AMPEROMETRIC DETERMINATION OF SELECTED PERSISTENT ORGANIC POLLUTANTS AND HEAVY METALS USING HORSERADISH PEROXIDASE BIOSENSOR

by

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As the candidate's supervisor I have approved this thesis for submission

Signed _____ Name_____ Date _____

PREFACE

The experimental work described in this dissertation was carried out in the School of Chemistry, University of KwaZulu Natal, Durban, from March 2009 to November 2010, under the supervision of Dr J. Catherine Ngila.

This study represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any tertiary institution where use has been made of the work of others it is dully acknowledge in the text.

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DECLARATION 2- PUBLICATIONS

Philiswa N. Nomngongo, J. Catherine Ngila*, Vincent O. Nyamori, Everlyne A. Songa and Emmanuel I. Iwuoha. (2010). Determination of selected heavy metals using amperometric horseradish peroxidase (HRP) inhibition biosensor. *Analytical letters* (accepted)

Philiswa N. Nomngongo, J. Catherine Ngila*, Vincent O. Nyamori, Titus A. M. Msagati and Emmanuel I. Iwuoha. (2010). Amperometric determination of polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs) and polychlorinated biphenyls (PCBs) using horseradish peroxidase (HRP) biosensor based on kinetic inhibition. *International Journal of Environmental Analytical Chemistry* (submitted).

Philiswa N. Nomngongo, J.C Ngila and V. Nyamori. Determination of Selected Persistent Organic Pollutants Using Horseradish Peroxidase Inhibition Amperometric Biosensor. *Analitika 2010 International Conference on Analytical Sciences*, Stellenbosch, South Africa, December 5-9, 2010.

Philiswa N. Nomngongo, Catherine J. Ngila and Everlyne A. Songa. The electrochemical biosensor based on horseradish peroxidase immobilized on polyaniline for the detection of heavy metals. *The 10th International Symposium on Kinetics in Analytical Chemistry*, Cape Town, South Africa, December 02-04, 2009.

Signed:…….

ABSTRACT

Persistent organic pollutants and heavy metals are released into the environment through different anthropogenic processes. They are of concern because they tend to bioaccumulate in the food chain and show adverse health effects ranging from acute to chronic toxicity. These pollutants need therefore to be monitored to conserve the environment. Conventionally, samples are sent to a laboratory for analysis by standard techniques such as chromatography and spectroscopy. Although these conventional techniques display high accuracy and low detection limits, they are expensive, require the use of highly trained personnel and tedious sample preparation. In comparison, electrochemical methods such as biosensors are sensitive, low cost and simple to operate. In this thesis, the determination of selected persistent organic pollutants (polybrominated diphenyl ethers, polybrominated biphenyls and polychlorinated biphenyls) and heavy metals (Cd, Pb and Cu) was achieved by the use of amperometric inhibition biosensor based on horseradish peroxidase (HRP) immobilized on the surface of platinum-polyaniline modified electrode.

Polyaniline (PANI) film was electrochemically deposited on the platinum electrode surface. The film was characterized by cyclic voltammetry and spectrometric techniques. The CV results proved that the PANI was electroactive and exhibited a fast reversible electrochemistry. Characteristic Ultraviolet–Visible and Fourier Transform Infrared features of the polymer film were identified. They revealed that PANI film synthesized in this study is the conductive emeraldine salt.

Horseradish peroxidase based biosensor was constructed by electrostatic attachment of the enzyme onto Pt-PANI electrode surface. Spectrometric and cyclic voltammetric results indicated that the immobilized HRP retained its bioelectrocatalytic activity towards the reduction of hydrogen peroxide. The Pt/PANI/HRP biosensor showed a linear response over a concentration range of 0.05 to 3.17 mM with a detection limit of 36.8 nM. Apparent Michaelis-Menten constant (K_M^{app}) was calculated as 1.04 mM. This implied that the HRP biosensor had a high affinity for H_2O_2. Furthermore, the fabricated biosensor showed high sensitivity, good reproducibility, repeatability and long-term stability.

The Pt/PANI/HRP biosensor was applied to the determination of selected persistent organic pollutants and heavy metals. The latter was found to inhibit the HRP enzyme's activity.

The percentage inhibition of the investigated persistent organic pollutants decreases in the following order: 2,2´4,4´,6-pentabrominated diphenyl ether> 2-brominated biphenyl> 2-chlorinated biphenyl> 2,2´,4,5,5´-pentachlorinated biphenyl> 2,4,4´-trichlorinated biphenyl. In the case of heavy metals, the degree of inhibition of heavy metals was highest for Cd^{2+}, followed by Cu^{2+} and then Pb^{2+}. Kinetic study for the amperometric response to H_2O_2, recorded in the absence and presence of persistent organic pollutants and heavy metals revealed that for polybrominated diphenyl ethers, the inhibition process corresponded to a competitive type whereas for polybrominated biphenyls, polychlorinated biphenyls and heavy metals, it corresponded to the on-competitive type. The biosensor exhibited high sensitivity towards the determination of the metals and persistent organic pollutants as pollutants in real water samples, namely tap water and landfill leachate samples.

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I recognize that this research would not have been possible without the financial assistance of NRF through grant-holder, Dr Anna Soares (UKZN- PMB) and Prof E.I. Iwouha.

DEDICATION

This thesis is dedicated to my mother, Lulama, Sive, Azukile and Sisipho for the understanding and encouragement they provided during all years of study.

“And we know that all things work together for good to them that love God, to them who are the called according to his purpose”. Romans 8:28

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LIST OF SYMBOLS

A	Ampere
AAS	Atomic absorption spectroscopy
AE	Auxiliary electrode
APPI	Atmospheric pressure photoionization
ASV	Anodic stripping voltammetry
ATR	Attenuated transmittance resonance
ATSDR	Agency for Toxic Substances and Disease Registry
BFRs	Brominated flame retardants
BSEF	Bromine Science and Environmental Forum
CV	Cyclic voltammetry
DAD	Diode-array detector
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
DPV	differential pulse voltammetry
ECNI	Electron capture negative ion
ENFET	Enzyme linked field effect transistor
FAAS	Flame atomic absorption spectroscopy
FRs	Flame retardants
FTIR	Fourier transform infrared
GCE	Glassy carbon electrode
GC-ECD	Gas chromatography coupled with electron capture detector
GC-MS	Gas chromatography coupled with mass spectrometry
GFAAS	Graphite furnace atomic absorption spectroscopy
HBCDD	Hexabromocyclododecane
HPLC-MS	High performance liquid chromatography couple with mass spectrometry
HRP	Horseradish peroxidase
ICP	Inductively coupled plasma
ICP-AES	Inductively coupled plasma- atomic emission spectroscopy
ICP-MS	Inductively coupled plasma -mass spectrometry
ISE	Ion-selective electrode

ISFET	Ion-selective field effect transistor
LLE	Liquid-liquid extraction
Ltd	Limited
MCL	Maximum contaminant level
NTP	National Toxicology Program
PANI	Polyaniline
PBBs	Polybrominated biphenyls
PBDEs	Polybrominated diphenyl ethers
PBS	Phosphate buffer solution
PCBs	Polychlorinated biphenyls
POPs	Persistent organic pollutants
RE	Reference electrode
SCE	Saturated calomel electrode
SPE	Solid-phase extraction
TBBA	Tetrabromobisphenol A
USEPA	United States Environmental Protection Agency
UV-Vis	Ultraviolet visible
V	Volts
WE	Working electrode
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Environmental pollutants have been and are still released into the environment through different anthropogenic processes such as coal conversion, petroleum refining, mining, textile, pharmaceutical and paper and pulp manufacturing. The large-scale uses of herbicides, insecticides, and pesticides in agriculture (Clark *et al.* 2003; Doong *et al.* 2008) also have contributed as pollutants. The industrial discharges contaminate water, air, and land thus putting flora and fauna at risk. The pollutants can have toxic, mutagenic and carcinogenic characteristics, therefore they are considered to be hazardous to the environment (Clark *et al.* 2003; Doong *et al*. 2008). Environmental pollutants include persistent organic pollutants (POPs) and heavy metals. Heavy metals and POPs are of concern because they tend to bioaccumulate in the food chain and show adverse health effects ranging from acute to chronic toxicity (Chrysikou *et al*. 2008).

Over the past years, several studies have been conducted to determine levels of pollutants in the environment (Lim *et al*. 2008; Huang *et al*. 2007; Nguyen *et al*. 2005; Zhang *et al*. 2003; Chrysikou *et al*. 2008). The results show that the environment contains relatively high levels of POPs and heavy metals. Most POPs and particularly heavy metals are immobilized in the soil and precipitated in the sediments of fresh ecosystems. Persistent organic pollutants, due to their hydrophobicity, have a high affinity for organic matter in soils or sediments (Doong *et al.* 2008). However, these environmental pollutants could be mobilized (increased transport) when biogeochemical conditions changes. In view of the common occurrence of POPs and heavy metals in the environment, this work will focus on analysis for these two classes of pollutants.

1.1 PERSISTENT ORGANIC POLLUTANTS

Persistent organic pollutants (POPs) are a group of organic compounds that are known to be persistent in the environment. Due to their lipophilic properties, they tend to bioaccumulate through the food web (Chrysikou *et al.* 2008). This causes a risk of undesirable effects to human health and the environment. The increase of POPs in the environment poses concern because of their reproductive, developmental, immunologic and carcinogenic effects (Abelsohn *et al.* 2002; EPA 2002). A number of POPs disrupt the normal functioning of the endocrine system, and they are known as endocrine disruptors. Endocrine disrupting properties are found in several classes

of chemicals released into the environment. These chemicals include: (i) flame retardants such as polybrominated diphenyl ethers; (ii) pesticides, e.g. dichlorodiphenyltrichloroethane (DDT); (iii) metals such as tributyltin; (iv) pharmaceuticals, e.g. ethynyl estradiol; iv) plasticizers such as phthalates; (vi) phenols, e.g. bisphenol A; (vii) polyaromatic compounds such as polychlorinated biphenyls and (viii) surfactants, e.g. alkylphenol ethoxylates (Colborn, Dumanoski, and Myers 1996; Wua *et al.* 2008). The focus of this study will be on halogenated aromatic compounds, namely, flame retardants and polychlorinated biphenyls.

1.1.1 Flame Retardants

Flame retardants (FRs) are substances that are added to materials during or after manufacture to suppress or slow down the combustion process. Flame retardants slow down the combustion process at different stages, for example, during heating, decomposition, ignition or flame spread. Flame retardants are used in the manufacture of wire cables, construction materials, textiles, printed wiring boards, electrical connectors, furniture, and electronic equipment (BSEF 2000; De Wit 2002). Figure 1.1 shows some examples of end-products where flame retardants are typically used.

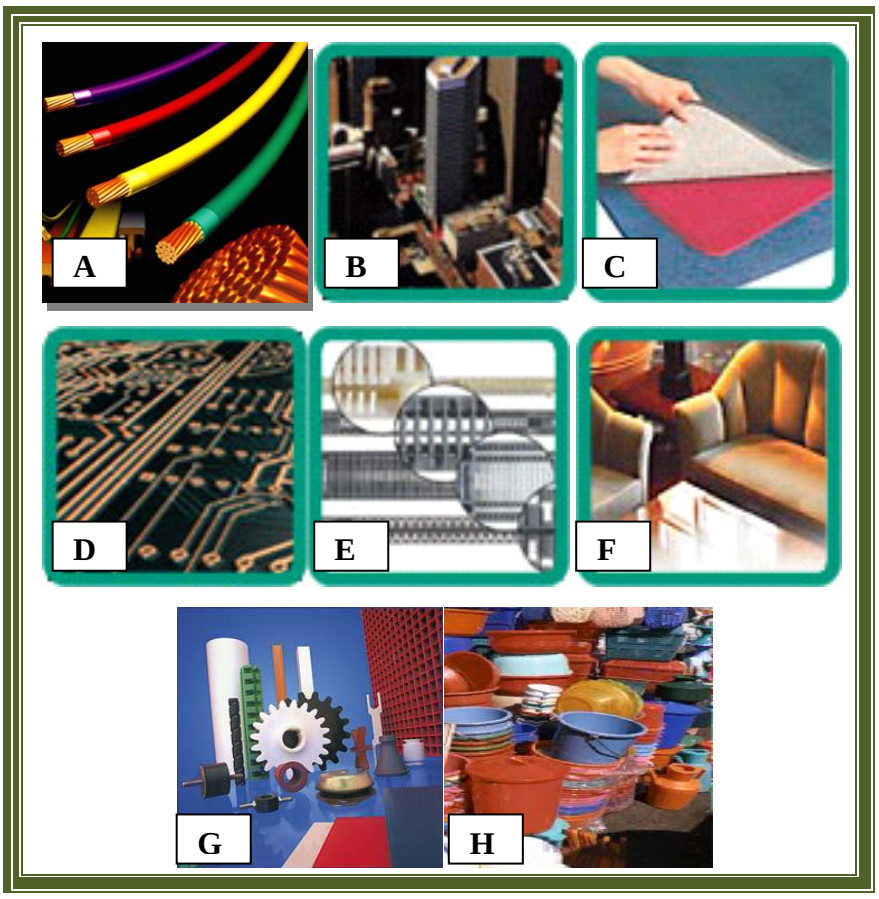


Figure 1.1. End-products in which flame retardants are used: A) wire cables, B) construction materials, C) textiles, D) printed wiring boards, E) electrical connectors, F) furniture and G-H various plastics (SpecialChem 2010).

1.1.1.1 *Brominated flame retardants*

Brominated flame retardants (BFRs) are bromine containing chemicals that are added to various materials to prevent fires (BSEF 2000). The bromine atom can be bonded on the aliphatic or aromatic moiety. However, aromatic BFRs are more thermally stable than aliphatic BFRs (Troitzsch 1998). Some of the BFRs are persistent and lipophilic compounds. They can bioaccumulate and are thus considered as a potential environmental health problem (de Wit 2002). The most commonly used BFRs include tetrabromobisphenol A (TBBPA), polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCDD) and polybrominated biphenyls (PBBs) (BSEF 2000; de Wit 2002; Sjodin *et al.* 2003) among others (see Figure 1.2A-D).

Depending on the industrial use, BFRs can be divided into two categories, additive and reactive. Reactive BFRs are a type of BFRs that bond covalently to the polymer matrix. Additive BFRs on the other hand are a type of BFRs that are added in the polymer matrix and a form very weak interaction with the material. Reactive BRFs include TBBPA whereas PBDEs, PBBs and HBCDD belong to additive BFRs. Due to the weak interaction with the polymer matrix, literature reports that additive BRFs are more likely to leach out of the products as compared to reactive BFRs (Alaee *et al.* 2003; Goosey 2006, Yogui and Sericano 2009). Therefore additive BFRs can be easily released to the environment. Due to the toxicity, lipophilicity and the increase of the levels of PBDEs in the environment, most of the studies carried out on BFRs focus on PBDEs. This study will focus on selected additive BFRs such as PBDEs and PBBs.

Polybrominated diphenyl ethers (PBDEs) are an important group of BFRs. They are synthetic BFRs (Alaee *et al.* 2003; Birnbaum and Staskal 2004). The major sources of PBDEs are industrial and domestic activities. Products containing PBDEs from domestic and industrial wastes are disposed of in landfills and incineration sites. During burning of the PBDE-containing products, they tend to leach out and produce toxic dioxins. This becomes a persistent source of emission of these compounds into the environment (Sjodin *et al.* 1999; Hale *et al.* 2003).

Polybrominated diphenyl ethers are structurally related to polychlorinated biphenyls (PCBs) and PBBs (Figure 1.2A, B and E). Therefore, they have similar properties and large number of congeners (Rahman *et al.* 2001). Similar to PCBs, PBDEs exist in 209 different congeners varying in both number and position of bromination. The PBDE congeners are named by number and position of the bromine atoms on the two phenyl rings under the IUPAC system established for the PCBs (Vonderheid 2009). The possible substitution on the phenyls ranges from 1-10 but there are three major commercially available PBDE mixtures that are commonly used. These include pentabrominated diphenyl ether (penta-BDE), octabrominated diphenyl ether (octa-BDE) and decabrominated diphenyl ether (deca-BDE). The penta-BDE compounds consist of a mixture of congeners that include mostly BDE- 47, BDE-99, BDE-100, BDE-153, and BDE-154. The main compound under octa-BDE class is BDE-183, whereas deca-BDE generally consists of BDE-209 (Birnbaum and Staskal 2004; Schecter *et al.* 2005; Sjodin *et al.* 1999; Hale *et al.* 2003; Rahman *et al.* 2001). The chemical for some congeners are presented in Table 1.

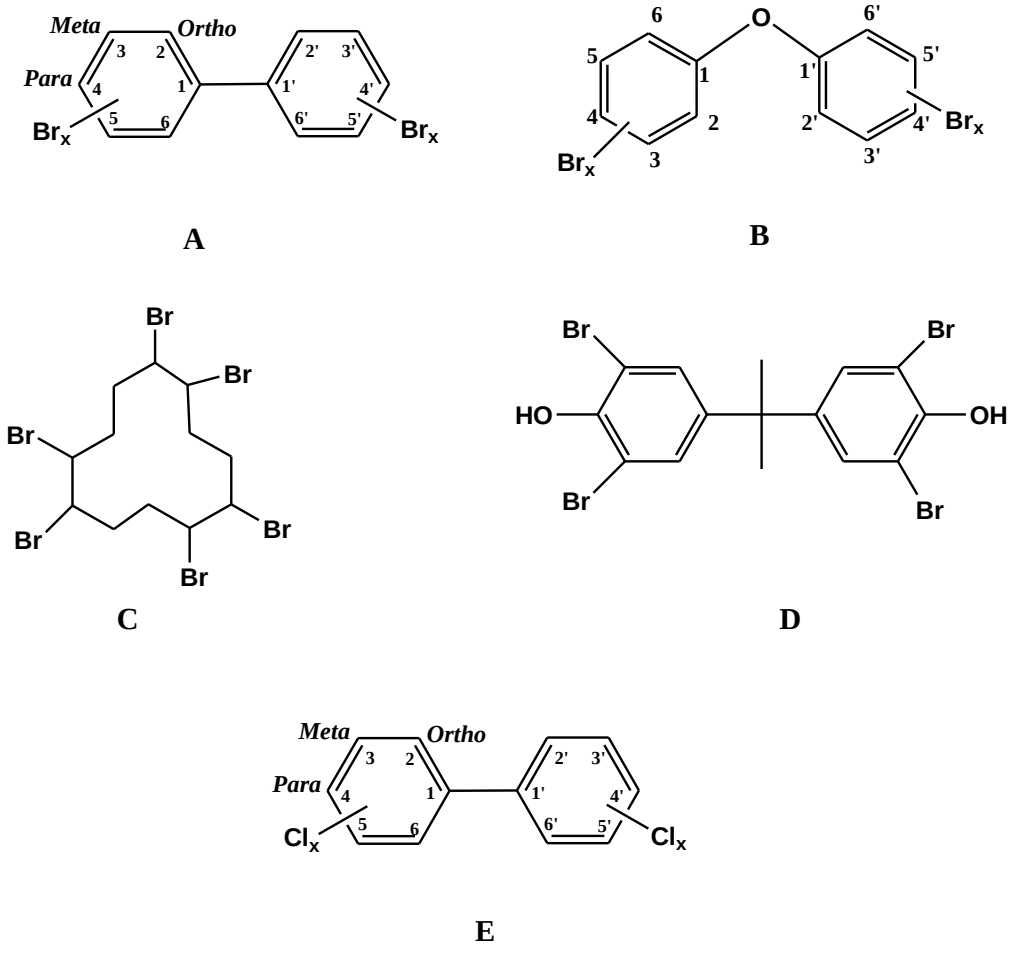


Figure 1.2. Chemical structures of (A) polybrominated biphenyls, (B) polybrominated diphenyl ethers, (C) hexabromocyclododecane, (D) tetrabromobisphenol A and (E) polychlorinated biphenyls.

Table 1. Major PBDE congers or mixtures.

PBDEs congeners	Chemical names
BDE-47	2,2',4,4'-tetrabromodiphenyl ether
BDE-99	2,2',4,4',5-pentabromodiphenyl ether
BDE-100	2,2',4,4',6-pentabromodiphenyl ether
BDE-153	2,2',4,4',5,5'-hexabromodiphenyl ether
BDE-154	2,2',4,4',5,6'-hexabromodiphenyl ether
BDE-209	Decabromodiphenyl ether

The increase of PBDEs in human tissue, breast milk and blood, pose a particular concern. As mentioned in Section 1.1, PDBEs are endocrine disruptors and therefore they interfere with the normal functioning of the endocrine system (Hallgren and Darnerud 1998; Hallgren and Darnerud 2002; Hall, Kalantzi and Thomas 2003; Gillner, Jakobsson 1996; Meerts *et al.* 2001, Meerts *et al.* 2002; Morse *et al.* 1993). They also cause reproductive and developmental toxicity including neurotoxicity (Birnbaum and Staskal 2003; Branchi *et al.* 2003; Branchi *et al.* 2002, Eriksson *et al.* 2002; Viberg, Fredriksson and Eriksson 2004) and cancer (National Toxicity Program (NTP). 1986). The toxicity of these compounds depends on the time of exposure. The immediate exposure of PBDEs (if it is swallowed or absorbed by the skin or inhaled) is slightly toxic. For long-term exposure, they can harm the brain and central nervous system (neurotoxin) and affect hormones. Among the three commercially available PBDEs, deca-BDE is known to be carcinogenic (Branchi et al 2003; Draggan 2004). Studies have shown an increase in levels of PBDEs in environmental samples and human tissues over time, although the levels in comparison are still lower than those for PCBs and DDT (de Boer 1989; Noren, and. Meironyte 2000; Darnerud *et al.*, 2001). The next section will focus on the second type of BFRs namely polybrominated biphenyls.

Polybrominated biphenyls (PBBs) are bromine containing flame retardant chemicals that were once added to plastics, carpets, electronic equipment, textiles and building materials during

their production but their manufacture was discontinued in late 1970s (ATSDR 2004). Even though the use of PBBs ceased years ago, they still persist and have the potential to contaminate the environment (Givens *et al.* 2007). Their environmental persistence and human health exposure consequences are still documented (Givens *et al.* 2007; Herzke *et al.* 2005; Vetter 2008, de Wit *et al.* 2006). Similar to PBDEs, PBBs are persistent and lipophilic substances (Liu *et al.* 2008, ATSDR 2004; Givens *et al.* 2007).

Current sources of PBBs may be residues remaining in and around products that formerly were manufactured, or secondary products (ATSDR 2004; Givens *et al.* 2007). Ingestion, inhalation and dermal absorption are primary routes of potential human exposure to PBBs. Exposure to the latter has been associated with adverse effects on the endocrine, immune and neurological systems.

1.1.2 Polychlorinated Biphenyls

The second class of POPs of interest in this study is polychlorinated biphenyls (PCBs). The PCBs are organic compounds that consist of a group of persistent environmental pollutants. Similar to PBDEs, depending on the number of chlorines and their location on the biphenyl ring, PCBs can have 209 possible congeners (Hu *et al.* 2009; Razaei et al 2008; Fonnum and Mariussen 2009; Carpenter 2006). These congeners are divided into three major structural classes (Figure. 1.2E). The chlorine substitution pattern (*para*-, *meta*- and *ortho*-substitution) makes it easier to differentiate between the three classes (Fonnum and Mariussen 2009).

PCBs were once commonly used in transformers, capacitors and the paper, glue, cement and paint industries as well as for insulating material for windows (Hu *et al.* 2009; Razaei *et al.* 2008; Fonnum and Mariussen 2009). Due to the environmental and health problems associated with PCBs, their production was banned in the 1970s in North America and other industrialized countries (ATSDR 2000). PCBs have been released into the environment through burning, evaporation, leaks and dumping (Breivik *et al.* 2002). They are very persistent, non-combustible and thermostable. Similar to other POPs, PCBs are lipophilic and resistant against biological breakdown. Hence, they tend to accumulate in the fauna and flora and become toxic (Fonnum and Mariussen 2009; Broding *et al.* 2008). Although the use of PCBs was banned in most of countries, they are still present in the environment (Rezaei *et al.* 2008; Hu *et al.* 2009). Possible routes of PCB exposure to living organisms could be ingestion, inhalation and dermal absorption

(Carpenter 2006). These compounds are known to cause alteration of the immune system, adverse alteration of the nervous system, skin, thyroid, liver, kidney, pancreas, and the cardiovascular system (Carpenter 2006). Therefore, people who are exposed to PCBs are at high risk of cancer, infections, reduced cognitive function accompanied by adverse behavioral effects, hypothyroidism, infertility, ischemic heart disease, hypertension, diabetes, liver disease, asthma and arthritis (Carpenter 2009, Broding *et al.* 2009; Fonnum, Mariussen and Reistad 2006; Lilienthal *et al.* 2009; Fonnum and Mariussen 2009). In this study levels of pentabrominated diphenyl ether, octabrominated diphenyl ether mixture of isomers, monobrominated biphenyl and monochlorinated biphenyls in landfill leachate water were investigated.

The second class of pollutants of interest to this project, as mentioned in the introduction, is heavy metals.

1.2 HEAVY METALS

Heavy metal is a general collective term that refers to the group of metals and metalloids with an atomic density greater than 6 g cm^{-3}. Unlike most POPs, heavy metals occur naturally in the earth's crust. Therefore, a range of background concentrations can be detected in water, living organisms, sediments and soils (O'Connel *et al.* 2008). On the other hand, toxic levels of the heavy metals can exceed the background concentrations found in nature. For this reason, studies have been carried out to determine their effect on flora and fauna (Swarup *et al.* 2007; Chary, Kamala and Raj 2008; Zhuang *et al.* 2009; Gopalakrishnan *et al.* 2008). Heavy metals can also enter into the environment through anthropogenic activities (industrial activities and domestic processes). These include burning or mining, fossil fuels, incineration of wastes, automobile exhausts, smelting processes, refining ores, fertilizer industries, tanneries, batteries, paper industries and pesticides (Sud *et al.* 2008). These sources increase the risk of exposure to heavy metals to living organisms and hence their toxicity, bio-accumulation and persistency in the environment cause concern (Meena *et al.* 2005).

Heavy metals are toxic to plants and animals even in relatively low concentrations (Mohan and Singh 2002, Mohan *et al.* 2006). They tend to accumulate in the food chain through uptake at the primary producer level and then through consumption at consumer level (Chary, Kamala and Raj 2008). Metals enter the human body either through inhalation, ingestion (food and water) and absorption by the skin (Yantasee *et al.* 2007). Exposure to heavy metals can

result in damaged or reduced mental and central nervous system functioning, lower energy levels, and damage to blood composition, lungs, kidneys, liver, and other vital organs (Amarasinghe and Williams 2007). Heavy metals include chromium, copper, lead, mercury, manganese, cadmium, nickel, zinc and iron (Raikwar *et al.* 2008; Meena *et al.* 2005). However, lead, cadmium and mercury being the most toxic, present major hazards to ecosystems and are a serious danger to humans. Furthermore, these metals have the ability to accumulate in living matter. In this work, only lead and cadmium have been determined in tap water as mercury is not typically found in drinking water systems. In addition, copper (an essential metal in limited levels) was also analyzed due to its common occurrence in water and other environmental systems. The next section will review each of the metals.

1.2.1 Cadmium

Cadmium is commonly found in ores together with zinc, copper and lead and can be produced as a by-product of zinc refining (Jarup 2003; Godt *et al.* 2006; Lenntech 2009). Since cadmium and zinc have similar physiochemical properties, cadmium tends to displace zinc in some important biological functioning of the human body (enzymatic and organ functions). Therefore, in this way cadmium derives its toxicological properties. Cadmium exposure may cause kidney damage, lung cancer and bone defects (Jarup 2003; Godt *et al.* 2006; Lenntech 2009). Cadmium can contaminate the environment through natural (volcanic eruption) and anthropogenic activities and therefore the latter increases the chances of occurrence of cadmium in the biosphere, hydrosphere and atmosphere (Jarup 2003; Godt *et al.* 2006; Staessen *et al.* 1999; Min, Ueda and Tanaka 2008).

1.2.2 Lead

Lead is a type of heavy metal that has specific toxicity and cumulative effects. Depending on the level and duration of exposure, lead poisoning is common. The health problems of lead include malfunction in the synthesis of haemoglobin, gastrointestinal tract, joints and reproductive system, acute or chronic damage of the nervous system and anemia (Jarup 2003; Lenntech 2009). The major sources of lead in the environment are industrial discharges from batteries, insecticides and plastic water pipe industries, among others (Meena *et al.* 2005; Jarup 2003).

1.2.3 Copper

Copper is an essential element to humans. However, when it is taken in excess it causes anemia, liver and kidney damage, jaundice, stomach and intestinal irritation and lung cancer (Meena *et al.* 2005; Lenntech 2009). Copper enters the environment through industrial emissions and agricultural fertilizers (Fischer *et al.* 2008). In drinking water the main sources of copper are the pipes used to transport water and as an additive to control algal growth (Lenntech 2009).

1.3 MOTIVATION FOR THE STUDY

The levels of pesticides, such as DDT and heavy metals in South African environment have been well documented (Bouwman *et al.* 2006; Bouwman *et al.* 2008; Batteman *et al.* 2008, Moodley *et al.* 2007; Janganyi *et al.* 2005). However, there is still lack of information concerning the levels of POPs such as PBDEs, PBBs and PCBs. The monitoring of the presence of PBDEs, PBBs and PCBs in domestic and industrial landfill leachates is of great importance. This is because these compounds can easily be leached into groundwater from solid waste landfill sites (Odusanya *et al.* 2009)**.** Therefore, people who use groundwater as their main source of water, particularly in villages, will be exposed to these compounds. For this reason, information about the levels of these compounds in different areas of the country is very important. This will help South Africa in formulating a water quality protection plan, in relation to halogenated POPs (WRC 2005).

Recently, Odusanya *et al.* (2009) reported the levels of PBDEs in landfill leachate samples from different regions in Gauteng province. Poler and coworkers (2008) carried out investigations on the levels of BFRs in bird egg samples from Western Cape, Free State and North West Provinces. The levels and trends of airborne PCBs monitored at three locations (urban, industrial and residential sites) in KwaZulu Natal (Durban), has been investigated by Batterman *et al.* (2009). Although few studies have been conducted on these pollutants, they do not cover the whole country. Also further investigation on different environmental samples such as sediments, sewage sludge and biota need to be conducted. This will assist in estimating the total amount of POPs released into the South African environment (Odusanya *et al.* 2009).

The presence of POPs and heavy metals in the environment is of concern because of their toxic effects on living organisms. The existing methods for detection of POPs utilize gas

chromatography (GC) (Vonderheide 2009) and high performance liquid chromatography (HPLC) (Vonderheide 2009), both coupled to different types of detectors such as electron capture detector (ECD) (Wang *et al.* 2006) or mass spectrometry (MS) (Tadeo *et al.* 2009) for GC, (diode array detector) DAD/UV-Vis (Vilaplana, Ribes-Greus and Karlsson 2009) and MS (Bacaloni *et al.* 2009) for HPLC. For the detection of heavy metals, conventional spectroscopic and voltammetric techniques have been utilized. These include inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (Bettinelli *et al.* 2000); inductively coupled plasma mass spectrometry (ICP-MS) (Rahmi *et al.* 2007); atomic absorption spectroscopy (AAS) (Tuzen *et al.* 2008) and anodic stripping voltammetry (ASV) (Brainina *et al.* 2004). Most of these instruments except for voltammetry are expensive. Although the conventional spectroscopic methods have high accuracy and the lowest detection limits, they are sophisticated and require skilled operators. They also require sample preparation before analysis, which is time-consuming and tiresome.

