CHAPTER 2
LITERATURE REVIEW

2.1. WHAT IS A BIOSENSOR?

The term “biosensor” is short for “biological sensor.” The device is made up of a transducer used to detect, usually a small quantity of specific analyte. It is composed of two parts. The first one is a biological element (e.g., enzyme, an antibody or a nucleic acid) that recognizes selectivity of the desired analyte (e.g., glucose, urea, toxic metal ions etc.). The bioelement interacts with the analyte being tested and the biological response is converted into an electrical signal by the transducer. The biological recognition system translates information from the biochemical domain, usually an analyte concentration, into a chemical or physical output signal with a defined sensitivity. 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 second component is a transducer that converts the recognition event into a readable signal (Koyum et al., 2012; Yoo et al., 2010; Yalcin et al., 2014). A commonly cited definition of a biosensor is: “A chemical sensing device, in which a biologically derived recognition is coupled to a transducer, to allow the quantitative development of some complex biochemical parameter or other words, it is a device used to detect and usually quantify a specific analyte.” The main function of a detector (transducer) is to transform the signal generated by interaction between the sensing element and the analyte of interest. The choice of the detector is dictated by the type of the physicochemical interaction between the sensing element and the analyte or by the biosensor application. Many types of transducers had been reported in the literature, including mechanical, thermal, electrical, optical or acoustic transducers (Grieshaber et al., 2008).

2.2. OPERATING PRINCIPLE AND VARIOUS DETECTION METHODS

A biosensor (Figure 2.1) is an analytical device which uses a biological or a biologically derived recognition system associated with a physico-chemical transducer to estimate the presence and/or concentration of the target substance by translating biological reactions into a quantifiable and a processible physical signal (Arya et al., 2006). The two most important characteristics of a biosensor are its
sensitivity and selectivity towards a target molecule (analyte). Selectivity depends only on properties of receptor element of the biosensor since that is where the analyte interacts with biosensor. Sensitivity is determined by both the biological compound and the transducer. For high sensitivity, it is important to have an excellent recognition of the analyte by the receptor element as well as a very efficient transduction of the signal to the output system.

Figure 2.1 A schematic diagram of the electron transfer in a typical biosensor

Level of glucose and dopamine can be monitored intermittently or continuously by spectroscopic, electrochemical, chromatographic and fluorimetric techniques. The electrochemical (Niu et al., 2012; Wang et al., 2014) methods are based on amperometry or potentiometry, whereas photonic methods are mainly polarimetry (Cote and Cameron, 1997; Patskovsky et al., 2008), spectrophotometry (Abbaspour et al., 2009), near-infrared Raman spectroscopy (Campanella et al., 2004), and other analytical techniques such as fluorimetry (Ammeraal et al., 1991), chromatography (Boduroglu et al., 2005), and titrimetry (Singh et al., 2000). Chromatographic method offers the selective detection of several biomolecules in a mixture (Singh et al., 2000), but this method is more time consuming. The titrimetric method also has several drawbacks such as long reaction time and difficulty in the determination of the end-point. To overcome this, high temperature and back titration are often used. Compared to the other methods, electrochemical methods have several advantages such as high selectivity and sensitivity, less time consuming, low cost and easy procedures (Atta et al., 2011).
2.3. TYPES OF BIOSENSOR

Biosensors can be grouped according to their biological element or their transduction element. Biological elements include enzymes, antibodies, microorganisms, biological tissue, and organelles. Antibody-based biosensors are also called immune sensors. When the binding of the sensing element and the analyte is the detected event, the instrument is described as an affinity sensor. When the interaction between the biological element and the analyte is accompanied or followed by a chemical change in which the concentration of one of the substrates or products is measured, the instrument is described as a metabolism sensor (Perumal et al., 2014; Mohanty et al., 2006). Finally, when the signal is produced after binding the analyte without chemically changing it but by converting an auxiliary substrate, the biosensor is called a catalytic sensor (Shaikh et al., 2012). The method of transduction depends on the type of physicochemical change resulting from the sensing event. Often, an important ancillary part of a biosensor is a membrane that covers the biological sensing element and has the main functions of selective permeation and diffusion control of analyte, protection against mechanical stresses, and support for the biological element. The most commonly used sensing elements and transducers are described below.

