

# CHAPTER 1

## Introduction to Biosensors

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## 1.1 INTRODUCTION

Bioelectronics deals with the application of the principles of electronics to biology and medicine. Biosensor is a special type of bioelectronic device commonly used in bioanalysis. A sensor can be viewed as the “primary element of a measurement chain, which converts the input variable into a signal suitable for measurement.” Over the past decade, many important technological advances have provided us with the tools and materials needed to construct biosensor devices. Since the invention of the Clark oxygen electrode sensor, there have been many improvements in sensitivity, selectivity, and multiplexing capacity of modern biosensors. *Biosensor* can be defined as a compact analytical device incorporating a biological or biologically derived sensing element either integrated within or intimately associated with a physicochemical transducer. Two fundamental operating principles of a biosensor are “biological recognition” and “sensing.” Therefore, a biosensor can be generally defined as a device that consists of three basic components connected in series: (1) a biological recognition system, often called a bioreceptor, (2) a transducer, and (3) microelectronics. The basic principle of

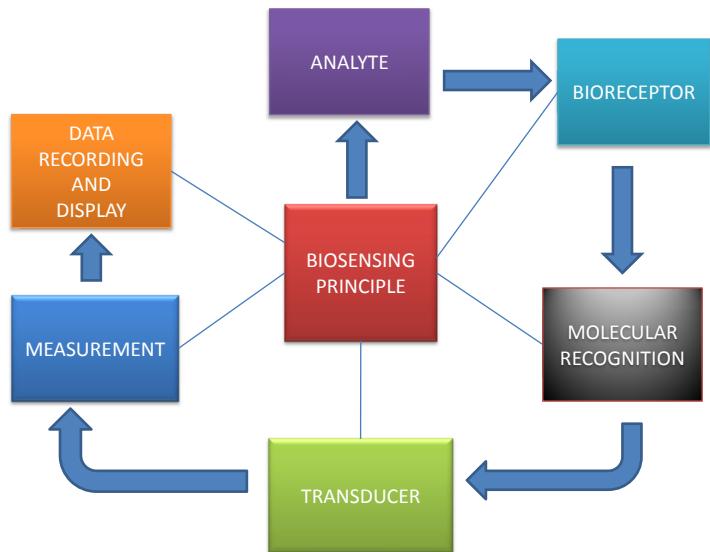
a biosensor is to detect this molecular recognition and to transform it into another type of signal using a transducer. The main purpose of the recognition system is to provide the sensor with a high degree of selectivity for the analyte to be measured. The interaction of the analyte with the bioreceptor is designed to produce an effect measured by the transducer, which converts the information into a measurable effect such as an electrical/optical signal. According to IUPAC recommendations 1999, a biosensor is an independently integrated receptor transducer device, which is capable of providing selective quantitative or semiquantitative analytical information using a biological recognition element (Thévenot et al., 1999). The purpose of a biosensor is to provide rapid, real-time, accurate, and reliable information about the analyte of interrogation. Ideally, it is a device that is capable of responding continuously, reversibly, and does not perturb the sample. Biosensors have been envisioned to play a significant analytical role in medicine, agriculture, food safety, bioprocessing, environmental and industrial monitoring (Luong et al., 2008).

## 1.2 BASIC PRINCIPLE OF A BIOSENSOR

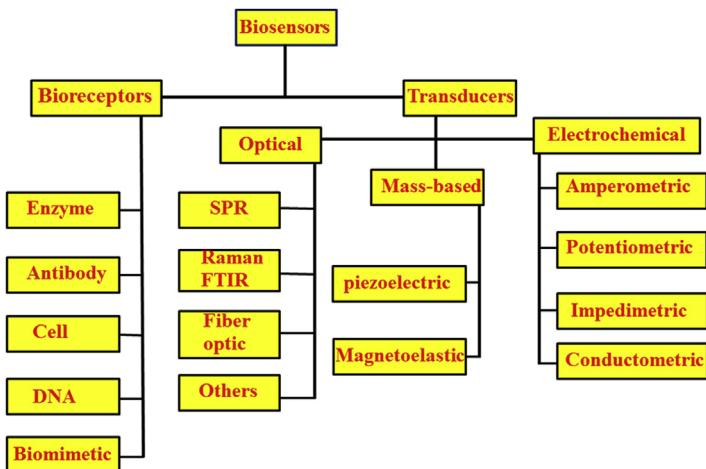
The term *biosensor* is short for *biological sensor* and is a device made up of a transducer and a biological element that may be an enzyme, an antibody, or a nucleic acid. The biological element or bioelement interacts with the analyte being tested and the biological response is converted into an electrical signal by the transducer. Every biosensor has a biological component that acts as the sensor and an electronic component that detects and transmits the signal. In other words, the biological material is immobilized and a contact is made between the immobilized biological material and the transducer. The analyte binds to the biological material to form a bound analyte, which in turn produces the electronic response that can be measured. Sometimes the analyte is converted to a product that could be associated with the release of heat, gas (oxygen), electrons, or hydrogen ions. The transducer then converts the product-linked changes into electrical signals, which can be amplified and measured. If the bioelement binds to the analyte, the sensor is called an *affinity sensor*. If the bioelement and the analyte give rise to a chemical change that can be used to measure the concentration of a substrate, the sensor is called a *metabolic sensor*. If the biological element combines with analyte and does not change it chemically but converts it to an auxiliary substrate, the sensor is called a *catalytic sensor*. Figure 1.1 represents the basic principle of biosensor.

## 1.3 COMPONENTS OF A BIOSENSOR

A biosensor consists of three main elements: a bioreceptor, a transducer, and a signal processing system (David et al., 2008). Biosensors can be classified by their bioreceptor or their transducer types. The classifications of biosensors based on bioreceptors and transducers are represented in Figure 1.2.



**Figure 1.1** Basic principle of biosensors.



**Figure 1.2** Classification of bioreceptors and transducers.

### 1.3.1 Bioreceptors

Bioreceptors or the biological recognition elements are the key to specificity for the biosensor technologies. The bioreceptor or biological recognition element is the significant distinguishing feature of a biosensor. The bioreceptor comprises the recognition system of a sensor toward the target analyte. A bioreceptor is molecular species that

utilize a biochemical mechanism for recognition. They are responsible for binding the analyte of interest to the sensor surface for the measurement. Bioreceptors can generally be classified into five major categories: enzyme, antibody/antigen, nucleic acid/DNA, cellular structure/cell, and biomimetic. The sampling component of a biosensor contains a biosensitive layer that can contain bioreceptors or be made of bioreceptors covalently attached to the transducer. The most common forms of bioreceptors used in biosensing are based on:

1. Antibody—antigen interactions
2. Nucleic acid interactions
3. Enzymatic interactions
4. Cellular interactions (i.e., microorganisms)
5. Interactions using biomimetic materials (i.e., synthetic bioreceptors)

The enzymes and antibodies are the main classes of bioreceptors that are widely used in biosensor applications.

#### **1.3.1.1 Enzyme bioreceptors: biosensors**

Enzymes have been the most widely used bioreceptor molecules in biosensor applications. Enzymes are often used as bioreceptors because of their specific binding capabilities as well as their catalytic activity. In biocatalytic recognition mechanisms, the detection is amplified by a catalytic reaction.

With the exception of a small group of catalytic ribonucleic acid molecules, all enzymes are proteins. Some enzymes require no chemical groups other than their amino acid residues for activity. Others require an additional component called a cofactor, which may be either one or more inorganic ions, such as  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ , or  $\text{Zn}^{2+}$ , or a more complex organic or organometallic molecule called a coenzyme. The catalytic activity provided by enzymes allows for much lower limits of detection than would be obtained with common binding techniques. As expected, the catalytic activity of enzymes depends upon the integrity of their native protein conformation. If an enzyme is denatured, dissociated into its subunits, or broken down into its component amino acids, its catalytic activity is destroyed. Enzyme-coupled receptors can also be used to modify the recognition mechanisms. For instance, the activity of an enzyme can be modulated when a ligand binds at the receptor. This enzymatic activity is often greatly enhanced by an enzyme cascade, which leads to complex reactions in the cell.

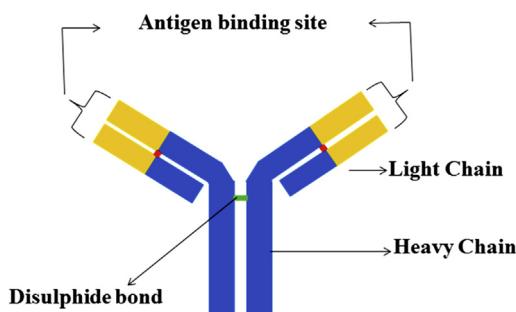
Enzymes are chosen for biosensors, since they are the natural proteins, catalyzing a specific substrate molecule into a product without being consumed in the reaction. The enzyme recognizes a particular target analyte in a similar way to a key fitting a lock. Enzymes are highly selective and sensitive compared with chemical reactions, they are fairly fast acting compared with other biological receptors, and they can be used in combination with different transduction mechanisms. The mechanisms of operation of these bioreceptors can involve: (1) conversion of the analyte into a sensor

detectable product; (2) detection of an analyte that acts as enzyme inhibitor or activator; or (3) evaluation of the modification of enzyme properties upon interaction with the analyte.

### 1.3.1.2 Antibody bioreceptor: immuno sensors

An immuno sensor exploits antibodies as a bioreceptor to detect the specific antigen. Immunoassays are the most specific analytical techniques, provide extremely low detection limits, and can be used for a wide range of substances. Such assays become extremely useful for identifying and quantitating the proteins (Warsinke et al., 2000; Ghindilis et al., 1998). The term *immunoassay* is used for tests based on the immunoreactions, while the term *immuno sensor* is specifically employed to describe whole instruments, i.e., immunoreactions-based biosensors. Schematic representation of Y-shaped structure of an antibody is shown in Figure 1.3.

Antibodies or immunoglobins are heavy globular plasma proteins (150 kDa). They are also called glycoproteins and are composed of two heavy chains and two light chains forming the well-known Y shape (Figure 1.3). They are produced in the animals by the immunological response to the foreign agents, so-called antigens. The antibody binds the target antigen with high affinity and is therefore able to detect the analyte even in the presence of other interfering substances. Antibodies employed in the immuno sensor development are of two kinds: polyclonal and monoclonal. Polyclonal antibodies are highly sensitive but less specific since they can recognize different epitopes (the small site on an antigen to which a complementary antibody may specifically bind) on their target antigens, subjecting them to cross-reactivity. Monoclonal antibodies, on the other hand, are identical, because they are produced from one type of immune cell and bound to the same epitope of their specific antigen, making them highly specific. Because of their specificity, monoclonal antibodies are excellent as the primary antibody in an immunoassay, or for detecting specific antigens in the presence of interfering molecules, and give significantly less background staining than polyclonal antibodies.



**Figure 1.3** Schematic representation of Y-shaped structure of an antibody.

### 1.3.1.3 Nucleic acid bioreceptors

Another biorecognition mechanism involves hybridization of DNA or RNA. The use of nucleic acid sequencing for the specific diagnostics application has developed since early 1953 and is still growing widely (Liu et al., 2012). The highly specific affinity binding's reaction between two single-strand DNA (ssDNA) chains to form double-stranded DNA (dsDNA) is utilized in nucleic acids-based biosensors, which appoint the nucleic acids as the biological recognition element.

#### DNA (*genosensors*)

DNA biosensors based on nucleic acid recognition processes are rapidly being developed toward the goal of rapid and inexpensive testing of genetic and infectious diseases. The development of DNA biosensors has attracted considerable attention due to their potential applications, including gene analysis, clinical diagnostics, forensic study, and more medical applications. A genosensor is a biosensor (electronic) that can detect the individual nucleotides that comprise a genome (DNA) molecule. Automated genosensors enable rapid, nondestructive sequencing of DNA molecules. Genosensors (or DNA biosensors) are devices that combine, as a biological recognition agent, a ssDNA called a DNA probe, with a transducer. The selectivity of this device is due to the former, whilst its sensitivity is provided by the latter. Genosensors make use of the hybridization event to detect a target DNA sequence. The determination of nucleic acid sequences from humans, animals, bacteria, and viruses is the departure point for solving different problems: investigations into food and water contamination caused by microorganisms, detection of genetic disorders, tissue matching, forensic applications, etc. A genosensor consists of a substrate modified with specific oligonucleotides (probe DNA) that can detect complementary DNA sequences (target DNA) through hybridization. Among the different types of genosensors, depending on the technique employed for the transduction, three main classes can be distinguished: optical, piezoelectric, and electrochemical. Among the various methods for DNA detection, electrochemical techniques offer great advantages with simplicity, rapidness, relatively low cost, and high sensitivity and are suitable for the development of inexpensive and portable devices. Electrochemical genosensors are based on electrochemical transduction to detect the hybridization event. These devices can be exploited for monitoring sequence-specific hybridization events directly measuring the oxidation signal of DNA electroactive bases, DNA electroactive indicators forming complexes with DNA nitrogenous bases, or with the aid of oligonucleotides labeled with enzymes. Impedance spectroscopy is an electrochemical technique that is rapidly developing as a tool for studying DNA hybridization. Impedance spectroscopy is an effective method for probing the interfacial properties (capacitance, electron transfer resistance) of modified electrodes. One advantage of this technique is that oligonucleotide labeling is not required for DNA detection. The success of biosensors based on “label-free” impedance sensing has been widely demonstrated.

### Principle of the DNA biosensor

DNA is especially well suited for biosensing applications because the base-pairing interactions between complementary sequences are both specific and robust. In a typical configuration, a single-stranded probe sequence is immobilized within the recognition layer, where base-pairing interactions recruit the target DNA to the surface ([Figure 1.4](#)). The repetitive, essentially uniform structure of DNA makes its assembly on the recognition surface well defined. It is at this interface that the critical dynamics of target capture take place to generate the recognition signal; therefore, immobilizing nucleic acid probe sequences in a predictable manner while maintaining their inherent affinity for target DNA is crucial to overall device performance. This recognition event depends ultimately on the method of signal transduction, whether it be optical, mechanical, or electrochemical. DNA techniques, including hybridization, amplification, and recombination, are all based on the double helix structure of the DNA. Nucleic acid hybridization is the underlying principle of DNA biosensors. General DNA biosensor design is illustrated in [Figure 1.4](#).

Target DNA is captured at the recognition layer, and the resulting hybridization signal is transduced into a usable electronic signal for display and analysis. In the case of electronic and electrochemical biosensors, signal transduction is greatly simplified because the incoming signal is already electronic in origin.

### DNA Hybridization Sensors

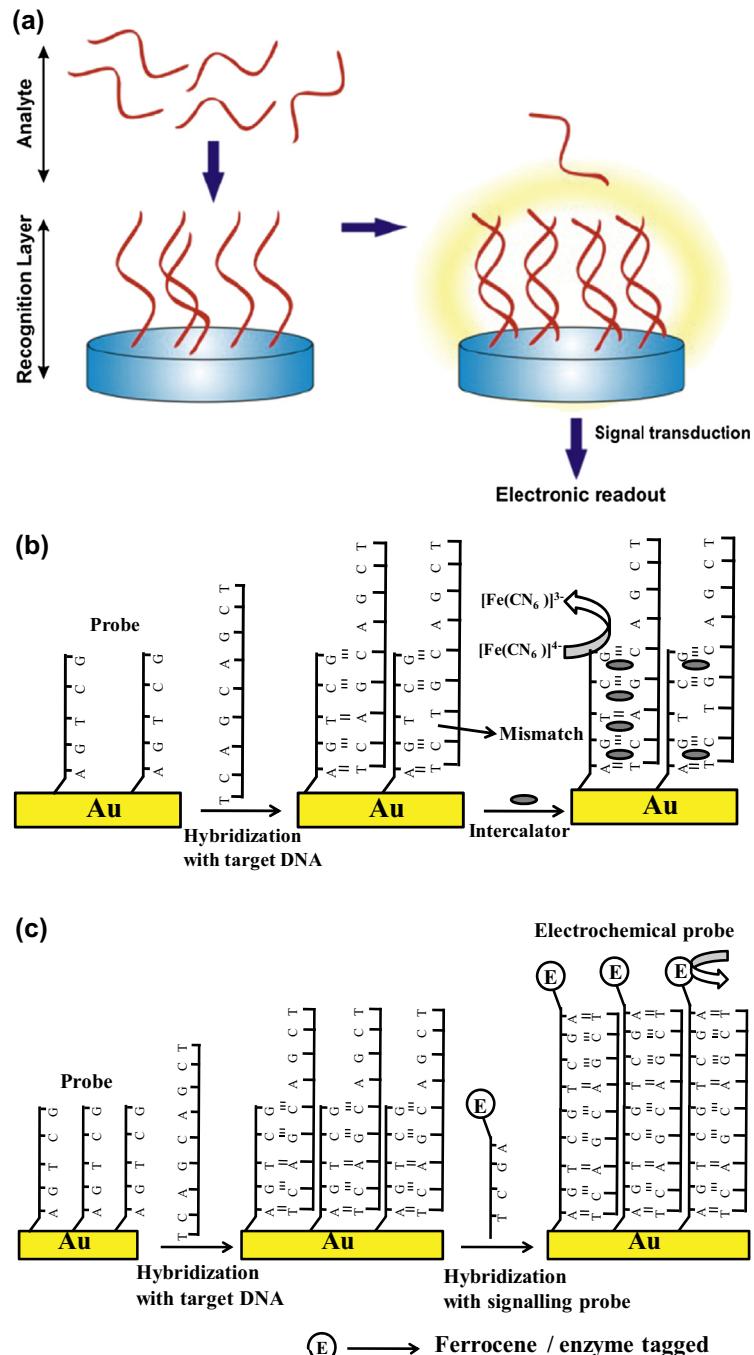
- Diagnostic test for mutations
  - Monitoring gene expression (sequence)
  - Screening for targets known to play a role in disease
  - Assessment of medical treatment
  - Environmental investigations
  - Biological warfare agent detection
- DNA hybridization can be used to identify specific DNA molecules.
1. Hybridization: the process of base-pairing between complementary ssDNA or RNA from two different sources.
  2. Probe: a labeled, defined sequence used to search mixtures of nucleic acids for molecules containing a complementary sequence. The general structure of DNA and DNA hybridization is shown in [Figure 1.5](#).

### Electrochemical DNA Biosensor

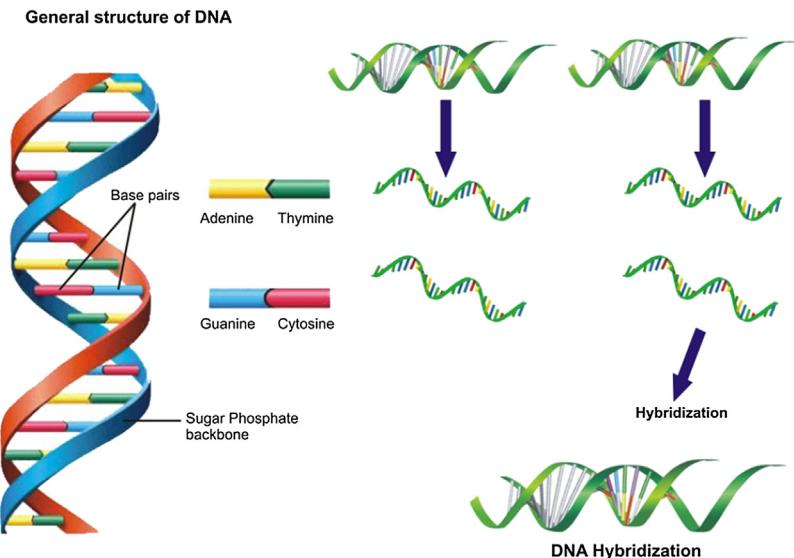
Electrochemical biosensors are based on electrochemical transduction to detect the hybridization event. The immobilization of DNA onto the electrodes plays an important role in the fabrication of DNA electrochemical biosensors.

