POLYMERIC TYROSINASE NANOBIOSENSOR SYSTEM FOR THE DETERMINATION OF ENDOCRINE DISRUPTING BISPHENOL A

VIRGINIA BUSISWA MATYHOLO

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University of the Western Cape

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Supervisor: Prof. Emmanuel I. Iwuoha

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Dedication

To my mother Nondumiso Pamla

To my aunt Zodwa Gqibela

To my late grandfather and grandmother, Ben and Nongqubela Gqibela

To my late Uncle Mabhelandile Michael Gqibela and Cousin Andisiwe Guleni

To my late grandfather and father, Velile and Pumzile Matyholo

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Declaration

I declare that ***Polymeric tyrosinase nanobiosensor system for the determination of endocrine disrupting bisphenol A*** is my own work**,** that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Virginia Busiswa Matyholo **May 2011**

Signature ----------

Supervisor: Prof. Emmanuel I. Iwuoha

Keywords

Poly(2,5-dimethoxyaniline)

Poly(4-styrenesulfonic acid)

Bisphenol A

Tyrosinase; Biosensor; Glassy carbon electrode

Cyclic voltammetry

Steady-state amperometry

Differential pulse voltammetry

Square wave voltammetry

Electrochemical impedance spectrometry

Abstract

Bisphenol A (4,4'-isopropylidenediphenol) also known as BPA, is a xenoestrogenous compound considered to be potentially harmful to human health and the environment. It's a typical product of the industrial society produced in large quantities worldwide, 90% or more being used as a monomer for the production of polycarbonate, epoxy resins, and unsaturated polyester-styrene resins. Thus, BPA is present in baby bottles, foods and beverage containers, as well as in lacquers coating metal products, such as food cans or water supply pipes. Human exposure to BPA eventually released from these materials is of increasing concern due to its endocrine-disrupting potential. Therefore, the development of a simple, rapid and sensitive method for BPA detection is very important and interesting. Herein, a sensitive and low cost electrochemical biosensor for quantitative determination of BPA is reported. Firstly, the electrochemical behavior of BPA at the bare glassy carbon electrode and tyrosinase glassy carbon electrode Tyr/GCE were investigated by voltammetric techniques. Electrochemical oxidation of bisphenol A led to the GCE deactivation as a result of electropolymerized film deposited on electrode surface. The fouling of the electrode surface by the electropolymerized film was evaluated by monitoring the electrode response of phosphate buffer.

Electrochemical activation and optimum pH response of free tyrosinase on GCE was evaluated at fixed concentration of 0.001 M bisphenol A in phosphate buffer ranging from pH 4.2-9.2. The optimum pH was found to be pH 7.2 meaning the tyrosinase enzyme was active at this pH. The biosensor was then constructed by electrochemically depositing poly(2,5-dimethoxyaniline)

(PDMA) doped with poly(4-styrenesulfonic acid) (PSS) onto GCE surface. Voltammetric are based on cyclic voltammetry (CV), square wave voltammetry (SWV), and differential pulse voltammetry (DPV). Analyses spectroelectrochemistry was used to monitor the changes in UV-Vis properties of undoped PDMA and doped PDMA film, electrochemical impedance spectrometry used to monitor charge transfer resistance, time constant, exchange current and impedimetric response of bare GCE, undoped PDMA film, doped PDMA-PSS film and biosensor. Bare GCE showed small time constant and exchange current when compared to biosensor because the electron transfer is faster due to direct interaction of electron to the GCE surface, however biosensor showed small semi-circle when its compared to bare and PDMA-PSS influenced by the presences of tyrosinase. Scanning electron microscopy (SEM) for the polymer surface morphology using undoped PDMA and doped PDMA-PSS screen printed carbon electrodes (SPCEs). The SEM images showed modification of the conducting film surface structure when the dopant is present and when it absent on the morphology of conducting polymer. The developed biosensor was then applied for BPA detection followed by the investigation of the kinetics of the tyrosinase-BPA interactions by employing square wave voltammetry, electrochemical impedance spectroscopy and steady state amperometry. Under optimized conditions, the detection limit for BPA by the developed biosensor was found to be 0.013 mM within a concentration range of 0.02-0.28 mM with the response time reached 95% within 12 seconds. The biosensing principle was based on the determination of the sensor response to BPA by amperometric, impedimetric and voltammetric methods.