2.3.1. BIOSENSOR BASED ON BIO-RECOGNITION METHOD

Here, the biosensors are classified according to the nature (molecules, whole-cells, etc.) or function (affinity or catalysis) of the bio recognition element. Isolation and purification are mandatory for the best performance of the sensor. Several molecules or whole cells can function as bio recognition elements some of which are described below:

2.3.1.1. Enzymatic biosensors

Enzymes are proteins with high catalytic activity and selectivity towards substrates. Their commercial availability at high purity levels makes them very attractive for mass production of enzyme sensors. Their main limitations are pH, ionic strength, chemical inhibitors, and temperature, which affect their activity. Most enzymes lose their activity when exposed to temperatures above 60ºC. Most of the enzymes used in biosensor fabrication are oxidases that consume dissolved oxygen and produce hydrogen peroxide (Newman et al., 2006; Prodromidis et al., 2002). Enzymes have been immobilized at the surface of the transducer by adsorption,
covalent attachment, and entrapment in a gel or an electrochemically generated polymer, in bilipid membranes or in solution behind a selective membrane. Enzymes are commonly coupled to electrochemical and fiber optic transducers.

2.3.1.2. Antibody biosensors

Antibodies are proteins that show outstanding selectivity. They are produced by b-lymphocytes in response to antigenic structures, that is, substances foreign to the organism. Molecules larger than about 1 kDa can stimulate an immune response. Smaller molecules like vitamins or steroids can be antigenic (also called haptens) but they do not cause an immune response unless they are conjugated to larger ones like bovine serum albumin. Many antibodies are commercially available and commonly used in immunoassays (North et al., 1985). Antibodies are usually immobilized on the surface of the transducer by covalent attachment by conjugation of amino, carboxyl, aldehyde, or sulfhydryl groups. The surface of the transducer must be previously functionalized with an amino, carboxyl, hydroxyl, or other group (Long et al., 2013). Antibodies share similar limitations with enzymes. Furthermore, binding may not be reversible and regeneration of the surface may require drastic changes in conditions like low pH, high ionic strength, detergents, etc. Therefore, efforts are being made to produce low cost, single use sensors. Probably the main potential advantage of immune sensors over traditional immunoassays is that they could allow faster and in-field measurements. Immuno sensors usually employ optical or acoustic transducers (Conroy et al., 2009).

2.3.1.2. DNA Based Biosensors

DNA biosensors are commonly employed to detect specific sequences of DNA. They can reach high levels of selectivity and affinity based on the hybridization between a DNA target and its complementary probe, which is present either in solution or on a solid support. These systems can be based on optical or electrochemical detection (Conroy et al., 2009; Iui et al., 1997; Hu et al., 2008).

2.3.2. BIOSENSOR BASED ON TRANSDUCTION METHOD

Based on the signal transduction method, biosensors are classified as optical, colorimetric, electrochemical, acoustic, piezoelectric, etc. Of these optical and electrochemical sensors are the most important and therefore they are briefly described below.
2.3.2.1. Optical biosensors

Fiber optic probes on the tip of which enzymes and dyes (often fluorescent) have been co-immobilized are used. These probes consist of at least two fibers. One is connected to a light source of a given wavelength range that produces the excitation wave. The other, connected to a photodiode, detects the change in optical density at the appropriate wavelength. Surface plasmon resonance transducers, which measure minute changes in refractive index at and near the surface of the sensing element, have been proposed. Surface plasmon resonance (SPR) transducers have been proposed. SPR measurement is based on the detection of the attenuated total reflection of light in a prism with one side coated with a metal. When a p-polarized incident light passes through the prism and strikes the metal at an adequate angle, it induces a resonant charge wave at the metal/dielectric interface that propagates a few microns. The total reflection is measured with a photo detector, as a function of the incident angle (Fan et al., 2008; Patel et al., 2010; Haes et al., 2002).

2.3.2.2. Electrochemical biosensors

An electrochemical biosensor is a self-contained integrated device, capable of providing quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor), which is retained in direct spatial contact with an electrochemical transduction element (Thevenot et al., 2001). The biochemical signals can be used to generate a current/charge or may change conductivity between the two electrodes. The corresponding transduction device can be potentiometric, amperometric, and conductometric/impedimetric.

