CHAPTER 2 LITERATURE REVIEW

2.1 Immunoassays and Immunosensors

Immunoassays are biological tests which allow the detection of antibody-antigen binding. Because of the specific bonds formed between the antigen and antibody, immunoassays are capable of directly identifying specific analytes in complex solutions [20]. Immunoassays are widely used in clinical diagnoses and in the field of biochemistry [21, 22]. The detection of the analyte typically depends on the identification of a label which is activated to signal the presence of the antibody-antigen binding. This can often take the form of a radioisotope (radioimmunoassay, RIA) [23], a chemiluminescent compound (chemiluminescence immunoassay, CLIA) [24], or enzymes (enzyme immunoassay, EIA [25], or an enzyme-linked immunosorbent assay, ELISA [26]). ELISA is one of the most frequently used methods of detecting antigens or antibodies against viruses or bacteria in the field of clinical immunology [27]. However, although ELISA is used for a variety of applications, it has limitations in detecting certain human diseases or weak concentrations of contaminants contained within various foods and agricultural products. False negative results are a consequence when ELISA testing fails to detect a signal when the contaminant concentrations in the sample falls below a certain threshold. One such example is the ELISA test for the *Salmonella* Typhimurium bacteria which causes illnesses via food contamination. The limit of detection (LOD) is 10^6-10^7 CFU mL^{-1} [28, 29] which implies a strong requirement for strategies to overcome this shortcoming in the ELISA technique.

Greater sensitivity, speed and selectivity in detection of the antigen-antibody binding reaction may be permitted through the introduction of biosensor technology. Direct observation of immune reactions would allow new insights and serve to overcome previous limitations.

2.1.1 Biosensor Technology

Biosensors comprise two parts: the first is a biological detector molecule and the second is a signal transducer which indicates when the analyte has formed a bond with the receptor molecule (Figure 2.1). The receptor molecules are typically selected from a range including antibodies, peptides, enzymes, micro-organisms or oligonucleotides (DNA) [30].

When a ligand is bound to the receptor, a signal is given in the form of a physical or chemical change which can then be detected by the transducer. The signal may occur through a change in pH, refractive index, electron transfer, heat transfer or the release of specific ions or gases [31]. The resulting signal can be amplified and processed, allowing a quantification of the receptor-ligand binding to take place [32].

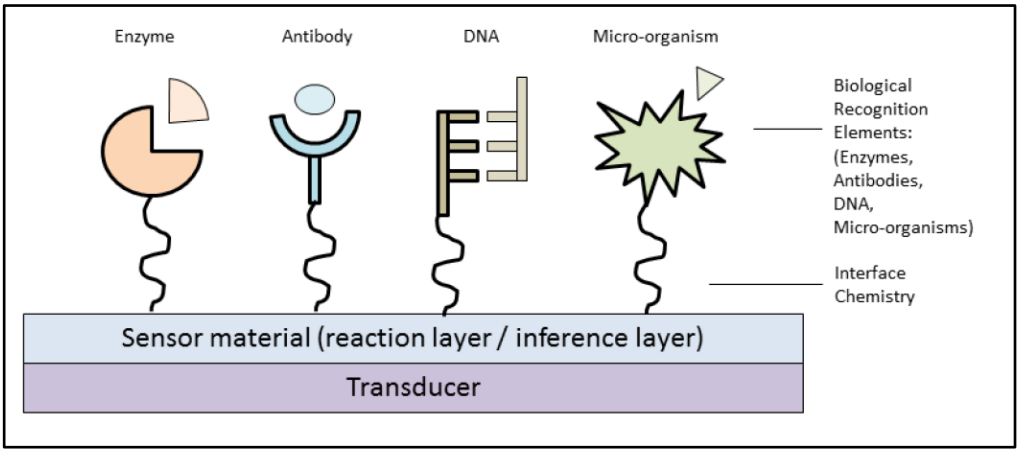

Figure 2.1 Components of a biosensor.

Transducers typically take four possible forms, depending upon the type of change they are able to detect. The changes can take place in electrochemical properties (amperometric, potentiometric, conductimetric), in mass (piezoelectric or acoustic waves), in heat (calorimetric), or in optical properties (luminescent, fluorescent, reflective, ellipsometric, surface plasmon resonance (SPR), waveguide) [33].

2.1.2 Immunosensors

Immunosensors are biosensors which detect and quantify the binding interactions between antibodies and antigens [3]. Immunosensors work in a manner similar to regular immunoassays, and are usually based on the solid-phase principle whereby antibodies or antigens are immobilized at the sensor surface [2]. Immunosensors can detect and measure a wide range of analytes, including hormones [34], drugs [35] and microorganisms [36] as medical diagnostic markers or contamination in foods, and also pollutants such as pesticides [37].

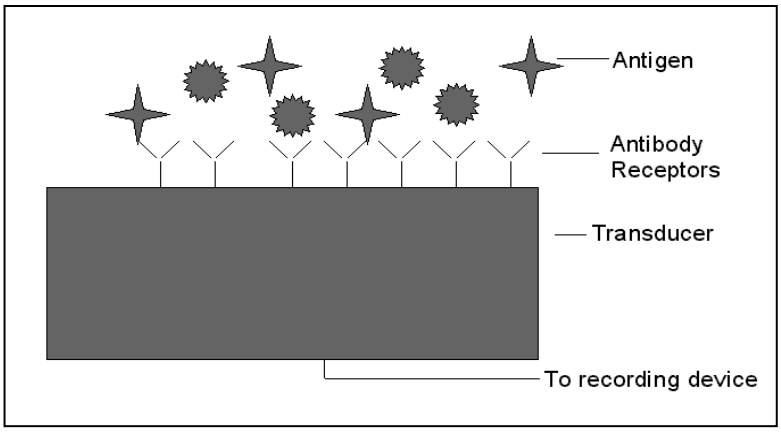

Figure 2.2 Diagrammatical representation of an immunosensor device.

An immunosensor's sensitivity and specificity are based on the affinity and specificity of the binding agent and are also determined to a certain extent by the background noise attributable to the transducer [38]. One way to improve sensitivity is to incorporate new labels within the immunosensor design which is considered indirect detection [39]. Indirect sensors do not, by definition, directly monitor the antibody-antigen reaction, but the signals generated are typically of a magnitude or intensity in proportion to the target analyte (usually an antigen, though sometimes the antibody can be the target). The primary objectives in terms of immunosensor design in recent years have been to enhance sensitivity, selectivity, practicality and speed, while minimizing costs and simultaneously allowing for the use of a wide range of transducer systems [40].

2.1.3 Electrochemical Immunosensors

Electrochemical biosensors are used to analyze biological samples in which a biological reaction or process results in the creation of an electrical signal. Detection of this electrical signal by the biosensor confirms the recognition of the biological event causing the signal [41]. Electrochemical biosensors comprise biological elements as the sensitive components, an electrode, a conversion device and an electric current as the signal to be detected [42]. The substrate of the sensor typically has three electrodes [43]. The first is a reference electrode; the second is a working electrode and the third is a counter electrode. The reaction involving the target analyte occurs on the working electrode, causing an electron transfer across the surface. The current, which is a flow of electrons at a rate in proportion to the concentration of the analyte, can be quantified. Electrochemical immunosensors are of a type which usually offer high levels of sensitivity, rapidity and control [4]. Electrochemical biosensors can be categorized into three kinds – amperometric, potentiometric or conductometric [44]– depending on whether the sensor measures current, potential or resistance.

I. Potentiometric Transducers

The underlying principle of a potentiometric transducer relies on the accumulation of a membrane potential created by the selective binding of ions to a sensing membrane [45]. The transducer allows the change in potential to be determined and hence the concentration to be calculated through a logarithmic relationship. Potentiometric transducers were first reported in 1975 for the purpose of monitoring an immunochemical reaction and measuring the potential change when the antibody-antigen bond was formed on the electrode [46].

II. AmperometricTransducers

Amperometric transducers are widely used in biosensing [47]. They are designed to measure the flow of current through an electrochemical cell at a constant voltage. The current is produced as a consequence of the redox reaction of the analyte at the electrode, and is of a magnitude in direct proportion to the concentration of the analyte at the surface of the electrode. Amperometric detection for immunosensors was first

reported in 1979 [48], when an electrochemical immunoassay was applied to detect human chorionic gonadotropin (hCG) . The test worked by immobilizing a monoclonal antibody to hCG at oxygen electrode, where hCG within the sample and catalase-labelled hCG could bind. The limit of detection (LOD) in this particular assay was 20 IU/L.

III. ConductimetricTransducers

Chemical reactions often produce or consume ionic species and in doing so can change the electrical conductivity of a sample solution [49]. Such chemical systems can therefore be tested using conductimetric transducers. This type of biosensor can operate by immobilizing an appropriate enzyme over a set of metal electrodes [50] – typically comprising gold, silver, chromium, copper or nickel – and then assessing the conductance change when an electrical field is applied to a solution which contains the target analyte. One example of conductimetric immunosensor is the immunoassay for pesticides using a conductive polymer-based immunosensor with electro conductive solid support [51].

2.1.4 Mass-Detecting Immunosensors

Piezoelectric biosensors offer a means of detecting changes in the mass of coated quartz crystals through measuring the frequency of oscillation [52]. The crystals are coated using an adsorbent which is able to interact selectively with the analyte, resulting in a change of mass. The mass is related to the oscillation frequency, which is in turn proportional to the concentration of the analyte. Sensors using piezoelectric crystals can be used to monitor interactions between antibodies and antigens when the crystals are coated with the antibody and the extent of antigen binding can be measured. One particular example gave an LOD of 43 mg/L for IgG [53].

2.1.5 Optical Immunosensors

An optical transducer can be used to detect the response from ultraviolet or visible radiation chemiluminescence [54]. An optical transducer can be modified to operate either with or without the use of a labelled reagent to produce the signal. Those which do use labels typically require an enzyme or fluorophore to produce the signal [55], but must be highly sophisticated on account of the low light levels which must be detected. Accordingly, direct optical sensors which do not use labelling form the majority of the immunosensors currently in use [2]. The most common types are those using attenuated total internal reflection[56], ellipsometry [57] , SPR [57] and monomode dielectric waveguides [58] . It is also possible to incorporate labels within these immunosensors in order to produce greater sensitivity [59].