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Abbreviations

PDMA	Poly(2,5-dimethoxyaniline)
PSS	Poly(4-styrenesulfonic acid)
DMA	2,5-dimethoxyaniline
CP	Conducting polymer
BPA	Bisphenol A
EDCs	Endocrine disrupting compounds
GCE	Glassy carbon electrode
Tyr	Tyrosinase
PBS	Phosphate buffer solution
EPA	Environmental protection agency
PPy	Polypyrrole
PTh	Polythiophene
PANI	Polyaniline
PPh	Polyphenylene

SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
UV-Vis	Ultra violet-visible
Rpm	Rotations per minute
EIS	Electrochemical impedance spectrometry
CV	Cyclic voltammetry
DPV	Differential pulse voltammetry
SWV	Square wave voltammetry
GC	Gas chromatography
CE	Capillary electrophoresis
LC	Liquid chromatography
MS	Mass spectrometry
HPLC	High performance liquid chromatography

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background information

Exogenous agents that interfere with the biosynthesis, secretion, transport, binding, action, metabolism, or elimination of endogenous hormones responsible for homeostasis, reproduction, and developmental process in the body are known as endocrine-disrupting compounds (Coille *et al.* 2002). Bisphenol A was identified to raise concern since about 1992 although it has been recognized since the 1930's and even earlier than that, exogenous substances are capable of interfering with the endocrine system (Fatoki *et al.* 2009). The endocrine system is one of the major systems in the human body. Endocrine disruptors are an endogenous reproductive developmental toxicant that causes adverse health effects in an intact organism, or its progeny, secondary (consequent) to changes in endocrine activity. Endocrine disrupting chemicals have also been described as a large and varied group of chemicals that are able to cause endocrine-mediated abnormalities in invertebrates, fish and avian, reptilian and mammalian species. Four classes of EDCs commonly differentiated in literature include natural estrogens (e.g. estrone, 17β-estradiol and estriol), synthetic estrogens (e.g. ethinylestradiol and tamoxifen), phytoestrogens (e.g. genistein and coumestrol) and xenoestrogens (e.g. bisphenol A, nonylphenol and DDT) (Fatoki *et al.* 2009). Concerns have been raised in particular about bisphenol A which is considered to be potentially harmful to human health and the environment. Bisphenol A (4,4'-

isopropylidenediphenol) (Figure 1.1.1) is a typical product of the industrial society produced in large quantities worldwide, 90% or more being used as a monomer for the production of polycarbonate, epoxy resins, and unsaturated polyester-styrene resins (Qiu *et al.* 2010). BPA is essentially a key building block of polycarbonate plastic, which is a lightweight, high-performance plastic that possesses a unique balance of toughness, dimensional stability, optical clarity, high heat resistance and excellent electrical resistance. These qualities make it an ideal material used in a wide variety of common products including digital media (e.g. CDs, DVDs), electronic equipment, automobiles, construction glazing, sports safety equipment and medical devices. The durability, shatter-resistance and heat-resistance of polycarbonate also make it an ideal choice for tableware as well as reusable bottles and food storage containers that can be conveniently used in the refrigerator and microwave (APME). The final products of BPA are used as coatings on cans, as powder paints, as additives in thermal paper, in dental fillings, and as antioxidants in plastics. Thus, BPA is present in baby bottles, foods and beverage containers, as well as in lacquers coating metal products, such as food cans or water supply pipes (Qiu *et al.* 2010). It has also been used as an inert ingredient in pesticides, as a fungicide, antioxidant, as flame retardant, rubber chemical and polyvinyl chloride stabilizer (Rodriguez-Mozaz *et al.* 2005). Human exposure to BPA eventually released from these materials is of increasing concern due to its endocrine-disrupting potential. In fact, BPA was one of the first chemicals discovered to mimic or inhibit estrogens in humans and wildlife (Yin *et al.* 2011). The BPA was acknowledged to have estrogenic effect since 1938 (Dodd's and Lawson, 1938). Soon after, BPA was established to be estrogenic in MCF-7 human breast cancer cell culture at low concentrations like 2.0-7.0 ppb (Krishnan *et al.* 1993). Ever since then, several studies have been reported on the estrogenic effects of BPA on humans and animals both in *vitro* and in *vivo*

