment. Here, multivariate regression analysis of LIBS data is used to differentiate the live bacterial pathogens *E. coli O157:H7* and *S. enterica* in various foods (eggshell, milk, bologna, ground beef, chicken, and lettuce) and surfaces (metal drain strainer and cutting board). Qassem et al. (2011) investigated the effect that adverse environmental and metabolic stresses have on the laser-induced breakdown spectroscopy (LIBS) identification of bacterial specimens. Single-pulse LIBS spectra were acquired from a nonpathogenic strain of *Escherichia coli* cultured in two different nutrient media: a trypticase soy agar and a MacConkey agar with a 0.01% concentration of deoxycholate. A chemometric discriminant function analysis showed that the LIBS spectra acquired from bacteria grown in these two media were indistinguishable and easily discriminated from spectra acquired from two other nonpathogenic *E. coli* strains. Samuels et al. (2003) also used laser-induced breakdown spectroscopy to study bacterial spores, molds, pollens, and proteins. Biosamples were prepared and deposited onto porous silver substrates. LIBS data from the individual laser shots were analyzed by principal components analysis and were found to contain adequate information to afford discrimination among the different biomaterials.

9.2.4 Need of Sensors

Environmental pollution in various media is a serious health concern worldwide. Hence there is a continuing need to develop a cost-effective, accurate, fast, reliable, noninvasive, and nondestructive methods or tools for fast, analytical techniques used in comprehensive monitoring programs. Humans have sensors to understand and detect the environment around them. Therefore, it is equally important to design and develop biosensor-based measurement techniques that can accurately detect various contaminants from a wide spectrum. However, biosensors have several limitations for environmental analysis including (1) response time, (2) sensitivity, (3) selectivity, (4) compatibility, (5) affinity, (6) stability, (7) lifetime, etc.

9.2.4.1 Biological Sensor

A biosensor is a device that can be used to convert the existence of a molecule or compound into a measurable and useful signal. Biosensors use excitation to translate changes into recognizable signals. In 1962 Clark and Lyons developed a fast and more precise biosensor for glucose measurement. Biological sensors are analytical

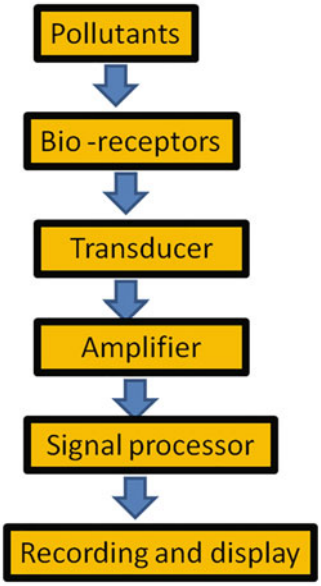


Fig. 9.1 Biological sensor (source of picture: modification of Costa et al. 2012)

devices that detect biochemical and physiological changes. A biosensor is defined by the International Union of Pure and Applied Chemistry (IUPAC) as a self-contained integrated device that is capable of providing specific quantitative or semiquantitative analytical information using a biological recognition element (biochemical receptor), which is retained in direct spatial contact with a transduction element. Transducers are essential to convert the particular biological and chemical changes into electrical data, which can identify different biochemical components of a complex compound to isolate the desired biochemical compounds (Fig. 9.1).

