1 Fundamentals of Biosensors

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1.1 INTRODUCTION TO BIOSENSORS

Today, the importance of monitoring various samples for quality assurance or assessing risk factors or, more seriously, for disease diagnosis cannot be overemphasized. Several analytical techniques, both simple and sophisticated, have made it possible to achieve the same and thereby raise the standards of living of human beings. However, most analytical techniques demand skilled labor and sophisticated instrumentation and are tedious, time-consuming, and expensive. Consequently, there is an aspiration for portable, reliable, fast, and relatively inexpensive detection techniques. With the advent of biosensor technology, such aspirations have begun to come to fruition. A biosensor combines the exquisite selectivity of a biological component with the processing power of a transducer. The biosensor is an interdisciplinary field. It demands an exciting amalgamation of knowledge from several fields viz. the principles of basic sciences (physics, chemistry, biology) with the fundamentals of micro/nanotechnology, electronics, computer sciences, and design. The biosensor as an analytical tool has revolutionized the conventional analyses paradigm and justly finds its applications in the field of clinical diagnostics and biomedical applications, environmental applications, food and processing industries, and much more [1].

As a first step towards understanding a biosensor, it is important to take into account the main difference between a chemical sensor and a biosensor. According to International Union of Pure and Applied Chemistry (IUPAC), a chemical sensor is a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal. As per IUPAC recommendations from 1999, a biosensor is an independently integrated receptor-transducer device, capable of providing selective, quantitative or semi-quantitative analytical information using a biological recognition element. As a matter of realization, the basic difference between a biosensor and a chemosensor is that a biosensor employs a biological component as a recognition system specific to the analyte of interest and converts the biological signal into a measurable signal. In contrast, a chemosensor converts a chemical or physical property of a specific analyte into a measurable signal. In general, a biosensor is an analytical tool consisting of a biological recognition element, specific to the analyte of interest, in close association with a transduction element, which converts the biochemical signal into a quantitative or semi-quantitative electrical or other suitable signal.

1.1.1 History of Biosensors

The origin of biosensor technology is linked to the development of pH and oxygen electrodes. In this path, M. Cremer demonstrated the proportional relationship between an acid concentration and the rising electric potential of a glass membrane. This led to the concept of pH (hydrogen ion concentration) in 1909 by Søren Peder Lauritz Sørensen. Soon enough, an electrode for pH measurements was realized by W.S. Hughes in 1922. Another significant development was that of Griffin and Nelson, who first demonstrated the immobilization of an enzyme, invertase, on aluminium oxide and charcoal between 1902 and 1922. Thereafter, Leland C. Clark Jr. developed a simple and small amperometric electrode for detection of oxygen and published his definitive paper on the subject in 1956[2]. This followed the development of an amperometric enzyme electrode by Clark and Lyons for the detection of glucose. The concept was illustrated by an experiment in which glucose oxidase was entrapped at a Clark oxygen electrode using a dialysis membrane. The decrease in measured oxygen concentration was proportional to glucose concentration. Clark and Lyons coined the term "enzyme electrode" in their published article [3], and later Updike and Hicks [4] expanded the experimental detail necessary to build functional enzyme electrodes for glucose. This event was followed by the discovery of the first potentiometric biosensor for urea detection in 1969 by Guilbault and Montalvo Jr. Finally, in 1975, with the introduction of a commercial glucose biosensor by Yellow Springs Instruments (YSI), which employed Clark's technology, the biosensor technology marked its first appearance in the market.

Table 1.1 showcases the important milestones in the development of biosensors during the period 1970–1992. Since then, the amalgamation of several fields has led to the emergence of more developed and sophisticated biosensors. The era of the biosensor is an interdisciplinary one. The utility of these sensors is well pronounced in the fields of clinical diagnosis, agriculture, biotechnology, environmental monitoring, military applications, and others.

TABLE 1.1
Important Milestones in the Development of Biosensors During the Period 1970–1992 (Adapted from [5])

1970	Discovery of ion-sensitive field-effect transistor (ISFET) by Bergveld
1975	Fiber-optic biosensor for carbon dioxide and oxygen detection by Lubbers and Opitz
1975	First commercial biosensor for glucose detection by YSI
1975	First microbe-based immunosensor by Suzuki et al.
1982	Fiber-optic biosensor for glucose detection by Schultz
1983	Surface plasmon resonance (SPR) immunosensor by Liedberg et al.
1984	First mediated amperometric biosensor: ferrocene used with glucose oxidase for glucose detection
1990	SPR-based biosensor by Pharmacia Biacore
1992	Handheld blood biosensor by i-STAT

1.2 GENERAL CONFIGURATION OF A BIOSENSOR

As per the conventional configurations, the biosensor consists of five components, namely, the biocatalysts/biorecognition element, transducer, amplifier, processor, and display (Figure 1.1). The biocatalyst is a biological component, also called a biorecognition element or bioreceptor. This biorecognition element usually interacts specifically with the target analyte of interest present in the samples. Typically, the bioreceptor is incorporated into the system by immobilization. Examples of biorecognition elements include enzyme, antibody, organelle, bacterial cell or other cells, whole slices of mammalian or plant tissues, and nucleic acids. Because the biological component is involved in identifying the target analyte in the sample, it is primarily responsible for conferring the specificity to the developed biosensors.

The next important component is the transducer, which converts the biorecognition signal into a measurable electrical signal. The transducer is generally placed in intimate contact with the recognition layer. It measures the physicochemical changes produced in the biorecognition layer by the presence of the analyte in the sample, generating a signal that is either proportional or inversely proportional to the analyte concentration. The typical transducers used in the system could be electrochemical, piezoelectric, calorimetric, or optical.

The function of the amplifier is to amplify the low electrical signal output from the transducer. Additionally, this unit removes the background noises generated within the electronic components of the transducer by subtracting the "reference" baseline signals.

The main function of a processor is to receive the input signal and provide the appropriate output signal. The signal produced at this level is usually converted to a digital form after being processed through a microprocessor stage, where the data is processed, converted to concentration units, and output to a display device or data store [5].

1.3 CHARACTERISTICS/SALIENT FEATURES OF A BIOSENSOR

1.3.1 Selectivity

Selectivity of a biosensor system refers to the extent to which a bioreceptor can discriminate a particular analyte of interest under given conditions in mixtures – simple or complex – without interference from other components in the mixture. Specificity, on the other hand, refers to the ultimate selectivity, i.e. 100% selectivity and 0% interference. This means that the bioreceptor is specific to a particular analyte of interest, recognizing no other. However, in cases where this ideal performance cannot be realized due to similarity between the analyte of interest and other components in the sample mixture or the lack of tools for specific sensing of the target, a selective sensing approach comes in handy. For example, although sugar-binding proteins – lectins – can be used to detect specifically carbohydrate-containing biomolecules, the individual lectins are not specific to individual glycosylated biomolecules viz. glycoproteins and glycolipids [5].

1.3.2 Sensitivity

Sensitivity refers to the minimum amount of analyte that can be detected by the system. In biosensors, the detection limits are commonly discerned by following two approaches: limit of detection (LOD) and limit of quantification (LOQ). Determination of LOD requires knowledge of limit of blank (LOB). LOB is the highest apparent analyte concentration expected to be found when replicates of a sample containing no analyte (blank) are tested. Although the blank samples are devoid of analyte, it can produce an analytical signal that might otherwise be consistent with a low concentration of analyte. LOB is estimated by measuring replicates of a blank sample and calculating the mean result and the standard deviation (SD).

$$\mathrm{LOB} = \mathrm{mean}_{\mathrm{blank}} + 1.645\ (\mathrm{SD}_{\mathrm{blank}}) \tag{1.1}$$

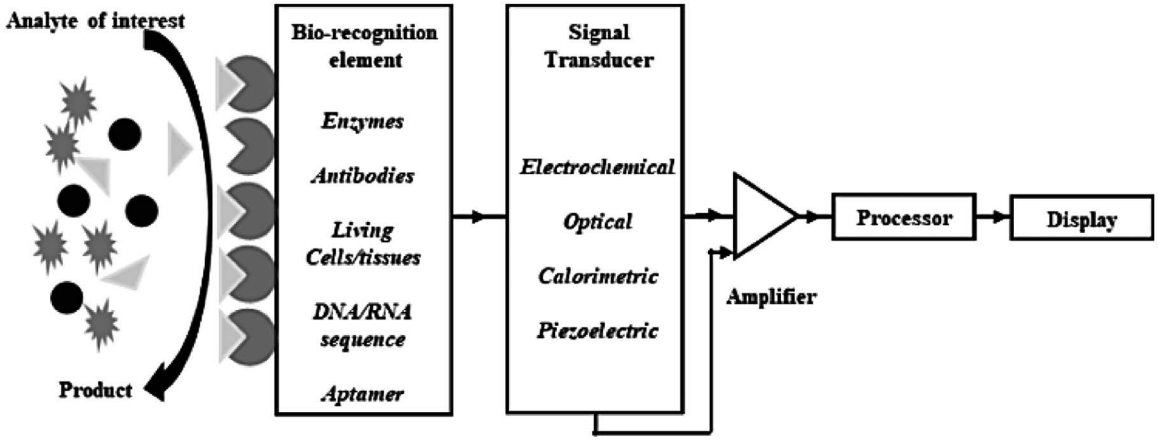

FIGURE 1.1 General configuration of a biosensor.

LOD is the lowest analyte concentration likely to be reliably distinguished from the LOB and at which detection is feasible. It is therefore greater than LOB. LOD is determined by utilizing both the measured LOB and test replicates of a sample known to contain a low concentration of analyte. The mean and SD of the low concentration sample is then calculated and used to calculate LOD.

$$\text{LOD} = \text{LOB} + 1.645\ (\text{SD}_{\text{low concentration sample}}) \tag{1.2}$$

LOQ is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met. The LOQ may be equal to or greater than LOD [6].

Additionally, for a linear calibration curve ($y = a + bx$), it can be assumed that the response (y) is linearly related to the concentration (x) of the analyte in a limited range. This model is used to discern the sensitivity b, LOD, and LOQ from the following relations:

$$\text{LOD} = 3 \times \frac{\text{SD}_a}{b} \tag{1.3}$$

$$\text{LOQ} = 10 \times \frac{\text{SD}_a}{b} \tag{1.4}$$

where SD_a is the standard deviation of the response and b is the slope of the calibration curve. The SD_a can be estimated by the standard deviation of y-intercepts, of the regression lines.

1.3.3 Reproducibility

It refers to the property of a system to generate identical responses under identical set-up conditions viz. sample nature, pH, temperature, etc. Reproducibility of a biosensor largely defines the reliability of the biosensor [5].

1.3.4 Stability

Stability refers to the property of a system to be independent of ambient interferences in the form of environmental conditions like humidity, temperature, pH, or other interferences from the sample matrix. These interferences can cause a shift of the output signal to a value that is not accurate or precise. The biorecognition element used in a biosensing system plays a major contributor to stability. For example, the most popularly used biorecognition element – enzymes – are prone to denaturation when subjected to mild changes in ambient conditions. This may demand specific storage conditions for the biosensing system. Stability as a performance factor defines the shelf life of the biosensor system in question [5].

1.3.5 Response Time

This refers to the total time taken by the biosensor to produce the output signal, qualitative or quantitative, that can be recorded and subjected to interpretation. Chemical sensors usually have a very short response time as compared to their counterpart biosensors. However, a few biosensors based on the enzyme catalase for peroxides have exhibited response time of few seconds [5].

1.3.6 Recovery Time

It refers to the interval time required by the biosensor between two subsequent sample analyses. It is desirable to have as low a recovery time as possible. Usually a recovery time of within few minutes is desirable [5].

1.3.7 Linearity and Dynamic Range

Linearity reflects the accuracy of the measured response for a set of measurements with different analyte concentrations to a straight line, mathematically represented as $y = a + bx$, where a is the concentration of the target analyte, y is the output signal, and b is the sensitivity of the biosensor. In this context, the term "linear range" is used to describe the range of analyte concentration for which the biosensor produces a linear response. However, another term, "dynamic range" of the sensor, is used to describe the maximum and minimum values of the analyte concentration that can be measured. Dynamic range can be linear or nonlinear [5].

1.3.8 Effect of Physical Parameters

A biosensor should be independent of physical parameters such as temperature, pH, ionic strength, etc., so as to assure that the performance of the biosensing system is not significantly compromised. This attribute can pave the way for sample analyses with minimal pre-treatment [5].

1.3.9 Additional Factors

A biosensor should possess the following beneficial features for successful commercial applications. (a) If the cofactors or coenzymes involved in the biorecognition reaction are not avidly associated with the biocatalysts, then these should be preferably co-immobilized with the biocatalysts to reduce the operational steps involved in the practical use of the biosensor. (b) For *in vivo* applications, the biosensor probe should be tiny, having no toxic or antigenic effects. (c) The biosensor or its sensing part should be sterilizable without destroying its function for monitoring an analyte in the fermentation setup. (d) The complete biosensor should be inexpensive, small, portable, and capable of being used by semi-skilled operators. (e) Finally, there should be a market for the developed biosensor. It should also fulfil the legislative and other statutory requirements for the country. Further, the biosensor product should meet the overall satisfaction of the customers

to encourage them to abandon traditional laboratory testing. Moreover, the World Health Organization (WHO) has suggested the ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, Deliverable to end users) criteria, particularly for the point-of-care (PoC) devices for disease diagnosis in underdeveloped and developing countries. Therefore, any biosensor that complies well with the ASSURED criteria may have better market potential.

1.4 CLASSIFICATION OF BIOSENSORS

Biosensors can be classified based on either of the two important elements i.e. based on the bioreceptor or based on the transducer used in the biosensor. Additionally, biosensors can be classified based on the biological mechanism or biorecognition principle they exploit for sensing – catalytic biosensor or affinity biosensor. However, the classification of biosensor based on the type of transducers is most widely reported in the current literature. Nonetheless, all three modes of classification (Figure 1.2) are briefly described in the following sections.

1.4.1 Classification of Biosensors Based on the Biorecognition Principle

Based on the biorecognition principle, biosensors can be classified into catalytic biosensors and affinity biosensors.