Steps involved in electrochemical DNA hybridization biosensors include:

- Formation of the DNA recognition layer
- Actual hybridization event
- Transformation of the hybridization event into an electrical signal



**Figure 1.4** (a) General DNA biosensor design (b) Electrochemical detection of DNA hybridization based on intercalative redox active probe (c) Electrochemical detection using redox active probe.



**Figure 1.5** General structure of DNA and DNA hybridization.

There are basically two different pathways for electrochemical detection of DNA hybridization:

1. Label-free: A decrease/increase in the oxidation/reduction peak current of electroactive DNA bases such as guanine or adenine is monitored.
2. Labeled: A decrease/increase in the oxidation/reduction peak current of the electrochemical label, which selectively binds with dsDNA/ssDNA, is monitored.

### 1. Label-free electrochemical detection of DNA hybridization

This pathway is based on intrinsic DNA signals. Guanine and adenine are the most electroactive bases of DNA because they can easily be adsorbed and oxidized on carbon electrodes. ([Kerman et al., 2004](#)). Guanine and adenine oxidation signals on carbon electrodes can be observed at around 1.0 and 1.3 V in 0.50 M acetate buffer solution (pH 4.80), respectively, as reported by [Jelen et al. \(1997\)](#). Monitoring the changes in these signals upon duplex formation enabled the detection of hybridization. The electrochemical signals obtained from free adenine and guanine bases decreased on binding to their complementary thymine and cytosine bases after hybridization.

### 2. Label-based electrochemical detection of DNA hybridization

#### a. Intercalative redox active probe

The electrochemical detection of DNA hybridization based on a redox-active label is illustrated in [Figure 1.4\(b\)](#). Basically, the hybrid modified electrode is immersed in a solution that contains a redox-active and DNA-binding molecule.

After a period of time for the interaction between the DNA and the molecule, an electrochemical technique is applied to the electrode to measure the surface species. If the redox-active molecule is an intercalator such as daunomycin, it would be inserted between the double helix structure of the dsDNA with the help of its planar aromatic ring, and an enhancement in the redox signal would be observed. On the contrary, if the molecule had an affinity toward ssDNA, such as methylene blue, then a high signal would be observed from the probe-modified electrode. These changes in the peak potential current of the labels for the probe and hybrid molecules provide the basis for detection of the label-based hybridization. If the base pair stack is intact, current can flow. Thus, this method was used to sense changes and perturbations in DNA, viz., damage, mistakes, mismatches, and even protein binding. Several metal complexes such as cobalt phenanthroline, cobalt bipyridine, and ruthenium bipyridine, anticancer agents such as echinomycin and epirubicin, and organic dyes such as methylene blue were used as labels for the detection of hybridization.

A DNA biosensor for the detection of hepatitis B virus (HBV) was developed by covalently immobilizing ss HBV DNA fragments to a gold (Au) electrode surface via a carboxylate ester to link the 3-hydroxy end of the DNA to the carboxyl of the thioglycolic acid monolayer. The surface hybridization of the immobilized HBV probe with its target DNA fragment was detected by using the electrochemical signal of osmium bipyridine. The formation of the hybrid on the Au electrode resulted in a substantial increase in the peak current of osmium bipyridine in comparison with those obtained at a bare or probe modified electrode.

**b.** Redox-active probe as label

This method consists of three critical components (capture probe, target, and signaling probe) as illustrated in [Figure 1.4\(c\)](#). The signaling probe, tagged with a ferrocene/enzyme/metal nanoparticle, serves to label the target upon hybridization. Electrons flow to the electrode surface only when the target is present and are specifically hybridized to both the signaling and capture probes ([Yu et al., 2001](#)). This DNA chip assay is called eSensor™ and was developed by Motorola Life Sciences Inc. This assay was successfully used to detect 86% of the HPV types contained in clinical samples ([Vernon et al., 2003](#)).

#### **1.3.1.4 Aptasensors**

Biosensors that employ aptamers as a recognition element are called aptasensors. Aptasensors will be more stable and well adapted to the conditions of real samples because of the specific properties of aptamers. Aptamers are single-stranded RNA or DNA molecules that bind to their target molecules with high specificity and affinity. Aptamers have been developed for different applications. Their use as biological recognition elements

in biosensors promises progress for fast and easy detection of proteins. Aptamers can rival antibodies in a number of applications. Aptamers are very small in size (30–100 nucleotides) in comparison to other biorecognition molecules like antibodies or enzymes. This allows efficient immobilization of aptamers at high density. Therefore, production, miniaturization, integration, and automation of biosensors can be accomplished more easily with aptamers than with antibodies. Once selected, aptamers can be synthesized with high reproducibility and purity. Aptamers can be classified as:

- DNA or RNA aptamers
- Peptide aptamers

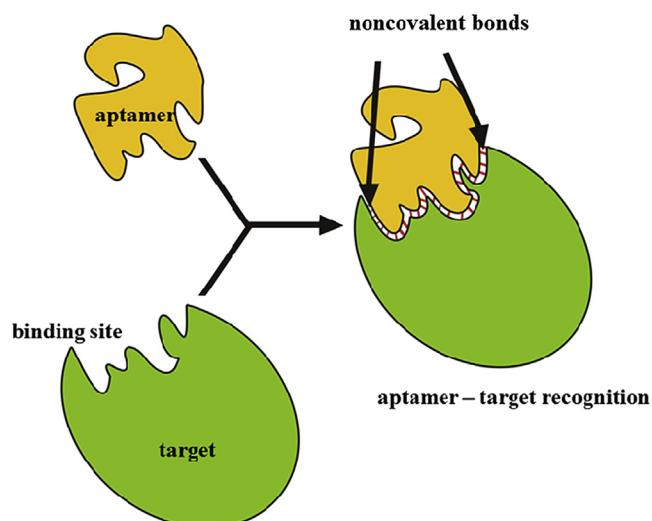
DNA aptamers are highly chemically stable enabling reusability of the biosensors. In contrast, RNA aptamers are susceptible to degradation by the endogenous ribonucleases typically found in cell lysates and serum. Therefore, biosensors using RNA aptamers as biorecognition elements can be used only for single-shot measurements in biological surroundings. DNA and RNA aptamers can be modified chemically to undergo analyte-dependent conformational changes.

- Development of aptasensors has been carried out with various detection methods:
- Label-free methods such as surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) measurements
  - Labeled methods such as electrochemistry, fluorescence, chemiluminescence, field effect transistors

The aptamer–target interaction is illustrated in [Figure 1.6](#).

Aptamers are typically isolated from combinatorial libraries by a process of in vitro evolution called systematic evolution of ligands by exponential enrichment (SELEX).

**Figure 1.6** Simplified schematic diagram illustrating aptamer–target interaction.



## The SELEX process

Aptamers are high-affinity ligands selected from DNA or RNA libraries via SELEX process. SELEX is a process used for in vitro selection of aptamers, which are highly specific in binding as well as function due to the nucleotide sequence and shape.

Basic steps involved in SELEX:

1. Library generation
2. Binding and separation
3. Amplification

### 1. Library generation

A library is created, containing around  $1 \times 10$  oligonucleotides. These are single-strand nucleic acids consisting of a random sequence region flanked by a binding site.

### 2. Binding and separation

The library is incubated with the immobilized target molecule. A few nucleic acids will bind to this target and then be considered aptamers. Unbound nucleic acids are filtered out of the solution and the bound nucleic acids are separated from the target—this is called elution.

### 3. Amplification

The bound nucleic acids are then copied using polymerase chain reaction (PCR) to create a new library. This new library will be used in a new round of SELEX to further optimize the quality of aptamers. Repeated selection and amplification steps allow identification of the highest binding species, through competitive binding.

The SELEX process is represented in [Figure 1.7](#).

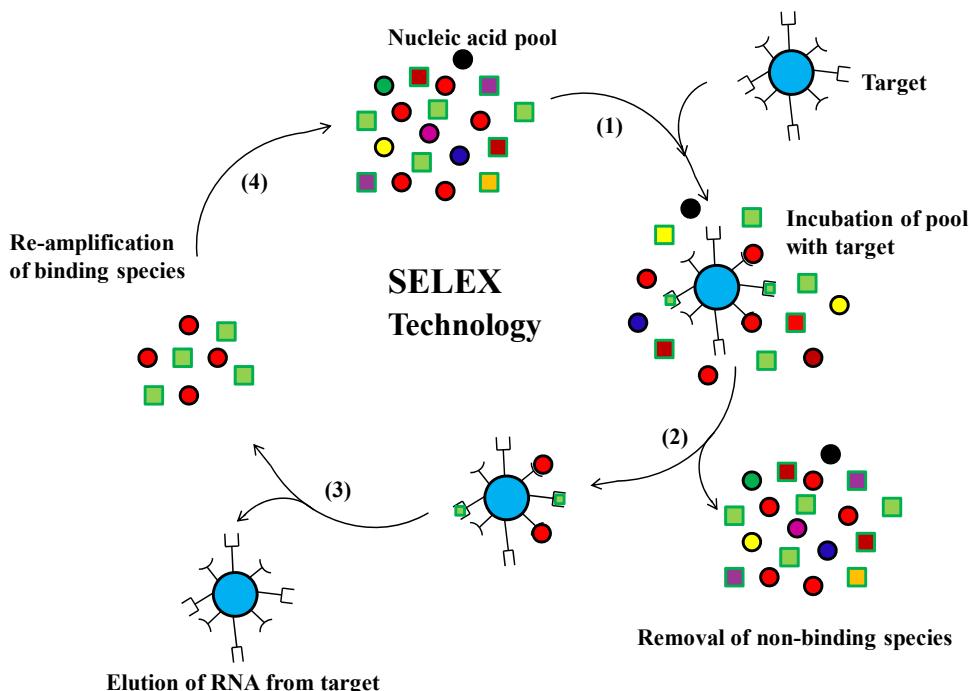
## Classification of aptasensors based on transduction

### 1. Electrochemical aptasensors

Electrochemical aptasensors facilitate simple, effective, and rapid detection of biomolecules, which are important in medicine, environment, and food applications. Electrochemical aptasensor makes use of an electrode surface as the platform to immobilize biological sensing aptamer, for which the analyte-binding event is monitored based on electrochemical current variations. Electrochemical transduction of biosensors using aptamers as bioreceptors includes methods like Faradaic impedance spectroscopy (FIS), differential pulse voltammetry, alternating current voltammetry, square wave voltammetry, potentiometry, or amperometry. In principle, it can be differentiated between either a positive or negative readout signal, i.e., an increase or a decrease of response following upon receptor–target interaction.

### 2. Optical aptasensors

Optical aptasensors include label-based aptamers (using fluorophore, luminophore, enzyme, nanoparticles) or label-free detection systems (e.g. surface plasmon resonance). Aptamers have also been widely used as biorecognition elements in



**Figure 1.7** The SELEX process.

optical bioassays. Of these assay formats, fluorescence and colorimetry are the two most important techniques. Fluorescent detection is widely used due to the ease of labeling aptamers with fluorescent dyes and the inherent capability for real-time detection. Surface plasmon resonance-based biosensors rely on the change of optical parameters upon changes in the layer closest to the sensitive surface.

### 3. Mass sensitive aptasensors

A mass sensitive biosensor is defined as any device that measures the property that scales proportionally to mass associated with its sensitive surface assembled with capture probes. Mass sensitive aptasensors are a class of label-free bioassays, including surface plasmon resonance (SPR), quartz crystal microbalance (QCM), and surface acoustic wave (SAW) devices. SPR can determine the binding constants of aptamers and their targets; this technology is often used in SELEX process and performed robustly, precisely, and rapidly. The SPR method is also applied for aptamers-based sensors. In this sensing format, a selective surface is formed by immobilizing the aptamer on the surface. The target is then injected at a constant flow rate while the instrument measures changes in the resonance angle that occur at the surface.

### Applications of aptasensors

Aptamers have been developed for different applications. Their use as new biological recognition elements in biosensors promises progress for fast and easy detection of proteins. Proteins are detected mostly by antibodies in analytical formats like ELISA, Western blotting, microarrays, and also biosensors. Aptamers are equal to monoclonal antibodies concerning their binding affinities. Aptamers can distinguish between chiral molecules and are able to recognize a distinct epitope of a target molecule. They have been widely used for detection of protein biomarkers such as thrombin, immunoglobulin E, and C-reactive protein.

For example, protein kinase C isozymes were detected by using radiolabeled aptamers. Similarly, fluorescently labeled aptamers were shown to mimic the abilities of antibodies in ELISA-like applications and in cell sorting. [Potyrailo et al. \(1998\)](#) described an aptamer-based biosensor that can be used to detect the free and nonlabeled nonnucleic acid targets. An anti-thrombin aptamer (15-mer 5'- GGTTGGTGTG GTTGG) that binds selectively to the blood clotting factor thrombin was chosen as an example of the use of an aptamer for biosensing. It was synthesized using a DNA synthesizer. It was further labeled with fluorescein isothiocyanate (FITC) and covalently immobilized on a microscope cover slip. The protein binding was detected by monitoring the evanescent-wave induced fluorescence anisotropy of the immobilized aptamer. Thrombin can be stripped by rinsing the aptamer-coated glass slide with a phosphate buffer solution (PBS) followed by guanidinium hydrochloride. The sensor was thus regenerated by equilibration with PBS.

Single-walled carbon nanotube field effect transistor (SWCNT-FET) biosensor for thrombin using its aptamer as biorecognition element has been developed ([So et al., 2005](#)). The biggest merit of using DNA (RNA) aptamers in FET-type sensors lies in their small size. In the case of immunological field effect transistors (ImmunoFETs), which use an antibody–antigen binding recognition step, there is a high possibility that the recognition binding occurs outside the electrical double layer in physiological salt concentrations. In this respect, the antibody (10 nm) is much larger than the electrical double layer, such that most of the protein charges will be at a distance greater than the Debye length (3 nm in 10 mM ionic concentrations), making them impossible to detect. Since aptamers (1–2 nm) are much smaller than protein antibodies, it is possible that the aptamer–protein binding event can occur inside the electrical double layer in millimolar salt concentrations.

For the experiment, the SWNT-FETs were prepared using standard chemical vapor deposition technique. Aptamer immobilization was performed by first modifying the sidewall of the carbon nanotube with CDI-Tween. While the Tween component was bound to the carbon nanotube sidewall through hydrophobic interactions, the carbodiiimidazole (CDI) moiety was used to covalently attach the 3'-amine group of the thrombin aptamer. Then, the devices were allowed to react with a (100 pM) thrombin

aptamer solution overnight. The electrical transfer characteristics of the SWNT-FET were measured at each process stage. The immobilization of the thrombin aptamer caused a rightward shift in the gate threshold voltage, presumably due to the negatively charged DNA backbone. This shift, together with a small concomitant decrease in the conductance, was observed in all of the devices functionalized with DNA aptamers. The lowest detection limit of the sensor used in this work was around 10 nM.

The fast response, high sensitivity, and relatively simple fabrication of these SWNT-FET sensors, combined with the small size, economy, stability, and high selectivity of aptamers, could provide a cost-effective point-of-care testing tool and a new method for high-throughput screening.

#### **1.3.1.5 Microbial biosensors**

A microbial biosensor is an analytical device that immobilizes microorganisms onto a transducer for the detection of target analytes. Microorganisms such as bacteria and fungi can be used as biosensors to detect specific molecules or the overall “state” of the surrounding environment. Compared to enzyme-based biosensors, microbial biosensors require no purification, which is time consuming and expensive. Microorganisms consist of numerous enzymes as the bioelements. The enzymes in the living cells can produce a response to the analytes specifically and selectively. Furthermore, proteins that are present in cells can also be used as bioreceptors for the detection of specific analyte. Microorganisms have been integrated with a variety of transducers such as amperometric, potentiometric, calorimetric, conductometric, colorimetric, luminometric and fluorimetric, to construct biosensor devices.

#### **Principle of microbial biosensors**

The use of microorganisms as biological elements in biosensors is based on the measurement of their metabolism, in many cases accompanied by the consumption of oxygen or carbon dioxide, and is also measured electrochemically. Integrating the microorganisms onto the transducer is the basic requirement of achieving a reliable microbial biosensor. Immobilization determines not only the quality of the signal transferred from microorganisms to the transducer but also the reusability of the microbial biosensor.

The appearance of the microbial sensors was the logical extension of the enzyme electrodes development. The signal generation mechanism is analogous in general terms for both of the microbial and enzyme biosensors. The microbial cell is treated as the “bag of enzymes.” In microbial biosensors, the analyte enters the cell and is converted using the intracellular enzymes. As a result, the cosubstrates are consumed and the reaction products that could also be electrochemically active are generated. The registration of the oxygen level, medium ionic composition, and other parameters in the immobilized cells layer can be used as the indicators of the cells’ metabolic state and the background for the electrochemical determination of biologically active compounds.

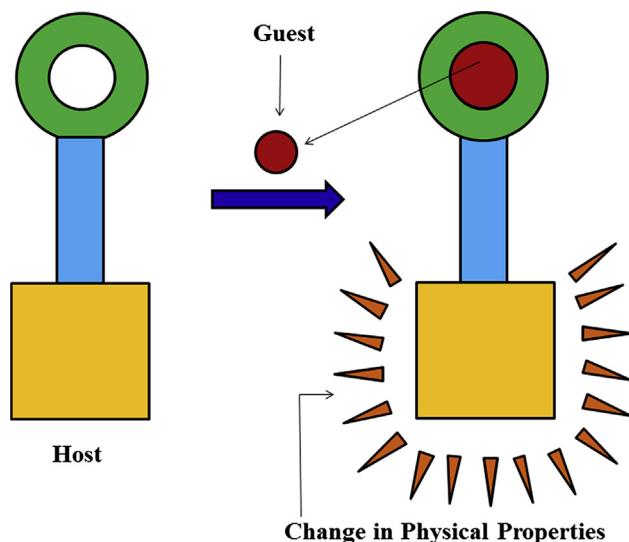
### Applications of microbial biosensors

Microbial biosensors have become one of the most useful means of monitoring environmental, food, and clinical samples. These biosensors provide a rapid, accurate, and inexpensive way for diagnosis of hormones, pathogens, and DNA, which are important parameters of a living individual. Akyilmaz et al. fabricated a novel microbial biosensor for the determination of epinephrine by immobilizing white rot fungi (*Phanerochaete chrysosporium* ME446) in gelatin using glutaraldehyde cross-linking agent on a Pt electrode, which achieved a linear range of 5–100 µM and a detection limit of 1.04 µM. In this biosensor, epinephrine was turned into epinephrine quinone through a redox activity catalyzed by lactate in the fungal cells, causing an increase in the current.