For this reason rapid and simpler methods are required for the analysis of these environmental pollutants. Electrochemical methods offer an alternative approach for the detection of both classes of pollutants, namely POPs and heavy metals. This is because electrochemical methods are simple to use and have low cost. In view of this, this project undertook to develop an electrochemical method based on amperometric detection using horseradish peroxidase (HRP) immobilized onto a platinum electrode modified with polyaniline (PANI). These techniques will be reviewed in Chapter 2.

1.4 HYPOTHESIS

The detection of POPs and heavy metals can be achieved through monitoring the decrease in amperometric current, produced through biocatalytic reduction of hydrogen peroxide by horseradish peroxidase (HRP) enzyme. The presence of POPs or heavy metals in the working solution should inhibit the reduction of hydrogen peroxide which in turn should show a decrease in the measured signal (current).

1.5 OBJECTIVES OF THE STUDY

The main objective of this project was the fabrication of an electrochemical biosensor through electrostatic attachment of the HRP enzyme onto the surface of a platinum electrode, modified

with polyaniline (PANI) doped with chloride ions. This was followed by application of the biosensor in the determination of selected persistent organic pollutants and heavy metals in tap water and landfill leachate water samples.

The specific objectives/ research activities include:

1. Electrochemical synthesis of PANI doped with chloride ions using cyclic voltammetry (CV).
2. Characterization of PANI films using cyclic voltammetry (CV), and Fourier transform infrared (FTIR) and UV-Vis spectroscopy.
3. Fabrication of an electrochemical biosensor by using electrostatic attachment method onto the electrode, that is, the enzyme, horseradish peroxidase (HRP), is attached electrostatically onto the surface of a platinum electrode modified with a PANI film.
4. Characterization of the fabricated PANI/HRP biosensor by CV, FTIR and UV-Vis spectroscopy.
5. Optimization of analytical parameters affecting the amperometric technique for quantitative detection of POPs and heavy metals. The parameters include operating potential, reproducibility, repeatability, selectivity and stability of the biosensor.
6. Investigation of the effects of POPs and heavy metals on the catalytic activity of HRP towards the reduction of hydrogen peroxide.
7. Application of the developed biosensor for rapid monitoring of POPs and heavy metals in tap water and landfill leachate samples.
8. Comparison of the results obtained by the proposed biosensor method with data obtained from standard analytical methods such as GC-MS and GC-ECD for POPs and ICP-OES for heavy metals.

1.6 THESIS OVERVIEW

Chapter 1 presents the introduction or background on halogenated aromatic compounds which fall under POPs as well as heavy metals. The motivation for this study, hypothesis and objectives are also included.

Chapter 2 reviews the literature on the analytical techniques used for the detection of brominated flame retardants and heavy metals, types of biosensors, and enzyme mobilization methods and

their kinetics in relation to their inhibition principles. This chapter also highlights the use of conducting polymers in the fabrication of the biosensor, their synthesis and characterization.

Chapter 3 reports on the results and discussion for the characterization of the electrochemically synthesized polyaniline (PANI). The characteristic properties of PANI were investigated by, CV, FTIR and UV-Vis spectroscopy.

Chapter 4 reports on the preparation and characterization of Pt/PANI/HRP biosensor. The results and discussion for the characterization of the HRP biosensor are discussed in details. The characteristic properties of PANI/HRP were investigated by FTIR and UV-Vis spectroscopy. The optimization of the buffer pH is also discussed.

Chapter 5 reports the application of amperometric horseradish peroxidase (Pt/PANI/HRP) inhibition biosensor for the determination of selected heavy metals. The kinetic studies of horseradish peroxidase inhibition by heavy metals are discussed in details. Comparison of the results obtained by Pt/PANI/HRP biosensor and inductively coupled plasma-optical emission spectrophotometry in tap water and leachate samples are also reported.

Chapter 6 reports the application of amperometric horseradish peroxidase (Pt/PANI/HRP) inhibition biosensor for the determination of selected POPs. Inhibition kinetics and analytical characteristics (selectivity and sensitivity) are discussed. The use of Pt/PANI/HRP biosensor and gas chromatography–mass spectrometry (used to confirm the results) for the determination of POPs in landfill leachate is also included.

Chapter 7 presents the summary of the major findings of this work and the conclusions drawn from the results obtained as well as further studies suggested.

CHAPTER 2

REVIEW OF ANALYTICAL TECHNIQUES FOR DETERMINATION OF PERSISTENT ORGANIC POLLUTANTS AND HEAVY METALS

In this chapter, analytical methods that have been used in the determination of persistent organic pollutants (POPs) and heavy metals in environmental and human samples are reviewed. These include chromatographic (gas chromatography and high performance liquid chromatography), spectroscopic (atomic absorption spectroscopy, inductively coupled plasma-optical emission spectroscopy and inductively coupled plasma-mass spectrometry) and electroanalytical techniques (anodic stripping voltammetry, biosensors, cyclic voltammetry and differential pulse voltammetry). Biosensors are discussed in detail.

2.1 BIOSENSORS

2.1.1 Introduction to Biosensors

Biosensors are analytical sensing devices consisting of a biological component in close contact with a suitable physicochemical transducer, which is able to convert the biological recognition reaction or the biocatalytic process into a measurable signal. The biological recognition elements include enzymes, tissues, antibodies, DNA, whole cell, nucleic acid, receptors and microorganisms (Chaplin 2004a; Freire *et al.* 2003; Eggins 1996; Andreescu and Sadik 2004; Egggins 2002).

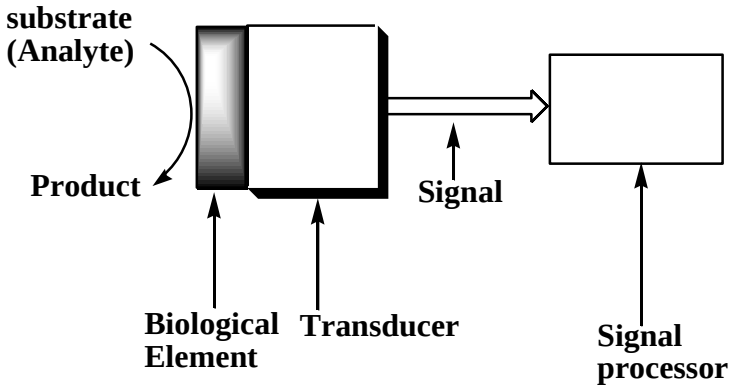


Figure 2.1. Schematic diagram showing the main components of a biosensor**.**

The important parts of the biosensor are shown in Figure 2.1. The biological element binds to the substrate and converts the substrate into product. The biological element must be highly specific, stable under storage conditions and immobilized. The second compartment is the transducer. The transducer acts as a boundary, measuring the physical change that occurs with the reaction at the bioreceptor and then transforming that energy into a measurable electrical signal. Signals from the transducer are passed to a signal processor where they are amplified and analyzed (Chaplin 2004a; Eggins 1996; Eggins 2002; Andreescu and Sadik 2004). The advantages of using biosensor over traditional analytical techniques include the possibility of portability and miniaturisation and the ability to measure pollutants in complex matrices with minimal sample preparation (Andreescu and Sadik 2004; Chaubey and Malhotra, 2002; Dennison and Turner 1995; Parellada *et al.* 1998). Biosensors are used in a number of applications such as environmental and bioprocess control, quality control of food, agriculture, military, and particularly medical applications (Andreescu and Sadik 2004; Rodriguez-Mozaz *et al.* 2006). Biosensors can be classified into different groups, according either to signal transduction or biological recognition elements or the combination of the two (Thevenot *et al.* 1999).

2.1.2 Biological Recognition Elements

In terms of biological recognition elements, biosensors can be divided into two different classes, namely biocatalytic recognition element and biocomplexing (bioaffinity) recognition element (Thevenot *et al.* 1999). Concerning the biocomplexing (bioaffinity) recognition element, the principle of the biosensor is based on the interaction of the analyte with macromolecules or organized molecular assemblies that have been isolated from their original biological environment (Roe 1992). Biocomplexing (bioaffinity) recognition elements mostly employ antibodies, antigens, proteins, nucleic acids, lectins, cell membrane receptors and hormone receptors (Schoning and Poghossian 2002). The interaction of the analyte with the macromolecule can be divided into two categories. These include antibody- antigen interaction and receptor/antagonist/agonist.

In the case of the biocatalytic recognition element, the principle of biosensor is based on the catalysis of chemical reaction by macromolecules. There are three types of biocatalysts that are commonly used, namely, enzymes, whole cells and tissues. Enzyme based biosensors are the

most developed and widely used sensors (Thevenot *et al.* 1999, Thevenot *et al.* 2001). This is because enzymes show very attractive properties. These include a variety of measurable reaction products arising from the catalytic process, which include protons, electrons, light, and heat (Chambers *et al.* 2008). This project focuses on biocatalytic recognition element using enzyme as the biocatalyst.

2.1.2.1 *Enzymes*

Enzymes are macromolecules composed of protein that function as biological catalysts. They speed up specific reactions that are important to the organisms without undergoing any net chemical change during the reaction. The specificity of enzymes is an important property that makes them excellent diagnostic and research tools. Enzymes differ in biological functions and therefore they exhibit different specificity. For instance, hydrolytic and detoxifying enzymes have broad specificities while enzymes associated with complex functions (like synthesis of new enzymes) are more specific (Plowman 1972). There are four ways in which enzymes exhibit specificity. These include (Hui *et al.* 2006):

(i) Absolute specificity where the enzyme will catalyze only one type of reaction.

(ii) Group specificity- the enzyme acts only on molecules that have specific functional groups such as amino, phosphate and methyl groups.

(iii) Linkage specificity- the enzyme acts on a particular type of a chemical bond regardless of the rest of the molecular structure.

(iv) Stereochemical specificity where an enzyme will only act on a particular steric or optical isomer.

Enzymes are named and classified according to the kind of chemical reaction they catalyze (Aehle 2007; Webb 1993; Chaplin 2004b). Table 2 shows the six classes (groups), type of reaction catalyzed and examples of each group.

Table 2. Classification of enzymes (Chaplin 2004b)

Group	**Type of reaction catalyzed**	**Examples**
Oxidoreductases	Catalyze oxidation and reduction reactions involving the transfer of hydrogen atoms or electrons. They include	Dehydrogenases; Oxidases, Peroxidases, Oxygenases
Transferases	Involve the transfer of functional groups	Transaminases, transketolases, transaldolases and transmethylases
Hydrolases	Hydrolysis reactions (cleavage and introduction of water)	Esterases, carbohydrates nucleases, deaminases, amidase, and proteases glycosidases, phosphatases and lipases,
Lyases	Catalyze the elimination reaction in which a group of atoms is removed from the substrate to form a double bond.	Aldokases, decarboxylases, dehydrates and some pectinases
Isomerases	Involve the isomerization (intramolecular rearrangements)	Epimerases, racemases and intramolecular transferases
Ligases	Catalyses the reaction involving the joining of two molecules through hydrolysis of pyrophosphate bond in ATP or other triphosphates	Glutathione synthase

Enzymes were the first biological components to be incorporated in biosensors (Clark and Lyons 1962). They remain the most widely used biological component and the best understood in biosensor technology. For instance, biosensors based on the enzyme horseradish peroxidase have been extensively and successfully fabricated for the detection of hydrogen peroxide (Yin *et al.* 2009; Liu and Ju 2002; Wang and Wang 2004; Ansari *et al.* 2009) and its inhibitors (Songa *et al.* 2009; Hani *et al.* 2001). Other enzymes that have been widely studied and used in biosensors include glucose oxidase (Liu and Ju 2003; Liu and Li 2006), tyrosinase (Dempsey *et al.* 2004; Shan *et al.* 2008), urease (Vostiar *et al.* 2002; Rajesh *et al*. 2005) and acetylcholinerase

(Stoytcheva 2002). Enzymes act as biocatalysts thus enabling the detection of analytes in two ways (activation and inhibition) (Wanekay *et al*. 2008).

Enzyme Kinetics: According to the following reaction (equation 2.1.1), the presence of the enzymes ensures the transformation of the substrate into the reaction product:

$$\mathbf{E+S} \underset{\mathbf{k_{-1}}}{\overset{\mathbf{k_1}}{\rightleftharpoons}} \mathbf{ES} \xrightarrow{\mathbf{k_2}} \mathbf{E+P} \qquad (2.1.1)$$

where E represents the enzyme, S is the substrate, P is the reaction product and k_1, k_{-1} and k_2 are the rate constants of the reaction (Gunzler and Williams 2001). The Michealis-Menten mechanism proposed that the enzyme reacts reversibly with the substrate to form an enzyme-substrate complex (ES). The complex subsequently decomposes to release free enzyme and convert the substrate to a reaction product (equation 2.1.1). If the steady state hypothesis is applied to the ES complex, equation (2.1.2) can be obtained. This equation can be used to estimate Michealis-Menten constant (Gunzler and Williams 2001).

$$V = \frac{V_{\max}[S]}{K_M + [S]} \qquad (2.1.2)$$

where V is the rate of conversion, $V_{\max}$ is the maximum rate of conversion, [S] is the substrate concentration, and K_M is the Michaelis-Menten constant. According to equation (2.1.2), plotting V against [S] results in a hyperbolic graph (Figure 2.2). This shows that the rate of reaction is proportional to [S] at low substrate concentrations, but eventually reaches the plateau level ($V_{\max}$) (Gunzler and Williams 2001).

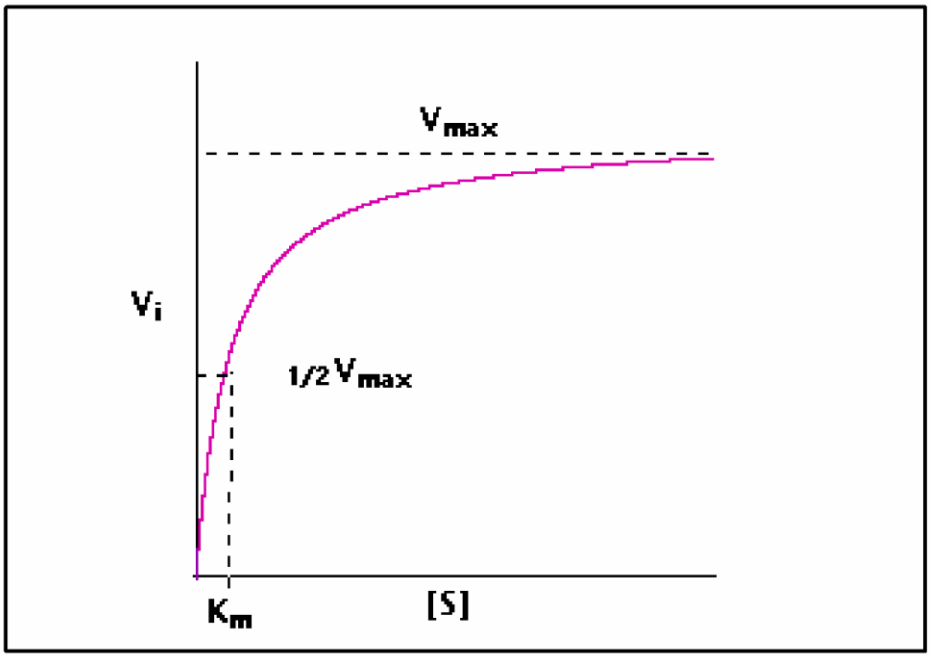


Figure 2.2. Enzyme velocity against substrate concentration (Kimball 2003)

When one is dealing with experimental data, it is more convenient to take the inverse of Michaelis-Menten equation and this yield to linear expression in 1/V against 1/[S]. The plot of 1/V versus 1/[S] is commonly known as Lineweaver-Bulk plot (equation 2.1.3) (Gunzler and Williams 2001).

$$\frac{1}{V} = \frac{1}{V_{\max}} + \left(\frac{K_M}{V_{\max}\,[S]} \right) \qquad (2.1.3)$$

Figure 2.3 shows a typical Lineweaver-Burk plot. The slope and the y-intercept of the plot are equal to $\frac{K'_M}{V_{\max}}$ and $\frac{1}{V_{\max}}$, respectively and the values of K_M and $V_{\max}$ can be calculated from the slope and the y-intercept.

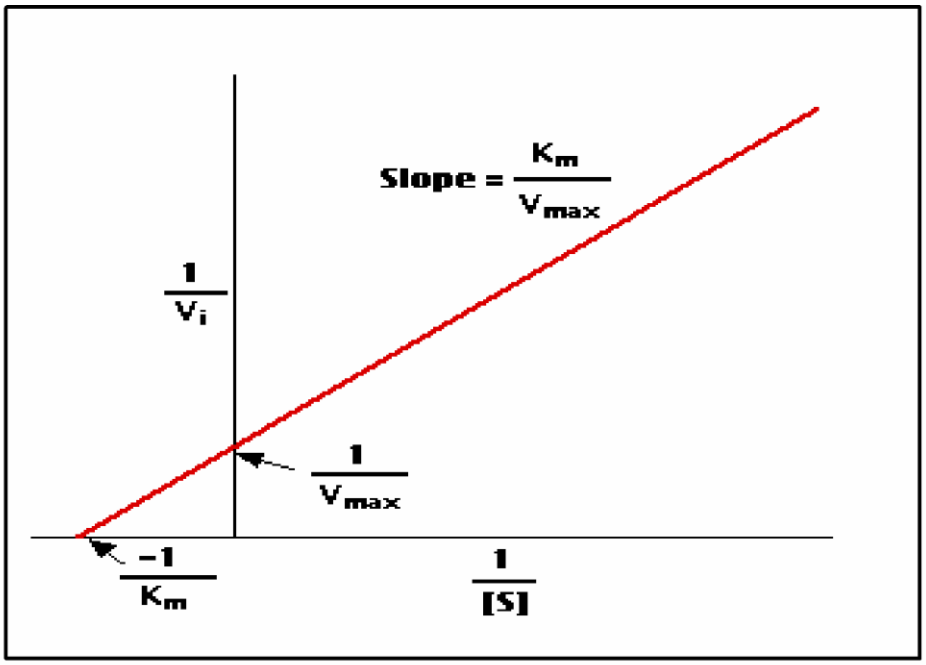


Figure 2.3. Typical Lineweaver-Burk plot (Kimball 2003).

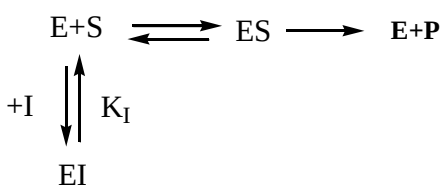
Michealis-Menten is known to be inversely proportional to the affinity of the enzyme for the substrate (Wang *et al.* 2009); hence some information about the enzyme can be deduced from the size of K_M obtained during the analysis. For instance, a small K_M shows that the enzyme requires small amount of substrate to become saturated. Thus, V_{max} is reached at relatively low substrate concentrations. Large K_M on the other hand shows that the enzyme needs high substrate concentrations to attain V_{max} (Plowman 1972).

Enzyme Inhibition: This refers to a phenomenon whereby the active sites of the enzyme are occupied by or react with molecules other than the substrate that leads to enzyme activity inhibition (Gunzler and Williams 2001). Enzyme inhibitors are substances that bind to the active sites of the enzymes and thus slows down the enzyme activity. The binding of the inhibitor to the immobilized enzymes results in a decrease of the biosensor signal. The type of inhibition mechanism varies from one inhibitor to another. Enzymatic inhibition can either be reversible or irreversible (Amine *et al.* 2006; Plowman 1972).

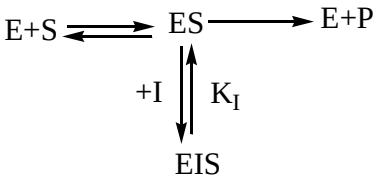
Reversible Inhibition: This type of inhibition is divided into three classes; these include competitive, non-competitive and uncompetitive inhibition. Kinetic mechanism of each class is

presented in Scheme 2.1. In competitive inhibition, the inhibitor competes with the natural substrate for the active binding sites of the enzyme. An example of competitive inhibition is the inhibition of polyphenol oxidase by benzoic acid reported by Shan *et al.* (2007). In addition, Pohanka *et al.* (2007) reported that Reactivator HI-6 is a competitive inhibitor of cholinesterase.

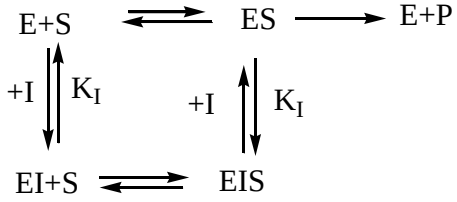
Uncompetitive inhibition occurs when the inhibitor binds only to the enzyme-substrate complex (not to free enzyme) (see Scheme 2.1). Tavakoli *et al.* (2005) reported that paraoxon and ethylparathion are uncompetitive inhibitors of choline oxidase. Non-competitive inhibition on the other hand, occurs when the inhibitor binds to both the free enzyme and enzyme-substrate complex (see Scheme 2.1). The inhibitor decreases the activity of the enzyme but it does not affect the binding of the substrate **(**Amine *et al.* 2006**).** Literature reports quite a number of inhibitive based biosensor that shows noncompetitive inhibition (Ilangovan *et al.* 2006; Malitesta, and Guascito 2005; Wang *et al.* 2009; Han *et al.* 2001; Tsai *et al.* 2003; Berezhetskyy *et al.* 2008; Ghica and Brett 2008; Mohammadi *et al.* 2005; Stoytcheva 2001; Songa *et al.* 2009).



Competitive inhibition



Uncompetitive inhibition



Non-competitive inhibition

Scheme 2.1. Kinetic mechanisms for reversible enzyme inhibition: where E is the enzyme, S is the substrate, P is the product ES is the enzyme-substrate complex, K_I is the inhibition constant, I is the inhibitor, EI is the enzyme-inhibitor complex and EIS is the enzyme-inhibitor-substrate complex (Amine *et al.* 2006).

Kinetic study and mechanism of the inhibitory: Calibration curves to the substrate for the enzyme electrode recorded in the substrate concentration in the absence and presence of the inhibitor helps in identifying the inhibition mechanism (type of reversible inhibition) (Figure 2.4). For competitive inhibition, higher substrate concentrations are needed in order to achieve the same enzyme responses that were reached in the absence of the inhibitor. In this case, V_{max} in the absence of the inhibitor will be equal to V_{max} in its presence and K_M values will be different (Figure 2.4B). For noncompetitive inhibition, the K_M values remain unchanged and V_{max} values will be different (Figure 2.4) (Kimball 2003).

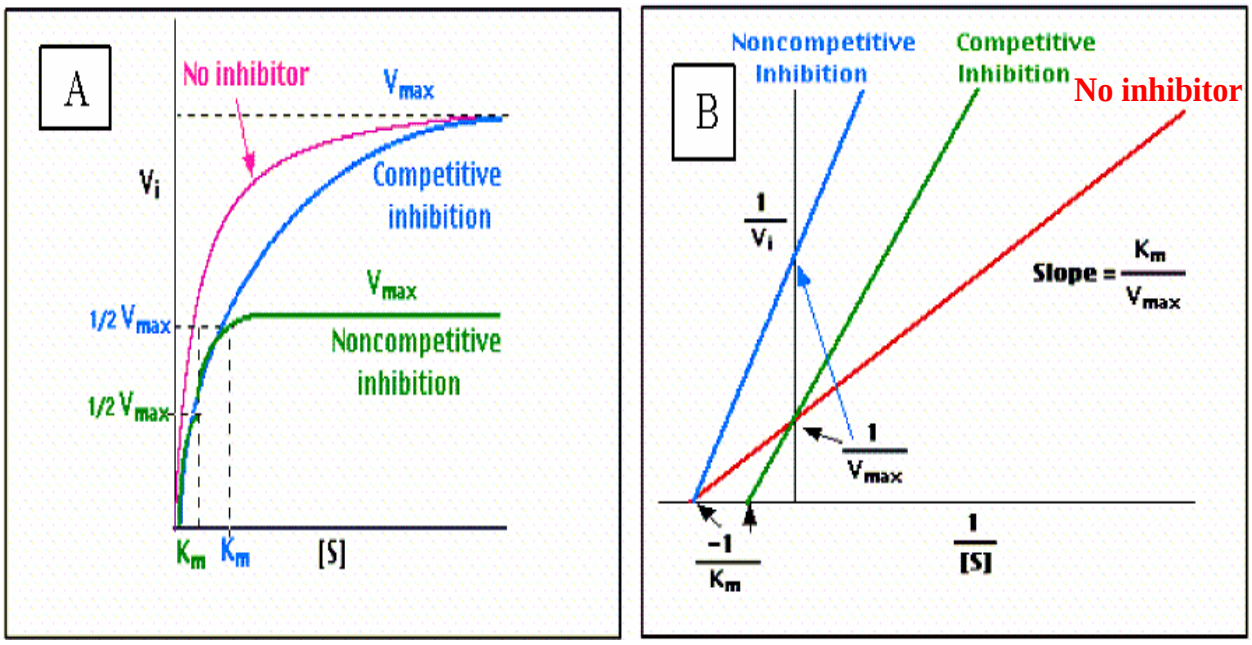
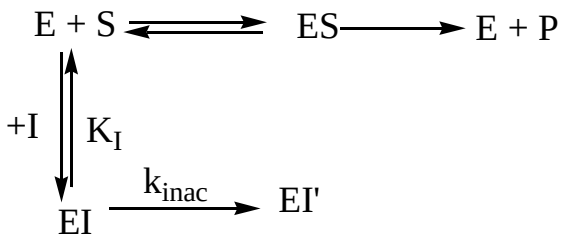


Figure 2.4. (A) Enzyme velocity against substrate concentration for noncompetitive and competitive inhibition. (B) Lineweaver-Burk plots for noncompetitive and competitive inhibition (Kimball 2003).

Irreversible inhibition: This phenomenon arises when the inhibitor reacts covalently with enzyme active sites. Irreversible inhibitors generally lead to the destruction of enzyme activity. Scheme 2.2 presents a kinetic mechanism for irreversible enzyme inhibition (Amine *et al.* 2006). An example of this type of enzyme inhibition was reported by Han *et al.* (2001) whereby HRP

immobilized on MB–β-CDP modified GCE was incubated with different concentrations of $HgCl_2$ for various times (from 1 to 30 min). At about eight minutes the irreversible inactivation of HRP enzyme was observed.



Scheme 2.2. Kinetic mechanisms for irreversible enzyme inhibition where k_{inac} is an inactivation constant (Amine *et al.* 2006).

2.1.3 Transduction Element

The next section discusses the second classification of biosensors, transduction element (transducer). Biosensors are classified according to four modes of detection (transduction element). The four transduction elements include electrochemical (measures the change in electric distribution), piezoelectric (measures the change in mass), optical (measures the change in light intensity) and thermal (measures change in heat) biosensors. Among other transducers, electrochemical transduction is the most widely used method, usually employing potentiometric, amperometric and conductometric techniques (Chaplin 2004a).

2.1.3.1 *Electrochemical biosensors*

Electrochemical biosensors are types of biosensors that use electrochemical methods for transduction. There are three widely used electrochemical biosensors these include potentiometric, amperometric and conductometric biosensors. However, between the three types of electrochemical biosensors, amperometric biosensors are more attractive because of their sensitivity and wide linear range (Freire *et al.* 2003).

Potentiometric biosensors: The principle of potentiometric biosensor is based on the measurement of the potential at a working electrode with respect to the reference electrode. Potentiometric biosensors are composed of two electrodes, working (indicator) electrode and

reference electrode. The electrodes function under equilibrium conditions and monitor the accumulation of charge (at zero current) created by selective binding at the electrode surface (Chaubey and Malhotra 2002). Potentiometric biosensors use ion-selective electrodes (ISEs) and ion-selective field effect transistors (ISFETs) in order to convert the biological reaction into an electrical signal. ISE or ISFET is modified by immobilizing an enzyme and the electrode becomes selective to specific enzyme substrates. Recently potentiometric biosensors based on ISFETs have been developed and the resulting biosensor is known as enzyme linked field effect transistors (ENFETs) (Chaubey and Malhotra 2002; Gerard *et al.* 2002; Eggins 2002).

Conductometric biosensors: The principle of conductometric biosensors is based on the change of conductivity of the electrolyte solution occurring between a pair of electrodes as a result of the presence of a biological component (Gerard *et al.* 2002; Mello and Kubota 2002; Chaubey and Malhotra 2002; Eggins 2002)

Amperometric biosensors: The principle of an amperometric biosensor is based on the measurement of the current at constant applied potential that is generated by oxidation or reduction of the redox species at the surface of the electrode. The measured current is proportional to the concentration of the analyte. An amperometric biosensor employs three electrodes, working, reference and counter (auxiliary) electrodes (Dzyadevych *et al.* 2008; Freire *et al.* 2003). Amperometric biosensors can be classified into three generations, first second and third generation (Freire *et al.* 2003, Eggins 2002) (Figure 2.5).

The first generation amperometric biosensor is based on the measurement of the concentration of natural substrates and products of enzyme reaction without the need of a mediator. The electronic coupling between redox enzymes and electrodes for the construction of amperometric biosensors can be based on the electroactivity of the enzyme substrate or product (Figure 2.5A). Most first generation biosensor functions are due to either absorption of oxygen during biocatalytic reaction (at -0.7 V) or biocatalytic generation of hydrogen peroxide (at +0.65 V). The main drawback of this type of biosensor is its use of high applied potentials. This problem can be eliminated by the use of electron mediators (e.g. ferrocene derivates, ferrocyanide, conducting organic salts and quinines) and this gives rise to the second generation amperometric biosensor (Rodriguez-Mozaz *et al.* 2006; Rodriguez-Mozaz *et al.* 2004; Freire *et al.* 2003).

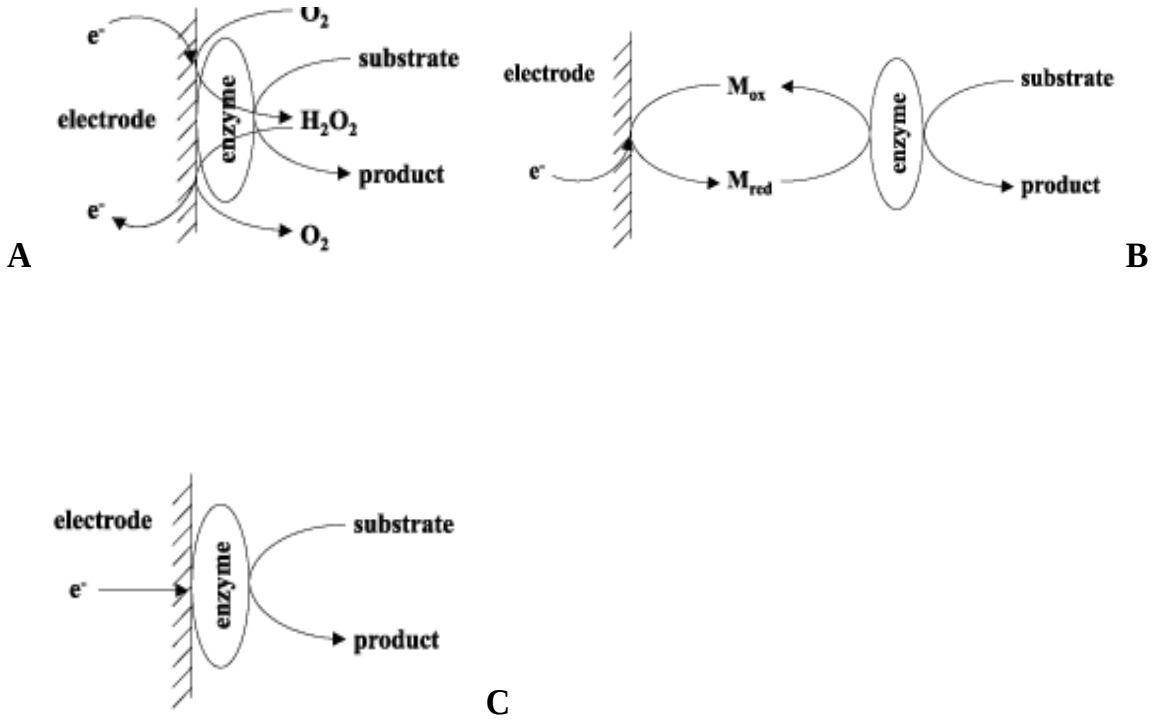


Figure 2.5. The schematic representation of the three generations of amperometric biosensor. A=1st Generation; B = 2nd Generation; C = 3rd Generation (Freire *et al.* 2003).