- *Catalytic biosensor*: This refers to the usage of enzymes or may be even living cells as biocatalysts with a transducer to produce a signal proportional to the target analyte concentration. This signal is brought about by the reaction catalyzed by the particular enzyme/cell, which may be heat change (exothermic or endothermic), release/uptake of gases viz. ammonia or oxygen, change in proton concentration, changes in light emission, etc. Enzyme biosensors are widely used biosensors that have been developed due to their substrate specificity and catalytic properties.
- *Affinity biosensor*: This refers to the usage of antibodies or nucleic acids (e.g. aptamers) as bioreceptors that specifically bind to the target analyte. An affinity biosensor works on the principle of receptor-ligand interactions with a high differential selectivity in a nondestructive fashion. Clearly, the structure of the analyte remains chemically unaltered during the detection process using this type of sensor.

1.4.2 Classification of Biosensors Based on the Biorecognition Element

The biorecognition element/bioreceptor/bioelement is largely responsible for conferring selectivity/specificity on the biosensor, and is therefore a distinguishing feature of biosensor technology. Based on the biorecognition element, a biosensor can be classified into different types such as enzyme-based biosensor, immunosensor, nucleic acid biosensor, cell/tissue-based or microbial biosensor, and biomimetic. The enzymes, antibody, nucleic acids, whole cells, biomembrane, and even organelles can be used either in isolation or in combination to act as the bioreceptor that brings about biochemical recognition of the target analyte.

1.4.2.1 Enzyme-Based Biosensors

An enzyme-based biosensor utilizes enzyme(s) as the biorecognition element. An enzyme is a biocatalyst that increases the rate of chemical reaction without itself being changed in the overall process. Virtually, all cellular reactions/processes are mediated by enzymes. Most enzymes are proteins, with

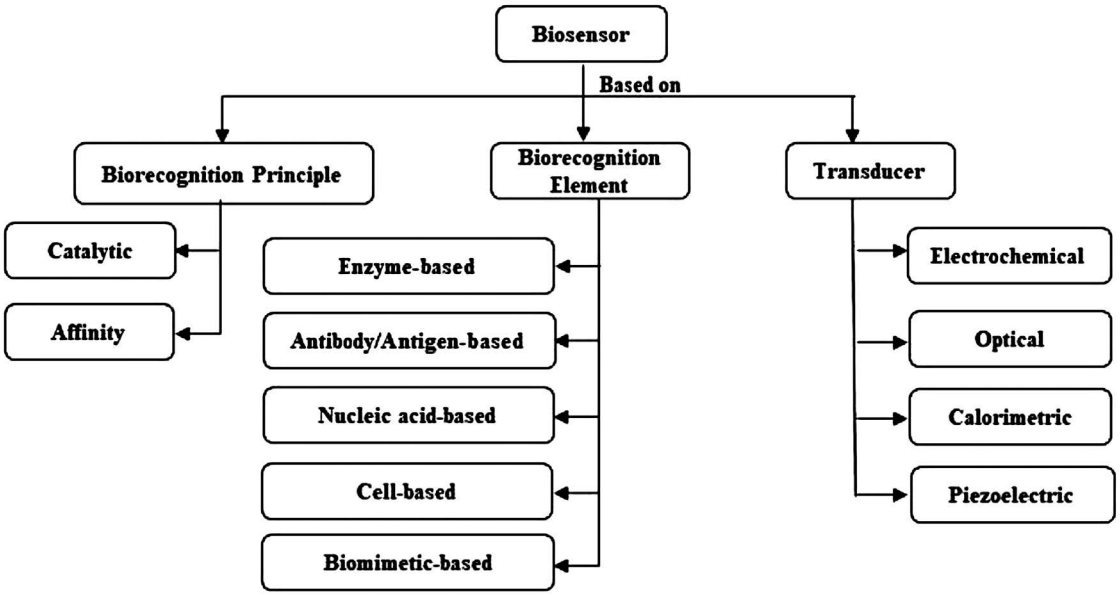

FIGURE 1.2 Classification of biosensors.

the exception of a small group of nucleic acid-based enzymes, such as ribozymes. The catalytic activity of the enzyme depends on the integrity of its native protein conformation. This means that denaturation of the enzyme – dissociation into its constituent subunits or amino acids – renders the enzyme nonfunctional.

Enzymes that are composed entirely of amino acid residues are called simple enzymes, while the enzymes that require a nonproteinaceous component attached to them in order to become functional are called conjugated enzymes, and this nonproteinaceous component is called a cofactor. In the case of conjugated enzymes, the protein component is called the apoenzyme, which when combines with the cofactor to form a fully functional enzyme, which is called the holoenzyme. Cofactors may include metal ions, or complex organic groups, in which case they are called coenzymes. Cofactors that are tightly associated with the apoenzyme are called prosthetic groups.

Enzyme-based biosensors were the earliest developed biosensors, introduced by Clark and Lyons in 1962 for amperometric detection of glucose. Example of the most exploited enzyme bioreceptors include glucose oxidase (GOD) and horseradish peroxidase (HRP). Currently, enzymes are widely used as bioreceptors because of their substrate specificity and usual high turnover rates.

The enzyme is usually immobilized or intimately associated with the transducer surface for signal detection. The catalytic activity of the enzyme accounts for the fast activity and possible signal amplification of the biosensor, thereby contributing sensitivity to the biosensor. This specific catalytic reaction of the enzyme makes enzyme-based biosensors able to detect much lower limits of target analyte than with normal binding techniques.

An enzyme as a bioreceptor can be utilized to detect the analyte of interest mostly by one of the following mechanisms: (a) detection of an analyte that gets converted by the enzyme into a detectable product, (b) detection of an analyte that acts as an enzyme inhibitor or activator, and (c) detection of an analyte that brings about modification of enzyme properties upon interaction.

An enzyme catalyzed reaction, and therefore the performance of the enzyme-based biosensor, is influenced by several factors such as the concentration of the substrate, pH, temperature, and presence of inhibitors or activators [1].

1.4.2.2 Immunosensors

An immunosensor typically utilizes an antibody as a bioreceptor. Antibodies are antigen-binding glycoproteins, synthesized by the body in different amino acid sequences, which confers a binding site for different antigen. They are collectively called immunoglobulins. An antibody is a Y-shaped immunoglobulin, constituting two heavy chains and two light chains. Both the chains have a variable region at their N-terminal end and a constant region at their C-terminal end. The variable region contains the specific amino acid sequence information for binding with the corresponding antigen/target with high affinity. This high affinity of the antibody only for its target antigen confers the property – specificity – which becomes the basis of utilizing antibodies as bioreceptors. By employing antigen-antibody interactions as a biorecognition mechanism, specificity and extremely low detection limits can be attained. Antibodies used in immunosensors can be either monoclonal or polyclonal. Monoclonal antibodies are produced from one type of immune cell and bind to the same epitope of their specific target antigen, making them specific. Polyclonal antibodies can recognize different epitopes on their target antigens, making them highly sensitive but less selective due to possible cross-reactivity. Immunosensors occupy a significant role in clinical diagnostics and health care, where high sensitivity, specificity, and rapid detection are particularly desired. The immunosensors also find applications in food quality and environmental monitoring. Of note, immunosensors developed for bacteria and pathogen detection have captured attention due to their application in POC [1].

1.4.2.3 Nucleic Acid/DNA Biosensors

Nucleic acid sensor utilizes nucleic acids as the bioreceptor, immobilized on the transducer surface. Nucleic acids are biopolymers (oligonucleotides) consisting of nucleotides as the monomeric unit. Each nucleotide comprises three components: a 5-C sugar, a phosphate group, and a nitrogenous base. Ribonucleic acid (RNA) contains ribose sugar, whereas deoxyribonucleic acid (DNA) contains deoxyribose sugar. Nucleic acids typically function as information molecules inside the cell, viz. acting as genetic material and encoding proteins. Single-stranded nucleic acids can form double-stranded structures by forming hydrogen bonds between the appropriate nitrogenous bases of nucleic acid strands. Typically, adenine pairs with thymine (DNA) or uracil (RNA) by forming two hydrogen bonds, and guanine pairs with cytosine by forming three hydrogen bonds. Base paring is sequence specific, employing the complementarity between the two sequences to form the double-stranded structure.

For developing biosensors, nucleic acids are immobilized in the recognition layer using linkers such as thiol or biotin, while the transducer used confers sensitivity. A genosensor consists of an immobilized modified oligonucleotide of known sequence (probe) that can detect the complementary nucleic acid sequence (target) through hybridization. The transducer converts the hybridization signal into a usable detectable signal: optical, electrochemical, or even piezo electrical. DNA can be used as a bioreceptor because base-pairing interactions between complementary sequences are specific and robust, making nucleic acid hybridization the principal basis of nucleic acid biosensors. The important property of DNA as a bioreceptor lies in its ability of thermal denaturation followed by renaturation under suitable conditions, making the sensor easily reusable.

DNA biosensors have been envisioned to play an important role in clinical diagnostics, gene analysis, forensic studies, etc. A few examples include diagnostic tests for mutations, monitoring gene expression, screening for targets associated with a disease, assessment of medical treatment, environmental investigations, and detecting biological warfare agents [1].

1.4.2.4 Cell-Based Biosensors

Cell-based biosensors utilize living cells as the bioelement, immobilized onto the transducer surface. The ability of the living cells to respond to the intracellular and extracellular microenvironment and their corresponding response in terms of certain measurable parameters are exploited in the cell-based biosensors. Microbial cells are particularly explored for use in biosensing for detection of specific molecules in the sample matrix or the overall state of the environment. The enzymes and other proteins present in the microbial cells can produce a response to the analytes selectively. The analyte of interest enters the living cell and undergoes some conversion, into a detectable product, or creates indirect changes in pH, ionic concentration, oxygen level in the biosensing layer, and measurement of other parameters, which could indicate the presence of the analyte in the sample matrix. Since the cell-based biosensors are more tolerant to inhibition by certain solutes and variation of pH and temperature values within a range, they can be more favored as compared to their isolated enzyme counterpart. This means that a longer shelf life can be expected. In addition, proteins and enzymes need not be isolated or purified in the case of cell-based biosensors, thereby reducing the cost of the biosensor.

The environmental conditions in which the microorganisms are immobilized on the transducer surface such that the microbes can stay alive for a longer time determine the limit of detection of such a biosensor. The main challenges faced include maintaining stability of the immobilized cells, which is a function of the narrow range of pH and temperature at which the cells remain tolerant and functional, biocompatibility of the immobilization matrix, and other factors. The cell-based biosensors suffer from poor selectivity as compared to the isolated bioreceptors (enzymes, antibodies, and others) due to the multireceptor behavior of the whole cells. Cell-based biosensors find applications in environmental monitoring, disease diagnosis, drug detection, etc. [1].

1.4.2.5 Biomimetic-Based Biosensors

Natural receptor elements such as enzymes, antibodies, nucleic acids, cells, tissues, etc., have been widely explored, characterized, and optimized to develop biosensors. Although they are robust when judged on some parameters, their scope for biosensing applications is restricted by limited stability under harsh conditions, high production costs, or limited availability. Under such circumstances, exploration of biomimetic receptors that overcome the limitations of natural bioreceptors while retaining their inherent properties of selectivity is of interest. Biomimetic sensors utilize a biomimetic molecule as a biorecognition element. A biomimetic receptor is a synthetic molecule that mimics the natural receptors. Few examples of synthetic receptors include genetically engineered proteins and cells, molecularly imprinted polymers (MIP), synthetic peptides, oligonucleotides viz. locked nucleic acids (LNAs) and peptide nucleic acids (PNAs), and aptamers. Aptamers are of particular interest as they are one of the most widely explored biomimetic receptor so far.

Biosensors that utilize an aptamer as a biorecognition element are referred to as aptasensors. Nucleic acid aptamers are artificial nucleic acid ligands reported for the first time in the early 1990s. These are short, single-stranded oligonucleotides that fold into a well-defined, three-dimensional structure and are capable of binding various molecules (targets) with high affinity and specificity. Aptamers are potential alternatives to antibodies, as they retain the desirable properties of antibodies as a bioreceptor, while overcoming the limitations of thermal stability, have a low cost, and have a wide range of applications [1]. A detailed description of aptamers is discussed in Chapter 4 of this book.

1.4.3 Classification of Biosensors Based on the Transducer Elements

This mode of classifying biosensors is by far the most widely acceptable form. This section discusses various classes of biosensors based on the type of transduction principles being used for their development.

1.4.3.1 Electrochemical Biosensors

According to IUPAC, an electrochemical 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 retained in direct spatial contact with an electrochemical transduction element [7]. Electrochemical enzyme biosensors contain a redox enzyme that selectively reacts with the target analyte and produces an electrical signal that is related to the concentration of the analyte being studied. Electrochemical biosensors combine the sensitivity of electrochemical transducers with the specificity of biological recognition processes [8]. The reaction being studied electrochemically typically generates a measurable charge accumulation or potential (potentiometry), alters the conductive properties of the medium between electrodes (conductometry), or produces a measurable current (amperometry). Therefore, electrochemical biosensors are classified as potentiometric, conductometric, and amperometric biosensors.

