CHAPTER 3

Enzymatic Biosensors

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3.1 ENZYMATIC BIOSENSORS

As mentioned in Chapter 1, biosensors can be divided into several categories based on the transduction process, such as electrochemical, optical, piezoelectric, and thermal/calorimetric biosensors (Serra et al., 2010). Among these, electrochemical biosensors are widespread and successfully commercialized as devices. Moreover, according to the receptor type, biosensors can also be classified as enzymatic biosensors, microbial biosensors, genosensors, and immunosensors (Turner, 2013). This chapter focuses on the enzyme and its mimetic-based electrochemical biosensors since they have attracted attention due to the potential applications in our daily life, such as health care (Marli and Paiva, 1990), food safety (Perez-Lopez and Merkoci, 2011) and environmental monitoring (Mozaz et al., 2004). Health care is the main area for monitoring blood glucose levels in diabetics using glucose biosensors. In addition, the reliable detection of urea has potential applications for patients with renal disease either at home or in the hospital (Eggenstein et al., 1999). Biosensors are also commonly used in industries for monitoring fermentation broths or food processing procedures through detecting concentrations of glucose and other fermentative end products (Terry et al., 2005).

Definition

Enzymatic biosensors can be defined as an analytical device having an enzyme as a bioreceptor integrated or intimately associated with the physical transducer to produce a discrete or continuous digital electronic/optical signal that is proportional to the concentration of analyte present in the sample. This chapter describes the enzyme-based electrochemical biosensors for the measurement of clinically important biomarkers, beginning with a history of biosensors.

3.2 HISTORY OF BIOSENSORS

Professor Leland C. Clark (Figure 3.1) is the father of the biosensor. In 1956, he constructed an oxygen electrode and applied it to measure the various analytes present in the body. Especially, he entrapped glucose oxidase at an oxygen electrode using a dialysis membrane and estimated glucose concentration by monitoring the decrease in oxygen concentration (Clarke, 1956). This is the basis of numerous variations on the basic design and many other reaction (oxidase) enzymes were immobilized by various researchers. Guilbault and Montalvo described a urea sensor based on urease immobilized at an ammonium-selective liquid membrane electrode (Guilbault and Nagy, 1973). In 1976, Clemens et al. developed an electrochemical glucose biosensor in a bedside artificial pancreas, which was later marketed by Miles (Elkhart) as the Biostator (Clemens et al., 1976). A new semi-continuous catheter-based blood glucose analyzer was introduced by VIA Medical (San Diego, California). In 1976, La Roche (Basel, Switzerland) introduced the Lactate
Analyser LA 640 in which the soluble mediator, hexacyanoferrate, was used to shuttle electrons from lactate dehydrogenase to an electrode.

In 1984, a much-cited article on the use of ferrocene and its derivatives as an immobilized mediator for use with oxidoreductases was published. Then in 1987, MediSense (Cambridge, Massachusetts) launched the screen-printed enzyme electrodes as crucial components in the construction of inexpensive enzyme electrodes and a pen-sized meter for home blood glucose monitoring. The electronics were redesigned into popular credit card— and computer mouse—style formats, and MediSense’s sales grew exponentially, reaching $175 million per annum by 1996, when they were purchased by Abbott. Boehringer Mannheim (now Roche Diagnostics), Bayer and Life Scan now have competing mediated biosensors, and the combined sales of the four companies dominate the world market for biosensors and are rapidly displacing conventional reflectance photometry technology for home diagnostics (Table 3.1).

**First-, second- and third-generation biosensors**

Depending on the level of integration, biosensors can be divided into three generations (Corcuera and Cavalieri, 2003), which are based on the method of attachment of the bio-recognition element or the bioreceptor molecule to the base of the transducer element. The three generations of biosensors are schematically depicted in Figure 3.2. In the first-generation biosensors, the biorecognition element is either bound or entrapped in a membrane, which in turn is fixed on the surface of the transducer (based on Clark biosensors). The mediated or second-generation biosensors use specific mediators between the reaction and the transducer to improve sensitivity. This involves the adsorption or covalent fixing of the biologically active component to the transducer surface and permits the elimination of semipermeable membrane. In the case of direct or third-generation biosensors, there is direct binding of the bioreceptor molecule to the sensor element.
and thus the bioreceptor molecule becomes an integral part of the biosensor. Thus no normal product or mediator diffusion is directly involved. Conducting polymers–based biosensors fall under this category.

### 3.3 ENZYMATIC AND NONENZYMATIC BIOSENSORS FOR VARIOUS DISEASES

Despite significant progress in prevention, diagnosis, and treatment in the last century, infectious and other diseases/disorders have remained significant global health problems. Major challenges for management of diseases include injudicious use of antimicrobials, proliferation of multidrug-resistant (MDR) pathogens, and ease of rapid disease dissemination due to overpopulation and globalization. Timely diagnosis and initiation of targeted antimicrobial treatment are essential for successful clinical management of diseases.

#### Table 3.1 Some Key Events in the History of Biosensor Development

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1916</td>
<td>First report on the immobilization of proteins: Adsorption of invertase on activated charcoal</td>
</tr>
<tr>
<td>1922</td>
<td>First glass pH electrode</td>
</tr>
<tr>
<td>1956</td>
<td>Invention of the oxygen electrode</td>
</tr>
<tr>
<td>1962</td>
<td>First description of the biosensor: An amperometric enzyme electrode for glucose</td>
</tr>
<tr>
<td>1969</td>
<td>First potentiometric biosensor: Urease immobilized on an ammonia electrode to detect urea</td>
</tr>
<tr>
<td>1970</td>
<td>Invention of the ion-selective field-effect transistor (ISFET)</td>
</tr>
<tr>
<td>1972–75</td>
<td>First commercial biosensor: Yellow Springs Instrument’s glucose biosensor</td>
</tr>
<tr>
<td>1975</td>
<td>First microbe-based biosensor</td>
</tr>
<tr>
<td>1980</td>
<td>First fiber-optic pH sensor for in vivo blood gases</td>
</tr>
<tr>
<td>1982</td>
<td>First fiber-optic-based biosensor for glucose</td>
</tr>
<tr>
<td>1983</td>
<td>First surface plasmon resonance (SPR) immunosensor</td>
</tr>
<tr>
<td>1984</td>
<td>First mediated amperometric biosensor for the detection of glucose</td>
</tr>
<tr>
<td>1987</td>
<td>Launch of the MediSense ExacTech blood glucose biosensor</td>
</tr>
<tr>
<td>1990</td>
<td>SPR–based biosensor system</td>
</tr>
<tr>
<td>1992</td>
<td>i-STAT launches hand-held blood analyzer</td>
</tr>
<tr>
<td>1996</td>
<td>Glucocard launched</td>
</tr>
<tr>
<td>1998</td>
<td>Launch of LifeScan FastTake blood glucose biosensor</td>
</tr>
<tr>
<td>2001</td>
<td>LifeScan purchases Inverness Medical’s glucose testing business for $1.3 billion</td>
</tr>
<tr>
<td>2003</td>
<td>i-STAT purchased by Abbott for $392 million</td>
</tr>
<tr>
<td>2004</td>
<td>Abbott acquires Therasense for $1.2 billion</td>
</tr>
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</table>
Biosensors offer the possibility of an easy-to-use, sensitive and inexpensive technology platform that can identify biomarkers rapidly and predict effective treatment. Advantages include small fluid volume manipulation (less reagent and lower cost), short assay time, low energy consumption, high portability, high throughput, and multiplexing ability. Recent advances in micro- and nanotechnologies have led to development of biosensors capable of performing complex molecular assays required for many of the diseases. In parallel, significant progress has been made toward the understanding of pathogen genomics and proteomics and their interplay with the host. Biosensor-based immunoassays may improve the detection sensitivity of pathogen-specific antigens, while multiplex detection of host immune response antibodies (serology) may improve the overall specificity. Further system integration may facilitate assay developments that can integrate both pathogen-specific targets as well as biomarkers representative of host immune responses at different stages of diseases.

A biosensor is a device that diagnoses and measures various diseases such as AIDS, tuberculosis, malaria, neurodegenerative disorders, cardiovascular diseases, oxidative stress, ischemia reperfusion injury, cancer, Parkinson’s, and amyotrophic lateral sclerosis (ALS) by producing measurable signals to the biomarkers of the various diseases (Arora, 2013). The biological recognition element immobilized or attached with the biosensor electrode is responsible for producing the changes that react with the biomarkers. If the enzymes are used as bioreceptors, then they are called as enzymatic biosensors. Although enzymes are highly selective and sensitive, activity of the biosensor decreases over time as the lifetime of the biological enzyme decreases. Therefore, nonenzymatic sources such as metal nanoparticles, carbon nanotubes, graphene, metal oxides, inert
electrodes, etc. are used as detection elements to react with the biomarkers (Si et al., 2013). The following discussion explains the different types of biomarkers and their determinations using biosensors.

3.4 BIOMARKERS FOR DIAGNOSIS OF DISEASES

Biomarkers are the species/substrates that provide a dynamic and powerful approach to understanding the spectrum of diseases with applications in observational and analytic epidemiology, randomized clinical trials, screening, diagnosis, and prognosis (Mayeux, 2004). A biomarker is generally defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological, pathogenic processes, or pharmacologic responses to a therapeutic intervention” Strimbu et al. (2010). For many of these measures, there is a normal range, and values outside of this range can serve as an indicator of disease or as an early sign of potential disease. Clinically, these values can help evaluate response to treatment. It is necessary to distinguish between disease-related and drug-related biomarkers. Disease-related biomarkers give an indication of the probable effect of treatment on patients (risk indicator or predictive biomarker), if a disease already exists (diagnostic biomarker), or how such a disease may develop in an individual case regardless of the type of treatment (prognostic biomarker). Predictive biomarkers help to assess the most likely response to a particular treatment type, while prognostic markers show the progression of disease with or without treatment. In contrast, drug-related biomarkers indicate whether a drug will be effective in a specific patient and how the patient’s body will process it.

3.4.1 Types of biomarkers

Biomarkers can be classified based on different parameters. They can be classified based on their characteristics such as molecular biomarkers (CT, PET, MRI) or imaging biomarkers. Molecular biomarkers can be used to refer to nonimaging biomarkers that have biophysical properties, which allow their measurements in biological samples (e.g., plasma, serum, cerebrospinal fluid, bronchoalveolar lavage, and biopsy) (Karley et al., 2011). These include nucleic acids-based biomarkers such as gene mutations or polymorphisms and quantitative gene expression analysis, peptides, proteins, lipid metabolites, and other small molecules (viz., low molecular weight) as biomarkers, viz., $O_2^-$, NO, $NO_2^-$, $NO_3^-$, urea, and cholesterol. Biomarkers can also be classified based on their application such as diagnostic biomarkers (i.e., cardiac troponin for the diagnosis of myocardial infarction), staging of disease biomarkers (i.e., brain natriuretic peptide for congestive heart failure), disease prognosis biomarkers (cancer biomarkers), and biomarkers for monitoring the clinical response to an intervention (HbA1c for antidiabetic treatment) (Schwarzenbach et al., 2011). Another category of biomarkers includes those used in decision making in early drug development. For instance, pharmacodynamic (PD) biomarkers mark a certain pharmacological response and are of special interest in dose optimization studies.
3.4.1.1 Protein biomarkers
Proteins are fairly large molecules called nanomachines, made up of strings of amino acids linked like a chain (Kohler and Seitz, 2012). There are 20 amino acids, and proteins range in length from a few to more than a thousand amino acids. Different combinations of amino acids link to form tens of thousands of proteins. Proteins usually contain thousands of atoms precisely arranged in a three-dimensional (3D) structure that is unique for each type. As a protein is made, it “folds” itself into a complex, 3D shape, like a piece of ribbon that has been crumpled up. Each protein has one folded shape and consistently folds into it, usually in less than a second. That complicated folded shape dictates how the protein works and also how it interacts with other entities. The specific sequence of amino acids that makes up each protein is coded by a gene in the deoxyribonucleic acid (DNA) of living cells. A protein cannot be synthesized without its messenger ribonucleic acid (mRNA) being present, but a protein can persist in the cell when its mRNA is no longer present. However, mRNA may be present in abundance but the message is not translated into proteins. There is, thus, no good correlation between mRNA and protein in a cell at any given time. Protein synthesis is a very complicated process. Ribosomes are the cell’s protein factories. RNA bridges in the ribosomes are not just support structures but also a part of the protein-forming machinery. Peptides are small proteins that play a central role in almost all biological processes. They function as biochemical messengers (e.g., insulin, calcitonin, and angiotensin) or occur as metabolites of proteins.

3.4.1.2 DNA biomarkers
Genetic information is contained in the cells in the form of DNA, which consists of two strands that resemble a ladder coiled into a spiral shape—the double helix. It is a macromolecule composed of a linear array of nucleotides, each of which comprises a base plus a pentose sugar and phosphate. Only four nucleotide bases are normally found in DNA: cytosine (C), thymine (T), adenine (A), and guanine (G). The information content of the DNA is embodied in the sequential arrangement of nucleotides. The assembly of higher-order structures comprising multiple proteins bound at distinct DNA sites initiates readout of information encoded in the DNA. DNA thus contains the instructions for making proteins. There is a need to assess DNA damage because of the impact that different insults on genetic material may have on human health (Levenson and Melnikov, 2012).

3.4.1.3 RNA biomarkers
Ribonucleic acid (RNA) is the other major nucleic acid besides DNA, but unlike DNA, it is single stranded. It contains ribose instead of deoxyribose as its sugar—phosphate backbone and uracil (U) instead of thymine (T) in its pyrimidine bases (Nilsson et al., 2015). Like DNA, RNA can be assembled from nucleotides using DNA sequence as a template and RNA polymerase. The structure of an RNA molecule is also determined by its
DNA-derived sequence. If proteins are the hardware, RNA is the software controlling how the genes are expressed to make proteins. RNA is unique in being able to store and transmit information as well as process that information. Classically, RNAs can be classified into mRNAs, which are translated into proteins, and nonprotein-coding RNAs (ncRNAs) (Kwok et al., 2015). mRNA is the short-lived intermediary in the transfer of genetic information from DNA to protein. It is transported out of the nucleus and translated into protein on the cytoplasmic ribosomes. Transcriptome is the complete set of mRNA molecules of a cell, tissue, or an organism. Transcription preserves the whole information content of the DNA sequence that it has been transcribed from since the RNA has the same base-pairing characteristics. ncRNA genes produce functional RNA molecules rather than encode proteins and include transfer RNAs (tRNAs) and ribosomal RNAs (rRNA). rRNAs are highly structured and conserved molecules found in all living organisms and are well established as phylogenetic markers. During the last two decades several ncRNAs have emerged, having a diverse range of functions, from structural through regulatory to catalytic (Jain, 2010). A dominating category is that of small nucleolar (sno) RNAs, which act as guides to direct pseudouridylation and 2-O-ribose methylation in rRNA. Other categories are microRNAs (miRNAs), antisense transcripts, and transcriptional units containing a high density of stop codons and lacking any extensive open reading frame. Profiling of human mRNA in serum has been found to be useful for detection of oral squamous cell carcinoma. Human mRNAs are present in saliva and can be used as biomarkers of oral cancer (Markopoulos et al., 2010). Saliva harbors both full-length and partially degraded forms of mRNA. RNA enters the oral cavity from different sources, and association with macromolecules may protect salivary RNA from degradation. However, RNA is unstable and the degradation process is likely to start before the cells are shed from the tissue, limiting its value as a biomarker. The results of measurements of transcript levels in biopsies of oral tissue need to be interpreted with caution. To address the problem of RNA instability, RNA is immediately stabilized after the blood draw by PAXgene (PreAnalytiX). Total RNA is then extracted from PAXgene-stabilized blood and subjected to microarray analysis (Herai et al., 2014). Combining RNA stabilization of peripheral blood with bead-based oligonucleotide microarray technology is not only applicable to small single-center studies with optimized infrastructure but also to large-scale multicenter trials that are mandatory for the development of predictive biomarkers for disease and treatment outcome.