2.3.2.2.1. Potentiometric biosensors

Potentiometry is commonly used to measure glucose concentrations greater than $10^{-5}$ M, which is in the physiological range in most cases. The potential difference between the reference electrode and the indicator electrode is measured at zero current flow. The ideally non polarizable reference electrode provides a constant potential, while the indicator electrode shows an erratic potential depending on the concentration of the analytes. The zero current potential applied between the two electrodes is recorded as a function of the concentrations of target analytes in a logarithmic manner (Wang et al., 2008). Nernst equation describes potential of electrochemical cell as a function of concentrations of ions taking part in the reaction.
where, $Q$ is a reaction quotient, $n$ is number of electrons exchanged. For constant temperature expression $RT/F$ has a constant value. To simplify calculations, it is often combined with conversion factor between natural logarithm (denoted here by $\ln$) and decimal logarithm (denoted here by $\log$) to form value of $0.0591$ (for $25^\circ$C).

The capability for their continuous measurement is also an interesting possibility for environmental applications. The apparatus is inexpensive, portable, and is well suited for in situ measurements. The main disadvantage is the high limit of detection and the poor selectivity (Pohanka et al. 2008).

2.3.2.2.2. Amperometric sensors

Amperometry is based on the measurement of the current resulting from the electrochemical oxidation or reduction of an electroactive species. It is usually performed by maintaining a constant potential at platinum, gold or carbon based working electrode or an array of electrodes with respect to a reference electrode, which may also serve as the auxiliary electrode, if currents are low ($10^{-6}$ to $10^{-9}$ A).

The resulting current is directly correlated to the bulk concentration of the electroactive species or its production or consumption rate within the adjacent biocatalytic layer (Pohanka et al. 2008). As biocatalytic reaction rates are often chosen to be first-order dependent on the bulk analyte concentration, such steady-state currents are usually proportional to the bulk analyte concentration. This signal-transduction mechanism is frequently used for enzymatic and catalytic biosensors (Thevenot et al., 1999). The main advantage of this class of transducer is the low cost and hence the electrodes are dispensed after use. The high degree of reproducibility with these electrodes eliminates the cumbersome requirement for repeated calibration.

The type of instrument used for these measurements is also easy to obtain and can be inexpensive and compact, allowing for the possibility of in situ measurements. Limitations for this signal transduction mechanism include the potential interferences to the response if several electroactive compounds generate false current. These effects have been eliminated in clinical applications through the use of selective membranes, which carefully control the molecular weight or charge of the compounds that have access to the electrode (Habermuller et al., 2000).
2.3.2.2.3. Conductometric / Impedimetric Biosensors

It is used to measure the ability of an analyte (e.g. electrolyte solutions) or a medium (e.g. nanowires) to conduct an electrical current between electrodes or reference nodes. The measured parameter is the electrical conductance/resistance of the solution. When electrochemical reactions produce ions or electrons, the overall conductivity or resistivity of the solution changes and the same is measured and calibrated to a proper scale. Conductance measurements have relatively low sensitivity. The electric field is generated using a sinusoidal voltage (AC), which helps in minimizing undesirable effects such as Faradaic processes, double layer charging and concentration polarization (Iqbal et al., 2012; Renault et al., 2008; Janata et al., 2009). Table 2.1 shows the common biological elements and transducer used for the biosensor fabrication.

Table 2.1 Biological elements and transducers commonly used in the fabrication of biosensors