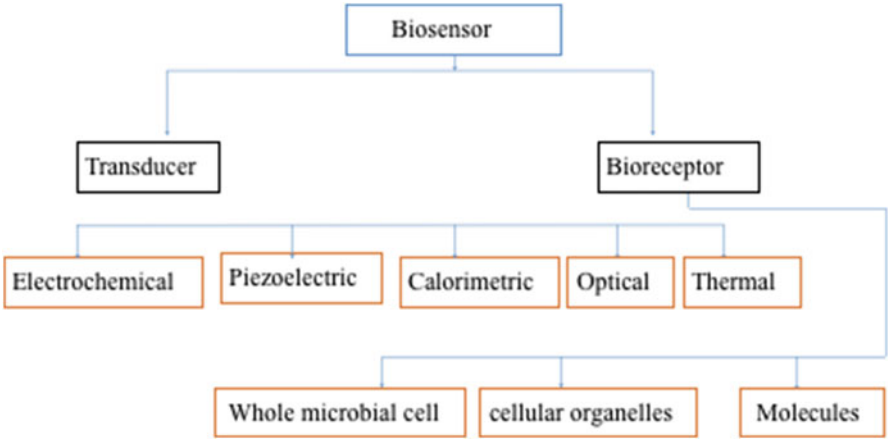
Basically, biosensors can be divided into two broad classes: (1) based on the signal transduction they employ and (2) based on the biological signaling mechanism they utilize. In the transducing element, class biosensors can be characterized as electrochemical, bioluminescence, optical, piezoelectric, and thermal sensors. A wide range of biological recognition elements have been used in biosensors constructed for potential environmental applications. Whole microbial cells, cellular organelles, and molecules such as enzymes, antibodies, different kinds of receptors, or DNA are the most common biorecognition elements of microbial origin.

Based on Transduction

Electrochemical Biosensors: Electrochemical biosensors measure the current produced from oxidation and reduction of an electroactive species in a biochemical reaction. These are generally based on biocatalysis of a reaction that produces or consumes electrons (such enzymes are rightly called redox enzymes). The sensor substrate usually contains an electrode that is used as the transduction element. These biosensors have the ability to even operate in turbid media. Electrochemical biosensors emerge as the most commonly used biosensors in monitoring and diagnosis of samples in environmental and clinical analysis (Vargas et al. 2018).

Optical biosensors are more suitable for direct monitoring systems. An optical biosensor is a compact analytical tool containing a biorecognition sensing element integrated with an optical transducer system. The basic objective of an optical biosensor is to produce a signal that is proportionate to the concentration of a measured substance (analyte) (Damborsky et al. 2016). The optical biosensor can use various biological materials, including enzymes, antibodies, antigens, receptors, nucleic acids, whole cells, and tissues as biorecognition elements. Optical-based biosensor is able to provide a direct, real-time, and label-free detection of many biological and chemical substances (Fan et al. 2008, Bhatta et al. 2012). The surface plasmon resonance (detailed in Sect. 9.2.4.2) or fluorescence that is integrated with optical fiber is a most popular method available for optical-based biosensing (Caygill et al. 2010).

Piezoelectric biosensors are developed by coating the surface of the biosensor with a selectively binding biologically active substance (Maraldo and Mutharasan 2007). Piezoelectricity can be explained as a linear interaction between mechanical and electrical systems in noncentric crystal or similar structure, which was first discovered by Curie brothers in 1880 (Pohanka 2018). Piezoelectric biosensors are a group of analytical devices working on the principle of affinity interaction. In piezoelectric biosensor, the transducer is made of piezoelectric material (e.g., quartz) and the biosensing material that coated on the piezoelectric material which vibrate at the natural frequency.



Based on Bioreceptor

Microbial Biosensors: A microbial biosensor consists of a transducer in conjunction with immobilized viable or nonviable microbial cells. They may be categorized into one of the two groups. The first group of biosensors is viable organisms, targeted to measure an integral toxicity, genotoxicity, estrogenicity or other general parameters of the sample. They essentially include whole microorganisms as biorecognition