2.1.6 Nanostructured Materials for Electrochemical Immunosensors

One of the specific goals of using an electrochemical immunosensor is to raise the level of sensitivity beyond that of conventional immunoassay. This can be accomplished by improving the electrochemical signal by using nanomaterials [40]. Carbon nanotubes [5], gold nanoparticles [6], graphene [7] and many types of nanoparticles offer potential enhancements in electrochemical signal performance according to a number of reports. Examples of the works that use nanomaterials for immunosensors are described as following;

Figure 2.3 below shows a report of the immobilization of biotinylated capture antibodies onto streptavidin-modified magnetic particles[60]. The technique uses a sandwich-type immunoassay in which the analyte target (prolactin, PRL) and the anti-prolactin antibodies are labelled using alkaline phosphatase (AP). A bioconjugate is duly produced and subsequently captured at the surface of the carbon SPE using a magnet. It is possible to quantify the PRL using differential pulse voltammetric (DPV) determination of 1-naphtol which is formed upon 1-naphtyl phosphate additions. The limit of detection (LOD) was found to be 3.74 ng/mL.

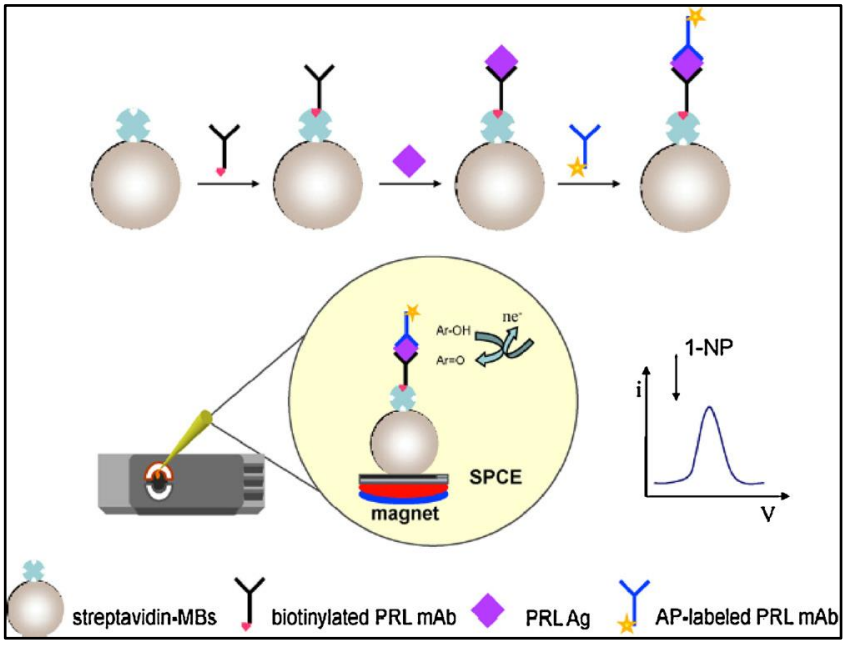

Figure 2.3 Streptavidin-modified magnetic particles with magnetized carbon SPE for use as an immunosensors [60].

In Figure 2.4 below, describes work that uses a DNA barcode and monoclonal antibody conjugated with magnetic nanoparticle modified polystyrene latex for ultrasensitive electrochemical immunosensors [61]. The resulting conjugate is an electrochemical label for *S. entericaserovar Typhimurium* detection. In the system, the antigen and biotinylated-pAb are magnetically collected. The biotinylated immunocomplex is then able to bind onto the avidin modified screen printed electrode (SPE), before silver enhancement amplifies the signal. The DPASV method allows quantification at an LOD of 12 CFU mL^{-1}.

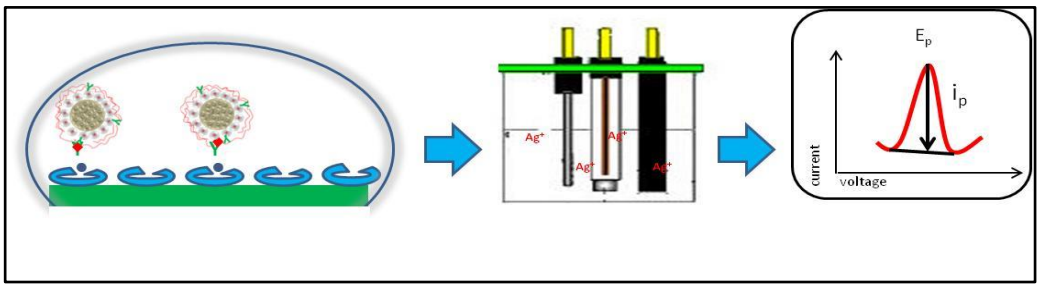

Figure 2.4 Silver amplification of biobarcode labelling for immuno-detection on SPE [61].

2.2 Label-Free and Label-Based Electrochemical Biosensors

2.2.1 Label-Free Electrochemical Biosensors

The benefits of label-free electrochemical immunosensors lie in the simplicity of their approach to targeting biomolecules [62]. By way of illustration in Figure 2.5, Kerman and colleagues described a label-free immunosensor that able to detect the hCG hormone (human chorionic gonadotropin) [63]. The system used a carbon paste electrode (CPE) modified using lysine residues of protein A. A monoclonal antibody for hCG could then be immobilized on the protein A-linked CPE and an analysis performed through the application of square wave voltammetry (SWV). The peak currents for both β-hCG-mAb and hCG were measured at ~ 0.6 V (vs Ag/AgCl). This immunosensor offered an LOD of 20 pM in human urine and 15 pM in synthetic hCG.

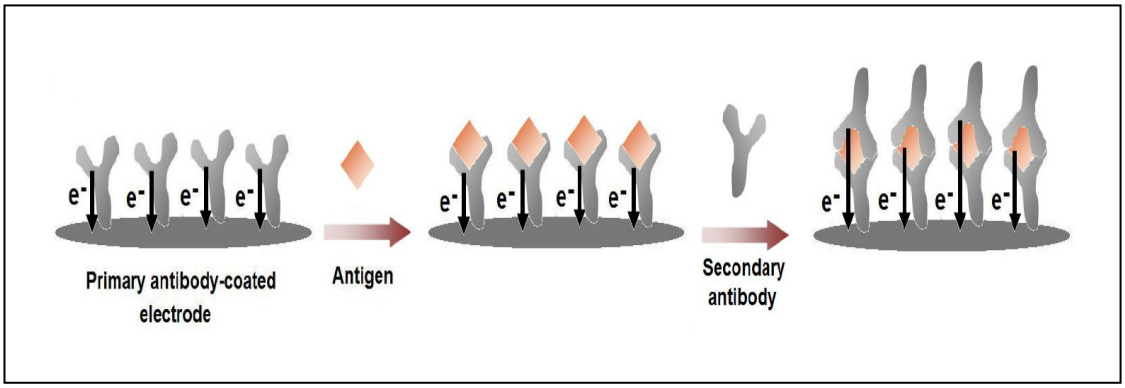

Figure 2.5 Depiction of label-free voltammetric immunosensors [63].

In addition to their applications in direct detection through label-free immunosensors, nanomaterials such as carbon nanotubes (CNTs) have also served to improve sensitivity [64]. A report has shown that electrochemical immunosensors which incorporate microelectrode arrays which have transducer surfaces in the form of modified single-

walled carbon nanotubes (SWCNTs) can be used for cancer marker detection [65]. In this particular application, the analyte is a prostate-specific antigen (T-PSA). The signal current is produced via the oxidation of Tyr and Trp amino residues, augmented through the interaction of T-PSA and T-PSA-monoclonal antibody which are covalently immobilized on SWCNTs. The LOD for T-PSA is shown to be 0.25 ng/mL.

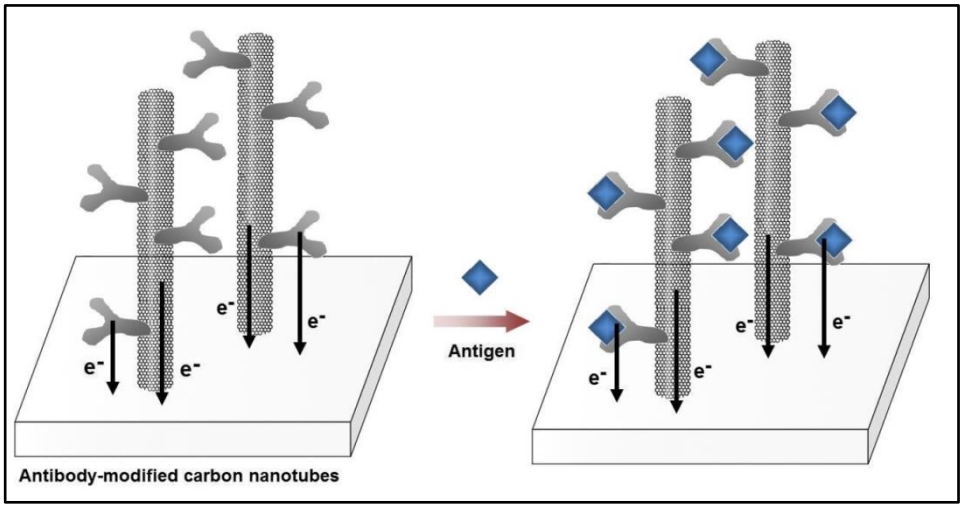

Figure 2.6 Depiction of label-free immunosensors using single-walled carbon nanotubes (SWCNTs)[65].

2.2.2 Label-Based Electrochemical Immunosensors

In a label-free electrochemical immunosensor, the reaction between the antigen and antibody typically causes a change in the electrochemical signal, but the magnitude of the change is usually minimal. For this reason, in order to achieve success in the design of an ultrasensitive electrochemical immunosensor or immunoassay, it is essential to find a means of amplifying the signal for detection [66]. Labelling the antibody is a common method of creating an amplified electrochemical signal, with enzyme-labelled antibodies frequently used when a stronger measurement signal is required [67].

Therefore, the global research focus has been upon the creation of innovative nanoscaled labels which can offer enhanced sensitivity and thus a greater ease of detection [68]. A number of different label types have been devised in recent times to provide an amplified electrochemical signal for immunosensors.

One such example involves the use of ligand-conjugated enzyme labels and nanolabels containing metals. According to the reports, labels typically contain bioactive enzymes such as horseradish peroxidase and alkaline phosphatase [69], or electroactive substances including thionine [66], methylene blue or ferrocene [70] derivatives. Metal ions may also be used [6, 69].