Second generation amperometric biosensor uses mediators as electron carriers from the active enzyme centre to the electrode (Figure 2.5B**).** When using this kind of amperometric biosensor, the experiments can be carried out at low potential so that the response of the analyte is not affected by the influence of oxygen (in case oxidase has been used) and any other interference. When constructing a second generation biosensor, it is important that the mediator being used has the following characteristics:

(i) The reduced form of the mediator should not react with oxygen;

(ii) The process of electron transfer between the mediator and enzyme should be fast;

(iii) Mediator should not be influenced by pH;

(iv) Mediator should be non-toxic and

(v) The reduced and oxidized form should be stable.

The advantages of using mediators are as follows:

(i) The measurements are less dependent on oxygen concentration;

(ii) The working potential of the enzyme electrode is determined by the oxidation potential of the mediator and

(ii) Mediator uses low oxidation potentials.

This eliminates the interference of unwanted species and lastly if the oxidation of the reduced mediator does not involve protons, it can make the enzyme electrode relatively pH insensitive (Chaubey and Malhotra 2002; Eggins 2002; Dzyadevych *et al.* 2008**).** However, the main limitation of the second generation biosensor is that the redox mediators combined with redox enzymes facilitate not only the electron transfer between electrode and enzyme but also with a variety of interfering reactions (Freire *et al.* 2003). This leads to development of the third generation.

Third generation amperometric biosensors are based on direct electron transfer between enzyme and electrode surface. The electron transfer occurs during the catalytic reaction i.e. when the substrate is converted to products (Figure 2.5C**).** In this case, the enzyme acts as an electrocatalyst because it facilitates the electron transfer between the electrode and the substrate molecule. In this process, there is no necessity of a mediator. The contact between the enzyme and the electrode surface can be facilitated by the redox active polymer (such as polyaniline). The electrode surface is first modified with the polymer film, followed by immobilization of the biological material. Third generation amperometric biosensors offer high sensitivity because they are able to operate at a potential range closer to the redox potential of the enzyme itself and they become less exposed to interfering reactions. The combination of an enzyme and the electrode surface improves the sensitivity of this type of biosensor (Dzyadevych *et al.* 2008; Freire *et al.* 2003).

2.1.4 Immobilization Materials and Methods

The most important step in the construction of the biosensors is the immobilization of the biological component on the electrode surface. Immobilization of enzymes involves retaining the enzymes in its active form on the electrode surface or modified electrode surface. Immobilization obtains both the stabilization of the biological element and the closeness between the biological element and the transducer. Various methods can be used for immobilizing the enzyme onto the electrode surface, and this depends on the nature of the electrode and properties and stability of an enzyme (Freire *et al.* 2003). The methods of immobilization are divided into two types:

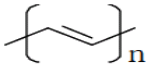
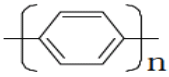
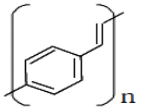
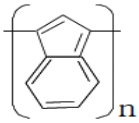
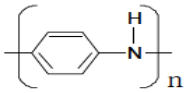
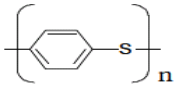
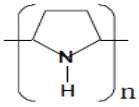
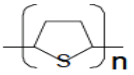
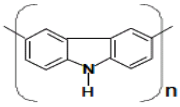
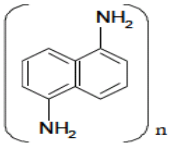
chemical and physical methods. The chemical immobilization method is classified into two categories: covalent bonding onto an electrode surface and cross-linking between the molecules. Physical immobilization methods include adsorption at a solid surface and entrapment within a membrane **(**Eggins 1996; Eggins 2002; Thevenot *et al.* 2001; Marcoy and Barcelo 1996).

A number of materials have been used for the modification of electrodes and to improve the stability of biological element used in the fabrication of the desired biosensors or chemical sensor (Malhotra, Chaubey and Singh 2006). These include membranes, gels, carbon, graphite, silica and conducting polymers (Gerard *et al.* 2002; Malhotra, Chaubey and Singh 2006). In this work, conducting polymers are the materials of choice for enzyme immobilization.

2.1.4.1. *Conducting polymers*

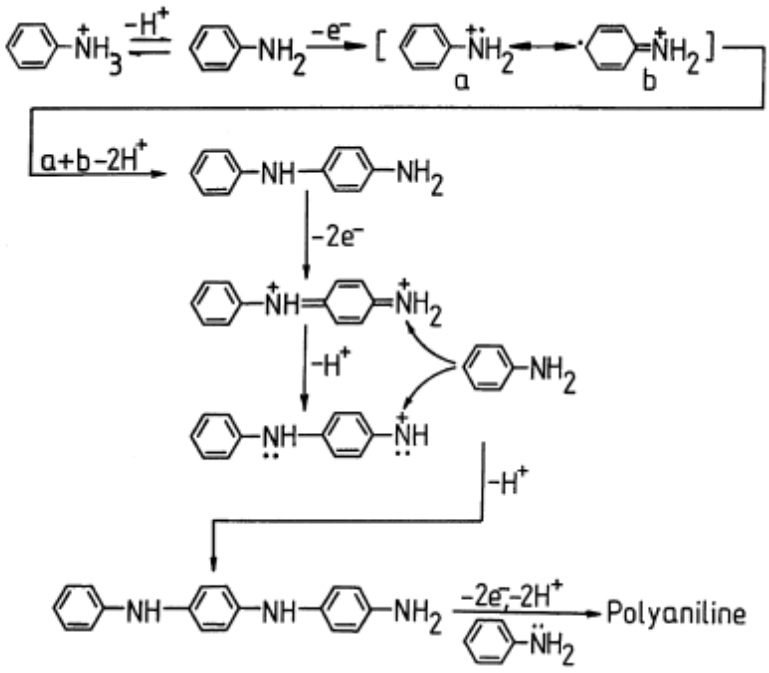
Conducting polymers are mainly organic compounds that have a π-electron backbone which is responsible for the movement of electrons from one end of the polymer to the other (Rahman *et al.* 2008). The backbone of the conducting polymer has single and double bonds alternating along the polymer chain (Gerard *et al.* 2002). In uncharged or neutral state, the conducting polymers are almost insulators. The formation of charge carriers upon oxidation (*p*-doping) or reduction (*n*-doping) of the conjugated backbone of conducting polymers results in their intrinsic conductivity (Lange *et al.* 2008). Conducting polymers can be synthesized chemically and electrochemically. However, the most preferred method is electrochemical polymerization. This is because reactions can be carried out at room temperature and the thickness of the polymer film can be controlled by varying either current or potential (Gerard *et al.* 2002). Common conducting polymers and their chemical structures are presented in Table 3 (Gerard *et al.* 2002; Rahman *et al.* 2008). Among the conducting polymers, polyaniline is the most widely studied and in this study it is the polymer of choice.

Table 3. Names and structures of some common conducting polymers **(**Gerard *et al.* 2002)

Conducting Polymer	Structure
Polyacetylene	
Polyparaphenylene	
Polyparaphenylene vinylene	
Polyazulene	
Polyaniline	
Polyparaphenylene sulfide	
Polypyrrole	
Polythiophene	
Polycarbazole	
Polydiaminonaphthalene	

Polyaniline is a conducting polymer that has been widely studied for electronic and optical applications. It can be obtained either by chemical or electrochemical oxidation of aniline in acidic and non-acidic media (Karykin *et al.* 1996). Electrochemical method is a better method compared to chemical because it provides a fine control of the initiation and termination steps. Also, its reaction is cleaner compared to the chemical method and the PANI is relatively in a purer form. Scheme 2.3 indicates the mechanism for electrochemical polymerization of aniline to

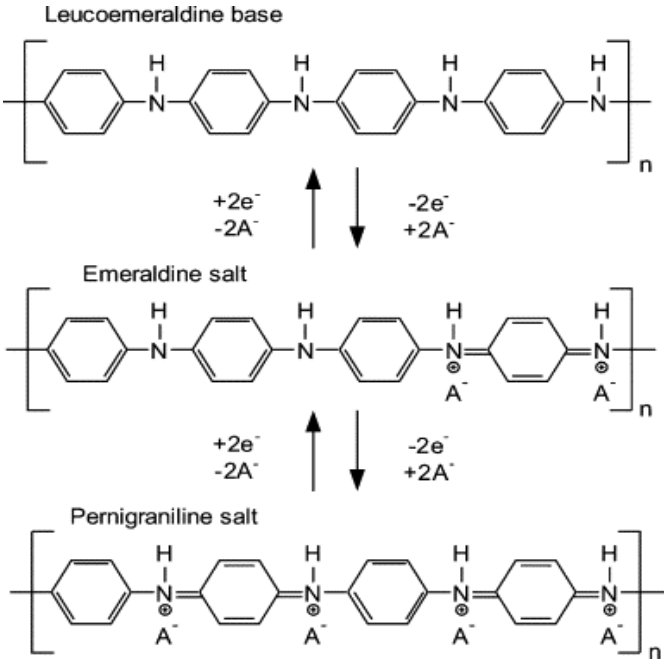
polyaniline as proposed by Wei *et al*. (1990). The formation of PANI proceeds stepwise. The first step is oxidation of aniline to form a radical cation. The formation of the radical cation is due to electron transfer from the 2s energy level of the aniline nitrogen atom. There are two resonance forms of aniline radical (Scheme 2.3). The aniline radical denoted by *b* is known to be very reactive. This is because of its important substituent inductive effect and the absence of steric hindrance. The second step involves the reaction between and the radical cation and the resonance form *b* to form a dimer. The reaction takes place in an acidic medium. This kind of reaction is known as 'head to tail" reaction. In the third step, the dimer is oxidized to form a new radical cation which then reacts with either the radical cation monomer or the radical cation dimer to form a trimer or tetramer, according to the mechanism proposed in step two and this adds up to the polymer (Nicolas-Debarnot and Poncin-Epaillard 2003).



Scheme 2.3. Mechanism for electrochemical polymerization of aniline to polyaniline (Nicolas-Debarnot and Poncin-Epaillard 2003)

Polyaniline can exist as three different states, namely fully reduced leucoemaraldine, half-oxidized emeraldine and fully oxidized pernigraniline **(**Wallace *et al.* 2003; Mazeikiene *et al.* 2007; Gospodinova and Terlemezyan 1998) as per Scheme 2.4. The fully reduced and fully oxidized states of PANI are insulators (Bhadra *et al.* 2009; Bhada *et al.* 2006) and half oxidized

emeraldine state is the state with highest conductivity. The conductivity of this form is commonly known to be due to its polaronic structure (Mazeikiene *et al.* 2007 Bhadra *et al.* 2009).



Scheme 2.4. Different forms of polyaniline (Mazeikiene *et al.* 2007 Bhadra *et al.* 2009).

Electrochemical characterization of PANI: It is normally performed in acidic media using cyclic voltammetry (CV). This is done in order to determine the changes in oxidation states of the polymer (Gospodinova and Terlemezyan 1998). The cyclic voltammograms of PANI film reveals two redox processes that correspond to the transitions from leucoemeraldine to emeraldine (0.20 V) and from emeraldine to pernigraniline (0.60 V) (Gospodinova and Terlemezyan 1998). Electrochemical characterization of PANI helps to determine the surface concentration and diffusion coefficient. These can be obtained by sweeping the modified electrode at different scan rates. The peak current increases linearly with increase in scan rate up to a certain level for example 5 to 100 mVs^{-1} (Kumar and Chen 2007). At higher scan rates the plot of peak currents against scan rate deviates from linearity and the peak current becomes proportional to the square root of the scan rate; this behavior is an indication of a diffusion controlled process (Kumar and Chen 2007).

Spectroscopic characterization of PANI: Fourier transform infrared (FTIR) and UV-Vis (ultraviolet and visible) spectroscopy have been used to provide qualitative indication of the intrinsic redox states of polyaniline.

UV-Vis spectroscopy is the spectroscopic technique that is based on the observation of the excitation of electrons. In this technique the excitation of electrons occurs when the frequency of the incident electromagnetic radiation matches the difference in energy between two electronic states. The excitation of the electron or vibrational and rotational transitions leads to generally broad spectrum. UV-Vis spectroscopy is normally used as a quick method for identification of certain classes of the analyte such as substituted aromatic rings (Gunzler and Williams 2001). In this work, the UV-Vis spectroscopy was used for quick identification of PANI intrinsic redox states.

UV-Vis spectrum of PANI differs from one intrinsic redox state to the other. For instance, leucoemaraldine spectrum shows the lowest absorption band around 345 nm and very weak absorption band around 635 nm. Kang *et al.* (1998) assigned the absorption bands to the π-π* transition. UV-Vis spectrum for emeraldine on the other hand shows two absorption bands around 330 nm and 630 nm. The absorption band at 330 nm is assigned to a charge-transfer excitation-like transition from the highest occupied energy level to the lowest unoccupied energy level. The absorption peak at 630 nm on the other hand, is attributed to the exciton absorption of the quinoid rings (Cho *et al.* 2004). Pernigraniline state spectrum exhibits absorption bands around 320 and 530 nm (McCall *et al.* 1990; Kang *et al.* 1998). The absorption band at 530 nm is assigned to donor–acceptor interaction between the quinoid fragments of PANI and the counter anions (Nekrasov *et al.* 2001).

Fourier transform infrared spectrum provides little information about molecular structure; however, it provides more information about functional groups present in an analyte of interest (Gunzler and Williams 2001). To get more information about the analyte, infrared (IR) spectroscopy can be used together with UV-Vis spectroscopy can be used. IR spectroscopy is based on the absorption of different IR frequencies by a sample positioned in the path of an IR beam. Different analytes have characteristic absorption peaks. IR spectroscopy was used for the characterization of PANI film.

Studies suggests that IR spectrum of PANI shows two characteristic absorption modes at about 1500 cm^{-1} and 1600 cm^{-1}, these peaks are attributed to C=C aromatic ring stretching. The

absorption peak at 1600 cm^{-1} is assigned to quinonoid ring and 1500 cm^{-1} peak to the benzenoid ring (Paul and Pillai 2000). The intrinsic redox states of PANI show a slightly different IR spectrum. The IR absorption band for leucoemaraldine (LE) is known to exhibit a low intensity ratio of 1600 cm^{-1}:1500 cm^{-1} peaks. This is because LE state is dominated by benzenoid units. The IR spectrum of emeraldine state (EM) on the other hand shows the almost equal amounts of the two units (benzenoid and quinonoid) and the intensities for 1600 cm^{-1} and 1500 cm^{-1} are almost equal. The IR spectrum of pernigraniline exhibits an enhanced quinonoid to benzenoid band intensity **(**Kang *et al*. 1998; Paul and Pillai 2000, Sun *et al.* 1990). A summary of IR absorption bands for polyaniline is presented in Table 4 (Kang *et al.* 1998).

Table 4. Assignment for IR absorption bands from polyaniline (Kang *et al.* 1998)

Frequency (cm^{-1})	**Assignment**
3460	NH_2 asymmetric stretching
3380	NH_2 asymmetric stretching, NH stretching
3310	H-bonded NH stretching
3170	=NH stretching
2930	Impurity or sum frequency
2850	Impurity or sum frequency
1587	Stretching of N=Q=N
1510	Stretching of N=B=N
1450	Stretching of benzene ring
1380	C-N stretching in QB_tQ
1315	C-N stretching in QB_cQ, QBB, BBQ
1240	C-N stretching in BBB
1160	A mode of N=Q=N
1140	A mode of $Q=N^+H$-B or B-NH-B
1220	C-H in-plane bending of 1,4-ring
1105	
1010	
1115	C-H in-plane bending of 1,2,4-ring
1060	
960	
910	C-H out-of-plane bending of 1,2,4-ring
895	
850	
830	C-H out-of-plane bending of 1, 4-ring
740	C-H out-of-plane bending of 1,2- ring
690	
645	Aromatic ring deformation
530	
500	

Abbreviations: B, Benzenoid unit; Q, quinonoid unit; t, trans; c, cis

2.1.4.*2 Physical adsorption*

Physical adsorption of the biological component is the oldest and simplest immobilization method. The procedure involves the deposition of the enzyme onto the electrode material and the immobilization is based on van der Waals interactions. Physical adsorption method does not need functionalization of the electrode material neither the chemical modification of the enzyme. It is non-destructive to the enzyme activity (Mello and Kubota 2002; Andreescu and Marty 2006). The resulting biosensors suffer from a poor stability (operation and storage) due to the loss of adsorbed biological components especially when there are changes in pH, ionic strength and temperature (Andreescu and Marty 2006; Mello and Kubota 2002).

2.1.4.3 *Physical entrapment*

The physical entrapment of the enzyme involves the mixing of the biological element with the monomer solution which is then polymerized to a film, by doing this the enzyme is being trapped. The advantage of using physical entrapment is that the thickness of the polymer/enzyme layer can be controlled. The method is simple and rapid but it requires higher amounts of the enzyme (Cosnier 1999; Andreescu and Marty 2006; Eggins 1996; Eggins 2002; Marcoy and Barcelo 1996).

2.1.4.4 *Covalent immobilization*

These are mainly immobilization method is obtained by the reaction between a nucleophilic functional group (not essential for catalytic activity of an enzyme) in the biological component and the support matrix. These nucleophilic functional groups include NH_2, COOH, OH, and SH. The procedure involves modification of the transducer with a bi-functional cross-linker such as glutaraldehyde, carbodiimide/succinimide or aminopropyltriethoxysilanes. The cross-linker reacts with amino, carboxyl or hydroxyl groups and on the other end with the enzyme. In this way, the nucleophilic functional groups are directly bonded to a membrane or onto the surface of the transducer. The advantage of using this method includes high operation and storage stability. However, a high concentration of the enzyme is required. When the immobilization method is done properly it can have higher lifetime compared to others (physical adsorption and

entrapment), typically 4 to14 months **(**Eggins 1996; Eggins 2002; Mello and Kubota 2002; Andreescu and Marty 2006).

2.1.4.5 *Cross-linking*

Cross-linking is the immobilization method whereby the biomaterial (enzyme) is chemically bonded to solid supports or to another supporting material such as gel. Bifunctional agents such as glutaraldehydrate are normally employed to bind the biomaterial. This method is useful for the stability of the adsorbed enzymes. However this method has some limitations in that it damages the enzyme, limits the diffusion of the substrate and it has a poor mechanical strength (Eggins 1996; Eggins 2002).

2.1.5 Biosensor Applications

Biosensor and immunosensors for determination of POPs has been reported (Liu *et al.* 2010, Gavlasova *et al.* 2008; Premkumar *et al.* 2002; Marrazza *et al.* 1999, Mascini 2001; Pribyl *et al.* 2006). Due to the large range of biological activity and high persistence of POPs, different biosensors have been developed to determine POPs (e.g. PCBs and PBDEs) in the environment (Gavlasova *et al.* 2008; Liu *et al.* 2010; Shelver *et al.* 2008**).** These include whole cell biosensor based on optical detection (Liu *et al.* 2010, Gavlasova *et al.* 2008; Premkumar *et al.* 2002) and DNA biosensor based on electrochemical detection (Marrazza *et al.* 1999, Mascini 2001). Other methods for the determination of POPs such as piezoelectric immunosensors for PCBs and magnetic particle immunoassay for PBDEs have also been reported (Pribyl *et al.* 2006; Shelver *et al.* 2008).

Recent developments have shown electrochemical biosensors to offer ultra-trace detection capabilities and can be used as alternative methods for heavy metals based on inhibition enzyme activity (Ilangovan *et al.* 2006; Malitesta and Guascito 2005). Biosensors based on various enzymes have been reported in the detection of heavy metals in the environment. These include; urease biosensor for the detection of Cd^{2+}, Cu^{2+} Cr^{3+}, $Zn^{2+,}$ Ni^{2+} and Pb^{2+} (Ilangovan *et al.* 2006; Tsai *et al.* 2003); alkaline phosphatase for Cd^{2+}, Co^{2+}, Zn^{2+}, $Ni^{2+}$and Pb^{2+}(Berezhetskyy *et al.* 2008); glucose oxidase for $Cd^{2+,}$ Cu^{2+}, Zn^{2+} and Pb^{2+}(Ghica and Brett 2008); glucose oxidase combined with invertase and mutarose for mercury (Mohammadi *et al.* 2005); acetylcholinesterase for Cu^{2+}, Cd^{2+}, Mn^{2+} and Fe^{3+} (Stoytcheva 2002); and nitrate

reductase for Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} (Wang *et al.* 2009). Horseradish peroxidase (HRP) biosensor has so far been only reported for the detection of mercury (Han *et al.* 2001).

2.2 ELECTROANALYTICAL TECHNIQUES

2.2.1 Introduction

Electroanalytical techniques are based on the transformation of chemical information into an analytically useful signal (Rahman *et al.* 2008**)**. Electroanalytical techniques use two or three electrode electrochemical cell systems (working electrode (WE), auxiliary electrode (AE) and reference electrode (RE) (Figure 2.6). The use of the three electrodes along with the potentiostat allows accurate application of potential functions and the measurement of the resultant current. The WE is used to monitor the response of the analyte. They are constructed from a wide range of conduction materials; these include mercury, silver, gold, gold, platinum, graphite, carbon paste and glassy carbon. The AE is used to control the potential applied to the working electrode and to complete the circuit for carrying the current generated at the WE. The most commonly used AE are platinum wire or sometimes graphite. The reference electrode is an electrode whose potential is known and is constant. The potential of the RE electrode is taken as the reference against which the potentials the other electrodes are measured. The most commonly used RE are saturated calomel electrode (SCE) and silver/silver chloride (Ag/AgCl) electrode (Schollz 2002).

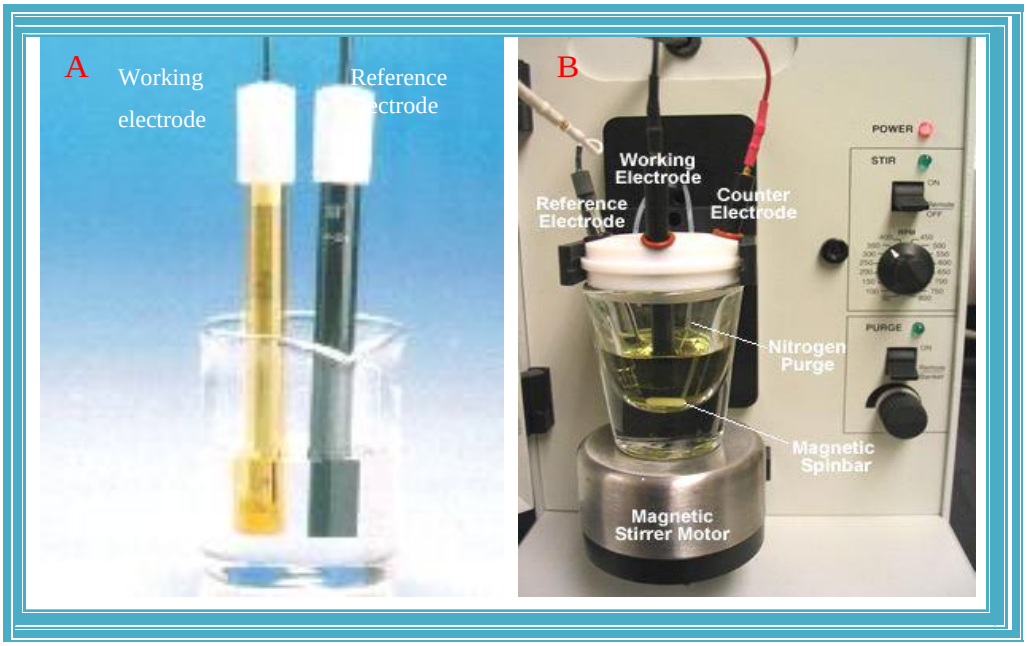


Figure 2.6. Photographs taken of the instruments used in this work: showing an electrochemical cell consisting of (A) two Metrohm electrodes; and (B) three electrodes of BASi voltammetric cell.

Electroanalytical techniques are capable of determining trace concentrations of an electroactive analyte such as environmental pollutants. They can also provide useful information regarding physical and chemical properties of an electroactive analyte. These methods include cyclic voltammetry, stripping voltammetry, differential pulse voltammetry, and amperometry (discussed in Section 2.1).

2.2.2 Cyclic Voltammetry

Cyclic voltammetry (CV) is a commonly used electrochemical technique. In this work, CV will be used for electrochemical synthesis and characterization of polyaniline and for investigation of the potential at which hydrogen peroxide is being reduced. It utilizes the three electrodes along with the potentiostat which allows accurate application of potential functions and the measurement of the resultant current. Cyclic voltammetry is widely used in the study of redox behavior of electrochemically active species, electrochemical properties of the analyte, and the kinetics of electrode reactions. Cyclic voltammetry offers the possibility of identifying reactive intermediates or subsequent products (multi-oxidation state electroactive species like

polyaniline) (Gunzler and Williams 2001). It also serves as method for teaching the concept of electrochemistry. Figure 2.7 is the typical cyclic voltammogram for a reversible redox process.

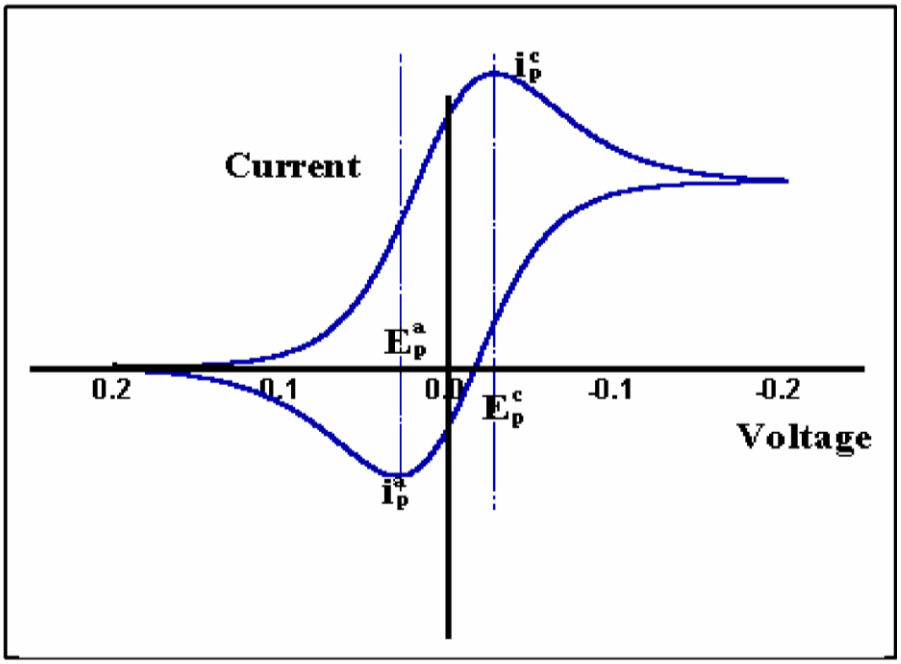


Figure 2.7. Typical cyclic voltamogram (www.cartage.org.lb/.../CyclicVoltammetry.htm accessed 27 January 2010).

The important parameters in a CV are peak currents (i_p^c and i_p^a) and peak potentials (E_p^c and E_p^a) of the cathodic and anodic peaks. For the reversible electrochemical reaction, the CV must have the following characteristics: the separation potential (ΔE_P) should be equal to 59 mV. The separation potential can be calculated from equation (2.2.1):

$$\Delta E_p = E_p^a - E_p^c = \frac{59}{n} mV \qquad (2.2.1)$$

The second characteristic is that the peak positions are not affected by the change of the scan rate. Thirdly, the ratio of peak currents is equal to 1 ($\frac{i_p^a}{i_p^c} = 1$). Lastly, the peak currents are

proportional to square root of the scan rate. In some cases electrochemical reactions show irreversible or quasi-reversible processes. For quasi-reversible process, ΔE_P becomes greater than 59 mV/n and although current intensity increases with the scan rate; peak current is not proportional to the square root of scan rate. For irreversibility on the other hand, the reduction product cannot be reoxidized, meaning that the anodic peak is not observed (Gunzler and Williams 2001).

2.2.3 Differential Pulse Voltammetry (DPV)

The principle of DPV is comparable to that of normal pulse voltammetry (NPV) in that potential is also scanned with a series of pulses. Unlike NPV, each potential pulse is fixed to small amplitudes (10 to 100 mV) and is superimposed on a slowly changing base potential. In each pulse the current is measured at two points: the first point is taken just before the application of the pulse while the second is taken at the end of the pulse just before it decreases back to baseline. The difference between the currents is amplitude of the pulse (ΔI_{pulse}), therefore the DPV results from a plot of ΔI_{pulse} versus potential (Figure 2.8) (Monk 2002). The DPV is more sensitive compared to CV. Therefore, DPV was used for investigation of the accurate potential at which hydrogen peroxide is being reduced and to get an accurate potential.

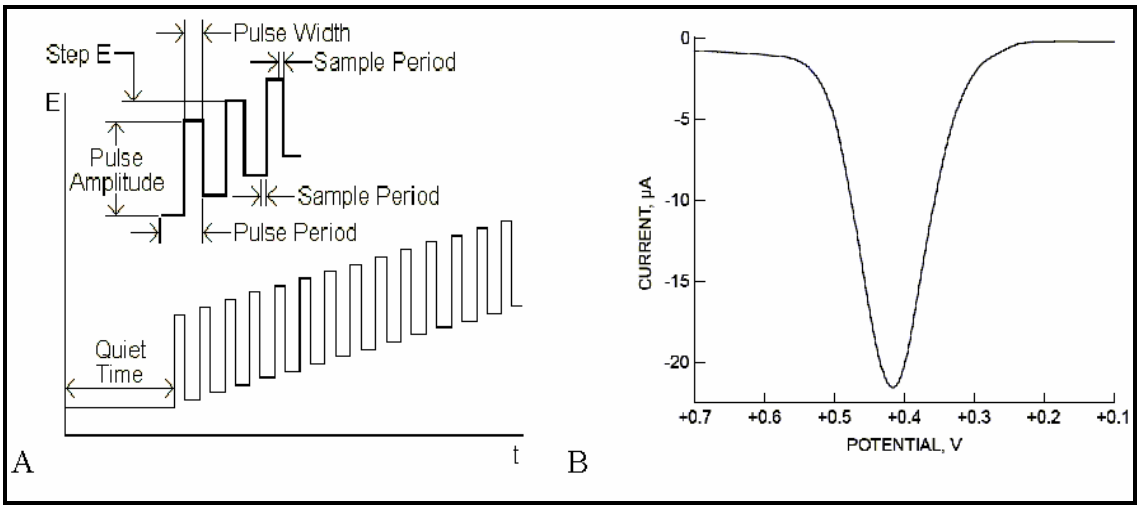


Figure 2.8. Typical potential wave form for differential pulse voltammogram (A) and differential pulse voltammogram (B) (Bionalytical Systems manual 2009).

2.2.4 Anodic stripping voltammetry

Anodic stripping voltammetry (ASV) involves two steps, in the first step a negative potential is applied and the metal ion is preconcentrated on the surface of the electrode. In the second step, positive potential is applied and the metal ion is stripped off the electrode. The electrochemical signal is normally observed during the second step. Due to the preconcentration of the analyte, ASV offers high sensitivity, selectivity, low detection limits and a wide linear range (Monk 2002; Gunzeler and Williams 2001). For this reason Environmental Protection Agency (EPA) has recommended ASV to be one of the standard techniques for heavy metal analysis (McGaw and Swain 2006; Berezhetskyy *et al.* 2008).