elements. The most often reported cell-based biosensors include genetically modified bacteria with artificially constructed fusions of particular regulatory system (native promoter) with reporter genes. The presence of an effector (nonspecific such as DNA damaging agents, heat shock, oxidative stress, toxic metals, organic environmental pollutants) results in transcription and translation of fused target genes, generating recombinant proteins that produce some measurable response. Frequently used reporter genes are lux (coding for luciferase) and gfp (coding for green fluorescence protein), expression of which correlates with luminescence- or fluorescence-based light emission. Colorimetric determination of target gene expression is possible by fusing it to reporter genes coding for β-galalactosidase (lacZ) or alkaline phosphatase (phoA). Recently, *E. coli* biosensor capable of detecting both genotoxic and oxidative damage has been developed by introducing plasmids with fusion of katG (gene encoding for an important antioxidative enzyme) promoter to the lux reporter genes, and another with recA (gene encoding crucial enzyme for DNA repair) promoter with the gfp reporter gene (Mitchell and Gu 2004). Microbial biosensors have been widely used in the environmental, food, and diagnostics industry due to its advantages of low cost, stability, and fast response. Compared to enzymes, the microorganisms that are used as bioelements can make use of the enzyme to specifically respond to the analytes without time-consuming and expensive purification. Based on its attractive properties, several directions for the development of the microbial biosensors have shown great promise.

DNA/Nucleic Acid Sensor: Genetic information can be used as a biorecognition part of various biosensors. Identification of pathogen from a human tissue or blood samples are common analytes for these biosensors. This biosensor principal is based on recognition of the complementary strand by ssDNA to form stable hydrogen bond between two nucleic acids to become dsDNA. In order to achieve this, ssDNA is used as probe to immobilized in bioreceptor and complementary sequences present in the target of interest. The highly specific affinity binding's reaction between target to the probe's single strand DNA, which results in hybridization of complementary ssDNA to form dsDNA. Subsequently biochemical reaction that allows transducer amplified the signal into electrical one. Sometimes linker such as thiol or biotin is needed in the effort to immobilize the ssDNA onto the sensing surface. The nucleic acid biological recognition layer, which incorporates with transducer, is easily synthesizable, highly specific, and reusable after thermal melting of the DNA duplex (Fig. 9.2). Moreover, Yeh et al. (2011) have reported optical biochip for bacteria detection based on DNA hybridization with detection limit of 8.25 ng/ml. However, electrochemical transduction is the most abandoned method used to study DNA damage and interaction, as reported in the literature. The development of electrochemical DNA biosensor has received a great deal of attention lately, and this has largely been driven by the need to develop rapid response, high sensitivity, good selectivity, and experimental convenience (Liu et al. 2012).

9.2.4.2 Surface Plasmon Resonance (SPR)

The phenomenon of surface plasmon resonance biosensor was first reported by Wood (1902). The application of biomolecule interaction was first reported by

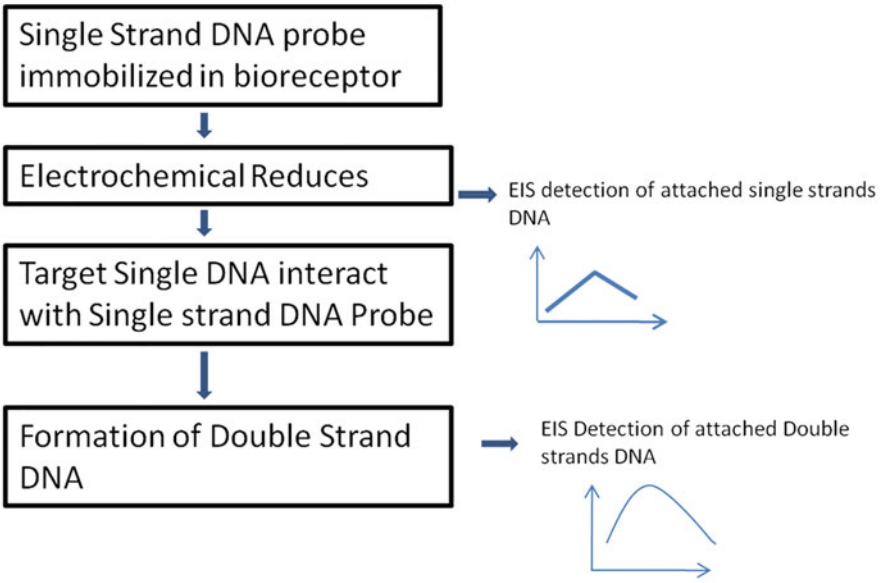


Fig. 9.2 Schematic diagram of the fabrication of the impedimetric DNA biosensor and the detection of target DNA (source: modification of Q. Gong et al. 2015)