Advances in the field of nanotechnology serve to expand the possibilities for nanomaterial labels to be used for signal amplification in immunosensors. Nanostructures using metals or semiconductors such as gold, silver, carbon nanotubes

and quantum dots have already been applied either directly or indirectly as labels to enhance electrochemical change detection in immunosensors [71].

I. Metal Nanoparticle Labels

An illustration below shows work that described an electrochemical immunosensor using magnetic beads and gold nanoparticle labels [72]. Anti-IgG antibody-modified magnetic beads are attached to a carbon paste transducer surface using a magnet within the sensor. The gold nanoparticle labels are then immobilized at the magnetic bead surface through a process of sandwich immunoassay. The signal provided via the gold nanoparticles is proportional to the target IgG concentration in the sample solution and can be determined through electrochemical stripping analysis.

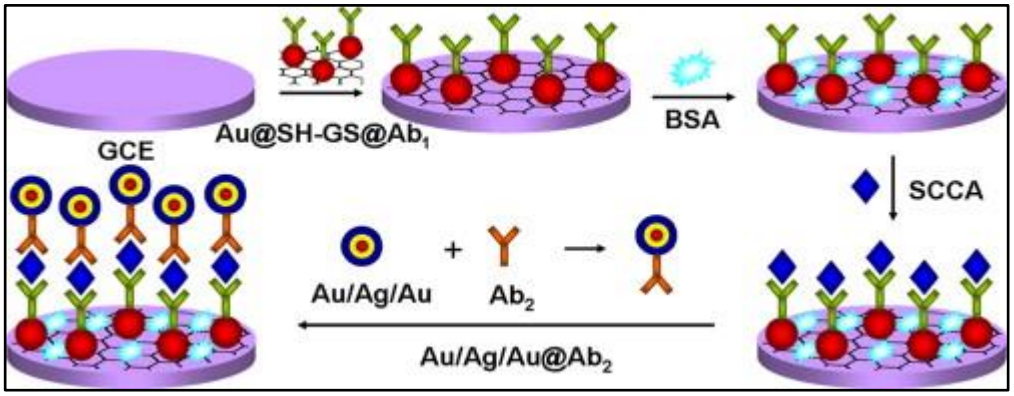

Figure 2.7 Depiction of an immunosensor to detect SCCA [72].

A further example involves the detection of the squamous cell carcinoma antigen (SCCA) in human serum [73]. For this process, Au/Ag/Au core/double shell nanoparticles (Au/Ag/Au NPs) are prepared and used as novel enzyme-mimetic labels in the creation of a sandwich-type electrochemical immunosensor for SCCA. Gold nanoparticle decorated mercapto-functionalized graphene sheets (Au@SH-GS) form a platform for the immobilization of the primary antibody (Ab1), while Au/Ag/Au NPs are used as labels for the secondary antibody (Ab2). On account of the strong electrocatalytic activity of Au/Ag/Au NPs towards the reduction of hydrogen peroxide (H_2O_2), electrochemical amperometric responses to SCCA are seen following the immuno-reaction. The electrochemical immunosensor demonstrates a wide linear range between 0.5 pg/mL and 40 ng/mL with an LOD at 0.18 pg/mL for SCCA.

II. Quantum Dot Labels

The use of quantum dot labels in immunoassay takes advantage of their multiplexing capabilities. It is thus possible to make simultaneous measurements of proteins through the application of various inorganic nanocrystal tracers.

For example, Liu and colleagues has reported the multi-protein electrical detection in combination with the amplification derived from electrochemical stripping transduction [74]. To work with multi-analytes, an electrical sandwich immunoassay relies on dual binding events in which the antibodies are connected to the nanocrystal tags and the magnetic beads. Each individual bio-recognition event produces its own voltammetric peak; the location and magnitude of this peak can be used to determine the type and concentration of the target antigen. This process has been applied in the simultaneous immunoassay of 2-microglobulin, IgG, bovine serum albumin and C-reactive protein in connection with ZnS, CdS, PbS and CuS colloidal crystals.

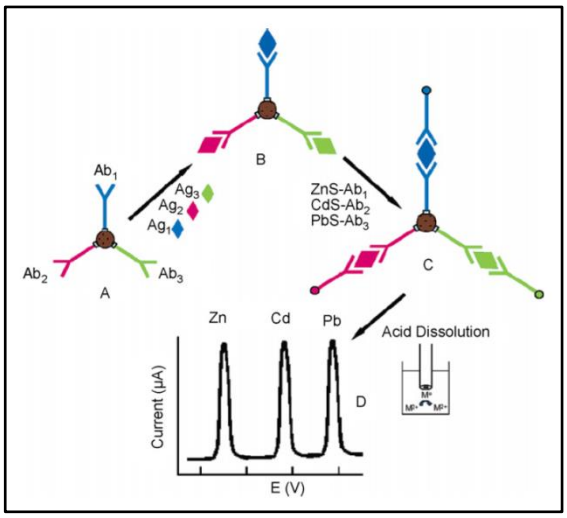

Figure 2.8 Depiction of multiprotein electrical detection based on inorganic colloid nanocrystaltracers [74].

III. Redox Labels

Immunosensors can also use redox labels to provide the signal allowing detection of the reaction. The signal can be amplified by electrochemical recycling of the redox label. This recycling can also be carried out using an enzyme whereby the redox probe acts as the substrate [75].

Chunglok and collegues have reported the use of a redox label, methyene blue, adsorbed on carbon nanotubes as labels [70]. Primary antibodies were collected magnetically in a process used to detect mouse IgG as a model analyte. A calibration curve using direct MB-MWNT reduction produced a dynamic range of 0.1 pg mL^{-1} to 100 pg mL^{-1}. Furthermore, using an Fe $(CN)_6^{3-/4-}$ redox couple in bulk solution to mediate the charge transfer from MB permitted the lowering of the accessible concentration range by two orders of magnitude.

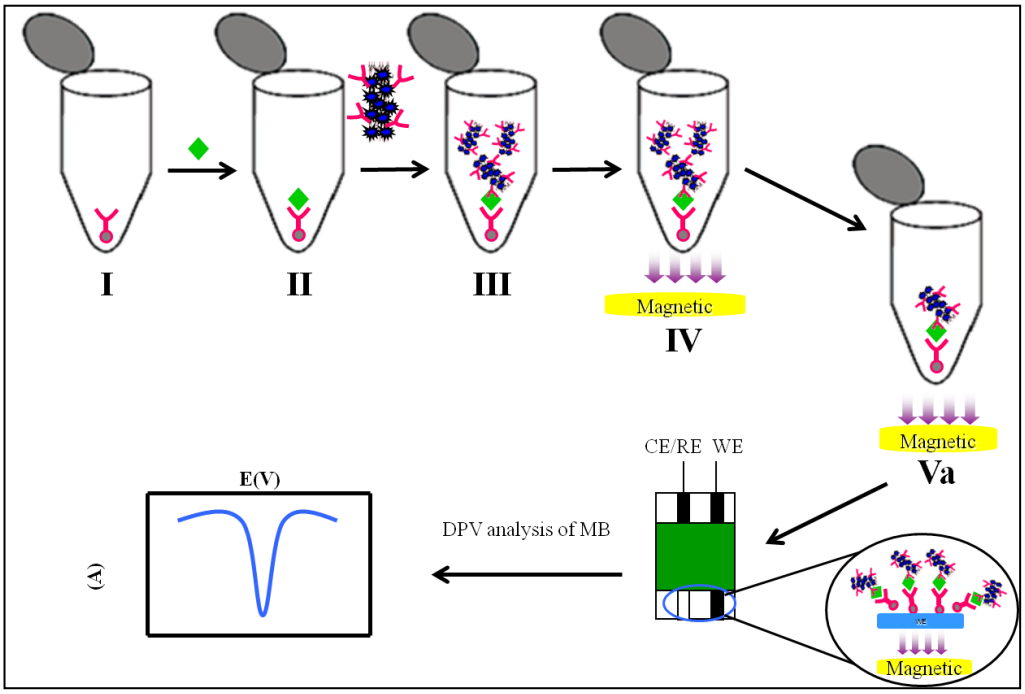

Figure 2.9 Depiction of a magnetic sandwich immunoassay [70].

IV. Enzyme Labels

Enzyme labels used for an optical immunoassay ELISA act as catalysts to produce detectable color species [26], but with an electrochemical immunosensor, the enzymatic product must be electro active in order to be measured using either voltammetric or amperometric methods [76].

The sensitivity of the platform in an electrochemical immunosensor can be enhanced using enzyme labels because it can generate detectable products in significant quantities. Conventionally, hydrolases such as alkaline phosphatase or β-galactosidase and oxidases such as peroxidase, glucose oxidase and laccase have been used for this purpose [40].

One example of the use of an enzyme label is the application of alkaline phosphatase which catalyses the dephosphorylation of p-aminophenyl phosphate (pAPP) and produces p-aminophenol (PAP) whose electrochemical reaction can be detected. The LOD can be subsequently enhanced through electrochemical recycling [77].

Amperometric immunosensors have been developed using various redox enzyme labels. Figure 2.10 (A) shows the workings of an enzyme-linked immunosensor. In this system, an analyte with an enzyme label is introduced to a sample which contains the target analyte. An immunoreaction brings the enzyme to the electrode surface where it acts as a catalyst to convert the substrate to an electrochemically active product. The resulting electrochemical reaction serves to produce the current which can then be detected and measured [78].

The use of an amperometric immunosensor applying the sandwich format is also demonstrated in Figure 2.10 (B). In this system, the analyte and an immobilized receptor interact at the electrode surface to create a binary complex. This binary complex is then bound to an enzyme-labelled secondary antibody. Further interaction with an enzyme substrate results in the formation of electrochemically active product, whose subsequent reaction creates the current which is to be detected.

Accordingly, researchers have sought to discover the most appropriate kinds of enzyme substrates in order to achieve the best possible outcomes in terms of the required level of sensitivity.