(Christiansen *et al.* 2000; Mueller 2002; Markey *et al.* 2005) etc. Due to the widespread use of BPA, with a global production of over 1 million tons/year, it has become an environmental contaminant mainly present in raw sewage or wastewater effluents at concentrations up to ppb (μg L^{-1}) and at ppt (ng L^{-1}) levels in river water and sediments (Marchesini *et al.* 2005). Of particular concern are the high levels found for this compound in river waters (up to 0.4 μg L^{-1}).

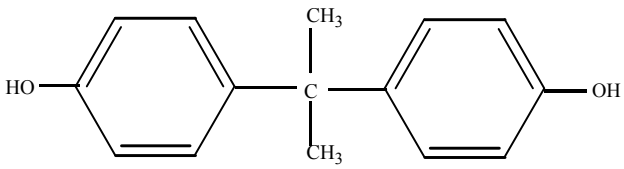

Figure 1.1.1: Structure of bisphenol A

This substance can enter the environment through leaching from final products and during manufacturing processes. The current release of BPA into streams and rivers occurs mainly through discharges of sewage treatment plants, which has led to elevated levels in both surface waters and sediments. The levels of BPA are expected to rise as a result of its extensive use and environmental ubiquity. BPA may decompose microbial in water, with a half-life of between 2.5 and 4 days (Staples *et al.* 1998). The primary source of exposure to BPA for most people is through the diet. While air, dust, and water are other possible sources of exposure, BPA in food and beverages resulting from the protective internal epoxy resin coatings of canned foods and from consumer products such as polycarbonate containers accounts for the majority of daily human exposure. The degree to which BPA leaches from polycarbonate bottles into liquid may depend more on the temperature of the liquid or bottle, than the age of the container. BPA has been reported to be detectable in breast milk (Schönfelder *et al.* 2002) . The reported results on BPA content in the blood of pregnant women, in umbilical blood at birth and in placental tissue,

and all samples examined were found to contain BPA at levels within the range shown to alter development (Schönfelder *et al.* 2002). The findings indicate that the environmental monitoring of BPA is very important and should be more prevalent. The analytical methods commonly used for the separation and detection of BPA are optical methods (Wang *et al.* 2006), high performance liquid chromatography (HPLC) (Katayama *et al.* 2001), gas chromatography (GC), or gas chromatography (GC) coupled with mass spectrometry (GC-MS) (Pulgar *et al.* 2000), Liquid Chromatography coupled with mass spectrometry (LC-MS) (Jiménez-Díaz *et al.* 2010) and capillary electrophoresis (Mei *et al.* 2011). These methods are highly sensitive and specific, but they are quite expensive, time-consuming, need skilled operators, require difficult pre-concentration and extraction steps and thus do not allow rapid processing of multiple samples. For that reason, the sensitive, rapid, and precise determination of phenolic compounds and its derivatives are of growing interest in environmental control and protection (Li *et al.* 2005; Mita *et al.* 2007). Moreover, the equipment is usually located far away from the possibly polluted sites. Recently these difficulties have been overcome by the introduction of sensor and biosensor technologies. Electrochemical biosensors are considered to be the most attractive techniques for such applications. The use of electrochemical devices offers many advantages compared to the above mentioned methods. Electrochemical sensors can operate in turbid media and are more amenable to miniaturization, which is regarded as crucial importance for monitoring purposes. In addition, the excellent compatibility of electrochemical sensors with flow injection analytical systems (FIA) increases the potential for assay automation, to which more and more attention has been paid in many fields including food analysis and environmental monitoring. A good combination of support material and immobilization method is of fundamental importance to achieve the desired performances from the sensing system. With regard to the fabrication of