3.5 GLUCOSE OXIDASE-BASED GLUCOSE BIOSENSORS FOR DIABETES

Diabetes is a chronic disease with no cure. It occurs when the pancreas does not produce enough insulin or the body cannot effectively use the insulin it produces (Zhong et al., 2015). This leads to hyperglycemia, characterized by increased blood glucose concentration. Blood glucose monitoring is an integral part of diabetes management, and in this
regard self-monitoring of glucose has been considered by many as one of the most important advances in management of the disease ever since the discovery of insulin (St John et al., 2010). The most commercially successful devices for glucose monitoring are the blood glucose biosensors, the initial concept of which was proposed by Clark and Lyons, which led to the first commercial glucose analyzer launched by Yellow Spring Instrument Co., Ohio. There are now over 40 blood glucose meters in the market, yet many challenges need attention. Most of the meters are based on the glucose oxidase modified electrodes (Pahurkar et al., 2015).

Glucose oxidase (GOX/GOD) is a flavoprotein that catalyses the oxidation of β-D-glucose to D-glucono-δ-lactone and hydrogen peroxide (H₂O₂) using molecular oxygen as an electron acceptor. The mechanism is that GOD accepts electrons in the process of glucose oxidation and thereby changes its active site to an inactivated reduced state (Bankar et al., 2009). It is normally returned to the active oxidized state by transferring these electrons to molecular oxygen, resulting in the production of H₂O₂. This method is widely used for the determination of free glucose in body fluids (diagnostics), in vegetal raw material, and in the food industry. The earliest approaches to the construction of glucose biosensors have already been explained in the preceding paragraphs.

GOD can be immobilized close to an electrode and the depletion of oxygen can be monitored, using a Clark oxygen electrode as depicted in the schematic in Figure 3.3. From this reaction scheme, it is apparent that it is also possible to measure glucose via the oxidation of the hydrogen peroxide produced by the enzymatic reaction, since this is directly proportional to the concentration of glucose (Chen et al., 2013). This can also be measured amperometrically at a potential of approximately +0.7 V versus Ag/AgCl, when a platinum working electrode is used.

The first really successful blood glucose biosensor for home use was a mediated device based on a disposable, screen-printed sensor design. It was developed by a company originally

Figure 3.3 Working mechanism of Clark oxygen electrode.
called Genetics International, in conjunction with the Universities of Cranfield and Oxford. The company was renamed MediSense, and the product that was launched was the ExacTech device (Figure 3.4). It was the first personal blood glucose meter that allowed people with diabetes to test their blood. The device effectively rejected common interferences such as ascorbic acid and paracetamol when present at their physiological concentrations. These principles formed the basis for the commercial success of the Therasense range of sensors.

3.6 NONINVASIVE GLUCOSE BIOSENSOR

Pendragon (Zurich, Switzerland) has developed a noninvasive continuous glucose monitoring system that uses impedance spectroscopy. It measures how changes in blood composition affect the impedance pattern of the skin and underlying tissue. The device itself is the size of a wrist watch and is fixed with an open resonant circuit that lies against the skin. The device is optimized to measure the indirect effects of glucose molecules on the impedance pattern; these measurements are then calculated into glucose concentrations. The device is claimed to be suitable for use by both Type 1 and Type 2 diabetics. However, as is the case with the Cygnus and Minimed devices, Type 1 diabetics must follow a warning from the device with a finger stick test. Differences in the thickness of skin and underlying tissues of the patient require a two-point calibration process. This provides individual user data about absolute offset and the ratio between impedance changes and glucose changes. Variations on impedance due to temperature changes are self-corrected.
3.7 IMPLANTABLE GLUCOSE BIOSENSORS

An implantable glucose measurement system is seen as a key component of such a closed-loop glycemic control system. A major historical advance in the in vivo application of glucose biosensors was reported by those who described the first needle-type enzyme electrode for subcutaneous implantation. Several other implantable sensors from manufacturers are in development and are claimed to be close to market (Vaddiraju et al., 2010). This device will be equipped with alarms to give warnings of impending hypoglycemia and hyperglycemia. Ultimately the aim is to link the sensor to an insulin infusion pump to provide closed-loop control of blood glucose levels. Figure 3.5 shows one of the implantable glucose measurement systems.

3.8 CHOLESTEROL BIOSENSOR

Cholesterol is a waxy steroid metabolite found in the cell membranes and transported in the blood plasma (Turner, 2013). It is an essential structural component of mammalian cell membranes. In addition, cholesterol is an important component for the manufacture of bile acids, steroid hormones, and fat-soluble vitamins including vitamins A, D, E, and K. Cholesterol is the principal sterol synthesized by animals, but small quantities are synthesized in other eukaryotes, such as plants and fungi. Cholesterol holds a significant character and crucial functioning in a number of biomolecules present in animal cells. However, the controlled level of cholesterol in the blood is a highly important parameter for the prevalence of the major life threatening diseases (Arya et al., 2008). In clinical diagnosis, cholesterol is an important indicator in human blood for hypertension, myocardial infarction, and arteriosclerosis. The normal range of total cholesterol in blood plasma, of which one-third is free cholesterol and two-thirds are cholesterol ester, is

![Figure 3.5 implantable glucose sensor.](image-url)
3.96 ± 0.8 mM (153 ± 31 mg per 100 mL) (Rahman et al., 2014). The value may be up to 8 mM for abnormal patients, and the high values have a relationship to the precursors of bile acid and steroid hormones. Therefore, the estimation of cholesterol quantity is of interest to both the biological science and food industries. In previous studies, the sensing process is usually based on spectrophotometry (Huang et al., 1963). However, the analysis involves complicated steps, hence, it is necessary to develop a sensing technique that may offer a faster response and higher selectivity. This is a strong motivation toward the fabrication of robust, facile, cheap, and invasive cholesterol biosensors possessing good sensitivity, electrical conductivity, and thermal stability that could be exploited for the clinical routine diagnosis of cholesterol concentrations. Electrochemical biosensors have been shown to be very effective tools for analysis of biologically important molecules. They are very simple, fast, inexpensive, portable, and capable of reliable response in wide concentration ranges.

Cholesterol oxidase (ChOx) is one such water-soluble enzyme that is catalytically active at the membrane interface from which cholesterol, the substrate, is accessed (Lolekha et al., 2004). Cholesterol is oxidized to cholest-5-en-3-one by the flavin cofactor (Figure 3.6). The reduced cofactor is recycled by oxygen to form hydrogen peroxide. This product is the basis of the serum cholesterol assays because hydrogen peroxide can be coupled to colorimetric assays using horseradish peroxidase (HRP). However, the cholest-5-en-3-one intermediate is not particularly stable.

![Figure 3.6 Schematic diagram of cholesterol oxidase (ChOx) enzymatic reaction.](image-url)
Recently, a cholesterol biosensor was developed using cholesterol oxidase functionalized on multiwall carbon nanotubes film—modified glassy carbon electrode (ChOx/MWCNTs/GCE) (Li et al., 2011; Figure 3.7). The electrocatalytic behavior toward cholesterol was investigated by cyclic voltammetry. This sensor showed excellent performance with a sensitivity of $1261.74 \mu$A mM$^{-1}$ cm$^{-2}$, a linear range of $4.68 \times 10^{-5} - 2.79 \times 10^{-4}$ M, and a detection limit of $4.68 \times 10^{-5}$ M.

### 3.9 OXIDATIVE STRESS BIOMARKERS

Oxidative stress is defined as an imbalance between the systemic manifestation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and a biological system’s ability to readily detoxify the reactive intermediates (Sies, 2015). It results in cellular damage linked to the atherosclerosis, hypertension, heart failure, myocardial infarction, malignancy, and neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease, and ALS (Figure 3.8).

As a consequence of these conditions, the levels of ROS such as superoxide anion radical ($O_2^{•−}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($•OH$) are increased (Lushchak, 2014). They inactivate the mitochondrial enzymes, directly damage DNA, DNA repair enzymes, lipid peroxidation, cyt $c$ release, and transcription factors leading to apoptosis/cell death. Figure 3.9 illustrates the ROS/RNS-induced oxidative damage in proteins, lipids, and DNA.

Further, it also inactivates the endothelium-derived relaxing factor (EDRF), nitric oxide (NO) due to the reaction of NO with $O_2^{•−}$ forming peroxynitrite ($ONOO^{−}$) (Li et al., 2014). In addition, half-life of the NO is very short and therefore immediately oxidized into its stable metabolites nitrite ($NO_2^{−}$) and nitrate ($NO_3^{−}$). They playing a significant role in the events including vasodilation, modulation of cellular respiration, and ischemic stress.
3.10 SUPEROXIDE ANION RADICAL BIOSENSOR

Superoxide anion radical $O_2^{•−}$, a signaling molecule, is involved in cell growth, differentiation, proliferation, DNA repair, and apoptosis at the physiological concentration. At the same time, it also causes oxidative damage at above the physiological levels. Dismutation of $O_2^{•−}$ either by spontaneous process or through a reaction catalyzed...
by superoxide dismutases, produces H$_2$O$_2$, which in turn may be fully reduced to H$_2$O or partially converted to $\cdot$OH, one of the strongest oxidants in nature (Eqn (3.1); Shilin et al., 2007). Thus, the detection of O$_2$$\cdot$$^-$ is necessary to understand the radical’s role in signaling pathways/degenerative processes, and more accurately diagnose the diseases in which it is involved.

\[
\begin{array}{ccc}
\text{hv or other energy} & \text{H}_2\text{O} & \text{O}_2 \\
\text{O}_2 & +e^- & \text{O}_2^- \\
\text{O}_2^- & +e^- & \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 & +e^- & \cdot\text{OH} \\
\text{H}_2\text{O}_2 & +e^- & \text{H}_2\text{O} \\
\end{array}
\]

(3.1)

Recent attempts have concentrated on electrochemical biosensors for the determination of O$_2$$\cdot$$^-$ due to their direct, real-time measurements and capability for in vivo detection. Electrochemical biosensors for the determination of O$_2$$\cdot$$^-$ based on its reduction by cyt $\epsilon$ as a biological recognition element were reported using the reoxidation of cyt $\epsilon$ on an electrode surface (Prieto-Simón et al., 2008; Wang et al., 2014; Shipovskov et al., 2004; Fujita et al., 2009). However, cyt $\epsilon$ is not a specific enzyme for the reduction of O$_2$$\cdot$$^-$. Indeed, the tissue/cell extract contains cyt $\epsilon$ oxidases, peroxidases, and oxidants including H$_2$O$_2$ and ONOO$^-$ that can also reoxidize the reduced cyt $\epsilon$. Consequently, the cyt $\epsilon$-based methods are not suitable for assessment of O$_2$$\cdot$$^-$ levels in biological systems.

Superoxide dismutases (SODs) are ubiquitously distributed in aerobic organisms and play an important role in cell protection mechanisms against oxidative damage from ROS by specifically catalyzing the dismutation of the O$_2$$\cdot$$^- to O$_2$ and H$_2$O$_2$ (Mruk et al., 2002). They comprise a family of metalloproteins primarily classified into four groups: copper-zinc-containing SOD (Cu,ZnSOD) (Salem et al., 2014), manganese-containing SOD (MnSOD) (Haikarainen et al., 2014), iron-containing SOD (FeSOD) (Tessarollo et al., 2015), and nickel-containing SOD (NiSOD) (Kim et al., 2003). The structures of the SODs in the SOD family are known to be relatively different. The Cu,ZnSOD (SOD) is the first SOD characterized and analyzed by X-ray methods. The third loop provides the Greek key connection across the $\beta$-barrel. In the SOD, the metals are bound by sequences connecting the barrel strands and are on the opposite sides of the dimer, with the Cu atoms having a histidine (HIS)-rich environment separated by 33.8 Å (Figure 3.10; Antonyuk et al., 2009). The catalytic rate of SOD is one order of magnitude higher than that of the MnSOD and FeSOD, respectively. The Cu and Zn ions are bound at the base of the active site cavity and are bridged by the imidazolylolate ring of HIS 61.

SOD is a selective scavenger of O$_2$$\cdot$$^-$, is abundantly found in the intracellular cytoplasmic space of aerobic organisms, and plays a central role in protecting cells from O$_2$$\cdot$$^-'-induced oxidative stress. It protects the organism against the toxic effects of the
O₂⁻ by efficiently catalyzing its dismutation to O₂ and H₂O₂ via a cyclic oxidation/reduction electron-transfer mechanism. SOD-immobilized electrodes have paved an elegant way to detect O₂⁻. SOD shows high rate constants up to the orders of 10⁹ M⁻¹ s⁻¹, and is distinguished by its high specificity to O₂⁻ dismutation, offering great potential for the sensitive and selective quantification of O₂⁻. To construct the selective and sensitive biosensor for O₂⁻, much effort has been paid to the direct electron transfer of SOD. We have reported SOD-based biosensor for O₂⁻ by immobilizing SOD on a SAM of cysteine on gold electrode. Tian et al. (2005) also reported carbon fiber microelectrode-based biosensor for O₂⁻ using the direct electron transfer of SOD. Endo et al. (2002) developed an O₂⁻ sensor using mediator-based electron transfer between SOD and the electrode surface. Campanella et al. (1998) have investigated the physical entrapment of SOD on carrageenan gel and measured the transduction signal using amperometric H₂O₂ electrode. Liu et al. (2008) used gold nanostructures for the direct electron transfer of SOD for the quantification of O₂⁻ without using any mediators. We have recently used for the first time nanocomposites based on the integration of the highly porous nature of biocompatible conducting polymer PPy with GNP/CNT for the biofunctionalization of SOD for sensitive and selective O₂⁻ determination.

### 3.10.1 Construction of O₂⁻ biosensor

Pyrrole was first electropolymerized onto the bare Pt electrode to form PPy matrix by following the reported procedure using cyclic voltammetry (CV). The optimum of 10 cycles for pyrrole electropolymerization was used owing to its increased conductivity and large surface area as reported earlier for the effective incorporation of GNP.
GNP of diameter around 6–10 nm was prepared according to a previously published route. The GNP was characterized by ultraviolet-visible (UV-vis) spectroscopy, showing a strong absorption at 525 nm, characteristic of gold plasmon resonance. The obtained PPy-Pt electrode was then immersed in GNP solution for 12 h to form a GNP-PPy-Pt electrode and then carefully rinsed with double-distilled water. Further, the GNP-PPy-Pt electrode modified with the SAM of cysteine was prepared by dipping the GNP-incorporated PPy-Pt electrode into a 1-mM cysteine solution for 10 min and rinsed with double-distilled water to remove the nonchemisorbed cysteine. Then, 5 µL of SOD1 (0.2 g mL⁻¹) solution was dropped onto the SAM–GNP–PPy nanocomposite–modified electrode by employing 5 µL of glutaraldehyde (GA) (2.5%) solution as cross-linking agent to obtain an SOD–functionalized SAM–GNP–PPy–Pt electrode. After that, the electrode was left for at least 24 h at room temperature for efficient cross-linking and stored at 4 °C when not in use.

3.10.2 O₂⁻⁻ sample preparation

The O₂⁻⁻ solutions were prepared by the addition of aliquots of KO₂ stock solution. The O₂⁻⁻ was also generated by the reaction of xanthine with xanthine oxidase (XOD) in 0.1 M phosphate buffer solution (PBS) at room temperature. The reaction for the formation of O₂⁻⁻ is as follows. The PBS containing 25 µM of xanthine and 40 U mL⁻¹ of catalase was added to the electrochemical cell. The function of catalase was to remove the coproduct H₂O₂.

\[
\text{Xanthine} + \text{O}_2 \rightarrow \text{Uric acid} + \text{O}_2^{\cdot -} + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 \rightarrow \frac{1}{2}\text{O}_2^{\cdot -} + \text{H}_2\text{O}
\]

3.10.3 Calibration and measurement of O₂⁻⁻

The electrochemical response of the SOD1-SAM–GNP–PPy–Pt electrode as an O₂⁻⁻ sensor was studied in 0.1 M PBS using the xanthine-XOD system (in situ generation of O₂⁻⁻) as shown in Figure 3.11. Before the addition of xanthine, a steady background response current was observed. After the addition of xanthine (2–500 µM), the current increased both anodically and cathodically (as indicated by arrows). Similar to KO₂ systems, the addition of SOD1 decreased both the anodic and cathodic currents by >95%, due to its scavenging of O₂⁻⁻. These data clearly indicate that the anodic/cathodic currents’ response observed after the addition of xanthine was due to the oxidation/reduction of O₂⁻⁻ via SOD1 confined on the electrode surface.

Using the analytical characteristics of this O₂⁻⁻ biosensor at the optimized conditions, the cathodic currents versus O₂⁻⁻ concentrations are plotted as shown in the inset of Figure 3.11. The calibration curve thus obtained exhibits a linear range for the O₂⁻⁻ concentration from 0.2 to 100 µM (\(r^2 = 0.9939\)) with a detection limit of 0.2 µM.
The SOD1-SAM-GNP-PPy-Pt electrode exhibited excellent analytical performance, for instance, wider linear detection range, shorter response time, and especially high sensitivity, compared to the other reported methods.