Wu *et al.* (2008) developed a novel sensor for simultaneous detection of Pb, Cd and Zn, based on the differential pulse anodic stripping response at a bismuth/poly(*p*-aminobenzene sulfonic acid) (Bi/poly(*p*-ABSA)) film electrode. Several methods based on the determination of heavy metals using ASV has been reported (Legeai and Vittori 2006; Xu *et al.* 2008; Injang *et al.* 2010; Kokkinos *et al.* 2008; Renedo and Martínez 2007).

2.3 CHROMATOGRAPHIC TECHNIQUES

Qualitative and quantitative analysis of POPs is generally achieved by employing gas or liquid chromatography coupled with different detectors.

2.3.1 Gas Chromatography

Gas chromatography (GC) is a separation technique that separates volatile organic compounds. A gas chromatograph consists of a carrier gas, an injection port, a separation column containing the stationary phase, column oven, a detector and a data recording system. The GC can be coupled with different detectors, namely electron capture detector (ECD), flame ionization detector (FID), mass spectrometry detector (MSD). The FID is the universal detector since it responds to almost every component in the mobile phase whereas ECD is a selective detector because it is selective to halogenated compounds (Gunzeler and Williams 2001). Mass spectrometry (MS) principle as a detector is based on ionizing chemical compounds to generate charged molecules or molecule fragments and detection of their mass-to-charge ratio. MS can be used for investigation of isotopic distribution of an element, the elemental or molecular

composition of a sample and the structure of a compound or its molecular mass (Gunzeler and Williams 2001).

The organic compounds are separated due to differences in their partitioning behaviour between the mobile gas phase and the stationary phase in the column (Gunzeler and Williams 2001**)**. Gas chromatography is known as a primary analytical technique for POPs such as brominated flame PCBs (Vonderheide 2009; Yu and Hu 2007). This is due to the volatility of their congeners **(**Vonderheide 2009; Riess and van Eldik 1998**)**.

Identification and quantification of PBDEs in environmental and human samples by means of GC has been extensively studied (Tadeo *et al.* 2009; Wang *et al.* 2006; Salgado-Petinal *et al.* 2006; Jakobsson *et al.* 2002; Dirtu *et al.* 2008; Vizcaino *et al.* 2009 and Brown *et al.* 2007). For instance, Tadeo *et al.* (2009) determined PBDEs in human hair by GC-MS. The hair was cut from sixteen individuals and five different congeners of PBDEs were detected. It was found that BDE-209 was the dominant compound because it was found in twelve out of sixteen hair samples. The other compounds that were detected were BDE-47, BDE-99, BDE-100, and BDE-190. The total amount of PBDEs detected in human hair samples ranges from 1.4 to 19.9 ng g^{-1}. Wang *et al.* (2006) used GC coupled with ECD for detection of PBDEs in real samples such as water (river water and wastewater) and milk (skimmed, semi-skimmed and whole fat milk) samples. In river water and skimmed milk samples no PBDEs were detected. They were, however, detected in wastewater, semi-skimmed milk and whole fat milk. The concentrations ranged from 134-215, 13-484 and 21 ng L^{-1} for wastewater, whole fat milk and semi-skimmed milk, respectively. Five different PBDEs congeners were detected. These included BDE-47, BDE-100, BDE-99, BDE-154 and BDE-154. Other GC methods have been reported by Salgado-Petinal *et al.* (2006), Jakobsson *et al.* (2002), Dirtu *et al.* (2008) Vizcaino *et al.* (2009) and Brown *et al.* (2007).

The production of PBBs was banned in the 1970s but recent studies show that in some parts of the environment they are still present. Gieron *et al.* (2010) carried out GC analysis of PBBs in fish from the Baltic and North Seas and selected food products from Poland. The results showed that the fish in both areas were contaminated with PBBs and the latter did not show PBBs in the selected food samples. Von der Recke and Vetter (2008) performed PBB analysis using gas chromatography in combination with electron capture negative ion mass spectrometry (GC/ECNI-MS) in marine biota samples. Their GC/ECNI-MS results showed that blubber of

seals, harbour porpoises and fish originating from the North Sea, the Baltic Sea, as well as coastal waters of Iceland and North America, were contaminated with residues of PBBs. They reported that hexabromobiphenyls (hexaBBs) were dominant (high concentration) in all the samples, followed by pentabromobiphenyls (pentaBBs) and heptabromobiphenyls (heptaBBs). On the other hand, octabromobiphenyls (octaBBs) were only detected in selected samples while nonabromobiphenyls (nonaBBs) and PBB 209 were not detected at all.

Similar to PBBs, the production of PCBs has been banned, however, trace amounts of these compounds has been detected in the environment. Determination of PCBs in environmental samples such as air, soil, sediment, leachate and water, as well as plant and animal tissues, has been carried out using GC-MS and GC-ECD (Numata *et al* 2006; Vane *et al.* 2007, Sporring *et al.* 2005; Cornelissen *et al.* 2009; Lopez *et al.* 2007; Kowalski *et al.* 2010). Popp *et al.* (2005) determined PCBs in environmental water samples. The water samples were spiked with PCB standards. The results obtained showed that water samples contained PCB 209, PCB 1, PCB 52 and PCB 77. The lower PCBs (PCB 1, PCB 52 and PCB 77) were found in larger amount compared to higher PCBs (PCB 209). The reason for this variation might be due to solubility. Shu *et al.* (2003) also carried out similar analysis by using GC-ECD.

2.3.2 Liquid Chromatography

GC is known as a standard method for detection of POPs, but there are some limitations especially when analyzing compounds with high molecular weight (higher PBDE, PBB and PCB congeners from the octa- to deca-mixtures) (Yu and Hu 2007). These compounds require high separation temperatures (> 350 °C) that can induce thermal reactions (Riess and van Eldik 1998). Therefore, liquid chromatography (LC) is used to overcome these problems because high temperatures are not applicable. Liquid chromatography is a chromatographic method that is based on a different distribution rate between a stationary and a liquid mobile phase. The separation of the analytes depends on their sorption affinities. The widely use liquid chromatography is high performance liquid chromatography (HPLC). HPLC separates compounds that are dissolved in solution and it has ability to separate different sample components (good resolution) at a given time. HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. The latter include mass spectrometry detector (MSD), Ultraviolet-Visible detector (UV-Vis) and refractive index, among

others. The UV-Vis detector detects absorption of chromophoric analytes based on molecular structure. It is more sensitive than refractive index but less sensitive than mass spectrometry. The different components in the mixture pass through the column at different rates due to differences in their partitioning behaviour between the mobile liquid phase and the stationary phase. The selectivity is not based only on the different types of the stationary phase but also to the mobile phase (Gunzeler and Williams 2001).

HPLC is normally used in the investigation of degradation products that are nonvolatile compounds or in the analysis of high PBDE, PBB and PCB congeners (Vonderheide 2009, references). For instance, Vilaplana *et al.* (2009) developed an analytical method based on HPLC–UV for the determination of BFRs in styrenic polymers. They were able to determine the highest PBDE congener (deca-BDE). HPLC technique is not limited to higher congeners only. HPLC-MS can be used for detection of PBDEs (from lower to higher congeners). Bacaloni *et al.* (2009) determined PBDEs using liquid chromatography–negative ion atmospheric pressure photoionization tandem mass spectrometry (LC/NI-APPI/MS/MS) from environmental water and industrial effluents. Five different PBDE congeners were detected; these include BDE-47, BDE-99, BDE-100, BDE-153 and BDE-154. HPLC analysis of PBBs and PCBs has been reported by (Von der Recke and Vetter. 2007).

In general chromatographic methods are robust and well established, but they are expensive and require specialized personnel and instrumentation. For instance, they require extensive clean-up and/ or a preconcentration step, which can be accomplished by the use of solid-phase extraction (SPE) and liquid-liquid extraction (LLE) techniques. The sample preparation step is time consuming and tiresome. Electrochemical techniques offer some advantages over chromatographic techniques, for example they are fast, low-cost instrumentation, minimum sample pretreatment and high sample throughput (Rodriguez-Mozaz *et al.* 2007).

2.4 SPECTROPHOTOMETRIC TECHNIQUES

Analysis of heavy metals has been achieved by spectrophotometric techniques. These techniques include inductively coupled plasma- optical emission spectroscopy, ICP-OES; inductively coupled plasma-mass spectrometry, ICP-MS and atomic absorption spectroscopy, AAS.

2.4.1 Atomic Absorption Spectrophotometry

This technique is based on absorption of resonant radiation by the ground state atoms of the analyte. The absorption is proportional to the concentration of the analyte. In this technique, the analyte is vapourized by the use of either a flame or furnace. When a flame is used the technique is known as flame atomic absorption spectroscopy (FAAS). On the other hand when a furnace is used (commonly graphite furnace) the technique is known as graphite furnace atomic absorption spectroscopy (GFAAS). Atomic absorption spectroscopy is commonly used for the determination of metals in liquid form (Gunzeler and Williams 2001).

Determination of heavy metals in different environmental (water, sedments and soils), food plant and human samples using FAAS and GAAS has been reported (Ghaedi *et al.* 2008; Tuzen 2003a; Tuzen 2003b; Tuzen *et al.* (2008), Karadede and Unlu 2000). Jahromi *et al.* (2007) reports the determination of Cd in water samples by GAAS.

2.4.2 Inductively Coupled Plasma-Optical Emission Spectrophotometry and Inductively Coupled Plasma-Mass Spectrometry

Inductively coupled plasma (ICP) is a high temperature source used primarily for generating atomic vapour from an aqueous sample. Generally, ICP is used for the determination of trace metals in environmental samples. Inductively coupled plasma is commonly combined with atomic emission spectroscopy (AES) and mass spectrometry (MS). The advantage of using ICP-AES or ICP-MS is that the combination of these instruments eliminates the sample preparation time required in the absence of an ICP. The latter produces singly-charged positive ions for most of the elements and this makes it an effective ionization source for MS. The advantage of using ICP-MS over ICP-AES is that ICP-MS has the ability to distinguish between the mass of the various isotopes of an element where more than one stable isotope occurs **(**Bradford and Cook 1997).

Analysis of heavy metals in environmental samples using either ICP-OES or ICP-MS has been reported (Karami *et al.* 2004; Bettinelli *et al.* 2000; Rahmi *et al.* 2007). Determination of heavy metals (Al, As, Cd, Co Cr, Cu, Mn, Mo, Ni, Pb, Se, Sn, Sr, and Zn) in fish, water, sediment and tissues have been carried out using ICP-OES (Meche *et al.* 2010; Demirak *et al.* 2006). Pereira *et al.* (2010) reported the simultaneous determination of Ag, As, Ba, Bi, Ca, Cd,

Cr, Fe, K, Li, Mg, Mn, Mo, Ni, Pb, Rb, Se, Sr, Tl, V and Zn in crude oil by ICP-MS and ICP-OES.

Due to wide linear dynamic range, low detection limit (parts per billion, ppb to parts per trillion, ppt) and multi-element analysis capability of these techniques (ICP-OES, ICP-MS and AAS), Environmental Protection Agency (EPA) has recommended them to be the standard techniques for heavy metal analysis (McGaw and Swain 2006; Berezhetskyy *et al.* 2008). However these techniques are not only unsuitable for in-situ analysis, they are also expensive, time consuming during sample preparation, sophisticated and require skilled operators (Ilangovan *et al.* 2006; Malitesta, and Guascito 2005; Wang *et al.* 2009; Han *et al.* 2001; Tsai *et al.* 2003; Berezhetskyy *et al.* 2008; Ghica and Brett 2008; Mohammadi *et al.* 2005; Stoytcheva 2002). For these reasons the development of alternative techniques is necessary.

CHAPTER 3

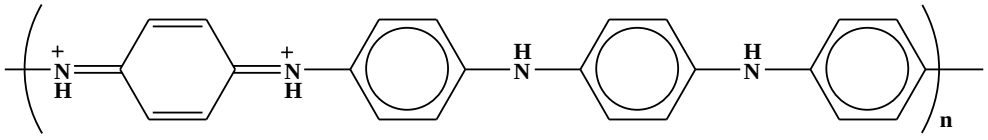
ELECTROCHEMICAL SYNTHESIS AND CHARACTERIZATION OF POLYANILINE

3.1 INTRODUCTION

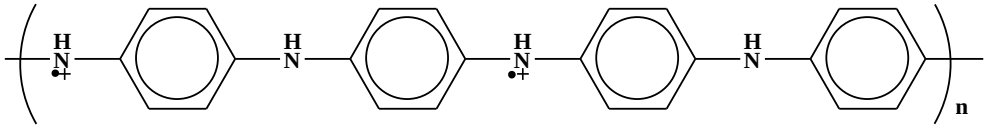
Conducting polymers have been widely studied and they still remain the subject of intense research by many researchers worldwide (Malinauskas 2001). They are used in various electronic devices such as chemical sensors, electrochromic displays and light emitting diodes, among others. The conductivity polymer of choice in this study is polyaniline (PANI). Polyaniline is one of the most widely studied conducting polymers. This is because, its monomer (aniline) is cheap and its synthesis is simple. Polyaniline displays good electrochemical properties, high conductivity and highest environmental stability (Mu *et al.* 1997; Baba *et al.* 2004). Due to the excellent properties that PANI possesses, it is used in battery electrodes and electrochromic devices. It has been found to be the most attractive material for chemical sensor and biosensor interfaces. This is because it can act as a mediator for electron transfer in redox or enzymatic reactions and can also be used as biocompatible matrix for enzyme immobilization (Luo *et al.* 2004). Advantages of using PANI as biocompatible matrix in biosensor application include remarkable signal amplification and elimination of electrode fouling (Mathebe *et al.* 2004). Due to the latter, measurements are performed at low applied potential of the electrodes.

The high conductivity and electroactivity of PANI qualifies it to act as mediator for enzyme based electrodes. In addition to this, PANI exhibits two redox couples in a potential range suitable to facilitate an efficient enzyme–polymer charge transfer (Arora *et al.* 2007). It connects electrons directly from the enzyme active site to the electrode surface and it can undergo redox cycling (Luo *et al.* 2006). As mentioned in Chapter 2, PANI exists in three different redox states; these include fully reduced leucoemaraldine, half-oxidized emeraldine and fully oxidized pernigraniline (Mazeikiene *et al.* 2007; Gospodinova and Terlemezyan 1998). The fully reduced and fully oxidized states of PANI are insulators (Bhada *et al.* 2009; Bhada *et al.* 2006) and half oxidized emeraldine state is the state with highest conductivity. The conductivity of emeraldine salt form is commonly known to be due to its polaronic structure. Due to the latter, emeraldine salt has two possible electronic structures (Mazeikiene *et al.* 2007; Bhada *et al.* 2009). These include polaronic and bipolaronic structures. In a polaronic structure, the cation

radicals are located at nitrogen atoms and they are separated by more than one phenyl ring (Scheme 3.1). A cation radical of nitrogen acts as a hole and because of the presence of the latter, polaronic structure serves as a charge carrier (Dhand *et al.* 2010; Mazeikiene *et al.* 2007). In the case of bipolaronic structure, the two cation radical bearing nitrogen atoms are separated by one phenyl ring (Scheme 3.1). The bipolaronic structure contains quinoid rings (Mazeikiene *et al.* 2007).



ES (b), emaraldine salt, bipolaronic form



ES (p), emaraldine salt, polaronic form

Scheme 3.1. Electronic structures of emeraldine salt (Mazeikiene et al. 2007).

The aim of this work was to electrochemically synthesize polyaniline by cyclic voltammetry (CV) method on platinum electrode to form Pt/PANI. The PANI film was characterized by CV, Ultra–Visible (UV-Vis) Spectrophotometry and Fourier Transform Infrared (FTIR) Spectrometry.

3.2 EXPERIMENTAL

3.2.1 Reagents

All chemicals used in this work were of analytical grade unless otherwise stated. Aniline (99%), hydrochloric acid (37% HCl) and N,N-dimethylformamide (DMF) (99%) were obtained from Sigma-Aldrich (South Africa).

3.2.2 Instrumentation

All electrochemical experiments were performed using BAS100W or BASi epsilon (BASi cell stand) Electrochemical Analyzer (Bioanalytical Systems, West Lafayette, IN). A 15 mL electrochemical cell with a conventional three electrode system, consisting of a platinum electrode as the working electrode (A = 0.0177 cm^2), platinum wire as the auxiliary electrode, and silver-silver chloride (Ag/AgCl) (saturated 3 M NaCl) electrode as the reference electrode, was used. Aniline solutions were degassed with argon or nitrogen gas for electrochemical experiments. All measurements were performed at room temperature (20-25 °C). All Fourier Transform Infrared (FTIR) and ultraviolet- visible (UV-Vis) spectra were recorded on PerkinElmer Spectrum 100, FTIR spectrometer equipped with diamond window Attenuated Total Reflectance (ATR) sampling accessory and PerkinElmer Lambda 35 UV-Vis spectrometer, respectively.

3.2.3 Electrochemical Synthesis and characterization of Polyaniline

The procedure for electrochemical polymerization of PANI carried out according to Mathebe *et al.* (2004) and Morrin *et al*. (2004). Aniline was distilled before use. The platinum electrode was first polished thoroughly with successive alumina slurries, particle size of 1.0, 0.3 and 0.05 µm and was rinsed with distilled water after each polishing step. After polishing, the electrode was then sonicated with ethanol followed by distilled water in an ultrasonic bath for 10 min. The PANI was electrochemically deposited onto the platinum electrode surface. Aniline (0.2 M) solution was prepared in 1.0 M HCl. Before the electropolymerisation process was started, the solution was degassed by bubbling argon or nitrogen for 10 min. In order to form the polyaniline film; the working electrode was scanned from -0.2 V to +1.1 V at 50 mVs^{-1} for 20 cycles. The PANI modified platinum (Pt/PANI) electrode was taken out from the solution, and then it was rinsed with distilled water in order to remove traces of aniline. The electrochemical behavior of Pt/PANI electrode in 1.0 M HCl solution was investigated by CV at different scan rates in the same potential range.

3.2.4 Spectrometric characterization of PANI

3.2.4.1 *Ultraviolet-visible spectrophotometry*

After electrochemical synthesis of PANI, the film was dissolved in DMF and placed in a quartz cuvette of 1 cm path length. The UV-Vis spectrum was recorded from 200 to 900 nm.

3.2.4.2 *Fourier transform infrared- attenuated total reflectance spectrometry*

After electropolymerization, the PANI film was scraped gently from the platinum electrode surface. The film was then placed onto the surface of the ATR and the spectrum was recorded from 3500-400 cm^{-1}.

3.3 RESULTS AND DISCUSSION

3.3.1 Electrochemical Synthesis of Polyaniline

The PANI film was electrochemically synthesized in acid media by sweeping the potential from −0.2 V to +1.1 V versus Ag/AgCl (3 M NaCl) at a scan rate of 50 mV s^{-1} for 20 cycles. Formation of PANI was signified by a green film formed on the surface of the Pt electrode. Literature reports that the formation of a green colour is an indication of the formation of the conducting form of PANI known as emeraldine salt (Sherman *et al.* 1994). Figure 3.1 shows the cyclic voltammograms (CV) for the electrochemical synthesis of PANI.

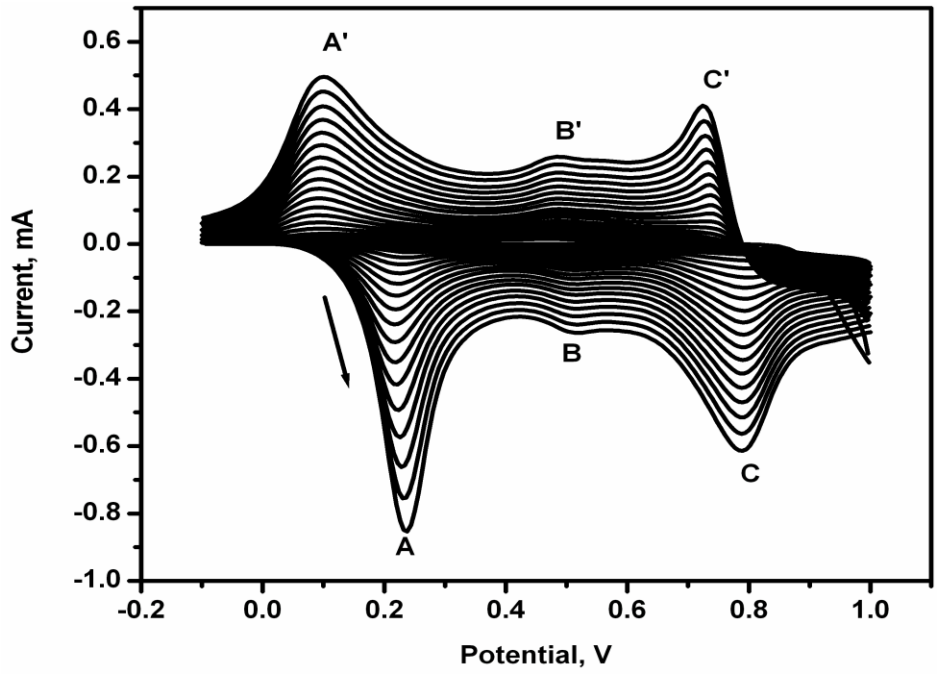


Figure 3.1. The CV (20 cycles) recorded during electropolymerization of PANI film in a solution containing 0.2 M aniline and 1 M HCl at a scan rate of 50 mV s^{-1}.

Electropolymerisation of polyaniline had been reported in the literature (Mu *et al.* 1997; Baba *et al.* 2004; Pournaghi-Azar and Habibi 2007; Ndangili *et al.* 2009; Martha *et al.* 2006; Mathebe *et al.* 2004). It was observed that as the number of CV cycles increased the current also increased. The increase in current as results of repeated potential scans indicated the deposition of PANI at the Pt electrode. This phenomenon also confirmed that PANI was conducting. The increase in current was in agreement with other workers (Mathebe *et al.* 2004; Ndangili *et al.* 2009). Cyclic voltammograms for electropolymerisation of PANI shows three oxidation peaks at 0.238, 0.489 and 0.801 V *versus* Ag/AgCl and their reduction counter peaks at 0.0562, 0.454 and 0.711 V *versus* Ag/AgCl, respectively. The first redox couple (A, A′) was assigned to the transformation of PANI leucoemaraldine (LE) state to partially oxidized emeraldine (EM) state while the third redox couple (C, C′) was assigned to the transition of PANI from EM to pernigraniline (PE) state (Pournaghi-Azar and Habibi 2007; Ndangili *et al.* 2009; Martha *et al.* 2006; Luo *et al.* 2006; Mathebe *et al.* 2004). The redox couple in the middle (B, B′) was attributed to the defects in the linear structure of PANI or formation of a dimer and/or hydrolysis

products (Lapkowski 1990; Zhang and Lian 2007; Seo *et al.* 2007). The redox peaks of the PANI film obtained in this study are similar to literature results (Mu *et al.* 1997; Baba *et al.* 2004; Pournaghi-Azar and Habibi 2007; Ndangili *et al.* 2009; Martha *et al.* 2006; Luo *et al.* 2006; Zhang, and Lian 2007; Seo *et al.* 2007).

3.3.2 Electrochemical Characterization of Pt/PANI Electrode in 1.0 M HCl

3.3.2.1 *Cyclic voltammetry*

Figure 3.2 shows (A) cyclic voltammograms of PANI film in 1.0 M HCl at various scan rates and (B) a plot of peak current against scan rate. From CV in Figure 3.2A, it can be seen that the redox couple (B, B′) which was due to the formation of dimer and/or hydrolysis products disappears (not visible) and only characteristic peaks of PANI (A, A′ and C, C′) are visible. This means that the redox couple B, B′ is less important than the other two (Songa *et al.* 2009). As expeceted, the peak currents increased with increasing scan rate while the peak potentials for the anodic sweep indicated by A and C showed slight increase in positive potential. In the reverse scan (cathodic sweep) peaks A′ and C′ also show slight shifts in potential to more negative values with increased scan rate (Figure 3.2A). These obervations show that the polymer is electroactive and the peak currents are diffusion controlled **(**Mathebe *et al.* 2004). In order to calculate surface concentration of the PANI film, Γ^*_{PANI}, Brown-Anson equation (3.3.1) (Bard and Faulkner 2000) was used.

$$I_P = \frac{n^2 F^2 \Gamma^*_{PANI} A}{4RT} \upsilon \qquad (3.3.1)$$

where n is the number of electrons (n = 2) transferred, F is the Faraday constant (96584 C mol^{-1}), Γ^*_{PANI} is the surface concentration of the PANI film (mol cm^{-2}), A is the surface area of the electrode (0.0177 cm^2), υ is the scan rate (V s^{-1}), R is the gas constant (8.314 J mol K^{-1}), and T is the absolute temperature of the system (298 K).

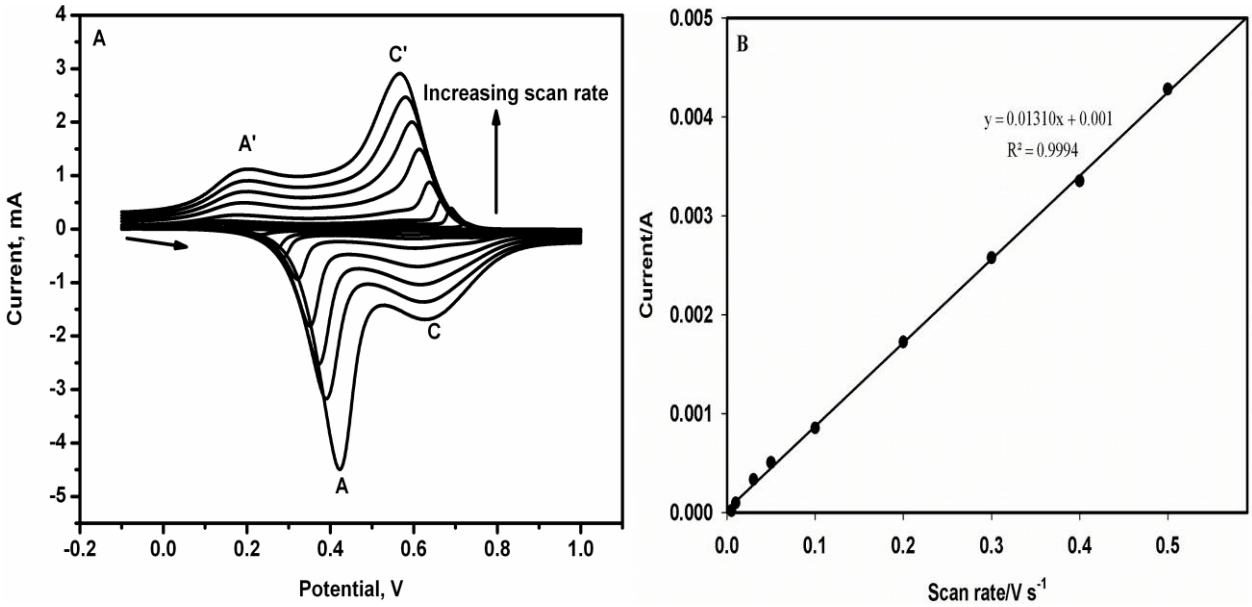


Figure 3.2. (A) The CV of PANI film at the gold electrode (Pt/PANI) in 1 M HCl at various scan rates. Scan rates: 5, 10, 30, 50, 100 200, 300, 400, and 500 mV s^{-1}. (B) Plot of current *versus* scan rate.

The redox couple A, A′ in Figure 3.2A is known to undergo one electron transfer (Singh *et al.* 2008; Mazeikiene *et al.* 2007) and peak A gave the best correlation coefficient (R^2 = 0.9994) for the linear plot of current versus scan rate compared to the other peaks (Table 5). A graph of peak current *versus* scan (Figure 3.2B) was obtained and the slope of the curve was used to calculate the surface concentration of the PANI film. The surface concentration was calculated as 7.8×10^{-7} mol cm^{-2}. The surface concentration obtained in our study was comparable to that reported by Mathebe *et al.* (2004) (1.85×10^{-7} mol cm^{-2}).

The Randles-Sevcik equation (3.3.2) was used to calculate the diffusion coefficient of the electrons within the polymer (Gau *et al.* 2005).

$$i_p = 2.69\times10^5 n^{3/2} A D_e^{1/2} C v^{1/2} \qquad (3.3.2)$$

where i_p is the peak current (A), n is the number of electrons appearing in half-reaction for the redox couple, A is the area of the electrode (cm^2), D is the diffusion coefficient (cm^2 s^{-1}), C is the concentration (mol cm^{-3}) and v is scan rate (V s^{-1}). Equation (3.3.2) was used to plot peak current

versus the square root of the scan rate and the slope of the linear regression (Table 5) was used to estimate the diffusion coefficient of the electrons (D_e) as 4.07×10^{-8}cm^2 s^{-1}.

Table 5. Summary from the calibration curves of current against scan rate and square root of scan rate.

Peaks	**Current *versus* scan rate**		**Current *versus* square root of the scan rate**	
	Slope/A V^{-1} s	**R^2**	**Slope/A (V^{-1} s)$^{1/2}$**	R^2
I	0.001310	0.9994	0.1086	0.9879
I'	0.0002362	0.9580	0.01424	0.9333
III	0.0007701	0.9906	0.03115	0.9452
III'	0.005980	0.9988	0.08854	0.9768

3.3.3 Spectrometric Characterization of polyaniline

3.3.3.1 *Ultraviolet-visible spectrophotometry*

Ultraviolet-Visible (UV-Vis) spectrophotometry has been used to provide qualitative indication of the intrinsic redox states of polyaniline. Figure 3.3 is a UV–Vis absorption spectrum of PANI dissolved in DMF. The absorption spectrum of PANI shows two characteristic absorption peaks at 340 nm and 660 nm. The first absorption peak was assigned to π–π^* electron transition within the benzenoid rings and the second was attributed to the exciton absorption of the quinoid rings **(**Laska and Widlarz 2005; Kan *et al.* 2006). These characteristic peaks are the indication of the formation of conductive emeraldine state (Sun *et al.* 1990). The results obtained in this study are in close agreement with the literature values (Laska and Widlarz 2005; Kan *et al.* 2006; Singh *et al.* 2008; Mazeikiene *et al.* 2007; Kang *et al.* 1998).