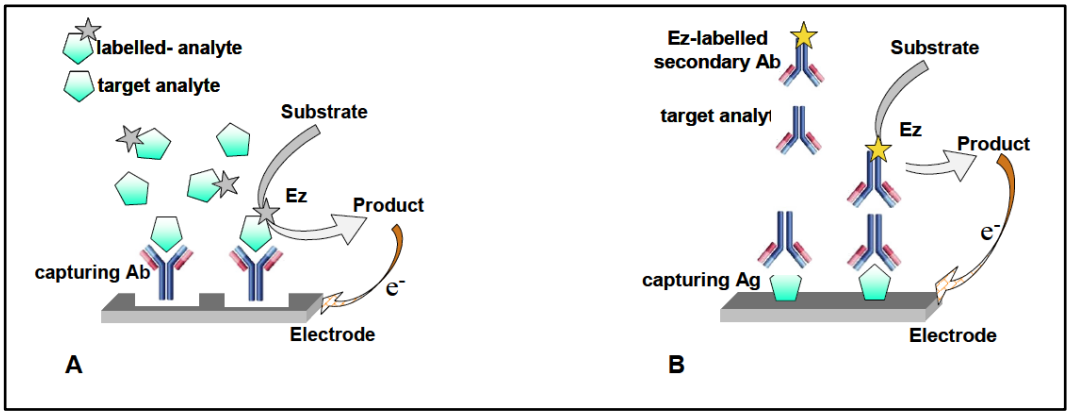

Figure 2.10 Depiction of immunoassay formats applying amperometric detection. (A) Biosensor for antigen detection using a competitive immunoassay system, with redox-enzyme-labelled antigens and the natural substrate of the enzyme, Ez. (B) Biosensor detects specific antibodies using an indirect immunoassay format [78].

2.3 Enzyme-Nanoparticle Label Based Electrochemical Immuno-sensors

It is considered desirable for immunosensors to exhibit a high degree of sensitivity; as a consequence, a number of signal amplification methods have been explored. One common strategy is to use functionalized nanomaterials as tracers to load a large quantity of enzymes [8]. Nanomaterials which have been examined for this role include carbon nanotubes [11, 15], carbon nanospheres [79] and gold nanoparticles [80]. These materials can serve as nanocarriers in order to load multiple enzymes and thereby cause the signal to be amplified, increasing the sensitivity of the immunosensor.

One example comes from Fu *et al.*, whose research tested enzyme HRP encapsulated in silica nanoparticles as trace labels to allow the electrochemical detection of IgG [81]. This method serves to enhance the sensitivity of the chemiluminescence immunoassay. The large surface area of MSN carriers allows an increase in the quantity of HRP bound in each stage of the sandwich immunoreactions. Therefore the conjugates are able to provide a stronger signal which improves sensitivity. Traditional sandwich immunoassay typically involves only one or two enzyme molecules to become bound to

each antibody, so the quantity increase offered in the new system offers a substantial improvement. The new approach represents a cost effective and efficient method of carcinoembryonic antigen (CEA) detection within samples. Analysis of the data collected demonstrated a linear response in the range of 0.1–40 ng/mL.

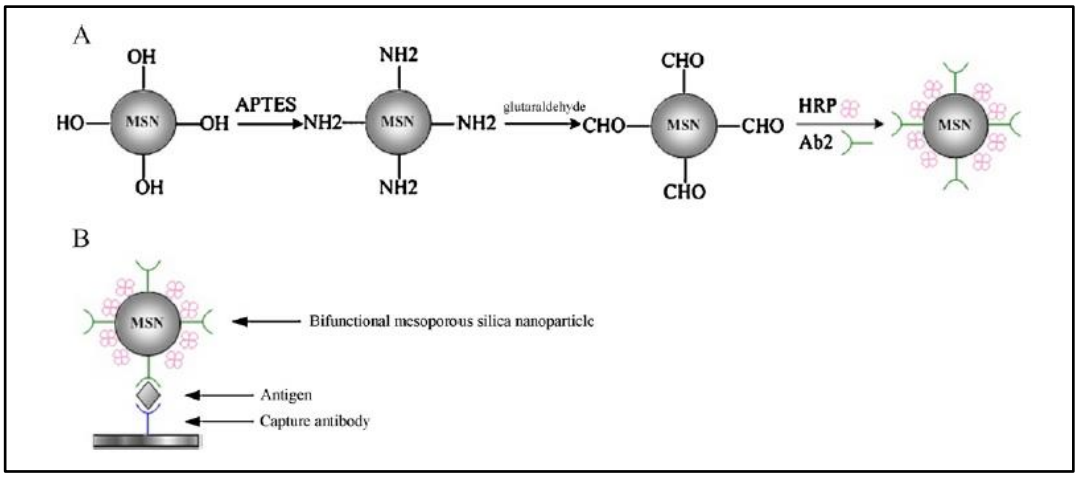

Figure 2.11 Depiction of the preparation procedure for bi-functional MSN and its use in immunoassay [81].

Further studies by Zhang *et al.* examined enzyme HRP functionalized AuNPs-graphene as a nanolabel enabling the electrochemical detection of CEA [82]. This system required a glassy carbon electrode treated with a Prussian Blue (PB) coating. Gold nanoparticles were then electrochemically deposited upon the surface along with the anti-CEA antibodies whose specific role was to capture the analyte. The immunoassay was then carried out using horseradish peroxidase-conjugated anti-CEA as secondary antibodies attached on the nanogold-enwrapped graphene nanocomposites (NGGN) surface (HRP-anti-CEA-NGGN). This method provided both strong signal amplification and a low LOD of 0.01 ng/mL CEA.

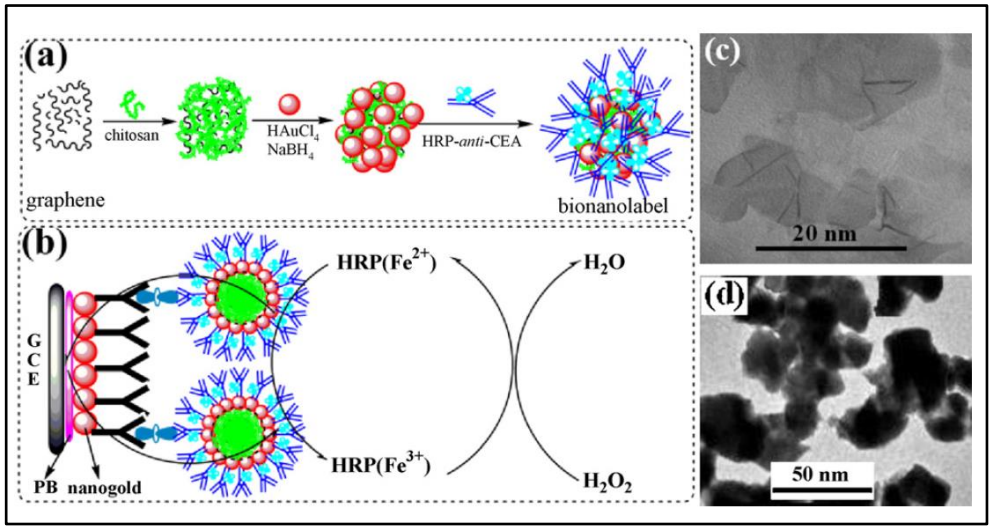

Figure 2.12 (a) Fabrication process of the HRP-anti-CEA-NGGN bio-nanolabel, (b) measurement protocol, and TEM images of (c) chitosan-protected graphene and (d) the prepared NGGNs [82].

Ju*et al.* investigated glucose oxidase-functionalized nanocomposites as labels for tumor markers where ultrasensitive multiplex measurement is required [83]. The immunosensor was arranged through coating layer-by-layer colloidal Prussian blue (PB) and gold nanoparticles in order to capture antibodies on screen-printed carbon electrodes. The glucose oxidase-functionalized nanocomposites and the labelled antibodies were prepared via one-pot assembly of glucose oxidase and antibodies on gold nanoparticles attached to carbon nanotubes. The PB which was immobilized on the immunosensor surface served as a mediator in catalyzing the reduction of H_2O_2 formed via the enzymatic cycle. Under analysis, this system offered detection limits ranging from 1.4 to 2.2 pg/mL of the target analyte.

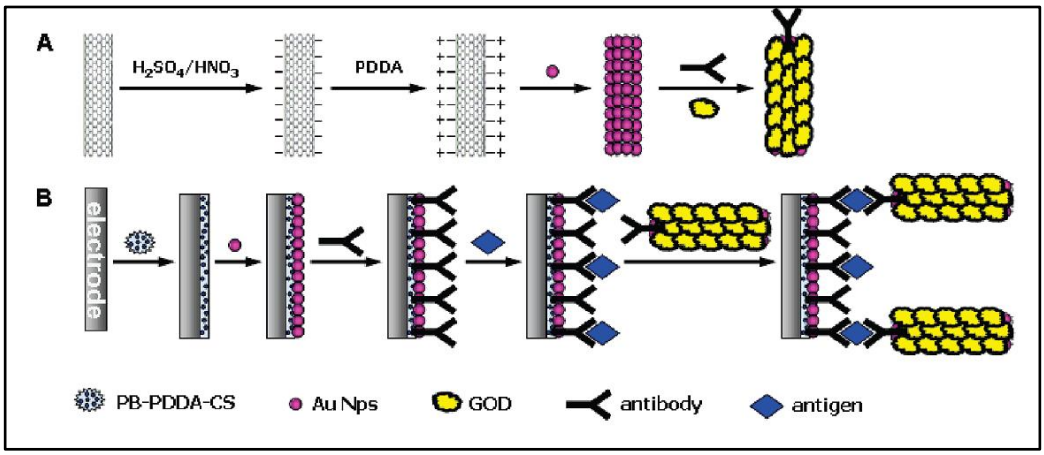

Figure 2.13 Depiction of (A) Preparation procedure of GOD-Au Nps/CNTs-Ab2 Tracer and (B) Preparation of immunosensors and sandwich-type electrochemical immunoassay [83].

2.4 Carbon Nanostructures for Enzyme Label Based Immunosensors

Carbon is an especially valuable material in the field of nanotechnology on account of the various physical forms it can take and the chemical properties they possess. For example, carbon is found in 2-dimensional form as graphene and carbon nanotubes and 0-dimensional form as fullerenes or nanodiamonds. The variety available permits various applications as nanolabels with enzymes. The following section describes the applications involving carbon nanotubes and graphene nanoparticles as nanolabels.