### 3.11 THIOL BIOSENSOR

CySH, one of the important sulphhydryl thiol—containing amino acids, plays an important role in pharmaceutical, food industries and in neurological disorders including motor neuron, Parkinson’s and Alzheimer’s diseases. H$_2$O$_2$ formation by the auto-oxidation (disulphide formation) of CySH is reported to cause oxidative stress. Park and Imlay (2003) described the CySH-promoting oxidative DNA damage by driving the Fenton’s reaction. Therefore, the detection and determination of CySH are very important from the biological and pharmacological standpoints. Numerous efforts have been made to develop highly sensitive methods for its detection. Han et al. (2002) measured the total concentration of CySH in human plasma using colorimetric method. Recently, the electrochemical oxidation of CySH has been the subject of many investigations. CySH can be easily oxidized by suitable oxidizing agents or at Pt, Au, carbon, and Hg electrodes.

![Figure 3.11](image)

*Figure 3.11* Typical electrochemical responses of the SOD1-SAM-GNP-PPy-Pt electrode to various concentrations of xanthine (the rate of O$_2$•$^-$ generation is 2.6 µM min$^{-1}$ for 25 µM of xanthine containing 0.002 U of XOD) in 0.1 M PBS (pH 7.0) containing 100 µM DTPA; scan rate of 50 mV s$^{-1}$. Inset: A linear calibration plot of cathodic peak currents against O$_2$•$^-$ concentrations. Each point represents the mean (±0.01 SD) of three measurements.
Unfortunately, the catalytic direct oxidation of CySH at the above solid electrode surface is kinetically slow and needs an overpotential. Hassan et al. (2007) developed a new, fast, simple, and highly selective potentiometric biosensor to determine the CySH in *Tricho-
sporon jirovecii* yeast cells. Deng et al. constructed the boron-doped CNT-modified electrode for the electrocatalytic determination of CySH using chronoamperometric method. Ardakani et al. constructed a carbon paste electrode modified with quinizarine for the measurement of cysteine in the presence of tryptophan and measured the CySH in blood samples and CySH tablets. The common biological interfering substrates perhaps interfere and reduce the sensitivity of the CySH detection. Therefore, it is important to look for the electrocatalytic oxidation that might decrease the overpotential and increase the sensitivity of CySH detection. Earlier, we studied the thiol oxidase—peroxidase activities of SOD. Based on that, we have developed a novel method for the measurement of CySH.

\[
\begin{align*}
2\text{CySH} & \xrightarrow{\text{SOD}} \text{CyS} - \text{SCy} + \text{H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 & \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\end{align*}
\]

The electrochemical biosensor employed for the measurement of CySH is a biennial-
matic system comprising of SOD coimmobilized with horseradish peroxidase (HRP) (Dharmapandian et al., 2010). The biennial cysine biosensor was fabricated by the following procedure. 10 μL of SOD (0.2 mg mL\(^{-1}\)) was first applied onto the PPy-Pt electrode and the electrode was dried at room temperature. Then the enzyme was cross-linked with 2.5% gluteraldehyde by Dip dry method. Further, 10 μL of HRP (0.2 mg mL\(^{-1}\)) was coimmobilized on SOD-PPy-Pt electrode. And then the HRP-SOD-PPy-Pt electrode was immersed in 0.2 mM thionine for 24 h and allowed to dry at room temperature.

The SOD oxidase—peroxidase activities with CySH first generate H\(_2\)O\(_2\). This generated H\(_2\)O\(_2\) is further detected using HRP in the presence of thionine as shown in the below mechanism:

\[
\begin{align*}
\text{E-Cu(II)} + \text{CySH} & \rightarrow \text{E-Cu(I)} + \text{CyS} \\
\text{E-Cu(I)} + \text{O}_2 & \rightarrow \text{E-Cu(II)} + \text{O}_2^{\cdot -} \\
\text{CyS}^{\cdot -} + \text{CySH} & \rightarrow (\text{CyS-CyS})^{\cdot -} + \text{O}_2 \rightarrow \text{O}_2^{\cdot -} + \text{CyS-CyS} \\
\text{E-Cu(II)} + \text{O}_2^{\cdot -} + 2\text{H}^+ & \rightarrow \text{E-Cu(I)} + \text{H}_2\text{O}_2 \\
\text{E-Cu(I)} + \text{H}_2\text{O}_2 & \rightarrow \text{E-Cu(II)} - \cdot \text{OH} \\
\text{E-Cu(II)} - \cdot \text{OH} + \text{HCO}_3^{\cdot -} & \rightarrow \text{E-Cu(II)} + \cdot \text{CO}_3^{\cdot -} \\
\text{HRP} + \text{H}_2\text{O}_2 & \rightarrow \text{HRP-I} + \text{H}_2\text{O} \\
\text{HRP-I} + (\text{TH})_{\text{red}} & \rightarrow \text{HRP-II} + (\text{TH})_{\text{ox}} \\
\text{HRP-II} + (\text{TH})_{\text{red}} & \rightarrow \text{HRP} + (\text{TH})_{\text{ox}} \\
(\text{TH})_{\text{ox}} + 2\text{e}^- & \rightarrow (\text{TH})_{\text{red}}
\end{align*}
\]
The obtained electrochemical responses of the CySH biosensor for the PBS and cysteine in the presence and absence of bicarbonate (HCO₃⁻) are shown in Figure 3.12. There is no significant change in the electrochemical response for CySH (curves c and d) in 0.1 M PBS. But in the presence of HCO₃⁻ (curves a and b), the electrode responds with a remarkable increase in current at the cathodic peak potential at −0.37 V. From this result, it is clearly revealed that the bienzymatic electrode exhibited a synergistic electrochemical response with cysteine due to the bicarbonate-dependent peroxidase activity stimulated by thiol oxidase activity of SOD (Karunakaran et al., 2005).

The detection limit of 10 μM CySH was obtained by using the thionine-mediated HRP-SOD-PPy-Pt electrode. The long-term stability of the TH-HRP-SOD-PPy-Pt electrode was evaluated by measuring the electrochemical response with CySH over a period of 4 weeks. The CV response was found to be stable and reproducible.

**3.12 NITRIC OXIDE BIOSENSOR**

Nitric oxide (NO) is an important messenger molecule regulating the biological processes, viz., blood vessel relaxation, neuronal cell-to-cell communication, and immune function (Szabo, 1996; Ahanchi et al., 2007). Its level is significantly altered in serum as well as in exhaled breath during oxidative stress, airway inflammation, and various diseases such as asthma and primary cilia dyskinesia (Nevin and Broadley, 2002; Zitt, 2005). Therefore, the measurement of NO is essential in human physiology. Commercially.
available NO analyzers function based on the chemical reaction of NO with ozone (O₃), and the concentration of NO is measured with respect to the luminescent intensity (Robinson et al., 1999; Buchvald et al., 2005). These analyzers involve high costs, corrosive chemicals, and are also an indirect method for the determination of NO.

Recently, electrochemical biosensor techniques have shown great promise for their simplicity, high sensitivity, good selectivity, fast response, and long-term stability for the direct determination of NO (see above). Ciszewski and Milczareck (2004) reported the electrochemical detection of NO using polymer-modified electrodes. Using manganese(III)meso-tetrakis(N-methylpyridinium-4-yl) porphyrin-modified ITO electrode, Trofimova et al. (2005) measured the NO concentration. A chemically modified ultramicroelectrode was fabricated by Rievaj et al. (2004) for the NO measurement in blood samples. Earlier research reports suggest that NO being a small molecule similar to O₂⁻ penetrates into the active site of the SOD and gets oxidized (Rajesh et al., 2010). Therefore, based on the NO oxidase activity of SOD, an electrochemical NO biosensor developed using SOD functionalized on carbon nanotubes (CNT)-polypyrrole (PPy) nanocomposite-modified Pt electrode has been described as below. It exhibits excellent electroanalytical properties such as linearity, sensitivity, selectivity, and stability.

### 3.12.1 Fabrication of nitric oxide biosensors

The Pt electrode modified with PPy and CNT nanocomposite has been used as an immobilization matrix for SOD. Initially the Pt electrode is polished with alumina powder (size 0.05 and 1.0 μm) and then PPy is formed by electropolymerization of 0.4 M pyrrole using 0.1 M KCl as supporting electrolyte and cycling the potential between 0.0 and +0.9 V versus Ag/AgCl with a scan rate of 50 mV s⁻¹ for 10 complete cycles. After PPy is coated on the Pt electrode, CNT is integrated by dropping 25 μL of CNT solution (1 mL of 0.5 wt% Nafion-ethanol solution containing 2 mg of CNT) on the PPy-Pt electrode and dried at room temperature. Then, 10 μL of SOD1 solution is dropped onto the CNT-PPy-Pt electrode by employing the 5 μL of glutaraldehyde as a cross-linking agent to obtain an SOD-CNT-PPy-Pt electrode (Figure 3.13). This SOD-modified electrode should be immersed in 0.1 M PBS to remove the loosely adsorbed SOD and is stored at 4 °C when not in use. This NO biosensor morphological characterization using scanning electron microscopy and electrochemical characterization using cyclic voltammetry confirms the formation of nanocomposite and attachment of SOD, respectively.

### 3.12.2 Measurement of NO in breath and endothelial cells

Figure 3.14 explains the typical electrochemical responses obtained for the SOD-PPy-Pt and SOD-CNT-PPy-Pt electrodes in the absence (curves a and b) and presence (curves c and d) of 10 μM NO at a scan rate of 50 mV s⁻¹ in 0.1 M PBS at pH 7.0. Before
the addition of NO, there were no changes observed in the current response. However, after the addition of NO, both the SOD-PPy-Pt and SOD-CNT-PPy-Pt electrodes exhibited significant increase in current anodically at the potential, +0.8 V. It is attributed to the electrochemical oxidation of NO to NO$_2^-$ via a cyclic redox reaction of SOD active site Cu(I/II) moiety (Figure 3.14; Madasamy et al., 2012).

Figure 3.15 illustrates the electrochemical responses obtained for the SOD1-CNT-PPy-Pt electrode at the various NO concentrations using a scan rate of 50 mV s$^{-1}$. The observed anodic currents versus NO concentrations were plotted as shown in Figure 3.15. The calibration curve thus obtained exhibits a linear range of response over the concentration of NO from 0.1 μM to 1 mM ($r^2 = 0.999$, n = 3) with a detection limit of 0.1 μM and the sensitivity of 1.1 μA μM$^{-1}$.

The analytical applicability of the NO biosensor has been investigated for human exhaled breath and endothelial cell culture samples as described below. The human
Exhaled breath consists of NO, acetone, ammonia, ethanol, isoprene, CO, CO₂, and O₂. Here Nafton membrane-coated NO biosensor is used for the selective measurement of NOₓ. Exhaled breaths of four healthy individuals of different ages from 24 to 32 years were used for the study by following the American Thoracic Society guidelines. The subject exhaled through the mouth using a mouthpiece by slow exhalation for 20 s at a constant flow rate, 50 mL s⁻¹ into an electrochemical cell containing 1 mL of deaerated 0.1 M PBS. After that, CV was run for the sample (exhaled NO in the form of dissolved NOₓ) and the corresponding current response was observed. Then, the concentration of NO was estimated by interpolating the obtained current response into the linear plot prepared by the standard NO solutions and the measured values are shown in Table 3.2. Each reading represents the average of three measurements. The mean ± standard deviation (SD) of 24.5 ± 0.5 ppb of NO was observed in the exhaled breath of normal human. The measured values are in good agreement with the earlier reported data (Buchvald et al., 2005).

![Figure 3.15](image.png)

**Figure 3.15** Typical electrochemical responses obtained for the SOD1-CNT-PPy-Pt electrode in 0.1 M PBS containing (a) 20, (b) 40, (c) 60, (d) 80, (e) 100, (f) 120, and (g) 150 µM NO solution at scan rate of 50 mV s⁻¹ versus Ag/AgCl. Inset: A linear calibration plot of anodic peak currents against NO concentrations (y = −0.9998x − 10.707, r² = 0.999).

<table>
<thead>
<tr>
<th>Age (Yrs)</th>
<th>Weight (Kg)</th>
<th>Concentration of NO ± SD (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>70</td>
<td>23 ± 0.46</td>
</tr>
<tr>
<td>26</td>
<td>55</td>
<td>20 ± 0.40</td>
</tr>
<tr>
<td>29</td>
<td>55</td>
<td>25 ± 0.50</td>
</tr>
<tr>
<td>32</td>
<td>65</td>
<td>30 ± 0.60</td>
</tr>
</tbody>
</table>

**Table 3.2** Determination of NO Level Present in the Exhaled Breath
The measurement of NO release from H₂O₂-treated endothelial cells assisted for proving the dose-dependent activity. After 8 h stimulation of cells by 250 μM H₂O₂, the anodic peak current at +0.8 V clearly increased compared to control and it was found that the concentration of NO released from the endothelial cell was 20.3 ± 0.4 μM. The anodic peak current of the 500 μM H₂O₂ stimulation was also investigated and it was found that 50.2 ± 1.0 μM of NO was released. The determined concentrations of the NO levels generated from the endothelial cells were in agreement with the reference method.

### 3.13 Nitrite Biosensor

There are several enzymatic biosensors reported for the determination of NO₂⁻ by means of its electroreductive reaction using heme proteins and nitrite reductases. Dai et al. (2008) and Hong and Dai (2009) reported the NO₂⁻ biosensor using hemoglobin-modified electrodes. The immobilization of nitrite reductase enzyme on the methyl viologen-modified glassy carbon electrode has been reported by Quan et al. (2006) for the measurement of NO₂⁻. However, these electroreductive reactions involved in the above-reported methods gave NO as a product causing interference by its reaction with oxygen. Hence, the researchers focused on the electrocatalytic oxidation of NO₂⁻ for its selective measurement without any interference. Therefore, for the first time the anodic oxidation of NO₂⁻ using the nitrite oxidase activity of SOD is described (Rajesh et al., 2011). Since the active channel of SOD is narrow to pass NO₂⁻, SOD was used as a specific biorecognition element for the determination of NO₂⁻ (Karunakaran et al., 2004). The electrochemical biosensor (SOD-CNT-PPy-Pt) for the measurement of NO₂⁻ has been fabricated as described in the section.

**Determination of NO₂⁻**

Figure 3.16 displays the electrochemical responses obtained for the SOD1-PPy-Pt and SOD-CNT-PPy-Pt electrodes in the absence (curves a and b) and presence (curves c and d) of 200 μM NO₂⁻ at a scan rate of 50 mV s⁻¹ in 0.1 M PBS (pH 7.0). Before the addition of NO₂⁻, no change was observed in the current response. However, after the addition of 200 μM NO₂⁻, SOD-PPy-Pt and SOD-CNT-PPy-Pt electrodes exhibited significant increases in current anodically at the potential, +0.8 V. These increases are ascribed to the electrochemical oxidation of NO₂⁻ to NO₃⁻ via a cyclic redox reaction of SOD active site Cu(I/II) moiety. The electrochemical responses of the NaR-SOD-CNT-PPy-Pt electrode in 0.1 M PBS (control) and various NO₂⁻ concentrations using the same scan rate are shown in Figure 3.17. As the concentration increases, the anodic current response also increases linearly at +0.8 V. The observed anodic peak currents versus NO₂⁻ concentrations were plotted as shown in the inset of Figure 3.17. The calibration curve thus obtained exhibits a linear range of response.
over the concentration of NO₂⁻ from 100 nM to 1 mM but for clarity here we have shown from 50 to 500 µM (r² = 0.9953, n = 3) with a detection limit of 50 nM and sensitivity of 98.5 ± 1.7 nA µM⁻¹ cm⁻².