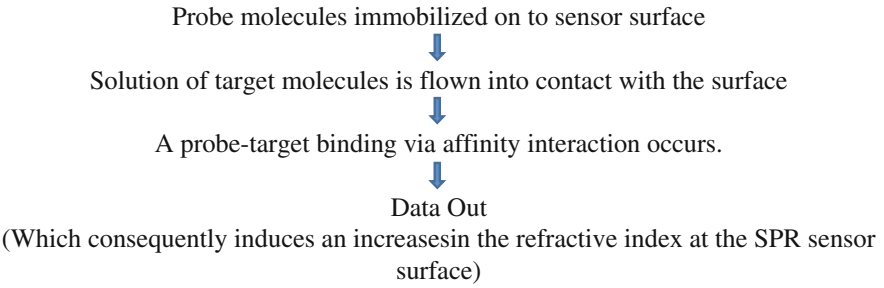


Fig. 9.3 Flow diagram show surface plasmon resonance system

Liedberg et al. 1983, and the complete phenomena of excitation of surface plasmon were explained by Otto (1968). Surface plasmon resonance biosensor (SPR), a modern, cutting edge sensor technology, can perform rapid detection of pathogen (Fig. 9.3). SPR is the optical sensor that provides sensitive, label-free, and real time (few seconds or minutes) monitoring of reaction and has been proven to be one of the most powerful technologies to determine specificity, affinity, and kinetic parameter during the binding of macromolecules in many bond types including protein–protein, DNA–protein, lipid–protein, polysaccharides–protein, and virus protein, among others. Identification of biomolecules on SPR was made possible by immobilizing a capturing agent, such as antibodies, enzyme, peptide, and DNA on

a metal surface, allowing the sample solution flow in excess over that surface, while using SPR spectroscopy to measure the changes in the SPR angle, which is the angle of minimum reflectivity, it can be determined by varying the incidence angle and recording the reflected light intensity during the biological binding reaction between various biomolecules. So far, numerous studies have advanced the potential of SPR sensors by increasing the effectiveness of the techniques.

SPR system offers a simple means of identifying bacteria, even a very small number of bacteria in real time, without any markers. The bacteria interact with specific ligands grafted on the chip, to bring about a local change in the refractive index in the vicinity of surface and then a plasmonic resonance signal. Using SPR system in imaging allows numerous different probes to be attached to the chip's surface measuring, so that numerous pathogens can be simultaneously identified in the course of a single test. SPR-based biosensors have been reported by many researchers for the detection of food-borne pathogens such as *L. monocytogenes* (Koubova et al. 2001), *Salmonella* (Koubova et al. 2001; Oh et al. 2004), and *E. coli O157:H7* (Subramanian et al. 2006; Waswa et al. 2007). Also, commercially available optical biosensors use SPR for monitoring and identifying pathogens and their toxins especially in environmental pollution.

9.2.4.3 Carbon Dioxide Sensor

Carbon dioxide sensor is a device for the measurement of elevated CO_2 gas level from biomedical studies to food-packaging processes. As the role of these gases, in the determination of air quality by biochemical reactions, Now a days, development of different types of CO2 sensors such as optical sensors, polymer opal films, polymer hydrogels, etc., by using different fabrics, such as solid electrolyte, mixed oxide capacitors, polymers with carbonate solution and so on, have been investigated (Lai et al. 2011). Among them, solid electrolyte-type CO_2 sensors are of particular interest from the viewpoint of low-cost, high-sensitivity, high-selectivity, and simple element structure (Santonico et al. 2017). There are needs of efficient CO_2 sensors that can intelligently monitor the gas concentration changes. Hence, a CO_2 sensor incorporated into package can efficiently monitor product quality. Although much progress has been made so far in the development of sensors monitoring CO_2, most of them are not versatile and suffer from limitations such as high equipment cost, bulkiness, and energy input requirement, including safety concerns. Latest approaches, for more compatible with industrial demand, would consist of printable sensor membranes on the packaging material and should provide information about analytes at any given stage in the packaging and delivery process, to sense the physical and biological (microbiological) changes (Mheen and Kwon 1984). Chu and Syu (2017) design a carbon dioxide based an optical sensor for the sensing films coated on filter paper. Ethyl cellulose (EC) doped with platinum (II) meso-tetrakis (pentafluorophenyl) porphyrin (PtTFPP) and 7-amino-4-trifluoromethyl coumarin serve as the oxygen-sensing material and reference blue emission dye for the pH indicator, respectively. The sensing layer includes the pH-sensitive fluorescent indicator 1-hydroxy-3,6,8-pyrenetrisulfonic acid trisodium salt immobilized within the ethyl cellulose. The carbon di oxide sensitive materials