2.4.1 Carbon Nanotubes

I. General Considerations

The first observation of multiwalled carbon nanotubes has been credited to Iijima, a Japanese electron microscopist, in 1991[84]. However, has been some that hold the belief that in the 1950s the first carbon nanotubes were discovered [85]. At this time, Radushkevich and Lukyanovich published a paper in the Soviet Journal of Physical Chemistry showing TEM evidence hollow graphitic carbon fibers that are 50 nanometers in diameter [86]. Due to language barriers, the paper was in Russian, and the fact that during the cold war, access to the Russian journals was problematic, this paper is not well known and cited [85]. The chemical composition and atomic bonding configuration of carbon nanotubes are especially simple, yet permit the greatest diversity among nanomaterials in terms of structure and properties[87]. CNTs have attracted considerable interest since their initial discovery on account of their unique electronic, mechanical and structural characteristics which enable their use in a variety of specific roles [88].

II. General Characteristics and Properties of CNTs

CNTs are considered to be novel nanostructures which are constructed through a bottom-up approach of chemical synthesis. CNTs consist of sp^2 carbon units which form a seamless structure in the form of a hexagonal lattice. In size, the structure typically has a diameter of several nanometers and a length of many microns. The structure of CNTs is closed to form two distinct regions – the tube and the cap – which have clearly defined and different properties. Any topological defects will usually appear in the form of pentagons. It is possible to create a single-walled CNT (SWCNT) by producing a cylinder from a rolled graphene sheet along an (m, n) lattice vector in the graphene plane. The indices, m and n, are critical nanotube parameters indicating diameter and chirality respectively [13].

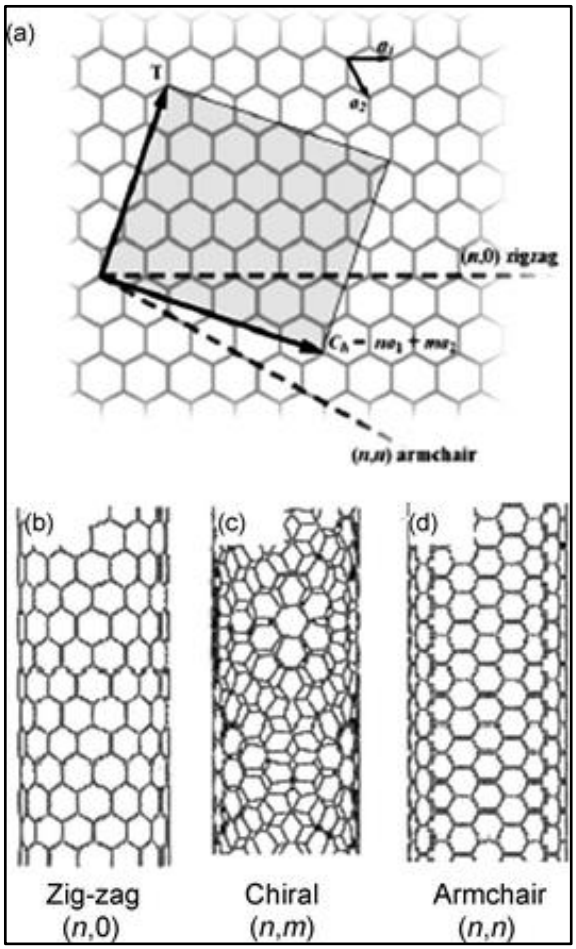

Figure 2.14 Depiction of a number of physical structures of CNTs.

Ando and colleagues described the physics of carbon nanotubes in their report[89]. Briefly, Ch is the lattice vector and is defined as Ch = na1 + ma2 = (n, m) where $0 \leq |m| \leq n$. The length of the chiral vector Ch is in direct proportion to the nanotube's diameter, while the chiral angle θ between Ch and the zigzag direction of the lattice (n, 0) relates to the indices, n and m . CNTs in which $n=m$ $(\theta = 30°)$ are termed armchair nanotubes on account of their distinctive cross-section shape. CNTs in which $m=0$ $(\theta=0°)$ are zigzag nanotubes while other CNT arrangements, in which $n \neq m$ $(0 < \theta < 30°)$ are known as chiral nanotubes.

Double-walled and multi-walled CNTs (MWCNT) can also be created. When this takes place, CNTs with differing chiralities may comprise two or more nanotube walls (Figure 2.15). A MWCNT is an arrangement in which a number of graphene sheets are rolled to form concentric cylinders with a layer spacing of 0.3–0.4 nm. The typical diameter of MWCNTs falls within a range of 2–100 nm and each layer has walls which run parallel to the central axis. These nanotubes can be combined in various ways allowing for a large number of differing types to be formed [13].

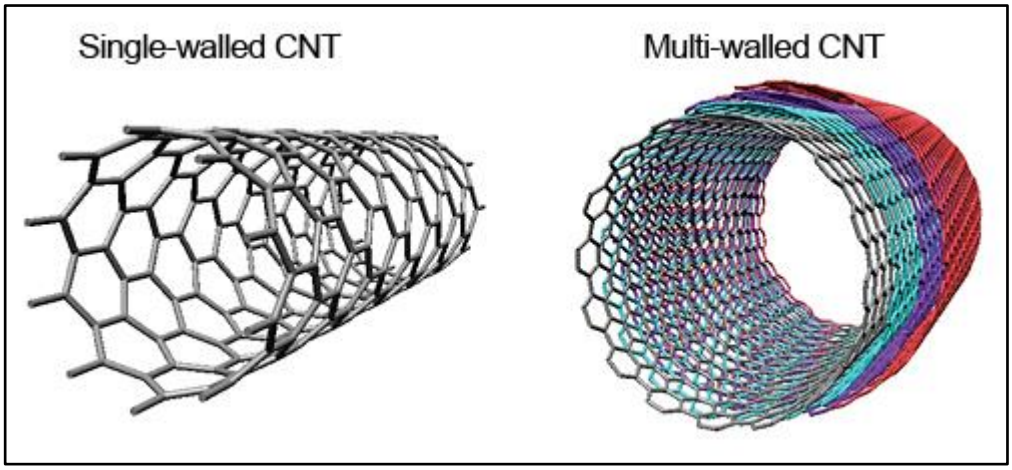

Figure 2.15 Depiction of a single-walled carbon nanotube (SWCNT) and a multi walled carbon nanotube (MWCNT).

III. CNTs-Enzyme Labels for Electrochemical Immunosensors

A number of researchers have paid carbon nanotubes close attention because of their unique properties [11, 13-15, 70]. CNTs have an especially high ratio of surface area to weight which has allowed them to become more widely used as a platform for signal amplification [15].

Wang and colleagues reported an important example of the use of multi-walled carbon nanotubes (MWCNTs) which were subsequently derivatized using copies of alkaline phosphatase and secondary antibodies [90]. The outcome was femtomolar levels of detection for proteins in buffer. A technique of alternating electrostatic layer-by-layer deposits of alkaline phosphatase interspersed with oppositely charged polyions on multi-walled carbon nanotubes was applied in creating alternative detection particles and thus obtaining detection limit of 70 aM for IgG.

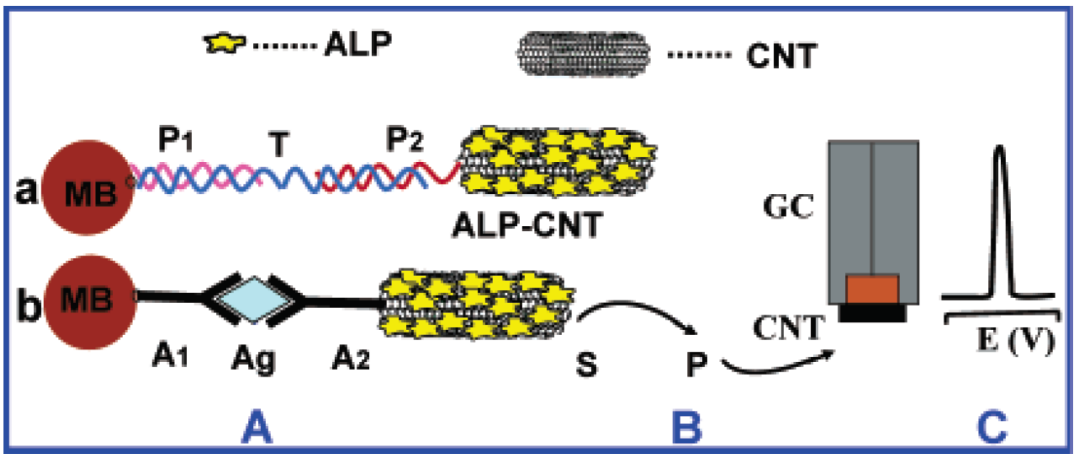

Figure 2.16 Depiction of the analytical protocol [90].

Over recent years, layer-by-layer (LBL) techniques have become accepted as the most appropriate strategies for creating practical multi-layer films with unique properties based on the template of carbon nanotubes. Keller *et al.* (1994) note that electrostatic LBL adsorption has been shown to be one of the most effective uses of enzyme-coated nanotubes for producing ordered, stable and bio-compatible multi-layer films [91]. The electrostatic LBL self-assembly procedure requires the alternating assembly of oppositely charged layers, and may be proved applicable for the ultrasensitive detection of proteins to enable diseases to be diagnosed and for treatments to be prescribed; this process would be facilitated by the sensitive enzyme catalytic reactions and amplification properties afforded by CNTs [9, 10, 92]. It has been suggested that the biocatalytic activity might be enhanced by the addition of a greater number of enzyme layers to the CNTs layer-by-layer assembly.

Munge and colleagues made further investigations in this area, testing a signal amplification strategy founded upon a stepwise LBL assembly of alkaline phosphatase (ALP) multi-layers on a CNT template [11]. Electrostatic interaction was shown to provide a high level of sensitivity in the electrochemical detection of IgG, while the detection limit was demonstrated to be extremely low at 5.4 aM of DNA and 67 aM of protein.