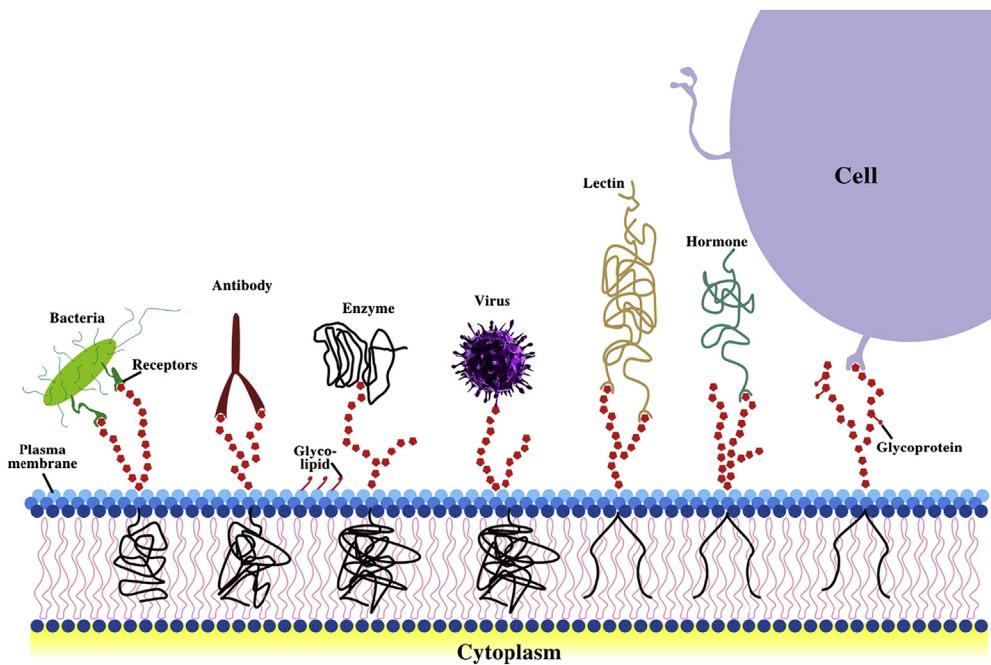
An amperometric biosensor based on *Candida tropicalis* cells immobilized in gelatin by using glutaraldehyde was developed for the determination of ethanol in the range from 0.5 to 7.5 mM.

### 1.4 MOLECULAR RECOGNITION

The success of a biosensor depends on molecular recognition. Molecular recognition is the ability of one molecule to “recognize” another through bonding interactions and molecular geometry. It refers to the specific interaction between two or more molecules through non-covalent binding such as hydrogen bonding, metal coordination, hydrophobic forces, van der Waals forces,  $\pi-\pi$  interactions, halogen bonding, and electrostatic and electromagnetic effects. The host and guest involved in molecular recognition exhibit molecular complementarities. This plays an important role in biological systems and is observed in between receptor–ligand, antigen–antibody, DNA–protein, sugar–lectin, RNA–ribosome, etc. The host–guest interaction is illustrated in Figure 1.8.



**Figure 1.8** The host–guest interaction.



**Figure 1.9** The molecular recognition involved in biological systems.

In the molecular recognition, a host interacts with a guest to produce a detectable change that can be read by an instrument. An important example of molecular recognition is the antibiotic vancomycin that selectively binds with the peptides with terminal D-alanyl-D-alanine in bacterial cells through five hydrogen bonds. The vancomycin is lethal to the bacteria since once it has bound to these particular peptides, they are unable to be used to construct the bacteria's cell wall. Molecular recognition forms the basis for many processes in biology:

- Receptor—substrate binding
- Enzyme catalysis
- Assembly of multiprotein complexes
- Active and passive ion transport across membranes via ion pumps, ionophores, and channels

The molecular recognition involved in biological systems is represented in [Figure 1.9](#).

### 1.4.1 Binding forces and interactions involved in molecular recognition

#### 1.4.1.1 Electrostatic and hydrophobic interaction

The electrostatic interaction between charged molecules and oppositely charged surfaces is an effective approach exploited in molecular recognition phenomenon. For example, cytochrome *c*, which carries eight positive charges, is attracted toward cytochrome *c*

oxidase in electron transport chain (ETC) in mitochondria. Similar to this, hydrophobic interaction is a good alternative for biorecognition of biomolecules with a lipophilic property. Lipophilic membrane-bound enzymes can be directly immobilized on hydrophobic surfaces for fabrication of biosensors.

#### **1.4.1.2 Covalent bonding**

Covalent bonds having high kinetic lability or reversibility have been regularly incorporated into host molecules. The essential point of dynamic covalent capture is that a molecular recognition event is followed by the formation of a reversible covalent bond between two molecules. Covalent bonds with the highest energy form the strongest chemical bonds contributing to protein structures. For example, covalent bonds between cysteine side chains, yielding disulfide bridges ( $-\text{CH}_2-\text{S}-\text{S}-\text{CH}_2-$ ) can be important determinants of protein structure and also in molecular recognition process.

#### **1.4.1.3 Hydrogen bonding**

Hydrogen bonding between a protein and its ligands such as protein, nucleic acid, substrate, and effector (or) inhibitor provides a directionality and specificity of interaction that is a fundamental aspect of molecular recognition. Although the H-bonding energy ( $\sim 1 \text{ kcal mol}^{-1}$ ) is 100 fold less than the covalent bond ( $\sim 100 \text{ kcal/mol}$ ), it is the multiplicity of the H-bond conferring rigidity to the protein/DNA structure, geometry and specificity to intermolecular interactions. Specificity in molecular recognition is driven by the interaction of complementary hydrogen bonding groups on interacting surfaces. Hydrogen bonding is a specific and very important type of intermolecular interaction.

#### **1.4.1.4 Specific interaction**

The specific interaction-mediated immobilization of biomolecules using antibody–protein A/G interaction or DNA hybridization can prevent deterioration in functionality due to the improved orientation and stability.

#### **1.4.1.5 Molecular recognition between biotin and avidin**

The specific interaction based on molecular recognition of avidin–biotin provides a facile approach for the immobilization of biomolecules on solid-state surface. The avidin (streptavidin)-based system is ideally suited for the well-controlled immobilization of biomolecules due to the specific and strong interaction between avidin and biotin. Since avidin has a nearly cubic shape with four biotin binding sites grouped in two pairs at opposite ends of the avidin molecule, it has been used for anchoring biotinylated biomolecules such as proteins and DNA thereby acting as a biocompatible linker between biotin and biotinylated biomolecules.

#### **1.4.1.6 Molecular recognition between protein A/G and antibody**

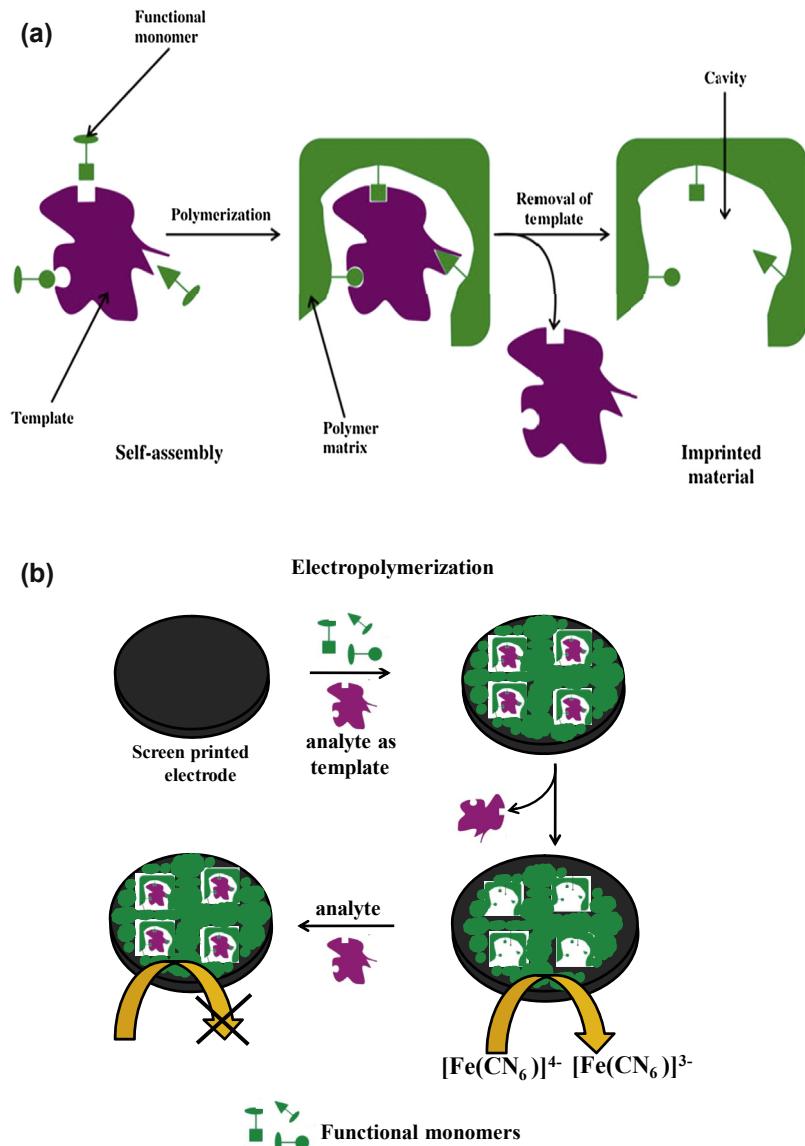
Protein A/G is a recombinant fusion protein that combines the immunoglobulin (IgG) binding domains of both protein A and protein G. Protein A/G thus has the additive properties of proteins A and G useful for biosensing applications. Pathogenic bacteria, notably *Streptococcus* and *Staphylococcus*, have proteins A and G on their surface that bind IgG (Kato et al., 1995). Therefore, proteins A and G could be used as immunological tools and are the most extensively studied of these antibody-binding proteins. Protein A contains five highly homologous Fc- binding domains, each of  $\sim 60$  amino acid residues, designated A–E. It binds to the Fc portion of IgG with an affinity that varies with the species and subclass of IgG. Protein G has a broader specificity than protein A for IgGs from different sources, and its IgG-binding domains are able to bind to both the Fab and the Fc portions of the antibody molecule, with relative affinities that are markedly species dependent. A detailed understanding of the binding mechanisms of these proteins is important, not only for providing us with the structural basis for their pathological and immunological functions but also as a contribution toward understanding the general rules of protein–protein interactions (Kato et al., 1995).

### **1.4.2 Molecular recognition based on geometry**

The active binding site of receptors has a unique geometric structure that is particularly suitable for a substrate. A substrate that has a complementary shape to the active site is recognized by selectively binding to the enzyme, while an incorrectly shaped molecule that does not fit the binding site is not recognized. This technique is based on the system used by enzymes for substrate recognition, which is called the “lock-and-key” model.

#### **1.4.2.1 Molecular recognition by imprinting**

Molecular imprinting is a technique to create template-shaped cavities in polymer matrices with memory of the template molecules to be used in molecular recognition. Through molecular imprinting, it is possible to develop tailor-made polymers that are selective for different compounds. This technique allows the formation of specific recognition and catalytic sites in macromolecules by the use of templates. In chemistry, molecularly imprinted materials are prepared using a template molecule and functional monomers that assemble around the template and then get cross-linked to each other. The functional monomers, which are self-assembled around the template molecule by interaction between functional groups on both the template and monomers, are polymerized to form an imprinted matrix. Then the template molecule is removed from the matrix under certain conditions, leaving behind a cavity complementary in size and shape to the template. The obtained cavity can work as a selective binding site for a specific template molecule. The molecular imprinting process is shown in Figure 1.10.



**Figure 1.10** (a) The molecular imprinting process (b) The MIP based sensor for analyte determination.

#### 1.4.2.2 Molecular imprinting process

Molecular imprinting is a technique to synthesize highly cross-linked polymers capable of selective molecular recognition. In a molecular imprinting process, one needs:

1. Template
2. Functional monomers
3. Cross-linkers

4. Initiator
5. Porogenic solvent
6. Extraction solvent

Molecular imprinting is a way of creating recognition sites in polymeric materials. The compound is used as a template. The process starts with dissolution of template, functional monomer, cross-linking agent, and initiator in a porogenic solvent. Functional monomers are chosen to interact with the template molecule since the formation of a stable template–monomer complex is fundamental for the success of molecular recognition. Monomers are positioned spatially around the template, and the position is fixed by copolymerization with cross-linking monomers. The polymer obtained is a microporous matrix possessing microcavities with a three-dimensional structure complementary to that of the template. Thus the removal of the template molecules from the polymer, by washing with solvent, leaves binding sites that are complementary in size and shape to the template. Consequently, the resultant polymer recognizes and selectively binds the template molecules. Molecules identical to the original template fit into the recognition sites and are bound strongly, while molecules that differ in structure are unable to bind.

The choice of monomer is very important in order to create highly specific cavities designed for the template molecule. Typical functional monomers are carboxylic acids (acrylic acid, methacrylic acid, and vinylbenzoic acid), sulphonic acids (2-acrylamido-2-methylpropane sulphonic acid), and heteroaromatic bases (vinylpyridine, vinylimidazole). The extensive use of methacrylic acid (MAA) is due to its capability to act both as hydrogen bond and proton donor and as hydrogen bond acceptor. The cross-linker is important in controlling the morphology of the polymer matrix, serves to stabilize the imprinted binding sites, and imparts mechanical stability to the polymer matrix in order to retain its molecular recognition capability. Different cross-linkers have been used. Ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TRIM) are the most commonly used cross-linkers. The nature and volume of the solvent play an important role in the molecular imprinting process. The most common solvents used in molecular imprinting are toluene, chloroform, dichloromethane, or acetonitrile. The solvent is used to bring all the components (monomer, template, initiator, and cross-linker) into one phase in the polymerization and is responsible for creating the pores in macroporous polymers. The solvent should produce large pores to assure good flow-through properties of the resultant molecularly imprinted polymer and increase the volume of the solvents, enlarging the pore volume of the polymer. Due to this reason, “porogenic” solvents are used in molecular imprinting.

Recently, a novel, highly sensitive, and selective electrochemical antibody-free cortisol sensor have been developed by using molecularly imprinted polymer (MIP) ([Manickam et al., 2015](#)). The general scheme for determination of analytes using MIP

is shown in [Figure 1.10\(b\)](#). Cortisol, an important steroid hormone in the body, plays a key role in several stress-related diseases including post-traumatic stress disorder. MIPs are synthetic polymers having highly specific recognition sites selective toward the target analyte and considered to be the versatile, stable, and cost-effective alternatives for natural antibodies. The cortisol-specific MIP film was prepared by *in situ* electropolymerization of pyrrole monomer onto the electrode surface in the presence of cortisol as a template. After removing the cortisol by electrochemical overoxidation, the specific imprinted sites created on the polymer matrix were used to detect cortisol using  $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$  as a redox mediator. The MIP-based cortisol sensor exhibited a good detection limit of  $1 \text{ pM mL}^{-1}$  cortisol.

### 1.4.3 Types of molecular recognition

- Static molecular recognition
- Dynamic molecular recognition

#### 1.4.3.1 *Static molecular recognition*

Static molecular recognition is similar to the interaction between a lock and key. It is a 1:1 type complexation reaction between a host molecule and a guest molecule to form a host–guest complex. To achieve advanced static molecular recognition, it is necessary to make recognition sites that are specific for guest molecules.

#### 1.4.3.2 *Dynamic molecular recognition*

In dynamic molecular recognition the binding of the first guest to the first binding site of a host affects the association constant of a second guest with a second binding site. In the case of positive allosteric systems, the binding of the first guest increases the association constant of the second guest; while for negative allosteric systems the binding of the first guest decreases the association constant with the second guest. The dynamic nature of this type of molecular recognition is particularly important since it provides a mechanism to regulate binding in biological systems. Dynamic molecular recognition is also being studied for application in highly functional chemical sensors and molecular devices. Static and dynamic molecular recognition are represented in [Figure 1.11](#).

## 1.5 CLASSIFICATION OF BIOSENSORS BASED ON TRANSDUCERS

The transducer is a component of biosensors, which has an important role in the signal detection process. A transducer is the device that converts biorecognition signal events into detectable signals. The detectable signals can be electrochemical (potentiometry, conductometry, impedimetry, amperometry, voltammetry), optical (colorimetric, fluorescence, luminescence, interferometry), calorimetric (thermistor), mass change (piezoelectric/acoustic

**Figure 1.11** Types of molecular recognition. **Static:**



**Dynamic:**



wave), or magnetic in nature. Although there are new types of transducers constantly being developed for use in biosensors, electrochemical transducers are widely used in point-of-care devices since they are portable, simple, easy to use, and cost effective, and in most cases disposable. The electrochemical instruments used with the biosensors have been miniaturized to small pocket-sized devices, applicable for home use or clinical analysis. Indeed, electrochemical sensors are the smallest of all the sensors, including optical and piezoelectric, which provide the electrochemical biosensors with an advantage of portability and simple instrumentation. In addition, the sensitivity and response of the electrochemical sensors are higher than optical or piezoelectric sensors. Finally, electrochemical sensors are cost effective. All these factors give us immense motivation to explore the field of electrochemical biosensors.

Biosensors can be classified based on the transduction methods that they employ. Transduction can be accomplished through a large variety of methods. Most forms can be classified in one of three main classes:

1. Optical detection methods
2. Electrochemical detection methods
3. Mass-based detection methods

### 1.5.1 Optical detection methods

This method is based on optoelectronics, which is the study and application of electronic devices that interact with light and thus is usually considered a subfield of photonics. In this context, *light* often includes invisible forms of radiation such as gamma rays, X-rays, ultraviolet, and infrared. Optoelectronic devices are electrical-to-optical or optical-to-electrical transducers, or instruments that use such devices in their operation.

1. Systems involved in optoelectronics
  - a. Optoelectronic components (devices)
  - b. Optical devices such as waveguides and optical fibers
  - c. Light-emitting devices like LEDs and laser diodes
  - d. Light-receiving devices like photodetectors and solar cells
  - e. Display instruments such as LCDs and LED display

### 1.5.2 Optical biosensors

Optical biosensors are a powerful detection and analysis tool that has vast applications in biomedical research, health care, pharmaceuticals, environmental monitoring, homeland security, and the battlefield. In the most commonly used form of an optical biosensor, the transduction process induces a change in the phase, amplitude, polarization, or frequency of the input light in response to the physical or chemical change produced by the bio-recognition process. Some of the advantages offered by an optical biosensor are selectivity, specificity, remote sensing, isolation from electromagnetic interference, fast, real-time measurements, multiple channels/multi parameters detection, compact design, minimally invasive for in vivo measurements, choice of optical components for biocompatibility, and detailed chemical information on analytes. The main components of an optical biosensor are light source, optical transmission medium (fiber, waveguide, etc.), immobilized biological recognition element (enzymes, antibodies, or microbes), and optical detection system. Optical biosensors can be broadly classified based on the different parameters. Generally, there are two detection protocols that can be implemented in optical biosensing:

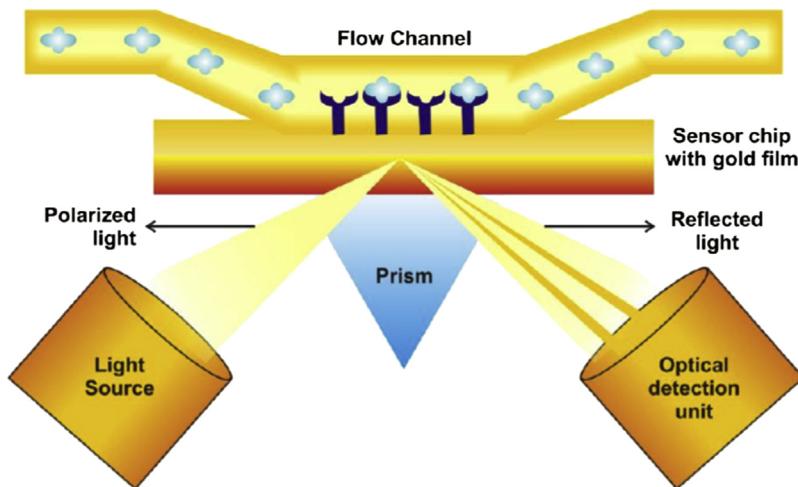
1. Fluorescence-based detection
2. Label-free detection

In fluorescence-based detection, either the target molecules or biorecognition molecules are labeled with fluorescent tags, such as dyes; the intensity of the fluorescence indicates the presence of the target molecules and the interaction strength between target and biorecognition molecules. Fluorescence-based detection is extremely sensitive, with the detection limit down to a single molecule. In label-free detection, target molecules are not labeled or altered and are detected in their natural forms. This type of detection is relatively easy and cheap to perform and allows for quantitative and kinetic measurement of molecular interaction.