3.14 NITRATE REDUCTASE-BASED BIOSENSOR FOR NITRATE

Nitrate reductases are widespread in both eukaryotes and prokaryotes. They are broadly classified into assimilatory and dissimilatory NaR, based on their important role in nitrogen assimilation and dissimilation (Campbell, 1999). Eukaryotic NaR is part of the sulfite
oxidase family of molybdoenzymes. They transfer electrons from NADH or NADPH to nitrate. Prokaryotic NaR belongs to the DMSO reductase family of molybdoenzymes (Elliott et al., 2004). They are diverse enzymes, in terms of active site constitution, subunit structure, and cell localization. They are usually homodimers or homotetramers of subunits whose molecular weight is approximately 95–100 kDa or 50 kDa, respectively. Each subunit contains FAD (the site for NAD(P)H oxidation), a b-type cytochrome and a molybdenum-pterin group (the site for nitrate reduction) in a 1:1:1 stoichiometry. Each cofactor domain constitutes an autonomous structural element and even isolated it retains its partial activity. The molybdenum domain of the NaR is responsible for NO$_3^-$ reduction into NO$_2^-$, with a pyridine nucleotide as the natural enzyme regenerator (Fischer et al., 2005). First, NO$_3^-$ molecule binds to the reduced Mo(IV) active site moiety. Upon binding, the oxygen closest to Mo attacks the metal center, thereby displacing the equatorial hydroxo/water ligand from Mo, thus forming the reaction intermediate. The position of the reaction intermediate is chosen by a planarity restrain for the nitrogen, NO$_3^-$ oxygen, Mo, and the apical oxygen bonds according to the stereochemistry. Further, the reactions of model compounds and reasonable distances to adjacent atoms are also considered. Once the reaction intermediate is formed, the electrons of the Mo d-orbital flip over to the Mo–ONO$_3^-$ bond, thereby forming the second Mo–O bond and causing the oxidation of Mo(IV) to Mo(VI) (Figure 3.18). Upon oxidation of the Mo center to Mo(VI), the bond between the NO$_3^-$ oxygen and nitrogen is broken, and NO$_2^-$ will be released (stages 4 and 5). After completion of the reductive half-reaction, the Mo is regenerated [Mo(IV)] for the next cycle. Upon product formation, the Mo center can be regenerated by the reductive half reaction, where two electrons derived from NAD(P)H are transferred via an intramolecular electron transport chain to the Mo.

![Figure 3.18](image.png)  
*Figure 3.18* Reaction mechanism of NO$_3^-$ reduction into NO$_2^-$ by NaR.
In the literature, several enzymatic (Kirstein et al., 1999; Cosnier et al., 1994) and nonenzymatic (Gamboa et al., 2009; Groot and Koper, 2004) electrochemical biosensors were reported for the $\text{NO}_3^-$ determination. The enzymatic determination of $\text{NO}_3^-$ using nitrate reductase (NaR)–modified electrode is novel and highly selective. NaR is a multidomain enzyme containing flavin adenine dinucleotide (FAD), two heme-Fe and molybdopterin, which catalyzes the two-electron reduction of $\text{NO}_3^-$ to $\text{NO}_2^-$ (Quan et al., 2005).

$$\text{NO}_3^- + \beta-\text{NAD}(P)H \xrightarrow{\text{NaR}} \text{NO}_2^- + \text{H}_2\text{O} + \beta-\text{NAD}(P)$$

### 3.14.1 Measurement of $\text{NO}_3^-$

The biosensing electrodes were prepared as mentioned earlier using NaR instead of SOD (Figure 3.19). Figure 3.20 shows the electrochemical responses obtained for the bare Pt, NaR–PPy–Pt, and NaR–SAM–GNP–PPy–Pt electrodes in the absence (curves a, b, and d)
and presence (curves c and e) of 500 μM NO$_3^-$ in 0.1 M PBS (pH 7.0) at a scan rate of 50 mV s$^{-1}$ versus Ag/AgCl. The current responses obtained for the bare Pt before and after NO$_3^-$ addition were same (curve a). Then, the current responses of the NaR-PPy-Pt (curve b) and NaR-SAM-GNP-PPy-Pt (curve d) electrodes were not changed before the addition of NO$_3^-$ but show their characteristic redox peak at the potential, −0.76 and −0.62 V. However, after the addition of NO$_3^-$, the current is significantly increased in both NaR-PPy-Pt (curve c) and NaR-SAM-GNP-PPy-Pt (curve e) electrodes cathodically as well as anodically. This is due to the enhanced electrocatalytic activity of the NaR toward NO$_3^-$ reduction into NO$_2^-$ via a cyclic redox reaction of its active site Mo(IV/VI) moiety as shown in Figure 3.21.

Typical CVs were obtained for several concentrations of NO$_3^-$ in 0.1 M PBS at 50 mV s$^{-1}$ for NaR-SAM-GNP-PPy-Pt. The observed cathodic currents versus NO$_3^-$ concentrations are plotted. The calibration curve thus obtained for NaR-SAM-GNP-PPy-Pt electrode exhibits a linear range of response for the NO$_3^-$ concentrations from 1 μM to 1 mM ($r^2 = 0.9937$, $n = 3$) with a detection limit of 0.5 μM.

The biosensor electrodes were prepared as mentioned earlier using NaR instead of SOD. Further, the NaR-SAM-GNP-PPy-Pt electrode shows the high sensitivity, 84.5 nA μM$^{-1}$. Moreover, the present NO$_3^-$ biosensor shows a wider linear detection range, lower detection limit, and higher sensitivity than the earlier reported NO$_3^-$ biosensors.

### 3.14.2 Analytical applications for biological samples

Beetroot juice contains a high level of NO$_3^-$, substantially decreases blood pressure (BP) levels, inhibits platelet aggregation, and prevents ischemia-induced endothelial dysfunction. The measurement of NO$_3^-$ concentration in beetroot supplement provides supporting information to prove its natural remedy effect. Therefore, Madasamy et al. (2013)

![Figure 3.21](image)

**Figure 3.21** Schematic illustration of the electrochemical reduction of NO$_3^-$ by NaR during the measurement of NO$_3^-$.
attempted to measure the NO$_3^-$ in the beetroot supplement using highly sensitive NO$_3^-$ biosensor. For the measurement, 1 mL of beetroot sample was taken in an electrochemical cell and monitored the current response of the NaR-SAM-GNP-PPy-Pt electrode. The cathodic peak current obtained at $-0.76$ V for the supplement was interpolated with the calibration curve giving the concentration of NO$_3^-$ present in the sample. The accuracy of the biosensor was investigated by comparing the results obtained using standard spectrophotometry (Griess) method as shown in Table 3.3. The observed results were comparable.

Further, using this biosensor the concentration of NO$_3^-$ released from lipopolysaccharide (LPS)-induced apoptosis in human breast cancer cells (MCF-7) was also measured. After 24 h stimulation by 50 ng LPS, the observed cathodic peak current at $-0.76$ V obviously increased compared to control and it was found that 86.5 ± 1.73 μM of NO$_3^-$ was released from the cells. Likewise, the cathodic peak current for the 100 ng LPS stimulation was observed and it was found that 200.4 ± 4.0 μM of NO$_3^-$ was released. Thus, LPS dose dependently stimulated NO$_3^-$ release was observed from the cancer cells, and the values were in agreement with the reference method.

Table 3.3 Measurements of NO$_3^-$ in Beetroot Supplements Using Present Biosensor and Griess Method

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Nitrate Conc. by Griess Method (μM)</th>
<th>Nitrate Conc. by Biosensor (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>592.5 ± 14.80</td>
<td>598.7 ± 11.97</td>
</tr>
<tr>
<td>02</td>
<td>594.3 ± 14.85</td>
<td>602.4 ± 12.05</td>
</tr>
<tr>
<td>03</td>
<td>590.5 ± 14.76</td>
<td>600.0 ± 12.0</td>
</tr>
</tbody>
</table>

3.15 APOPTOSIS MARKER

Apoptosis or programmed cell death is a normal, highly conserved physiological process and is an active field of biochemical and biomedical research. This regulated process is responsible for the removal of damaged or infected cells from the cellular population, which links apoptosis to the cell cycle, replication, and DNA repair (Taylor et al., 2008). Moreover, apoptosis is one of the main mechanisms governing accurate embryonic development and the maintenance of tissue homeostasis (Elmore, 2012). Apoptosis in cells can be characterized by specific morphological and biochemical changes, viz., nuclear shrinkage, chromatin condensation, DNA fragmentation, and membrane blebbing (Rastogi and Sinha, 2009). To date, research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Wang and Youle, 2009). In both pathways of cell death, translocation of cyt c release from mitochondria to cytosol is one of the most important regulatory steps of apoptosis.
Once cyt c has been released into the cytosol, it is able to interact with a protein called Apaf-1 (Figure 3.22). This leads to the recruitment of pro-caspase 9 into a multiprotein complex with cyt c and Apaf-1 called the apoptosome. Formation of the apoptosome leads to activation of caspase cascades, which further leads to activation of apoptosis (Jiang and Wang, 2004). Such cyt c release has been documented for apoptosis induced by chemotherapeutic drugs, oxidative stress, UV irradiation, serum, glucose deprivation (Kaufmann and Earnshaw, 2000; Kannan and Jain, 2000).

In addition to its well-established role as an electron shuttle between mitochondrial respiratory complexes and biomarker of apoptosis, the antioxidant role of cyt c has been linked to its propensity to catalyze the oxidation of ROS, especially O$_2^•$ to molecular oxygen. Thus, cyt c can act as an ROS scavenger (Atlante et al., 2000; Pasdois et al., 2011). At early stages of apoptosis, a mitochondrion-specific phospholipid, cardiolipin, binds cyt c in between the outer and the inner mitochondrial membranes. Cyt c-bound cardiolipin undergoes a conformational change and acquires peroxidase activity. The catalytic cyt c peroxidizes cardiolipin and cardiolipin peroxidation products in turn are responsible for membrane permeabilization and cyt c release.

In the last few decades, it has been confirmed that cyt c is released into the blood circulation following myocardial infarctions. In myocardial infarctions, the oxygen supply to regions of the heart becomes interrupted; when such events occur, cells die, releasing cyt c, into the circulating blood (Pérez-Pinzón et al., 1999). Moreover, the utility of cyt c as an in vivo marker of disease and injuries has been predominantly examined in clinical

![Figure 3.22](image.jpg): Mitochondrial-mediated apoptotic signaling. Cyt c release from the mitochondrion leads to formation of the apoptosome and activation of pro-caspase-9. Active caspase-9 cleaves and activates caspase-3, which leads to apoptosis.
studies including fulminant hepatitis, debilitating brain injury, neurological disease, such as influenza-associated encephalopathy and ALS (Renz et al., 2001).

The techniques that are currently used by most laboratories to measure cyt c release include ELISA, Western blot, and flow cytometry (Kim et al., 2007; Ott et al., 2002; Christensen et al., 2013; Adachi et al., 2004). Despite providing high sensitivity and selectivity, these traditional analytical methods still have some drawbacks, such as time-consuming, sophisticated, expensive equipment, limitations in colored sample analysis, and the demand for skilled professionals. To minimize limitations imposed by traditional methods, electrochemical biosensors/immunosensors combined with the high specificity of conventional methods also present several advantages including the possibility of point-of-care testing development.

3.15.1 Cytochrome c oxidase and reductase-based cytochrome c biosensors

Cyt c is a heme-containing metalloprotein located in the intermembrane space of mitochondria. It plays a key role in the biological respiratory chain, whose function is to transfer electrons between cytochrome c reductase (CcR) (complex III) and cytochrome c oxidase (CcO) (complex IV). Mitochondria, besides their primary physiological function to generate ATP through oxidative phosphorylation, are also an important source for the production of cellular ROS. Recent findings have implicated that oxidative stress can cause mitochondrial dysfunctions, protein oxidation, and excessive cellular damage, all of which ultimately releases the cyt c from mitochondria into cytosol of the cells. This translocation of cyt c from mitochondria to cytosol is a critical event in the activation of intracellular signaling; it results in a cascade of caspase activation and leads to programmed cell death—apoptosis. Thus, the quantification of cyt c release as a biomarker of apoptosis is of great importance in clinical diagnosis and therapeutic research.
Recently, amperometric sensors for the direct determination of cyt c with good detection limits were reported (Zhao et al., 2008; Liu and Wei, 2008). However, they suffer from lack of selectivity for the quantification of cyt c, especially in cells or biological samples, due to the fact that the interaction of the recognition elements, viz., single-strand DNA-functionalized GNP and lauric acid—modified lipid bilayer (negative charge) with the cyt c (positive charge) is purely based on electrostatic interactions. These methods are prone to interferences by other positively charged species present in the samples and hence are not applicable for the quantification of cyt c release in biological systems. Enzymatic biosensors for the determination of cyt c have also been investigated by incorporating cytochrome c oxidase (CcO) (Li et al., 1996; Ashe et al., 2007). However, the CcO-based biosensors are capable of determining only the reduced form of cyt c (Fe^{2+}) by mediating electron transfer between the cyt c (Fe^{2+}) and the electrode. But in apoptotic cells, only the oxidized form of cyt c (Fe^{3+}) triggers the time-dependent caspase activation and serves as a proapoptotic molecule (Pasdois et al., 2011). Moreover, in permeabilized cell models, the cytosolic cyt c (Fe^{2+}) is rapidly oxidized (Fe^{3+}) by the mitochondrial CcO (Brown and Borutaite, 2008), thus making it difficult for the CcO-based biosensors to quantify the apoptotic form of cyt c (Fe^{3+}). Further, upon immobilization, it is reported that the electron transfer is blocked in active centers of the CcO (Hrabakova et al., 2006). Consequently, the analytical applications of CcO-based biosensors are limited. Thus, there is a real need for simple, rapid, selective, and inexpensive methods for cyt c (Fe^{3+}) measurement for point-of-care and research applications. Therefore, in this section we have described an alternate method for the detection of mitochondrial cyt c release for the first time using CcR functionalized with nanocomposites-decorated electrodes. Two nanocomposite platforms were used for the fabrication of biosensor: (1) CNT-incorporated PPy-Pt and (2) SAM-functionalized GNP in PPy-Pt for biofunctionalization of CcR.

### 3.15.2 Determination of cyt c

Typical CVs obtained for CcR-PPy-Pt (a), CcR-SAM-GNP-PPy-Pt (b) and CcR-CNT-PPy-Pt (c) electrodes in 0.1 M PBS in the presence of 500 µM of cyt c containing 0.5 mM HQ were compared in Figure 3.24. Upon addition of cyt c, the current increased cathodically at −0.45 V and also anodically at −0.35 V, which was attributed to the redox reaction of cyt c by the CcR, see Figure 3.24. CcR-SAM-GNP-PPy-Pt and CcR-CNT-PPy-Pt electrodes showed a huge increase in currents at −0.45 and −0.34 V versus Ag/AgCl than that of the CcR-PPy-Pt. The remarkable increase in currents can be attributed to the large number of CcR firmly functionalized on the electroactive nanoporous surfaces provided by the GNP-PPy/CNT-PPy nanocomposite than in the only microporous PPy matrix. Further, the CcR-CNT-PPy nanocomposites-based biosensor exhibited nearly a two-fold increase in current response than the CcR-GNP-PPy biosensor. This higher increase in the current for CNT-PPy platform
may be explained due to its high electrical conductivity and fast electron transfer. Also, the nanoscale contours of these nanotubes perhaps penetrated slightly into the CcR thereby lowering the electron transfer distance between the electrode and the various active sites of the CcR.

The CVs were obtained for several concentrations of cyt c in 0.1 M PBS containing 0.5 mM HQ using these two cyt c biosensors at 50 mV s\(^{-1}\) as shown in Figure 3.25. The current responses to cyt c obtained with the CcR-CNT-PPy-Pt biosensor were linear from 1 to 1000 µM \((r^2 = 0.997)\), with a detection limit of 0.5 ± 0.03 µM and sensitivity of 0.46 ± 0.003 µA µM\(^{-1}\) cm\(^{-2}\).