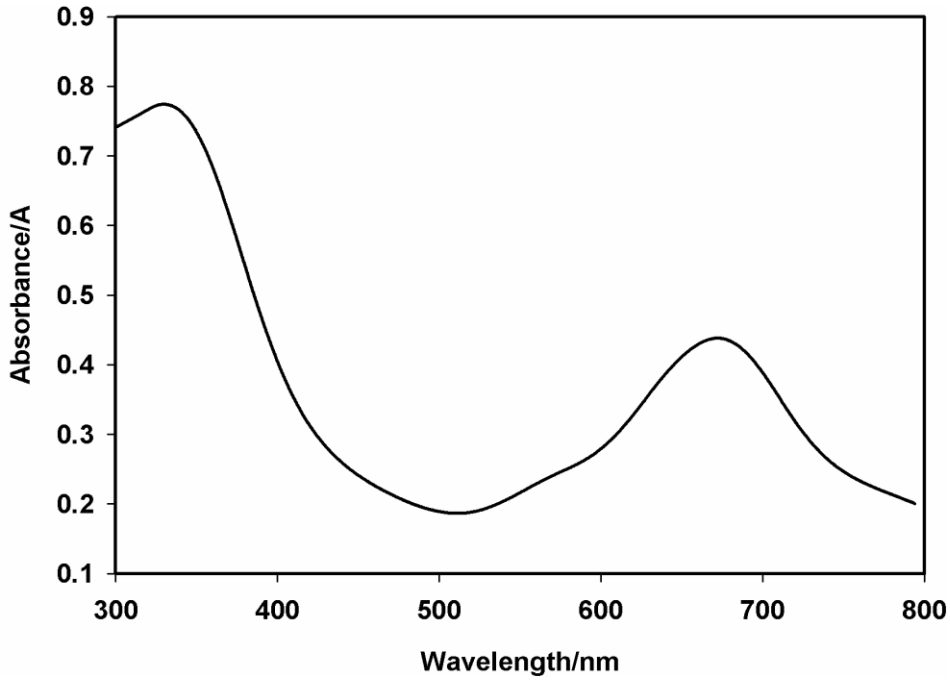


Figure 3.3. The UV-Vis spectrum of polyaniline

3.3.3.2 *Fourier transform infrared-attenuated total reflectance spectrometry*

Figure 3.4 shows the FTIR-ATR spectrum of electrochemical synthesized PANI film which is in close agreement with the spectra that is reported by Kang *et al.* (1998) and Sun *et al.* (1990). The absorption bands for PANI film at 1582 cm^{-1} and 1493 cm^{-1} are main the characteristic peaks and they are assigned to C=C bond stretching quinoid and benzinoid rings, respectively (Lakshmi *et al.* 2009; Kim *et al.* 2001). The presence of the two absorption bands suggested that PANI had amine and imine units in its backbone (Huang *et al.* 2003). It can be seen from the spectrum that the intensities for 1582 cm^{-1} and 1493 cm^{-1} are almost the same. This shows that the amounts of the two units (benzenoid and quinonoid) are almost equal. This phenomenon is attributed to formation of conducting intrinsic redox state of PANI which is emeraldine state (Kang *et al.* 1998; Paul and Pillai 2000; Sun *et al.* 1990). The absorption band at 3229 cm^{-1} was assigned to N-H stretching of the amine group of polyaniline. The bands at 1304 and 1158 cm^{-1} were assigned to benzenoid ring C–N stretch and quinoid ring C–N stretch, respectively. The absorption band at 825 cm^{-1} was attributed to the out-of-plane bending vibration of C–H on the 1,4-disubstituted ring (Kang *et al.* 1998).

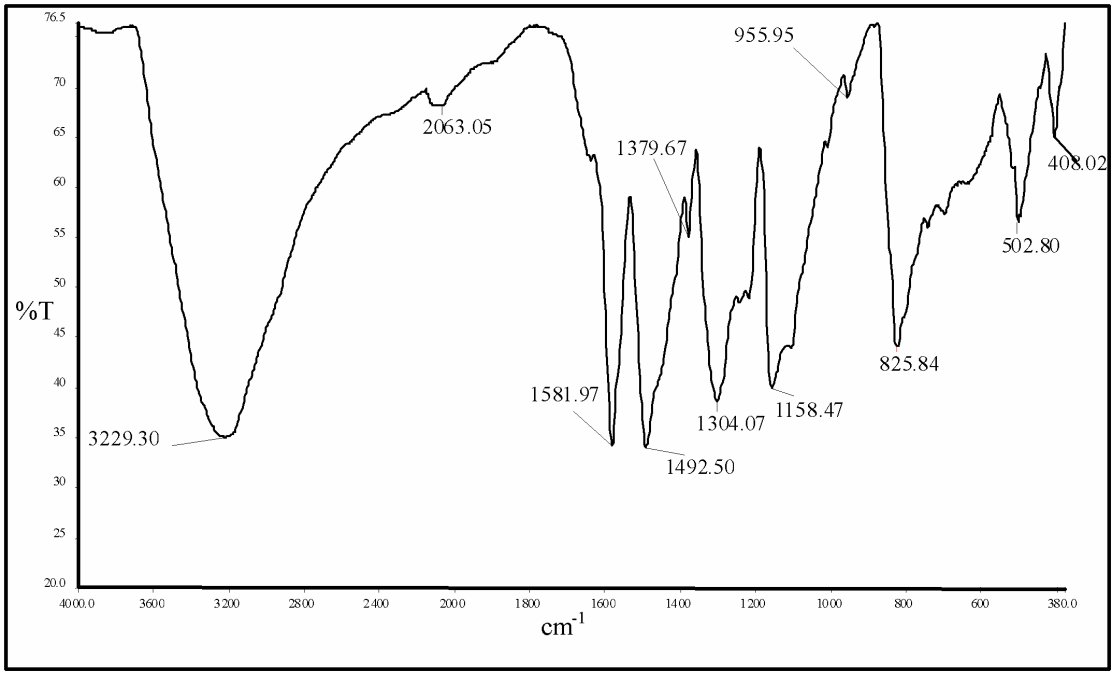


Figure 3.4. The FTIR-ATR spectrum of polyaniline film

3.4 CONCLUSIONS

The synthesis of PANI was achieved by electropolymerization of aniline in hydrochloric acid solution. Cyclic voltammograms results showed that PANI has three different redox forms; these include leucoemeraldine, emeraldine and pernigraniline. Electrochemical characterization results showed two redox pairs of anodic and cathodic peaks. These peaks were assigned to two redox transitions between different redox forms. From the electrochemical characterization results, surface concentration of the PANI film and diffusion coefficient of electrons were calculated as 7.85×10^{-7} mol cm^{-2} and $4.07\times10^{-8} cm^2\ s^{-1}$, respectively. The UV-Vis and FTIR-ATR spectrometric results revealed that PANI film synthesized in this study exhibited similar properties to those reported in the literature. They also showed that the PANI film synthesized in this study is the conductive emeraldine salt.

CHAPTER 4

PREPARATION AND CHARACTERIZATION OF HORSERADISH PEROXIDASE BASED BIOSENSOR FOR THE DETERMINATION OF HYDROGEN PEROXIDE

4.1 INTRODUCTION

Horseradish peroxidase (HRP) is the hemeprotein that belongs to the large class of peroxidase. The heme group contains iron (III) protoporphyrin IX (ferriprotoporphyrin IX) as the prosthetic group (Figure 4.1) which acts as an active site of an enzyme (Lu *et al.* 2006).

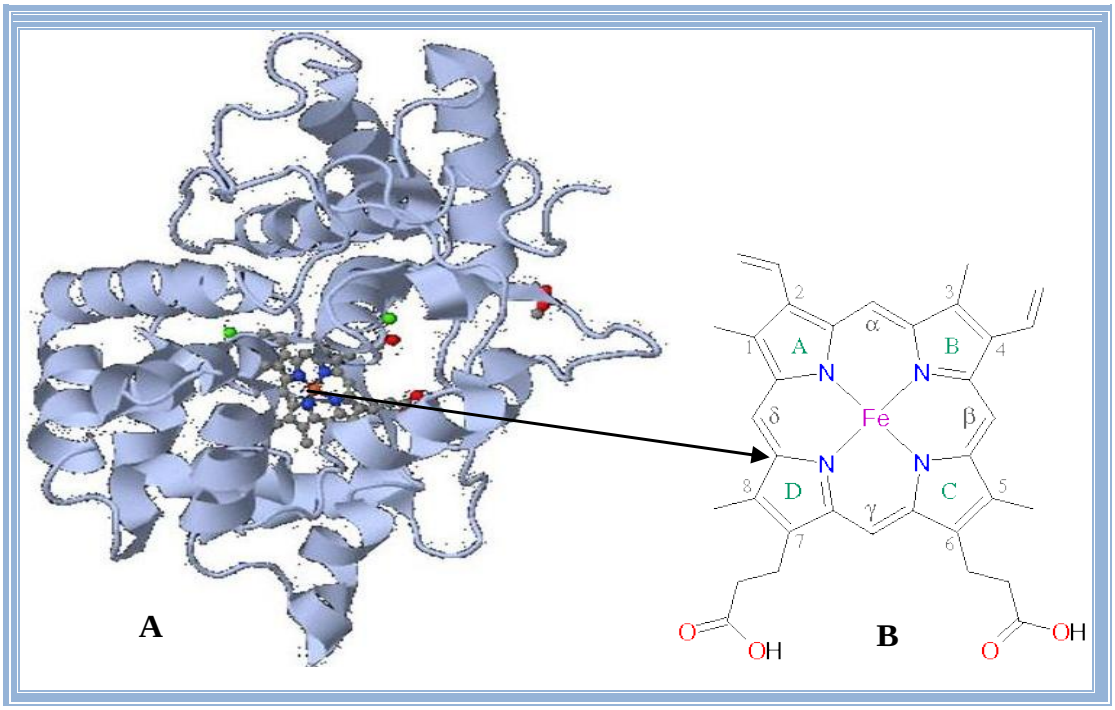


Figure 4.1. (A) X-ray crystal structure of ferric horseradish peroxidase and (B) some essential structural features of ferric horseradish peroxidase (RCSB PDB 2010; Promise 1997).

Horseradish peroxidase catalyzes the reduction of hydrogen peroxide (H_2O_2, substrate) and the oxidation of many of organic and inorganic compounds by H_2O_2. The catalytic cycle of

HRP in Figure 4.2 involves two intermediates. The HRP enzyme is oxidized by H_2O_2 to produce compound I (an oxidized form of the heme structure, the first intermediate) which contains an oxyferryl centre (Fe^{4+}=O) and a porphyrin p-cation radical. In the same step, H_2O_2 is reduced to water. Compound I oxidizes the substrate (electron donor) to produce compound II (secondary intermediate). Compound II gains an electron from the second substrate molecule to form the native ground state, HRP–Fe(III) (Berglund *et al.* 2002; Sun *et al.* 2004; Hamid and Khalil-ur-Rehman 2009).

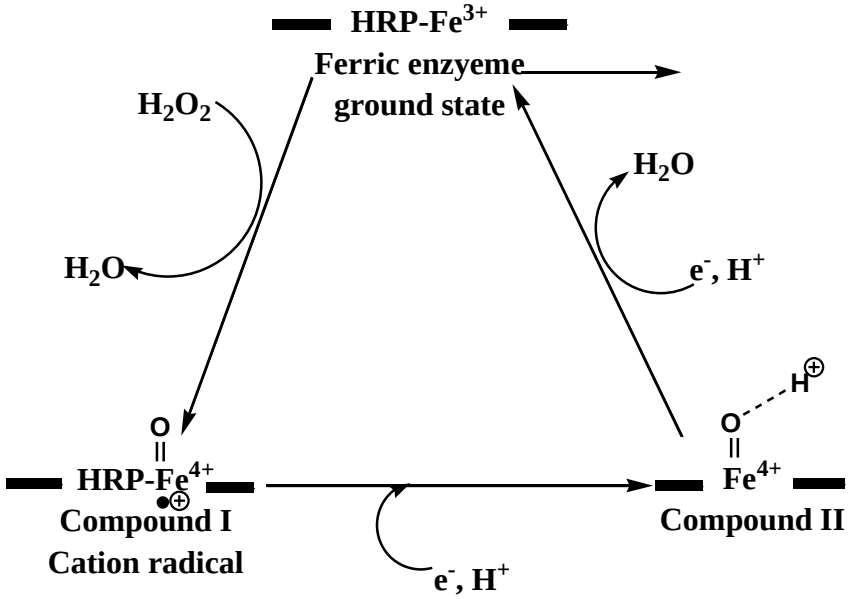


Figure 4.2. Schematic diagram drawn to illustrate catalytic pathway of HRP

Horseradish peroxidase enzyme displays some important features that make it suitable for many practical applications such as biosensors (Ruzgas *et al.* 1995). These include good stability in aqueous and organic phases, high reaction rates with substrates and oxidation of some substrates using oxygen instead of peroxide (Ruzgas *et al.* 1995). In biosensors, the HRP enzyme is immobilized on the surface of the electrode. Immobilized HRP enzyme shows distinctive bioelectrocatalytical properties in the reduction reaction of H_2O_2, by direct electron transfer from the electron-donating electrode through the HRP enzyme active sites and to H_2O_2 in solution (Ruzgas *et al.* 1995; Ferapontova and Gorton 2001). The efficiency of direct electron transfer can

be increased by immobilizing HRP on the surface of the electrode modified with a good conductive material.

Direct electron transfer between the bare electrode and the redox enzyme such as HRP is very important for fundamental studies and construction of biosensors (Wang *et al.* 2008). However, studies report that the electrochemistry of the HRP enzyme is difficult to investigate at a bare electrode (Hong *et al.* 2006). There are two reasons that have been stated, firstly, HRP molecules can be adsorbed onto the surface of the electrode. Therefore this will result in denaturation and loss of electroactivity and bioactivity. Secondly, the HRP active sites are deeply masked in a thick protein shell. This results to a long distance between the active sites and electrode surface which leads the slow electron transfer. Promoters or mediators can be used to obtain the electrochemical reaction of the enzyme (Sun *et al.* 2004; Hong *et al.* 2006; Yin *et al.* 2009). Various materials have been employed to modify the electrode as a link of electron transfer between the HRP redox center and the electrode surface. These include materials like gold nanoparticles, carbon nanotubes, conducting polymers (polyaniline and polypyrrole) and others (Lu *et al.* 2006; Yin *et al.* 2009; Wang *et al.* 2008; Razola *et al.* 2002; Baba *et al.* 2004). In this work, polyaniline was used as a promoter and microenvironment for HRP enzyme immobilization.

The aim of this chapter was to prepare and characterize a polyaniline-based amperometric biosensor incorporating HRP enzyme for the detection of hydrogen peroxide. Horseradish peroxidase was immobilized onto the platinum/polyaniline (Pt/PANI) electrode by electrostatic attachment. Stability of the HRP after immobilization was investigated by spectroscopic techniques (FTIR and UV-Vis). Electrocatalytic response of the biosensor (Pt/PANI/HRP) towards the reduction of H_2O_2 was investigated by cyclic voltammetric, differential pulse voltammetric and amperometric techniques. Amperometric results were further used to investigate the kinetics of the HRP enzyme electrode. The analytical characteristics of the determinations performed by the fabricated biosensor for detection of H_2O_2 were evaluated by amperometry.

4.2 EXPERIMENTAL

The details of the procedures for electrochemical polymerization and instrumentations (electrochemical analyzer, FTIR and UV-Vis) are discussed in Chapter 3.

4.2.1 Reagents

All the chemicals used in this work were of analytical grade unless otherwise stated. Horseradish peroxidase (E.C. 1.11.1.7, 169 Units mg^{-1} powder), disodium hydrogen phosphate (dehydrated) (99%) and sodium dihydrogen phosphate (hydrated) (99%) were obtained from Sigma-Aldrich (South Africa). Hydrogen peroxide (H_2O_2) (30% v/v) was obtained from Merck Chemical (PTY) Ltd. The H_2O_2 (30%) stock solution was stored in refrigerator at 4 °C. H_2O_2 solutions were freshly prepared using a 30% H_2O_2 solution. Phosphate buffer solution (PBS, 0.1 M, pH 7.0) was prepared by mixing an appropriate amount of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4. The pH was adjusted with 0.1 M NaH_2PO_4 or 0.1 M Na_2HPO_4. PBS was used as supporting electrolyte for electrochemical measurements.

4.2.2 Enzyme Immobilization by Electrostatic Attachment

The procedure of immobilization of HRP on the surface of platinum electrode modified with polyaniline used in this study was adopted from Morrin *et al.* 2004, Songa *et al.* 2009 and Mathebe *et al.* 2004. The preparation of the HRP based biosensor was as follows. After electrochemical deposition of PANI film onto the platinum electrode surface, the electrode was then transferred to an electrochemical cell containing PBS. The polymer film was reduced at a constant potential of – 500 mV until the current reached steady state value. A solution of HRP (50 µl of 2.0 mg ml^{-1}) in PBS was prepared before use. Immediately after reduction was complete, PBS was removed from the electrochemical cell and quickly replaced with HRP solution, with no stirring or degassing. The PANI film was oxidized for 20 min at + 0.65 V. During the oxidation process, the positively charged PANI film binds with the negatively charged heme protein of HRP *via* electrostatic interactions (Morrin *et al.* 2004). Schematic representation for the preparation of the Pt/PANI/HRP biosensor is shown in Figure 4.3. The HRP solution was cautiously recovered from the electrochemical cell and stored for later use. The biosensor was washed with double distilled water to remove any un-immobilized HRP and stored in PBS at 4 °C when not in use.

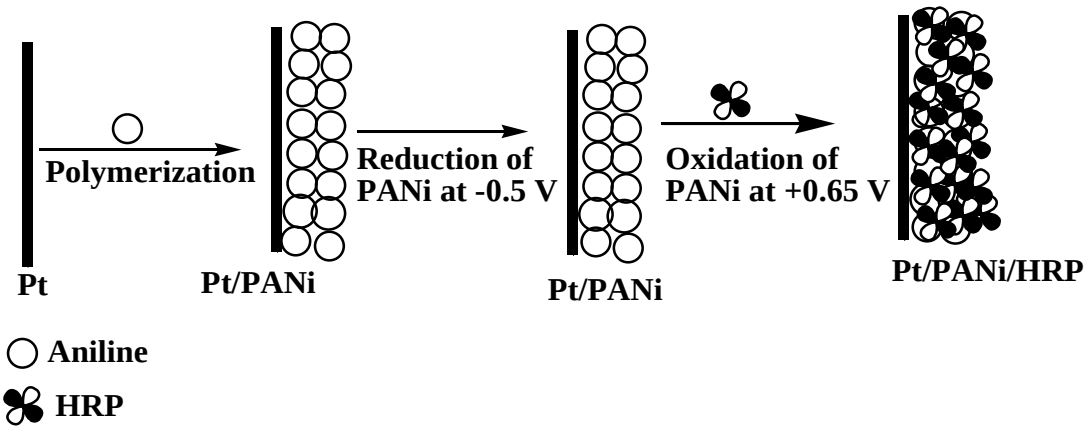


Figure 4.3. Schematic diagram drawn to show representation for the preparation of Pt/PANI/HRP biosensor.

4.2.3 Spectrometric characterization of PANI/HRP film

Spectrometric characterization of PANI/HRP film was performed as per Chapter 3. The PANI/HRP film and free HRP were dissolved in DMF-PBS mixture and PBS, respectively and the solutions were analyzed by UV-Vis spectrophotometry.

4.2.4 Electrocatalytic Reduction of H_2O_2 at the Pt/PAN/HRP Electrode

The Pt//PANI/HRP biosensor's response to H_2O_2 was studied at pH 7.0 in PBS. Cyclic voltammetric (CV), differential pulse voltammetric (DPV) and amperometric responses of the biosensor were recorded by consecutively adding small aliquots of 0.01 to 0.05 M H_2O_2. Amperometric measurements were performed in the stirred system by applying a potential of -200 mV to Pt/PANI/HRP electrode (working electrode). Current-time data were recorded after steady state current was reached. The difference between the steady state current (I_{ss}) upon addition of H_2O_2 and the background current (I_0) was reported as current I. After each experiment, the electrode was rinsed with double distilled water and kept in the working buffer solution at 4 °C.

4.3 RESULTS AND DISCUSSION

4.3.1 Spectrometric Characterization of PANI/HRP Film

4.3.1.1 *Fourier transform infrared-attenuated total reflectance spectrometry*

The stability of HRP after immobilization was investigated by FTIR-ATR. The FTIR-ATR of free HRP (A), PANI/HRP (B) and PANI (C) are present in Figure 4.4. In Figure 4.4A the absorption bands at 1642 and 1530 cm^{-1} were assigned to the stretching of amide groups (I and II) of HRP respectively (Sun *et al.* 2004; Sun *et al.* 2007; Ma *et al.* 2007; Songa *et al.* 2009). Literature reports that the amide I band (1700-1600 cm^{-1}) is due to C=O stretching vibrations of the peptide linkages. While the amide II band (1620-1500 cm^{-1}) on the other hand results from a combination of N–H in plane bending and C–N stretching vibrations of the peptide groups (Sun *et al.* 2004; Ma *et al.* 2007). It can be observed from Figure 4.4C that PANI did not have absorption peaks at the absorption region of amide I and amide II band as in the case of HRP and PANI/HRP. Hence, the IR peaks at 1643 and 1533 cm^{-1} for PANI/HRP film (Figure 4.4B) are thus attributed to those of amide I and II bands of HRP, occurring at similar positions as free HRP. The similarities of the two spectra of free HRP and PANI/HRP (Figure 4.4A and B) showed that HRP retained the essential feature of its secondary structure on the surface of platinum electrode modified with PANI. Sun *et al.* (2004) that when HRP is denatured it shows completely different spectral characteristics in the amide I and II regions).

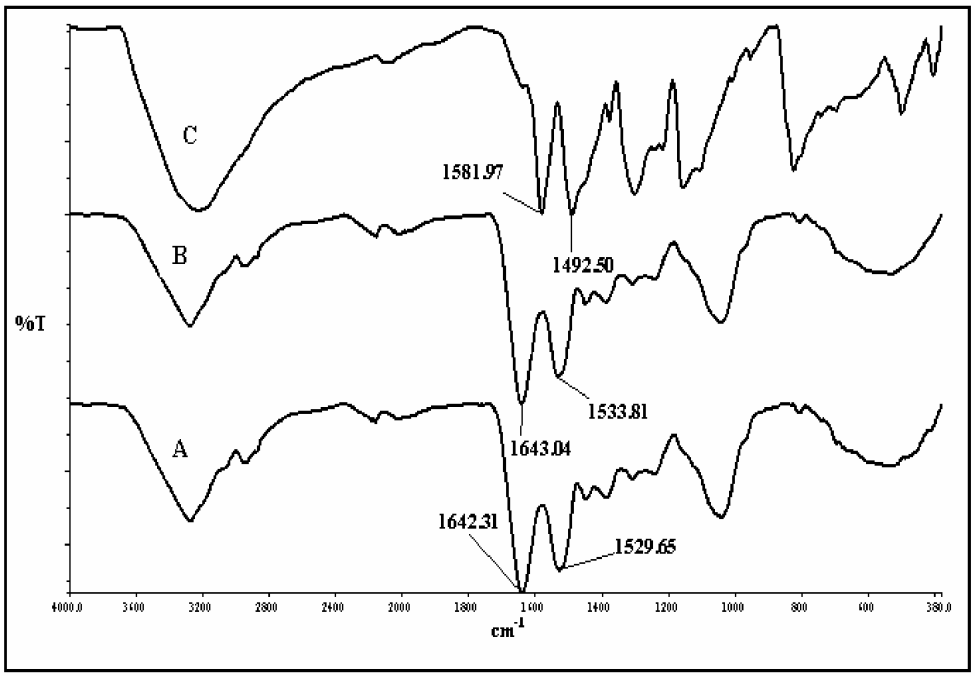


Figure 4.4. The FTIR-ATR spectra of (A) free HRP, (B) PANI/HRP film and (C) PANI film.

4.3.1.2 *Ultraviolet-visible spectrophotometry*

UV–Vis spectroscopy is an effective method to investigate the characteristic structure of proteins (Yin *et al.* 2009). For that reason, the possible change in the intense peak (Soret peak) of HRP was monitored by UV-Vis spectroscopy. Figure 4.5 shows UV-spectra of free HRP (PBS), PANI (in DMF) and PANI/HRP (in PBS-DMF mixture). The UV-Vis spectrum of free HRP in Figure 4.5A shows Soret absorption band at 400 nm (Yin *et al.* 2009), which was also observed in PANI/HRP spectrum (Figure 4.5B) at 404 nm. The slight shift in Soret band for PANI/HRP may be due to the interaction between PANI film and HRP during immobilization (Songa *et al.* 2009). The UV-Vis spectrum (Figure 4.5B) confirms that that the interactions of PANI film and HRP do not destroy the structure of the enzyme (Songa *et al.* 2009). Therefore it can be concluded, that HRP was attached and retained its biological activity after immobilization on PANI modified Pt electrode (Songa *et al.* 2009; Yin el at. 2009). The UV-Vis spectrum of PANI in Figure 4.5C does not have absorption band at 400 nm; this shows that the absorption band (404 nm) observed in Figure 4.5B is entirely due to presence of HRP.

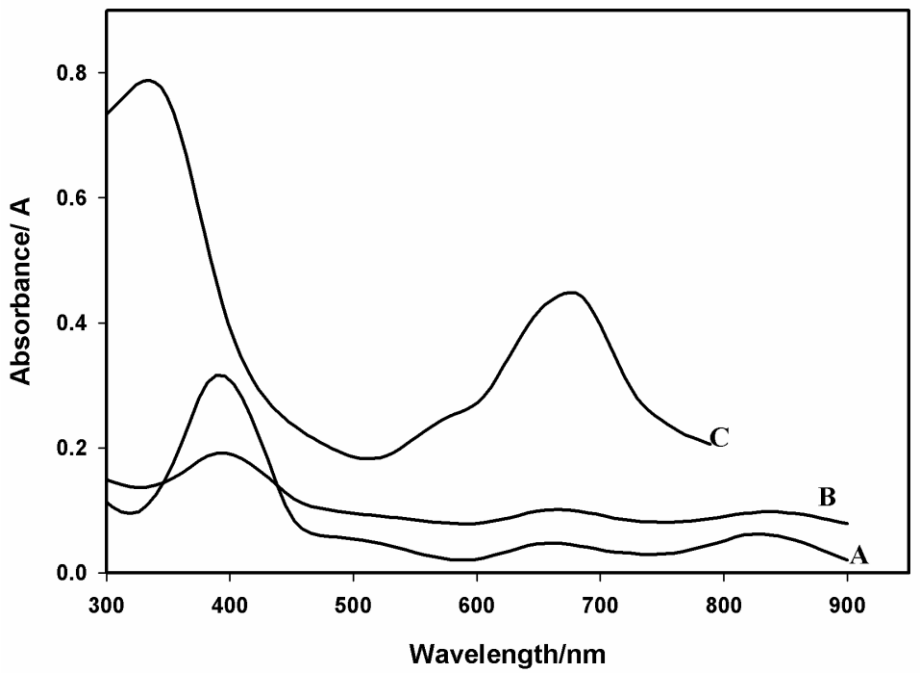


Figure 4.5. UV-Vis spectra of (A) free HRP in PBS, (B) PANI/HRP in PBS-DMF mixture and (C) PANI in DMF

4.3.2 Optimization of Buffer pH

The pH is one of the parameters which affects the response of the PANI/HRP modified electrode towards the reduction of H_2O_2. The effect of pH on HRP electrode response was investigated by CV in the pH range 5.5–8.5 in the presence of 1.0 mM H_2O_2. The pH dependence of the HRP electrode in the presence of 1.0 mM H_2O_2 is demonstrated in Figure 4.6. The HRP electrode response current achieved a maximum value at pH 7.02. Therefore, in order to obtain maximum sensitivity, 0.1 M PBS solution of pH 7.0 was used throughout this study.

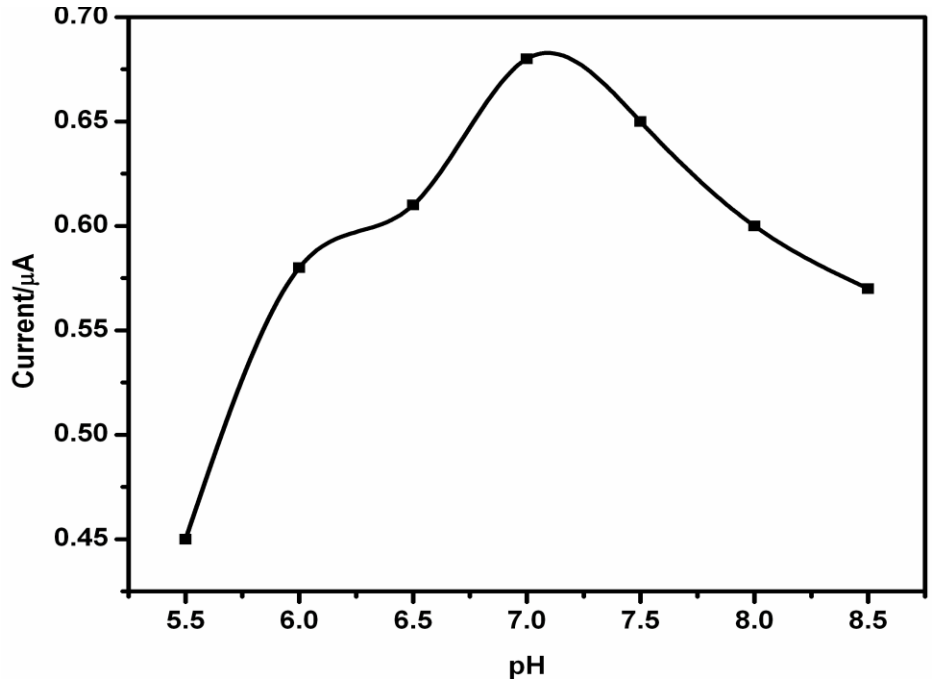


Figure 4.6. Effect of pH on the cyclic voltammetric current responses in 0.1 PBS (supporting electrolyte) containing 1.0 mM H_2O_2

4.3.3 Electrocatalytic Reduction of H_2O_2 at the PT/PAN/HRP Electrode

4.3.3.1 *Cyclic voltammetry and differential pulse voltammetry detection of H_2O_2*

The electrochemical behaviour of Pt/PANI/HRP electrode in the absence and presence of H_2O_2 in PBS (0.1 M, pH 7.02) was studied using CV and DPV. Figure 4.7 shows CV and DPV of the Pt/PANI/HRP electrode in different concentrations of H_2O_2 (0-6.9 mM) at scan rates of 10 and 20 mV s^{-1} (*vs.* Ag/AgCl) for CV and DPV, respectively.

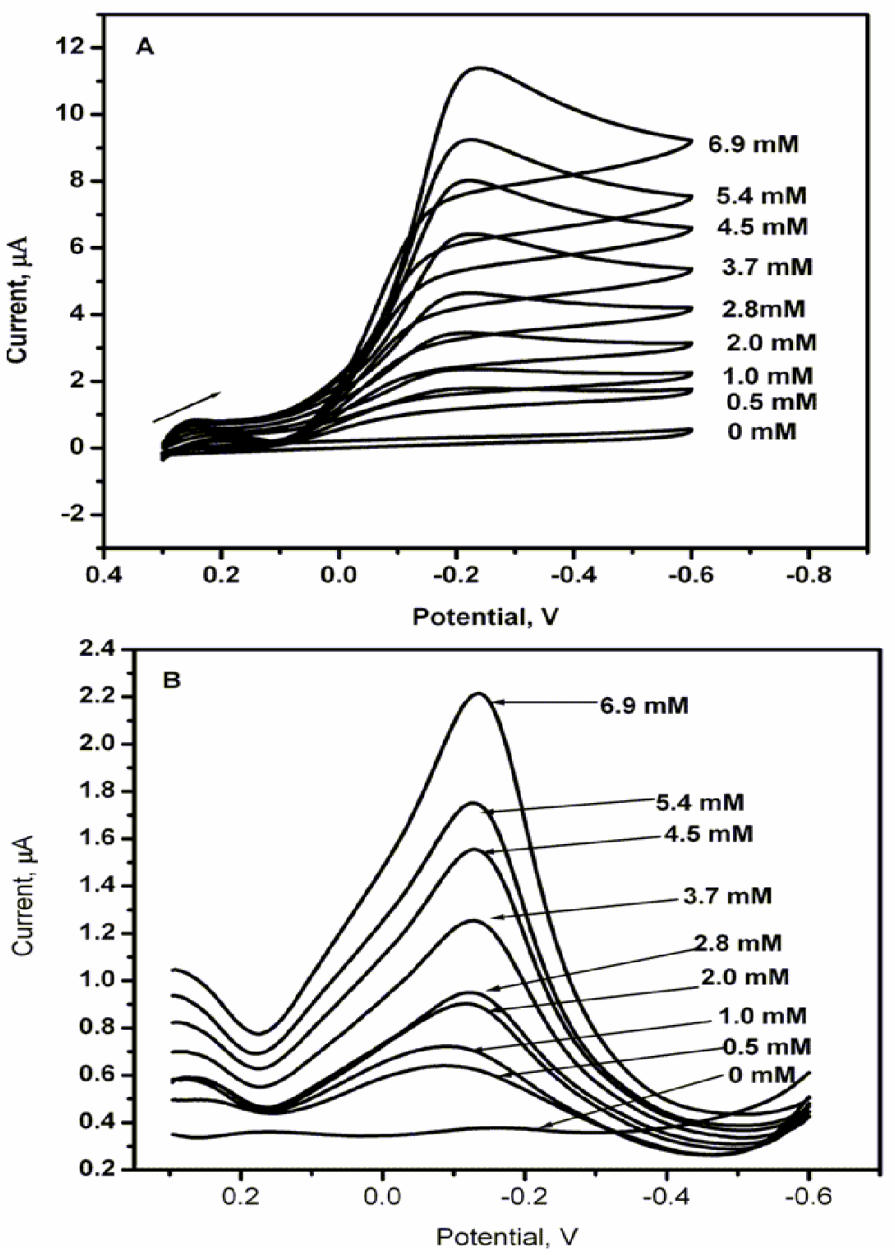


Figure 4.7. (A). Cyclic voltammograms and (B) Differential pulse voltammograms for the response of the biosensors (Pt/PANI/HRP) to different concentrations of H_2O_2 ranging from 0.5 to 6.9 mM made up in 0.1 M PBS (pH 7.02). CV experiments: scan rate, 10 mV s^{-1}; DPV experimental conditions were: scan rate 20 mV s^{-1} pulse width: 50 msec and pulse amplitude: 20 mV.