### 1.5.3 Optical label-free biosensor

#### 1.5.3.1 *Surface plasmon resonance-based biosensors*

Surface plasmon resonance (SPR) is an optical phenomenon that provides a noninvasive, label-free means of observing binding interactions between an injected analyte and an immobilized biomolecule in real time. In the conventional SPR biosensor configuration, a thin metallic film is coated on one side of the prism, separating the sensing medium and the prism. The SPR effect is sensitive to binding of analyte because the associated increase in mass causes a proportional increase in refractive index, which is observed as a shift in the resonance angle. Optical sensors based on excitation of surface plasmons, commonly referred to as SPR sensors. SPR biosensors use surface plasmon waves (electromagnetic wave) to detect changes when the target analyte interacts with the biorecognition element on the sensor. SPR biosensor was first demonstrated for biosensing in 1983

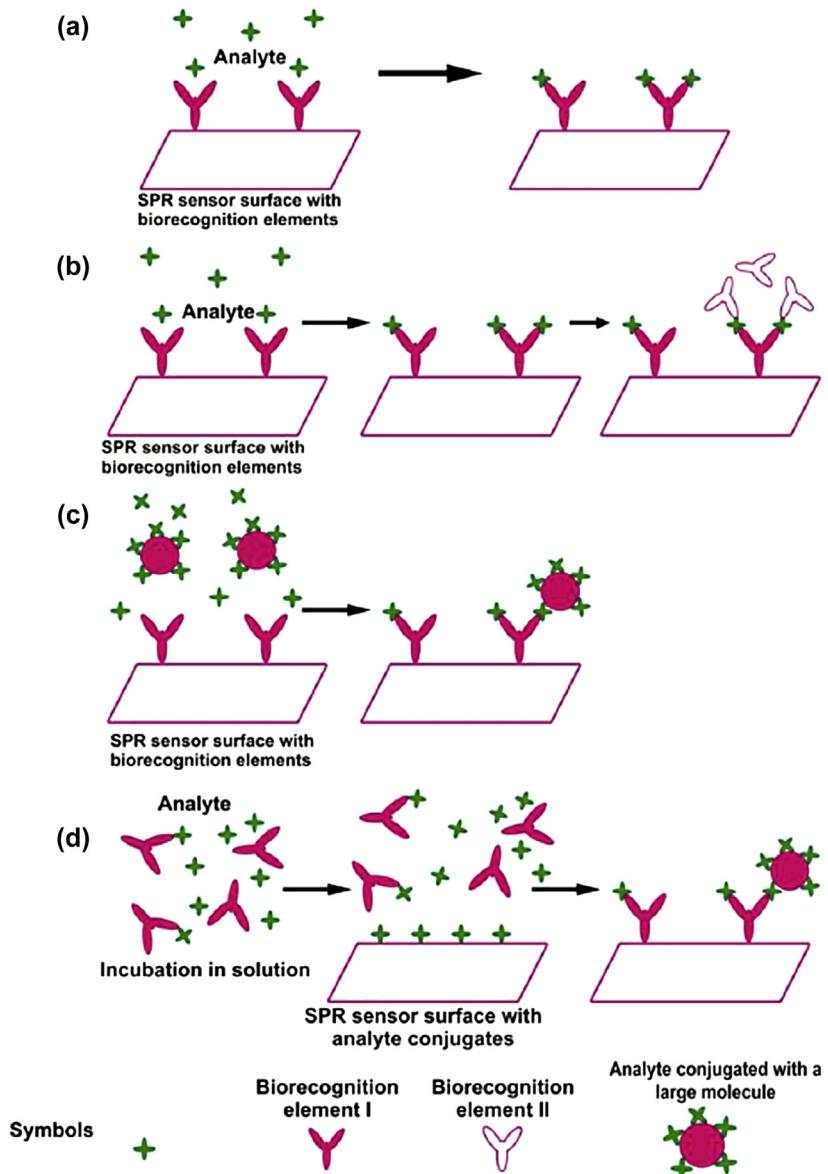


**Figure 1.12** Surface plasmon resonance principle.

by Liedberg et al. (1983). It becomes a very powerful label-free tool to study the interactions between the target and biorecognition molecules. The SPR transducer incorporates a thin metal film that supports a special mode of electromagnetic field—a surface plasmon polariton (SPP)—sometimes referred to as a surface plasma wave. The most commonly used metal is gold due to its chemical stability. In principle, when the SPR biosensor is exposed to any changes, it will induce changes in the refractive index, which is used to measure or observe the reaction. The SPR transducer is incorporated with the biomolecule/biorecognition element that recognizes and is able to interact with a specific analyte (Mol and Fischer, 2010). Hence, when a target analyte interacts with the immobilized biomolecule on the sensor surface, it produces change in the refractive index at the sensor surface. This change produces a variation in the propagation constant of the surface plasmon wave, and this variation is measured to produce a reading. A spectrophotometer is used to measure the absorption spectrum of the sample. Various biorecognition elements have been incorporated with SPR biosensors such as proteins, antibodies—antigens, nucleic acids, and enzymes. An important feature of SPR biosensors is that they are able to provide label-free sensing without radioactivity and fluorescence, which makes it highly attractive for real-time monitoring. SPR biosensors have become a central tool for characterizing and quantifying biomolecular interactions. The principle of surface plasmon resonance is represented in Figure 1.12. The main detection formats used in SPR biosensors are shown in Figure 1.13.

### Advantages of SPR biosensors

- High detection sensitivity
- Real-time detection
- Anti-interference capability



**Figure 1.13** Main detection formats used in SPR biosensors. (a) direct detection (b) sandwich detection (c) competitive detection format (d) inhibition detection format.

- Samples without pretreatment
- Rapid
- High throughput analysis
- Less reagents and samples

## Applications of SPR

### 1. Physical applications

SPR is used to measure dielectric properties, adsorption processes, surface degradation of thin organic monolayers (or) bilayers, and polymer films.

### 2. Biological applications

SPR sensors are used as biosensors for specific biological interactions including adsorption and desorption kinetics, antigen–antibody binding, and epitope mapping for determination of biomolecular structure and interactions of proteins, DNA, and viruses.

### 1.5.4 Fluorescence-based biosensors

Fluorescence is one of the most sensitive spectroscopic techniques, and its sensitivity makes it uniquely suited for the detection of very low concentrations of bioanalytes. Fluorescence is commonly used in signal transduction, especially when using enzymes and antibodies. Fluorescence requires an external light source (short-wavelength light) to initiate electronic transitions in an atom or molecule, which then produces luminescence (longer wavelength light). Fluorescence-based biosensors incorporated with fluorochrome molecules are used to produce light during the biorecognition event. Since most of the biological-sensing elements and most analytes do not possess intrinsic spectral properties, the biorecognition event is transduced to optical signal by coupling fluorescence optically responsive reagents to the sensing elements. For example, the nucleic acid or antibodies is used to tag with fluorochrome and convert the hybridization interaction between two complementary DNA strands into an optical signal. The major drawbacks of fluorescence technology are additional complexity of time-resolved instrumentation, in either the time or frequency domains or both, and not suitable for real-time monitoring.

Optical biosensors based on fluorescence detection often use the combination of a fluorescent bioreceptor associated with an optical transducer. Fluorescent biosensors may also be obtained by immobilizing whole cells on the surface of a sensor layer. This bioactive layer is usually placed in front of the tip of an optical fibers bundle to generate a fluorescent signal. The optical fibers are required to send the excitation radiation to the fluorescent bioelement and convey the fluorescence radiation up to a fluorimeter. In order to improve the simplicity and reliability of fluorescence-based biosensors, optically translucent supports are used because their optical properties enable detection of fluorescence emitted by the algal cells.

A common fluorescence technique used for biosensing is the sandwich assay. In this experiment, the analyte is selectively bound to a surface by a targeting molecule (like an antibody), which has been immobilized covalently on the surface of a well or other cell. By labeling the analyte molecule with a fluorescent tag, its surface concentration may be measured via highly sensitive fluorescence spectroscopy.

Fluorescence spectroscopy has been widely applied in analytical chemistry. It is a sensitive technique that can detect very low concentrations of analyte because of the instrumental principles involved. At low analyte concentrations, fluorescence emission intensity is directly proportional to the concentration. Fluorescent materials and green fluorescent protein have been extensively used in the construction of the fluorescent biosensor.

### **Fluorescent protein-based biosensors**

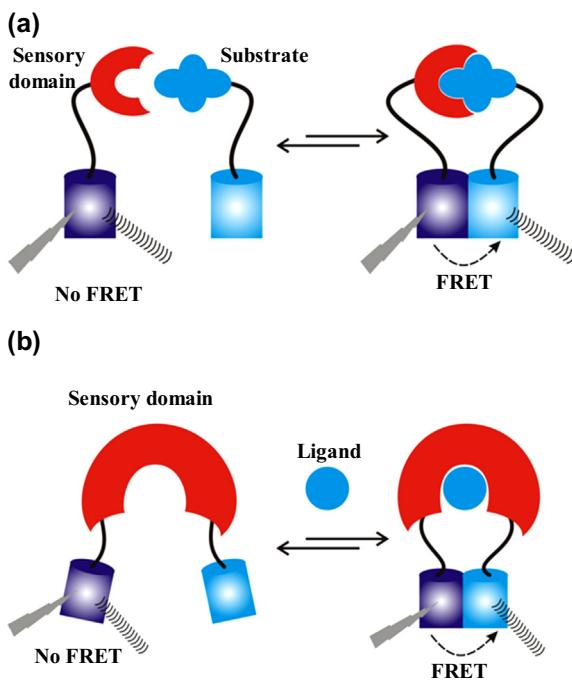
Fluorescent protein (FP)-based biosensors are relatively easy to construct using standard molecular biology techniques. In fluorescent protein-based biosensors, the sensing element consists of one or more polypeptide chains. The polypeptide chain acts as the molecular recognitions element (MRE) that undergoes conformational changes upon binding with the analyte, producing a change in fluorescence properties. Generally, FP-based biosensors can be classified into three types based on their structure:

- Type 1: Forster (or fluorescent) resonance energy transfer (FRET)-based biosensors
- Type 2: Bimolecular fluorescence complementation (BiFC)-based biosensors
- Type 3: Single FP-based biosensors

### **FRET-based biosensors**

FRET is the phenomenon of nonradiative energy transfer observed between an excited blue-shifted fluorescent chromophore (donor) and a chromophore with a red-shifted absorption spectrum (acceptor) through dipole–dipole coupling. FRET has proven to be extremely useful in the design of genetically encoded biosensors. FRET describes the energy transfer between two chromophores. A donor chromophore, in a higher energy state, may transfer energy to an acceptor chromophore through nonradiative dipole–dipole coupling. The efficiency of the energy transfer is determined by the distance and distance and orientation between the donor and acceptor proteins. Generally, FRET efficiency measured by a fluorescence emission spectrum is used to determine the proximity of the two chromophores. In FRET-based biosensors, two fluorescent proteins are genetically linked either to each end of a polypeptide chain i.e. molecular recognition element (MRE) or two separate polypeptides, the MRE and the analyte protein. The molecular recognition element is sensitive to the analyte. Upon interaction with the analyte, conformation of the sensor protein changes, thus altering the distance between two chromophores. This causes a change in the fluorescence intensities of the donor and acceptor FPs, which is measured in terms of FRET efficiency. Increased FRET efficiency indicates that the two FPs are aligned together while decreased FRET efficiency indicates that the donor and acceptor FPs are separated. FRET-based biosensors are widely used to detect a range of molecular events such as protein binding interactions, protein conformational changes, enzyme activities (such as proteolysis, phosphorylation, dephosphorylation, and GTPase activities), and concentration of biomolecules. The common examples of FRET-based biosensors are represented in [Figure 1.14](#).

**Figure 1.14** Two common examples of FRET-based biosensors.



In this FRET-based biosensor (a): one of the FPs is linked to the MRE and the other is linked to the analyte protein. When the sensory protein domain binds with the substrate, the donor and acceptor FPs are brought together, thus increasing the acceptor fluorescence intensity while reducing the donor fluorescence intensity. This strategy is commonly used to tag protein–protein interactions in live cells.

In this FRET-based biosensor (b): The donor FP and the acceptor FP are fixed to the opposite ends of the MRE. When the analyte binds to the MRE, the conformation of the sensor protein changes thus placing the donor and acceptor FPs side by side. This increases the FRET efficiency. This is usually used for the detection of glucose, maltose, glutamate, and cyclic nucleotides.

### Bimolecular Fluorescence Complementation (BiFC)-based biosensors

Bimolecular fluorescence complementation (BiFC)-based biosensors have been used to visualize a variety of protein–protein interactions in live cells. In this type of biosensor, the FP that is split up and MRE are linked to one portion and the analyte protein is linked to the other portion. When the two proteins interact, the two fragments fuse together, refolding properly into its three-dimensional structure and producing a fluorescence signal.

### Single FP-based biosensors

A single fluorescent protein coupled with an MRE makes up single FP-based biosensors. The MRE can be either exogenous or endogenous. Analyte binding to the MRE causes conformational changes of the fluorescent protein consequently altering its fluorescent properties.

### Advantages of molecular fluorescence for biosensing

- The technique is extremely sensitive
- Fluorescence measurements cause little (or) no damage to the host system

### 1.5.5 Chemiluminescence-based biosensors

Luminescence is the emission of light from an electronically excited compound returning to the ground state. The source of excitation energy serves as a basis for a classification of the various types of luminescence. Chemiluminescence occurs in the course of some chemical reactions when an electronically excited state is generated. Bioluminescence is a special case of chemiluminescence occurring in some living organisms and involves a protein, generally an enzyme. Chemiluminescence measurements consist of monitoring the rate of production of photons, and thus the light intensity depends on the rate of the luminescent reaction. Consequently, light intensity is directly proportional to the concentration of a limiting reactant involved in a luminescence reaction. With modern instrumentation, light can be measured at a very low level, and this allows the development of very sensitive analytical methods based on these light-emitting reactions. Chemiluminescence-based sensors have been developed with the aim of combining the sensitivity of light-emitting reactions with the convenience of sensors.

Optical fibers associated with a sensitive light detector appeared to be convenient transducers for designing biosensors involving these kinds of luminescent reactions. Recently, chemiluminescence and electrochemiluminescence detections have been used instead of fluorescence for the development of biochips and microarrays.

Chemiluminescence can be used to detect specific biochemical reactions that occur, and this property has contributed for chemiluminescence-based biosensor development. In the chemiluminescence biosensor, the reaction between analyte and the immobilized biomolecule that has been marked with chemiluminescence species will end in generating light as result of biochemical reaction. This emitted light can be detected using a photo multiplier tube (PMT). Chemiluminescence is an emerging tool for diagnostics with extremely high sensitivity along with the sample instrumentation, fast dynamic response properties, and wide calibration range. Chemiluminescence-based transduction has been widely applied for immunosensing and nucleic acid hybridization. This type of transduction has a detection limit of  $5.5 \times 10^{-13}$  M. However, chemiluminescence

transduction has some drawbacks such as less quantitative accuracy due to short lifetime, not suitable for real-time monitoring, and it is an expensive method.

### **Chemiluminescence-based biosensor for metal ion detection**

Using chemiluminescence measurements, Zn(II), Be(II), and Bi(III) were detected in trace levels ([Kamtekar et al., 1995](#)). This technique forms the basis in the development of a metal ion-based fiber-optic sensor. Inhibition of the native metalloenzyme, alkaline phosphatase, in the presence of some metal ions, and the reactivation of its apoenzyme by Zn(II) ions is used to determine metal ion concentrations. Alkaline phosphatase—catalyzed hydrolysis of a chemiluminescent substrate, chloro 3-(4-methoxy spiro [1, 2-dioxetane-3-2-tricyclo-[3.3.1.1]-decan]-4-yl) phenyl phosphate, generates light. By measuring the chemiluminescence signal strength in the presence or absence of metal ions, this reaction can be used to detect and determine metal ion concentrations. The strength of the chemiluminescence signal is directly proportional to the enzyme activity at a given chemiluminescence substrate concentration. The metalloenzyme alkaline phosphatase was successfully immobilized by covalent cross-linking on a silane-treated glass surface. Three metal ion analytes—Zn(II), Be(II), Bi(III)—that inhibit the enzyme were quantified.

## **1.6 PIEZOELECTRIC BIOSENSORS**

Piezoelectric-based biosensors are based on piezoelectricity (piezo means to squeeze or press), which is defined as the potential difference created across certain materials due to an applied mechanical stress. Although the piezoelectric effect was discovered by Pierre Curie in 1880, it was only in the 1950s that the piezoelectric effect started to be used for industrial sensing applications. Since then, this measuring principle has been increasingly used and can be regarded as a mature technology with an outstanding inherent sensitivity. It has been successfully used in various applications, such as in medical, aerospace, nuclear instrumentation, and telecommunication.

### **Principle of piezoelectric-based biosensors**

The piezoelectric biosensor is a class of micro electromechanical systems (MEMS) based on the principle of measurement of changes in oscillating crystal resonance frequency due to bioreceptor and analyte interactions. In piezoelectric MEMS biosensors, the transducer is made of piezoelectric material (like quartz) and the biosensing material is then coated on the piezoelectric material, which vibrates at the natural frequency. Piezoelectric materials have no center of symmetry and produce an electric signal when stressed mechanically (i.e., by applying some pressure on them). A crystal oscillates at a certain

frequency, which can be modulated by its environment. When the crystal is coated with some biosensing material, the actual frequency depends on the mass of the crystal and coating. The resonant frequency can be measured with great accuracy, hence making it possible to calculate the mass of analyte adsorbed on to the crystal surface. Quartz is the most commonly used piezoelectric since it is cheap, can be processed to yield single crystals, and can withstand chemical, thermal, and mechanical stress. Lithium niobate and lithium tantalite can also be used as piezoelectric materials (Tichý et al., 2010). Piezoelectric transduction is suitable for portable, label-free detection, and real-time biosensing (Nicu et al., 2005). This method is highly sensitive, and therefore with these devices, detection limits are down to the picogram level. Antibodies, enzymes, and antigens have been used as biological elements in these devices. Such types of biosensors have been utilized for the measurement of ammonia, hydrogen, methane, carbon monoxide, nitrous oxide, and other organophosphorous compounds.

### **Types of piezoelectric sensors**

In the case of a piezoelectric crystal resonator, the traveling wave is either a bulk acoustic wave (BAW) propagating through the interior of the substrate or a surface acoustic wave (SAW) propagating on the surface of the substrate. There are two main types of piezoelectric sensors:

- Bulk acoustic wave piezoelectric sensors
- Surface acoustic wave piezoelectric sensors

### **Bulk acoustic wave piezoelectric sensors**

Generally, acoustic wave sensors utilize a mechanical or acoustic wave as the sensing mechanism. As the acoustic wave propagates through (or) on the surface of the material, any changes to the characteristics of the propagation path affect the velocity and amplitude of the wave. Changes in velocity can be monitored by measuring the frequency and then can be correlated to the corresponding physical quantity that is being measured. Virtually all acoustic wave devices and sensors use a piezoelectric material to generate the acoustic wave. Applying an appropriate electric field to a piezoelectric material creates a mechanical stress. Conversely, by applying an appropriate mechanical stress, an electric field will be created. Piezoelectric acoustic wave sensors apply an oscillating electric field to create a mechanical wave, which propagates through the substrate and is then converted back to an electric field for measurement.