enzyme-based phenol biosensors, many examples can be found in the literature, where various sources of tyrosinase and a wide variety of matrixes including graphite (Sarapuu *et al.* 2010), carbon paste (Mita *et al.* 2007), conducting polymers (Dempsey *et al.* 2004; Li *et al.* 2006), biopolymers (Tembe *et al.* 2006), nafion membrane (Zhao *et al.* 2005) nanoparticles (Carralero Sanz *et al.* 2005; Alkasir *et al.* 2010), silica sol–gel composite films (Munjal *et al.* 2002) have been used, with different combinations of stability and sensitivity, depending on the application. Many biosensors for phenol determination have been developed in the past using the catalytic activity of the redox enzymes such as tyrosinase, peroxidase, laccase (Chang *et al.* 2002; Sulak *et al.* 2010) etc using different electrode materials, flow systems and sample pre-treatment techniques.

This study reports the development of simple and sensitive electrochemical tyrosinase based biosensors for quantitative determination of bisphenol A based on the use of modified glassy carbon electrode with poly(2,5-dimethoxyaniline) doped with poly(4-styrenesulfonic acid) (PDMA-PSS) and to complete the biosensor a protein such as enzyme tyrosinase was immobilized on top of polymer composites (Tyr/PDMA-PSS/GCE). The immobilization of tyrosinase was a crucial step in the fabrication of phenol biosensor. The previous reports on the immobilization methods included polymer entrapment (Sulak *et al.* 2010), electropolymerization (Chen *et al.* 2003), self-assembled monolayer (SAM) (Ding *et al.* 2003), and covalent linking (Dempsey *et al.* 2004). However, some of these immobilizations are relatively complex, require the use of solvents that are unattractive to the environment and result in relatively poor stability and bioactivity of tyrosinase. In this study drop coating has been chosen as immobilization method because it's simple method, precise and reproducible. In recent years there has been

increased interest in searching about simple and reliable method for immobilization of enzyme. For instance biocompatible nonmaterial's have their unique advantages when it comes to enzyme immobilization. They could retain enzyme activity well suitable to the desirable microenvironment, and they could improve the direct electron transfer between the enzyme's active sites and the electrode. Tyrosinase is a copper-containing monophenol monooxygenase enzyme that catalyzes the hydroxylation of monophenols into *o*-diphenols (monooxygenase, cresolase, monophenolase or hydroxylase activity) and also the two-electron oxidation of diphenols (e.g. catechol, BPA etc.) to *o*-quinones (catecholase or diphenolase activity) with molecular oxygen and formation of water (Bartlett 2008). The *o*-quinones generated are reduced electrochemically at appropriate potentials thus reduction currents obtained serve as analytical signals which are proportional to the concentrations of phenols or phenol derivatives e.g. BPA in solution. The tyrosinase mechanism and structure are shown below:

$$Phenol + \frac{1}{2}O_2 \xrightarrow{Monooxygenase} o-diphenol$$

$$o-diphenol + \frac{1}{2}O_2 \xrightarrow{Catecholase} o-quinone + H_2O$$

The drawbacks common to phenol sensors and biosensors include electrode fouling due to polymerization of radicals, and enzyme inactivation by the generated *o*-quinone. It has been identified that the electrochemical oxidation of phenolic compounds in general causes the inactivation of electrodes surface, through deposition of electropolymerized films which are produced when phenoxy radical attacks the unreacted substrate (Kuramitz *et al.* 2001). The common problem recognized for a lot of biosensors is the need of the critical operational and storage stability essential for commercial exploitation. For instance tyrosinase based biosensors instability in pure standard solution is mostly due to high instability of *o*-quinone in water.