<table>
<thead>
<tr>
<th>Biological elements</th>
<th>Transducers</th>
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<tbody>
<tr>
<td>Enzymes</td>
<td>Electrochemical</td>
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<td>Antibodies</td>
<td>Amperometric</td>
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<td>RECEPTORS</td>
<td>Potentiometric</td>
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<tr>
<td>Cells</td>
<td>Ion selective</td>
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<tr>
<td>Membranes</td>
<td>Field effect transistors</td>
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<tr>
<td>Tissues</td>
<td>Conductometric</td>
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<tr>
<td>Organisms</td>
<td>Optical</td>
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<tr>
<td>Organelles</td>
<td>Fiber optic (optrode)</td>
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<tr>
<td>Nucleic acids</td>
<td>Surface plasmon resonance (SPR)</td>
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<tr>
<td>Organic molecules</td>
<td>Fiber optic SPR</td>
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<td></td>
<td>Calorimetric</td>
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<td>Heat conduction</td>
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<td>Isothermal</td>
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<td>Isoperibol</td>
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<td></td>
<td>Acoustic</td>
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<td>Surface acoustic wave</td>
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<td>Piezocrystal</td>
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<td></td>
<td>Microbalance</td>
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2.4. BRIEF HISTORY OF ENZYMATIC BIOSENSORS

Currently, enzymatic glucose sensors dominate the biosensing industry, particularly with the ever advancing development of self-testing and continuous monitoring of blood glucose. In the history of enzymatic glucose sensors, it was developed by Clark and Lyons of the Cincinnati Children’s Hospital in 1962 (Wang, 2008), which relied on a thin layer of glucose oxidase (GOx) entrapped over an oxygen electrode via a semipermeable dialysis membrane. Measurements were made based on the monitoring of the oxygen consumed by the enzyme-catalyzed reaction (equation 2.2).

\[ \text{Glucose} + O_2 \rightarrow \text{Gluconic acid} + H_2O \]  \hspace{1cm} \text{2.2}

A negative potential was applied to the platinum cathode for a reductive detection of the oxygen consumption (equation 2.3).

\[ O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \]  \hspace{1cm} \text{2.3}

Clark’s original patent (Toghill et al., 2010) covers the use of one or more enzymes for converting electro-inactive substances to electro-active products. The effect of interference was corrected by using two electrodes, one of which was covered with the enzyme, the other a blank one and by measuring the differential current.

2.4.1. FIRST GENERATION GLUCOSE BIOSENSOR

The first generation of glucose biosensors was prepared by the catalytic principle of natural mediator (i.e. oxygen). The consumption of oxygen was followed by the electrochemical reduction at a platinum electrode. The cell current is proportional to the oxygen concentration and the current is measured (amperometric method of detection has been employed). The concentration of glucose is then proportional to the increase in current (oxygen concentration). Other than measuring the oxygen concentration, two other methods can also be used to measure the glucose concentration: measuring the acid (gluconic acid) production with a pH sensor or measuring the production of H2O2 with a peroxide sensor (Cash et al., 2010; Wang, 2001). The routine use of a first-generation glucose biosensor has been hindered by two major limitations. The first limitation originates from the fact that the amperometric monitoring of hydrogen peroxide requires high operating potential. The second limitation stems from the restricted solubility of oxygen in biological fluids.
that limits the enzymatic reaction, especially in the case of implantable glucose biosensors suitable for in vivo measurements.

2.4.2. SECOND GENERATION GLUCOSE BIOSENSOR

Due to the above mentioned limitations in first generation of glucose sensors, it was overcome by the development of second generation glucose sensors. In second-generation glucose biosensors, man-made mediators were doped into the enzyme membrane, which can decrease the interference of ambient oxygen. The improvements were achieved by replacing oxygen with a non-physiological electron acceptor, called redox mediator, which was able to carry electrons from the enzyme to the surface of the working electrode (Yoo et al., 2010). Using redox mediators, the measurements became insensitive to oxygen fluctuations and can be carried out at lower and more negative potentials. Organic and organometallic redox compounds have been used as electron mediators.

2.4.3. THIRD GENERATION GLUCOSE BIOSENSOR

The third generation of glucose biosensors involves direct electron transfer between the enzyme and the electrode without mediators. Instead of mediators with high toxicity, the electrode can perform direct electron transfers using organic conducting materials based on charge-transfer complexes (Khan et al., 1996). Using new electrode materials, such as conducting organic salt and conducting organic substances, the electrode can perform direct electron transfer. The modification of glucose oxidase with an appropriate electron relay has been successfully employed to the electrode with a long chain polymer having a dense array of electron relays, which is flexible enough to fold along the enzyme structure. Ultimately, this third generation of glucose biosensors would lead to implantable, needle-type devices for continuous in vivo monitoring of blood glucose (Wang et al., 2013). Such devices would offer improved control of diabetes in connection with an internal insulin release system. However, so far its full potential has not been reached.