1.4.3.1.1 Potentiometric Biosensors

Potentiometric measurements entail the determination of the potential difference between either an indicator and a reference electrode or two reference electrodes separated by a permselective membrane, in the absence of significant current between them. The transducer may be an ion-selective electrode (ISE), which is an electrochemical sensor based on thin films or selective membranes as recognition elements [9]. The most common potentiometric devices are pH electrodes; several other ions (Na^+, K^+, Ca^{2+}, NH_4^+, F^-, I^-, CN^-) or gas (CO_2, NH_3) selective electrodes are also available. The potential differences between these indicators and reference electrodes are proportional to the logarithm of the ion activity or gas fugacity (or concentration), as described by Nernst-Donnan. The response of a potentiometric biocatalytic sensor is either

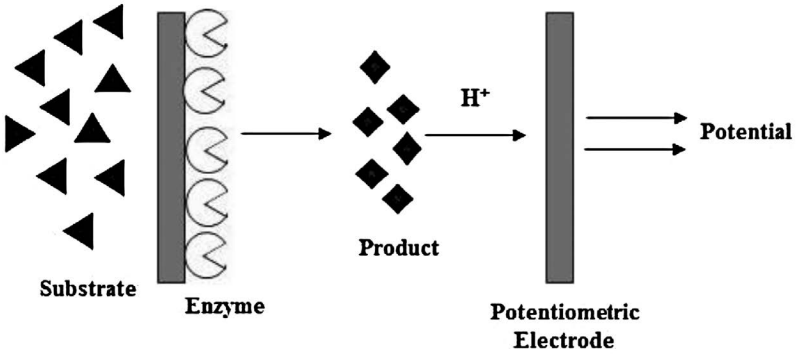

FIGURE 1.3 Schematic of potentiometric biosensor.

steady-state or transient. One significant feature of the ISE-based biosensors, such as pH electrodes, is the large dependence of their response on the buffer capacity of the sample and on their ionic strength [9]. The schematic representation of a potentiometric biosensor is shown in Figure 1.3. In these measurements, concentration and potential are related by the Nernst equation, as given in Equation 1.5.

$$E_{\text{cell}} = E^{0}_{\text{cell}} - \frac{RT}{nF}\ln Q \tag{1.5}$$

where E_{cell} is the observed cell potential at zero current that is called the electromotive force (EMF), E^{0}_{cell} is a standard potential contribution to the cell, R and T represent universal gas constant and absolute temperature (in degrees Kelvin), respectively, n is the number of charges, F is the Faraday constant, and Q is the ratio of ion concentration at the anode to ion concentration at the cathode [10].

There are many examples of potentiometric biosensors, such as the one developed for the detection of different pesticides [11]. Over the last decade, the ion selective field effect transistor (ISFET)-based potentiometric devices have received intensive interest due to their several advantages over the conventional potentiometric biosensors. A detailed account of the ISFET-based sensors has been illustrated in Chapter 14 of this book.

1.4.3.1.2 Conductometric Biosensors

Conductometry is the measure of the ability of ions in solution to carry current between two inert electrodes under an applied electric field. The ions are formed by the dissociation of the electrolyte. On the application of a potential difference between the two electrodes, a chaotic ion movement occurs, where anions move toward anodes, while cations move toward cathodes in the electrolytic cells (Figure 1.4). The conductivity (S) of the electrolyte solution depends on the ion concentration and mobility. The resistance of electrolyte solution is in direct proportion to the distance L between the immersed electrodes and reciprocal to their area A; therefore, conductivity (S) can be calculated using the following equation:

$$S = \chi \times \left(\frac{A}{L}\right) \tag{1.6}$$

where χ is specific conductivity. This shows that a conductometric measurement includes determining the conductivity of a solution between two parallel electrodes; it is a sum of all the ions within the solution.

Conductometric biosensors utilize a change in solution conductivity as the transduction mechanism for sensing. These biosensors are based on the fact that almost all biocatalytic reactions involve either consumption or production of charged species and therefore lead to an overall change in the ionic composition of the tested sample. The conductometric transducer is a small two-electrode device fabricated to measure the conductivity of the thin electrolyte layer close to the electrode surface. The interdigitated structure of conductometric electrodes has received increasing interest in sensor and biosensor research. Conductometric biosensors have advantages over other types of transducers – for example, they can be produced through low-cost thin-film standard technology, there is no need for a reference electrode, they

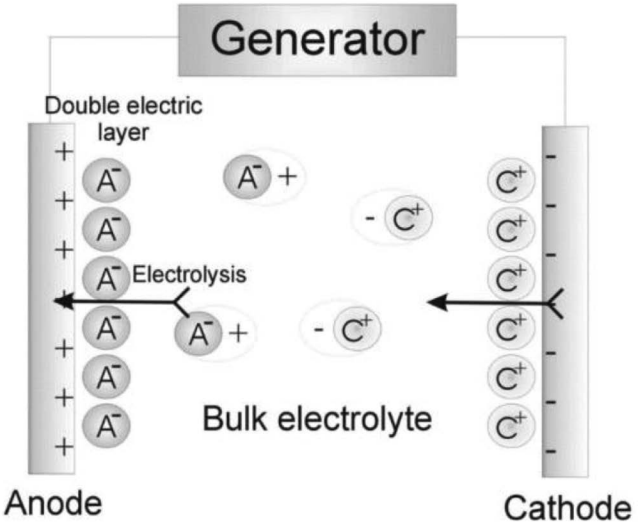

FIGURE 1.4 Ion migration in solution under an applied potential difference between two electrodes [12].

are insensitive to light, and differential mode measurements permit cancellation of a great number of interferences. The liquids analyzed are generally considered to have significant background conductivity, which is easily modified by different factors; that is why the selectivity of this method is supposed to be low. Interested readers may consult some of the prominent literature to understand the detailed theory and application of this type of electrochemical biosensor [12, 13].

1.4.3.1.3 Amperometric Biosensors

The amperometric transducer-based biosensors work by the production of a current when a potential is applied on the working electrode in an electrochemical setup in response to the analyte of interest [14]. Here we are describing amperometric biosensors citing the widely used biorecognition element enzymes. Normally, the enzymes are immobilized over the electrodes as a selective layer to transduce a biochemical signal into an electrical one under the influence of an appropriate applied potential [15, 16]. If the response arises from the electro-activity, mainly of a co-substrate (oxygen in most of the cases), product, or co-product (H_2O_2 in most of the cases with flavin adenine dinucleotide (FAD)-based redox enzymes) of the enzyme-catalyzed reaction the category of the biosensor is termed first generation. A Clark oxygen electrode-based glucose biosensor is the first example of this type [3]. The requirement of a high operation potential for the measurement of H_2O_2 and limited solubility of oxygen in aqueous samples causing fluctuations in the oxygen tension are some major drawbacks of first-generation biosensors.

Pairing of electrons between the redox active centers of the enzyme and the electrode through some specialized small electroactive molecules to generate the response represents the second-generation biosensors. These specialized molecules are termed "electron transfer mediators" (ETMs), which shuttle electrons between the redox center of the enzyme and the electrode. This approach leads to a considerable reduction of electrochemical interferences due to the involvement of the low redox potential of the enzyme in generating the electrical signal. Many second-generation amperometric biosensors have been developed. One prominent example is the ferrocene-mediated enzyme electrode for the determination of glucose using GOD with FAD in the redox center, which acts as a biorecognition molecule as reviewed in [10].The reaction involves the following steps, where M/M* are the oxidized and reduced forms of the mediator.

$$\text{Glucose} + \text{GOD} / \text{FAD} \rightarrow \text{Gluconolactone} + \text{GOD} / \text{FADH}_2$$

$$\text{GOD} / \text{FADH}_2 + 2\text{M} \rightarrow \text{GOD} / \text{FAD} + 2\text{M}^* + 2\text{H}^+$$

$$2\text{M}^* \rightarrow 2\text{M} + 2\text{e}^- \left(\text{transferred to electrode}\right)$$

The mediator is chosen in such a way that it possess a lower redox potential than the other electrochemically active interferents present in the sample. The redox potential of the mediator should be more positive for oxidative biocatalysts and more negative for reductive biocatalysts than the redox potential of the enzyme-active site. The ETMs should possess some critical characteristics, such as react rapidly with the reduced state of the enzyme, exhibit reversible heterogeneous kinetics, the overpotential for the regeneration of the oxidized mediator should be low and pH independent, stable in oxidized and in reduced forms, and the reduced form should not react with oxygen.

Ferrocene and its few derivatives are widely used as ETMs. Tetrathiafulvalene (TTF), tetracyanoquinodimethane (TCNQ), ferricyanide, N,N,N′,N′ tetramethyl-4-phenylene diamine (TMPD), and benzoquinone are also reported as ETMs in some assays. Organic dyes such as alizarin yellow, azure A and C, methylene blue, methyl violet, phenazines, prussian blue, thionin, and toluidine blue are known for their electron transfer-mediating properties. Many ETMs, however, suffer from a number of problems such as poor stability and pH dependence of their redox potentials (organic dyes). Conversely, many of the inorganic mediators are not easy to tune their redox potentials and solubility by the use of substituents. Mediators are generally added directly to the measuring solution or immobilized on the electrode surface. The first method is easier, though it is not suitable from the technological perspective [10, 17].

The second-generation biosensors, however, suffer from drawbacks, such as poor stability and reproducibility. The diffusion barrier between the enzyme-electrode interface and the leaching susceptibility of the free mediator from the interface to the sample solution are ascribed as the reasons for the poor performance of the second-generation bioelectrode-based biosensors.

In an effort to overcome the drawbacks of first-generation and second-generation biosensing principles, the concept of third-generation biosensors emerged. The principle of third-generation biosensors involves direct electron transfer (DET) between the redox center of the enzyme and the electrode without using any ETMs to generate the response. High selectivity and sensitivity are the main advantages of these biosensors, as they can operate in a potential window closer to the redox potential of the enzyme [18]. There are, however, limited redox proteins with a redox center in the periphery of the protein matrix, such as cytochrome c, a peroxidase that supports the DET principle. Different strategies have been explored to introduce DET-based enzyme electrodes that include nanofabrication of electrodes, immobilization of conductive polymer coupled redox proteins, cofactors tethered by the reconstitution process, etc. [14].

The development of first- to third-generation amperometric biosensors reflects a simplification and improvement of the signal transduction pathway. The electrical signal transduction pathways for the three generations of biosensors are depicted in Figure 1.5, citing the examples with an FAD-based redox enzyme.

The feasibility of electron exchange between the redox proteins and the electrodes can be explained by the electron transfer (ET) theory of Marcus [19]. The ET rate constant (K_{ET}) between a donor and acceptor pair is given by Equation 1.7,

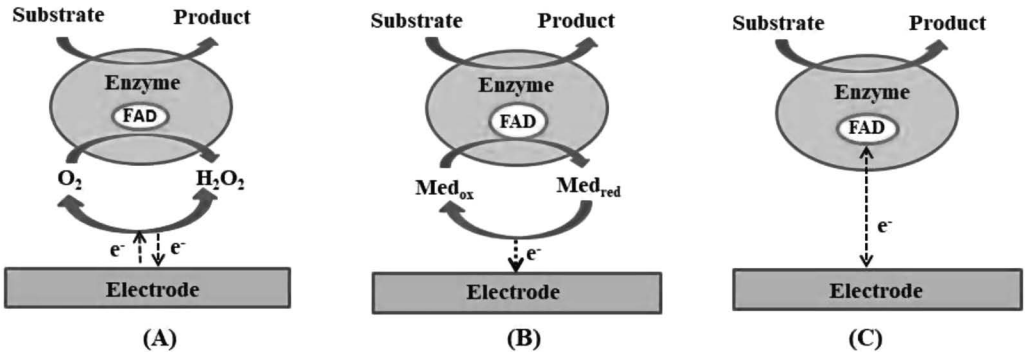

FIGURE 1.5 Three generations of amperometric bioelectrodes: A: first-generation, B: second-generation, and C: third-generation enzyme electrodes.

where d and $d°$ are the distance separating the electron and donor and the van der Waals distance, respectively; β is the electron-coupling constant; and $\Delta G°$ and λ are the Gibbs free energy change and the reorganization energy accompanying the ET process, respectively.

$$K_{ET} \propto \exp\left[-\beta\left(d-d^0\right)\right].\exp\left[-\frac{(\Delta G^0+\lambda)^2}{4RT\lambda}\right] \quad (1.7)$$

Thus, the electron exchange between two redox sites depends largely on three factors: the reorganization energy qualitatively representing the conformational rigidity of the redox compound in its oxidized and reduced form, the potential difference between the involved redox entities (since $\Delta G^o = \sim -nF\eta$, where, over potential $\eta = E - E^o$, the applied and standard potential, respectively), and separation between the redox sites.

The concept of protein film voltammetry (PFV) is beneficial for studying the principle of DET. In PFV a stable mono-/submonolayer film of redox protein on an electrode surface is prepared and studied by a variety of electrochemical techniques.

The thin film protects the chemistry of the active site of the redox proteins and facilitates fast electron transfer due to the proximal distance with the electrode [20, 21]. PFV has several advantages over conventional voltammetry, such as high sensitivity, sharp redox status of the entire sample, requirement of a tiny amount of sample, stoichiometry, and fast reactions.

Some common techniques used for characterizing amperometric biosensors are briefly discussed in the following sections.

1.4.3.1.3.1 Cyclic Voltammetry (CV) CV is a powerful tool for the determination of formal redox potentials, detection of chemical reactions that precede or follow the electrochemical reaction, and evaluation of ET kinetics. It scans a potential window in the forward and reverse directions and measures the resulting current. The rate of change of potential with time is referred to as the scan rate (ϑ). The data from the anodic (I_{pa}) and cathodic (I_{pc}) peak currents versus scan rate plots of the immobilized enzymes is extracted to understand the redox processes on the electrode surface, such as surface-controlled or diffusion-controlled process, electron transfer coefficient (α), reversible or quasi-reversible process, surface coverage area (Γ), ET rate constant (k_s), and number of electrons transferred in the reaction (n). The value of α can be estimated by measuring the anodic (E_{pa}) and cathodic (E_{pc}) peak potentials at various CV scan rates (ϑ) using Equations 1.8 and 1.9 [22]:

$$E_{pa} = E^{0'} + \frac{2.3RT}{(1-\alpha)nF\log\vartheta} \quad (1.8)$$

$$E_{pc} = E^{0'} - \frac{2.3RT}{\alpha nF\log\vartheta} \quad (1.9)$$

where $E^{0'}$ is the formal potential, ϑ is the scan rate, and R is the thermodynamic constant (R = 8.314 JK^{-1} mol^{-1}), F is the Faraday constant (F = 96,500 C mol^{-1}), T is the temperature in Kelvin, and n and α are the charge transfer number and the charge transfer coefficient, respectively, when $0.5 < \alpha < 1$, in general $n = 1$.