If the wave propagates through the substrate, the wave is called a bulk wave. The most commonly used BAW devices are the thickness shear mode (TSM) resonator and the shear-horizontal acoustic plate mode (SH-APM) sensor. The TSM, also widely referred to as a quartz crystal microbalance (QCM), is the best-known and simplest

acoustic wave device. The SH-APM device combines the best properties of both the BAW and SAW devices. A BAW resonator is an electromechanical device in which a standing acoustic wave is generated by an electrical signal in the bulk of a piezoelectric material. In the simplest configuration, a device will consist of a piezoelectric material (typically quartz or ZnO) sandwiched between two metallic electrodes. The natural frequency of the material and the thickness are used as design parameters to obtain a desired operating frequency. Two types of BAWs can propagate. They are the longitudinal waves, also called compressional/extensional waves, and the transverse waves, also called shear waves, which, respectively, identify vibrations where particle motion is parallel and perpendicular to the direction of wave propagation. Longitudinal waves have higher velocity than shear waves.

### Surface acoustic wave piezoelectric sensors

If the wave propagates on the surface of the substrate, it is known as a surface acoustic wave. SAW sensors are made by a thick plate of piezoelectric material, typically quartz, lithium niobate, or lithium tantalate where predominantly Rayleigh waves propagate along the upper surface. The most commonly used surface wave devices are the SAW sensor and the shear-horizontal surface acoustic wave (SH-SAW) sensor, also known as the surface transverse wave (STW) sensor. SAW-based sensors are built on single crystal piezoelectric materials like quartz, lithium niobate, lithium tantalate, langasite, or zinc oxide. In the case of single crystals, differently cut angles produce largely different results. The design of the sensor needs to be adapted for each application by selecting the appropriate design alternative.

### Applications of piezoelectric biosensors

The piezoelectric transducer is suitable for DNA and protein detection with a detection limit of  $1 \text{ ng cm}^{-2}$  ([Nirschl et al., 2009](#)). The piezoelectric sensor has been used in various applications such as cholera toxin diagnostic detection, hepatitis B, hepatitis C, and food-borne pathogen detection ([Skládal et al., 2004](#); [Serra et al., 2008](#); [Chen et al., 2008](#)). The piezoelectric transducer is a very sensitive method, noting that a detection limit of  $8.6 \text{ pg L}^{-1}$  was obtained for hepatitis B virus DNA and  $25 \text{ ng mL}^{-1}$  for cholera toxin detection ([Yao et al., 2008](#); [Chen et al., 2010](#)).

### Advantages of piezoelectric sensors

The piezoelectric MEMS acoustic wave biosensors have the following characteristic advantages, viz., high sensitivity, small size and portability, fast responses, robustness, high accuracy, and compatibility with integrated circuit (IC) technology. These sensors can be manufactured using standard photolithography and hence can be produced as relatively inexpensive devices.

## 1.7 MAGNETOELASTIC BIOSENSORS

Magnetoelastic sensor was based on a magnetoelastic film coated by a pH-sensitive polymer. Magnetoelastic sensors are made from amorphous ferromagnetic metal film ribbons ([Grimes et al., 2002](#)). These ribbons are usually iron rich alloys that have a high mechanical tensile strength and high magnetoelastic-coupling coefficient. Hence the ribbons are magnetostrictive—they can change the shape upon application of a magnetic field. Magnetoelastic sensors with a size of approximately 4 cm × 6 mm × 25 µm are widely used as antitheft markers.

### Principle of magnetoelastic biosensors

The principle is similar to the more well-known quartz crystal microbalance (QCM), but magnetoelastic biosensors are based on magnetoelasticity instead of piezoelectricity. A magnetoelastic material changes its dimensions when it is exposed to a magnetic field. A thin strip of a magnetoelastic material forms a resonator, similar to a tuning fork. Exposing the film to a short magnetic pulse excites the film and it starts to oscillate emitting a magnetic field. The frequency, amplitude, and damping of the emitted magnetic field give information about the status of the sensor and the coating or media surrounding it.

### Application in monitoring blood coagulation

The determination of blood coagulation time is an essential part of monitoring therapeutic anticoagulants. Magnetoelastic biosensors have been used for the monitoring of blood coagulation. The ribbon-like magnetoelastic sensor oscillates at a fundamental frequency, which shifts linearly in response to applied mass loads of changing elasticity. The magnetoelastic sensors emit magnetic flux, which can be detected by a remotely located pickup coil, so that no direct physical connections are required.

During blood coagulation, the viscosity of blood changes due to the formation of a soft fibrin clot. This change in viscosity shifts the characteristic resonance frequency of the magnetoelastic sensor enabling real-time continuous monitoring of this biological event. By monitoring the signal output as a function of time, a distinct blood clotting profile can be seen.

### Advantages of magnetoelastic biosensors

1. Wireless detection: The antenna-to-sensor range is a few decimeters in air and a few centimeters in a liquid.
2. Noninvasive and passive sensor: the sensor needs no battery or other power supply.
3. Low sensor cost: The sensor is made from a low-cost material, so it is well suited for disposable sensors.

## 1.8 FIELD EFFECT TRANSISTOR-BASED BIOSENSOR

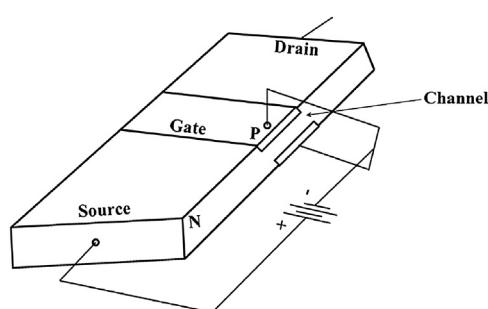
### Field Effect Transistor

A field effect transistor (FET) is one of the most commonly used semiconductor devices. All FETs have three semiconductor devices, called the source (S), the drain (D), and the gate (G). There is no physical contact between source and drain, but a current path, which is called a conduction channel, forms between the source and the drain. The gate-to-source voltage ( $V_{gs}$ ) will turn on (or off) the device, as a FET-type device can function as an on/off switch. The electric field strength, which serves as a control mechanism, is associated with the voltage applied to the gate. The current flow is determined by the actual motion of the carriers to be more exact, of the electrons for the *n*-type channel or the holes for the *p*-type channel. For an *n*-type FET, the applied gate voltage will cause electrons to pass through the channel from the source to the drain. If positive voltage is applied to the gate of an *n*-type FET, a channel is created and the charge effect on the conductance across the channel increases accordingly. In contrast, if negative gate voltage is applied, the *n*-type channel will pinch off. For a *p*-type FET, the opposite occurs, as positive (negative) gate voltage will turn off (on) the transistor device. The simplified diagram of the FET is shown in Figure 1.15.

A FET uses an electric field to control the electrical channel of conduction and hence the conductivity of the charge carriers in the channel. The flow of charge carriers between the source and the drain can be tuned by modifying the size and the shape of the conducting channel by applying an electric field to the gate.

### FET-based biosensor

A number of FET-based biosensors have been developed to study biomolecular interactions, which are the key drivers of biological responses in *in vitro* or *in vivo* systems. Among the many different biosensing systems, the FET-type biosensor is one of the most attractive electrical biosensors due to its advantages of sensitive measurements, portable instrumentation, easy operation with a small sample requirements, low cost



**Figure 1.15** The simplified diagram of the FET.

with mass production, and high speeds. In the biosensor configuration, the FET consists of a nanowire channel between the source and the drain terminals. The nanowire surface can be biofunctionalized so that a biomolecular binding event can create an electric field, similar to the control electric field applied to a conventional FET. The FET sensor is connected to an electronic circuit to monitor the specific conductance of this sensor surface. FET biosensors are adapted for the measurement of biomolecules interacting with such a sensor surface. As with other forms of sensing, the surface of a FET biosensor is modified to selectively recognize specific analytes.

### **Principle of FET based biosensor**

The principle of an ion-sensitive FET biosensor is based on a traditional metal-oxide semiconductor FET (MOSFET) structure. The metal gate of a FET-type biosensor is generally replaced by a biofilm layer material such as a receptor, enzyme, antibody, DNA, or other type of capturing molecule biologically specific for the target analyte. In response to target molecules in the solution, the biomodified gate (G) surface modulates the channel conductivity of the FET, leading to a change in the drain current. As a transducer, a FET whose conductance is modulated by the gate voltage through the field effect of a semiconductor can be used. In the FET conductance is also affected by surface voltage, surface charge, and work function other than the gate voltage (electrical signal). A biosensor can be obtained by a combination of FET and biological material with conductance changed by the biological reaction. In other words, the FET can be configured as a biosensor by modifying the gate terminal with molecular receptors or ion-selective membranes for the analyte of interest. The binding of a charged biomolecule results in accumulation of carriers caused by change of electric charges on the gate terminal. The dependence of the channel conductance on gate voltage makes FETs good candidates for electrical biosensors because the electric field generating from the binding of a charged biomolecule to the gate is analogous to applying a voltage to a gate.

### **Ion-selective field effect transistor**

An ion-selective field effect transistor (ISFET) selectively measures ion activity in an electrolyte. It works as an ion-selective electrode (ISE) with low output impedance. By combining the ISFET with a membrane that contains a biological material such as an enzyme (or) a microbe, a biosensor able to measure a specific organic substance can be obtained. The ISFET has been used to measure ion concentrations in a solution. Another form of FET utilizes a nanowire between two conducting materials. The nanowire has its atoms concentrated on its surface. Thus, any small changes in the charges present on the nanowire will cause a change in the flow of current. The electrical properties of one-dimensional material such as silicon nanowire, conducting polymer-based nanowires, metal oxide nanowires, and carbon nanotubes are sensitive to the recognizing element

attached to them. This is because the high surface-to-volume ratio associated with the one-dimensional materials.

One promising approach for the direct electrical detection of biomolecules uses nanowires configured as FETs. FETs readily change their conductance upon binding of charged target biomolecules to their receptors linked to the device surfaces.

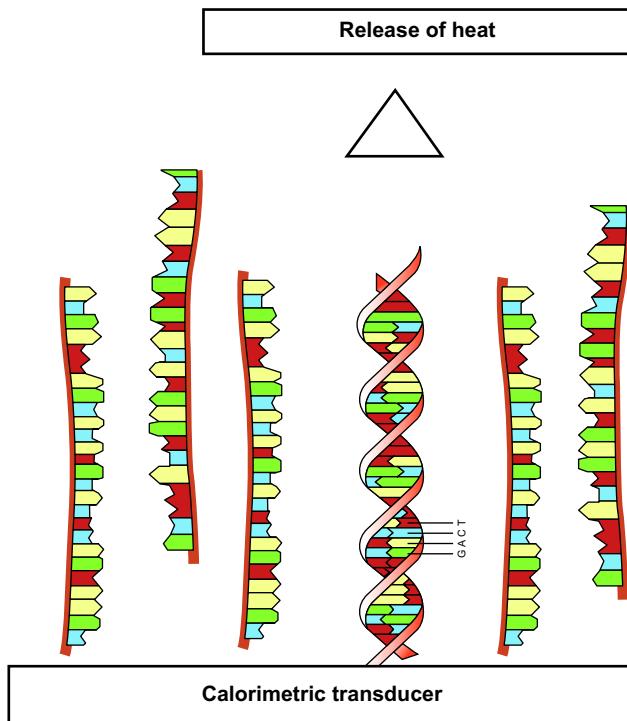
## 1.9 CALORIMETRIC BIOSENSOR

All chemical and biological reactions involve exchange of heat (Xie et al., 1999). Thus the general idea of generation and absorption of heat resulting in all biochemical reactions has contributed to the birth of calorimetric-based biosensing devices. Initially, calorimetric transduction has been employed for enzyme-based sensors and has subsequently been applied in DNA/cell and immunosensors. The principles of calorimetrics measured the changes in temperature in the reaction between biorecognition element and a suitable analyte. This change in temperature can be correlated to the amount of reactants consumed or products formed. In the calorimetric device, the heat change is measured using either a thermistor (usually metal oxide) or thermophile (usually ceramic semiconductor). The major advantages of this type of thermal detection are the stability, increase sensitivity, and possibility of miniaturization. This method is used for label-free screening of biomolecule interaction. Calorimetric technique is capable of rapidly detecting the DNA hybridization. Nowadays, this method has been used in food industry and environmental monitoring (Maskow et al., 2012; Kirchner et al., 2012). The calorimetric transduction is shown in Figure 1.16.

## 1.10 NONINVASIVE BIOSENSORS

The measurement of metabolites in media other than blood is becoming increasingly significant because of major demands for noninvasive analysis (Guilbault and Palleschi, 1995). Noninvasive sensing means there is no invasion of the body to collect body fluids such as whole blood, serum, cerebrospinal fluid. Several amperometric biosensors have been developed and applied for the noninvasive determination of metabolites in body fluids. Noninvasive electrochemical sensors can detect target analytes in tears, saliva, sweat, and skin interstitial fluid. Advantages of saliva or sweat analysis are the ease of sample collection and that samples can be collected more frequently with much less stress on the patient. The best example of a noninvasive biosensor is an alcohol biosensor with a hydrogen peroxide-based electrode utilizing immobilized alcohol oxidase.

Noninvasive techniques include infrared, Raman spectroscopy, polarimetry, light scattering, photoacoustic spectroscopy, polarization technique, and impedance. In infrared spectroscopy, absorption or emission data in the region of spectrum are compared to known data for glucose. In Raman spectroscopy, laser light is used to



**Figure 1.16** The calorimetric transducer.

stimulate emission from transitions close to the level excited. Photoacoustic spectroscopy deals with the laser excitation of fluids to generate an acoustic response and a spectrum as the laser is tuned. In scatter technique, the scattering of light can be used to indicate a change in the material being examined. For polarization technique, the presence of glucose in a fluid is known to cause a polarization preference in the light transmitted.

### 1.10.1 Saliva-based sensors

Saliva is a complex biofluid comprising numerous constituents permeating from blood via transcellular or paracellular paths. Hence sialochemistry offers an excellent noninvasive alternative to blood analysis for monitoring the emotional, hormonal, nutritional, and metabolic states of the human body (Bandodkar and Wang, 2014). Saliva is also readily available compared to blood and requires fewer pretreatment steps. An ideal salivary sensor must conform well to the complex anatomy of the mouth with minimal inconvenience to the wearer. Saliva is an excellent matrix for detection of many substrates, and the number of analytes detectable in saliva is continuously increasing. Saliva is used as a matrix for glucose detection and measurement of alcohol and lactate.

### 1.10.2 Tear-based sensors

Tears can be used as an attractive fluid for noninvasive monitoring. Tears are a complex extracellular fluid containing proteins/peptides, electrolytes, lipids, and metabolites from lacrimal glands, ocular surface epithelial cells, Meibomian glands, goblet cells, and blood. Due to a correlation between glucose levels in tears and blood, tear-based sensors are extensively used for continuous diabetes management.

### 1.10.3 Sweat-based sensors

Human sweat contains abundant information about a person's health status and thus is an excellent biofluid for noninvasive chemosensing. For example, sodium, lactate, ammonium, and calcium levels in sweat are indicators of electrolyte imbalance, and cystic fibrosis, physical stress, osteoporosis, and bone mineral loss, respectively. Sweat has also been used for monitoring a person's intoxication level and signs of drug abuse. Noninvasive electrochemical sensors for monitoring sweat can mainly be divided in to two types: fabric/flexible plastic-based sensors and the epidermal-based sensors.

### 1.10.4 Breath sensors

Breath diagnostics involve the analysis of a human breath sample to monitor, diagnose, and detect diseases and conditions. Exhaled breath contains a complex mixture of nitrogen, oxygen, carbon dioxide, water, and trace amounts of various volatile organic compounds like NO, acetone, isoprene, and ammonia. Many of these species are formed as the by-products of metabolic processes and can be used as biomarkers for various diseases. Examples of such biomarkers are acetone for diabetes mellitus (type I), ammonia for renal disease, NO for asthma, etc.

Breath analysis is a noninvasive method of disease detection. Most breath sensors are chemoresistive based on measuring the resistance of the sensor material in the presence of biomarkers. For example, metal oxide sensors, viz.,  $\text{Fe}_2\text{O}_3$ ,  $\text{SnO}_2$ ,  $\text{CdO}$ ,  $\text{ZnO}$ ,  $\text{TiO}_2$ , and  $\text{WO}_3$ , showed sensitivities less than 5–10 ppm for acetone detection. The major drawback is that they either lacked satisfying sensitivity or showed cross-sensitivity to other gases. Recently, Cr-doped  $\text{WO}_3$  nanoparticle deposited on Pt electrode as sensing material exhibited both high sensitivity and good selectivity for acetone detection in a single breath sample, i.e., a noninvasive diagnostic tool for monitoring the diabetes (Wang et al., 2010). The device responded to 1.8 ppm or higher concentrations of acetone gas as required for diabetes diagnosis. As acetone is a reducing gas, it lowered the electrical resistance of *n*-type semiconducting oxide, as sensing material. Similarly, considerable efforts have been made to fabricate  $\text{NO}_x$  sensor using different semiconducting oxide ( $\text{SnO}_2$ ,  $\text{ZnO}$ ,  $\text{WO}_3$ ,  $\text{TiO}_2$ ) thin films and nanostructured materials. Nanowires and nanotube field effect transistor (FET)-based gas sensors were also developed for their low-level detection. In the case of breath analysis, sensors have to detect NO concentrations in

the 1–100 ppb range, be selective against CO, CO<sub>2</sub>, and hundreds of volatile organic compounds, and detect NO in a vapor stream. Recently, a high-sensitive potentiometric sensor for NO<sub>x</sub> detection with ppm detection levels using yttria-stabilized zirconia (YSZ) with WO<sub>3</sub>-sensing electrode and Pt-zeolite/Pt as the reference electrode has been accomplished (Mondal et al., 2011).

Breath analysis also has the potential to detect cancer at an earlier stage by analyzing volatile biomarkers in exhaled breath (Queralto et al., 2014). Since the composition of volatile organic compounds (VOCs) in exhaled breath reflects the metabolic state of the body, the metabolomic approach to cancer screening provides an opportunity to detect early tumor-related perturbations in biochemical pathways associated with the cancerous cell growth. Unlike genomics and proteomics, metabolomics permits the study of the ultimate, downstream phenotypic response of biological systems to genetic change. Common VOCs, such as acetone and isoprene, are found in relatively high concentrations in ppm levels; however, cancer discriminatory VOCs are typically found in low parts per billion levels. Published studies indicate that there is no single molecule that can be correlated to cancer, but instead relative concentrations of a number of compounds are required for cancer detection. Mass spectrometry (MS) has been a very useful analytical tool to identify specific biomarkers in breath. For example, lung cancer showed around 30 VOCs, including alcohols, alkane derivatives, alkenes, ketones, esters, aldehydes, and aromatics, and breast cancer contains alkanes, alkenes, aromatics, alcohols, esters, and ketones (e.g., ethylenecyclopropane, 1,4-pentadiene, 2-methyl-1,3-butadiene, 1,2,4,5-tetramethylbenzene, 2-hexyl-1-octanol, n-octadecyl trifluoroacetate, 2,5-di-t-butyl-1,4-benzoquinone). But its potential use as a bedside diagnostic tool is limited due to its long analysis time, need for qualified operators, and high cost. So, an *electronic nose* (array-based sensor) is employed. It consists of cross-reactive sensor arrays coupled with a pattern recognition algorithm to detect VOC biosignatures in exhaled breath. Unlike MS techniques, array-based sensors do not attempt to identify individual VOC components of exhaled breath. Instead, they look for a difference in the overall chemical profile of healthy controls and patients with disease. This approach is similar to the mammalian nose containing a large number of olfactory receptors that detect and discriminate thousands of odorants based on cross-responsive patterns. There are a variety of different array-based sensors, including metal-oxide sensors, conducting polymer sensors, electrochemical sensors, metal-oxide field effect transistors, nanoparticle sensors, etc. Among them, conducting polymer composite sensors and nanomaterials-based sensors such as gold nanoparticle are sensitive to many VOCs with fast response time and exhibit a good linear response with respect to the analyte concentration. They can reach detection limits of 1–100 ppb for many breath biomarkers for lung cancer. The advantages of using array-based sensors include low cost, fast analysis, and portability (Queralto et al., 2014).