In the absence of H_2O_2, no significant current was observed. However, increasing the amount of H_2O_2 showed increased cathodic peak current intensity due to the reduction of H_2O_2 at -200 mV. In order to confirm whether the change in current intensity observed was due to the

enzymatic catalytic reduction of H_2O_2, control experiments in the absence of HRP were carried out. At both the bare electrode (Pt) and PANI-modified surface (Pt//PANI) no H_2O_2 reduction current was observed at -200 mV. This is because the reduction reaction of H_2O_2 at both electrodes is very slow and usually occurs at higher potentials. The difference in the observations made for the control experiments (Pt and Pt/PANI) compared to that for Pt/PANI/HRP confirms that the increase in the cathodic current was due to the direct electron transfer between the HRP molecules and the electrode (Wang and Wang 2004). Moreover, PANI provides a suitable platform for the immobilization of HRP on the platinum electrode surface and also mediates in electron transfer between HRP and the electrode (Gerard *et al.* 2002). Thus, the reduction peak is an indication of the electrocatalytic activity of the enzyme (immobilized HRP) towards the reduction of H_2O_2 (Sun *et al.* 2004). The relatively low catalytic reduction potential value for H_2O_2 (-200 mV) due to the presence of HRP, ensures minimal risk of interfering reactions of other electroactive species in solution as well as low background current and noise levels (Tong *et al.* 2007; Wang and Wang 2004).

4.3.3.2 *Amperometric detection of H_2O_2*

The principle of an amperometric biosensor is based on the measurement of the current produced when hydrogen peroxide is reduced by HRP at a constant applied potential. The possible mechanism of PANI-mediated HRP reduction of H_2O_2 is presented Figure 4.8. In the first step of the mechanism, H_2O_2 in solution diffuses to the surface of the film where it is reduced by the immobilized HRP and the latter is oxidized to form HRP-I (also known as Compound-I). The latter in turn oxidizes PANI to give HRP-II (Compound II). The HRP resting state, Fe-III is then regenerated *via* the HRP-II intermediate, Compound-II. The partially oxidized $PANI^+$ (emeraldine) is then electrochemically reduced at the electrode to leucoemeraldine (fully reduced form) yielding an enhanced reduction current (Liu and Ju 2002). The magnitude of the reduction current produced by the electrode reaction depends on the bulk concentration of the substrate, H_2O_2.

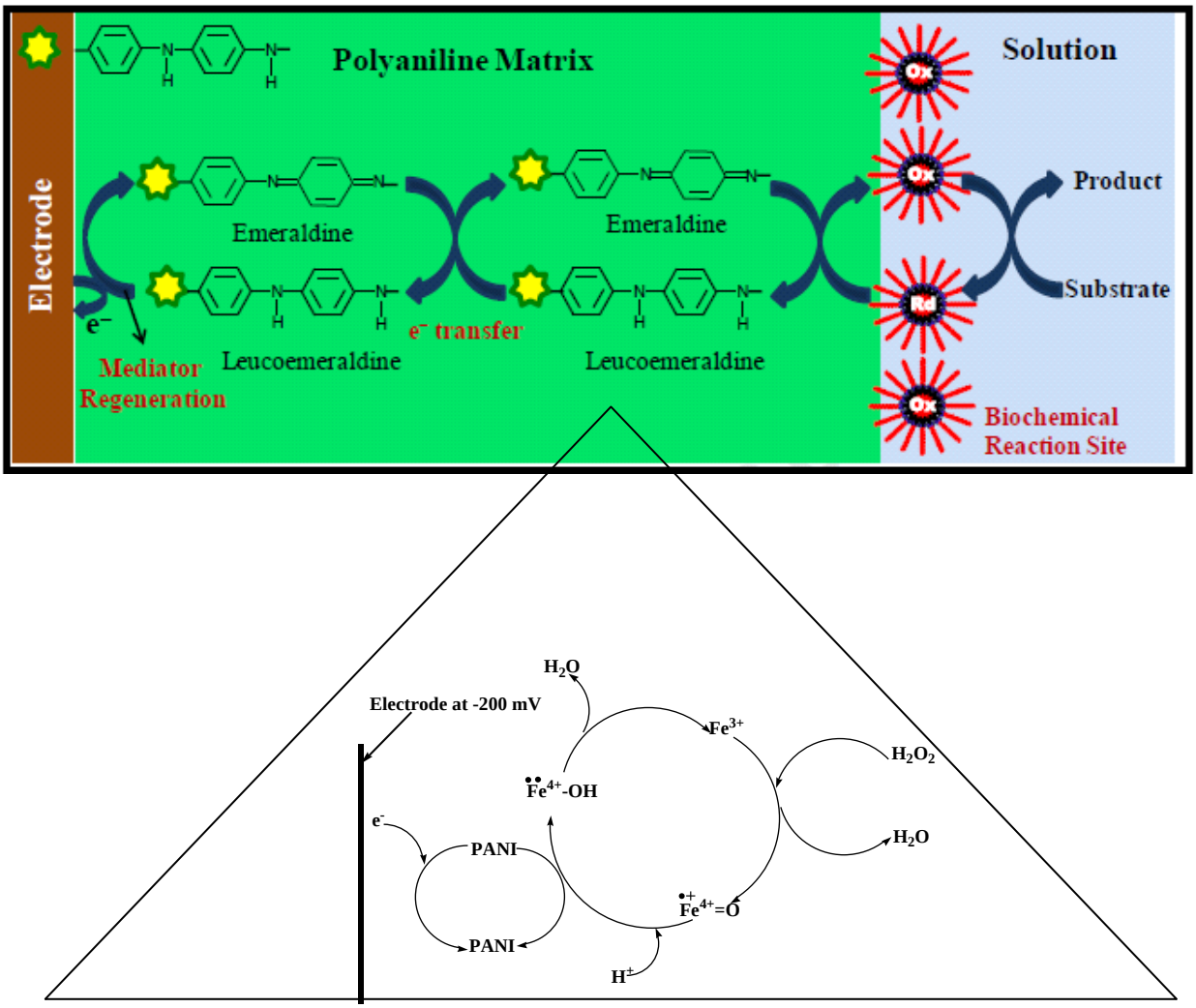


Figure 4.8. The possible mechanism of PANI-mediated HRP reduction of H2O2, where Fe^{3+} is the ferric HRP in resting state, $\overset{\bullet\oplus}{Fe}^{4+} = O$ stands for oxyferryl HRP-I (Compound I), $\overset{\bullet\bullet}{Fe}^{4+} - OH$ is the hydroxyferryl HRP-II (Compound II) and $PANI^{0/+}$ stands for leucoemeraldine/emeraldine cation radical redox couple (Dhand et al. 2010; Iwuoha et al. 1997).

Amperometric responses of Pt/PANI/HRP biosensor were investigated by consecutively increasing the concetration of H_2O_2 at a working potential of -200 mV. Figure 4.9 presents a typical steady state current-time plots obtained with the fabricated biosensor upon successive additions of 10 µL of 0.01 M H_2O_2 into 2.0 mL PBS with the calibration plot as an inset. It was observed that, upon the addition of H_2O_2 to the PBS, the reduction current rises sharply to reach

the steady state value. In addition, the biosensor attained 95% steady state current within 5 seconds after each addition of 0.010 M H_2O_2 aliquot. This observation implied a fast response of the fabricated biosensor.

The biosensor's responses (response currents) were linear to H_2O_2 in the range from 0.05 to 3.17 mM with a correlation coefficient of 0.9991 (n = 18); sensitivity of 1.75 μA mM^{-1}; and a detection limit of 36.8 nM (0.0368 μM) (estimated as signal-to-noise ratio of 3).

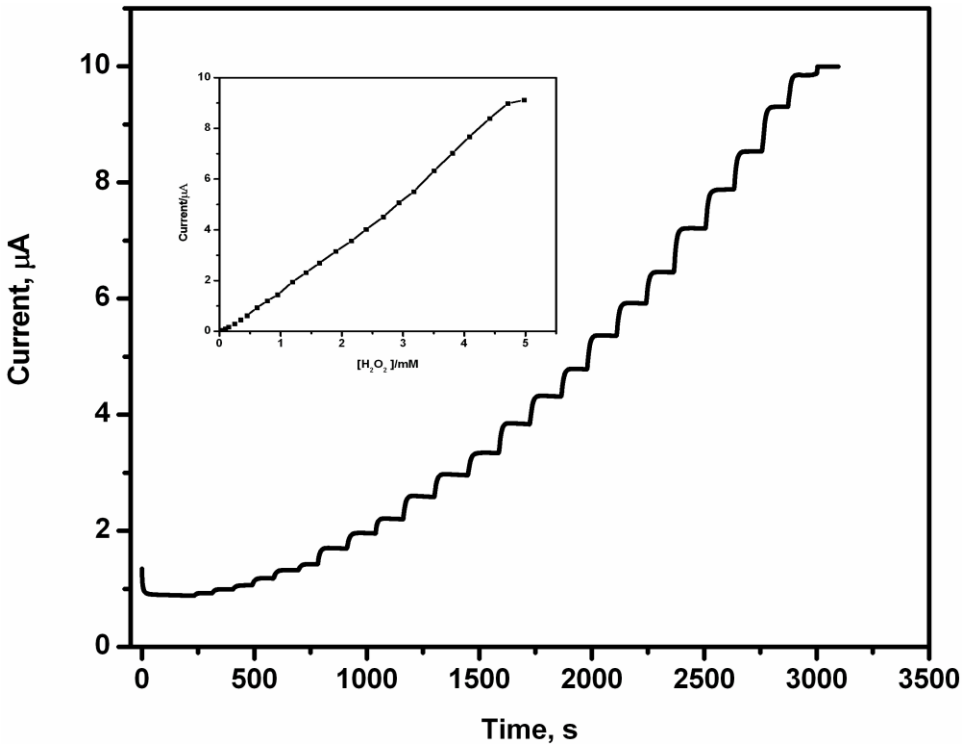


Figure 4.9. Amperometric responses of Pt/PANI/HRP biosensor to successive additions of 10 μL of 0.05 mM of hydrogen peroxide (inset shows the calibration curve). Potential: −0.2 V; supporting electrolyte: 0.1 M PBS (pH 7.02).

4.3.4 Kinetics of Pt/PANI/HRP Electrode

The data obtained from the amperometric experiments was used to calculate the kinetic parameters of the Pt/PANI/HRP electrode. A plot of the reciprocal of current *versus* the reciprocal of H_2O_2 concentration showed a linear relationship implying a kinetic behavior (Liu

and Ju 2002; Wang *et al.* 2009a). Using equation 4.3.1, Michaelis–Menten constant of the enzyme could be calculated as per previous studies (Liu and Ju 2002; Mathebe *et al.* 2004):

$$\frac{1}{I} = \frac{1}{I_{\max}} + \left(\frac{K'_M}{I_{\max}\,[H_2O_2]} \right) \qquad (4.3.1)$$

where I is the observed response current, $I_{\max}$ is the maximum steady state current that can be attained for the system (Pt//PANI/HRP biosensor), K_M^{app} is apparent Michaelis–Menten constant and $[H_2O_2]$ is the concentration of H_2O_2 in the bulk solution. Figure 4.10 shows the Lineweaver–Burk plot for the determination of Michaels-Menten constant for Pt//PANI/HRP biosensor.

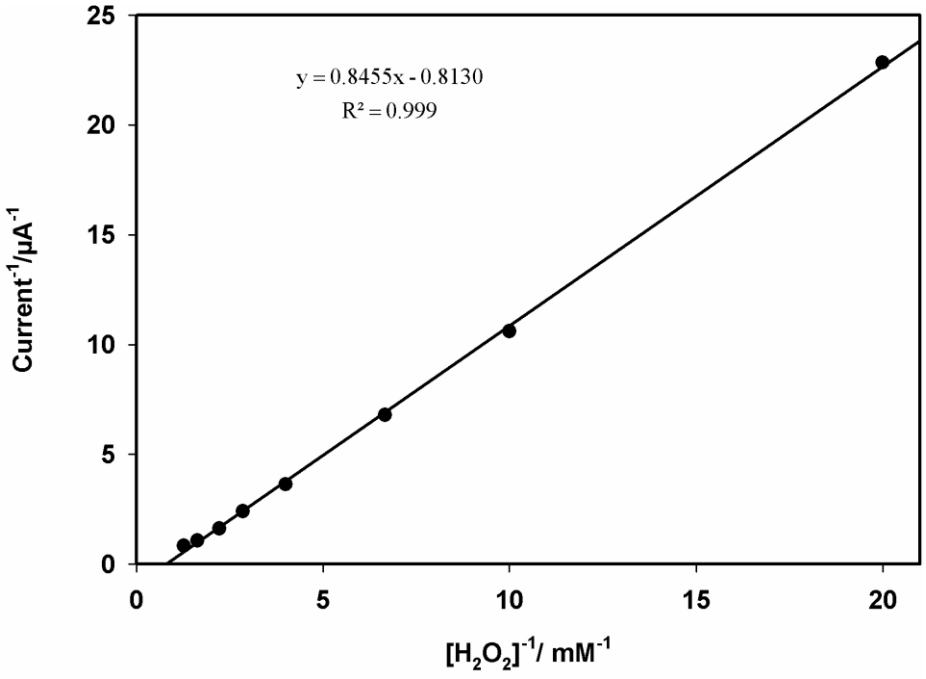


Figure 4.10. Lineweaver–Burk plot for determination of apparent Michaels-Menten constant and maximum current of Pt//PANI/HRP biosensor in PBS (pH 7.0)

The *slope* and the *y-intercept* of the Lineweaver–Burk plot are equal to $\frac{1}{I_{\max}}$ and $\frac{K_M^{app}}{I_{\max}}$, respectively. The values of $I_{\max}$ and K_M^{app} were calculated as 1.23 µA and 1.04 mM, respectively. The K_M^{app} value obtained in this study was relatively better (lower) than 27.1 mM for FTO/PANI/HRP obtained by Wang *et al.* (2009a); 3.69 mM for HRP-Au-CPE obtained by Liu and Ju (2002), and 1.7 mM obtained by Ndangili *et al.* (2009). Since K_M^{app} is inversely proportional to the affinity of the enzyme for the substrate (Wang *et al.* 2009b; Ansari *et al.* 2009), based on the value 1.23 mM it can be concluded that Pt/PANI/HRP electrode exhibited high affinity towards H_2O_2. The differences in the values obtained by different workers indicate that the affinity between H_2O_2 and HRP is plainly dependent on the immobilizing materials and procedure used (Wang *et al.* 2009b).

4.3.5 Reproducibility, Repeatability and Stability of Pt/PANI/HRP Biosensor

To evaluate the reproducibility of the HRP based biosensor, six biosensors were prepared under similar conditions separately. The amperometric responses of each biosensor to successive additions of H_2O_2 (0.05 to 0.30 mM) were recorded and gave a relative standard deviation (RSD %) of 3.8 % (n = 6). The precision (repeatability) of a selected biosensor was investigated by recording the response current using 0.05 mM H_2O_2 for replicate experiments (n = 10). The biosensor showed a high precision with a RSD of 2.1%.

The stability of the Pt/PANI/HRP electrode (stored in 0.1 M PBS at 4 °C in between measurements) was tested every five days for a period of one and half month (45 days) by amperometric measurements in the presence of 0.05 mM H_2O_2. Figure 4.11 shows the long-term stability of the biosensor.

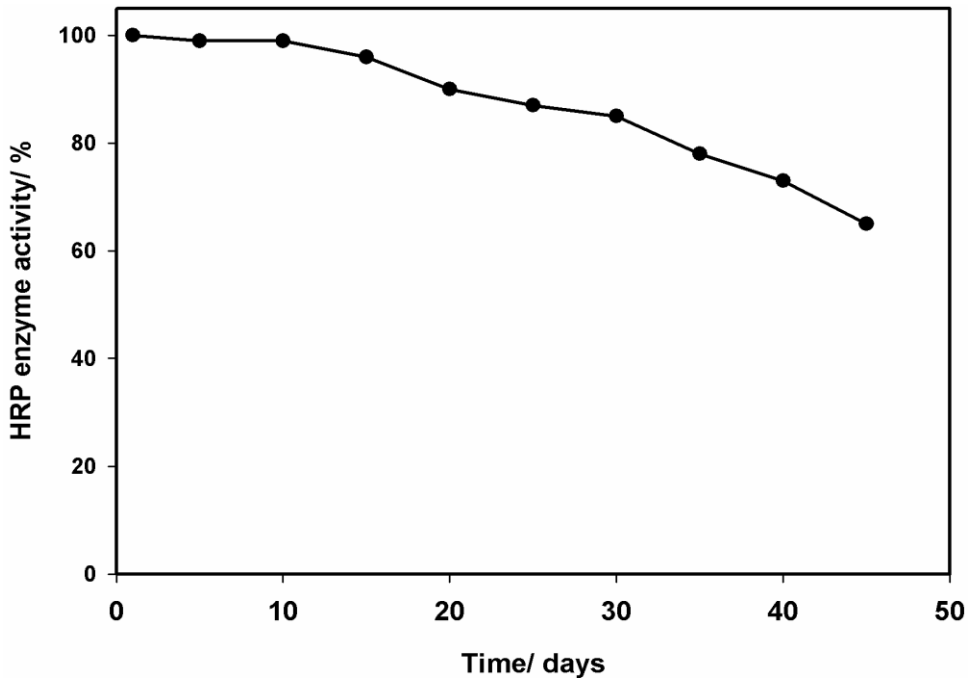


Figure 4.11. The storage stability of the Pt/PANI/HRP biosensor

It was observed from Figure 4.11 that the biosensor was stable in the first 15 days whereby it retained more that 98% of its initial response. Thereafter the response however declined marginally to about 85% in 30 days. A biosensor response of about 85% is normally considered to be stable and it can therefore be concluded that the HRP biosensor exhibited good stability. This implies that the HRP molecules were firmly immobilized in the PANI films which provided a biocompatible microenvironment (Sun *et al.* 2004). After 30 days the enzyme activity decreases gradually and it retained about 65% of its initial response. The decrease in enzyme activity could be due to the denaturation of the immobilized HRP enzyme during the long-term storage. It was concluded that the HRP enzyme electrode fabricated in this study can be used only for a period of 30 days.

4.4 CONCLUSIONS

In this chapter, an amperometric biosensor was fabricated by immobilizing HRP onto the surface of platinum electrode modified with polyaniline. Polyaniline film provided a microenvironment around HRP enzyme molecules and a pathway for electron transfer between HRP and the

platinum electrode surface. The spectroscopic (UV-Vis and FTIR) results showed that HRP enzyme immobilized on PANI film still keeps its electrocatalytic activity towards the reduction of H_2O_2. Electrochemical measurements showed that the immobilized HRP exhibits high biological activity. The resultant Pt/PANI/HRP biosensor exhibited fast amperometric response, low detection limit and wide linear range to H_2O_2. In addition, the biosensor exhibited high sensitivity, good reproducibility (% RSD = 3.8), precision (% RSD =2.1) and stability (30 days).

CHAPTER 5

DETERMINATION OF SELECTED HEAVY METALS USING AMPEROMETRIC HORSERADISH PEROXIDASE (HRP) INHIBITION BIOSENSOR

5.1 INTRODUCTION

Due to the high toxicity of heavy metals, it is crucial to detect ultra low levels especially in drinking water. The common techniques include spectrometric techniques such as inductively coupled plasma- atomic emission spectroscopy, ICP-AES **(**Bettinelli *et al.* 2000); Rahmi *et al.* 2007; Tuzen *et al.* 2008) as well as anodic stripping voltammetry (Brainina *et al.* 2004). Even though ICP techniques have low detection limits (ranges from parts per billion, ppb to parts per trillion, ppt (Berezhetskyy *et al.* 2008), however, they are unsuitable for in-situ analysis, expensive, sophisticated and require skilled operators. For these reasons, the development of alternative techniques such as electrochemical biosensor techniques, offer alternative methods because they are sensitive, low cost and simple to operate (Wang *et al.* 2009b).

Recent developments have shown the use of electrochemical biosensors as indirect methods for detection of Cd^{2+}, Cu^{2+}, Cr^{3+}, Zn^{2+}, Ni^{2+} and Pb^{2+} using urease biosensor (Ilangovan *et al.* 2006; Tsai *et al.* 2003); Cd^{2+}, Co^{2+}, Zn^{2+}, $Ni^{2+}$and Pb^{2+} using alkaline phosphatase (Berezhetskyy *et al.*2008); Cd^{2+}, Cu^{2+}, Zn^{2+} and Pb^{2+} using glucose oxidase (Ghica and Brett 2008); Hg^{2+} using glucose oxidase invertase and mutarose (Mohammadi *et al.* 2005); Cu^{2+}, Cd^{2+}, Mn^{2+} and Fe^{3+} using acetylcholinesterase (Stoytcheva 2002); and Cu^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+} using nitrate reductase (Wang *et al.* 2009b).

Horseradish peroxidase (HRP) biosensor has so far only been reported for the detection of mercury (Han *et al.* 2001). This study sought to extend its application for detection of other metals such as lead, cadmium and copper. We have chosen cadmium due to its similarities with mercury with regards to toxicity as both metals belong to the same group. In addition, we have also chosen copper and lead because of their common occurrence in environmental matrices (Pb from leaded petrol and Cu from wiring activities). Furthermore, copper is reported to show interaction with biological systems (Cecconi *et al.* 2002; Uriu-Adams and Keen 2005) and therefore it is interesting to see how it interacts with the HRP enzyme.

The main aim of this present work is to investigate the inhibition of HRP enzyme by Cd, Pb and Cu, a phenomenon that can be employed for their indirect determination. Kinetic studies were done to determine the nature of enzyme inhibition (whether it is reversible or irreversible and if reversible whether it is competitive or noncompetitive). The apparent Michealis-Menten constant (K_M^{app}) as well as maximum current ($I_{\max}$) values in the absence and the presence of metal inhibitor were investigated. The developed biosensor was applied to the determination of Cd, Pb and Cu in tap water and landfill leachate sample.

5.2 EXPERIMENTAL

The details of procedures for polymerization of polyaniline and preparation of the PT/PANI/HRP electrode has been discussed in earlier chapters (electrochemical instrument and polymerization of polyaniline in Chapter 3 Section 3.2; preparation of the PT/PANI/HRP electrode in Chapter 4 Section 4.2).

5.2.1 Reagents

All the chemicals used in this work were of analytical grade unless otherwise stated. Cadmium and copper stock solutions (1000 ppm) were obtained from KIMIX Chemicals & Lab Supplies. A lead stock solution (1000 ppm) was obtained from Saarchem-Holpro Analytic (PTY) Ltd. The details of other chemicals such as H_2O_2 and salts for phosphate buffer preparation are discussed in Chapter 4.

5.2.2 Instrumentation

The details of the electrochemical instrument are discussed in Chapter 3 Section 3.2. UV photolysis of the leachate water sample was carried out by UV digester 705 equipped with a 500 W Hg lamp from Metrohm (Herisau, Switzerland). Inductively coupled plasma optical emission spectroscopy (ICP-OES) analysis of Cd, Cu, and Pb was performed using an Optima 5300 ICP-OES system (Perkin Elmer LLC, 761 Main Avenue, Norwalk, USA) equipped with AS 93plus autosampler.

5.2.3 Determination of Heavy metals in Model Solutions

Amperometric measurements of HRP inhibition by cadmium, copper and lead were carried out in a cell containing 2.0 ml of 0.1 M PBS (pH 7.02) and constant concentration H_2O_2 (0.5 mM) with continuous stirring. The experiments were carried out at -0.20 V *versus* Ag/AgCl (3 M NaCl) and allowing the steady-state current to be attained. An appropriate volume (µl) of the inhibitor stock solution (10 ppm of each Cd^{2+}, Cu^{2+} and Pb^{2+}) was then added using a micropipette. After each experiment the enzyme electrode activity was regenerated by rinsing the electrode with distilled water.

5.2.4 Analysis of Heavy Metals in Tap Water and Landfill Leachate Samples

Water samples were collected as follows: Tap water was collected from the Laboratory Tap at University of Western Cape, Bellville, Cape Town. Landfill leachate sample was collected from the Marrianhill landfill (Ethekwini municipal solid waste deposit). The leachate water sample was collected in a polyethylene container and stored in the fridge at 4 °C (the leachate samples were not acidified).

Determination of heavy metals in tap water was achieved using standard addition method. The pH of the tap water samples was first adjusted from 8.90 to 7.04 before the analysis was carried out. The tap water sample (10 ml) was spiked with 0.1 ppm of each metal solution (Cd^{2+}, Cu^{2+} and Pb^{2+}) followed by amperometric analysis. For ICP-OES, the tap water sample was analysed without the addition of metal standards.

Leachate water sample is rich with organics; therefore prior to electrochemical analysis, the organics were removed by passing the water sample through a C-18 SPE column. The cartridges were first activated with 5 mL methanol followed by 5 mL water. The C-18 column retained the organics and the water sample containing inorganics was collected. The collected leachate water sample was spiked with 0.1 ppm of each metal solution (Cd^{2+}, Cu^{2+} and Pb^{2+}) followed by Pt/PANI/HRP biosensor analysis.

For ICP-OES analysis, the leachate samples were filtered with 0.45 µm pore size filter before they were subjected to UV digester. This procedure was done in order to destroy all dissolved organic matter in the landfill leachate sample. A UV digester 705 equipped with a 500 W Hg lamp from Metrohm was used. The quartz vessels were arranged concentrically around the

Hg lamp with a distance of 2.5 cm. A 10 mL aliquot of the leachate samples was placed in a quartz vessel and 100 µL H_2O_2 was added to each sample. The solution was irradiated with UV light for about 2 hour. The leachate water sample was then analyzed by ICP-OES.

5.3 RESULTS AND DISCUSSION

5.3.1 Detection of Heavy Metals (Cd^{2+}, Cu^{2+} and Pb^{2+}) in Model Solutions

Heavy metals such as Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+} and Pb^{2+} as well as some organic and inorganic compounds such as sodium azide, cyanide, L–cystine, dichromate, ethylenethiourea, hydroxylamine, sulfide, vanadate, p–aminobenzoic acid are well-known inhibitors of horseradish peroxidase (Zollner 1993). Figure 5.1 (I) shows amperometric responses of Pt/PANI/HRP to successive additions of 0.05 mM H_2O_2 (denoted by r) followed by addition of Cd^{2+} (denoted by i). It can be observed that the current for the electrocatalytic reduction of H_2O_2 decreases upon the addition of Cd^{2+}. This observation clearly showed that the catalytic activity of HRP at Pt/PANI electrode is inhibited by Cd^{2+} by binding to the enzyme's active sites. The inhibition of HRP activity was analyzed by monitoring the current decrease at -0.20 V using the following procedure: H_2O_2 was added to PBS (electrolyte solution) and the current response was recorded. After the stabilization of the steady-state current response, Cd^{2+} was added, leading to an immediate decrease in the biosensor response.

The other two heavy metal ions (Pb^{2+} and Cu^{2+}) showed the same behaviour. Calibration plots for the determination of the metals in the range 0.05 to 60.8 ppb were obtained. The linear ranges were established as 4.76-55.3 ppb for Cd^{2+} and Pb^{2+} while that for Cu^{2+} was 2.38-52.8 ppb. Limits of detection (LOD= $\frac{3\times SD}{m}$) and limits of quantification (LOQ= $\frac{10\times SD}{m}$) were calculated and their values are presented in Table 6. Where SD is standard deviation of the blank signal (n = 8) obtained in PBS (SD = 2.4×10^{-4} µA) and m is the slope of the calibration curve. The low LOD and LOQ values confirmed good sensitivity of the proposed biosensor method for the determination of heavy metals. Table 6 shows a summary of analytical response and linear regression characteristics of calibration curves for heavy metals. The inhibition of heavy metals to HRP enzyme is reversible, and the biosensor can be reused (beyond 10 runs) after rinsing with double distilled water.

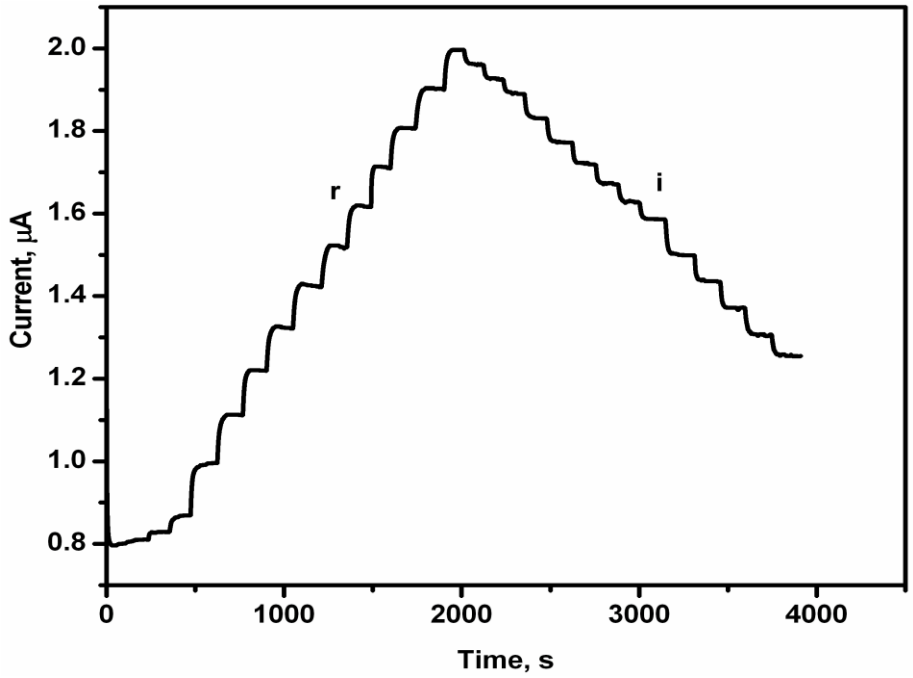


Figure 5.1. Typical amperometric responses of Pt/PANI/HRP biosensor to successive additions of 0.05 mM hydrogen peroxide (r) and Cadmium (i). Applied potential: −0.20 V; supporting electrolyte: 0.1 M PBS (pH 7.02).

Table 6. A summary of analytical characteristics and regression parameters of the calibration curves for the determination of heavy metals.