The surface coverage, Γ of the bioelectrode, can be calculated using the following equation:

$$\Gamma = \frac{Q}{n}FA \quad (1.10)$$

where A is the area of the electrode and Q is the charge obtained by integrating the peak current area.

k_s can be estimated using the following equations:

$$\log k_s = \alpha\log(1-\alpha) + (1-\alpha)\log\alpha - \frac{\log RT}{nF\vartheta} - \alpha(1-\alpha)\frac{nF\Delta E_p}{2.3RT} \quad (1.11)$$

(When $\Delta E_p > 200$ mV)

$$k_s = \frac{\alpha nF\vartheta}{RT} \quad (1.12)$$

When $\Delta E_p < 200$ mV)

The magnitude of k_s indicates the efficacy of the DET between the immobilized enzymes and electronic unit. The increasing height of the redox peak in CV with increasing concentration of substrate implies the involvement of the DET principle in sensing the substrate of interest by the constructed enzyme electrode [23, 24].

Again, for electrochemically reversible ET processes which involve freely diffusing redox species, the Randles–Sevcik equation (Equation 1.13) explains how the peak current i_p (*A*) increases linearly with the square root of the scan rate ϑ (V s^{-1}), where n is the number of electrons transferred in the redox process, A (cm^2) is the electrode surface area (generally represented as the geometric surface area), C^0 (mol cm^{-3}) is the bulk concentration of the analyte, and D_0 (cm^2 s^{-1}) is the diffusion coefficient of the oxidized analyte [25].

$$i_p = 0.446nFAC^0\left(\frac{nF\vartheta D_0}{RT}\right)^{1/2} \tag{1.13}$$

1.4.3.1.3.2 Chronoamperometry The response curve for biosensors can be determined through a chronoamperometry experiment. Chronoamperometry is a time-dependent technique where a square-wave potential is applied to the working electrode. The current of the electrode, measured as a function of time, fluctuates according to the diffusion of an analyte from the bulk solution toward the sensor surface. Chronoamperometry can therefore be used to measure current-time dependence for the diffusion-controlled process occurring at an electrode. This differs with analyte concentration. The resulting current occurring at the electrode is monitored as a function of time after applying the peak potential. The analysis of chronoamperometry data is based on the Cottrell equation (Equation 1.14), which defines the current-time dependence for linear diffusion control. The Cottrell equation describes the observed current (planar electrode) at any time following a large forward potential step in a reversible redox reaction (or to large overpotential) as a function of $t^{-1/2}$.

$$i = \frac{nFAC_0\sqrt{D_0}}{\sqrt{(\pi t)}} \tag{1.14}$$

where n = stoichiometric number of electrons involved in the reaction, F = Faraday's constant (96,485 C/equivalent), A = electrode area (cm^2), C_0 = concentration of electroactive species (mol/cm^3), and D_0 = diffusion constant for electroactive species (cm^2/s)

1.4.3.1.3.3 Differential Pulse Voltammetry (DPV) and Square Wave Voltammetry (SWV) DPV and SWV provide a unique alternative to sweeping voltammetry methods such as CV. The main advantage of these pulsed techniques is the higher sensitivity that they offer in terms of both potential and current. This enables the detection of electroactive species at very low concentrations (as low as 10^{-7} M) and facilitates the resolution of overlapping redox features of multiple electroactive species (typically, any redox peaks separated by >50 mV can be resolved). In DPV fixed-magnitude pulses superimposed on a linear potential ramp are applied to the working electrode at a time just before the end of the drop. Again, SWV is a specialized form of DPV, which is a large-amplitude differential technique in which a waveform composed of a symmetrical square wave, superimposed on a base staircase potential, is applied to the working electrode. Readers may consult some works on DPV [26, 27] and SWV [28–30] to understand the application of these techniques for biosensors.

1.4.3.1.3.4 Electrochemical Impedance Spectroscopy (EIS) EIS helps to understand the charge transfer behaviors of the thin-film layers on the electrode surface and to discern the performance of enzyme electrodes. It is commonly employed for analysis of enzymatic electrodes by overlaying a range of alternating current (AC) perturbation signals to an electrode that is under direct current (DC) bias. A Nyquist plot (a plot of the imaginary part of the impedance versus the real part of the impedance for different frequencies) is widely employed, which provides the variations of the frequency response to deduce limiting mechanisms connected with charge transfer [31]. The following example may help to understand the biosensing application of EIS. A DNA aptasensor for sensitive detection of a malaria biomarker, *Plasmodium falciparum* lactate dehydrogenase (PfLDH) using the impedimetric technique was developed. For this a specific aptamer, P38, was immobilized over graphene oxide (GO) on a glassy carbon electrode (GCE). With the help of an external redox probe ($[Fe(CN)_6]^{-3}$), the presence of PfLDH in a human serum sample was impedimetrically sensed down to a femtomolar level.

Here, in this paper, EIS experiments were performed in a background of 2.5 mM $[Fe(CN)_6]^{-3}$. The obtained spectra were represented in the form of Nyquist plots (Figure 1.6A); the arc radius of the semicircle part represents the magnitude of the charge transfer resistance (R_{ct}). The increase in R_{ct} (more the radius of the semicircle more is the R_{ct} value in the Nyquist plot) with increasing PfLDH concentration infers (Figure 1.6A) that PfLDH was successfully captured on the aptamer electrode surface. This is because the negatively charged DNA phosphate backbone of the aptamers prevented the communication of $[Fe(CN)_6]^{-3}$ ions with the electrode, which resulted in a prominent increase in the R_{ct}. The acquired Nyquist plots have been fitted to the Randles–Ershler-type equivalent circuit (Figure 1.6A inset), where R_s is the solution resistance, Z_w is the Warburg impedance, and CPE is the constant phase element. So R_{ct} can be calculated from this Randles–Ershler-equivalent circuit. The response data were generated by plotting the fitting results (Figure 1.6B), i.e. concentration of PfLDH with respect to R_{ct}, from which the concentration of PfLDH was detected up to as low as 0.5 fM [32].

1.4.3.1.4 Biofuel Cells (BFCs)-Based Biosensors BFCs are galvanic cells, can be used to measure potential, current or

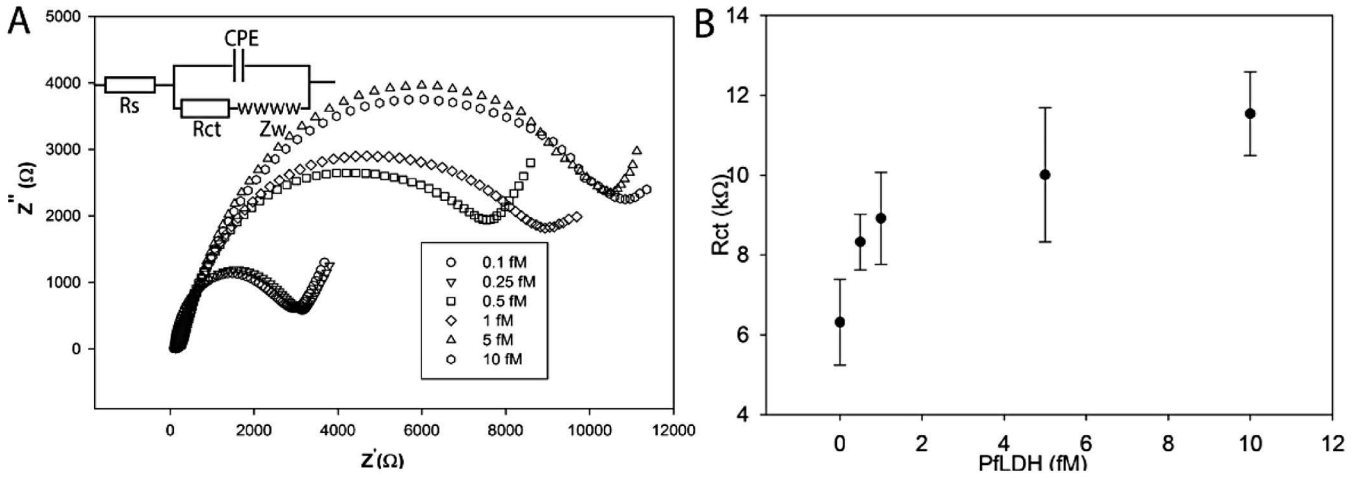

FIGURE 1.6 **(A)** Nyquist plots on the response of P38-GO-GCE toward increasing PfLDH concentration from 0.1 fM, to 10 fM. **(B)** Response curve of R_{ct} versus increasing PfLDH concentration [32], Copyright (2016), with permission from Elsevier.

both these signals simultaneously. The bio-catalysts used in the BFC converts chemical energy into these electrical energy forms. The basic differences between these cells and chemical fuel cells are the types of catalysts, fuels, and physical conditions used for their operations. BFCs mostly use enzymes and microorganisms as electrode catalysts. The fuels for BFC are mostly renewable in nature, such as glucose and other carbohydrates, such as alcohols. However, nonrenewable fuels such as hydrocarbons can also be used if the biocatalysts can electrochemically oxidize these compounds or metabolically convert these substances to produce current in the cell. As a whole, BFCs can encompass a wide range of compounds as a fuel substrate. These BFCs function under mild conditions, around physiological pH and room temperature, without using any hazardous or toxic chemicals or materials. Considering these facts, BFCs are acclaimed as green energy technology. The general configuration, principle of operation, and application potential of BFCs are illustrated in Chapters 10 and 13.

The scope of scaling down the size of BFCs to a chip-based platform supported by their self-powered attributes, along with their highly selective responses (potential or current) against a vast array of substrates under physiological operating conditions is the primary stimulus of exploring this energy-transducing technology for biosensing applications. There are two approaches for generating biosensing signal in BFCs: (a) Turn-on sensors. Here the target analyte, which may be a fuel or other substance, activates/initiates the bioelectrocatalytic reactions and generates or increases the electrical output. (b) Turn-off sensors. Here the target analyte, which may be an inhibitor or other substance, de-activates/reduces the bioelectrocatalytic reactions and correspondingly decreases the electrical output signal current [33]. The application of microbial BFCs as a sensor for biochemical oxygen demand (BOD), microorganism load, toxicants, etc., is widely known [34, 35]. Over the last decade, the research on the application of enzymatic BFC-based wearable sensors in the fields of health care and sports have made significant progress.

1.4.3.2 Optical Biosensors

The research and technological developments of optical biosensors have accomplished an exponential growth over the last decade. This is because there is a wide range of optical behaviors, such as fluorescence including various luminescence approaches, ultraviolet (UV)-visible absorption and reflections, internal reflection, and scattering spectroscopy that can be explored to develop the biosensor signals. Moreover, some of the optical behaviors (e.g. fluorescence) offer extremely high sensitivity to the biosensor. Additionally, the color-based detection could be achieved in a simple, low-cost, portable format that greatly boosts their PoC and point of need (PoN) applications relevant to health care, environmental monitoring, and food and agricultural industries, among others [36].

One of the earliest examples of an optical biosensor is a test strip for the detection of glucose in urine [37]. The strip was made of a cellulose pad that consisted of co-immobilized glucose oxidase and peroxidase. The H_2O_2 produced from the enzymatic oxidation of glucose reacts with o-tolidine in the vicinity of peroxidase to form a dye. The color intensity of the dye was proportional to the glucose concentration as envisioned by the naked eye. It represented a semi-quantitative measurement technique for glucose. There has been a parallel growth of technological innovation to process the color intensity of the detection strip to a concentration unit of the target analyte with the help of suitable apps in the interface of modern smart phones. One such example is the quantitative detection of urea in saliva [38]. A further illustration of smart phone-based detection devices has been discussed in a separate chapter of this book. We would like to highlight here working principles of few prominent optical biosensors.

1.4.3.2.1 Absorbance/Reflectance-Based Optical Biosensors

The optical detection principle of absorbance transducers is fundamentally based on the Beer-Lambert law:

$$\log \frac{I_0}{I} = A = \varepsilon Cl \tag{1.15}$$

where I_o is the intensity of incident light, I is the intensity of transmitted light, A is absorbance, ε is the molar absorbance of the analyte at a specific wavelength, C is the concentration of analyte, and l is the path length of light through solution. The common absorbance transducers utilize a single fiber or fiber bundle that brings light to the analyte-sensitive reagent phase, and the transmitted or reflected light is returned to a measurement instrument or detected through fiber(s), as shown in Figure 1.7A. The optical path length, absorption cross-section of the transducing molecule, and the illumination wavelength determine the extent of the absorption. Changes in the chemical environment can modify the absorption of the biorecognition element, and this modification is monitored as a change in transmitted intensity within the biosensor.

In case of a nontransparent sample, it becomes difficult to measure transmitted light acceptably, and in these cases the intensity of the reflected light may be used as a measure of the color of the recognition element, analyte, or product, as shown in Figure 1.7B [39].

1.4.3.2.2 Surface Plasmon Resonance (SPR) Biosensors

The name itself implies that SPR is a surface phenomenon that occurs on the surface of highly conducting metals, typically gold, which support to generate plasmons. Plasmons are collective charge density oscillations of electron in a metal. Surface plasmons (or surface plasmon polaritons) are surface electromagnetic waves that propagate parallel along a metal-dielectric interface (e.g. metal-air). The excitation of surface plasmons by light is denoted as an SPR for planar surfaces or localized surface plasmon resonance (LSPR) for nanometer-sized metallic structures. The LSPR, on the other hand, is highly sensitive to size, size distribution, and shape of the metal nanostructures, as well as the environment that surrounds them. The LSPR is the fundamental principle behind many color-based biosensor applications and different lab-on-a-chip sensors.

The velocity of the surface plasmons (and hence the light energy for resonance) changes with the change in the refractive index near the metal surface. The refractive index in turn is greatly dependent on the chemical environment of the metal-dielectric medium. In this affinity biosensor, biorecognition elements specific to analyte molecules (in liquid samples) are immobilized on the surface of the metal. The binding of the analyte to the biorecognition layer over the sensor surface gives rise to a refractive index change close to the sensor surface, which can be measured by the optical reader.

The refractive index change (Δn_b) caused by the binding of specific analyte molecules at the sensor surface can be expressed as (often referred to as the de Feijter formula):

$$\Delta n_b = \left(\frac{dn}{dc}\right)_{vol} \Delta c_b = \left(\frac{dn}{dc}\right)_{vol} \frac{\Delta\Gamma}{h} \tag{1.16}$$

where $(dn/dc)_{vol}$ is the refractive index increment, Δc_b is the wt/vol concentration of bound molecules within the sensitive layer with the thickness h, and $\Delta\Gamma$ is the corresponding surface concentration (mass per surface area). The $(dn/dc)_{vol}$ is a well-characterized property for most of the biochemical species and ranges typically from 0.1 to 0.3 cm^3g^{-1}. A change in the effective refractive index of the surface plasmon due to the capture of analyte can be expressed as

$$\Delta n_{ef} = K\Delta\Gamma \tag{1.17}$$

where K is a constant.