can both be excited with a 405 nm LED, and the two emission wavelengths can be detected separately. The proposed optical dual sensor can be used for the simultaneous sensing of carbon di oxide concentrations in environmental applications.

9.2.4.4 Immunosensor

An antibody-based biosensor was applied for the first time to detection in the 1950s, opening the doors to the possibility of immunodiagnosis (Donahue and Albitar 2010). Since then, there have been vigorous efforts made to develop immunosensor that is composed of antigen/antibody as bioreceptor as a tool for clinical diagnostics (Conroy et al. 2009; Orazio 2011). Hence, an immunosensor is highly specific, stable, and versatile. The specificity of an antibody toward the binding side of its antigen is a function of its amino acids (Fowler et al. 2008). Those days, there are two types of detection methods, which are frequently used in immunosensor, that is, optical and electrochemical. However optical detection transduction method has suffered from poor sensitivity when coupled with radioimmunoassay, the short half-life of radioactive agents, concerns of health hazards, and disposal problems. Electrochemical detection overcomes problems associated with other modes of detection of immunoassays and immunosensors (Fig. 9.4). In contrast, electrochemical immunoassays and immunosensors enable fast, simple, and economical detection, which are free of these problems Fowler et al. (2008). However, recent advance in science and technology has created an optical transduction method, a new path toward highly sophisticated automated instrument. Hence, optical and electrochemical detection methods are gaining mutual importance for development of

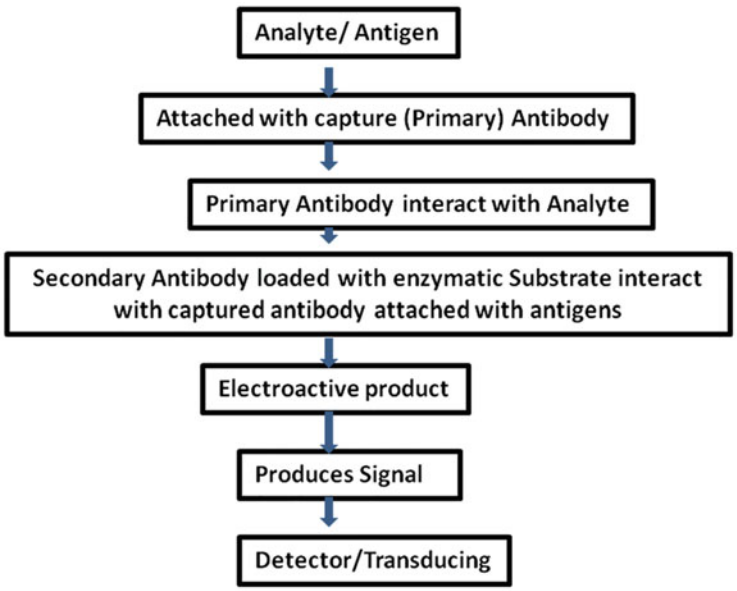


Fig. 9.4 Process of immunosensor (source of picture: modified from Cho et al. 2018)

immunosensor (Shankaran et al. 2007; Bhatta et al. 2012). The development of immunosensor for bacteria and pathogen detection has gained a great deal of attention due to its application in the point-of-care measurement (POC) (Braiek et al. 2012; Holford et al. 2012).