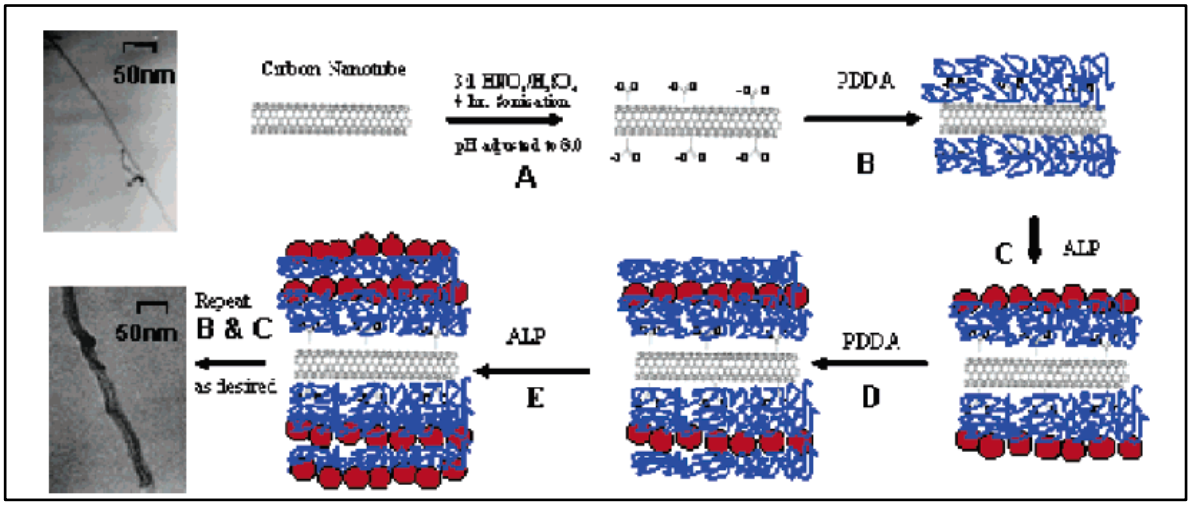

Figure 2.17 Depiction of the LBL electrostatic self-assembly of a protein-polyion on carbon nanotube template [11].

2.4.2 Graphene and Graphene Oxide

I. General Considerations

Graphene is the term for a single layer of graphite (Figure 2.18). The structure is atomically thin so as a result it can be thought of as an ideal 2-dimensional material [93]. It has particularly interesting properties and potential applications which have attracted considerable attention over recent years. The application of graphene in the field of sensor development is supported by the material's very high ratio of surface to volume and chemical properties for biological functionalization on its surface [18].

II. General Characteristics and Properties of Graphene Oxide

Graphene is a planar sheet of sp^2-bonded carbon atoms. The sheet has a thickness of one atom. The carbon atoms are arranged densely in a honeycomb lattice as shown in Figure 2.18. Graphene is a term which is derived from "graphite" and "alkene", while graphite comprises graphene sheets which are stacked together [16].

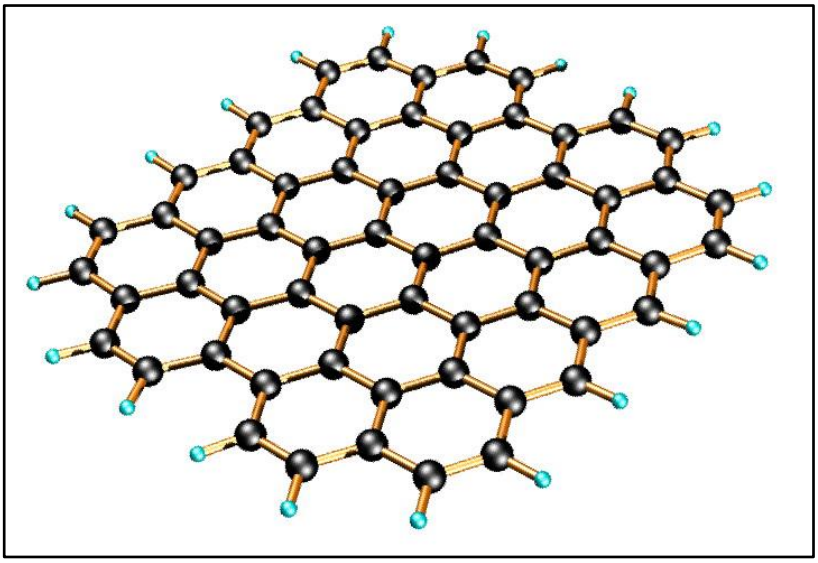

Figure 2.18 Depiction of the structure of a single graphene sheet.

The most frequently used large-scale method of producing graphene requires the production of graphite oxide. Methods pioneered by Staudenmeier and Hummers have been extended and developed in recent years to produce graphite oxide [17], which is a highly exfoliated material which can disperse in water to form monomolecular sheets of graphene oxide. The exact structures of graphite oxides have been widely studied. There exist many variations, depending on the particular synthesis method used in their creation and the degree of oxidation, but they are known to contain epoxide functional groups along the basal plane of the sheets as well as carboxyl and hydroxyl terminations on the edges of each layer (Figure 2.19).

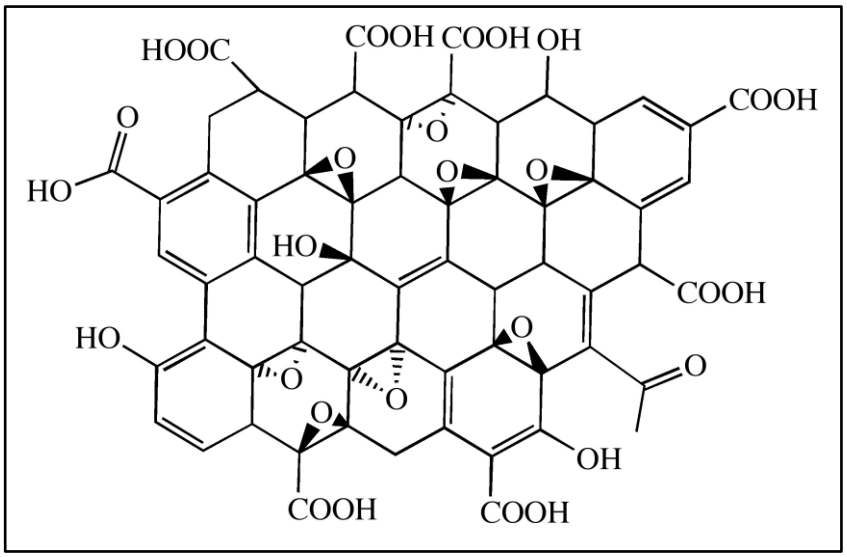

Figure 2.19 Chemical structure of graphene oxide

III. Graphene Oxide-Enzyme Labels for Electrochemical Immunosensors

The modification of electrodes using graphene oxide has become widespread because of the material's inherent properties of high electrical conductivity, a large specific surface area and excellent biocompatibility [94, 95]. Furthermore, nanocomposites of graphene oxide have been used as labels to improve sensitivity for better detection [96, 97]. This increased sensitivity was derived as a result of greater enzyme loading in combination with upgraded electrical conductivity of the bio-interface.

One example of the use of graphene involves the application of dendrimer functionalized-graphene as a nanocarrier to detect a-1-fetoproteins using an amperometric immunosensor [12]. By this system, enzyme-labelled antibodies are first combined with the dendrimer functionalized-graphene. To detect the target, the necessary high level of sensitivity is attained through a dual signal amplification method. Dendrimer functionalized-graphene is able to load greater quantities of enzyme-labelled antibodies, which enables the generation of stronger signals. Electron transfers can be accelerated by graphene due to its high electrical conductivity, and the resulting signals can also be amplified. The amperometric immunosensor is able to detect a-1-fetoproteins in the range of 1.0–100 ng mL1, while 0.45 ng mL1 represents the LOD.

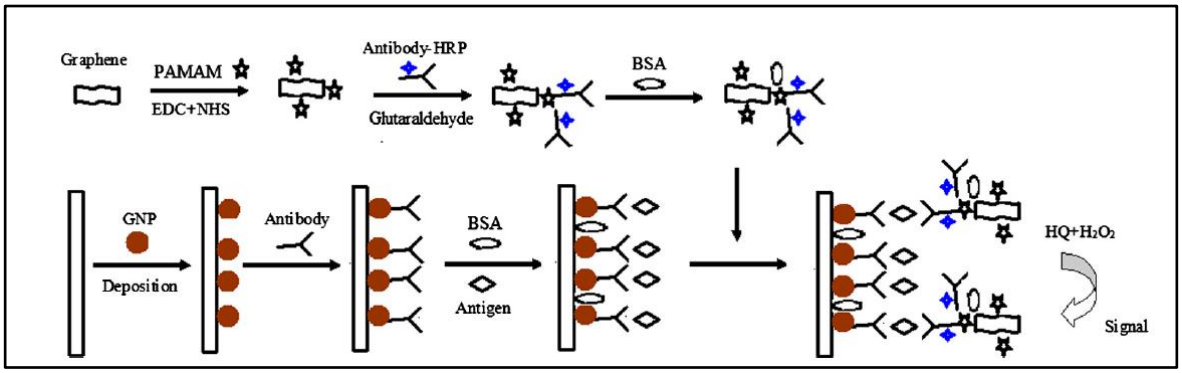

Figure 2.20 Depiction of the preparation of Gr-PAMAM-anti-AFP-HRP and the sandwich-type ELISA procedure based on signal amplification [12].

A further example concerns the use of a new estradiol immunosensor which uses the enzyme HRP and graphene oxide composite[98]. Horseradish peroxidase–graphene oxide–antibody (HRP–GO–Ab) conjugates are first produced using carboxylated graphene oxide to carry the antibody and horseradish peroxidase as the label to improve the catalytic activity for hydrogen reduction of the electrode. This immunosensor showed LOD levels of 0.02 ng/mL.

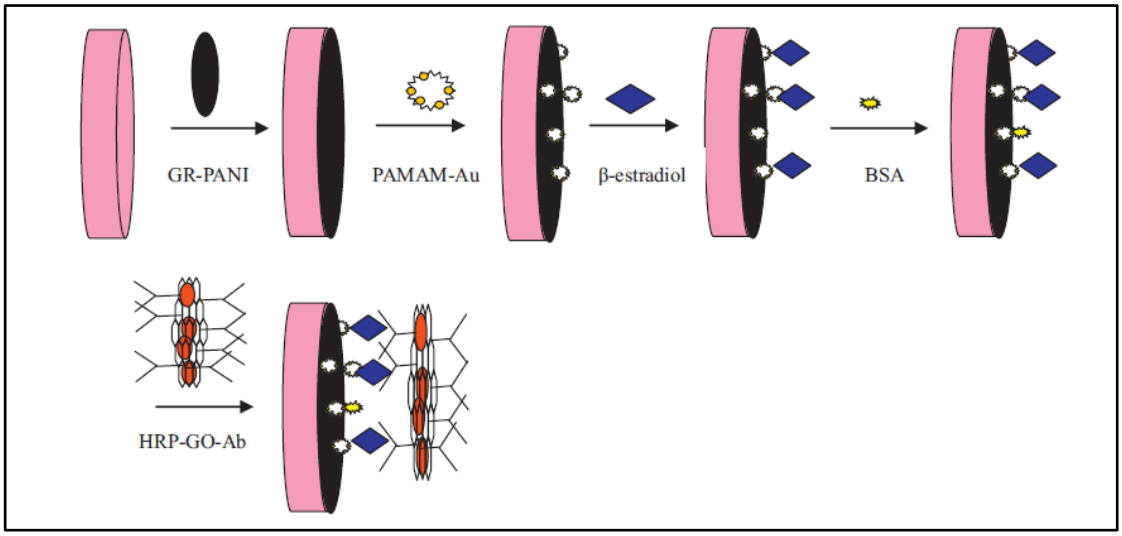

Figure 2.21 Depiction of an electrochemical immunosensor [98].