Redox mediators such as PDMA-PSS can achieve a two electron transfer scheme of *o*-quinone avoiding intermediate radicals and helping to prevent deactivation of the sensor (Dempsey *et al.* 2004). These intermediate radicals are formed in both enzymatic and electrochemical reaction that may readily react and polymerized to polyaromatic compounds that can inactivate the enzyme and cause fouling of electrode. To date researchers are faced with a major challenge in the area of biosensor, finding ways to minimize fouling and improving tyrosinase stability. To address this challenge of stability and activity of tyrosinase biosensor, conducting polymers are used as modifies and addition of various substances onto the electrode surface prior the immobilization of an enzyme (Chuang *et al.* 2006; Asav *et al.* 2009) etc. Conducting polymers enhance the conductivity of the biosensor by assisting in directing electron transfer between the enzyme's active sites and the electrode. Again during preparation of tyrosinase enzyme stock solution, bovine serum albumin used like protein carrier, as a stabilizing agent in enzymatic reactions and it enhances enzyme activity. Bovine serum albumin has a different effectiveness with regard to the stability of the signal generated, 2.5% glutaraldehyde was used as cross linking bonding to minimize the loose of higher or lower percentage of immobilized enzyme during the measurement. The biosensors modified with conducting polymer have high conductivity and good stability in air and aqueous solution.

1.2 Problem statement

Bisphenol A is one of the endocrine disrupting compounds yet 90% or more of it is being used as a monomer for the production of polycarbonate plastics, epoxy resins, and unsaturated polyester-styrene resins. The final products are used as coatings on cans, as powder paints, as additives in thermal paper, in dental fillings, and as antioxidants in plastics. Thus, BPA is present in baby

bottles, foods and beverage containers, as well as in lacquers coating metal products, such as food cans or water supply pipes. BPA leaching rate from the polymer matrix to the environment was determined to be increased by approximately 1000 folds when containers are aged so that prolonged use of these polymers could cause a high level exposure to BPA. The current release of BPA into streams and rivers occurs mainly via discharges of sewage treatment plants, which have led to elevated levels in both, surface waters and sediments. Another potential route of BPA exposure is polyvinyl chloride (PVC) pipes used in the supply of tap water. Human exposure to BPA eventually released from the final products and contaminated water is of increasing concern due to its endocrine disrupting potential. To prevent the noxious effects of BPA, an efficient monitoring system is required, so that immediate remediation can be activated. The analytical methods most frequently used for the determination of BPA are gas chromatography (GC) and liquid chromatography (LC), both coupled to mass spectrometry (MS). These methods are highly sensitive and specific, but instruments are rather complicated, quite expensive, time-consuming, need skilled operators, require laborious pre-treatment and extraction steps, not easy to employ for onsite measurements and thus do not allow rapid processing of multiple samples. These difficulties can be overcome by the introduction of sensor and biosensor technologies. Since the widespread use of BPA has raised concerns about its effects on humans, a sensitive, selective and a low-cost analytical method is strongly demanded. This research project focuses on developing simple and sensitive electrochemical sensor and tyrosinase based biosensor for the determination of BPA. Tyrosinase (polyphenol oxidize) based electrochemical biosensors have the potential to provide a simple, low cost, rapid and sensitive determination of BPA.

1.3 Objectives

1.3.1 Main objective

The main objective of this work was to develop simple and sensitive electrochemical sensors for the detection of bisphenol A.