## 1.11 ELECTROCHEMICAL BIOSENSORS

### 1.11.1 Introduction

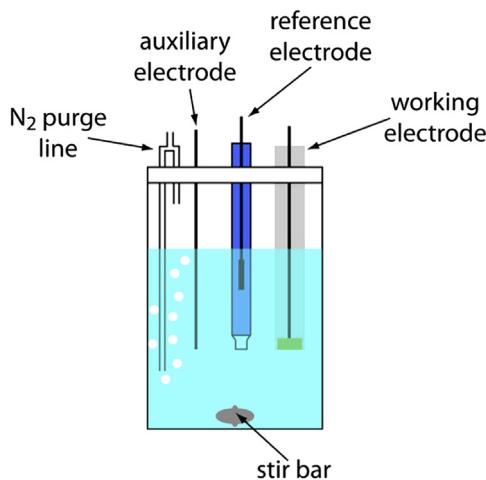
An electrochemical biosensor is a biosensor with an electrochemical transducer. An electrode is used as the transduction element in this biosensor. According to the 1999 IUPAC recommendation, an electrochemical biosensor is a self-contained integrated device that is capable of providing specific quantitative or semiquantitative analytical information using a biological recognition element (biochemical receptor) that is retained in direct spatial contact with an electrochemical transduction element. Electrochemical biosensors measure the current produced from oxidation and reduction reactions. Electrochemical biosensors are capable of achieving direct conversion of a biological event to an electrical signal, which makes them quite desirable and attractive for analyzing the content/concentration of a biological sample or analyte of interest. These rely on the detection of an electrical property (resistance, current, potential, capacitance, impedance), which are detected and measured using different methods such as potentiometry, conductometry, amperometry, or voltammetry.

### 1.11.2 Principle of electrochemical biosensors

Electrochemical detection is another possible means of transduction that has been extensively used in biosensors. The technique is complementary to optical detection methods such as fluorescence, the most sensitive of the optical techniques. Electrochemical detection is based on the chemical potential of a particular species in solution (the analyte), as measured by comparison to a reference electrode. In other words, many chemical reactions produce or consume ions or electrons that in turn cause some change in the electrical properties of the solution, which can be sensed out and used as measuring parameter. Therefore, the electrochemical response is dependent on the activity of the analyte species, not their concentration. Electrochemical biosensors measure the current produced from oxidation and reduction reactions. This current produced can be correlated to either the concentration of the electroactive species present or its rate of production/consumption. The resulting electrical signal is related to the recognition process by target and analyte and is proportional to the analyte concentration. Depending upon the nature of electrochemical changes detection during a biorecognition event, electrochemical biosensors are classified into five types: amperometric, potentiometric, voltammetric, impedimetric, and conductometric.

### 1.11.3 Electrochemical cell

An electrochemical cell is used in electrochemical sensor studies. The electrodes themselves play an important role in the performance of electrochemical cells and electrochemical biosensors. An electrochemical analyzer is an instrument used in analytical chemistry to study an analyte by measuring the potential and/or current in an electrochemical cell.



**Figure 1.17** Schematic representation of conventional three-electrochemical cell.

The electrochemical cell, where the experiments are carried out, consists of a working electrode (WE), a reference electrode (RE), and a counter electrode (CE).

The conventional electrochemical cell is a single-compartment glass cell, which needs to be cleaned extensively before each experiment. The type of cell used depends on the amount, type of the sample, the technique, and the analytical data to be obtained. The conventional electrochemical cell is represented in [Figure 1.17](#).

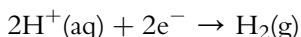
### Working electrode

The WE is an electrode where the reaction of interest is taking place ([Radhakrishnan et al., 2007](#)). In an electrochemical system with three electrodes, the working electrode can be referred to as either cathodic or anodic depending on whether the reaction on the working electrode is a reduction or an oxidation. The performance of the voltammetric measurements were strongly influenced by the working electrode material. Since the reaction of interest (reduction or oxidation) takes place on the working electrode, it should provide high signal-to-noise characteristics, as well as a reproducible response. Thus, its selection depends primarily on two factors: the redox behavior of the target analyte and the background current over the potential region required for the measurement. Other considerations also include the potential window, electrical conductivity, surface reproducibility, mechanical properties, cost, availability, and toxicity. There are many kinds of working electrodes: glassy carbon electrode, Pt electrode, gold electrode, mercury electrode, screen-printed electrode, silver electrode, Indium tin oxide-coated glass electrode, carbon paste electrode, etc. The commonly used WE materials for voltammetry include platinum, gold, mercury, and glassy carbon. Materials such as semiconductors and other metals are also used for more specific applications ([Zhou et al.](#)).

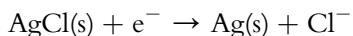
### Reference electrode

The RE is used to produce a constant potential at the electrochemical cell ([Gyorgy and Fritz, 2013](#)). Since the passage of current through an electrode can alter the potential, such effects are minimized for the reference electrode in three electrode systems by considering two factors. Firstly, it should have low impedance as potentiostats are less tolerant of high impedance REs. A high impedance RE not only shows the response of the potentiostats (slow rise time) but also increases the susceptibility of the system to environmental noise (in particular power line noise). Secondly, the reference electrode should be a nonpolarizable electrode since the passage of small currents does not alter the potential.

Reference electrodes should be constructed using half-cell components that are stable over time and, with changing temperature, present at well-defined values of activity. They should possess fixed, reproducible electrode potentials. The most familiar reference electrode is the standard hydrogen electrode (SHE), composed of an inert solid-like platinum on which hydrogen gas is adsorbed, immersed in a solution containing hydrogen ions at unit activity. The half-cell reaction for the SHE is given by



with a half-cell potential value of zero ( $E_0 = 0.000 \text{ V}$ ). The saturated calomel electrode (SCE) has the half-cell potential of  $E_0 = +0.241 \text{ V}$ . The Ag/AgCl electrode has the half-cell potential of  $E_0 = +0.197 \text{ V}$ . The most commonly used RE for aqueous solutions is the silver/silver chloride electrode (Ag/AgCl), with potential determined by the reaction.



### Counter electrode

The CE (also known as auxiliary electrode), is an electrode that is used to close the current circuit in the electrochemical cell. It is usually made of an inert material (e.g., platinum, gold, graphite, and glassy carbon) and it does not participate in the electrochemical reaction ([Thomas and Henze, 2001](#)). Because the current is flowing between the WE and the CE, the total surface area of the CE (source/sink of electrons) must be higher than the area of the WE so that it will not be a limiting factor in the kinetics of the electrochemical process under investigation.

In a two-electrode system, when a known current or potential is applied between the working and counter electrodes, the other variables may be measured. The counter electrode functions as a cathode whenever the working electrode is operating as an anode and vice versa. The counter electrode has a surface area much larger than that of the working electrode. The potential of the counter electrode is not measured against the reference electrode but is adjusted to balance the reaction occurring at the working electrode.

#### 1.11.4 Microfluidic electrochemical cell

A microfabricated electrochemical cell comprising gold microelectrodes was fabricated as a sensitive and a miniature alternative to the conventional electrochemical cell. A microfluidic electrochemical cell with microelectrodes can greatly facilitate sample handling, electrode cleaning, online/in-line detection and can enhance sensitivity by reducing interferences (Chand et al., 2013). In microfluidic electrochemical cell, three different materials were optimized as electrodes: gold film or graphite–epoxy composite as working electrode, silver–epoxy composite as pseudo-reference electrode, and graphite–epoxy composite as auxiliary electrode. The behavior of microelectrodes differs from conventional-sized electrodes in that nonlinear diffusion is the predominant mode of transport. This difference in mass transport from the bulk solution toward the electrode has several important implications that make microelectrodes very attractive in many areas of electroanalytical chemistry. These include reduced ohmic potential drop, a decreased time constant, a fast establishment of steady state signals, and an increased signal-to-noise ratio. The performance of the microfluidic cell was characterized by cyclic voltammetry.

The microfluidic electrochemical cell is represented in Figure 1.18.

#### Advantages of microfluidic cell

1. Reduction in solution consumption
2. Less waste is generated
3. Online/in-line detection
4. Enhanced sensitivity by reducing interferences.

#### 1.11.5 Lab-on-a-chip

A lab-on-a-chip (LOC) is a device that integrates one or several laboratory functions on a single chip of only millimeters to a few square centimeters in size. It deals with the handling of extremely small fluid volumes down to less than pico liters. The lab-on-a-chip device is shown in Figure 1.19.

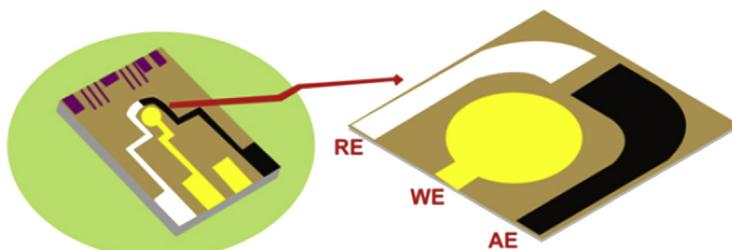
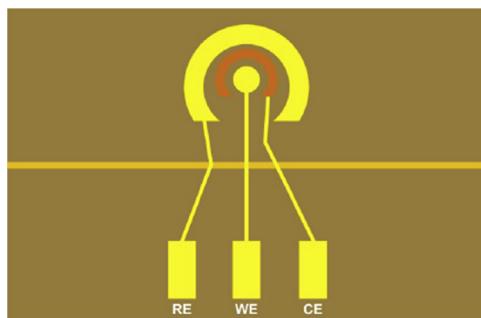


Figure 1.18 The microfluidic electrochemical cell.



**Figure 1.19** The lab-on-a-chip device.

### Advantages of LOC:

1. Low fluid volume consumption
2. Faster analysis and response times
3. Compactness of the systems due to integration of much functionality and small volumes
4. Lower fabrication costs

## 1.12 VARIOUS ELECTROCHEMICAL TECHNIQUES

### 1.12.1 Amperometric transducers

Amperometric transducers measure the currents resulting from the electrochemical oxidation or reduction of an electroactive species with the biorecognition element under a constant potential (voltage) applied to working electrode. The driving force for the electron transfer reaction of the electroactive species is the applied potential that forces the species to gain or lose electrons. The obtained current is a direct measure of the rate of the electron transfer reaction, which at the same time is representative of the recognition process and thus proportional to the analyte concentration.

### 1.12.2 Voltammetric transducers

Voltammetry is the most versatile technique in electrochemical analysis (Protti, 2001). In voltammetric technique, both the current and the potential are measured and recorded. The position of peak current is related to the specific chemical, and the peak current density is proportional to the concentration of the corresponding species. A remarkable advantage of voltammetry is the low noise, which can endow the biosensor with higher sensitivity (Bard and Faulkner, 2001). In addition, voltammetry is able to detect multiple compounds, which have different peak potentials, in a single electrochemical experiment, thus offering the simultaneous detection of multiple analytes. The graph of current

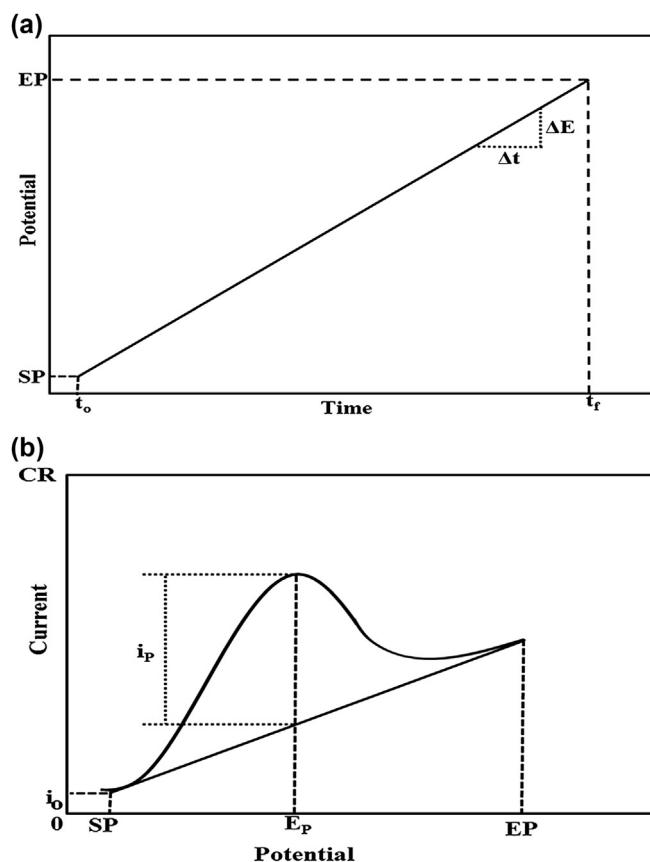
versus potential, called a voltammogram, provides information about the chemical reaction. Voltammetric methods include cyclic voltammetry (CV), linear sweep voltammetry, hydrodynamic voltammetry, differential pulse voltammetry, ac voltammetry, polarography, and stripping voltammetry.

### 1. Rapid scan voltammetry—Linear sweep voltammetry

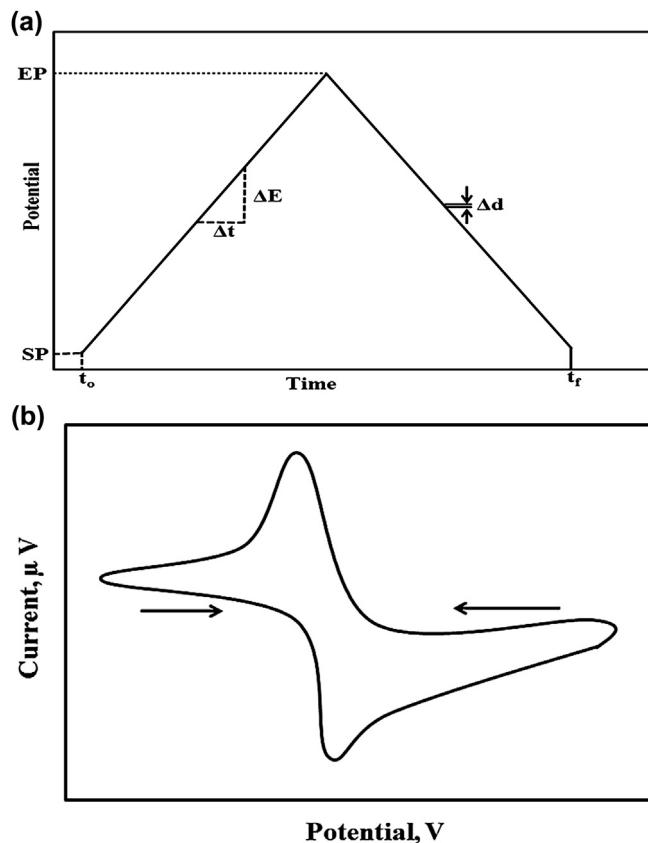
Rapid scan voltammetry is the simplest technique. At the working electrode the potential is rapidly scanned linearly ( $20\text{--}100 \text{ mV s}^{-1}$ ) as shown in [Figure 1.20\(i\)](#).

The scanning starts before the discharging potential and stops afterwards. The current flowing through the working electrode has two components:

- a. The faradic current
- b. The capacitive current



**Figure 1.20** (i) Linear sweep voltammetry (a) anodic scanning of potential (b) plot of voltammogram (SP, start potential; EP, end potential;  $t_0$  and  $t_f$ , starting and final times of the scanning;  $i_0$ , current at the beginning of the scanning;  $i_p$ , peak current;  $E_p$ , peak potential).

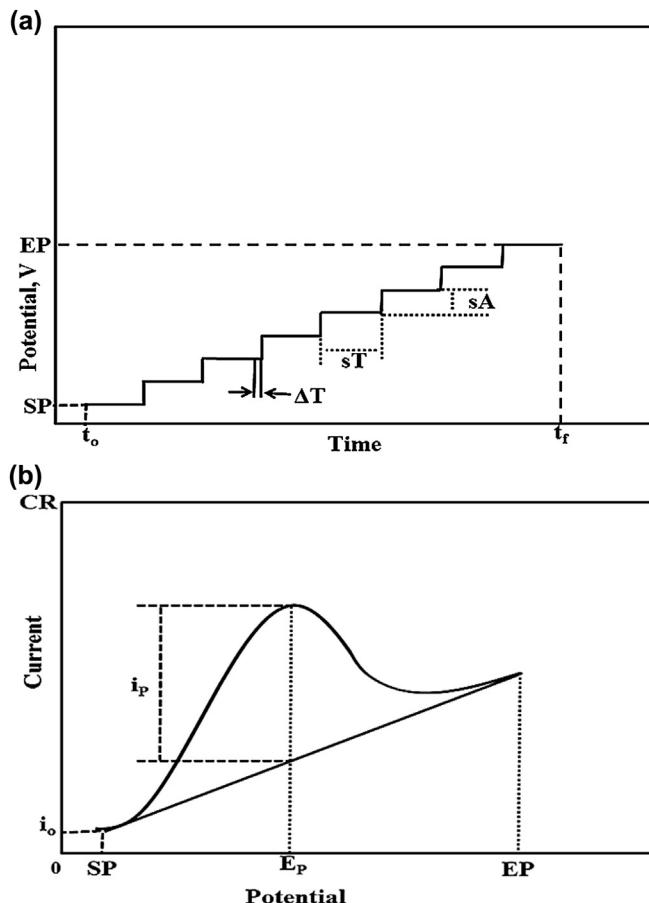


**Figure 1.20 Cont'd. (ii)** Cyclic voltammetry (a) potential scanning starting from an anodic sense (b) cyclic voltammogram of a reversible redox system (P, start potential; EP, end potential;  $t_0$  and  $t_f$ , starting and final times of the scanning).

The faradic current follows the Faraday laws and is due to the discharge of the electroactive compound (Aox). The capacitive current is produced by the growth of a double electric layer on the interface between the electrode and the solution. This double layer is due to the high concentration of the supporting electrolyte in the solution and acts as a condenser with high capacity. The total current flowing through the electrode is finally due to the sum of the charging current (capacitive current) of this condenser and the faradic current. In linear sweep voltammetry (LSV), capacitive current increases when the scan rate is increased and cannot be electronically compensated. Thus the performance of this technique is strongly restricted. Detection limits range at  $\text{mg L}^{-1}$  levels.

## 2. Cyclic voltammetry

Cyclic voltammetry (CV) is a technique devoted to the theoretical study of the behavior of redox couples. Cyclic voltammetry is similar to LSV that performs a

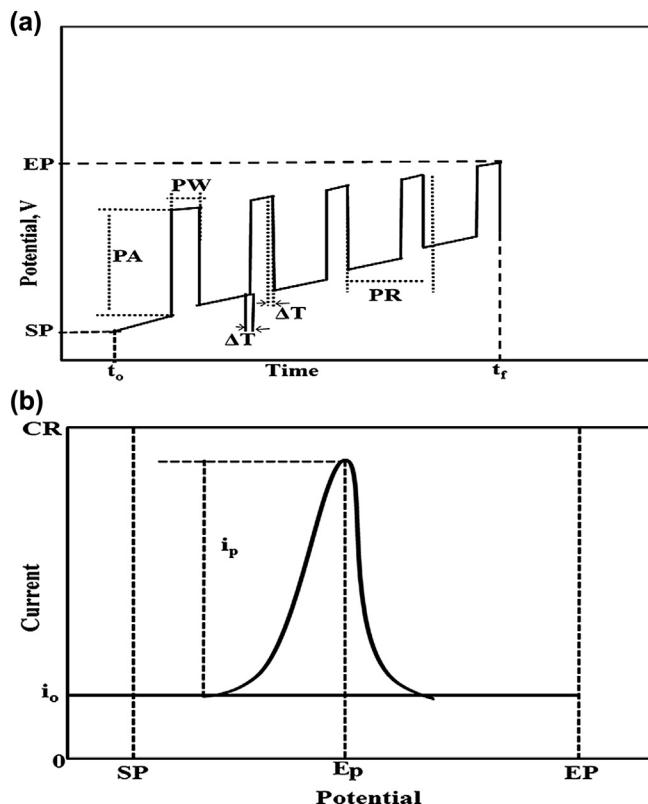


**Figure 1.20 Cont'd. (iii) Staircase voltammetry (a) anodic scanning of the potential (b) plot of voltammogram (SP, start potential; EP, end potential;  $t_0$  and  $t_f$ , starting and final times of the scanning;  $i_0$ , current at the beginning of the scanning;  $i_p$ , peak current;  $E_p$ , peak potential;  $\Delta T$ , sampling time; CR, Current range; sA, step Amplitude of potential; sT, step time ).**

triangular-shaped scanning at the working electrode as shown in Figure 1.20(ii). The plot of a cyclic voltammetry consists of a closed curve: reversible redox couples show both cathodic and anodic peaks, while irreversible redox systems show only one peak. Generally this technique is not used for quantitative analysis because of its poor sensitivity.