One final example is the use of an electrochemical immunosensor for the detection of clenbuterol (CLB)[99]. This system is constructed using glucose oxidase functionalized graphene oxide nanocomposites to label CLB. The immunosensor was develop through layer-by-layer assembly using colloidal Prussian blue (PB), multi-walled carbon nanotubes and CLB antibodies on a glassy carbon electrode. For the immunoassay process, PB serves as the redox mediator in reducing the H_2O_2 originating from the catalyst cycle of the glucose oxidase. The use of a high glucose oxidase to graphene oxide ratio allowed amplification of the signal. In ideal conditions, this particular immunosensor demonstrates a broad linear range from 0.5 to 1000 ng/mL with a value of just 0.25 ng/mL as the LOD.

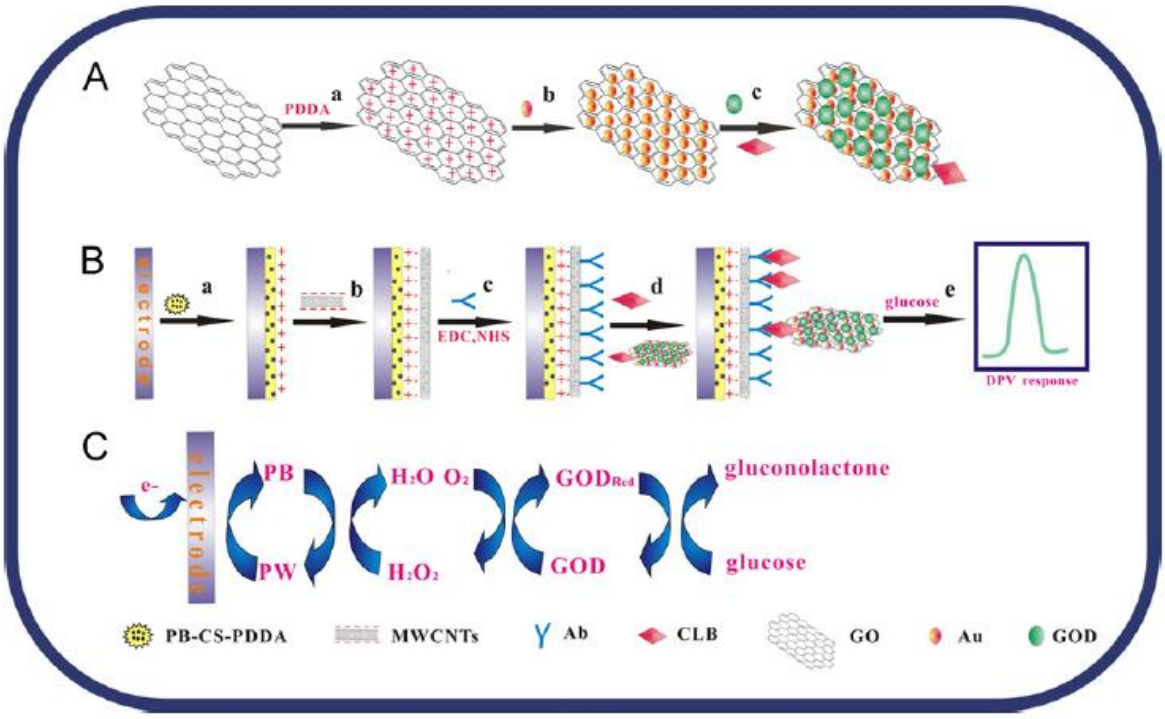

Figure 2.22 Depiction of an immunosensor used to detect clenbuterol [99].

2.5 Signal Amplification by Enzyme-Substrate Recycling Systems

Signal amplification is possible by increasing the quantity of detectable species in enzyme-labeled designs. Two of the ways in which it is possible to achieve this kind of increase are based on the magnification of the electron transfer process, and can be outlined as follows:

2.5.1 Recycling the Electrochemically-Transformed Compound

The first method uses catalytic enzymatic cycles and involves the regeneration of the active-site of the redox enzyme through the use of a charge transfer mediator which can be electro-transformed at the electrode surface [78] (Figure 2.23). To maintain the enzymatic cycle, the enzyme substrate must be held at excess concentration. The signal which is produced and detected to confirm the enzymatic cycle is linked to the presence, or in some cases the absence, of the sample analyte during the process on electrochemical immunoassay

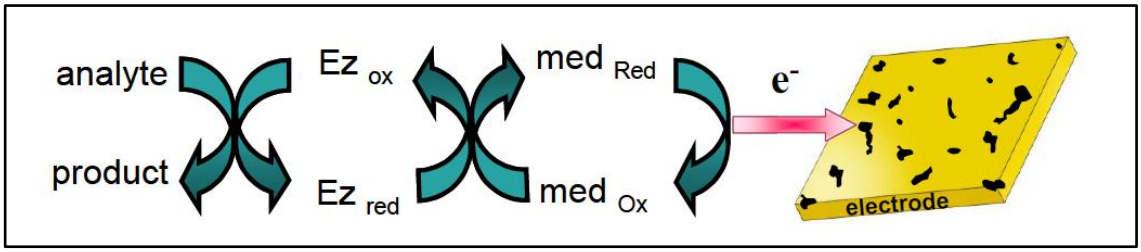

Figure 2.23 Depiction of the catalytic cycle using an enzymatic amperometric bio-electrode and an electrochemical mediator to regenerate the original oxidation state of the enzyme redox site [78].

2.5.2 Using a Bi-Enzymatic System

In the second method, the analyte is used as the substrate of the first enzyme (Ez-1). The analyte and the Ez-1 form a co-substrate, which is the electroactive species, and reacts with Ez1 forming resultant products, including the Ez-2 substrate[78] (Figure 2.24). Ez-2 proceeds to react with its substrate and regenerates the analyte, thereby maintaining the cycle and continuing to consume the Ez-1 co-substrate. This reaction is the one which can be electrochemically detected, so even when the amounts of analyte are minute, detection remains possible because the consumption of the electroactive species can be sustained by the ongoing cyclic reactions of the two enzymes; the bi-enzymatic amplification.

This method can be used to detect, for example, catecholamines IgG and cocaine [100]. This technique has also been applied to detect lactate through the use of the enzymes lactate oxidase and lactate dehydrogenase [101]. The process by which these biosensors function adheres to the principle that the product of the first enzymatic reaction forms the substrate of the second enzyme, which then serves to transform this product back to the original species. In this way, a single electrochemically active species is able to act as the co-substrate of the first enzyme and thus continue being consumed, which results in the multiplication of the detected signal.

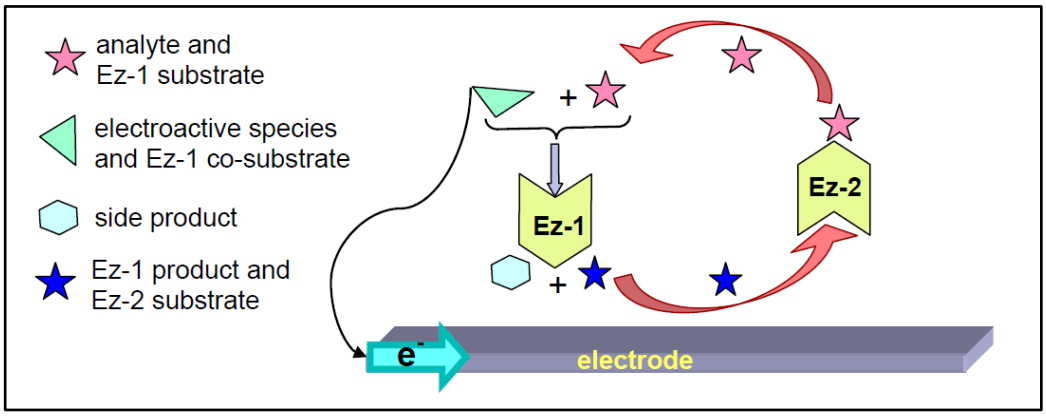

Figure 2.24 Depiction of a bi-enzymatic amperometric biosensor.The analyte is recycled as long as the electroactive species is consumed [78].

2.6 Layer by layer deposition: Theory

In 1966 Iler exhibited the notion that films of alternating positively charged alumina fibrils, along with a negatively charged silica colloids could be adsorbed onto hydrophilic glass [102]. By the 1990s, Dekker et. al had further developed this idea to include the deposition of solutions of oppositely charged polyelectrolytes [103]. The method became known as 'layer-by-layer' (L-b-L) deposition, and is currently employed in various production processes. The technique is fairly simple, and comprises: 1) derivatising a substrate with a stable surface charge excess, 2) immersing the substrate in a solution of oppositely charged polyelectrolyte (PE), 3) immersing the resulting substrate in water to remove weakly bound PE, and 4) immersing it into a solution of a secondary PE, which is oppositely charged to the first. Steps 2) to 4) can be repeated as many times as necessary to give the required thickness.

The reasons for this being such a popular technique, in addition to its simplicity, are: a) this method permits control of the resulting film thickness down to a level of a few angstrom, b) films of more than 1000 PE layers are possible, and c) resulting films are physically stable, and are permeable to solution species, enabling a film-confined catalyst to react with the substrate. The L-b-L technique has been applied to the deposition of many different charged species, including conducting polymers, DNA and proteins.

The main factor causing the adsorption of, for example, a positive PE onto a negative surface is electrostatic attraction. Using the zeta potential to measure amounts of adsorption, it can be observed that over-compensation of charge occurs [104]. In other words, the PE charge is not neutralized, but maintains a general positive charge, and can therefore adsorb a negative PE. During the generation of layers, the zeta potential oscillates systematically about zero, and when neutron reflectrometry experiments were performed it was found that the polymer layers were not universally planar, but in fact had penetrated each other [105].