### 3.15.3 Measurement of cyt c released from mitochondria

An important step in the mitochondrial pathway is the release of cyt c from mitochondria into cytosol. Earlier it was demonstrated that the cyt c translocation from the mitochondria into cytosol preceded doxorubicin (DOX)-induced apoptosis in various cell and animal models \((\text{Hrabakova et al., 2006})\). Therefore, we chose DOX for the induction of apoptosis in human lung carcinoma A549 cells. Recent findings revealed that the oxidized form of cyt c \((\text{Fe}^{3+})\) mainly induced the caspase activation, thereby causing apoptosis over the reduced form of cyt c \((\text{Fe}^{2+})\) \((\text{Pandiaraj et al., 2013})\). This clearly indicates that the measurement of only the oxidized form of cyt c \((\text{Fe}^{3+})\) in cytosol presumably serves as a marker for apoptotic process in cells. In this report, cyt c \((\text{Fe}^{3+})\) measurements were performed on the cytosolic fractions of DOX-treated and

![Figure 3.24 CV responses of (a) CcR-PPy-Pt, (b) CcR-SAM-GNP-PPy-Pt, and (c) CcR-CNT-PPy-Pt electrodes in the presence of 500 µM cyt c solution in 0.1 M PBS containing 0.5 mM HQ; scan rate of 50 mV s\(^{-1}\). (Reproduced from Pandiaraj et al., Bioelectrochemistry, 2013, by permission of Elsevier Science Ltd.).](image-url)
untreated human lung carcinoma apoptotic A549 cells using the CcR-CNT-PPy-Pt biosensor and Western blot. After 24 h exposure of cells with 1 μM DOX, the cyt c concentration in cytosolic fractions of the cells (3.63 ± 0.02 μM) was increased when compared to that in untreated cells (2.4 ± 0.02 μM). Treatment for 48 h resulted in further increase in cytosolic cyt c (5.3 ± 0.018 μM) levels. These results are quite comparable with the cell viability studies and Western blot analysis. Table 3.4 compares the electroanalytical performance of the present CcR-based biosensors with CcO-based biosensors.

**Table 3.4** Comparison of Electroanalytical Performances of CcR-Based Biosensors with CcO-Based Biosensors

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Linearity</th>
<th>Sensitivity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CcO-SAM-Au</td>
<td>5–200 mM</td>
<td>–</td>
<td>Li et al. (1996)</td>
</tr>
<tr>
<td>CcO-DDAB-Au</td>
<td>0.2–800 μM</td>
<td>–</td>
<td>Ashe et al. (2007)</td>
</tr>
<tr>
<td>CcR-SAM-GNP-PPy-Pt</td>
<td>5–600 μM</td>
<td>0.24 ± 0.004 μA μM⁻¹ cm⁻²</td>
<td>Pandiaraj et al. (2013)</td>
</tr>
<tr>
<td>CcR-CNT-PPy-Pt</td>
<td>1–1000 μM</td>
<td>0.46 ± 0.003 μA μM⁻¹ cm⁻²</td>
<td>Pandiaraj et al. (2013)</td>
</tr>
</tbody>
</table>

**Figure 3.25** Typical CV responses of the CcR-CNT-PPy-Pt electrode in 0.1 M PBS containing 0.5 mM HQ, without (a) and with 50, 100, 200, 300, 400, and 500 μM of cyt c (b–g) measured at scan rate of 50 mV s⁻¹. A linear calibration plot of cathodic peak currents against cyt c concentrations (inset of Figure 3.25). Each point represents the mean (±0.03 SD) of three measurements.
3.16 SIMULTANEOUS DETERMINATION OF BIOMARKERS

Due to the complexity of biological systems, especially the human body, a single biomarker alone is not effective enough for accurate diagnosis. A medical decision based on a single biomarker usually has a high possibility of being false positive or false negative (Wei et al., 2010). Recently, research has shown that a combination of multiple biomarkers generates improved accuracy instead of relying on a single biomarker. The combination of multiple biomarkers is not limited to numbers of biomarkers for single type, i.e., proteinomic and genomic. Those biomarkers in the combination could also include nucleic acids, proteins, and small molecules. Therefore, multiplexing detection of different types of biomarkers is essential for accurate diagnosis.

The following section describes a simultaneous determination of nitrite and nitrate in biological samples.

3.16.1 Simultaneous determination of nitrite and superoxide anion radical

In the literature, \( \text{NO}_2^- \) and \( \text{O}_2^{\cdot-} \) were determined by several independent methods. \( \text{NO}_2^- \) determination is mainly carried out by spectrophotometry (Griess reagent), ionic chromatography, capillary electrophoresis, and fluorescence methods (Moorcroft et al., 2001). Similarly, for the detection of \( \text{O}_2^{\cdot-} \), indirect methods (Fridovich, 1997) such as spectrophotometric measurement (Haseloff et al., 1991), chemiluminescence method (Reichl et al., 2001), and electron spin resonance spectroscopy (Harbor and Hair, 1978) were used. However, these strategies are ex situ detection techniques with low sensitivity and poor selectivity. Therefore, the researchers were moved to more suitable electrochemical measurement of \( \text{NO}_2^- \) and \( \text{O}_2^{\cdot-} \). In recent years, the enzymatic and nonenzymatic modified electrodes have been used for the individual electrochemical determination of \( \text{NO}_2^- \) and \( \text{O}_2^{\cdot-} \). \( \text{NO}_2^- \) biosensors have been widely reported using many heme proteins, viz., hemoglobin, nitrite reductase, and myoglobin (Wu et al., 1997; Yang et al., 2005; Astier et al., 2005; Sun et al., 2009). These biosensors are mainly ascribed to the electroreductive reactions of \( \text{NO}_2^- \) leading to the formation of several products depending on the electrode conditions and the nature of the catalyst employed. But, the anodic oxidation is a straightforward reaction, with \( \text{NO}_3^- \) being the final product. Hence, the anodic \( \text{NO}_2^- \) determination has attracted great attention because it offers several advantages especially with no interference (see above).

For an individual electrochemical determination of \( \text{O}_2^{\cdot-} \), SOD1, a selective scavenger of \( \text{O}_2^{\cdot-} \), has been the best approach for the detection of \( \text{O}_2^{\cdot-} \) compared to cyt c and hemin. Mostly, SOD1-immobilized electrodes have paved an elegant way to detect \( \text{O}_2^{\cdot-} \). SOD1 shows high rate constants, up to the order of \( 10^9 \text{M}^{-1} \text{s}^{-1} \), and is distinguished by its highly uncommon specificity to \( \text{O}_2^{\cdot-} \). Therefore, SOD1, a specific enzyme for \( \text{O}_2^{\cdot-} \) dismutation, offers a great potential for the sensitive and selective
quantification of $\text{O}_2^{\cdot-}$ using electrochemical biosensors. The SOD1-modified electrode is the biosensor used for the simultaneous determination of $\text{NO}_2^{\cdot-}$ and $\text{O}_2^{\cdot-}$.

In earlier sections, using the same SOD1-modified electrode, we have described the individual determination of $\text{NO}_2^{\cdot-}$ and $\text{O}_2^{\cdot-}$. Here, we have simultaneously added the concentrations of $\text{O}_2^{\cdot-}$ from 0 to 550 $\mu$M and $\text{NO}_2^{\cdot-}$ from 0 to 600 $\mu$M as shown in Figure 3.26. From these results, it is obvious that increases in the anodic/cathodic peak currents at the potential of $+0.1 \text{ V} / -0.035 \text{ V}$ due to $\text{O}_2^{\cdot-}$ and anodic peak current at the potential of $+0.68 \text{ V}$ due to $\text{NO}_2^{\cdot-}$ were observed with the increasing concentrations. The selective electrocatalytic reactions of SOD1 with $\text{O}_2^{\cdot-}$ and $\text{NO}_2^{\cdot-}$ are perhaps explained due its narrow positively charged active site channel Figure 3.26. These data clearly demonstrate that $\text{O}_2^{\cdot-}$ and $\text{NO}_2^{\cdot-}$ levels were simultaneously determined using the SOD1 immobilized on CNT-PPy nanocomposite electrode.

### 3.16.2 Simultaneous measurement of $\text{O}_2^{\cdot-}$ and $\text{NO}_2^{\cdot-}$ released from MCF-7 cancer cells

Earlier it was shown that $\text{NO}_2^{\cdot-}$ a stable end product of NO, and $\text{O}_2^{\cdot-}$ are generated by breast cancer cells when exposed to HMG-CoA reductase inhibitors (Kotamraju et al., 2007). In this study, we attempted to measure $\text{NO}_2^{\cdot-}$ and $\text{O}_2^{\cdot-}$ simultaneously by using our newly developed biosensor in MCF-7 cells stimulated with LPS. After 24 h stimulation by 50 ng mL$^{-1}$ of LPS, the cathodic peak current at the potential, $-0.035 \text{ V}$ for $\text{O}_2^{\cdot-}$ and the anodic peak current at the potential, $+0.68 \text{ V}$ for $\text{NO}_2^{\cdot-}$ were clearly seen. However at 48 h duration, the peak current due to $\text{O}_2^{\cdot-}$ increased but the
peak current due to $\text{NO}_2^-$ remained constant. The $\text{O}_2^-$ ($0.1 \pm 0.02 \text{ µM}$) and $\text{NO}_2^-$ ($4.2 \pm 0.015 \text{ µM}$) levels generated from the stimulated cancer cells at 48 h were determined from the calibration plots (Rajesh et al., 2010).

### 3.17 BIENZYMATIC BIOSENSOR

Measurement of various analytes using multienzymatic biosensors in a single experiment is a challenging research area. The previous sections describe the various biosensors for the determination of several clinically important biomarkers based on the single enzyme immobilized on the sensor surface. In this section, we describe the coimmobilization of two enzymes for the measurement of two substrates in a single experiment and denoted as a bienzymatic biosensor. It demonstrates the bienzymatic biosensor for the simultaneous determination of $\text{NO}_2^-$ and $\text{NO}_3^-$ ions using SOD1 and NaR coimmobilized on CNT-PPy nanocomposite-modified platinum electrode as shown in Figure 3.27 (Huangxian et al., 2011). Figure 3.28 shows the CVs obtained for the bienzymatic biosensor by increasing the concentration of $\text{NO}_2^-$ from 500 nM to 300 µM and $\text{NO}_3^-$ from 700 nM to 400 µM using a scan rate of 50 mV s$^{-1}$ in 0.1 M PBS (pH 7.0). The observed results exhibit the increase of well-distinguished anodic peak at $+0.8 \text{ V}$ ascribed to the electrochemical oxidation of $\text{NO}_2^-$ catalyzed by SOD1 and the cathodic peak at $-0.76 \text{ V}$ attributed to the $\text{NO}_3^-$ reduction catalyzed by NaR. These results indicate that the NaR-SOD1-CNT-PPy-Pt electrode is successfully used for the simultaneous measurement of $\text{NO}_2^-$ and $\text{NO}_3^-$. Further, the utility of the proposed bienzymatic biosensor for the biological samples was explored by using it for the simultaneous determination of $\text{NO}_2^-$ and $\text{NO}_3^-$ in human plasma. The simultaneous measured values of $\text{NO}_2^-$ and $\text{NO}_3^-$ in the plasma samples are given in Table 3.6. The mean ± standard deviation (SD) values of $510.3 \pm 3.9 \text{ nM}$ for $\text{NO}_2^-$ and $16.76 \pm 1.2 \text{ µM}$ of $\text{NO}_3^-$ were obtained.

![Figure 3.27](image-url)  
Figure 3.27 Schematic representation of the construction of bienzymatic biosensor NaR-SOD1-CNT-PPy-Pt electrode and illustration of reactions that take place during the simultaneous determination of $\text{NO}_2^-$ and $\text{NO}_3^-$. 
3.18 ENZYME INHIBITION-BASED BIOSENSORS

This method is based on an indirect usage of enzymes in which the catalytic action of the enzyme is inhibited by the presence of a given species in the medium (Monti et al., 2009). The inhibition phenomenon can be caused by different types of compounds, namely, heavy metal cations, inorganic species and organic compounds such as pesticides. In the literature, the enzyme inhibition methods reported so far are seldom applied to real samples, and in some cases, a separation step precedes the enzyme-inhibited reaction. Such reactions are nonspecific, so they should be applied only for solving problems connected with general screening purposes of natural samples. The enzyme inhibition mechanism is often complex. There are reversible and irreversible inhibition-based mechanisms involved. The following paragraphs clearly explain both cases.

3.18.1 Reversible enzyme inhibition method

Inhibitors structurally related to the substrate may be bound to the enzyme active center and compete with the substrate (competitive inhibition) (Crapo and Day, 2000). Morales et al. (2002) showed a competitive inhibition of tyrosinase by benzoic acid (Rajesh et al., 2011). If the inhibitor is not only bound to the enzyme but also to the enzyme–substrate complex, the active center is usually deformed and its function is thus impaired. In this case the substrate and the inhibitor do not compete with each other (noncompetitive inhibition). The inhibition of horseradish peroxidase was apparently reversible and noncompetitive in the presence of HgCl2 for less than 8 s incubation time (Hrbác et al., 2007). The inhibition of immobilized acetylcholinesterase with metal ions
(Cu$^{2+}$, Cd$^{2+}$, Fe$^{3+}$, Mn$^{2+}$) has a reversible and noncompetitive character (Balamurugan et al., 2015).

3.18.2 Irreversible enzyme inhibition method

For irreversible inhibitors, the enzyme—inhbitor interaction results in the formation of a covalent bond between the enzyme active center and the inhibitor (Webb, 2008). The term irreversible says that the decomposition of the enzyme—inhbitor complex results in the destruction of enzyme, e.g., its hydrolysis, oxidation, etc. This process usually proceeds stepwise, as for phosphorylated cholinesterases, and can be accelerated by particular reagents. The kinetics of the inhibition depends strongly on the biosensor configuration. In the case of a thin enzymatic layer, the kinetics observed is similar to that of the enzyme in solution. For native enzymes the inhibition is related directly to the incubation time. Han et al. (2001) have investigated an interesting case concerning the inhibition of peroxidase (Hrbác et al., 2007). There is an early phase of reversible inhibition (5 s) followed by irreversible inhibition. However, since the reversible inhibition lasted for just a few seconds, it was difficult to carry out the measurement of residual activity within that interval. Therefore, irreversible inhibition has to be dealt with in cases where longer incubation times must be used. There have been some initial attempts at the development and experimental verification of theoretical models for the inhibition of immobilized enzymes used for biosensors. When diffusion phenomena are taken into account, the model predicts that the percentage of enzyme inhibition ($\%$), after exposure to an inhibitor, is linearly related to both the inhibitor concentration ($I$) and the square root of incubation time ($t_{1/2}$).

3.18.3 Pesticide as inhibitors

The determination of pesticides has become increasingly important in recent years because of the widespread use of these compounds, which is due to their large range of biological activity and a relatively low persistence. The development of biosensors for pesticides is the subject of considerable interest, particularly in the areas of food and environmental monitoring. Several enzymes such as cholinesterase enzymes (AChE, BChE) and urease have been used in the design of direct electrochemical biosensors for the detection of pesticides (Larsen et al., 2007). Analytical devices based on the inhibition of cholinesterase have been widely used for the detection of organic phosphate compounds and carbamate pesticides.

3.18.4 Heavy metals as inhibitors

Enzymatic methods are commonly used for metal ion determination, as these can be based on the use of a wide range of enzymes that are specifically inhibited by low
concentrations of certain metal ions. For the inhibitive determination of trace mercury, a large number of enzymes have been used: horseradish peroxidase, urease, glucose oxidase, alcohol oxidase, glycerol 3-phosphate oxidase, and invertase. Cadmium ion could be monitored by enzymatic sensors since it was found that it induced inhibition of several enzymes such as urease (Lundberg, 2009) and butyrylcholinesterase (BChE) (Madasamy et al., 2014). For copper determination, a cholinesterase sensor has been used (Sheo et al., 2013). It has been reported that heavy metal ions induced reversible cholinesterase inhibition.

3.19 ENZYME MIMETIC (METALLOPORPHYRIN)-BASED BIOSENSORS

Because of cost and less stability of the biological enzyme, there has been great interest for the use of metalloporphyrins as an alternative for enzymes as a biorecognition element in biosensor applications (Arduini et al., 2009). Recently, enzyme mimetic-based biosensors have been reported for the determination of various biomarkers with high stability and reproducibility that enable researchers to easily fabricate biosensors. First, we briefly provide a general overview of porphyrin and its derivatives as enzyme mimetic. Then, the following section will emphasize the attractiveness of porphyrins for sensing applications due to their unique properties.

3.19.1 Introduction

As shown in Figure 3.29, a porphyrin is a macrocyclic tetrapyrrole containing methine bridges (QCH—), which is usually prepared by acid-catalyzed condensation of pyrrole and aldehyde building blocks followed by oxidative treatment.