2.5. NON-ENZYMATIC BIOSENSORS

Though the enzymatic glucose sensors are extensively studied and applied, the most common and serious problem with these sensors is insufficient long-term stability, which originates from the intrinsic nature of the enzymes, lower reproducibility and influence of oxygen limitation (Toghill et al, 2010). Further, a complicated procedure, including adsorption, cross-linking, entrapment, and electro
polymerization, is required for the immobilization of the enzyme on the solid electrode (Malitesta et al., 1990; Guerrieri et al., 1998; Tang et al., 2004; Cosnier, 1999), and this may decrease the activity of the GOx. Because the sensitivity of these glucose sensors essentially depends on the activity of the immobilized enzymes, reproducibility is still a critical issue in quality control (Dong et al., 2015). On the other hand, there are many advantages in using non-enzymatic sensors in the electrochemical methods for the detection of glucose including stability, simplicity, reproducibility, low cost, and no oxygen limitation (Zhang et al., 2015). Considering these aspects, the enzymeless glucose sensor is an attractive alternative technique. Numerous nanomaterials like ZnO nanoparticles (Hu et al., 2011), Au nanowires (Cherevko et al., 2009), NiO nanofibers (Zhang et al., 2012), flake like Pt-Pd (Niu et al., 2012), CuO microfibers (Cao et al., 2012) and ruthenium nanowires (Chi et al., 2009) have been used as non-enzymatic electrochemical glucose sensors. But the toxicities of the heavy metal elements involved prevented these electrodes from being put to practical applications. Even state-of-the-art technology for glucose sensors using Pt electrodes is not free from setbacks like poisoning by adsorbed intermediates (Sheng et al., 2015), low sensitivity and poor selectivity. Pt-Pb alloy nanoparticles electrodeposited onto multiwalled carbon nanotubes (MWCNTs) have been used as a testing electrode for glucose oxidation. It produced a much higher current density than either Au or Pt counterpart in both neutral and alkaline solutions.

During recent years, nanomaterials such as carbon nanotubes (CNTs) and transition metal nanoparticles (NPs) have been widely applied in sensors and biosensors. CNTs have become an attractive material of electroanalysis due to their high surface/volume ratio and chemical stability (Yang et al., 2015). Transition metal NPs, including Au, Pt, Pd, Cu, Ni and Ag are used to increase the electrocatalytic activities. Sensors and biosensors modified with metal NPs reveal good performances like enhanced mass transport, catalysis, good biocompatibility and control over the electrode microenvironment when compared with macro electrodes (Li et al., 2015; Wang et al., 2015; Li et al., 2014).

2.6. APPLICATION OF BIOSENSORS

One of the major driving forces for the development of biosensors is biomedical diagnosis. The most popular example is glucose oxidase-based sensor used by individuals suffering from diabetes to monitor glucose levels in blood. Biosensors
have also found potential applications in the agricultural and food industries. However, very few biosensors have been commercialized. There is multiple numbers of potential applications based on biosensors. A few examples are given below:

- Monitoring glucose for diabetic patients (historical market driver).
- Other health/medical-related targets.
- Food Industry.
- Determination of toxic substance levels.
- Environmental applications (e.g., detection of pesticides in ecosystems and water resident contaminants).

2.6.1. ELECTROCHEMICAL SENSING OF GLUCOSE IN DIABETES MANAGEMENT

Diabetes is one of the serious diseases which can cause severe complications such as lower limb amputations, blindness and cardiovascular disease, caused by the high levels of blood glucose from a total or partial lack of insulin. There are about over 100 million people suffering from diabetes in the world. According to the data released by the American Diabetes Association, 6% of the general U.S. population over age 40 has been found to have diabetes and an equal amount has not been diagnosed yet. Although there is no efficient way to cure diabetes, disease associated complications can be reduced through the tight control of blood glucose levels. Sensitive and accurate detection of glucose has many applications as the concentration of glucose is an informal indicator in many diseases such as diabetics and other endocrine metabolic disorder (Zhang et al., 2012; Jusoh et al., 2012; Liu et al., 2013). Treatment has been a far more sophisticated science, with self-testing becoming more compact and hence accurate determination requires a close monitoring of blood glucose levels (Xu et al., 2010; Zhu et al., 2013). As a result, exploration of fast and reliable methods for glucose concentration monitoring in the treatment of diabetes is of great interest. To facilitate the resolution of inherent problems with discrete blood glucose measurement, new commercial products focus on continuous glucose interrogation.