### 3. Staircase voltammetry

Staircase voltammetry is a different variant of the LSV technique that consists of a regular potential step scanning Figure 1.20(iii). The current is sampled just before the subsequent step. Thus the signal is less influenced by the capacitive current.



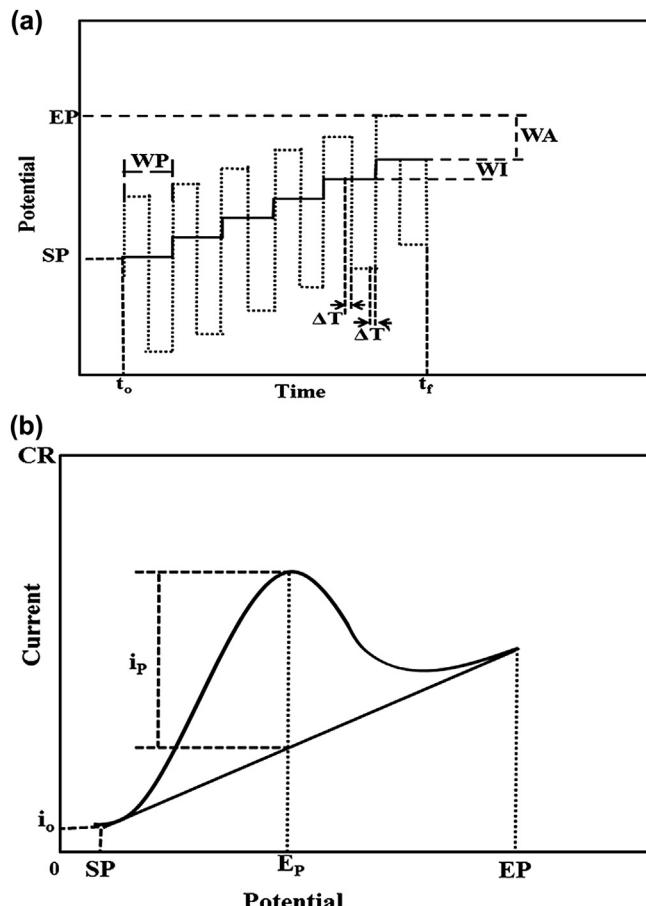
#### 4. Differential pulse voltammetry

If a series of periodical constant pulse of potential is superimposed to a linear scanning, a consistent enhancement of the signal is achieved as seen in Figure 1.20(iv).

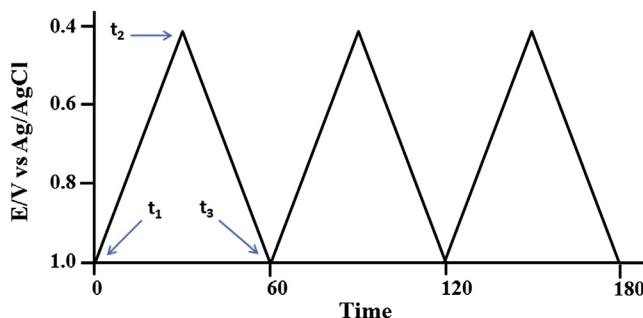
Moreover, if the difference between the current just before and at the end of the pulse is measured, a reading less influenced by the capacitive current can be performed. In this way this differential reading of the current generates a peak-shaped voltammogram. This differential pulse voltammetry technique is very sensitive and detection limits range near  $10-100 \mu\text{g L}^{-1}$ .

#### 5. Square wave voltammetry

Square wave voltammetry represents a further development of the preceding one. A rapid step scanning of potential is applied to the electrode and a high frequency square wave (20–100 Hz) is superimposed on each step as shown in Figure 1.20(v). The



**Figure 1.20 Cont'd. (v)** Square wave voltammetry (a) anodic scanning of the potential (b) plot of voltammogram (SP, Start potential; EP, End potential;  $t_0$  and  $t_f$ , starting and final time of the scanning;  $i_0$ , current at the beginning of the scanning;  $i_p$ , peak current;  $E_p$ , peak potential;  $\Delta T$ , sampling time; CR, Current range; WA, Wave amplitude; WP, Wave period; WI, Wave increment).



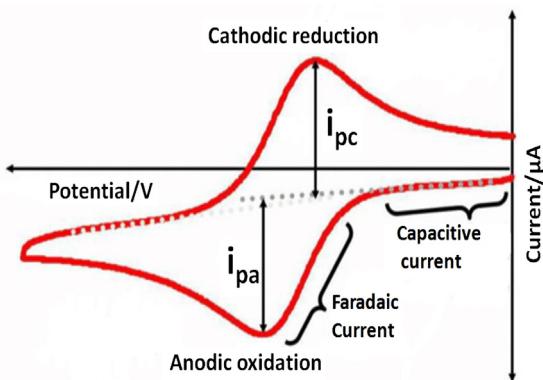
**Figure 1.20 Cont'd. (vi)** Triangle waveform used in cyclic voltammetry.

current is sampled two times at the end of the two half waves. If the amplitude of the wave is very small and the redox system is reversible, during the first half wave the electroactive compound can be reduced (or oxidized), while, in the second half wave, at the contrary, it can be oxidized (or reduced). The two currents are then summed up and thus the sensitivity is increased. The sensitivity of this technique can be increased by enhancing the amplitude of the square wave or the frequency. Detection limits range from 5 to 50  $\mu\text{g L}^{-1}$ .

### 1.12.2.1 Fundamentals of cyclic voltammetry

Among the voltammetric techniques, cyclic voltammetry is most widely exploited in electrochemical biosensor instrumentation. As mentioned earlier, cyclic voltammetry is very similar to linear sweep voltammetry. It is a technique used to investigate the behavior of redox couples. CV consists of cycling the potential of an electrode, called working electrode, which is immersed in an unstirred solution, and measuring the resulting current. A large variety of working electrodes has been used with voltammetry. The voltammetric technique termed polarography utilizes the dropping mercury electrode (DME). This electrode consists of mercury drops continuously extruding from the end of a capillary. The hanging mercury drop electrode (HMDE) is commonly used for CV. A significant advantage of mercury is its good negative potential range. Solid electrodes such as platinum, gold, glassy carbon, wax-impregnated graphite, and carbon paste are also commonly used in CV. Such electrodes have a better positive potential range than mercury. The significance of CV is its ability to generate a species during one scan and then probe its fate with subsequent scans. The potential of this working electrode is controlled versus a reference electrode such as a saturated calomel electrode (SCE) or a silver/silver chloride electrode ( $\text{Ag}/\text{AgCl}$ ). The controlling potential that is applied across these two electrodes can be considered an excitation signal. The excitation signal for CV is a linear potential scan with a triangular waveform as shown in [Figure 1.20](#). This triangular potential excitation signal sweeps the potential of the electrode between two values, sometimes called the switching potentials. The potential is varied linearly with time, and the rate at which the potential is varied is called the sweep rate. A cyclic voltammetry experiment starts by scanning the potential from  $t_1$  to  $t_2$  in a linear fashion. The scan direction is then reversed to the original potential at  $t_3$ .

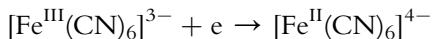
In a cyclic voltammetry experiment, the potential of an electrode is cycled from a starting potential ( $E_i$ ) to a final potential ( $E_f$ ) and then back to  $E_i$ . During the potential sweep, the potentiostat measures the current (between WE and CE) resulting from the applied potential. The resulting current–potential plot is termed a cyclic voltammogram. The cyclic voltammogram is a complicated, time-dependent function of a large number of physical and chemical parameters. [Figure 1.21](#) depicts the cyclic voltammogram obtained for a solution containing 1 mM  $\text{K}_3[\text{Fe}(\text{CN})_6]$  in 1 M  $\text{KNO}_3$  in water as



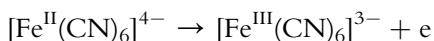
**Figure 1.21** Cyclic voltammograms of ferri/ferrocyanide redox couple on a bare Pt electrode surface.

the supporting electrolyte. Two distinct current peaks ( $i_{pa}$  and  $i_{pc}$ ) are observed, which correspond to the oxidation and reduction potentials for the  $[\text{Fe}(\text{CN})_6]^{4-/3-}$  redox couple.  $E_{pc}$  is the potential corresponding to the cathodic or reduction peak current ( $i_{pc}$ ), while  $E_{pa}$  is the potential associated with the anodic or oxidation peak current ( $i_{pa}$ ).

The initial potential of 1 V applied is chosen to avoid any electrolysis of  $[\text{Fe}(\text{CN})_6]^{3-}$  when the electrode is switched on. The potential is then scanned negatively, forward scan, as indicated by the arrow. When the potential is sufficiently negative to reduce  $[\text{Fe}^{\text{III}}(\text{CN})_6]^{3-}$ , cathodic current is increased due to the electrode process.



The electrode is now a sufficiently strong reductant to reduce  $[\text{Fe}^{\text{III}}(\text{CN})_6]^{3-}$ . The cathodic current increases rapidly until the concentration of  $[\text{Fe}^{\text{III}}(\text{CN})_6]^{3-}$  at the electrode surface is substantially diminished, causing the current to peak. The current then decays as the solution surrounding the electrode is depleted of  $[\text{Fe}^{\text{III}}(\text{CN})_6]^{3-}$  due to its electrolytic conversion to  $[\text{Fe}^{\text{II}}(\text{CN})_6]^{4-}$ . The scan direction is switched to positive at  $-0.15$  V for the reverse scan. The potential is still sufficiently negative to reduce  $[\text{Fe}^{\text{III}}(\text{CN})_6]^{3-}$ , so cathodic current continues even though the potential is now scanning in the positive direction. When the electrode becomes a sufficiently strong oxidant,  $[\text{Fe}^{\text{II}}(\text{CN})_6]^{4-}$ , which has been accumulating adjacent to the electrode, can now be oxidized by the electrode process.



This causes anodic current. The anodic current rapidly increases until the surface concentration of  $[\text{Fe}^{\text{II}}(\text{CN})_6]^{4-}$  is diminished, causing the current to peak. The current then decays as the solution surrounding the electrode is depleted of  $[\text{Fe}^{\text{II}}(\text{CN})_6]^{4-}$ . The first cycle is completed when the potential reaches 1 V.

The concentrations of the reduced form of the compound compared to its oxidized form at the electrode surface can be calculated based on the applied potential by using the following Nernst equation in Eqn (1.1):

$$E = E_0 + \frac{RT}{nF} \ln \frac{[a_0]}{[a_r]} \quad (1.1)$$

where R is the universal gas constant, F is Faraday constant, n is the number of electrons transferred per ion,  $a_0$  is the concentration of the oxidized form of the redox compound, while  $a_r$  is the concentration of the reduced form. The plot of a cyclic voltammetry consists of a closed curve: reversible redox couples show both as cathodic and anodic peaks, while irreversible redox systems show only one peak. The following relations can be useful to establish the standard potential of a reversible redox couple, and the number of electrons involved in the discharge process.  $E_0$  is the formal reduction potential and is given by Eqn (1.2).

$$E_0 = \frac{E_{pa} + E_{pc}}{2} \quad (1.2)$$

$$\Delta E_p = E_{pa} - E_{pc} = \frac{0.059}{n}$$

This equation only remains valid if the reaction is reversible. A reaction is considered reversible if the total analyte that is oxidized/reduced on the forward scan equals the total analyte that is reduced/oxidized on the reverse scan. From the graph, reversibility is observed if  $I_{pa} = I_{pc}$ . The reaction at the electrode surface is limited almost entirely by the diffusion of the redox couple from the bulk solution to the surface. Other forms of mass transport including migration and convection have little effect on the movement of the redox species in solution due to the high electrolyte concentration and short distances between the electrodes. Thus, this diffusion creates a concentration gradient of both the oxidized and reduced forms of the redox couple as one moves away from the electrode surface. The magnitude of the current for a given applied potential does not depend on the magnitude of the analyte concentration at the electrode surface but rather the concentration gradient as shown following in Eqn (1.3):

$$i = nFAD \left( \frac{\partial C}{\partial x} \right)_{x=0} \quad (1.3)$$

where A is the area of the electrode ( $\text{cm}^2$ ), D is the diffusion coefficient ( $\text{cm}^2 \text{ s}^{-1}$ ), C is the concentration ( $\text{mol cm}^{-3}$ ), and x is the distance from the electrode surface (cm). From this equation, it is observed that the current will reach its highest magnitude when the concentration gradient is at a maximum. Equation (1.4) is derived from the more

complex Butler–Volmer equation for measuring current from the redox reaction given below:

$$i = nFk_0 \left[ [a_0]e^{\frac{-\alpha nF}{RT}(E-E_0)} - [a_r]e^{\frac{1-\alpha nF}{RT}(E-E_0)} \right] \quad (1.4)$$

where  $k_0$  is the heterogeneous rate constant and  $\alpha$  is the transfer coefficient (which is 0.5 for a reversible reaction). The peak current of the forward scan can be used to determine variables related to the reaction as described by the Randles–Sevcik equation, Eqn (1.5):

$$i_p = (2.69 \times 10^5) n^{3/2} A D^{1/2} C v^{1/2} \quad (1.5)$$

where  $v$  is the scan rate of the experiment ( $V s^{-1}$ ). This equation is often used to calculate the active surface area of the working electrode. Due to surface roughness, it can be difficult to know exactly what surface area is interacting with the solution. By performing a CV experiment using a known redox compound concentration and scan rate, the area can be estimated using the above equation with a standard diffusion coefficient of  $1 \times 10^{-5}$  for small molecules.

### 1.12.3 Conductometric transducers

The biorecognition event that changes the ionic concentration can be monitored using conductometric biosensors. Most reactions involve a change in the ionic species concentration, which leads to change in electrical conductivity or current flow (Mikkelsen and Rechnitz, 1989). Normally a conductometric biosensor consists of two metal electrodes separated by a certain distance and an AC voltage applied across the electrode causes a current flow. During a biorecognition event, the ionic composition changes and the change in conductance between the metal electrodes are measured. The major advantages of conductometric device are nonrequirement of reference electrode, inexpensive, possibility of miniaturization, and direct electrical response (Grieshaber et al., 2008). Unfortunately, the conductometric transduction measurement is less sensitive compared to the other electrochemical methods and strongly dependent of the response upon buffer capacity.

### 1.12.4 Impedimetric transducers

Electrochemical impedance spectroscopy (EIS; including Faradaic impedance in the presence of a redox probe and non-Faradaic-capacitance methods) is considered as a rapid technique for the characterization of the structure and functional operation of biomaterial-functionalized electrodes (Yang and Bashir, 2008). The immobilization of biomaterials on electrodes produces changes in the capacitance and interfacial electron transfer resistance of electrodes, causing changes in the impedance. Hence, interfacial

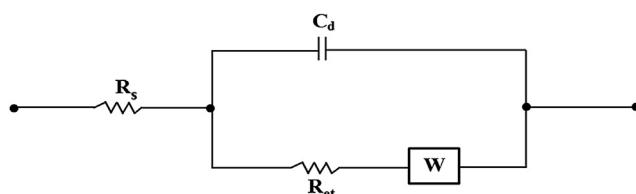
changes generated by biorecognition processes can be detected by this electrochemical technique. EIS is a widely used technique for probing the bioaffinity interactions at the sensor surfaces of electrically conducting polymers and can be employed to investigate label-free detection of analytes via impedimetric transduction. Though EIS can offer label-free detection compared to amperometry or potentiometry, its detection limits are inferior compared to the traditional methods.

#### **1.12.4.1 Electrochemical impedance spectroscopy**

Impedance spectroscopy or electrochemical impedance spectroscopy is a powerful electrochemical technique used to investigate the binding events that occur at the electrode surface. The same three electrode systems comprising of a WE, RE and CE are utilized for the EIS experiment. Electrochemical impedance is usually measured by applying an AC sine wave potential with low amplitude (5–10 mV peak to peak) superimposed on a DC potential to the electrochemical system. The AC signal scans the frequency domain, allowing the individual excitation of different processes with different time constants. Therefore, slow processes like chemical reactions and fast reactions like ionic conduction can be studied independently this way.

The measured impedance of the system can be modeled using an electrical circuit. The impedance consists of the electron transfer resistance between the redox compound and the electrode surface, the capacitance between the electrode and the charged ions in the solution, the solution resistance between the electrodes, and the impedance due to the rate of diffusion of the redox compound to the electrode. These elements are arranged in the circuit shown in [Figure 1.22](#).

Here,  $R_s$  is the solution resistance between the electrodes, which is typically much smaller than the other components.  $R_{et}$  is the charge transfer resistance, which accounts for the ability of the redox compound to interact with the electrode surface via electron transport.  $C_d$  is the capacitance between the electrode and the charged ions in solution. This capacitance is known as the double layer capacitance, which exists between any metal placed in an electrolyte solution.  $W$  is an element called the Warburg impedance, which accounts for the effects of mass-transfer limitations. The Warburg impedance itself has both a real and imaginary component and is frequency dependent. One can calculate



**Figure 1.22** Randles equivalent circuit model for the complex impedance of the three-electrode system in an ionic solution.

the total impedance of this circuit with respect to the values of the components and the frequency as shown in the following Eqn (1.6):

$$i_p = (2.69 \times 10^5) n^{3/2} A D^{1/2} C v^{1/2} \quad (1.6)$$

$$Z = \left( R_s + \frac{R_{et} + W}{1 + (R_{et} + W)^2 W^2 C^2} \right) - j \left( \frac{(R_{et} + W)^2 W C}{1 + (R_{et} + W)^2 W^2 C^2} \right) \quad (1.7)$$

$$Z = Z' + jZ'' \quad (1.8)$$

where  $j = \sqrt{-1}$ .

Impedance is usually expressed as a complex number, where the ohmic resistance is the real component and the capacitive reactance is the imaginary one. The most popular formats for evaluating electrochemical impedance data are the Nyquist and Bode plots. In the former format, the imaginary impedance component ( $Z''$ , out of phase) is plotted against the real impedance component ( $Z'$ , in phase) at each excitation frequency, whereas in the latter format, both the logarithm of the absolute impedance,  $|Z|$ , and the phase shift,  $\theta$ , are plotted against the logarithm of the excitation frequency. Faradaic impedance spectra presented in the form of a Nyquist plot is represented in Figure 1.23.