Porphyrin has a delocalized system involving 26 pi electrons and satisfies Huckel’s rule for aromaticity, i.e., (4n + 2) pi electrons. Aromaticity induces a ring current effect in 1H-NMR spectra so that peripheral protons (i.e., protons at mesopositions and

![Figure 3.29 Basic structure of porphyrin.](image-url)
b-positions) appear at lower field (6–9 ppm relative to tetramethylsilane; TMS), while inner pyrrolic NH protons (i.e., N–H) appear at high field (2.5 ppm relative to TMS). Porphyrins exhibit rapid tautomerism under ambient conditions with the two inner pyrrolic protons being effectively delocalized over the four available sites. Porphyrins are generally approximately planar although examples of nonplanarity are available. The pi electrons of porphyrins lead to their unique optical, electronic, magnetic, redox, catalytic, self-assembly, and other properties. These physicochemical properties endow the porphyrins with sensitivities to variation in local environments as well as to interactions with various potential analytes so that porphyrins make excellent components for fabrication of sensing electrodes (Morales et al., 2002). The following subsections discuss a few metalloporphyrins and their biosensor application as an enzyme mimetic.

### 3.19.2 Mn(III) porphyrin

Mn(III)TMPyP is a manganese porphyrin that acts as a superoxide dismutase (SOD) mimetic and peroxynitrite decomposition catalyst (Han et al., 2001). SOD mimetics described to date are unstable and are capable of catalyzing undesired side reactions in addition to the dismutation of the superoxide radical. Mn(III)TMPyP is an SOD mimetic with increased stability to pH and hydrogen peroxide. The rate constants for superoxide dismutation and peroxynitrite decomposition are $3.9 \times 10^7 \text{M}^{-1}\text{s}^{-1}$ and $\sim 2 \times 10^6 \text{M}^{-1}\text{s}^{-1}$, respectively. Mn(III)TMPyP protected and enhanced the growth of SOD null *Escherichia coli* with a doubling time of 60 min (as compared to 240 min of the control) at 25 μM.

### 3.19.3 Nitrite oxidase and superoxide dismutase activities of MnTMPyP

Earlier, electrochemically incorporated redox active metalloporphyrins, viz., hemin, cyt c, have been reported to exhibit electrocatalytic properties with $\text{NO}_2^-$ and $\text{O}_2^{\bullet^-}$. Here, cationic MnTMPyP-modified electrode has been prepared and studied for its possible enhanced electrocatalytic reaction with negatively charged anions, viz., $\text{NO}_2^-$ and $\text{O}_2^{\bullet^-}$ (Stoytcheva, 2002). Figure 3.30 shows the pathway of catalytic mechanism with the active site Mn.

Figure 3.31 exhibits the CVs obtained by simultaneously increasing the concentrations of $\text{NO}_2^-$ from 0 to 1000 μM and $\text{O}_2^{\bullet^-}$ from 0 to 500 μM. From the results, it is clearly evident that increases in the anodic peak current at the potential of +0.73 V due to $\text{NO}_2^-$ and anodic/cathodic peak currents at the potential of 0.0 V/−0.43 V due to $\text{O}_2^{\bullet^-}$ were observed with the increasing $\text{NO}_2^-$ and $\text{O}_2^{\bullet^-}$ concentrations. The cyclic voltammetric peaks of both $\text{NO}_2^-$ and $\text{O}_2^{\bullet^-}$ are not altered due to the addition of either of them. These data clearly demonstrate that $\text{NO}_2^-$ and $\text{O}_2^{\bullet^-}$ levels would be simultaneously determined using the MnTMPyP incorporated in PPy matrix electrode.
Figure 3.30 Schematic diagram shows the electrostatic attraction of negatively charged \( \text{NO}_2^- \) and \( \text{O}_2^- \) toward the metal active center of cationic MnTMPyP.

Figure 3.31 Simultaneous electrochemical responses of the MnTMPyP-PPy-Pt electrode in (a) control, (b) 100 \( \mu \text{M} \ \text{NO}_2^- + 50 \ \mu \text{M} \ \text{O}_2^- \), (c) 500 \( \mu \text{M} \ \text{NO}_2^- + 200 \ \mu \text{M} \ \text{O}_2^- \), and (d) 1000 \( \mu \text{M} \ \text{NO}_2^- + 500 \ \mu \text{M} \ \text{O}_2^- \) at scan rate of 50 mV s\(^{-1}\). Inset: A linear calibration plot of anodic peak currents measured at +0.73 V against \( \text{NO}_2^- \) concentrations. Similarly, a linear calibration plot obtained for different \( \text{O}_2^- \) concentrations versus cathodic peak currents at the potential, −0.43 V, in 0.1 M PBS. Each point represents the mean (±0.03 SD) for \( \text{NO}_2^- \) and mean (±0.02 SD) for \( \text{O}_2^- \) in three sets of measurements.
The anodic oxidation peak currents measured at the modified electrode were almost linearly related to the concentration of NO$_2^-$ in the range of 0.8–1000 μM ($r^2 = 0.9952$) as shown in the inset of Figure 3.29. The detection limit was found to be 0.8 ± 0.03 μM, with a sensitivity of 0.1 ± 0.003 μA μM$^{-1}$ cm$^{-2}$. Similarly, the observed cathodic currents versus O$_2^{−}$ concentrations are plotted (inset of Figure 3.29). The calibration curve thus exhibits a linear range for the O$_2^{−}$ concentrations from 0.6 to 1000 μM ($r^2 = 0.9971$) with a detection limit of 0.6 ± 0.02 μM and sensitivity of 0.12 ± 0.004 μA μM$^{-1}$ cm$^{-2}$.

3.19.4 Ni(II) porphyrin

The electropolymerized nickel tetrakis(3-methoxy-4-hydroxyphenyl) porphyrin (NiTMHPP) and similar porphyrinic compounds have become popular in the construction of nitric oxide sensors. Carbon fiber microelectrode, covered with electropolymerized nickel porphyrin derivative followed by a Nafion layer, “porphyrinic sensor,” is among the most widely used electrochemical sensors for the detection and monitoring of nitric oxide both in vitro and in vivo (McDonald and Tipton, 2012). Electropolymerized NiTMHPP grants the sensor electrocatalytic properties toward nitric oxide oxidation, typically reducing its overpotential by some 50–100 mV. It is known that the electrocatalytic action of NiTMHPP and similar compounds cannot be ascribed to easier oxidation of NO bound to nickel central atom of the porphyrin complex through axial coordination. This mechanism would require counterbalancing of the charge induced by Ni(II) oxidation to Ni(III) by diffusion of anions into the film. The electrocatalytic activity persists in the Nafion-coated porphyrinic sensor, although Nafion effectively suppresses the diffusion of anions and thus blocks nickel redox transitions.

3.19.5 Copper(II) chlorophyllin

Chlorophyllin copper complex is an isolate derived from natural sources. Unlike native plant chlorophyll, chlorophyllin copper complex is a water-soluble molecule. Water solubility is believed to enhance the bioavailability of chlorophyll. The synthesis of chlorophyllin copper complex from native chlorophyll involves supplanting the magnesium atom at the center of the ring with copper.

Previous sections focused on the enzymatic determination of NO, NO$_2^-$ using copper, zinc superoxide dismutase (SOD1), and NO$_3^-$ using nitrate reductase (NaR)-modified Pt electrodes. This is a costly, time-consuming, and tedious procedure and the enzymes are not very stable. In order to resolve these drawbacks, the highly stable metalloporphyrin-modified electrode would be the preeminent choice for the determination of NO metabolites. Different metalloporphyrins including nickel, iron, cobalt, manganese, and some other metal complexes were previously used for the
effective catalytic oxidation or reduction of NO. In this section, we have described the use of copper(II) chlorophyllin (CuCP) for the oxidation of NO and NO\textsubscript{2} since it contains copper at the center of the porphyrin with excellent electrocatalytic redox property as similar to that of SOD1 (Pundir and Chauhan, 2012). Furthermore, due to its high stability and low cost, it is a preferred target for the fabrication of electrochemical sensors.

### 3.19.5.1 Preparation of CuCP-modified ZnO-SPCE

Prior to fabricating the CuCP-based sensor, the SPCE is pretreated to remove the organic ink constituents or contaminants and to increase the surface functionalities (Haikarainen et al., 2014). Briefly, SPCE is dipped in 0.1 M PBS solution and the potential is cycled from −0.6 to +1.6 V (vs Ag/AgCl) for 40 cycles at a scan rate of 10 mV s\textsuperscript{−1}. After pretreatment, ZnO is incorporated by placing the mixture of 0.1 M zinc nitrate and 0.5 M urea solution on the working electrode surface of the SPCE and cycling the potential from −1 to +1 V for 10 complete cycles. Then, CuCP-modified ZnO-SPCE is prepared by placing the mixture of 0.1 M CuCP, NaOH, and KCl solution onto the ZnO-incorporated SPCE and cycling the potential from −1 to +1 V for 10 cycles. During this process, CuCP is electrodeposited onto the ZnO-SPCE. It is then gently washed with 0.1 M PBS and stored at 4 °C when not in use. The above fabricated biosensor electrode CuCP-ZnO-SPCE is highly reproducible, which could be confirmed from the cyclic voltammetric responses. All of the experiments were carried out at 27 ± 0.5 °C.

Figure 3.32(a) exhibits the typical electrochemical responses obtained for the CuCP-SPCE and CuCP-ZnO-SPCE in the absence (curves a and b) and presence (curves c and d) of 100 μM NO using a scan rate of 50 mV s\textsuperscript{−1} in 0.1 M PBS (pH 7.0). Before the addition of NO, there are no changes observed in the current response. However, after the addition of NO, these two electrodes exhibited significant increases in current anodically at the potential, +0.8 V. Further, it is clearly seen that the CuCP-ZnO-SPCE (curve d) shows higher current response than CuCP-SPCE (curve c). This is perhaps due to the n-type semiconducting ZnO that enhanced the electron transfer between the active site of the porphyrin CuCP and the electrode surface during the oxidation of NO via a cyclic redox reaction of its Cu(I/II) active site moiety. Figure 3.32(b) illustrates the electrochemical responses obtained for the CuCP-ZnO-SPCE in the presence of various NO concentrations at the same scan rate. The observed anodic peak currents versus NO concentrations were plotted as shown in the inset of Figure 3.32(b). The calibration curve obtained exhibits a linear range of response over the concentration of NO from 100 nM to 500 μM, but for clarity here we have shown from 50 to 500 μM (r\textsuperscript{2} = 0.9968, n = 3) with a detection limit of 100 nM and sensitivity of 85.4 nA μM\textsuperscript{−1}. 
Figure 3.32 (a) Electrochemical responses obtained for the CuCP-SPCE and CuCP-ZnO-SPCE in the absence (curves a and b) and presence (curves c and d) of 100 μM NO solution at scan rate of 50 mV s⁻¹ versus Ag/AgCl. (b) Electrochemical responses obtained for the CuCP-ZnO-SPCE in the presence of (curve a) control, (curve b) 50 μM, (curve c) 100 μM, (curve d) 200 μM, (curve e) 300 μM, (curve f) 400 μM, and (curve g) 500 μM of NO solution at scan rate of 50 mV s⁻¹ versus Ag/AgCl. Linear calibration curve (inset of Figure 3.32(b)) \[ y = -0.0983x - 10.019, r^2 = 0.9968. \]

3.19.5.2 Measurement of NO₂⁻ and NO₃⁻ using CuCP-modified ZnO-SPCE

Figure 3.33(a) represents the typical CV responses obtained for the CuCP-SPCE and CuCP-ZnO-SPCE in the absence (curves a and b) and presence (curves c and d) of 100 μM NO₂⁻ using a scan rate of 50 mV s⁻¹ in 0.1 M PBS (pH 7.0). Before the addition of NO₂⁻, there were no changes observed in the current response. However, after the addition of NO₂⁻, both electrodes exhibited significant increases in current anodically at the potential, +0.83 V. The electrochemical responses of the CuCP-ZnO-SPCE for the various concentrations of NO₂⁻ are shown in Figure 3.33(b), and the linear calibration is shown in the inset of Figure 3.33. The calibration curve thus
obtained exhibits a linear range of response over the concentration of \( \text{NO}_2^- \) from 100 nM to 1 mM, but for clarity here we have shown from 100 \( \mu \)M to 1 mM \( (r^2 = 0.9984, n = 3) \) with a detection limit of 100 nM and sensitivity of 96.4 nA \( \mu \)M\(^{-1}\).

The measurement of total \( \text{NO}_2^- \) and \( \text{NO}_3^- \) in blood is an index of endothelial nitric oxide synthase activity (Tessarollo et al., 2015). Moreover, the high altitude subjects suffer from the reduction of \( \text{NO}_2^- \) level in their blood, leading to several diseases at hypoxia conditions (Kim et al., 2003). Recent studies reported that the administration of \( \text{NO}_3^- \)-rich beetroot juice to human and several animal models promotes NO-like
bioactivity and regulates biological activities like reduction of blood pressure, vasodilation, cytoprotection, cardioprotection, and protection from ischemia-reperfusion injury (Lee and Russel, 2003; Mourzina et al., 2004; Evtugyn et al., 2003). Therefore, we have investigated here the natural remedy effect of beetroot supplement by measuring the total NO$_2^-$ and NO$_3^-$ levels in human blood plasma of four subjects before and after suppletions. The CuCP-ZnO-SPCE coated with cellulose acetate membrane was employed to measure total NOx (NO$_2^- +$ NO$_3^-$) concentration. First, the concentration of NO$_2^-$ was directly measured alone in the sample. Then, the total NOx was measured by reducing the NO$_3^-$ into NO$_2^-$ using NaR. Further, the measured values were validated with standard Griess method as shown in Table 3.5. Finally, the concentration of NO$_3^-$ could be deduced by subsequent subtraction of [NO$_2^-$] from the total [NO$_2^- +$ NO$_3^-$].

$$[\text{NO}_3^-] = [\text{NO}_2^- + \text{NO}_3^-] - [\text{NO}_2^-]$$

The level of NOx in blood after beetroot supplementation is obviously increased as shown in Table 3.3, confirming that the administration of exogenous NO$_3^-$ elevated the concentration of NO$_2^-$ and NO$_3^-$ in blood and showing its remedy effect against oxidative stress, thereby enhancing the NO-NO$_2^-$-NO$_3^-$ metabolic pathway.

<table>
<thead>
<tr>
<th>Individual</th>
<th>Total NO$_2^- +$ NO$_3^-$ Conc. by Griess Method ($\mu$M)</th>
<th>Total NO$_2^- +$ NO$_3^-$ Conc. by Sensor ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Beetroot Juice</td>
<td>After Beetroot Juice</td>
</tr>
<tr>
<td>01</td>
<td>28.4 ± 1.8</td>
<td>29.7 ± 1.7</td>
</tr>
<tr>
<td>02</td>
<td>24.5 ± 1.1</td>
<td>32.1 ± 2.0</td>
</tr>
<tr>
<td>03</td>
<td>21.9 ± 1.3</td>
<td>26.1 ± 1.8</td>
</tr>
<tr>
<td>04</td>
<td>24.7 ± 1.4</td>
<td>28.9 ± 1.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Conc. of NO$_2^-$ (nM mL$^{-1}$)</th>
<th>Conc. of NO$_3^-$ (µM mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>492 ± 3.0</td>
<td>20.80 ± 1.1</td>
</tr>
<tr>
<td>2</td>
<td>594 ± 3.0</td>
<td>19.75 ± 1.3</td>
</tr>
<tr>
<td>3</td>
<td>583 ± 5.0</td>
<td>19.26 ± 1.0</td>
</tr>
<tr>
<td>4</td>
<td>518 ± 4.3</td>
<td>14.40 ± 1.2</td>
</tr>
<tr>
<td>5</td>
<td>390 ± 2.4</td>
<td>9.40 ± 1.4</td>
</tr>
<tr>
<td>6</td>
<td>485 ± 6.0</td>
<td>16.95 ± 1.1</td>
</tr>
</tbody>
</table>
3.20 SCREEN-PRINTED FUNCTIONALIZED ELECTRODES AND ADVANTAGES

In the previous sections, we have described the determinations of clinically important biomarkers such as $O_2^-$, NO, $NO_2^-$, $NO_3^-$, cyt c, glucose, etc. For all the measurements, 1–2 mL of the sample is required, making it difficult to prepare the sample (blood plasma, serum, and cell cultures) for measurements. Therefore, in order to reduce the sample volume, we have developed electrochemical assays for the measurement of various biomarkers in a single drop of the biological sample using screen-printed electrode functionalized with enzyme, viz., SOD, CcO, CcR on CNT, and GNP. We previously immobilized SOD on Pt and screen-printed electrodes. The sensitivities of these two electrodes for nitrite determinations are $0.19 \mu A \mu M^{-1} cm^{-2}$ and $0.0964 \mu A \mu M^{-1} cm^{-2}$, respectively, were obtained. The main advantages of SPCE are (1) one drop of biological sample size, (2) disposable and low cost, (3) mass production of biosensors, (4) reproducible results, and (5) less wastage.