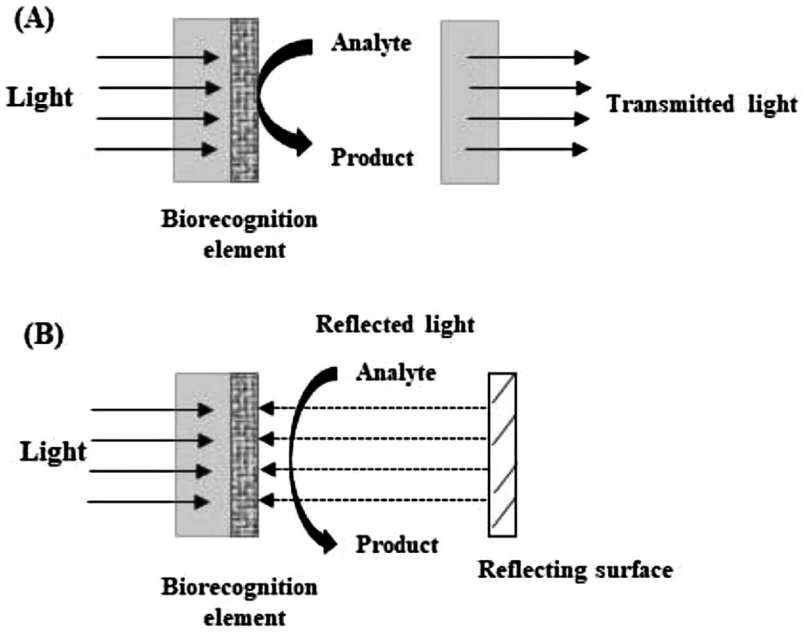

FIGURE 1.7 Absorbance-based optical transducer with **(A)** absorption configuration and **(B)** reflectance configuration [39].

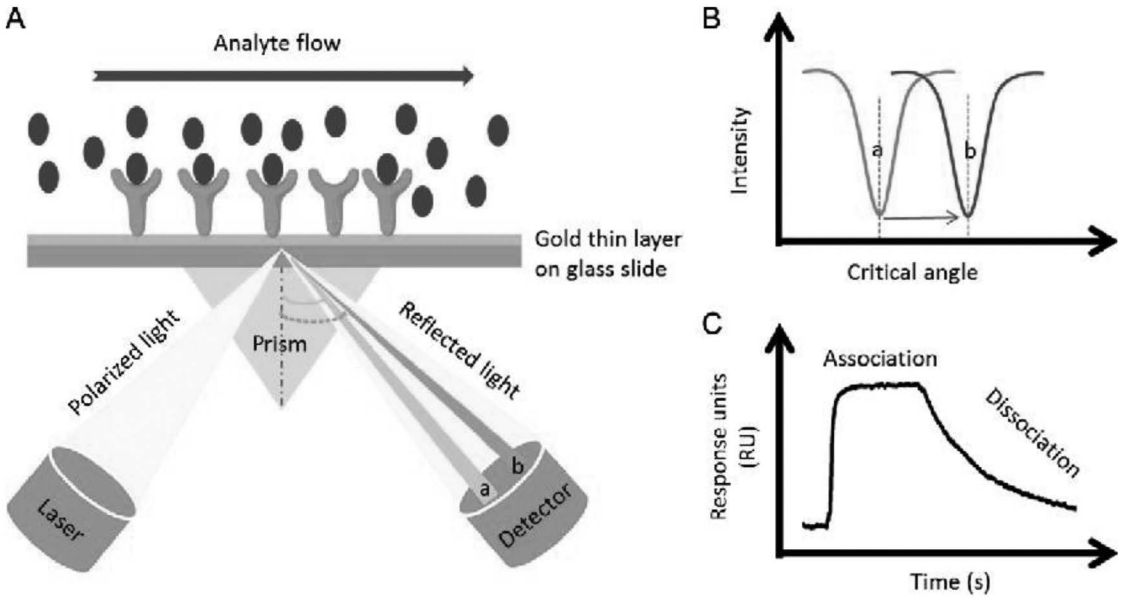

FIGURE 1.8 Schematic representation of SPR phenomenon for the measurement of analyte binding to immobilized ligand. **(A)** The ligand is immobilized on the sensor chip, which is composed of a gold thin layer on a glass slide. **(B)** Reflected light intensity shifts upon a critical angle change from "a" to "b," resulting from a binding interaction event. **(C)** Light intensity shifts are transformed into sensorgrams, a plot of response units (RU) versus time [40]. Copyright (2015), with permission from Elsevier.

The optical reader of the SPR sensor measures changes in a characteristic of a plasmon-coupled light wave resulting from changes in the effective refractive index. Different coupling mechanisms have been developed for coupling a light wave to a surface plasmon, among which the attenuated total reflection in prism couplers and diffraction on diffraction grating couplers are common.

Most of the SPR sensors are designed by using the Kretschmann configuration of the attenuated total internal reflection method. Here, a light wave (usually plane polarized) passed through a high refractive index prism is reflected at the prism base covered with a thin gold film (Figure 1.8). The light evanescently passes through the thin film and excited the plasmon at the outer boundary of the film if the incident light wave and plasmon are closely phase-matched. The phase-matching condition can be written as:

$$n_p \sin\theta = n_{ef} \tag{1.18}$$

where n_p is the refractive index of the coupling prism and θ is the angle of incidence on the metal film (in the prism).

The SPR biosensor's response can be generated by using either (a) wavelength modulation, where the angle of incidence is fixed and the coupling wavelength serves as a sensor output, or (b) angular modulation, where the coupling wavelength is fixed and the coupling angle of incidence serves as a sensor output, or (c) intensity modulation, where both the angle of incidence and the wavelength of incident light are fixed at nearly resonant values and the light intensity serves as a sensor output.

Since the development of the first commercial SPR sensors in 1990, many commercial products are now available in the market. SPR biosensors allow label-free, direct, highly sensitive, and real-time detection of chemical and biological analytes. It is a useful technique for measuring the affinity, stoichiometry, and kinetics of an interaction [41, 42].

1.4.3.2.3 Fluorescent-Based Optical Biosensors

The research on fluorescence-based biosensors and allied analytical techniques is increasing exponentially over the last two decades due to the emergence of various novel and efficient fluorescence probes. The major types of fluorescent probes explored for biosensor applications include organic, nucleic, and cell fusion dyes, fluorescent proteins, and small fluorescent nanoparticles such as quantum dot, metal nanocrystals, and carbon dots. These fluorescence probes absorb (excited with) electromagnetic radiation at their specific wavelengths and they emit fluorescence. The wavelength of the emitted fluorescent light is usually longer than the excitation wavelength. This difference between the absorbance and the emission peak wavelength is termed the Stokes shift after the name of Sir George Stokes [43, 44]. The time interval between absorption and emission in fluorescence is very short, usually on the order of 10^{-9} to 10^{-8} s. The Jablonski diagram, named after the physicist Aleksander Jablonski, commonly depicts the illustration of a single fluorescence event.

Fluorescence biosensor development normally involves coupling the target recognition with a change in fluorescence of the reporter/probe. Based on the nature of the sensing element, different designs of the fluorescent biosensors can be introduced. In single fluorophore-based architecture, the reporter/probe that is sensitive to the microenvironment is attached to the recognition element and then probes the presence of the target analyte in the sample by monitoring

the change in fluorescence. As the target molecule binds, the conformation of the recognition element changes, which further changes the microenvironment of the fluorophore. This change in fluorescence intensity may occur due to various mechanisms, such as dynamic quenching caused by the change in accessibility of the solvent, change in polarity caused by the surrounding environment, or a change in fluorophore-protein interactions. However, there can be a change in fluorescence intensity when the fluorophore directly interacts with the bound target molecule and hence can be a basis for designing a biosensor.

A two fluorophore-based design format was also widely used to develop a biosensor following the principle of FRET (Förster resonance energy transfer). FRET takes place by the direct excitation of an acceptor fluorophore by the energy donated by a donor fluorophore, which is excited by electromagnetic radiation in the appropriate wavelength. Transfer of energy takes place when the donor and acceptor are in close proximity (<10 nm distance) and the dipoles of both molecules are oriented appropriately [43, 44]. In these biosensors, fluorophore pairs are normally fused with the recognition element. A change in FRET is observed whenever there is a ligand-induced change in conformation that can alter the distance or relative orientation of the fluorophores of the FRET pair. In many cases, the fluorophore is coupled with a suitable quencher, which dissipates the absorbed energy in the form of heat. The quenchers can absorb energy over a wide range of wavelengths and can also dissipate this absorbed energy in the form of heat, and they remain dark. Due to these properties, quenchers can be very useful molecules for energy acceptors in FRET pairs.

The FRET sensor provides a signal based on the ratio of the acceptor to donor emission that enables the quantitative measurements of the target analyte even in a complex environment. One of the major constraints for developing the FRET-based sensor is to identify a suitable partner for the fluorophores to generate a noise-free effective signal. It may be mentioned that the relationship between the donor acceptor proximity is critical for the FRET phenomenon. FRET-based mechanisms have been employed in the genetically encoded biosensors such as for cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), sugars, phosphate, Ca^{2+}, and adenosine triphosphate (ATP) [44]. The ratio-metric signal-based sensors can also be designed by using an intermolecular charge transfer (ICT) approach. However, the ratio between two relatively broad signal emissions becomes difficult to determine in many ICT-based ion sensors.

Apart from exploiting the normal change in fluorescence intensity and FRET, FCS (fluorescence correlation spectroscopy), and FLIM (fluorescence lifetime imaging) are also explored for generating specific signals for biosensing applications. FCS analyzes small deviations from spontaneous fluorescence intensity of the sample to gain information on the kinetics of thermodynamic processes associated with reversible fluorescence changes, e.g. flow rate, diffusion coefficient, and molecular concentration [45]. FCS is usually useful to deal with low concentrations of molecules.

FLIM is used for imaging biological tissues and reactions taking place in living cells. This method gives information on changes in the local environment of the fluorophore or changes in its energy in response to the interactions with the local environment [45].

Fluorescent nanoparticles are increasingly used to develop various detection methods. As an example, a sol-gel encapsulated CdS quantum dots (QDs)-uricase/horseradish peroxidase (HRP) enzymes hybrid system has been used to detect uric acid. The hybrid system oxidizes uric acid to allantoin, CO_2, and H_2O_2. The produced H_2O_2 has the ability to quench the QDs fluorescence intensity, which is proportional to the uric acid concentration [46].

The information described here is certainly not a comprehensive coverage on the vast subject of fluorescence-based detections, but should provide the reader with at least a general appreciation of the breadth of prominent options available.

1.4.3.2.4 Luminescence Biosensors

Luminescence is defined as the radiation emitted by an atom or a molecule when these species return to the ground state from the exited state. On the basis of the source of excitation, the luminescence phenomenon is mainly classified as photoluminescence (fluorescence and phosphorescence) when the excitation source is energy from absorbed light, chemiluminescence (energy from chemical reactions), and bioluminescence (energy from biologically catalyzed reactions) [47]. When a molecule absorbs a photon in the visible region, exciting one of its electrons to a higher electronic excited state and then radiates a photon as the electron returns to a lower energy state, this process is called photoluminescence. If the molecule experiences internal energy redistribution after the initial photon absorption, the radiated photon may be a longer wavelength than the absorbed photon. Fluorescence and phosphorescence are special forms of photoluminescence. Chemiluminescence occurs on the emission of light by the release of energy from a chemical reaction. Bioluminescence is a type of chemiluminescence occurring in some living forms and involves a protein (enzyme). It is the result of certain oxidation processes (usually enzymatic) in biological systems like fireflies, jellyfish, glow worms, and mostly marine animals. Among different luminescence types, chemiluminescence and electro-chemiluminescence are growingly used in developing biosensors. The principle behind chemiluminescence-based sensors is the combination of light-emitting reactions with sensor capabilities. Optical transducers have been used for the design of these biosensors. They can be used to detect certain biochemical reactions that occur. The immobilized biorecognition element has been marked with a chemiluminescence species, and on reaction with the analyte, it generates light. Generally, photomultiplier tubes (PMTs) are used to detect this light. Chemiluminescence-based sensors have a high sensitivity, a fast dynamic response property, and a wide calibration range. These sensors have been used for immunosensing applications. However, they are expensive and difficult to use for real-time monitoring [47].

A comprehensive discussion on electrochemilunescene biosensors has been included in another chapter of this book.

The assembly of a luminescence-based transducer is similar to that of an absorbance-based transducer. Excitation light is directed to the recognition element, which is exposed to the analyte. The fluorescence is then collected by the detection system. Any change in luminescence intensity, phase, or lifetime can be related to the interaction of recognition element, analyte, and product. The principle of luminescence quenching is normally employed in a luminescence-based transducer. According to Stern and Volmer, the relationship between intensities or lifetimes in the absence and presence of a quencher is given by:

$$\frac{I_0}{I} = 1 + K_{SV}[Q] = \frac{\tau_0}{\tau} \tag{1.19}$$

where I_o and I are the luminescence intensities in the absence and presence of quencher Q, respectively; K_{SV} is the Stern-Volmer constant; $[Q]$ is the quencher concentration; and τ_0 and τ are the luminescence lifetimes in the absence and presence of quencher, respectively [39]. This type of transducer is best exemplified by the oxygen sensors based on luminescence quenching of ruthenium complexes [48, 49].

1.4.3.3 Calorimetric Biosensors

A large number of enzyme-catalyzed reactions are known to be exothermic in nature with a significant evolution of heat (normally $\Delta H \sim -10$ to -200 kJ mol^{-1}). The calorimetric biosensor thus relies on the heat generated in these reactions to produce the response signal for the target analyte of interest. The relations among the heat generated, enthalpy change, and temperature change under adiabatic conditions can be expressed by the following generic equations:

$$Q = -n_p \Delta H \tag{1.20}$$

$$Q = mC_p \Delta T \tag{1.21}$$

$$\Delta T = \frac{-\Delta H n_p}{mC_p} \tag{1.22}$$

where Q is the total heat evolved during a catalytic reaction, ΔH is the molar enthalpy change, n_p is the molar number of the product, ΔT is the temperature change, C_p is the heat capacity, and m is the mass of the system in which the reaction takes place.