2.6.2. FOOD INDUSTRY

Biosensors for the measurement of carbohydrates, alcohols, and acids are commercially available. These instruments are mostly used in quality assurance laboratories or at best, on-line coupled to the processing line through a flow injection
analysis system. Their implementation in-line is limited by the need of sterility, frequent calibration, analyte dilution, etc. Potential applications of enzyme based biosensors to food quality control include measurement of amino acids, amines, amides, heterocyclic compounds, carbohydrates, carboxylic acids, gases, cofactors, inorganic ions, alcohols, and phenols (Mello and Kubota 2002). Biosensors can be used in industries such as wine, beer, yogurt, and soft drink producers. Immunosensors have important potential in ensuring food safety by detecting pathogenic organisms in fresh meat, poultry, or fish.

2.7. CHARACTERIZING THE BIOSENSOR DEVICES

Characterization is necessary to establish understanding and control of nanoparticle synthesis and their applications.

2.7.1. CYCLICVOLTAMMETRIC AND CHRONOAMPEROMETRIC MEASUREMENTS

**Principles of cyclic voltammetry**

Cyclic voltammetry (CV) is a widely used electroanalytical technique. It has wide applications in the study of redox processes, electrochemical properties of analytes in solution, and for understanding the reaction intermediates. The common characteristics of all voltammetric techniques is that they involve the application of a potential (E) to an electrode and the monitoring of the resulting current (I) flowing through the electrochemical cell. In many cases, the applied potential is varied or the current is monitored over a period of time (t). Thus all voltammetric techniques can be described as some function of E, I, and t. The CV studies are carried out by scanning the potential of the working electrode in a cyclic manner and switching it in reverse. The flowing current can be measured through the working electrode. The resultant trace of current against potential is termed as a voltammogram. During cyclic voltammetry measurement, the potential is ramped from an initial potential, \( E_i \) to a more negative potential but, at the end of its linear sweep, the direction of the potential scan is reversed, usually stopping at the initial potential \( E_i \) (or it may commence an additional cycle) (Bieniasz, 2015). The potential is usually measured between the reference electrode and the working electrode and the current is measured between the working electrode and the auxiliary electrode, also known as the counter electrode. This data is then plotted as current versus potential as shown in Figure 2.2.
The forward scan produces a current peak for any analyte that can be reduced (or oxidized depending on the initial scan direction) through the range of the potential scanned. The current increases as the potential reaches the reduction potential of the analyte, but then decreases as the concentration of the analyte is depleted close to the electrode surface. If the redox couple is reversible, then reversing the applied potential makes it reach a potential that re-oxidizes the product formed in the first reduction reaction, thus producing a current of reverse polarity from the forward scan. The oxidation peak usually has the same shape as that of the reduction peak. As a result, the information about the redox potential and the electrochemical reaction rates of compounds can be obtained. For instance, if the electronic transfer at the surface is fast and the current is limited by the diffusion of species to the electrode surface, then the current peak will be proportional to the square root of the scan rate. The important parameters in a cyclic voltammogram are the peak potentials ($E_{pc}$, $E_{pa}$) and peak currents ($I_{pc}$, $I_{pa}$) of the cathodic and anodic peaks, respectively. If the electron transfer process is fast compared with other processes (such as diffusion), the reaction is said to be electrochemically reversible, and the peak separation is

$$\Delta E_p = |E_{pa} - E_{pc}| = 2.303RT/nF$$  \hspace{1cm} 2.4

Thus, for a reversible redox reaction at 25°C with $n$ electrons, $\Delta E_p$ should be $0.0592/n$ V or about 60 mV for one electron. In practice, this value is difficult to attain because of factors like cell resistance. Irreversibility due to a slow electron
transfer rate results in $\Delta E_p > 0.0592/nV$, greater, say, than 70 mV for a one-electron reaction (Kissinger and Heineman 1983). The diagnostic tests for electro-reversibility are listed in Table 2.2.