1.3.2 Specific objectives

i. To synthesize poly(2,5-dimethoxyaniline) doped with poly(4-styrenesulfonic acid) (PDMA-PSS) electrochemically.

ii. To characterize the synthesized PDMA-PSS by cyclic voltammetry (CV), UV-Vis spectroscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM).

iii. To develop an electrochemical biosensor for bisphenol A by immobilizing the enzyme tyrosinase by drop coating onto the surface of glassy carbon electrode (GCE) modified with PDMA-PSS.

iv. To characterize the bisphenol A biosensor by cyclic voltammetry, differential pulse voltammetry (DPV) and square wave voltammetry (SWV).

v. To investigate the electrochemical behavior of BPA on a bare glassy carbon electrode.

vi. To optimize the sensitivity and stability of the developed tyrosinase biosensor.

vii. To apply the developed biosensor for the determination BPA by differential pulse voltammetry, electrochemical impedance spectrometry, square wave voltammetry and steady-state amperometry.

1.4 Significance of the research

Tyrosinase is a binuclear copper containing metallo-protein which catalyzes the hydroxylation and oxidation of monophenols and diphenols (e.g. BPA) to *o*-quinone. The *o*-quinone thus generated can be reduced electrochemically at an appropriate potential and thus reduction currents obtained serve as good analytical signals for quantitative determination of phenol derivatives. The reduction current measured is usually proportional to the concentration of the phenols in solution. Tyrosinase-based biosensors utilize the reduction of *o*-quinone at moderately negative potentials, so that the interference from oxidizable species can be prevented. Thus, here we report the development of tyrosinase-modified electrodes for BPA detection. The development of this new biosensor method will provide a breakthrough in the screening of BPA and cost-effective monitoring of environmental pollution by BPA.

1.5 Thesis layout

Chapter 2 presents reviews on the health effects and environmental occurrence of Bisphenol A as well as the analytical techniques used for their detection; the recent developments in the use of conducting polymers and nanostructured conducting polymers as materials for biosensor construction, as well as the features and applications of biosensors. Various synthetic routes for nanostructured polyaniline materials and their improved features over the conventional polyaniline are discussed. The chapter also highlights the enzyme kinetics in relation to their inhibition principles.

Chapter 3 describes the various analytical techniques employed, detailed research methodology and general experimental procedures for the electrochemical synthesis of nanostructured

conducting polymer, biosensor construction, characterization and application of the developed biosensor for the determination of bisphenol A.

Chapter 4 presents the results for the characterization of the poly(2,5-dimethoxyaniline) (PDMA) electrochemically synthesized by the "soft template" method employing the structure-directing molecule poly(4-styrenesylfonic acid) (PSS). The results for the electrochemical characterization of the PDMA-PSS film are presented. The doping properties of PDMA-PSS films were investigated by electrochemical impedance spectroscopy (EIS) and UV-Vis spectroscopy and the results are presented in this chapter. In addition, the results obtained from the morphological characterization of PDMA-PSS by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) to estimate their sizes are presented. The features of the nanostructured materials were investigated in relation to their application as biosensor materials.

Chapter 5 presents the results for the characterization of the Tyr/PDMA-PSS biosensor as well as those for its optimization for the detection of BPA. The direct electron transfer of Tyrosinase immobilized on nanostructured PDMA-PSS film as well as the biosensor parameters were characterized by cyclic voltammetry (CV), square wave voltammetry (SWV) and Electrochemical impedance spectroscopy (EIS) and UV-Vis spectroscopy and inhibition analysis of BPA by Tyr/PDMA-PSS biosensor employing voltammetric and amperometric techniques the results discussed in this chapter.