A Nyquist plot contains information about the electrified interface and the electron transfer reaction. This plot commonly includes a semicircle region lying on the axis followed by a straight line. The semicircle portion, observed at higher frequencies, corresponds to the electron transfer-limited process, whereas the linear part is characteristic of the lower frequencies range and represents the diffusionally limited electrochemical process. In the case of very fast electron transfer processes, the impedance spectrum could include only the linear part, whereas a very slow electron-transfer step results in a large semicircle region that is not accompanied by a straight line. The electron transfer kinetics

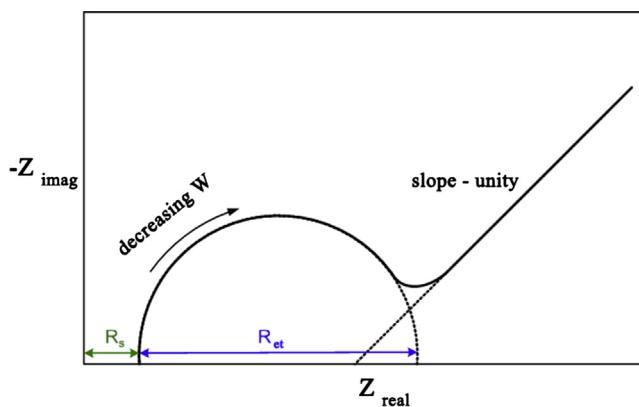


Figure 1.23 Schematic Faradaic impedance spectra presented in the form of a Nyquist plot.

and diffusional characteristics can be extracted from the spectra. The semicircle diameter equals to the electron transfer resistance,  $R_{et}$ . The intercept of the semicircle with the  $Z'$  axis at high frequencies ( $\omega \rightarrow \infty$ ) is equal to the solution resistance,  $R_s$ . Extrapolation of the circle to lower frequencies yields an intercept corresponding to  $R_s + R_{et}$ .

### 1.12.5 Potentiometric transducers

In potentiometric biosensors the biological recognition reaction causes a modulation of a redox potential, a transmembrane potential, or the activity of an ion. So the potentiometric biosensors utilize the measurement of a potential at an electrode in reference to another electrode (Bard and Faulkner, 1980). Mostly, it is comprised of a perm-selective outer layer and membrane or sensitive surface to a desired species (a bioactive material), usually an enzyme. The enzyme-catalyzed reaction generates or consumes a species, which is detected by an ion-selective electrode. Usually a high-impedance voltmeter is used to measure the electrical potential difference or electromotive force (EMF) between two electrodes at near-zero current. The basis of this type of biosensor is the Nernst equation, which relates the electrode potential ( $E$ ) to the concentration of the oxidized and reduced species. For the reaction:  $aA + ne^- \rightleftharpoons bB$ , the Nernst equation can be described as the following,

$$E = E^0 + \frac{RT}{nF} \ln \left( \frac{[C_A]^a}{[C_B]^b} \right) \quad (1.9)$$

where  $E^0$  is the standard redox potential,  $R$  is the gas constant,  $T$  is the absolute temperature,  $F$  is the Faraday constant,  $n$  is the number of exchanged electrons in the reaction, and  $C_A$  and  $C_B$  are the concentration of oxidized and reduced species, respectively. Since potentiometry generates a logarithmic concentration response, the technique allows the detection of extremely small concentration changes.

## 1.13 ELECTROANALYTICAL CHARACTERISTICS OF BIOSENSORS

The linear range, detection limit, sensitivity, selectivity, reproducibility, response time, and stability are the major characteristics of the biosensor.

### 1. Linear range

Linear range is defined as the range of analyte concentrations in which the sensor response changes linearly. The linear calibration curve for the particular analyte concentrations is prepared by plotting the various concentrations of the analyte along with the current responses obtained for each concentration.

### 2. Detection limit

Detection limit or lower limit of detection is the lowest quantity of a substance that can be distinguished by the sensor signal from the absence of that substance (control).

**3. Sensitivity**

The sensitivity is defined as the minimum magnitude of the input signal required to produce output having a specified signal-to-noise ratio. It is dependent on the standard deviation of the measurements. Generally, it is calculated from the slope of the calibration curve obtained by plotting the observed current in Y-axis and the concentrations of the particular analyte in X-axis. The higher value of slope represents the higher sensitivity of the biosensor.

**4. Selectivity**

Selectivity is expressed as the ratio of the signal output with the analyte alone to that with the interfering substance alone, at the same concentration of the analyte. It is investigated by measuring the biosensor response to interfering substance addition at their expected concentration into the measuring cell already containing the usual analyte concentration. It is dependent on the analyte concentration range and the choice of biological receptor and transducer. Many biological recognition elements (enzymes) are specific, however, some are nonselective. The measurement of analyte in biological media consists of various coexisting substrates that may be interfering with the measurement, and their influence may be restricted by the application of appropriate membranes.

**5. Reproducibility**

Reproducibility is a measure of the drift in a series of observations or results performed over a period of time. It is generally determined for the analyte concentrations within the usable range. It is investigated by constructing four different biosensors using the same procedure and measuring their electrochemical responses toward the oxidation/reduction of the analyte.

**6. Stability**

The operational stability of the biosensor may vary considerably depending on the sensor geometry and method of preparation. It is strongly dependent on the inner diffusion of the substrate and the operational conditions. It is investigated by the continuous or sequential contact of the biosensor with the analyte solution (various concentrations), temperature, buffer composition, and pH.

**7. Response time**

Response time of the biosensor is classified into steady state response time and transient response time. The time required to reach 95% of the steady state response of the biosensor is known as steady state response time. It is easily determined after the addition of each analyte into measurement cell. Transient response time corresponds to the first derivative of the output signal to reach its maximum value following the analyte addition. Both are dependent upon the analyte and the activity of the molecular recognition system, i.e., the higher activity, the shorter the response time.

## 1.14 MEMBRANES USED IN BIOSENSORS FOR SELECTIVITY

### 1.14.1 Ion-selective membranes

The main phenomenon responsible for generation of the response, i.e., the potential drop across the membrane, is an ion exchange between two phases: membrane/solution, which depends on the activity of the target ion (analyte) in these phases. The ion-selective membranes are usually composed of three or four components: (1) polymeric matrix, (2) plasticizer, (3) lipophilic salt, and (4) ionophore, all matched in adequate proportions. Complex formation constants for the different ions and the ionophore determine selectivity of the membrane. The plasticizer polarity influences extraction properties of the membrane, while a concentration of anionic sites in the membrane depends on the lipophilic salt content. The polymers such as poly (vinylchloride), polyurethane, polysiloxane, polyacrylamide, and cellulose used for the membrane fabrication play a role of a scaffold maintaining the liquid membrane. Since valinomycin is highly selective for potassium over sodium ions, it is the most frequently used natural ionophore. In nature, valinomycin is employed as a potassium ion-specific transporter through the cellular membrane, by binding and carrying the ions, resulting in reducing an electrochemical potential gradient across the membrane ([Pijanowska and Torbicz, 2008](#)). It is a macrocyclic molecule, obtained from the cells of *streptomyces* strains. Compounds that enhance transport of the ions across the lipid cellular membranes are lipophilic in nature. The great majority of ionophores are synthetic products. The design of synthetic carriers takes advantage of the different elements of molecular recognition. Cavities and clefts in the ionophores make them complementary to the size and charge of a particular ion. As mentioned above, the ion-selective membrane has a great impact on the parameters of potentiometric sensors: selectivity, sensitivity, and lifetime.

### 1.14.2 Nafion and cellulose acetate membranes

Immobilization of the biorecognition element is the key to the development of biosensors. Among the various enzyme immobilization protocols, an entrapment in polymer membranes is a general method for a variety of transducers. Nafion encapsulation of enzyme is a common practice to prepare biosensors. Nafion is a sulfonated tetrafluorethylene, negatively charged copolymer that has been widely used as a proton conductor for proton exchange membrane in fuel cells and biosensor applications, whereas cellulose acetate is used to form a neutral polymeric membrane. Polyelectrolyte nafion/cellulose acetate (CA) has found wide use for development of the enzyme-containing membranes. The main advantages of nafion in biosensor applications are its biocompatibility, excellent thermal and mechanical stability, mechanical strength, and antifouling properties. Nafion was an effective solubilizing agent for carbon nanotubes that yielded CNT-based biosensors exhibiting both the efficient electrocatalytic action of CNT toward hydrogen peroxide and the antifouling/discriminative properties of nafion films ([Lu et al., 2007](#)). The method

for membrane formation is a simple dipping of the electrode into the polyelectrolyte solution/CA in acetone or casting a small volume of the solution onto the electrode surface and allowing the solvent to evaporate. Optimal environment for glucose oxidase (GOD) in nafion/cellulose acetate membranes is achieved using an advanced immobilization protocol based on a nonaqueous immobilization route.

## 1.15 BIOSENSOR ELECTRODE FABRICATION TECHNIQUES

It has long been realized that advanced fabrication techniques are a key to the successful development of commercially viable biosensors in many applications. Fortunately, many technologies have been developed such as the microelectronics industry and therefore are available with much greater reliability and at a much lower cost than would otherwise be the case, although they obviously require certain modifications and considerable development.

### 1.15.1 Screen Printing

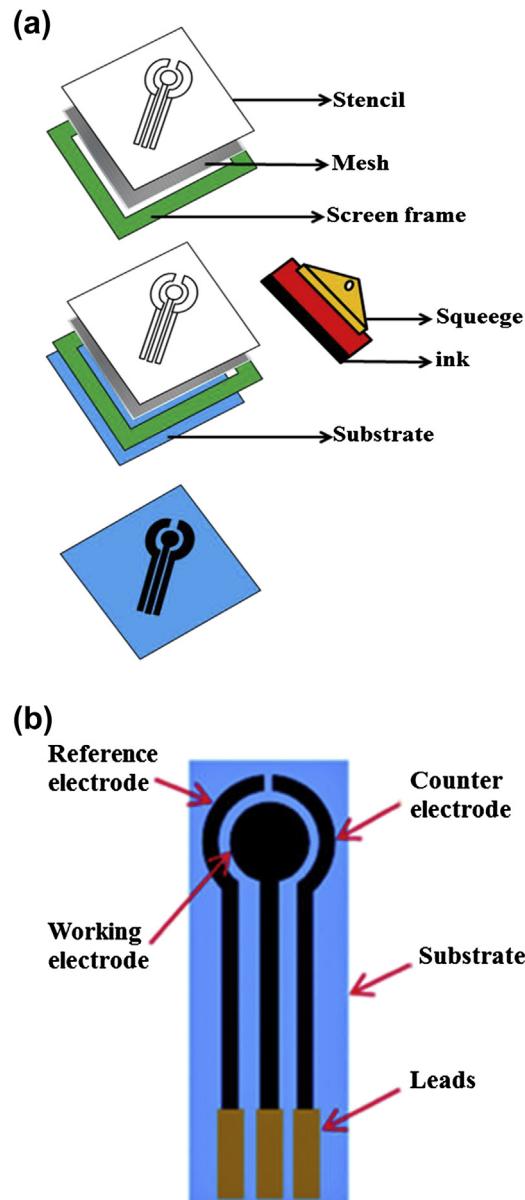
Screen printing is simply a process used for making multiple prints of the same design. Screen printing uses a stencil with open areas through which the ink passes and solid areas that act as a mask to prevent the passage of ink. Since the 1990s, screen-printing technology, adapted from the microelectronics industry, has offered large production of extremely inexpensive, highly reproducible, and reliable single-use sensors—a technique that holds great promise for on-site monitoring. Therefore, screen-printing technology is highly used for the serial production of disposable low-cost electrodes for the electrochemical determination of a wide range of substances. Screen-printed electrodes (SPEs) are devices that are produced by printing different inks on various types of plastic or ceramic substrates. Polyester screens are generally used for printing with patterns designed by the analyst in accordance with the analytical purpose in mind. The composition of the various inks used for printing on the electrodes determines the selectivity and sensitivity required for each analysis (Renedo et al., 2007).

Screen printing is the most commonly used fabrication method for base transducers.

Screen printing can be used with robust enzymes. A commercially available screen-printed electrode is shown in [Figure 1.24](#).

#### Substrates used in base transducers

- Polyester
- PVC
- Polycarbonate
- Polystyrene
- Alumina



**Figure 1.24** (a) Screen printing process (b) commercially available screen printed electrode.

### Screen print materials

Frames: Frames may be of wood, metal, or new environmentally friendly frames of recycled plastic. These are light weight, easy to clean, and easy to lock into hinge clamps. Frames will not soak up water, rot, or warp.

Screen mesh: Different mesh sizes are used for different applications in the screen-printing process. Multifilament and monofilament polyester are the products used for screen mesh. Mesh count is denoted by a number, which increases with the fineness of the mesh. A mesh count for monofilament is 160, which means there are 160 openings per inch.

Screen prep tape: This plastic tape is applied around the outer edges of the screen, making cleanup much easier.

XR blackout: This compound is ideal for touching up pinholes or register marks. Brushes on with a cotton swab or brush.

Artist's knife: An essential tool for cutting masking film.

Amber masking film: Used for artwork preparation and for handcutting positives. Easy stripping and very opaque.

Squeegees: A basic piece of equipment (rubber-like blade in a handle) used for flooding the screen with the ink and printing. Blades have varying degrees of hardness (durometer) for different printing jobs.

Hinge clamps: Features a wide-wing thumbscrew for firm and easy fastening to the screen frame. Easy to install, the clamp offers positive locking for perfect registry and precise control.

Spray adhesive: Applied to clean printing table to keep the paper in place and wrinkle-free during printing.

Screen strip: Mixes with water to form a solution that removes the stencil after printing.

### **Advantages of screen printing**

1. Cheap for small volumes—less than 1 billion sensors per year.
2. Film thickness can be high ( $>40\text{ }\mu\text{m}$ )
3. Other printing techniques give lower weights of deposit.

### **1.15.2 Liquid-handling techniques**

The ability to handle small volumes of liquids with high precision is one of the key areas in the development of some of the next generations of biosensors. As devices become smaller and more sophisticated, it becomes increasingly difficult to handle the analytical reagents involved in production. The liquid-handling biosensor devices allow detection of biomolecular interactions in liquid (Setford and Newmann, 2005). The use of labels is not required and the methods can be performed in a high-throughput manner. Some of the latest advances in transducer design, for example, make the production of 1 million measurement points on a  $1\text{ cm}^2$  chip a possibility. The most difficult aspect of the production of these devices is incorporating the biological reagents onto the surface of such arrays. Ink-jet techniques are suitable for depositing droplets of less than 1 nL in volume.

This can be achieved at very high speeds (kHz), but the resolution of the droplets is comparatively poor. In addition, although the volume appears very small, the droplet size of 50–100 µm is relatively large compared to the size of the transducer structures. Other liquid-handling techniques include automated syringe-type processes, the best known of which is often referred to as Cavro deposition and usually involves “touching off” a droplet onto a surface. Another method involves picking up reagents on a “pin” that possesses a concave head and depositing it onto the surface of the device, a technique adapted from pharmaceutical applications.

### **Liquid handling tools**

- Manual pipettes
- Electronic pipette and dispenser
- Automated systems

### **Manual pipettes**

Pipetting process

1. Spindle able to move between two mechanical stops
2. Aspirated volume varied by moving one stop
3. Pipette tip dipped into liquid
4. Spindle moves back and sucks in liquid
5. Tip placed above the target reservoir
6. Spindle pressed down to displace fluid

Limitations of manual pipettes

- Capillary forces suck in more fluid than wanted
- Liquid kept back at wall of tip due to adhesive forces
- Gas volume inside tip compressible (influenced by hydrostatic pressure of liquid)

Improvements in manual pipettes:

- Tips containing piston reduce gas dead volume
- Volume: 200 nl
- Accuracy: 10%

### **Syringe-based systems**

Pipetting process

1. Stainless steel plunger travels inside glass barrel actuated by stepper motor
2. Syringes having variable volumes available (2.5–500 µl)
3. Resolution: Stepper motor divides volume into 100,000 steps

Limitations of syringe-based systems:

- Adhesive forces
- Volume range below 200 nl is not accessible

## Nanopipette

1. Fabricated by silicon micromachining
  - a. Silicon/glass anodically bonded
  - b. Forming pump chamber
  - c. Piezodisk glued on the top of diaphragm
2. Liquid volume variable via nozzle size between 400 pl and 1 nl.
3. Nanopipette to be filled with system liquid.

### 1.15.3 Photolithographic techniques

#### Need for lithography

Simple layers of thin films do not make a device. To create a device such as a transistor, layers of thin films have to be patterned, etched, and coated. Lithography combines these processes and can create millions of devices in batch.

#### Lithography

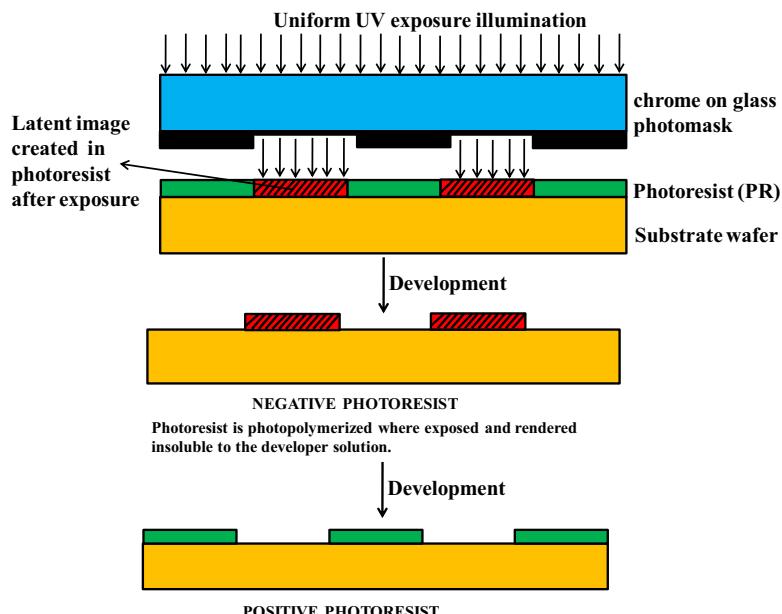
Lithography is the transfer of geometric shapes on a mask to a smooth surface. In modern semiconductor manufacturing, photolithography uses optical radiation to image the mask on a silicon wafer using photoresist layers. Other methods are electron beam, scanning probe, X-ray, and XUV lithography. Among the available lithographic techniques, photolithography is the most important technique in the cell fabrication.

#### Photolithography

Photolithography, also known as optical lithography, is a process by which images are photographically transferred to a matrix, i.e., microfabrication of a thin film over the substrate. It uses light to transfer a geometric pattern from a photomask to a light-sensitive chemical on the substrate. Photolithography is the process of transferring geometric shapes on a mask to the surface of a silicon wafer. This technique is widely used for fabrication of microfluidic cells and lab on chip ([Figure 1.25](#)).

#### Photolithographic process

1. Surface preparation—clean and dry wafer surface
2. Photoresist apply—apply a thin layer of photoresist to the wafer
3. Softbake—partial evaporation of photoresist solvents to promote adhesion
4. Alignment and Exposure—precise alignment of mask to wafer and exposure to UV light. Negative resist is polymerized.
5. Development—removal of unpolymerized resist
6. Hard bake—final evaporation of solvents
7. Develop inspection



**Figure 1.25** Photolithographic process.

8. Etch
9. Photoresist removal
10. Final inspection

The wafer is uniformly coated with a thick, light-sensitive liquid called photoresist. Photoresists are polymeric materials that can undergo a solubility change upon exposure with light of a specific wavelength. Portions of the wafer are selected for exposure by carefully aligning a mask between a UV light source and the wafer. In the transparent areas of the mask, light passes through and exposes the photoresist. Photoresist hardens and becomes impervious to etchants when exposed to UV light. The wafer is subjected to an etch process (either wet acid or plasma dry gas etch) to remove that portion of the nitride layer that is not protected by the hardened photoresist. This leaves a nitride pattern on the wafer in the exact design of the mask. The hardened photoresist is then cleaned and removed with another chemical.

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