In addition to Coulomb attraction, the additional forces at play can be observed. These include: thermodynamic hydrophobic effects, hydrogen bonding and Van der Waals interactions, and such additional forces lead to a process which delivers negative enthalpy. Furthermore, various ions and molecules of solvent shell water are released, and it is such events which produce the negative free energy of L-b-L deposition, pertaining to $\Delta G = H - T\Delta S$.

Polyelectrolytes of opposite charge also have the ability to form complexes in solutions, and these complexes are termed 'interpolyelectrolyte complexes' [106]. Furthermore, solid-state nuclear magnetic resonance spectroscopy has displayed that interpolyelectrolyte complexes are similar in their structures to L-b-L films, and thus L-b-L films may be interpreted as multiple layers of IPECs. In addition, it can be observed that the increase in entropy is the main reason why IPECs are formed [107].

When employing electrostatic attraction to form L-b-L films, greater film thicknesses can be achieved if the solution's ion concentrations are increased accordingly. It is expected that such a resulting increase in thickness is caused by the polyelectrolyte ion group shield increasing as a result of higher salt concentrations, which in turn leads to more coiled formation.

2.7 Methods Used in Electrochemistry

2.7.1 Cyclic Voltammetry

Qualitative information can be obtained about electrochemical reactions using the method of cyclic voltammetry (CV). The use of this technique is widespread and involves observation of the cycling of potential at a working electrode, then subsequently taking measurements of the current. The method requires the use of a reference electrode of constant potential. The working electrode potential can then be measured in comparison, with a signal created from the applied potential. The diagram in Figure 2.25 below depicts an excitation signal from cyclic voltammetry. In part (a), the forward scan shows the starting potential at a high level before ending at the low level (b). This is a negative scan. Point (b), at the vertex, represents the switching potential. From here, the positive scan from (b) to (d) commences in the reverse direction, and returns to the original starting potential. The broken line shows the subsequent cycle and indicates that the process can be repeated. [108].

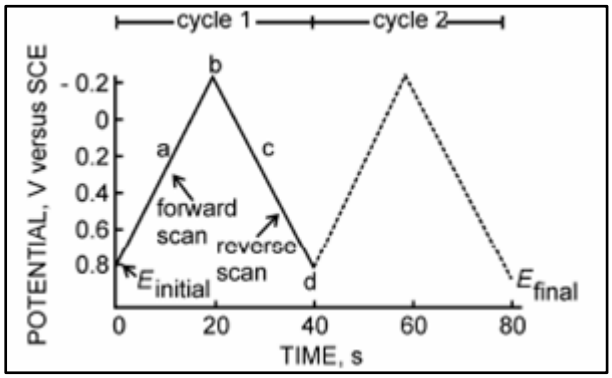

Figure 2.25 Triangular potential waveform; the typical excitation signal in cyclic voltammetry [108].

By measuring the current at the working electrode as the potential scans take place, a cyclic voltammogram, such as the example shown below in Figure 2.26 can be produced. The example shown was created from a reduction and oxidation process involving a single electron.

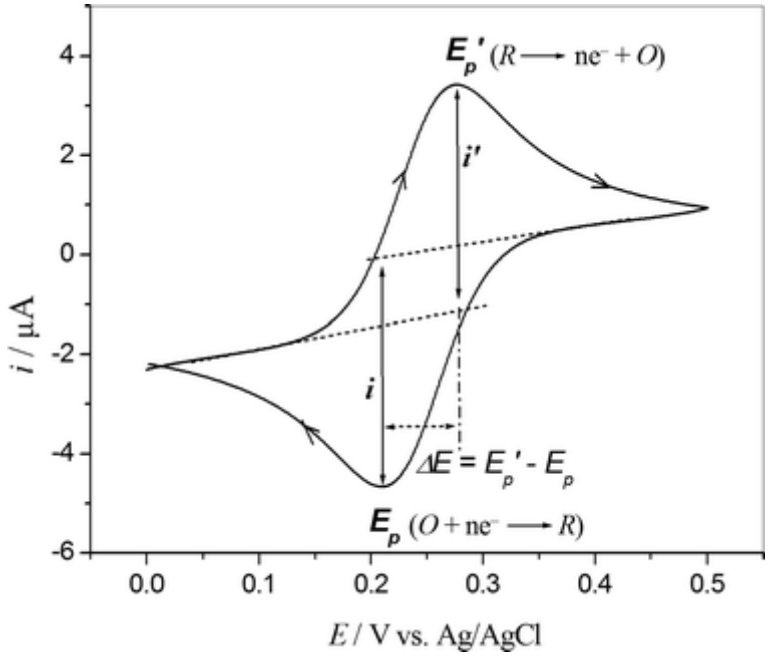

Figure 2.26 Voltammogram of a single electron oxidation-reduction [109].

If the following reversible reaction is considered; O+ne−⇌R

the CV process takes place as follows:

As the scan proceeds from the higher potential to the switching potential, the reduction process takes place. The current obtained is known as the cathodic current (I_{pc}),O+ne^- −>R. The cathodic peak potential (E_{pc}) occurs at 0.2 V. Following the switching potential, the positive scan is observed, producing the anodic current (I_{pa}). This is where the oxidation takes place; R−>O+ne^-. The anodic peak potential (E_{pa}) occurs at 0.3 V. This is achieved once the substrate at the electrode surface has been entirely oxidized [109].

2.7.2 Differential Pulse Voltammetry

In differential pulse voltammetry (DPV), the potential arises in the form of small pulses which have a constant amplitude of around 10–100 mV, which are superimposed in the form of a staircase wave. In each pulse period, the current can be measured on two occasions – at the commencement and the termination of each pulse, as shown in Figure 2.27a. This approach allows the current to be determined before each potential change, and the difference in current can then be plotted to give a function of potential. This is displayed in Figure 2.27b [110].

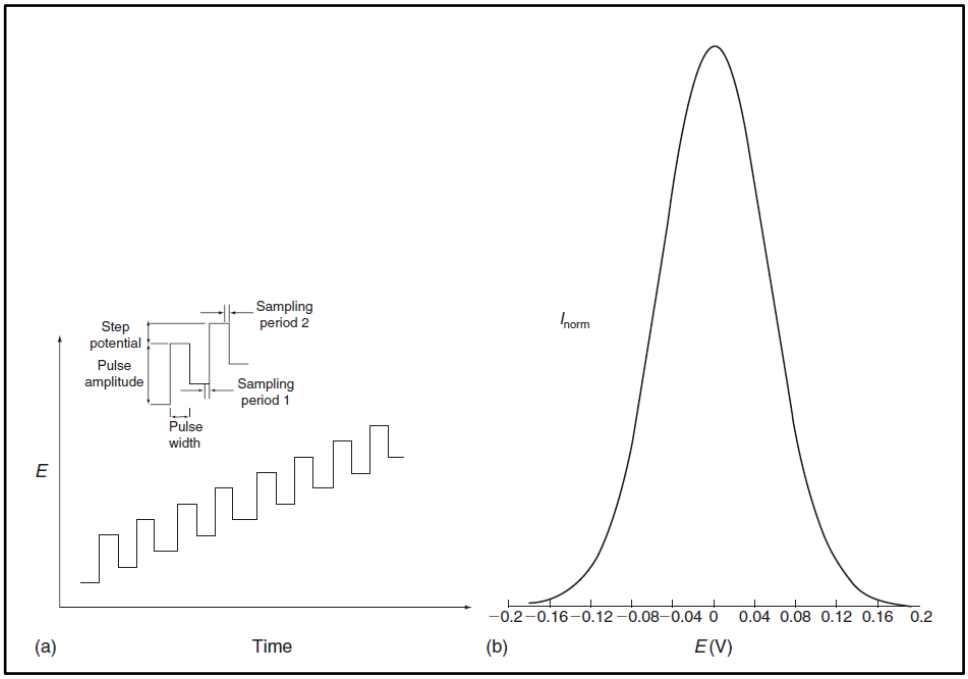

Figure 2.27 (a) Form of the potential; **(b)** Function of potential results in a simulated voltammogram for DPV [110].

2.7.3 Amperometry

Another electrochemical technique is amperometry. In this approach, the potential applied to the working electrode is related to measurements of time changes in the current flowing through the electrode system. In practice, a fixed potential can be applied to the electrochemical cell, while an oxidation or reduction reaction will then generate a corresponding current which can be measured as a function of time. The process of using an fixed potential amperometric sensor involves taking measurements in a stirred solution or using a rotating electrode. For this reason, the method is accordingly also known as hydrodynamic amperometry [111]. Figure 2.28 below shows the current response when presented as a function of time.

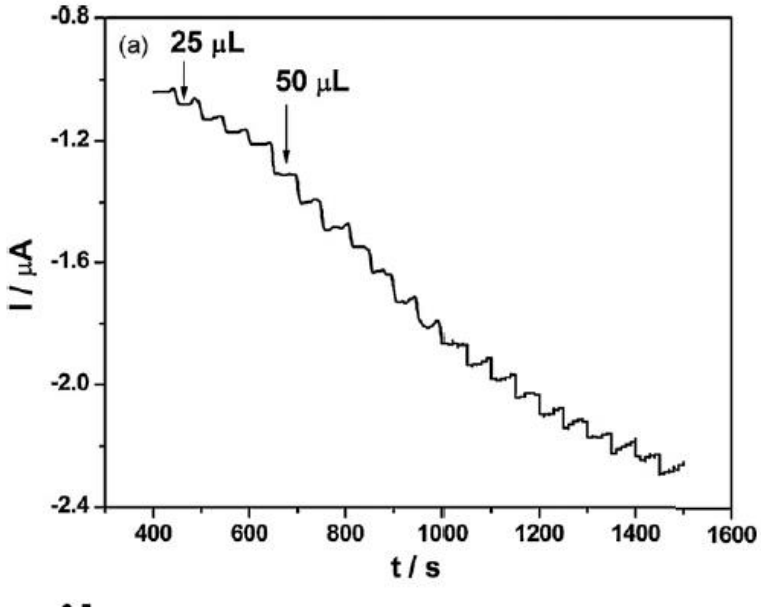

Figure 2.28 Amperometric biosensor response to repeated additions of 0.05 M and 0.1 M bisphenol A into stirred PBS (pH 7.4) [112].