The heat generated in the reactions and hence the temperature change, though in principle, could be measured by using the conventional mercury-based thermometer, practically their use is limited due to their low temperature sensitivity. More sensitive different temperature transducers are known and employed in enzyme calorimetric analyzers among which the thermopile (or thermocouple) and thermistor are more popular.

The potential difference (ΔV) of a thermopile transducer depends on the pair number of thermocouples (n), the Seebeck coefficient (ε), and the temperature difference (ΔT):

$$\Delta V = n\varepsilon\Delta T \tag{1.23}$$

Substituting ΔT from Equation 1.22:

$$\Delta V = n\varepsilon\frac{-\Delta H n_p}{mC_p} \tag{1.24}$$

Hence, for the adiabatic environment, the enthalpy change (ΔH) produced by an enzymatic reaction in a biosensor can lead to a direct change in potential (ΔV). Up to 80% of the heat generated in the reaction can be registered as a temperature change in the reaction under such adiabatically controlled conditions. A thermopile transducer has been reported for the detection of many analytes, including organophosphate pesticides such as dichlorvos [50].

The thermistor is a combination of *thermal* and *resistor*, implying that it is a type of resistor whose resistance is dependent on temperature. Hence, thermistors are widely used as temperature sensors, among other applications. Two major types of thermistors are available, depending upon the decrease or increase in resistance with increasing temperature. The thermistor whose resistance decreases as temperature rises (negative temperature coefficient, or NTC, type typically) is commonly used as a temperature sensor. The relationship between resistance and temperature is linear, assuming, as a first-order approximation,

$$\Delta R = k\Delta T \tag{1.25}$$

where ΔR is the change in resistance, ΔT is the change in temperature, and k is the first-order temperature coefficient of resistance. If k is positive, the resistance increases with increasing temperature, and the device is called a positive temperature coefficient (PTC) thermistor. If k is negative, the resistance decreases with increasing temperature, and the device is called an NTC thermistor.

The benefits of using a thermistor are accuracy and stability. Different versions of the thermometric biosensors are known. The conventional ET thermistor system has been progressively modified into mini, micro, and hybrid thermometric devices. A cross-section of a conventional enzyme thermistor instrument with its various components is shown in Figure 1.9.

The instrument contained an immobilized enzyme column for the reaction in a thermostated system, and the temperature at the point of exit was analyzed using a thermistor connected to a Wheatstone bridge. The components were assembled in a compact design for operation of the ET attached to the flow injection analysis. The reference probe contains an immobilized nonenzyme protein. The response signal can be refined by subtracting the signal of the reference column from the signal of the enzyme column [51].

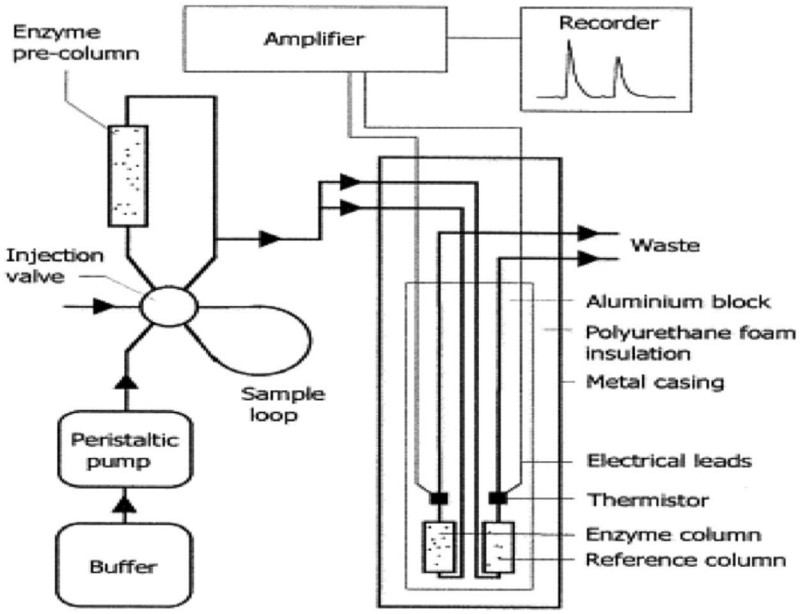

FIGURE 1.9 A cross-section of a conventional enzyme thermistor (ET) instrument showing its various components [51] Copyright (2001), with permission from Elsevier.

The low sensitivity of calorimetric biosensors for the reactions with low enthalpy change could be increased substantially through coupled reactions, which contribute to the heat output of the assay [52]. In the simplest case, this can be achieved by linking together several reactions in a reaction pathway, all of which contribute to the heat output. For instance, the sensitivity of the glucose analysis using glucose oxidase can be substantially increased by the co-immobilization of catalase in the column that exothermically degrades the hydrogen peroxide produced in the oxidase reaction. This highly exothermic reaction doubles the sensitivity of the sensor, reduces the deleterious effects of hydrogen peroxide in the reactor, and enriches the oxygen concentration in the reactor for the oxidase reaction. There have been reports of increasing the enthalpy change by using certain buffers like Tris that can increase the total enthalpy of the proton-producing reactions. Small amounts of organic solvents (around 5%, v/v) present in the aqueous buffer can also be used to increase the registered temperature changes by increasing the total enthalpy change in the reaction, which can be attributed to the lowering of heat capacity of the solvent.

The advantage of the calorimetric biosensors is that their application is not interfered with by the optical properties of the sample, viz. color and turbidity. The extensive application of calorimetry has, however, been restricted by the relatively high cost and complexity of the existing calorimeters. The design fabrication must ensure that the temperature of the sample stream remains constant (±0.01°C). Recently numerous inexpensive, less complicated devices for biochemical analysis have been developed merging the universality of calorimeters with the specificity of enzymatic reactions [53].

1.4.3.4 Piezoelectric Biosensors

Pierre Curie discovered the piezoelectric (Greek *piezo* means to squeeze or press) effect in 1880, and later it was used for sensing purposes in the 1950s. The Curie group perceived that anisotropic crystals, i.e. crystals without a center of symmetry, can generate an electric dipole when mechanically squeezed (Figure 1.10). The effect can also work in an opposite way in that an anisotropic crystal becomes deformed when voltage is applied on it [54]. The mechanical deformation is, however, a simple situation, and generally oscillation occurs in the common applications. In the case of oscillation, an alternating voltage is imposed on the crystal and then mechanical oscillation occurs. A piezoelectric sensor is used to measure changes in pressure, acceleration, temperature, strain, or force by converting them to an electrical charge. Many materials, both natural and man-made, exhibit piezoelectricity such as natural crystals: cane sugar, quartz, Rochelle salt, topaz, dry bone, tendon, silk, wood, enamel and man-made crystals: gallium orthophosphate ($GaPO_4$), and langasite ($La_3Ga_5SiO_{14}$).

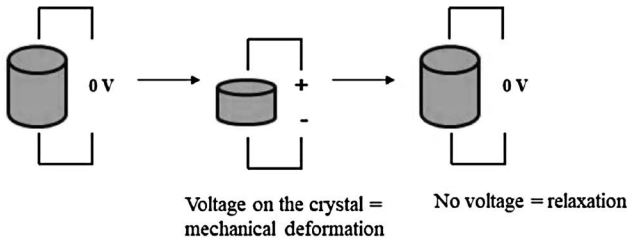

FIGURE 1.10 Piezoelectric effect when mechanical deformation is initiated by an applied voltage [54].

Frequencies of oscillations are determined in standard analytical applications. Analyte can be determined from the electricity produced on the crystal surface through interaction with either crystal alone or electrode. The bound mass on the crystal surface causes slowing of oscillation. For the common quartz crystals, the frequency shift (Δf) is directly proportional to the change of mass (Δm) on the crystal, as described by Sauerbrey [55] (Equation 1.26).

$$\Delta f = \frac{-2 f_0^2 \Delta m}{A\sqrt{(\rho_q \mu_q)}} = -2.3 \times 10^6 \; f_0^2 \frac{\Delta m}{A} \tag{1.26}$$

In this equation, f_0 is the fundamental mode of the crystal oscillation (in hertz), A is the piezoelectrically active area (in centimeters), ρ_q means density (2.648 g/cm^3), and μ_q means shear modulus (2.947×10^{11} g/cm·s^2) of quartz. The Sauerbrey equation is reliable for calculating frequency shift when the ambient environment is not unaltered. Upon a change in the surrounding environment, since viscosity has an impact on frequency shift, the equation described by Kanazawa and coworkers for quartz crystal should be consulted with [56] (Equation 1.27).

$$\Delta f = f_0^{3/2} \sqrt{\frac{\Delta(\rho_l \eta_l)}{\pi \rho_q \eta_q}} \tag{1.27}$$

The equation states that frequency shift is proportional to an increase of ambient viscosity η. The symbols' meaning is the same as for the Sauerbrey equation – the symbols with index l relate to the ambient liquid and q to quartz crystal.

Piezoelectric biosensors can be of two different types: bulk acoustic wave (BAW) piezoelectric sensors and surface acoustic wave (SAW) piezoelectric sensors. BAW is based on the principle that the wave propagates through the interior of the substrate. Conversely, SAW works on the principle that the wave propagates on the surface of the substrate. As the wave propagates through or on the surface of the substrate, its velocity continually changes. This change can be known by measuring the change in the frequency. This can be related to the physical mass being measured. These sensors apply an electric field that creates mechanical stress (wave). This moves through or on the substrate, and in the last step, is converted back to an electric field before we can measure it.

A quartz crystal microbalance (QCM) is one of the simplest and commonly used BAW devices. SAW sensors are composed of a thick layer of piezoelectric material, like quartz, lithium niobate, or lithium tantalite. Here, Rayleigh waves propagate along the upper surface. The surface transverse wave (STW) sensor is the most commonly used SAW device.

This technique is known for its excellent sensitivity, and hence it has wide applications in the medical, aeronautical, and telecommunications, fields. Quartz is a commonly used piezoelectric material as it is cheap and has the ability to withstand various types of stresses. Other potential materials are lithium niobate and lithium tantalite. These transducers are suitable for label-free and real-time biosensing. They can attained detection limits to the pico level and hence are suitable to measure various gases such as ammonia, hydrogen, methane, and carbon monoxide. Piezoelectric biosensors have been reported to detect various toxins, pathogens in food and water, hepatitis B and C, etc. They have also been used in protein and DNA detection. These sensors have good compatibility with integrated circuits (IC) technology and can be easily manufactured by photolithography, which renders them inexpensive [57].

A QCM-based biosensor was constructed for the determination of organophosphorus and carbamate pesticides in the nM level. The sensor had an immobilized enzyme acetylcholinesterase, which converted 3-indolyl-acetate to insoluble indigo pigment, providing alteration in the oscillations. The biosensor was used for the assay of pesticides, which inhibit the enzyme acetylcholinesterase. When the enzyme became inhibited, the precipitate was not formed [58].

1.5 BIORECEPTOR IMMOBILIZATION STRATEGIES

To efficiently capture the biochemical signal generated as a result of interactions between the bioreceptor and the target analyte, the bioreceptors are usually immobilized on the transducer surface of the biosensor device. This process empowers reusability of the bioreceptor with linked cost reduction of the developed devices/process. There are mainly four different techniques of bioreceptor immobilization, and their choice is based on their intended use. The different immobilization strategies for bioreceptors have been depicted in Figure 1.11 and Table 1.2.

1.5.1 Adsorption

Adsorption refers to the easiest technique of immobilization of the bioreceptor onto a surface by reversible surface interaction between the bioreceptor and the surface. The forces involved in this interaction are weak forces viz. van der Waals forces, ionic bonds, hydrogen bonds, and hydrophobic interactions. In this method the solid support onto which the immobilization is desired is placed in contact with a solution containing the dissolved bioreceptor at optimum pH, ionic concentration, etc., for an appropriate period of time to allow the adsorption to take place. The unbound bioreceptor molecules are removed from the surface by washing with a suitable buffer [59].

Adsorption offers the advantage of retention of bioreceptor activity (native conformation) during immobilization, as this process is not accompanied by chemical changes in the bioreceptor molecule or the surface. It is simple, low cost, and nondestructive to the bioreceptor. However, the disadvantage of this technique is that the immobilized bioreceptor is prone to desorption or leaching under conditions of changed pH, ionic strength, temperature, or polarity of the solvent, as only weak forces are involved to achieve the immobilization. Also, nonspecific interaction of other proteins or molecules

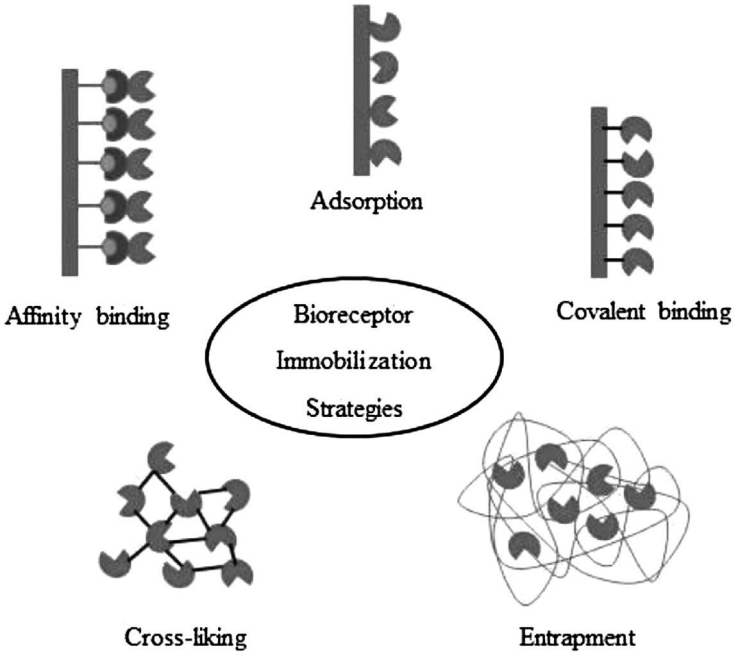

FIGURE 1.11 Bioreceptor immobilization strategies.

on the surface can cause contamination, inhibition, or signal interference. This is why biosensors employing immobilization of the bioreceptor by adsorption usually suffer from poor operational and storage stability. Importantly, the bioreceptor molecules may not exhibit homogeneity in orientation in the immobilized form; hence, there is a possibility of reduction of binding sites/active sites for the analytes of interest. In order to overcome the drawbacks, many strategies are emerged, among which, the immobilization on three-dimensional and/or porous structure of the matrix is widely used. Hydrogel polymers, such as agarose, collagen, gelatin, and polyacrylamide gel, and inorganic materials, such as mesoporous carbon, silica, and zeolite, which have a rigid porous structure for the retention of bioreceptor special enzyme molecules are mostly used as the enzyme immobilizing matrix surface.