**Table 2.2** Diagnostic tests for the electrochemical reversibility of a redox couple, carried out by cyclic voltammetry

1. $I_{pa}/I_{pc} = 1$
2. The peak potentials, $E_{pc}$ and $E_{pa}$, are independent of the scan rate $v$
3. The formal potential ($E^{0'}$) is positioned midway between $E_{pc}$ and $E_{pa}$, so $E^{0'} = (E_{pa} + E_{pc})/2$
4. $I_p$ is proportional to $v^{1/2}$
5. The separation between $E_{pc}$ and $E_{pa}$ is 0.0592/nV for an n-electron couple (i.e. $\Delta E_p = |E_{pa} - E_{pc}| = 0.0592/nV$)

For a reversible reaction, the concentration is related to peak current by the Randles-Sevcik expression (at 25°C) (Streeter et al., 2008):

$$I_p = 2.689 \times 10^5 n^{3/2} AD^{1/2} C^0 v^{1/2}$$

where $I_p$ is the peak current in amperes, $n$ is the number of electrons transferred, $A$ is the electrode area (cm$^2$), $D$ is the diffusion coefficient (cm$^2$s$^{-1}$), $C_0$ is the concentration in mol cm$^{-3}$, and $v$ is the scan rate in Vs$^{-1}$.

**Principles of amperometry**

Amperometry is based on measurement of the intensity of the current resulting from the electrochemical oxidation or reduction of an electroactive species under an imposed potential. The change in current is usually measured as a function of time. It is usually performed by maintaining a constant potential at platinum, gold or glassy carbon working electrode where the oxidation or reduction of a species takes place with respect to a reference electrode. A three electrode conventional system can be used as in voltammetry. During electrolysis, the working electrode may act as an anode or a cathode, according to the nature of the analyte. The potential of the working electrode is adjusted to a value of the plateau of the voltammetric wave. For a simple electron transfer process, it is always possible, by applying a sufficiently
high over potential, to make the rate of the electron transfer step greater than the mass transport. The current flowing in the cell will then reach a mass transport limited plateau value, which will increase in magnitude if the solution is stirred, or the electrode is rotated or vibrated as in the case of rotating disk electrode (RDE). The resulting current is directly correlated to the bulk concentration of the electroactive species or its production or consumption rate within the adjacent biocatalytic layer. As biocatalytic reaction rates are often chosen to be first order dependent on the bulk analyte concentration, such steady-state currents are usually proportional to the bulk analyte concentration. Figure 2.3 shows the waveform for a step experiment, which is the basis concept in chronoamperometry experiment. The potential of the working electrode is stepped from a value $E_1$ at which the oxidized or reduced species is electroinactive (no faradaic reaction occurs), to the value of $E_2$, where the oxidation or reduction process occurs. At this time, the surface concentration of the electroactive species is effectively zero.

![Figure 2.3](image)

**Figure 2.3** (a) The variation of applied potential in a potential step experiment, (b) The current response vs. time

At the same time, a large current is detected, which falls steadily with time (Figure 2.3a). This arises since the mass transport under this condition is controlled by the rate of diffusion of the electroactive species to the electrode surface. The initial process has created an extremely large concentration gradient as well as large flowing current, since there has been little time for any depletion of the electroactive species.

Chronoamperometry is often used to measure the diffusion coefficient of electroactive species or the surface area of the working electrode according to the Cottrell equation:
\[ i = nFACD^{1/2} \pi^{-1/2} t^{-1/2} \]

where, \( n \) is the number of electrons involved in redox reaction, \( F \) is the Faraday constant (96,500 C/mol), \( A \) is the electrode area (cm\(^2\)), \( C \) is the bulk concentration (mol/cm\(^3\)), \( D \) is the diffusion coefficient (cm\(^2\)/s) and \( t \) is the time (s). However, this Cottrell equation is only valid for mass transport-limited currents. It could also be applied to study the mechanisms of electrode processes (Grieshaber et al., 2008; Wang et al., 2008).