3.21 NANOCOMPOSITE-ENHANCED ELECTROCHEMICAL BIOSENSORS

In order to specifically recognize the biomarker, the immobilization of selective bio-recognition element, viz., enzyme, without affecting its native structures and bioactivities is the crucial step. Indeed, the direct electron transfer in redox biomolecules is very difficult at conventional electrodes, since with redox the active sites are deeply embedded in protein structure. Recently, conducting polymer, polypyrrole (PPy), has been widely used as a suitable host transducer for the immobilization of enzymes owing to its unique combination of high electronic conductivity with well-ordered polymer chain, porosity, and good environmental stability (Reiter et al., 2001). Further, the research has extended to modify PPy with other nanomaterials, viz., carbon nanotubes (CNTs), gold nanoparticles (GNPs) so as to obtain nanocomposite-enhanced immobilization features. These nanocomposite materials obtained by the integration of CNT/GNP with PPy have shown a greater electron transfer/catalytic activity of bioactive molecules due to synergic effect than the properties of the individual components in various biosensors. CNT are novel molecular nanowires with high mechanical, chemical, and electrical properties. The morphology of CNT is extremely important in establishing the direct electrical contact between the redox center of the protein and the electrode. CNTs also have a hollow core, which is suitable for storing immobilized proteins and enzymes without losing biological activity. Thus, the CNT-PPy nanocomposite promotes the electron transfer reactions thereby exhibiting enhanced electrochemical response to biomolecules. GNP s have also gained considerable attention in nanobiotechnology owing to their advantages in electron transport, high effective surface area, control over surrounding
environment, and biofunctionalization of enzymes. Modification of the surface of GNP with thiols provides a well-ordered, compact, and stable self-assembled monolayer (SAM) used to conjugate enzymes close to the electrode surface. These self-assembled GNP-PPy nanocomposites can further act as tiny conduction centers and facilitate the direct transfer of electrons between the biomolecules and base electrode (Pandiaraj et al., 2013).

3.21.1 Effect of GNP-PPy nanocomposite

Figure 3.34 compares the CVs of NaR-PPy-Pt (curve a) and NaR-SAM-GNP-PPy-Pt (curve b) electrodes in 0.1 M PBS containing 200 μM NO₃⁻. It is obviously seen that the cathodic peak current of the NaR-SAM-GNP-PPy-Pt electrode is 40 μA greater than that of the NaR-PPy-Pt electrode at −0.76 V. This observed increase in current suggests that the SAM of cysteine on GNP perhaps provided more surface area to couple NaR effectively. Further, GNP-PPy nanocomposite enhanced the direct electron transfer between the active site of the NaR and the base electrode.

3.21.2 Effect of CNT-PPy nanocomposite

The effect of CNT on the current response due to NO₂⁻ is shown in Figure 3.35. It exhibits a remarkable increase in the amperometric response from the modified electrode consisting of CNT when compared to that without CNT. Furthermore, with NO₂⁻ (500 μM), the increase in the anodic oxidation current observed at the SOD1-CNT-PPy-Pt electrode was fivefold greater than that of the CNT free

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![Figure 3.34](image-url) Typical CV responses of (a) NaR-PPy-Pt and (b) NaR-SAM-GNP-PPy-Pt electrodes in 0.1 M PBS containing 200 μM NO₃⁻ at scan rate of 50 mV s⁻¹ versus Ag/AgCl.
SOD1-PPy-Pt electrode (Figure 3.35). It is clearly evident that the CNT-PPy nano-composite facilitated the direct electron transfer between SOD1 and the base electrode (Madasamy et al., 2014a,b).

3.21.3 Characterization using SEM and CV

Figure 3.36(a)–(c) illustrate the scanning electron microscopic (SEM) images of bare Pt, PPy-Pt, and CNT-PPy-Pt electrodes, respectively. Image (b) reveals the typical highly porous morphology of PPy on Pt electrode surface. This high porous background of PPy perhaps provides a much larger surface area to bind more CNT and hence SOD1 at the electrode surface. Further, image (c) shows the integration of CNT in the PPy matrix.

Figure 3.35 Influence of CNT on the amperometric response of (a) SOD1-PPy-Pt and (b) SOD1-CNT-PPy-Pt electrodes to 500 μM NO₂⁻ solution in 0.1 M PBS (pH 7.0) containing 100 μM DTPA at scan rate of 50 mV s⁻¹.

Figure 3.36 SEM images of (a) bare Pt, (b) PPy-Pt, and (c) CNT-PPy-Pt electrodes.
The surface morphological changes of the GNP-PPy-Pt nanocomposite electrode were also investigated by SEM and energy dispersive X-ray spectroscopy (EDX) as shown in Figure 3.37. The SEM image of GNP-PPy-Pt electrode (Figure 3.37) reveals the incorporation of GNP onto the microporous matrix of PPy. This GNP-PPy nanocomposite increases the specific surface area available on the PPy-Pt electrode for the efficient functionalization of the enzymes.

3.21.4 Electrochemical characterization using CV

The SOD1-CNT-PPy-Pt electrodes were further electrochemically characterized and exhibited a quasireversible peak at the potential of +0.06 V versus Ag/AgCl (Figure 3.38). This observed quasireversible peak is attributed to the Cu$^{2+}$/Cu$^{+}$ redox reaction.
changes at the active site of SOD1 in agreement with the previously reported data. Hence, this clearly reveals that the SOD1 was immobilized on the electrode surface.

3.21.5 Optimization
The biosensors thus developed were optimized as follows.

3.21.5.1 Effect of pH
The electrochemical behavior of the NO biosensor was studied in the pH range of 3.0–10.0. For the pH study, a mixture of disodium hydrogen phosphate and citric acid buffer was used. The current response was decreased from pH 7.0 to 3.0 and also from pH 7.0 to 10.0. This may be due to the denaturation of immobilized SOD1. The maximum current response was observed at pH 7.0 as shown in Figure 3.39.

3.21.5.2 Effect of scan rate
Further, the influence of scan rate on the performance of the SOD1-CNT-PPy-Pt electrode in 0.1 M PBS containing 100 μM of NO solution was also investigated (Figure 3.40). It was observed that the anodic peak currents negatively increased linearly with increasing the scan rate of 50 to 300 mV s\(^{-1}\) and also that the characteristic CV remains unchanged. This indicates the favorable orientation of SOD1 at the CNT-PPy-Pt electrode leading to a facilitated electron transfer of SOD1, and the linear variation of anodic peak currents (IP) with scan rate (\(\gamma\))\(^{1/2}\) indicate that the electrochemical process is diffusion controlled.

3.21.5.3 Stability, Repeatability, and Reproducibility
The stability of the bienzymatic NaR-SOD1-CNT-PPy-Pt electrode was evaluated by monitoring the current response three times a day in the presence of 100 nM nitrite and 300 nM nitrate over four weeks, and the rest of the time it was stored at 4°C. The SOD1 and NaR were quite stable, as inferred from their electrocatalytical activity of 92% and 89.6%, respectively, after one month of storage and 83% and 76%, respectively, after two months of storage. Repeatability of the bienzymatic biosensor was tested by

![Figure 3.39](image.png)

**Figure 3.39** Effect of pH on the peak current of the SOD1-CNT-PPy-Pt in 0.1 M PBS at scan rate of 50 mV s\(^{-1}\) versus Ag/AgCl. Each point represents the average of three measurements.
measuring the decrease in current response during five successive CV measurements of nitrite and nitrate. The resulting standard deviations were 1.2% and 1.5% for nitrite and nitrate, respectively. Further, to ascertain the reproducibility of the experimental results, four different bienzymatic electrodes were constructed and tested toward the oxidation of 100 nM nitrite and reduction of 300 nM nitrate showing the SD of 3.57% and 2.98%, respectively, for NO$_2^-$ and NO$_3^-$ . Thus, it confirms that the NaR-SOD1-CNT-PPy-Pt electrode is reproducible.

### 3.21.5.4 Interferences and their elimination

Since the oxidation potential of NO$_2^-$ is high, other electroactive species such as ascorbic acid (AsA) and uric acid (UA) present in the biological samples (complex media) can also be oxidized and thus interfere with the NO$_2^-$ measurement. Upon addition of 250 mM UA into 0.1 M PBS containing 100 nM NO$_2^-$ and 300 nM NO$_3^-$ , no current change was observed in time versus current response of the NaR-SOD1-CNT-PPy-Pt electrode. Therefore, this does not interfere with the measurement. However, noticeable current change was observed upon addition of 250 mM AsA (Figure 3.41, curve b). Earlier, it was reported that CA membrane was perm-selective for NO$_2^-$ and NO$_3^-$ . Therefore, in the present study we have also used CA membrane to eliminate AsA and other possible interferences. CA membrane-coated bienzymatic biosensor was prepared by dropping 10 mL of cellulose acetate solution (cellulose acetate in acetone) onto the NaR-SOD1-CNT-PPy-Pt electrode and dried at room temperature. After CA membrane coating, again the similar time versus current response of the bienzymatic biosensor was investigated in the presence of 250 mM AsA and no change in current was found (Figure 3.41, curve a). CA membrane not only excludes interferences but also prevents the bienzymatic electrode from fouling due to the nonspecific adsorption of proteins (see below) and other materials typically present in the biological samples.
3.22 RECENT APPLICATIONS

3.22.1 Clinical Applications

Cardiovascular diseases are highly preventable, yet they are a major cause of human death worldwide (Dimmeler, 2011). One of the most important reasons for the increasing incidence of cardiovascular diseases and cardiac arrest is hypercholesterolemia, i.e., increased concentration of cholesterol in blood. Hence the diagnosis of cholesterol level in blood is important in clinical applications. Biomarkers have become increasingly important in this setting to supplement electrocardiographic findings and patient history because one or both can be misleading. Cardiac troponin is the only marker currently used routinely in this setting because it is specific from the myocardial tissue, easily detected, and useful for therapeutic decision making (Qureshi et al., 2012; Collinson et al., 2015). Determination of the level of other nonmyocardial tissue-specific markers might also be helpful, such as myeloperoxidase, copeptin, growth differentiation factor 15, and C-reactive protein (CRP). CRP, which reflects different aspects of the development of atherosclerosis or acute ischemia, is one of the plasma proteins known as acute-phase proteins and its levels rise dramatically during inflammatory processes occurring in the body. This increment is due to a rise in the plasma concentration of IL-6, which is produced predominantly by macrophages as well as adipocytes. CRP can rise as high as 1000-fold with inflammation. CRP was found to be the only marker of inflammation that independently predicts the risk of a heart attack.

**Biosensors for cardiovascular disease**

Early and accurate diagnosis of cardiovascular disease is crucial to saving lives, especially for patients suffering a heart attack. Accurate and fast quantification of cardiac muscle–specific biomarkers in the blood enables accurate diagnosis and prognosis and

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*Figure 3.41* Time versus current response of the bienzymatic biosensor obtained (a) after CA membrane coating and (b) before CA membrane coating upon addition of 250 μM AsA in 0.1 M PBS containing 100 nM NO₂⁻ and 300 nM NO₃⁻ at scan rate of 50 mV s⁻¹ versus Ag/AgCl.
timely treatment of patients (Sadana and Sadana, 2015). It is apparent that increasing incidences of cardiovascular diseases and cardiac arrest in contemporary society denote the necessity for the availability of cholesterol and other biomarker biosensors. Biosensors for cholesterol measurement comprise the majority of the published articles in the field of cardiovascular diseases. In the fabrication of cholesterol biosensor for the estimation of free cholesterol and total cholesterol, mainly cholesterol oxidase (ChOx) and cholesterol esterase (ChEt) have been employed as the sensing elements. Electrochemical transducers have been effectively utilized for the estimation of cholesterol in the system. Other cardiovascular disease biomarkers are also quantified. CRP measurement relies mainly on immunosensing technologies with optical, electrochemical, and acoustic transducers besides approaches to simultaneous analytes measurement. Silva et al. (2010) incorporated streptavidin polystyrene microspheres into the electrode surface of SPEs in order to increase the analytical response of the cardiac troponin T, and Park et al. (2009) used an assay based on virus nanoparticles for troponin I highly sensitive and selective diagnostic, a protein marker for a higher risk of acute myocardial infarction. The efforts directed toward the development of cardiovascular disease biosensors have resulted in the commercialization of a few cholesterol biosensors. A better comprehension of the bioreceptors immobilization and technological advances in the microelectronics are likely to speed up commercialization of the much-needed biosensors for cardiovascular diseases.

**Biosensors for cancer diagnosis**

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. This disease continues to increase globally largely because of the aging and growth of the world population alongside an increasing adoption of cancer-causing behaviors, particularly smoking (Basen-Engquist and Chang, 2011). Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, and lung cancer is the leading cancer site in males (Kawalec et al., 2015; Perez and Sinkam, 2015). Breast cancer is now also the leading cause of cancer death among females in economically developing countries. Solid cancers are a leading cause of morbidity and mortality worldwide, primarily due to the failure of effective clinical detection and treatment of metastatic disease in distant sites. Cancer can be caused by a range of factors, both genetic and environmental. Chemical, physical, and biological factors such as the exposure to carcinogenic chemicals, radiation, bacterial infections (e.g., stomach cancer), viral infections (e.g., cervical cancer), and toxins (aflatoxin, e.g., liver cancer) can lead to cancer development.

As the causes of cancer are so diverse, clinical testing is also very complex. The multifactorial changes (genetic and epigenetic) can cause the onset of the disease and the formation of cancer cells. However, no single gene is universally altered during this process, but a set of them, which poses difficulties to correct disease diagnosis. All the changes that take place, in the tumors from different locations (organ), as well within
tumors from the same location, can be so variable and overlapping that it is difficult to select a specific change or marker for the diagnosis of specific cancers. Therefore, a range of biomarkers can potentially be analyzed for diagnosis. These biomarkers shown in Table 3.7 can be produced either by the tumor itself or by the body in response to the presence of cancer (Gouvea, 2011).

The analysis of biomarkers in body fluids such as blood, urine, and others is one of the methods applied in the detection of the disease. Multimarker profiles, both presence and concentration level, can be essential for the diagnosis of early disease onset. These methods should provide information to assist clinicians in making successful treatment decisions and increasing patient survival rate. A range of biomarkers have been identified with different types of cancers. These include DNA modifications, RNA, proteins (enzymes and glycoproteins), hormones and related molecules, molecules of the immune system, oncogenes, and other modified molecules. The development of protein-based biomarkers for biosensors use in cancer diagnosis is more attractive than genetic markers due to protein abundance, recovery, and cost-effective techniques for the development of point-of-care devices.

For cancer diagnosis, multiarray sensors would be beneficial for multimarker analysis.

<table>
<thead>
<tr>
<th>Table 3.7 Biomarkers of Cancer (Gouvea, 2011)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
</tr>
<tr>
<td>Bladder</td>
</tr>
<tr>
<td>Cervix</td>
</tr>
<tr>
<td>Colon</td>
</tr>
<tr>
<td>Esophagus</td>
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<tr>
<td>Leukemia</td>
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<tr>
<td>Liver</td>
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<tr>
<td>Lung</td>
</tr>
<tr>
<td>Melanoma</td>
</tr>
<tr>
<td>Ovarian</td>
</tr>
<tr>
<td>Pancreas</td>
</tr>
<tr>
<td>Prostate</td>
</tr>
<tr>
<td>Solid tumors</td>
</tr>
<tr>
<td>Stomach</td>
</tr>
</tbody>
</table>
A range of molecular recognition molecules have been used for biomarker detection, with antibodies the most widely used (Al., 2014). More recently, synthetic (artificial) molecular recognition elements such as nanomaterials, aptamers, and phage display peptides, binding proteins and synthetic peptides, as well as metal oxide materials have been fabricated as affinity materials and used for analyte detection and analysis. Antibodies (monoclonal and polyclonal) have been applied in cancer diagnostic tests targeting cancer cells and biomarkers. Polyclonal antibodies can be raised against any biomarker or cells, and with the introduction of high-throughput techniques, applying these molecules in sensors has been successful (Sharon et al., 2005). The use of monoclonal antibodies, however, results in more specific tests (Scott et al., 2012). The drawbacks include that monoclonal antibodies are more difficult to maintain and can be more expensive than polyclonal antibodies. Replacing natural biomolecules with artificial receptors or biomimics has therefore become an attractive area of research in recent years. The advantages of using these molecules are that they are robust, more stable, and less expensive to produce and can be modified easily to aid immobilization on the sensor surface as well as add labels as markers for detection. Electrochemical affinity sensors based on antibodies offer great selectivity and sensitivity for early cancer diagnosis, and these include amperometric (Sarkar et al., 2002), potentiometric (Bohunicky and Mousa, 2011), and impedimetric/conductivity devices (Sevena et al., 2013). Amperometric and potentiometric transducers have been the most commonly used, but much attention in recent years has been devoted to impedance-based transducers since they are classified as label-free detection sensors. Electrochemical detection of rare circulating tumor cells has the potential to provide clinicians with a standalone system to detect and monitor changes in cell numbers throughout therapy, conveniently and frequently, for efficient cancer treatment. Different surface plasmon resonance (SPR)-based biosensors have been developed for cancer marker detection based on optical systems (Uludag and Tothill, 2012; Yang et al., 2014). In spite of the achieved developments in cancer biosensing, point-of-care testing is not yet available. In order to achieve this goal, challenges must be overcome such as development of reproducible biomarker assays, improvement in recognition ligands, development of multichannel biosensors, advances in sample preparation, device miniaturization and integration, development of more sensitive transducers, microfluidics integration, advanced manufacturing techniques, and cost reduction (Rasooly and Jacobson, 2006).