1.5.2 Covalent Binding

This involves immobilization of the bioreceptor onto the transducer surface through covalent bonds. The functional groups of bioreceptors, such as amine, hydroxyl, carboxyl, aldehyde, and sulfhydryl, are exploited for the covalent linkages with the solid transducer surfaces. The covalent immobilizations are frequently performed in two steps. First, the surface is activated using linker (also called carrier)

TABLE 1.2
Summary of Bioreceptor Immobilization Strategies

Immobilization Strategy	Characteristic Feature	Advantages	Disadvantages
Adsorption	Weak bonds	• Simple and easy • Limited loss of enzyme activity	• Desorption • Nonspecific adsorption
Covalent binding	Strong chemical bonds	• Stable • No diffusional barrier • Short response time	• High loss of enzyme activity
Entrapment	Confinement of bioreceptors within gel or polymer without chemical reaction	• Enzyme activity not compromised	• Diffusion barrier formed • Enzyme leakage may occurs
Crosslinking	Bond between bioreceptors via crosslinking agents	• Simple	• Likely to suffer from high loss of enzyme activity
Affinity	Affinity bond between affinity tag of bioreceptor and a functional group on the support	• Controlled immobilization	• Requirement of the presence of specific groups on the bioreceptor (e.g. histidine, biotin)

molecules such as glutaraldehyde or carbodiimide that act as the covalent linker between the bioreceptor and the surface. Second, addition of the bioreceptor (viz. enzyme) followed by formation of a covalent bond between the activated substrate and the bioreceptor molecule. The most widely used carrier molecules, particularly for enzyme immobilization, are hydrophilic in nature such as agarose, dextran, cellulose, and starch. These carrier molecules consists of ideal functional groups viz. hydroxyl groups that support the formation of covalent bonds. Also, their ability to form hydrogen bonds with water helps maintain a hydrophilic environment that favors bioreceptor/enzyme stability. The orientation and homogeneity of the linker/carrier molecules can be controlled as they form a self-assemble monolayer (SAM) [59]. This in turn allows for controlled homogeneity of the immobilized bioreceptor on the surface, unlike that of adsorption. Due to the immobilization of bioreceptors through covalent bonds, the leaching problem of the receptor could be overcome. The main disadvantage of this technique lies in the probable denaturation of the bioreceptors accompanied by the loss of their activity; additionally, this method is time consuming and complex as compared to absorption and is poorly reproducible.

1.5.3 Entrapment

Entrapment refers to confinement of the enzyme within a polymeric network without chemically interacting with the support matrix. The pore size of the matrix is controlled to prevent loss of bioreceptor while allowing movement of substrates and products. In this method, a bioreceptor is usually mixed with monomeric units in solution, which is then made to undergo polymerization. The bioreceptor molecules are entrapped within the polymer during the polymerization process. This method offers the advantage of minimizing bioreceptor leakage and denaturation. Additionally it helps to increase stability of the enzyme as the microenvironment of the entrapment matrix can be controlled to have optimal pH, ionic strength, or polarity. However, the disadvantage of this method is that the support matrix polymerization generates mass transfer resistance because of which the binding sites/active sites of the bioreceptor may not be accessible to the substrates efficiently. Special care needs to be taken in controlling the pore size of the matrix to minimize bioreceptor leaching. Also, this method is accompanied with low enzyme loading capacity. All these factors may contribute to serious kinetic implications. A few procedures to achieve entrapment include electrochemical polymerization, photopolymerization, sol-gel process, and microencapsulation.

In electrochemical polymerization, monomer polymerization takes place due to the application of appropriate potential or current. This application of potential or current may generate radical species that facilitate the polymerization process on the electrode surface solution while simultaneously entrapping the bioreceptor. Examples of electropolymerized films used for immobilization include polyaniline (PANI), polypyrrole, thiophenes, etc. In photopolymerization, monomers are activated to form cross-links or form polymers when exposed to light (photopolymers). In the sol-gel process, a metal alkoxide precursor such as tetramethoxysilane is suspended or dissolved in a suitable acidic solvent for hydrolization to produce silanol groups. This is followed by addition of a base to activate to hydrolized precursor to initiate a condensation reaction to form siloxane polymers. With appropriate time and temperature, gelation of the polymer occurs with bioreceptor entrapment. The sol-gel method offers the advantage of forming a stable nanoporous material with high loading capacity of the enzyme that aids in preserving enzyme activity and increasing biosensor sensitivity. However, this method is comparatively costly and is accompanied with matrix inhomogeneity. Encapsulation refers to entrapment of the bioreceptor inside a closed semi-permeable membrane. The nature of the membrane may be polymeric, lipoidal, or nonionic in nature. The membrane restricts the movement of the enzyme, thereby preventing loss of the enzyme, while allowing the diffusion of small molecules like substrates or products. This method maintains enzyme integrity, as the enzyme remains isolated from the external environment. However, control of membrane pore size is crucial to ensure retention of the enzyme and prevention of enzyme leakage, while allowing the entry and exit of substrate and product molecules [59].

1.5.4 Crosslinking

Immobilization by crosslinking refers to the creation of intermolecular cross-linkages between the bioreceptor molecules by covalent bond formation. This process requires the involvement of a multifunctional reagent like glutaraldehyde or toluene diisocyanate, which links the bioreceptor molecules together. Glutaraldehyde, for example, can crosslink enzyme molecules via the reactions of free amino groups of lysine residues of the individual enzyme molecules. This results in the formation of the three-dimensional complex structure or bioreceptor aggregates. The advantages of immobilization by crosslinking include minimization of bioreceptor leakage due to the involvement of covalent bonds and possible control of the bioreceptor microenvironment to maintain optimum pH, ionic strength, and other factors. The disadvantage of this technique is that the crosslinking agent can bring about certain modifications in the bioreceptor or denaturation that compromises its biological activity to various degrees [60].

1.5.5 Affinity

The strategy of affinity immobilization is employed with the main focus of obtaining a particular orientation of the enzyme or bioreceptor so as to leave its active site/binding site undisturbed and therefore accessible to the substrate/analyte. This can be achieved by creating affinity bonds between an activated support viz. lectin, avidin, metal chelates, and a specific interacting group or sequence of the bioreceptor viz.

carbohydrate residue, biotin, and histidine, respectively. Such groups or affinity tags are sometimes attached to the bioreceptor by genetic engineering methods [61].

1.6 APPLICATION OF BIOSENSORS

1.6.1 Application in the Food Industry

The important concerns relevant to the food industry include quality and safety of the food products, storage and shelf life, and processing. Quality control measures play an important role in approving any food product as suitable for consumption. This involves detection of food spoilage microorganisms or other molecules that may be indicative of degraded food quality and of food-borne pathogens. Because biosensors are specific, sensitive, rapidly responsive, and cost-effective as compared to the traditional methods, these have captured attention for practical application in assessing food quality. The enzyme-based biosensors and immunosensors are widely used for this purpose. Biosensors with different detection principles for detection of foodborne pathogens such as *Staphylococcus aureus* [62], *Salmonella typhimurium* [63], *Salmonella enteritidis* [64], *Escherichia coli* [65], and *Listeria monocytogenes* [66] are some examples.

1.6.2 Application in the Fermentation Industry

Biosensors play a pivotal role in the fermentation industry where continuous monitoring of metabolites, products, enzyme, antibody, biomass, or byproducts is essential. Several commercial biosensors are available to monitor various fermentation processes. A few examples of compounds usually monitored in the fermentation industry are listed in Table 1.3 [67].

1.6.3 Biomedical Applications

The application of biosensors in biomedical field has been rapidly growing since the commercial release of the glucose biosensor. Blood glucose monitoring has been occupying center stage in biosensor research since the first product launched in the market in the year 1975 by Yellow Springs Instruments (YSI). Following this, several new and improved products were introduced in the global market by different companies over the years [68]. Nova's StatStrip has introduced a glucose monitor for use in neonatal testing. StatStrip is the only glucose monitor with 6s analysis time that measures hematocrit on the strip. The company also provides a handheld device for the measurement of blood lactate using a very tiny drop of blood (0.7 μL) with an analysis time of 13s. Nova also commercializes a biosensor that measures creatinine with an analysis time of 30s [68]. Roche Diagnostics markets the Accu-Chek family of products/services for blood glucose monitoring. Its US Patent Number 6,541,216 describes an invention that allows the measurement of ketone levels in blood. In 2001, LifeScan, a part of the Johnson & Johnson companies, launched a glucose measuring device (OneTouch Ultra blood glucose) and the In Duo system, the world's first blood glucose checking and insulin-dosing system. After that, in 2003, LifeScan launched the OneTouch UltraSmart blood glucose monitoring system with a 3,000-record memory for the storage of health, medication, exercise, and meal information [68]. The acceptance and success of biosensors in this field are largely determined by a high level of precision in measuring analytes of clinical importance, capability to sense the analyte in real time, and high sensitivity. A few common applications of biosensors in biomedicine include detection and measurement of disease-specific biomarkers; biomolecules such as glucose, lactate, peroxides, cholesterol, and cytokines; and release of antibodies or other indicator biomolecules in various inflammatory diseases and tumors. For example, biosensors for detection of *Candida* infection [69], circulating tumor cells (CTCs) [70], and antibiotic sensitivity of bacteria [71] are reported.

1.6.4 Environmental Applications

Harmful environmental agents pose a serious threat not only to human health but to the entire ecosystem. The first and foremost step for appropriate treatment of these agents is to detect their levels and their sources. The attainable high sensitivity of the biosensors plays a crucial role in detecting

TABLE 1.3
Analytes of Interest in the Fermentation Industry

Fermentation Process	Compounds to Monitor
Microbial fermentation	Short-chain monocarboxylic and dicarboxylic acids-butyl esters of volatile (C1–C7) and nonvolatile (lactic, succinic, and fumaric) acids
Wine fermentation	Malolactic fermentation compounds
Rice wine	Total sugar content, alcohol, and pH
Wine	L-Lactic acid
Probiotic fermented milk	Oligosaccharides, improved fermentation rates, accelerated lactose hydrolysis
Grapes during yeast fermentation	Volatile flavor chemicals—acetates, ethyl esters, C4–C8 fatty acids
Fermented soybean foods	Proteases and ethanol, ethylene glycol, glucose, isopropanol, and mannitol
Cheese	Tyramine

the otherwise undetectable low level of harmful agents in the environment. Such major pollutants include heavy metals, pesticides, polychlorinated biphenyls, toxic organic wastes, nitrogenous compounds, endotoxin, and several pathogens. Considerable emphasis has been laid on detection of different toxic heavy metals such as Hg, Cd, Ni, Co, Zn, Pb, and Cu, and biosensors with different detection principles have been reported [72–75].

1.6.5 Applications in Biodefense

The world is witnessing increased threats of terrorism, including bioterrorism. Organisms or toxins used for such activities are termed biowarfare agents (BWAs), which include bacteria like *Bacillus anthracis* causing anthrax, toxins such as *Botulinum* neurotoxin and *Cholera* toxin, and viruses. The anthrax bioterrorist attack is predominantly due to the resistant spores, which have been observed to be lethal in 75% of infections. Biosensors hold application in detecting these agents by employing their important attributes of high selectivity. Some of the examples of potential bioterrorism agents against which biosensors have been developed in the literature with their detection limit in parenthesis are detection of *Anthrax* spores (1 μg/mL), *Botulinum* toxin (400 ng/mL), and ricin (400 ng/mL) with an assay time of 15–25 min following flow immunosensor systems, commercialized as a biowarfare agent detection device (BADD); detection of *Anthrax* spores (4,000 spores), *Botulinum* toxin (5 ng), ricin (10 ng), and smallpox (100,000 pfu) with an assay time of 15 min following fluorescent bead immunoassay commercialized by Response Biomedical Corp. [76] detection limits of the noted sensors are shown in the parentheses.

1.7 RECENT ADVANCES IN BIOSENSOR TECHNOLOGY

Recent advancement in biosensor technology comprises miniaturization of the devices, wearable and implantable design using smart materials, and analyzing and transmitting the response signal through modern communication technology, including Internet of Thing (IoT), machine learning, and smart phone technology. In these technology-driven research and developments, application of various nanomaterials for signal transduction and amplifications, and micro-scale domain technology, such as microfluidics and MEMS are increasingly used in biosensing applications to improve the sensitivity, render multiplexing capability in a chip format, and provide cost economy of the devices.

Nanomaterials can be one dimensional viz. surface nanofilms, two dimensional viz. nanofibers, or three dimensional viz. nanoparticles. A common property of the nanomaterials is their tremendously increased surface area as compared to their corresponding bulk materials. The increased surface area supports enhanced immobilization of the bioreceptors. Also, nanoscale quantum effects bring about certain properties viz. unique mechanical, magnetic, electrical, optical, and other properties, which can be used either to amplify or to transduce the biorecognition signal in the biosensor. The most popular nanomaterials used for biosensor applications include gold nanoparticles (AuNPs), carbon nanotubes, graphene, quantum dots, and others, among which, AuNPs are one of the most stable noble metal nanoparticles and most popularly used in biosensors due to their biocompatibility, suitable optical and electrical properties, and relatively simple mode of production and modification [77].