9.2.4.5 Nanosensor

Nanosensor is defined as a modified version of a biosensor or chemical sensor employing nanomaterial in an analytical device, helping in detection of toxic contaminants in environmental toxicant at very sensitive levels. Nanomaterial is defined as material with at least one dimension smaller than 100 nm. Owing to their incredibly small size, nanomaterials can be very versatile in many terms of their detection and monitoring. Nanoparticle-based biosensors are considered as potential tools for rapid, specific, and highly sensitive detection of the analyte of interest (Table 9.3). Nanomaterials can provide optical, catalytic, magnetic, and antimicrobial properties for sensing applications. Therefore, the integration nanotechnology in sensing platforms has provided significant enhancements in detection capabilities and functionality of these devices. However, multifunctional nanosystems have the potential to act simultaneously as a method for rapid microbial capture, detection, and decontamination. Thus, future developments are also expected in the

Table 9.3 Detection of biological contaminant by Nanobiosensor

Nanomaterials	Biological contaminants	Electrode/sensor	Reference
Functionalized AuNPs	Aflatoxin	Immunoelectrode	Sharma et al. (2010)
CNTs	Salmonella	Electrochemical biosensor	Jain et al. (2012)
Glyconanoparticles	Cholera toxin	Colorimetric bioassay	Schofield et al. (2007)
Silver core and a gold shell (AgAu)	AF B1	Immunodipstick assay	Liao and Li (2010)
Nanostructured zinc oxide	Mycotoxin	ITO glass plate	Ansari et al. (2010)
Magnetic nanoparticles and TiO_2 nanocrystals	Salmonella	Optical nanocrystal probes	Joo et al. (2007)
Oligonucleotide-functionalized Au nanoparticles	Escherichia coli O157:H7	Piezoelectric biosensor	Chen et al. (2008)
Liposomic and poly (3,4-ethylenedioxythiophene)-coated CNTs	Cholera toxin	Electrochemical immunosensor	Viswanathan et al. (2006)
Fe_3O_4 NPs	*Campylobacter jejuni*	Glassy carbon electrode	Huang et al. (2010)
AuNPs	Melamine	Colorimetric aptasensor	Yun et al. (2014)

Source: Kuswandi et al. (2017)

development of smart labels to indicate food spoilage or presence of harmful toxins. Thus, this area would benefit from fundamental advances in the development of low-cost and flexible nanosensors suitable for roll-to-roll manufacturing in large-scale production. The use of inexpensive materials such as paper or plastic and integration of all sensing reagents into a portable compact unit is also desirable for future deployment and rapid implementation of these devices. Method validation, comparability, stability, and interlaboratory studies to evaluate performance are also needed to ensure robustness and accuracy of these devices for real-world applications (Mustafa et al. 2017).

9.3 Conclusion

In this chapter, we summarized the recent progress in modern tools for monitoring of environmental pollution and assessment to promote for betterment of the public health and individual life quality. So our center of interest to detect of pathogens in the actual environmental samples is imperative. Design and development of detection methods with sensitivity, reproducibility, selectivity, and speediness are urgently required for screening their occurrence in correspondence with safety regulations at significant levels. The nucleic acid-based biosensors have potential to sense the samples (pollutant) in a very low concentrations, and it is time-effective upstream processes. Immunosensors have relatively fewer steps and required less assay time but needs specific antibodies that are complicated and non-economical. Using different signal amplification and background-reduction techniques coupled with the miniaturization with enhanced sensitivity, nucleic acid/antibody-based detection methods offer sensitive and selective tools for screening various forms of pathogens. Use of nanoparticles and nanomaterials will facilitate efficient techniques, multiplex detection systems, and nanomaterial-based research for simultaneously sensing relevant pathogens in a specific environmental scenario. It has been revolutionized the case of biological detection. The overall mechanism has become robust, smarter, less costly, and user friendly. The significant advantage includes rapid results because the approach to increase signal rather than the target analytes has revolutionized the paradigm of detection.

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