2.7.2. DIFFERENTIAL PULSE VOLTAMMETRY

Differential Pulse Voltammetry (DPV) is considered as a series of regular potential pulses super imposed on a linearly changing potential applied to the working electrode at a time just before the end of the drop. The resulting current is measured between the ramped baseline potential and pulse potential. A digital staircase potential is commonly used as the ramped baseline. DPV sampled the current twice in each pulse period, just before the pulse and at the end of the pulse application, at which when the charging current has decayed (Figure 2.4).

![Figure 2.4](image)

**Figure 2.4** Potential-excitation signal and voltammogram for differential pulse polarography. The current is sampled at the time intervals shown by black rectangles. When measuring a change in current, \( \Delta i \), the current at point 1 is subtracted from the current at point 2. The symbols in the diagrams are as follows: \( \tau \) is the cycle time; \( \Delta E_p \) is a fixed or variable pulse potential; \( \Delta E_s \) is the fixed change in potential per cycle, and \( t_p \) is the pulse time.

Differential pulse voltammetry is a similar technique to CV, where a small pulse is superimposed onto a single linear forward scan, with no reverse scan as shown in Figure 2.4. The current is measured just before each pulse (ia) and just before the end (ib) of each pulse and the resultant difference in current (ib-ia) is plotted versus the linear sweep voltage. DPV can be loosely thought of as the differential of the forward
scan of the corresponding CV. Again the peak current will be proportional to the concentration of the electroactive moieties of interest, but we can no longer determine if the system is reversible. The main advantage of using DPV is the elimination of background charging currents, resulting in generally higher sensitivity and better peak resolutions than CV (Farghaly et al., 2014).

2.7.3. ELECTROCHEMICAL IMPEDANCE SPECTROSCOPY

Impedance can be understood to have a similar concept of electrical resistance. Electrical resistance is the ability of a circuit element to resist the flow of electrical current. According to Ohm’s law, resistance is defined in terms of the ratio between voltage, $V$, and current, $I$:

$$ V = IR $$

This relationship is limited to only one circuit element which is the ideal resistor. However, the real world may contain circuit elements that exhibit much more complex behaviour. In this case, we use impedance, which is a measure of the ability of a circuit to resist the flow of electrical current that is made of combinations of resistors, capacitors or inductors. Electrochemical impedance is usually measured by applying an AC potential to an electrochemical cell and measuring the current through the cell. Impedance spectroscopy is a very versatile tool used in different fields (biosensors, electrode kinetics and mechanisms, semiconductor electrodes, corrosion, etc.). The Electrochemical Impedance Spectroscopy (EIS) is a more general concept of resistance. In direct current (DC) circuits, only resistors oppose the flow of electrons. In alternating current (AC) circuits, the capacitors (impedance) influence the flow of electrons in addition to resistors. Electrochemical impedance spectroscopy is usually measured by applying an AC potential with small amplitude (5 to 10 mV) to an electrochemical cell and measuring the current flowing through the working electrode. The advantage of EIS is that the electrochemical cell can be modeled by using a purely electronic model. An electrode - electrolyte interface undergoing an electrochemical reaction is treated as an electronic circuit consisting of a combination of resistors and capacitors (Lisdat and Schafer 2008; Macdonald, 2005; Vladikova, 2004). Frequently, a Randles circuit presented in Figure 2.5 is used.
It consists of a solution resistance ($R_s$) in series with the parallel combination of the double-layer capacitance ($C_{dl}$) and an impedance of the Faradaic reaction of interest, which consists of charge transfer resistance ($R_{ct}$) and the so-called Warburg diffusion element $W$. To obtain the Randles circuit parameters, the experimental data are fitted to the model circuit using the non-linear least-squares procedures, which are available in the modern EIS software. Typically, the result of such fitting is presented in the form of the Nyquist plot (Figure 2.5). Such plot displays the kinetic control in the region of high frequencies and a mass transfer (Warburg diffusion) control at low frequencies. The charge transfer resistance of our modified electrodes $R_{ct}$ is determined by measuring the diameter of a semicircle (Figure 2.6), which is proportional to the $R_{ct}$. 

Figure 2.5 Typical fitting Nyquist plot

Figure 2.6 Typical fitting according to Nyquist plot