Smart biosensors refer to the coupling of a biosensor with smart technology, especially for application in health care, food safety, and environmental monitoring. An amalgamation of nanotechnology, wireless technology, information technology, machine learning, material sciences, and biomedical sciences has made this concept of smart biosensors possible. One straightforward outcome is the integration of POC biosensors with smart phone technology. This has become possible with the advancements in various technologies viz. deep learning, which is derived from conventional artificial neural networks, wherein a sensory input is received and subjected to an iterative process of training until a desired/accurate output is reported. The applicability of deep learning can be envisioned with the emergence of wearable biosensors, invasive sensors, or embedded sensors in smart devices to collect medical data for disease diagnosis or prognosis. Artificial intelligence is concerned with the creation of intelligent machines that work in analogy with the human brain. It encompasses machine learning, which includes application of pattern-recognition algorithms to improve performance via experience. Pattern-recognition algorithms particularly involve training by using previously acquired data, and its assessment for retrieval of those data with particular characteristics aid in grouping data into classes with similar characteristics and interpretation of the final assignment of the class. In other words, pattern-recognition trains the device to identify the presence or absence of substances in the sample, characteristic composition, analysis, and designation of a reported outcome as readout [78].

1.7.1 Wearable Biosensors

As the name suggests, wearable biosensors are meticulously designed miniaturized biosensors that can be worn on the skin in the form of a temporary skin tattoo or bands, on eyes as contact lenses, or on tooth enamel. Noninvasive monitoring of biofluids (sweat, tears, saliva, and interstitial fluid) is the characteristic feature of such sensors and has great value in the ever-booming health care industry, as they can provide continuous real-time monitoring of targets, management of chronic diseases, and alert the user or medical professionals in case of emergency. In order to achieve this, wearable biosensor platforms must be in direct contact with the noninvasive sample fluid without causing any discomfort to the user. Multidisciplinary research has brought about this possibility by integrating smart materials with the necessary flexibility, stretchability and biocompatibility, and miniaturization technologies which facilitate data processing and transmission for real-time monitoring of the target analyte (biomarkers, metabolites, hormones, etc.). Although several proofs-of-concept

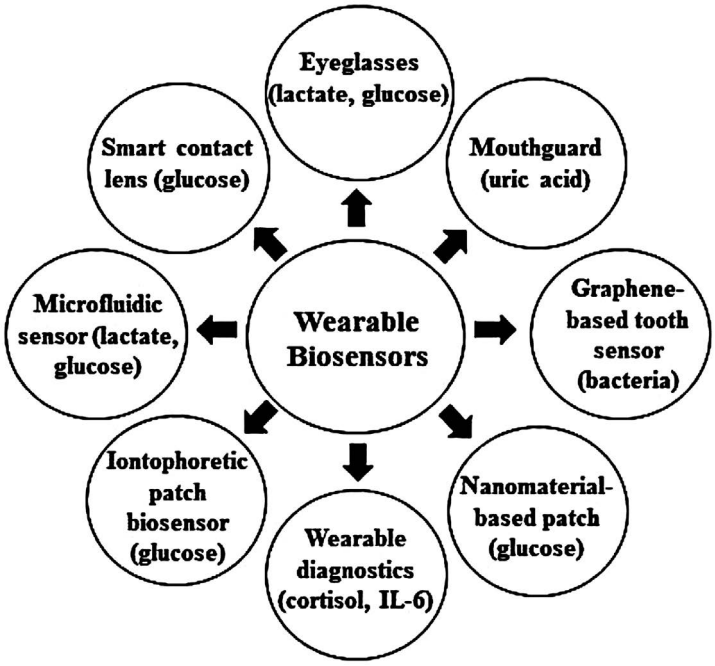

FIGURE 1.12 Representative examples of wearable biosensors [79].

have been forwarded in the literature, a thorough validation for correlation of analyte concentrations in noninvasive body fluids and a gold-standard sample fluid in most cases, i.e., blood, is needed to improve reliability. Additionally, the limited arena of smart materials and miniaturization technologies best suited for the development of wearable biosensors hampers its commercialization so far. Figure 1.12 illustrates a few examples of wearable biosensor, and the target analytes are shown in parentheses [79].

1.7.2 Implantable Biosensors

Most implantable biosensors use the amperometry-based principle, which is envisioned to measure and remotely transmit a record of specific molecular level of a biological analyte within the human body. Implantable biosensors generally use enzymes as the biorecognition molecule to enable the detection of biochemicals of interest within the body under a suitable impressed potential [80]. Trauma management and diagnostics are the foremost aims of research for implantable amperometric biosensor technology [81]. A recently developed dual responsive electrochemical cell-on-a-chip microdisc electrode array (ECC MDEA 5037) transducer was used in a wireless, implantable biosensor system for the continuous measurement of interstitial analytes. Preliminary studies with the MDEA 5037 in a rat hemorrhagic shock model have shown discordance between blood and interstitial lactate levels [82]. Lactate can accumulate more readily in the muscles particularly during periods of compensation and increased peripheral resistance during moderate to severe hemorrhage as blood oxygen delivery will be even further reduced, thus causing a rapid spike in interstitial lactate levels. Now, this lactate will diffuse back into the blood and eventually make its way to the liver. It is hypothesized that under conditions of diminished peripheral perfusion, lactate levels in the tissues will be discordant with systemic lactate levels and that the amount and duration of the tissue lactate levels will be a better indicator of the extent of hemorrhagic shock in the trauma patient. Continued examination of interstitial compartments using biosensors will aid in understanding the temporal relationships among markers of stress in these environments and how they relate to shock-like states. Potentiostat is a vital instrument for an implantable amperometric enzyme biosensor. However, for implantation, the potentiostat is closely connected with two-way telemetry and communications. Three general formats of implantable amperometric biosensors are being followed. The first is an implantable but tethered biotransducer with outwardly located power, electronics and communications components with the external components being mounted outside but on the subject's body [83]. The second is a fully integrated discrete but otherwise fully implanted device [84], and the third is an application-specific integrated circuit (ASIC) where all components are likewise fully implanted [85]. Representative examples of these three formats are shown in Figure 1.13.

Implantable amperometric enzyme biosensors have tremendous technological potential to influence patient management and compliance among diabetics but also to address the management of hemorrhaging victims of trauma. However, they face some major challenges, such as (a) enzyme stability, (b) biomolecular interferences, (c) the performance of molecular mediators used in Generation II biotransducers, and (d) internal calibration. Modern nano-biomedicine approaches such as biomaterials biomimicry, programmed anti-inflammatory drug delivery, and regenerative medicine are examples of tactics being developed to quiet the foreign body response. The

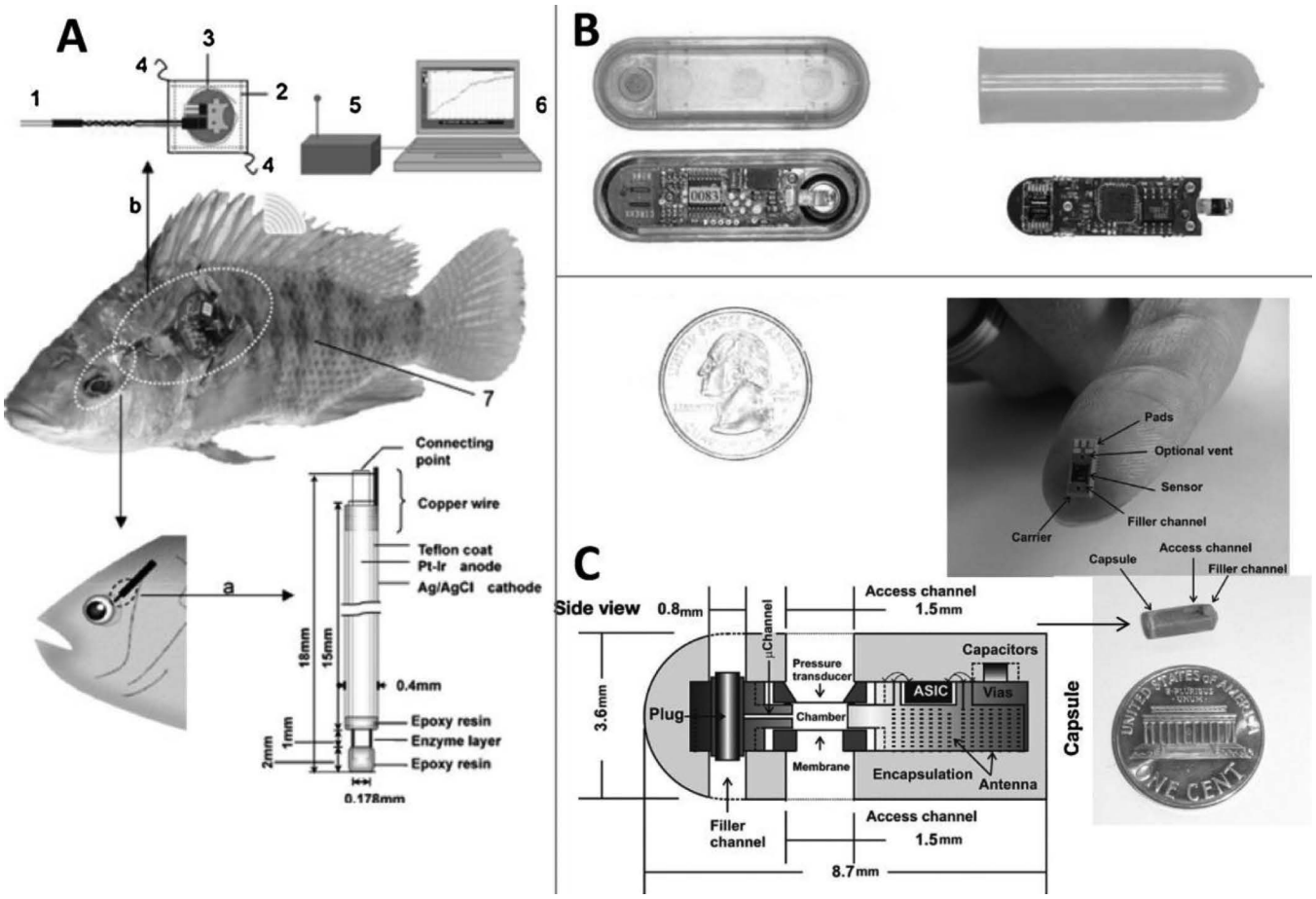

FIGURE 1.13 Examples to illustrate three general formats for implantable biosensor systems. **(A)** The tethered biotransducer with externally located power, electronics, and communications components. **(B)** The fully integrated discrete but otherwise fully implanted biosensor system. **(C)** The application-specific integrated circuit (ASIC) [80] Copyright (2012), with permission from Elsevier.

efforts to address the inherent factors that limit device bioanalytical performance include enzyme engineering to promote stability, hybrid biomaterials to address endogenous interferences, and the emergence of reagentless, third-generation biosensors hold considerable promise. Finally, fully implantable ASIC devices with a small footprint and wireless communication capabilities are being developed [80].

1.7.3 Engineered Enzymes for Biosensor Development

Genetically engineered acetylcholinesterases (AChEs) have been extensively exploited in enzyme inhibition-based biosensors for the detection of pesticides, like carbamate insecticides and organophosphate [86–88]. Currently great attention is given to protein engineering in order to improve the complete performance of bioelectronics. Specifically, there exist two strategies: (a) improving the biomolecular recognition between enzyme and substrate and (b) increasing the rate of electron transfer between enzyme and electrode. The first strategy can enhance the functional performance of amperometric sensors by increasing their selectivity as well as sensitivity. To address the second strategy, the enzyme is modified, keeping in mind the two key parameters: the turnover number, k_{cat}, and the Michaelis constant, K_M. The first reports on the effect of deglycosylation of redox enzymes were on recombinant horseradish peroxidase (r-HRP) overexpressed in *E. coli* [89]. Bioengineering of GOD extracted from *Penicillium amagasakiense* to make the enzyme less oxygen-dependent demonstrated a promise for making the enzyme more suitable for biosensor applications. Another example of bioengineering of a sugar oxidizing enzyme is the mutation of two ascomycete CDHs to increase the affinity for glucose and at the same time largely decrease the affinity for maltose, which is of vital importance for glucose biosensors. Another recent work in which a FAD-GDH was successfully fused with cyt *c* to mimic CDH and other flavohaemo proteins with direct electron transfer properties to obtain enzymes with modified properties [90]. To make the active site more accessible, eliminating the amino acids that are not vital for the enzyme functionality is another approach that facilitates the electron transfer easy.

1.8 MARKET POTENTIAL FOR BIOSENSORS

The market potential of biosensors is driven by their demand in diverse fields such as medicine, pharmacology, health

care, food and agriculture, environmental monitoring, and biodefense. Additionally, with the advantages of biosensors to detect analytes over the established conventional techniques, compounded with possible miniaturization and low production costs, remote monitoring and reduced health care expenses, promising growth in the biosensor market seems visible. According to a recent analysis (Frost & Sullivan), the global biosensor market is expected to grow at a 12% compound annual growth rate (CAGR) during 2018–2023, from revenues of $17.7 billion in 2018, to reach $31.2 billion by the end of 2023. The patent publication in the field is on the rise, with 56% of total patents published by the United States alone from 2016 to 2018. The key areas of innovation include POC diagnostics, wearable biosensors, and noninvasive monitoring. Numerous companies are working worldwide in the area of biosensors for commercialization of their products.

Many factors influence the commercial success of biosensor devices. From the end-user point of view, low cost, simple, and reliable performances of the product are essential factors for penetrating the developed product in the market. It may be mentioned that the WHO prescribed the ASSURED [91] criteria for applications of the diagnostic devices in developing countries. The biosensors should comply with all the performance criteria as discussed elsewhere in this chapter. Addressing these issues may expedite the process of bridging the gap between academia (for proof-of-concept) and industry (to translate the concept to technology) for commercialization of the biosensors. However, few concerns associated with biosensor research need to be identified before developing the product such as the market for biosensors, advantages of biosensors over the existing analytical methods, ease of manufacturing and usage, and last but not the least, hazards and ethics associated with the particular biosensor research in question. Moreover, some other issues, such as government support in terms of ease of doing business and customer perceptions, should encourage for developing and marketing biosensor products for the decentralization of laboratory testing.

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