Health care

About 3% of the population worldwide suffers from diabetes, a leading cause of death, and its incidence is growing fast. Diabetes is a syndrome of disordered metabolism resulting in abnormally high blood sugar levels. Without diligent monitoring of blood glucose concentrations, diabetic individuals are at greater risk of heart disease, stroke, high blood pressure, blindness, kidney failure, neurological disorders, and other health-related
complications. Optimal management of diabetes involves patients measuring and recording their own blood glucose levels. Under normal physiological conditions, the concentration of fasting plasma glucose is in the range of $6.1 - 6.9$ mM L$^{-1}$, so the variation of the blood glucose level can indicate diabetes mellitus, besides other conditions (Pickup, 1989; Turner and Pickup, 1985). The American Diabetes Association recommends that insulin-dependent Type 1 diabetics self-monitor blood glucose 3–4 times daily, while insulin-dependent Type 2 diabetics monitor once daily.

**Biosensors for glucose measurement**

Glucose can be monitored by invasive and noninvasive technologies. Requirements of a sensor for in vivo glucose monitoring include miniaturization of the device, long-term stability, elimination of oxygen dependency, convenience to the user, and biocompatibility. Long-term biocompatibility has been the main requirement and has limited the use of in vivo glucose sensors, both subcutaneously and intravascular, to short periods of time. In order to address the problem, microdialysis or ultrafiltration technology has been coupled with glucose biosensors. The current invasive glucose monitors commercially available use glucose oxidase-based electrochemical methods and the electrochemical sensors are inserted into the interstitial fluid space. Most sensors are reasonably accurate, although sensor error including drift, calibration error, and delay of the interstitial sensor value behind the blood value are still present (Castle and Ward, 2010). This type of biosensor has been used widely worldwide for home glucose testing, bringing diagnosis to on-site analysis.

Noninvasive glucose sensing is the ultimate goal of glucose monitoring, and the main approaches being pursued for glucose sensor development are: near infrared spectroscopy, excreted physiological fluid (tears, sweat, urine, saliva) analysis, microcalorimetry, enzyme electrodes, optical sensors, and sonophoresis and iontophoresis, both of which extract glucose from the skin. Despite the relative ease of use, speed, and minimal risk of infection involved with infrared spectroscopy, this technique is hindered by the low sensitivity, poor selectivity, frequently required calibrations, and difficulties with miniaturization. Problems surrounding direct glucose analysis through excreted physiological fluids include a weak correlation between excreted fluids and blood glucose concentrations. Exercise and diet that alter glucose concentrations in the fluids also produce inaccurate results. Nevertheless, the drawbacks of in vivo biosensors must be solved before such an insulin modulating system can be achieved.

### 3.23 VETERINARY

#### 3.23.1 Detection of pathogens in meat

An immune competitive assay that detected pathogens in spiked meat extracts at 104 CFU mL$^{-1}$ after a 3 h enrichment was developed. The application of techniques like this will help reduce or eliminate contamination of pathogens and toxicants in foods (Abdalhai et al., 2014).
3.23.2 Detection of drugs (ractopamine residues) in swine

Ractopamine (RCT) is a beta-adrenergic agonist licensed for growth promotion in pigs in the United States, but it is illegal in Europe. Due to RCT’s molecular structure, many of the existing screening and confirmatory tests for beta agonist compounds fail to detect RCT and its metabolites. A screening assay based on optical biosensor detection of RCT and its metabolites following sample extraction was developed (Ferguson et al., 2002). Detection limits well below 1 ng mL$^{-1}$ or g were achieved in urine and tissue samples.

3.23.3 Determination of immunoglobulin G in bovine colostrum and milk

An automated biosensor-based assay has been developed for the determination of IgG in bovine milk and colostrum using either goat or rabbit anti-bovine IgG or protein G as ligand. The method is configured as a direct and nonlabeled immunoassay, with quantitation against an authentic IgG calibrant (Abernethy et al., 2010). Whole colostrum or milk is prepared for analysis by dilution into buffer. Analysis conditions including ligand immobilization, flow rate, contact time, and regeneration were optimized, and nonspecific binding considerations were evaluated.

3.24 FOOD AND AGRICULTURE

3.24.1 Vitamin analysis in food products

Biosensor-based analysis is becoming more and more important in the food industry, and one of the fields of application is in vitamins analysis. The method for vitamin analysis is a label-free, inhibition assay (Kalman et al., 2006). The SPR biosensor monitors interactions of a specific binding protein with the vitamin immobilized on a CM5 sensor chip. The prepared samples are mixed with a fixed concentration of the vitamin binding protein by the autosampler and injected over the chip surface. The vitamin present in the sample binds to the protein and subsequently inhibits it from binding to the surface of the sensor chip. The higher the concentration of the vitamin is in the sample, the higher the level of inhibition, and hence the lower the response of the biosensor (O’Kane and Wahlström, 2011). A regeneration step prepares the chip surface for the next sample. Quantification is performed by multilevel calibration with the vitamin standards.

3.24.2 Detecting antibiotics in food: regulatory and quality control

The presence of banned antibiotics in honey is one recent example of why food consumer groups are insistent on better quality assurance and increased testing (Al-Waili et al., 2012). Methods for detecting chemical contaminants, e.g., streptomycin and chloramphenicol, that combine reliability and throughput at the required sensitivity are in demand (Pikkemaat, 2009). Biosensors can analyze the presence of antibiotics reliably, effectively, and quickly.
3.24.3 Amperometric biosensor in food analysis

Histamines can accumulate in seafood when bacteria spoilage begins and cause histamine poisoning without altering the fish’s normal appearance and odor. Therefore, a histamine biosensor using immobilized enzyme diamine oxidase (DAO) has been developed for the rapid monitoring of the histamine levels in tiger prawn (*Penaeus monodon*) (Perez et al., 2013). The histamine biosensor has a response time of less than 1 min, and optimum pH operation was 7.4. The reusable biosensor is simple and can be used for direct histamine determination without further pretreatment, and is suitable for routine analysis of tiger prawns to monitor spoilage.

3.24.4 Agriculture

Concentrations of herbicides, pesticides, and heavy metals in agricultural lands are increasing, which is a matter of great concern worldwide. Biosensors can be used to measure the levels of pesticides (Cesarino et al., 2012), herbicides (Glover et al., 2002), and heavy metals (Verma and Singh, 2005) in the soil and groundwater. Biosensors can also be used to forecast the possible occurrence of soil disease, which has not been feasible with existing technology. The biological diagnosis of soil using biosensors means opening the way to reliable prevention and decontamination of soil disease at an earlier stage than currently possible.

3.25 BIOMEDICAL APPLICATIONS

3.25.1 Detection of viral agents

The use of biosensors to detect specific viruses in biological samples offers a great diagnostic tool for medical applications. Identifying viruses in clinical materials during the acute phase of infections could provide necessary information for the treatment of infections by human immunoglobulin (hIg) or interferon (IF). A real-time detection system for viruses in general has been developed using an optical biosensor and a model virus: herpes simplex virus type 1 (HSV-1) (Inoue et al., 1999). The HSV-1 virus was found to propagate in Vero cells and, when diluted in minimum essential medium (MEM) with 10% fetal bovine serum (FBS), could be detected with an SPR sensor with high sensitivity and a detection limit of 10 infectious units (50% tissue culture infective dose [TCID50] units). This real-time viral detection and titration system has sensitivity high enough for clinical purposes.

One of the reasons for the traditionally low success for the direct identification of viruses by simple immunological assays is the large variability of their surface epitopes. An SPR biosensor with a modified silicon surface with broadly reactive serotype antibodies to 3Cpro has been developed (Ostroff et al., 2001). Although these antigenicities have previously been determined from ELISA methods, the SPR-based technique is superior...
in that it allows a fast and straightforward screening of antigens while simultaneously providing kinetic data for the Ag—Ab interaction.

### 3.25.2 Detection of human immunodeficiency virus

The human immunodeficiency virus (HIV) has been the target of intense research in the past two decades. SPR has been used in a number of HIV studies (Shafiee et al., 2013). It provides a means of looking at the interaction between macromolecules as it occurs in real time, providing information about the kinetics of the interaction, in addition to estimating affinity constants.

HIV-1 proteinase was immobilized on the sensor surface by direct amine coupling. A large number of inhibitors and noninteracting reference drugs were applied to the sensor surface in a continuous flow of buffer to estimate binding constants. The optimized assay could correctly distinguish HIV-1 inhibitors from other compounds in a randomized series, indicate differences in their interaction kinetics, and reveal artifacts due to nonspecific signals, incomplete regeneration, or carryover (Markgren et al., 1998).

The steady-state binding level and the time course of association and dissociation could be observed by measuring the binding of inhibitors injected in a continuous flow of buffer to the enzyme immobilized on the biosensor surface. Characterization of another set of HIV-1 protease inhibitors using binding kinetics data from an SPR biosensor–based screen has also been reported.

### 3.25.3 Detection of bacterial pathogens

Several physicochemical instrumental techniques for direct and indirect identification of bacteria such as IR and fluorescence spectroscopy, flow cytometry, chromatography, and chemiluminescence have been reviewed as feasible biosensor technologies (Fournier et al., 2014). *Staphylococcus aureus* is a pathogen that commonly causes human infections and intoxication. A sandwich immunoassay with fluorescein isothiocyanate conjugated with anti-(protein A) IgG was used to monitor the Ag—Ab reaction. In a different approach, an optical biosensor based on resonant mirrors was used in the detection of whole cells of *S. aureus* (Cowan-1). The bacterium cells, which express protein-A at their surface, were detected through their binding to human IgG immobilized on an aminosilane-derivatized sensor surface. A fiber-optic evanescent-wave sensing system that features all-fiber-optical design and red semiconductor laser excitation has been developed and tested. A 650-nm laser was used because biological matrices demonstrate minimal fluorescent background in the red; this helps reduce the background signal of nonessential biomolecules. The fiber directs the fluorescent signal of a sandwich immunoassay to detect *Salmonella* back to a charge-coupled device (CCD) fiber spectrophotometer.

A very different detection approach for *Salmonella typhimurium* involved immune magnetic separation and a subsequent enzyme–linked assay with alkaline phosphatase.
The magnetic microbeads coated with anti-Salmonella were used to separate Salmonella from sample solutions at room temperature for 30 min. A sandwich complex with alkaline phosphatase and the Salmonella immobilized on the magnetic beads was formed, separated from the solution by a magnetic filtration, and incubated with a p-nitrophenyl phosphate substrate at 37 °C for 30 min to produce p-nitrophenol by enzymatic hydrolysis. Salmonella was detected by measuring the absorbance of p-nitrophenol at 404 nm, with a linear response of 2.2–104 and 2.2–106 CFU mL⁻¹.

### 3.25.4 Detection of parasites

Detection of antibodies specific for the parasite Leishmania donovani in human serum samples is based on an evanescent-wave fluorescence collected by optical fibers that have the purified cell surface protein of L. donovani immobilized on their surface (Nath et al., 1997). The sensing fibers are incubated with the patient serum for 10 min and then incubated with goat anti-human IgG. Fluorescence was proportional to L. donovani–specific antibodies present in the test sera.

### 3.25.5 Detection of toxins

Ricin, a potently toxic protein, has been detected with an evanescent-wave fiber-optic biosensor with a detection limit of 100 pg mL⁻¹ and 1 ng mL⁻¹ for buffer solutions and river water, respectively (Narang et al., 1997). This detection was based on a sandwich immunoassay scheme, using an immobilized anti-ricin IgG on the surface of the optical fiber. Two coupling methods were used. In the first, the antibody was directly coated to the silanized fiber using a cross-linker; the second method utilized avidin-coated fibers incubated with biotinylated antiricin IgG to immobilize the antibody using an avidin-biotin bridge. The assay using the avidin-biotin–linked antibody demonstrated higher sensitivity and a wider linear dynamic range than the assay using the antibody directly conjugated to the surface. The linear dynamic range of detection for ricin in buffer using the avidin-biotin chemistry is 100 pg mL⁻¹ to 250 ng mL⁻¹.

The lipopolysaccharide (LPS) endotoxin is the most powerful immune stimulant known and a causative agent in the clinical syndrome known as sepsis. Sepsis is responsible for more than 100,000 deaths annually, in large part due to the lack of a rapid, reliable, and sensitive diagnostic technique. An evanescent wave fiber-optic biosensor was developed for the detection of LPS from E. coli at concentrations as low as 10 ng mL⁻¹ in 30 s (James et al., 1996). Polymyxin B covalently immobilized onto the surface of the fiber-optic probe was able to bind fluorescently labeled LPS selectively. Unlabeled LPS present in the biological samples was detected in a competitive assay format, by displacing the labeled LPS. The competitive assay format worked in buffer and in plasma with similar sensitivities. This method might also be used with other LPS capture molecules, such as antibodies, lectins, or antibiotics, to simultaneously detect LPS and determine the LPS serotype.
3.25.6 Blood factors

SPR was used to determine absolute heparin concentration in human blood plasma (Gaus and Hall, 1998). Protamine and polyethylenimine (PEI) were used to modify the sensor surface and were evaluated for their affinity to heparin. Heparin adsorption onto protamine in blood plasma was specific with a lowest detection limit of 0.2 U mL\(^{-1}\) and a linear detection range of 0.2–2 U mL\(^{-1}\). Although heparin adsorption onto PEI in buffer solution had indicated superior sensitivity to that on protamine, in blood plasma it was not specific for heparin and adsorbed plasma species to a steady-state equilibrium. By reducing the incubation time and diluting the plasma samples with buffer to 50%, the nonspecific adsorption of plasma could be controlled and a PEI pretreated with blood plasma could be used successfully for heparin determination. Heparin adsorption in 50% plasma was linear between 0.05 and 1 U mL\(^{-1}\) so that heparin plasma levels of 0.1–2 U mL\(^{-1}\) could be determined with a relative error of 11% and an accuracy of 0.05 U mL\(^{-1}\).

3.25.7 Congenital diseases

SPR and biospecific interaction analysis (BIA) have been used to detect the Delta F508 mutation (F508del) of the cystic fibrosis transmembrane regulator (CFTR) gene in homozygous as well as heterozygous human subjects (Feriotto et al., 1999). The detection method involved the immobilization on an SA5 sensor chip of two biotinylated oligonucleotide probes (one normal, N-508, and the other mutant, Delta F508) that are able to hybridize to the CFTR gene region involved in F508del mutation. A hybridization step between the oligonucleotide probes immobilized on the sensor chips and (1) wild-type or mutant oligonucleotides, as well as (2) ssDNA. These nucleic acid samples were obtained using asymmetric polymerase chain reaction (PCR), performed using genomic DNA from normal individuals and from F508del heterozygous and Delta 508del homozygous patients. The different stabilities of DNA/DNA molecular complexes generated after hybridization of normal and Delta F508 probes immobilized on the sensor chips were then evaluated. The results strongly suggest that the SPR technology enables a one-step, nonradioactive protocol for the molecular diagnosis of F508del mutation of the CFTR gene. This approach could be of interest in clinical genetics because the hybridization step is often required to detect microdeletions present within PCR products.

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