Metal ion	**Linear range/ppb**	**Slope/μAppb^{-1}**	**Correlation Coefficients (R^2)**	**LOD/ppb**	**LOQ/ppb**
Cd^{2+}	4.76-55.3	7.945×10^{-3}	0.9985	0.091	0.30
Pb^{2+}	4.76-40.5	2.100×10^{-2}	0.9995	0.033	0.12
Cu^{2+}	2.38-52.8	7.230×10^{-3}	0.9990	0.10	0.33

5.3.1.1 *Inhibition studies*

The values of the steady state current in the absence (I_0) and in the presence (I_i) of an inhibitor were determined from the recorded amperograms in Figure 5.1. The inhibition percentage ($I\%$) was calculated from the following expression (Nwosu et al. 1992):

$$I\% = \frac{I_0 - I_i}{I_0} \times 100\% \qquad (5.3.1)$$

The $I\%$ values obtained were used to compare the inhibitory effects of the different metal ions on the activity of the immobilized HRP. Inhibition plots showing the effects of heavy metals on the activity of the immobilized HRP are presented in Figure 5.2. The order of inhibition was found to increase from Pb^{2+} (32.8%), Cu^{2+} (43.4%) to Cd^{2+} (51.1%) in the presence of 0.95 mM H_2O_2. It should be noted that the higher the $I\%$ value the higher the degree of enzyme inhibition by the heavy metal.

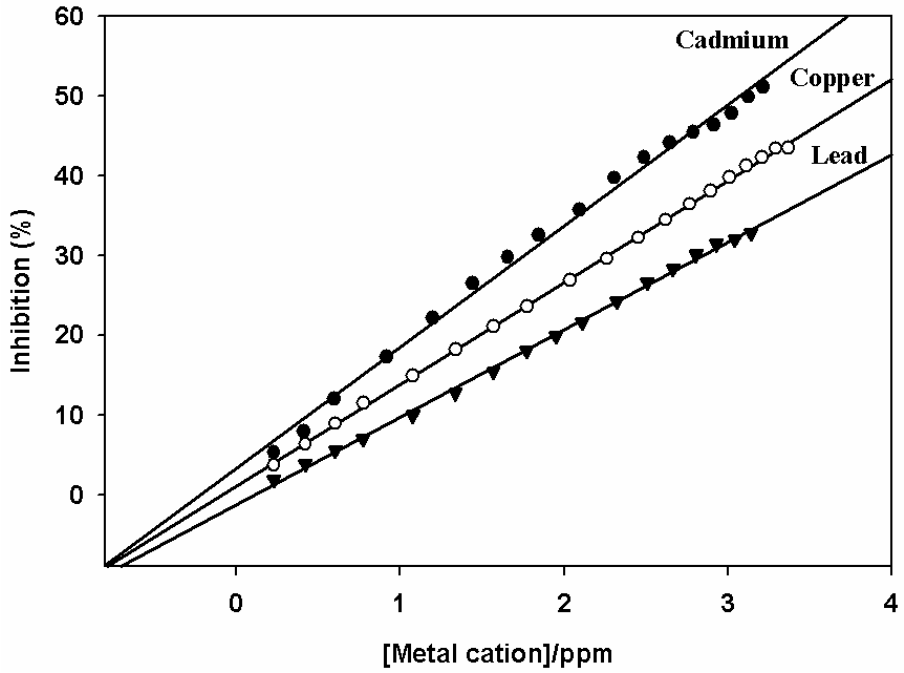


Figure 5.2. Plots showing the Inhibition of Pt/PANI/HRP biosensor by heavy metals; $Cd^{2+,}$ Cu^{2+} and Pb^{2+} .

There are two approaches that can be used for inhibition studies; incubation method and direct method (without incubation) and in this study the latter was employed. It was observed that when using the direct method, it is difficult to attain the concentration that causes 50% inhibition (IC_{50}) (Songa *et al.* 2009); this worked reasonably well for Cd^{2+} (51.1%) with the value for IC_{50} calculated as 3.13 ppm but was not successful for Pb^{2+} and Cu^{2+}. Therefore unlike Cd^{2+}, in order to attain the 50% inhibition (IC_{50} value) for Pb^{2+} and Cu^{2+}, either a higher concentration of the metal can be used or the incubation method can be employed.

After successfully obtaining the IC_{50} value for Cd^{2+} using direct approach, the results from the latter approach were used to investigate the inhibition kinetics and the type of inhibition (competitive or non-competitive).

5.3.1.2 *Investigation of inhibition kinetics and mechanism*

The amperometric response of the biosensor to various H_2O_2 concentrations (in 0.1 M PBS, pH 7.02) in the absence (0 ppm Cd^{2+}) and presence (3.13 ppm Cd^{2+}, IC_{50}) of an inhibitor was recorded at -200 mV (Figure 5.3A). The response of the biosensor to various H_2O_2 concentrations in the presence of an inhibitor was done as follows; HRP electrode was first incubated in PBS containing 3.13 ppm Cd^{2+} for 20 min followed by the successive addition of different concentrations of H_2O_2. A fast response of the biosensor to the additions of different concentrations of H_2O_2 was observed in the absence of Cd^{2+} whereas the presence of 3.13 ppm Cd^{2+} gave a slow response. Studies suggest that during incubation, Cd^{2+} slowly binds to the enzyme and leads to gradual conformational changes (Keyhani *et al.* 2003, Tayefi-Nasrabadi *et al.* 2006). Therefore, it can be suggested that the difference in background current could be due to the conformational changes of HRP. Figure 5.3B shows the Lineweaver–Burk plots for enzymatic reactions. The *slope* and the *y-intercept* values of the linear plots were used to calculate apparent Michealis-Menten constants K_M^{app} and maximum current (I_{max}) in the absence and the presence of Cd^{2+} (Table 7).

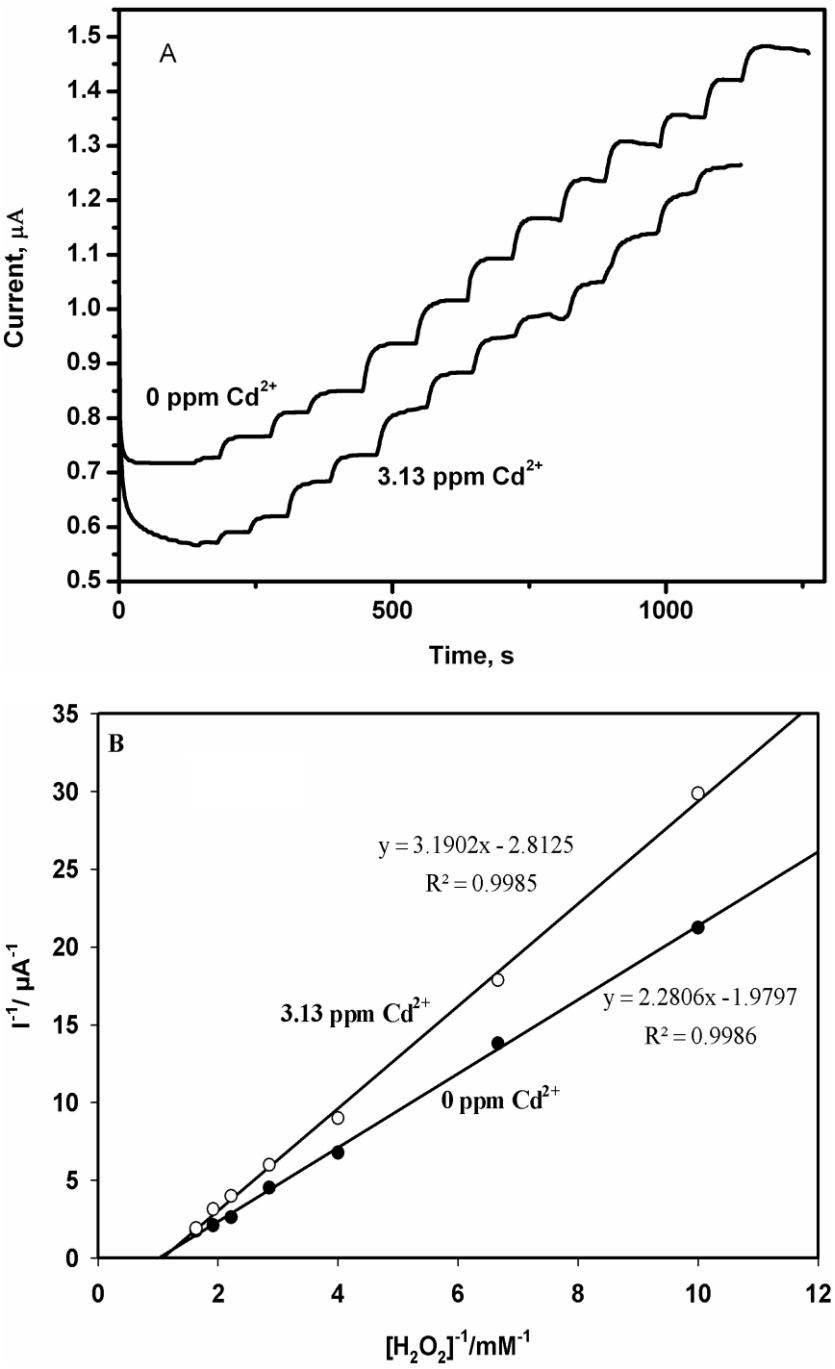
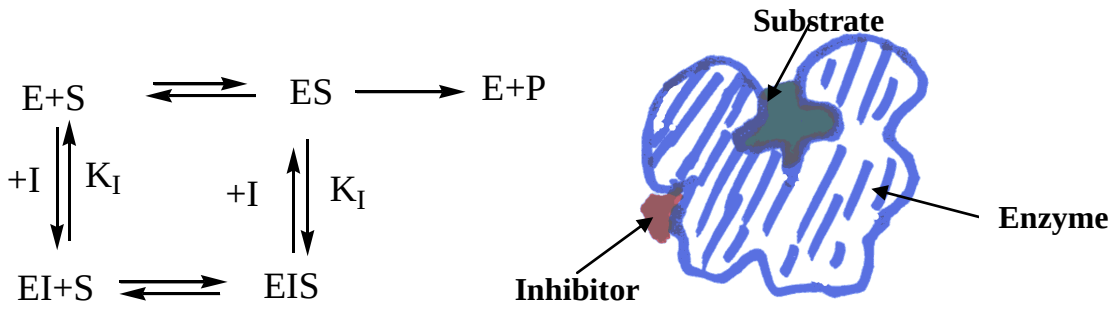


Figure 5.3. (A) Pt/PANI/HRP biosensor response to successive additions of H_2O_2 in the absence and presence of heavy metals, at applied potential of -0.20 V. (B) Lineweaver- bulk plot for HRP response to H_2O_2 in the absence and presence of heavy metals.

Table 7. Apparent Michealis-Menten constants (K_M^{app}) and I_{max} in the absence and the presence of Cd^{2+} (3.125 ppm).

Kinetic parameters	***0 ppm Cd^{2+}***	***3.125 ppm Cd^{2+}***
Slope μA^{-1}mM	2.28	3.19
y-intercept μA^{-1}	1.98	2.81
K_M^{app}/mM	1.16	1.13
I_{max}/μA	0.505	0.356

The K_M^{app} values for Cd^{2+} in the absence (1.16 mM) and the presence (1.13 mM) of the inhibitor were not significantly different (based on statistical t-test, n =3, p = 0.05), whereas the I_{max} values decreased. The decrease in I_{max} indicates that the type of inhibition in this study is reversible and also non-competitive inhibition and the inhibition constant was found to be 27.8 μM (Amine *et al.* 2006; Stoytcheva 2002). Non-competitive inhibition occurs when the inhibitor binds to both the enzyme and enzyme-substrate complex. The possible mechanism of non-competitive inhibition can be seen in Scheme 5.1 (Amine *et al.* 2006**)**; where E represents HRP enzyme, S represents H_2O_2, ES represents Compound I containing an oxyferryl centre with the iron in the ferryl state ($Fe^{IV}=O$), and a porphyrin π cation radical; EIS presents HRP-heavy metal-H_2O_2 complex; I represents heavy metal cation and P represents H_2O.



Scheme 5.1. Schematic diagram drawn to show mechanisms for reversible non-competitive enzyme inhibition.

5.3.1.3 *Selectivity of Pt/PANI/HRP biosensor*

In order to show the selectivity of Pt/PANI/HRP biosensor, the response of possible interferences in tap water (drinking water) components on the determination of heavy metals were investigated. A range of cations were measured to examine whether they interfered with the determination of heavy metal cations. The effect of possible interferences in drinking water such as Mg^{2+}, Zn^{2+}, Ca^{2+}, K^{+}, Fe^{3+} and Na^{+} was investigated by Pt/PANI/HRP biosensor under the same working conditions. Table 8 presents the possible interferences tested with the biosensor. It can be observed that most cations had minimal effect (<5%) on the determination of heavy metals apart from Fe^{3+} which also inhibit HRP activity. However, at the same concentration (1.0 ppm), the degree of inhibition is still less than that of Cd^{2+} (22.8 %), Pb^{2+} (11.9%) and Cu^{2+} (14.8 %).

Table 8. Interference studies showing the effects of cations on the response of Cd^{2+}, Pb^{2+} and Cu^{2+}.

Interferent	Concentration (ppm) Added	%Inhibition
Fe^{3+}	1.0	7.46
Fe^{2+}	1.0	2.01
Zn^{2+}	1.0	1.86
Mg^{2+}	1.0	2.43
Na^{+}	1.0	2.58
K^{+}	1.0	2.06
Ca^{2+}	1.0	2.56

Apart from the degree of inhibition of the interfering species, the selectivity of the biosensor was also evaluated by two methods; mixed and separate solutions (Stefan *et al.* 2001; Macca and Wang 1995) with respect to Cd^{2+}, Cu^{2+} and Pb^{2+}. For the mixed solution method, the concentration of the interfering cation was 1.0 ppm and the concentration of the analyte was 0.5 ppm. In the case of separate solution method, the concentration of the analyte and that of interfering cation were equal (1.0 ppm). Amperometric selectivity coefficients and response ratios were calculated using equations (5.3.2) and (5.3.3) for mixed solutions and separate solutions (Macca and Wang 1995), respectively.

$$K_{i,j}^{amp} = \left(\frac{\Delta I_j}{\Delta I_t - \Delta I_j} \right) \times \frac{c_i}{c_j} \tag{5.3.2}$$

$$R_{i,j} = \frac{\Delta I_j}{\Delta I_i} \tag{5.3.3}$$

where ΔI_t, ΔI_j and ΔI_i are current valued recorded for mixed solution, interfering cation and analyte of interest, respectively; c_j and c_i are concentration of the interfering cation and the analyte, respectively. The $K_{i,j}^{amp}$ and $R_{i,j}$ values in Table 9 suggest that Fe^{3+} is a strong interferent while the other cations species are relatively not interfering with the determination of cadmium, copper and lead. Therefore, the results suggest that Pt/PANI/HRP electrode can be used for the determination of cadmium, copper and lead in the presence of other cations except Fe^{3+}.

Table 9. Interference studies showing the effects of cations on the response of Cd^{2+}, Pb^{2+} and Cu^{2+} using mixed (amperometric selectivity coefficients, $K_{i,j}^{amp}$) and separate (response ratio, $R_{i,j}$) solution methods.

Interferent, j	Amperometric selectivity coefficient, $K_{i,j}^{amp}$			Response ratio, $R_{i,j}$		
	Cd	Cu	Pb	Cd	Cu	Pb
Fe^{3+}	0.80	1.33	2.28	0.56	0.74	0.89
Fe^{2+}	4.06×10^{-3}	4.72×10^{-3}	4.50×10^{-3}	1.10×10^{-2}	1.35×10^{-2}	1.61×10^{-2}
Zn^{2+}	5.69×10^{-3}	6.63×10^{-3}	6.31×10^{-3}	1.42×10^{-2}	1.88×10^{-2}	2.25×10^{-2}
Mg^{2+}	3.64×10^{-3}	4.23×10^{-3}	4.03×10^{-3}	9.12×10^{-3}	1.21×10^{-2}	1.24×10^{-2}
Na^{+}	3.64×10^{-3}	4.23×10^{-3}	4.03×10^{-3}	6.15×10^{-3}	1.12×10^{-2}	1.44×10^{-2}
K^{+}	4.15×10^{-3}	4.15×10^{-3}	3.95×10^{-3}	8.95×10^{-3}	1.19×10^{-2}	1.42×10^{-2}
Ca^{2+}	3.15×10^{-3}	3.66×10^{-3}	3.49×10^{-3}	7.90×10^{-3}	1.05×10^{-2}	1.25×10^{-2}

5.3.2. Application of Pt/PANI/HRP for Analysis Heavy Metals in Tap Water and Landfill Leachate Samples

The performance of the biosensor (Pt/PANI/HRP) was tested using both tap water and landfill leachate water samples. The latter were collected from Sensor Lab at the University of Western Cape (Cape Town, South Africa) and Marrianhill landfill in Durban (South Africa). The quantification of Cd^{2+}, Pb^{2+} and Cu^{2+} in water samples was achieved by employing the standard addition method. For tap water sample, the sample preparation was not required. This is because the possible interferences that are normally present in drinking water samples did not have much effect on the catalytic activity of immobilized HRP. The procedure involved initially measuring the response of the biosensor after subsequent additions of 0.05 mM H_2O_2 followed by metal-spiked tap water (0.1 ppm metal ion solution), into the PBS solution containing H_2O_2 (0.5 mM). On addition of the water sample, current intensity was observed to decrease and the magnitude of the decrease was proportional to the amount of metal spiked. The decrease in the response current was most likely due to the inhibition of the enzyme by the metal ions. A possible dilution

effect due to increased volume of added tap water was checked by setting a control experiment where deionized water was used instead of metal spiked tap water. The inhibition effect was not observed with the deionized water. In order to double-check the effect of metal inhibition, H_2O_2 was added into the mixture and resulted in an increase in current intensity. Similar observations have been made by Han *et al.* (2001); Stoytcheva (2002) and Ghica and Brett (2008).

The concentrations of Cd^{2+}, Pb^{2+} and Cu^{2+} (0.4507 ppb Cd^{2+}, 0.2201 ppb Pb^{2+}, 41.77 ppb Cu^{2+}) in the tap water sample (analysed in triplicate for each metal) were calculated from the calibration curves and the results are presented in Table 10.

Table 10. Comparison between the two analytical techniques: Amperometric biosensor and ICP-OES for determination of heavy metals in Tap water sample.

	Tap water sample		**Landfill leachate water sample**	
Cation	**Amperometric Biosensor**	**ICP-OES**	**Amperometric Biosensor**	**ICP-OES**
Cd^{2+}	0.46 ± 0.004	0.34 ± 0.05	ND	ND
Pb^{2+}	0.22 ± 0.0008	ND	ND	ND
Cu^{2+}	41.8 ± 0.07	41.5 ± 0.2	14.6 ± 0.09	14.0 ± 0.03

Concentrations were determined in ppb; ND: not detected, ± standard error ($SE_{\bar{x}} = \frac{s}{\sqrt{n}}$, where s is the sample standard deviation and n is the size of the sample).

The values obtained were compared against the allowed MCLs by USEPA in drinking water. The MCLs are given as 5 ppb, 15 ppb and 1300 ppb for cadmium, lead and copper, respectively. The World Health Organization (WHO 2004) on the other hand has given the guideline values for cadmium, lead and copper in drinking water as 3.0 ppb, 10 ppb and 2000 ppb, respectively. It can be seen that the concentrations of Cd^{2+}, Pb^{2+} and Cu^{2+} obtained in this study are much lower than the USEPA and WHO guideline values, implying that the drinking water was not contaminated and therefore safe for human consumption.

In the case of landfill leachate water samples, sample preparation was performed. Solid phase extraction (amperometric biosensor) and UV digestion (ICP-OES) were used to minimize organic interferences. After removal of organics the same procedure that was applied for tap water samples was followed. Summary of the concentrations found by the fabricated biosensor are presented in Table 10.

Results of heavy metals obtained by amperometric biosensor and ICP-OES were compared statistically by Student t-test (two-tailed). At 95% confidence interval, the results by the two analytical techniques for the determination of Cu^{2+} were not significantly different (p = 0.035 < 0.05). However, the two methods differed significantly for the determination of cadmium, at 95% confidence interval (P = 0.116 > 0.05). The reason for this discrepancy could be the difference in limits of detection (LOD) capabilities by the two techniques. The detection limit for cadmium was 2.70 ppb with the ICP-OES and 0.091 ppb (91 ppt) with the biosensor. Thus the detection limit for Cd^{2+} obtained with the biosensor is lower by a magnitude of 30 to that by ICP-OES. It should be noted that the concentration of Cd^{2+} in the tap water sample was less than the LOD of ICP-OES (LOD = 2.70 ppb cited from ICP-OES operation manual). This explains the poor precision when the tap water sample was analyzed by ICP-OES in triplicate (3 repeated runs gave 0.41, 0.25 and 0.35 ppb). Lead on the other hand, was not detected probably because its level was lower than 90 ppb (conc. of Pb^{2+} was 0.22 ppb by the biosensor). It can be concluded that the Pt/PANI/HRP biosensor technique is suitable for determination of ultra trace levels of heavy metals in drinking water.

The amount of copper in landfill leachate samples (Table 10) showed good correlation between the results obtained with the biosensor and the standard technique. Applying the Student t test, it was possible to verify that the averages obtained by the both methods which are not significantly different in the confidence level of 95% (P = 0.009 < 0.05). Cadmium and lead were not detected in both methods.

5.4 CONCLUSIONS

An inhibition amperometric biosensor for the determination of selected heavy metals was fabricated on the basis of inhibition to horseradish peroxidase, which was immobilized on platinum-polyaniline electrode. Inhibition of HRP activity by heavy metals followed a noncompetitive reversible mechanism. The HRP biosensor exhibited fast response, high

sensitivity towards the determination of heavy metals (LOD of 0.091, 0.033 and 0.10 ppb for Cd^{2+}, Pb^{2+} and Cu^{2+}, respectively). The fabricated biosensor was applied to the determination of heavy metals in real samples (landfill leachate and tap water). The evaluation of the amperometric biosensor measurements against the standard technique (ICP-OES technique) verified the suitability of the biosensor for rapid analysis of heavy metals. Moreover, the amperometric biosensor requires minimal sample preparation as compared to the tiresome sample pretreatment procedures required prior to metal determination by the conventional ICP-OES method.

CHAPTER 6

AMPEROMETRIC DETERMINATION OF PERSISTENT ORGANIC POLLUTANTS USING HORSERADISH PEROXIDASE BIOSENSOR BASED ON KINETIC INHIBITION

6.1 INTRODUCTION

Pollution caused by persistent organic pollutants (POPs) released into the environment, poses a considerable risk to the environment and human health even at extremely low concentrations. These pollutants are described by marked persistence against chemical or biological degradation, high environmental mobility and strong tendency for bioaccumulation in the food chain (Katsoyiannis and Samara 2006). Persistent organic pollutants consist of a wide range of compounds which are produced by industrial activities. These include polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs) and polychlorinated biphenyls (PCBs), among others. The environmental health effects of these compounds have been discussed in Chapter 1.

Many methods have been proposed for the determination of POPs. The existing methods for detection of POPs utilize gas chromatography, GC (Vonderheide 2009) and high performance liquid chromatography, HPLC (Vonderheide 2009), both coupled to different types of detectors such as electron capture detector, ECD (Wang *et al.* 2006) or mass spectrometry, MS (Tadeo *et al.* 2009) for the GC method; and DAD/UV-Vis (Vilaplana *et al.* 2009) and MS (Bacaloni *et al.* 2009) for the HPLC method. Although the conventional chromatographic methods have high accuracy and low detection limits, they are however sophisticated and require skilled operators. They also require a sample preparation step before detection, which is time consuming and tiresome. For this reason, rapid and simpler methods are required for analysis of these environmental pollutants. Electrochemical biosensors offer an alternative method. This is because electrochemical techniques are easy to use, require minimal sample preparation and are easily miniaturized.

During the last decade, biosensors have been applied as useful monitoring devices in environmental programmes (Rodriguez-Mozaz *et al.* 2006). This is due to the advantages they possess; such as minimizing the sample pretreatment, reducing cost and time of analysis as well

as displaying sufficient sensitivity and selectivity. Recently, attention has turned towards the enzyme inhibition based biosensors (Yang *et al.* 2008). The latter is used to determine the concentration of inhibitors in the sample by measuring the degree of inhibition with lower limits of detection.

The aim of this chapter was to explore the application of Pt/PANI/HRP biosensor for the kinetic determination of PBDEs, PBBs and PCBs. The inhibitory effect on horseradish peroxidase (HRP) activity towards the reduction of hydrogen peroxide (H_2O_2, substrate) by selected POPs has been examined. The proposed biosensor was used to analyze for POPs in landfill leachate samples collected from Marrianhill landfill. The results obtained by the proposed biosensor were cross-checked using gas chromatography–mass spectrometry (GC–MS) technique.

6.2 EXPERIMENTAL

6.2.1 Reagents

All chemicals used in this work were of analytical grade unless otherwise stated. Iso-octane, dichloromethane (DCM), methanol, 2,2′,4,4′,6-pentabromodiphenyl ether (BDE-100), octabromodiphenyl ether (octaBDE) mixture of isomers, 2,4,4'-trichlorobiphenyl (PCB-28), 2,2',4,5,5'-pentachlorobiphenyl (PCB-101), 2-chlorobiphenyl (PCB-1), 2-bromobiphenyl (PBB-1) were obtained from Sigma-Aldrich (South Africa). The details of other chemicals such as H_2O_2 and salts for phosphate buffer preparation are discussed in Chapter 4, Section 4.2.

6.2.2 Instrumentation

Gas chromatography–mass spectrometry analysis was performed on ThermoFinnigan Trace GC equipped with ThermoFinnigan ion trap mass spectrometer detector Polaris-Q (Thermo Electron Corporation, Waltham, MA) equipped with an autosampler. The separation of the analytes was achieved using fused silica SGE forte GC capillary column coated with BPX5 (stationary phase 5 % Phenyl Polysilphenylene-siloxane, 0.25 mm i.d., 0.25 µm film thickness, 30 m length). The temperatures for the GC-MS interface and ion source were 280 °C and 250 °C, respectively. The mass spectrometer was operated in the electron ionization (EI) mode at 70 eV and helium (flow

rate 1 mL min^{-1}) was used as carrier gas. Full scan data acquisition was performed over the mass range of *m*/*z* 150-596. The GC oven temperature program is presented in Table 11**.**

Table 11. Gas oven temperature program

Initial temperature/°C	Ramp/°C min^{-1}	End temperature/°C	Hold time/ min
80	-	80	2
80	50	140	1.5
140	20	220	1
220	2	280	0
300	30	300	10

6.2.3. Determination of POPs in Model Solutions

An aliquot (200 µL) of BDE-100 stock solution (50 ppm in iso-octane) was first dissolved in 200 µL methanol followed by 9.8 mL PBS and the resulting concentration was 1.0 ppm (BDE-100). A concentration of 0.1 ppm was prepared by further diluting 1.0 ppm in a 10 mL volumetric flask. Amperometric measurements for enzyme inhibition by BDE-100 were carried out in an electrochemical cell containing 2.0 mL of 0.1 M PBS (pH 7.02) and constant concentration H_2O_2 (0.5 mM) with continuous stirring. The experiments were carried out at -0.20 V *versus* Ag/AgCl (3 M NaCl) while allowing the steady-state current to be attained. The desired volume of the inhibitor stock solution (1.0 ppm BDE-100) was then added using a micropipette with continuous stirring until a steady-state current was obtained. After each experiment the enzyme electrode activity was regenerated by rinsing the electrode with distilled water. Stock solutions (100 ppm) of PCB-1, PCB-28 and PCB-101 were prepared by dissolving 1.0 mg of each compound in 100 µL methanol and then diluting it to 10 mL in a volumetric flask using PBS (0.1 M, pH 7). The stock solution (1000 ppm) of PBB-1 was prepared by dissolving 75 µL in 100 µL of methanol and then diluting it to 100 mL in a volumetric flask using PBS (0.1 M, pH 7).

Working solutions of PCB-1, PCB-28, PCB-101 and PBB-1 were prepared by serial dilution of the stock solutions in PBS.

6.2.4 Analysis of POPs in Landfill Leachate Sample

Landfill leachate sample was collected from the Marrianhill landfill (Ethekwini municipal solid waste disposal). The leachate water sample was collected in a polyethylene container and stored in the fridge at 4 °C. Determination of POPs in the leachate sample was performed by two methods, that is, amperometric biosensor and GC-MS methods. The latter was performed for the validation of the results obtained by the fabricated amperometric biosensor. For amperometric analysis, the determination of POPs in leachate water sample was achieved using standard addition method. The landfill leachate was filtered to remove the solid particles and then spiked with 0.1 ppm of each standard solution followed by amperometric measurements.

For GC-MS analysis, the landfill leachate sample was filtered to remove the solid particles. The samples were extracted by subjecting them to C-18 solid phase extraction cartridge. Prior to use, the cartridge were activated with 5 mL methanol followed by 5 mL water. After sample loading the SPE cartridges was rinsed with 5 ml water. The SPE cartridge was eluted with 5 ml hexane or DCM and the extract was concentrated to about 2 ml by bubbling nitrogen followed by GC-MS analysis.

6.3 RESULTS AND DISCUSSION

6.3.1 Detection of Selected Brominated Flame Retardants and Polychlorinated Biphenyls in Model solution

The effect of selected POPs (three BFRs and three PCBs) on the catalytic activity of the immobilized HRP was evaluated by amperometry. The three BFRs include two polybrominated diphenyl ethers (BDE-100 and octaBDE) and one polybrominated biphenyl (PBB-1). Polychlorinated biphenyls on the other hand include PCB-1, PCB-28 and PCB-101. Amperometric measurements were carried out in a cell containing 2 mL PBS, at -200 mV under continuous magnetic stirring. Figure 6.1 shows the typical amperometric Pt/PANI/HRP biosensor's response to successive additions of 10 µL of 0.01 M H_2O_2 (r) followed by successive additions of aliquots (µL) of BDE-100 standard solutions (0.1 ppm) (i) in PBS.

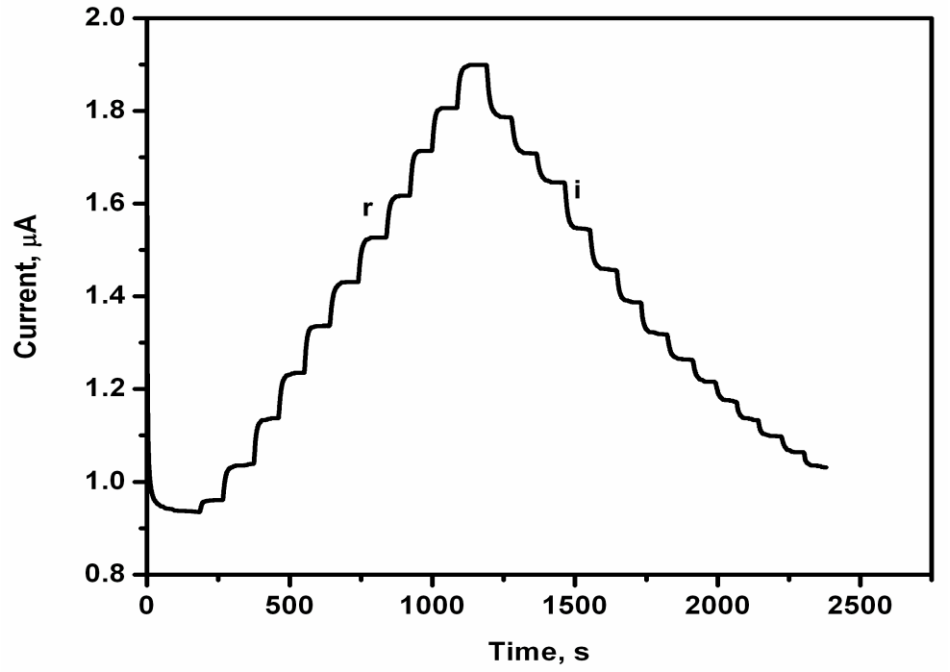


Figure 6.1. Typical amperometric responses of Pt/PANI/HRP biosensor to successive additions of 0.01 mM H_2O_2 (r) and BDE-100 (i). Applied potential: −0.20 V; supporting electrolyte: 0.1 M PBS (pH 7.02).

It can be seen that after each addition of BDE-100, the intensity of the response current decreased. The latter indicated that BDE-100 inhibits the activity of HRP. The other four POPs (PBB-1, PCB-1, PCB-28 and PCB-101) showed the same behavior. All the POPs were measured in the range of 0.1 to 44.2 ppb and the linear ranges, limits of detection (LOD= $\frac{3\times SD}{m}$), limits of quantification (LOQ= $\frac{10\times SD}{m}$) as well as regression coefficients for each compound are presented in Table 12. The abbreviation *SD* refers to standard deviation of the blank signal (n = 8) obtained in PBS (SD = 4.8×10^{-5} μA) and m is the slope of the calibration curve. The biosensor was used to perform several measurements for each analyte before its performance level declined. This suggests that the type of inhibition is reversible because HRP recovers its activity when BDE-100 is not present in the supporting electrolyte. That is, the background current of the Pt/PANI/HRP biosensor returned to the original value when a new PBS (without BDE-100) was measured (Vidal *et al.* 2008; Songa *et al.* 2009). The response time of the

fabricated biosensor reaches 95 % of its maximum response at about 5-7 seconds. As seen in Table 12, the fabricated Pt/PANI/HRP biosensor exhibits a long linear range, relatively high sensitivity and low LODs for all the tested POPs.

Table 12. A summary of analytical characteristics and regression parameters for calibration curves for determination of POPs.

POPs	**Linear range/ppb**	**Sensitivity/μAppb^{-1}**	**LOD/ppb**	**LOQ/ppb**	**R^2**
BDE-100	0.424-25.8	$9.38\times10^{-3} \pm 0.005$	0.014	0.048	0.9983
OctaBDE	Not detected	-	-	-	-
PBB-1	0.862-13.4	$7.56\times10^{-3} \pm 0.002$	0.018	0.059	0.9991
PCB-1	0.930-18.9	$6.24\times10^{-3} \pm 0.004$	0.022	0.072	0.9966
PCB-28	0.730-15.7	$8.29\times10^{-3} \pm 0.006$	0.016	0.054	0.9939
PCB-101	0.930-27.1	$6.95\times10^{-3} \pm 0.005$	0.019	0.063	0.9959

The biosensor shows high sensitivity towards the determination of BDE-100 in aqueous media. However, due to high hydrophobicity of octaBDE, no observable results were obtained. OctaBDE dissolves only in selected hydrophobic organic solvents (DCM). Although the biosensor was found to respond to BDE-100, it was concluded that in aqueous media only, can hydrophilic PBDEs be analyzed using the fabricated biosensors. Therefore, there is a need for an organic-phase biosensor for the detection of highly hydrophobic PBDEs. Organic phase biosensors are known to offer a favorable environment for detection of water-insoluble analytes and less interference from water-soluble analytes (Wu *et al.* 2004; Wu *et al.* 2007). Since it was not possible to determine octaBDE in aqueous media, methanol was used as supporting electrolyte (instead of PBS). Electrocatalytic reduction of H_2O_2 was found to be very slow and the biosensor was not as stable as in aqueous media. The extent of stability was found to affect

both reproducibility and repeatability. Therefore, the determination of octaBDE mixture of isomers was not successful. Wu *et al.* (2007) reported that the major challenge in the construction of an organic phase biosensor is that the biosensor demonstrates poor stability. Construction of organic phase biosensor for detection of highly hydrophobic PBDEs requires more time so that all analytical parameters are optimized.

6.3.1.1 *Inhibition studies*

The values of the steady state current in the absence (I_0) and presence (I_i) of an inhibitor were determined from the recorded amperograms similar to that presented in Figure 6.1. The inhibition percentage ($I\%$) was calculated as per equation (5.3.1) in Chapter 5. In order to obtain the inhibitors concentration that causes 50% inhibition (IC_{50}), 1.0 ppm was used instead of 0.1 ppm. The obtained $I\%$ values were used to compare the inhibitory effect of POPs on immobilized HRP enzyme's activity. Calibration curve constructed by plotting the $I\%$ of HRP activity against the concentration of BDE-100, is shown in Figure 6.2. Similar calibration curves were obtained for the other POPs in this study (results shown here).

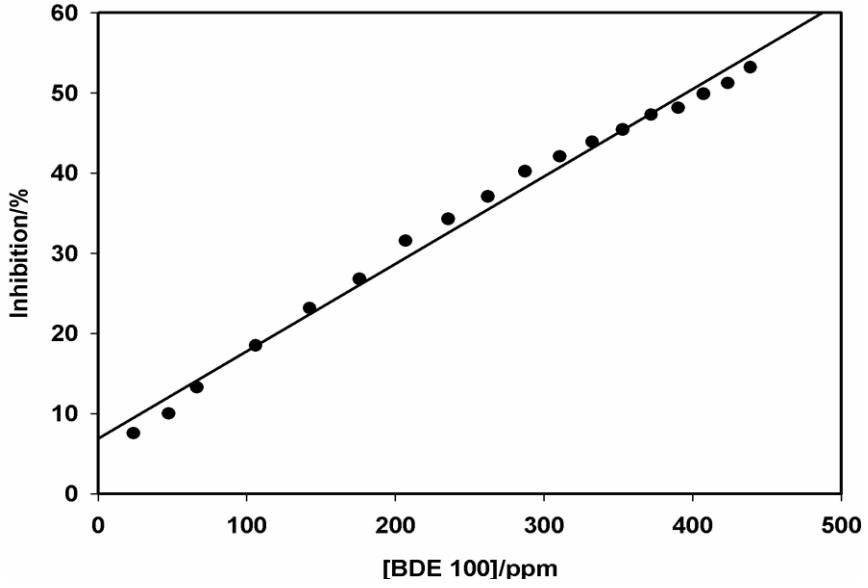


Figure 6.2. Calibration curve showing the inhibition of HRP activity by BDE-100

It was observed that the degree of inhibition increased with an increase in concentration of an inhibitor. The sequence of inhibition to HRP activity is as follows; BDE-100 > PCB-101 > PCB-28 > PCB-1 > PBB-1. The inhibition percentages as well as the inhibitor concentration leading to 50% inhibition (IC_{50}) are presented in Table 13.

Table 13. Inhibition percentages and the inhibitor concentration leading to 50% inhibition (IC_{50}).

POPs	**Degree of inhibition/%**	**IC_{50}/ppm**
BDE-100	53.2 ± 1.9	0.407 ± 0.291
PBB-1	50.8 ± 0.97	0.487 ± 0.132
PCB-1	51.7 ± 1.3	0.531± 0.154
PCB-28	52.3 ± 0.57	0.542± 0.113
PCB-101	52.8 ± 1.2	0.506 ± 0.511

6.3.1.2 *Investigation of inhibition kinetics and mechanism*

The inhibition mechanism was studied by investigating the relationship between the Pt/PANI/HRP biosensor's response current to H_2O_2 (substrate) concentration in the absence and presence of the inhibitor. The biosensor's response to various H_2O_2 concentrations in the presence of an inhibitor was done as follows; HRP electrode was first incubated in PBS containing a known concentration (IC_{50}) of each compound for 20 min followed by the successive addition of different concentrations of H_2O_2. The biosensor's response to various H_2O_2 concentrations recorded in the absence and in the presence of BDE-100 (Figure 6.3A) were interpreted using the Lineweaver-Burk plots (Figure 6.3B). In the absence of the 0.407 ppm (IC_{50}) BDE-100, fast response to the additions of different concentrations of H_2O_2 was observed

whereas in the presence of BDE-100 it was slow. The experiment was repeated for other POPs and similar behavior was observed. Sariri *et al.* (2006a&b) and Zaton and de Aspuru (1995) suggested that the inhibition of HRP activity by phenyl containing compounds could be due to the incorporation of the phenyl group into enzyme molecules at the heme periphery thus leading to a decrease in HRP activity. The *slope* and the *y-intercept* values of the linear plots were used to calculate kinetic parameters (apparent Michealis-Menten constants (K_M^{app}) and maximum current (I_{max}). The K_M^{app} and I_{max} values in the absence and presence of selected POPs are presented in Table 14.

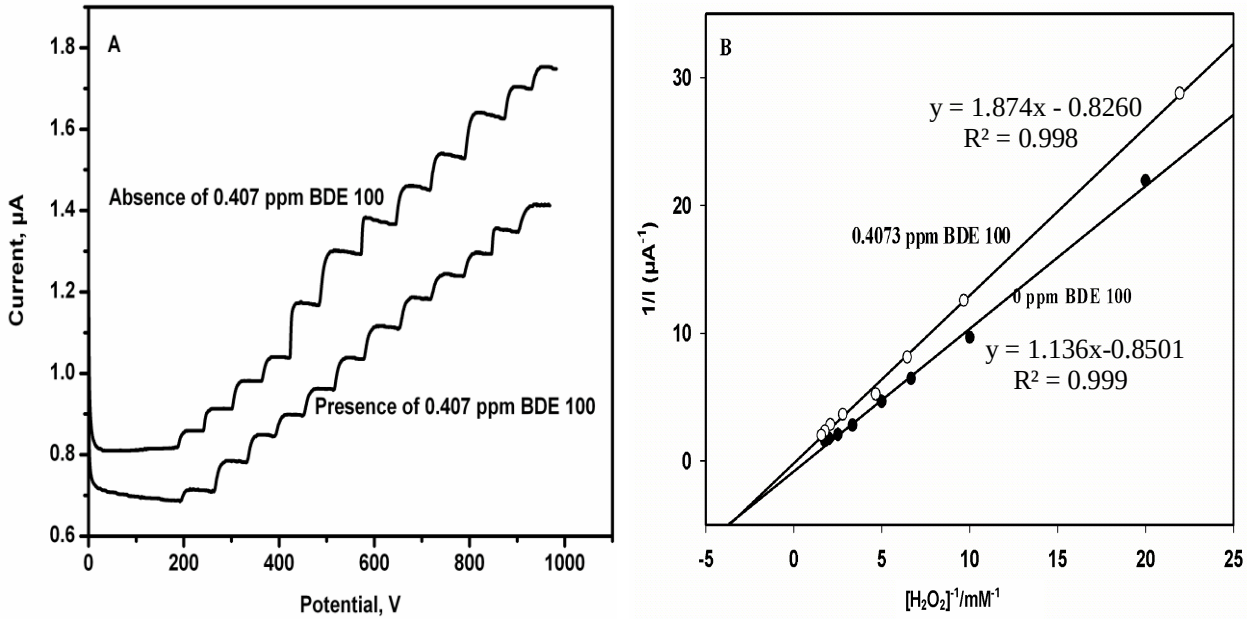
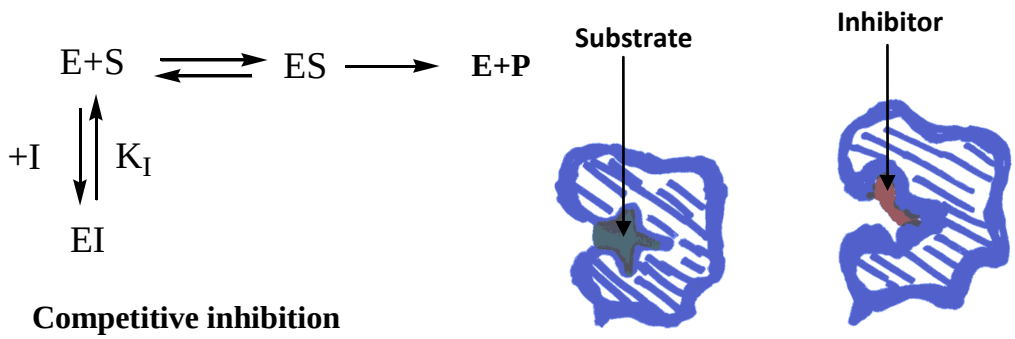


Figure 6.3. (A) Pt/PANI/HRP biosensor response to successive additions of H_2O_2 in the absence and presence of BDE 100, at applied potential of -0.20 V. (B) Lineweaver- bulk plot for HRP response to H_2O_2 in the absence and presence of BDE-100.

Table 14. Apparent Michealis-Menten constants and maximum current in the absence and the presence of POPs.

POPs	Kinetic parameters			
	K_M^{app} /mM		I_{max} /µA	
	Absence of inhibitor	Presence of inhibitor	Absence of inhibitor	Presence of inhibitor
BDE-100	1.34 ± 0.03	2.27 ± 0.09	1.18 ± 0.02	1.21 ± 0.08
PBB-1	2.97 ± 0.13	3.01 ± 0.07	1.16 ±0.03	0.984 ± 0.14
PCB-1	3.03 ± 0.09	2.99 ± 0.15	1.24 ±0.05	0.978 ± 0.07
PCB-28	3.05 ± 0.02	3.10 ± 0.11	1.12 ±0.06	0.991 ± 0.03
PCB-101	3.01 ± 0.05	3.07 ± 0.06	1.18 ±0.10	0.986 ± 0.08

Enzyme kinetic parameters (K_M^{app} and I_{max}) in Table 14 were used to estimate the inhibition mechanism of POPs to HRP activity. It can be observed in Table 14 that the presence of BDE-100 affected the value of K_M^{app} but the I_{max} values remain unchanged (1.2 µA) irrespective of the absence or presence of the inhibitor. This indicates that BDE-100 bound to the same HRP active sites (causing a decrease in the amount of free HRP) available for H_2O_2 binding, thus increasing the K_M^{app} (Amine *et al.* 2006). Based on these results it was concluded that the inhibition mechanism in this case is competitive inhibition. The possible inhibition mechanism in Scheme 6.1 suggests that BDE-100 (inhibitor, I) binds only to free enzyme (E) active sites rather than enzyme–substrate (ES) complex (Li *et al.* 2010).



Scheme 6.1. Schematic diagram drawn to show the mechanisms for reversible competitive enzyme inhibition

Polychlorinated biphenyls and polybrominated biphenyls, on the other hand affected the activity of HRP in a different manner. The K_M^{app} values in the absence and the presence of each biphenyl were not significantly different (based on statistical student *t*-test, n =3, P = 0.001< 0.05), whereas the I_{max} values were affected (Table 14). The decrease of I_{max} values suggested that the inhibition mechanism is non-competitive (Amine *et al.* 2006). This implied that PCBs and PBBs bind to HRP active sites different from where H_2O_2 binds (Scheme 5.1 presented in Chapter 5 Section 5.3). The PBBs and PCBs can be either bound to free HRP or to HRP-H_2O_2 complex (ES). This results in the conformational change of HRP at active sites, thus leading to a decrease in the overall rate of the reaction I_{max} (Amine *et al.* 2006).

Inhibition constant (K_i) is an absolute value dependent only on the inhibitor-enzyme affinity. Generally, the smaller the K_i value, the stronger the binding, and hence the more effective an inhibitor is. Apparent K_i values for the competitive and non-competitive inhibitor were calculated from the relationship reported by Besombes *et al.* 1995 and Tanimoto de Albuquerque and Ferreira 2007, using equation (6.3.1) and (6.3.2), respectively.

$$K_i = \frac{K_M^{app}[I]}{K_M^{'app} - K_M^{app}} \qquad (6.3.1)$$

$$K_i = \frac{I_{max}^{'}[I]}{I_{max} - I_{max}^{'}} \qquad (6.3.2)$$

where $K_M^{'app}$ and $I_{\max}^{'}$ are Michaelis-Menten constant and maximum current in the presence of an inhibitor, respectively. The K_i values (Table 15) suggested that the immobilized HRP enzyme presents high affinity to the POPs, particularly, BDE-100, which explains the high degree of inhibition of the HRP activity by POPs (Table 15).

Table 15. The K_i values in nM for HRP inhibition by POPs using H_2O_2 as a substrate

POPs	**Inhibition constant, K_i/nM**
BDE-100	0.983
PBB-1	11.7
PCB-1	10.5
PCB-28	9.22
PCB-101	7.88

6.3.1.3 *Selectivity of Pt/PANI/HRP biosensor*

Selectivity of the biosensor is very important for the construction and application of the biosensor. Amperometric measurements of Cu^{2+}, Fe^{2+}, Cd^{2+}, Pb^{2+} and phenol as interferents were measured under the same working conditions. The effect of the interferents on the detection signal was investigated by adding about 0.5 mM H_2O_2 in PBS, 0.5 ppm BDE-100 or PCB-101 and different concentration levels of interferents. Phenol did not interfere with the biosensor response except for Cu^{2+}, Fe^{2+}, Cd^{2+} and Pb^{2+} which also inhibits the activity of HRP.

6.3.2. Analysis of Selected Brominated Flame Retardants and Polychlorinated Biphenyls in Landfill Leachate Samples

6.3.2.1 *The Pt/PANI/HRP biosensor*

Landfill leachate samples were examined in order to demonstrate the applicability of the Pt/PAN/HRP biosensor. The leachate sample was first filtered using 0.45 µm pore size filter to remove insoluble residue. Determination of POPs in the leachate samples were achieved by employing the standard addition method. The sample was spiked with POP standard so as to obtain a final concentration of 0.10 ppm and analyzed by Pt/PAN/HRP biosensor. Amperometric measurements were carried out as discussed in Section 6.3.1, but for this experiment PBS contained 0.5 mM H_2O_2 instead of 0.95 mM. The concentrations of the detected POPs (PCB-28 and PCB-101) were calculated from the calibration curve. Their concentrations were calculated as 0.28 ± 0.03 and 0.31 ± 0.02 ppb for PCB-28 and PCB-101, respectively. The concentration values obtained for PCBs were compared with the allowed maximum contaminant level (MCL) (0.5 ppb) set by United States Environmental Protection Agency (USEPA 1991). The values obtained from the leachate sample were lower than the values recommended by USEPA.

6.3.2.2 *Gas Chromatography- Mass Spectrometry*

The validation of the results obtained by the fabricated amperometric biosensor was performed by using gas chromatography coupled to mass spectrometry (GC–MS) as the standard method. The presence of the target analytes detected by GC-MS in the leachate sample was confirmed by comparing their mass spectra to the National Institute of Standards and Technology (NIST, MS Search 2.0) 2002 library. Full scan EI spectra of components found in the leachate sample with fragmentation indicative of PCB-101 is presented in Figure 6.4. The mass spectra were assigned according to Safe and Hutzinger (1972), Medina *et al.* (2009) and Ramos *et al.* (2007). In the EI spectrum an intense molecular ion (M^+) is found at *m/z* 326 ($[C_{12}H_5{}^{35}Cl_4{}^{37}Cl]^+$). A less intense molecular ion is observed at m/z 324 ($[C_{12}H_5{}^{35}Cl_5]^+$). Loss of one (^{35}Cl), two ($2 \times {}^{35}Cl$, in the case of M^+ at *m/z* 326 and 324) and four chlorine atoms ($3 \times {}^{35}Cl$ and ^{37}Cl) molecules leads to formation of the major fragment ions at m/z 291 ($[C_{12}H_5{}^{35}Cl_4]^+$), 256 ($[C_{12}H_5{}^{35}Cl_2{}^{37}Cl]^+$), 254 ($[C_{12}H_5{}^{35}Cl_3]^+$) and 184 ($[C_{12}H_5{}^{35}Cl]^+$), respectively.

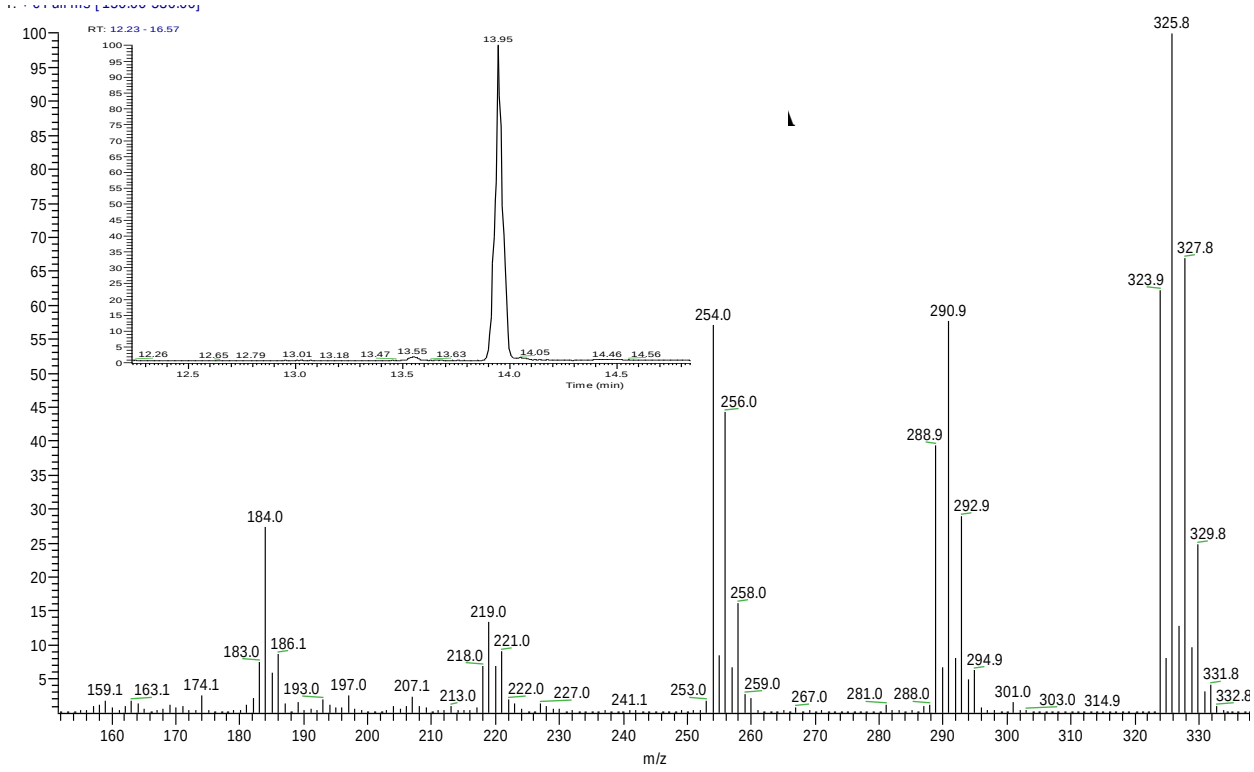


Figure 6.4. Electron ionization mass spectra of PCB-101. Inset: gas chromatogram of PCB-101

In the mass spectrum of PCB-28 (Figure 6.5), the molecular ion was observed at m/z 258 ($[C_{12}H_7{}^{35}Cl_2{}^{37}Cl]^+$), along with ions at m/z 256 ($[C_{12}H_7{}^{35}Cl_3]^+$), 186 ($[C_{12}H_7Cl]^+$) and 151($[C_{12}H_7]^+$). The fragment ion at *m/z* 186 was due to the loss of two chlorine atoms (2 × ^{35}Cl (in the case of M^+ at *m/z* 256) or ^{35}Cl and ^{37}Cl (in the case of M^+ at *m/z* 258)). The loss of three chlorine atoms (2 × ^{35}Cl and ^{37}Cl) leads to the formation of fragment ion at *m/z* 151. The type of fragmentation obtained for both compounds was found to be in close agreement (100%) to data given in the NIST library.

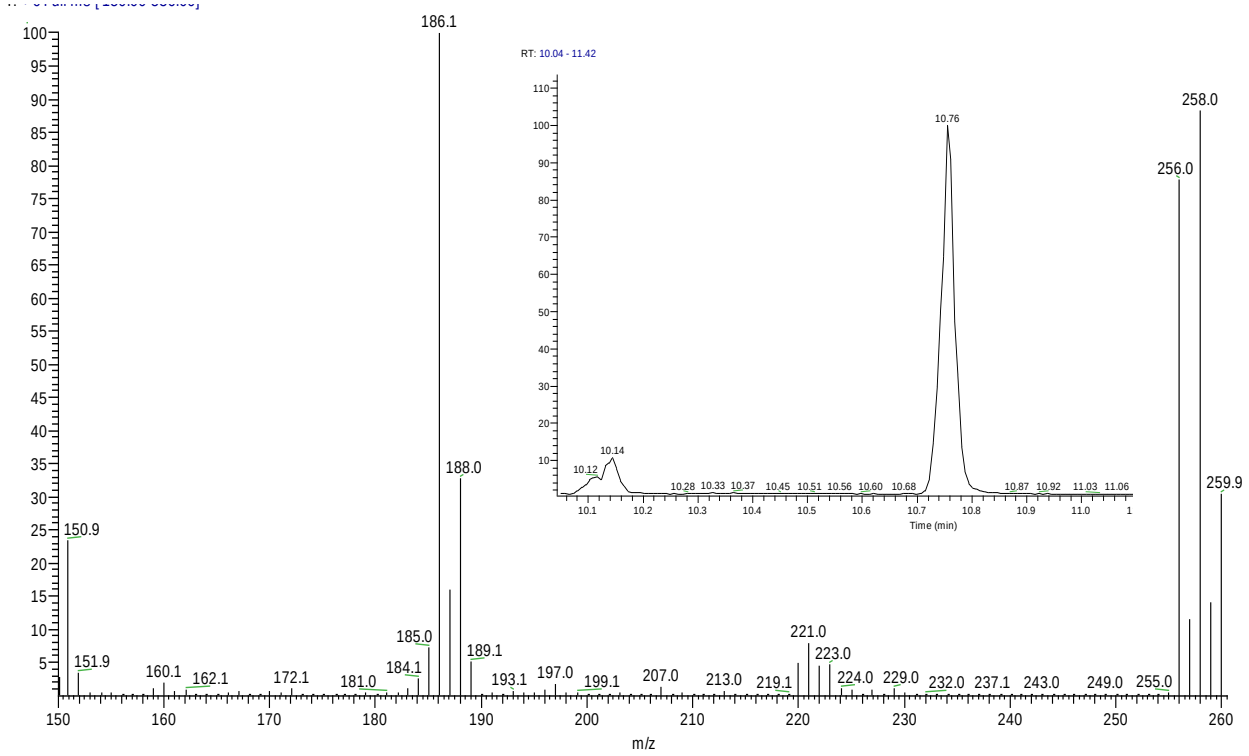


Figure 6.5. Electron ionization mass spectra of PCB-28. Inset: gas chromatogram of PCB-28.

The results obtained with the proposed biosensor corroborated well with those obtained by GC-MS. Both methods recognized (detected) the presence of the two PCB congeners (PCB-28 and PCB-101). However, it was necessary to spike the individual PCB congeners, one at a time, so as to differentiate their specific effects on the HRP activity. Notwithstanding this limitation, the Pt/PANI/HRP biosensor was established to be robust with higher throughput and is thus suitable for rapid monitoring of POPs in the environment. The Pt/PANI/HRP biosensor can also be used as a screening method for different halogenated aromatic hydrocarbons. The advantage of using the biosensor as a screening method is that it allows the analysis of a large number of samples such that the samples that give positive response will then be re-analyzed by chromatographic methods in order to determine the target POPs responsible for the enzyme inhibition (Suwansa-ard *et al.* 2005).

6.4 CONCLUSIONS

This chapter reports the application of a Pt/PANI/HRP biosensor for the determination of selected POPs. The determination of the latter was achieved by enzyme inhibition mechanism. The degree of inhibition was found to increase with an increase in concentration of the inhibitor. The percentage inhibition of the investigated inhibitors decreases in the following order: 53.2 %, 52.8 %, 52.3 %, 51.7 and 50.8 % for BDE-100, PCB-101, PCB-28, PCB-1 and PBB-1, respectively. The inhibition mechanism was found to be competitive type for PBDEs but non-competitive type for PBBs and PCBs. Thus, the proposed amperometric biosensors was fast, sensitive and showed good linear relationship as well as low limits of detection in the determination of selected POPs. The LODs and LOQs obtained are low enough to detect trace amounts of PBDEs, PBBs and PCBs in environmental matrices. Therefore the use of HRP amperometric biosensors for the determination of selected PBDEs, PBBs and PCBs in landfill leachate samples has been demonstrated. The presence of PCBs in the real samples was detected by the Pt/PANI/HRP biosensor and GC-MS analysis. The concentration values (0.28-0.31 ppb) obtained from the leachate samples were lower than the values recommended by USEPA (0.5 ppb). The evaluation of the amperometric biosensor measurements against the standard technique (GC-MS technique) verified the suitability of the biosensor for rapid analysis of heavy metals. Moreover, the amperometric biosensor requires minimal sample preparation as compared to the tiresome sample pre-treatment procedures required prior to metal determination by the conventional GC-MS method.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

7.1 CONCLUSIONS

An inhibition amperometric biosensor for the determination of POPs and heavy metals based on HRP enzyme immobilized on a Pt/PANI electrode has been studied. The principle of the inhibitive biosensor was based on the measurement of the reduced current intensity occurring as a result of inhibition of the catalytic activity of the immobilized HRP enzyme.

The horseradish peroxidase enzyme was immobilized on the PANI-modified Pt electrode by electrostatic attachment. The UV-Vis and FTIR results confirm that the incorporation of HRP in the PANI film did not destroy its structure. The results also showed that HRP was attached and retained its biological activity after immobilization on PANI modified Pt electrode. Polyaniline did not only serve as a point of attachment for HRP enzyme but it also facilitated the electron transfer between the redox active centre of the enzyme and the electrode surface. Electrochemical measurements (CV, DPV and amperometry) showed that the immobilized HRP exhibited high biological activity. The fabricated Pt/PANI/HRP biosensor exhibited fast amperometric response (5-7 s), low detection limit (36.8 nM) and wide linear range to H_2O_2 (0.05 to 3.17 mM). In addition, the biosensor exhibited high sensitivity, good reproducibility (%RSD = 3.8), precision (%RSD = 2.1 and stability (30 days) and then it can said to be suitable for routine analysis of hydrogen peroxide.

In the presence of constant H_2O_2 concentration, POPs and heavy metals inhibited the HRP activity towards the reduction of H_2O_2. The degree of inhibition was found to increase with an increase in concentration of the inhibitor. The percentage inhibition of the investigated inhibitors decreases in the following order 53.2 %, 52.8 %, 52.3 %, 51.7 and 50.8 % for BDE-100, PBB-1, PCB-1, PCB-101 and PCB-28, respectively. The inhibition mechanism was found to be the competitive type for PBDEs but was of the non-competitive type for PBBs and PCBs. In the case of heavy metals, the degree of inhibition of heavy metals was higher for Cd^{2+} (51.1%) followed by Cu^{2+} (43.4 %) and Pb^{2+} (32.8 %). From the Lineweaver-Burk plot, the type of inhibition was found to be reversible and non-competitive character.

The HRP biosensor exhibited high sensitivity towards the detection of POPs (LOD of 0.014 to 0.022 ppb) and heavy metals (LOD of 0.033 to 0.10 ppb). The biosensor was applied for

the determination of POPs and heavy metals in real samples (landfill leachate and tap water). The comparison of the proposed amperometric biosensor measurements with that of standard methods such as GC–MS and ICP-OES, verified the suitability of the biosensor for rapid analysis of these environmental pollutants (POPs and heavy metals), as compared with the tiresome sample pre-treatment methods required by the conventional method of analysis for metals (ICP-OES) and POPs (GC-MS).

7.2 FUTURE WORK

Horseradish peroxidase based inhibition biosensor for aqueous media are well developed. However, due to the instability of the enzyme and the immobilization matrix they are usually not suitable for highly hydroscopic samples (Konash and Magner 2006). Organic phase biosensors are known to offer a favorable environment for detection of water-insoluble analytes and less interference from water-soluble analytes (Wu, Choi and Wu 2004; Wu *et al.* 2007). Therefore future developments will include searching for ways to construct an organic phase HRP biosensor for determination of highly hydroscopic POPs in different environmental matrices. Future work will also include the use of screen-printed electrodes to produce disposable PANI/HRP coated screen-printed electrodes. These electrodes can be used for on-site application and analysis of field samples.

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