Chapter 6 summarizes the major findings of this study and the conclusions drawn from the results of the research. The conclusions drawn have been used to formulate the recommendations for further studies.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Few years ago phenols were known to be among the top 50 chemicals produced in manufacturing industries worldwide (Chang *et al.* 2002). Phenolic compounds are important contaminants of ground and surface waters, causing problems to human health and showing adverse effects on wildlife not only at the level of the individual, but also at that of the population and the community by interfering with endogenous hormones in the body. There are several ways in which these chemicals can affect the endocrine system for example, they can bind to hormone receptors and either mimic or inhibit the action of natural hormones, or they can affect their synthesis and metabolism and more ways will be discussed in this study. It has been known for some time that the normal operation of the endocrine (hormonal) system can be disrupted by a number of anthropogenic and naturally occurring chemicals, thereby affecting those physiological processes which are under hormonal control. BPA compound is a known endocrine disruptor; a substance possesses properties which are suspected to cause endocrine disruption in an intact organism. The concept of a biosensor dates back to 1962 when Clark and Lyons described the development of the first "enzyme sensor" (Zhang *et al.* 2000). Since then, various biosensors have been developed to detect a wide range of biochemical parameters and

there has been incredible activities witnessed in this area of biosensors. This chapter discusses about the conducting polymer material used for biosensor fabrication and their properties when used as mediators for electron transfer between electrode surface and the active sites of the enzyme immobilized. Tyrosinase enzyme is used as bio-component and the PDMA doped with PSS as conducting polymer. The biosensor was constructed for the quantitative determination of bisphenol A endocrine disrupting compound in waste water.

2.1.1 Endocrine disrupting compounds

A group of chemicals known collectively as endocrine disrupting compounds are suspected of interfering with the normal function of hormonal regulation and endocrine system, consequently causing adverse health effects in humans and wildlife (Dempsey *et al.* 2004). These are exogenous agents that interfere with the synthesis, secretion, a transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior (USEPA, 1997). The endocrine system is one of the major systems in the human body. These chemicals have been acknowledged as concern to the environment since about 1992. Although, it has been identified since the 1930's and even earlier that exogenous substances are capable of interfering with the endocrine system.

In literature EDCs are commonly differentiated in four classes which include natural estrogens (e.g. estrone, 17-β-estradiol and estriol), synthetic estrogens (e.g. ethinylestradiol and tamoxifen), phytoestrogens (e.g. genistein and coumestrol) and xenoestrogens (e.g. bisphenol A, nonylphenol and DDT) (Coille *et al.* 2002). In industries, for example bisphenol A is used in paper mill, nonylphenol is a detergent, and DDT is a pesticide. The estrogenic hormones are

extremely responsible for the growth and development of female sexual characteristics and reproduction in both humans and animals (Yin *et al.* 2011). The word "estrogen" includes a group of chemically similar hormones: estrone, estradiol and estriol. More particularly, the estradiol and estrone forms are produced primarily in the ovaries in premenopausal women, while estriol is produced by the placenta during pregnancy. Several factors contribute to exoestrogens becoming a contentious issue: for example, epidemiological studies have found significant increases in the incidence of breast, prostate and testicular cancer and decreasing sperm counts and semen volume have been observed as well as longer times to conception (Yin *et al.* 2011). There are many different types of EDCs worldwide and the table below illustrates a brief summary of some of most well known EDCs: Endocrine system of human controls the important functions ranging from gender differentiation during fetal development to the 'adrenalin rush' of extreme sports. Endocrine system utilize molecules named hormones to send signals to regulate sexual development, metabolism, puberty, a woman's menstrual cycle, bone growth and a host of other body functions (Fatoki *et al.* 2009). Several chemicals creates no direct threat to the human and wildlife but creates indirect by interacting with endocrine system of the organism leading to unnatural, untimely and perhaps excessive release or suppression of hormones, a phenomenon known as endocrine disruption. Other harmful health effects EDCs on wildlife and humans include dermal toxicity, immune toxicity, carcinogenicity, neurobehavioral abnormalities, altered or reduced sexual behavior, attention deficit/hyperactivity disorder, altered thyroid and adrenal cortical function, pathological changes to the spleen, damaged digestive systems, amongst others (Fatoki *et al.* 2009).

Table 2.1.1 : Some endocrine disrupting compounds

Category	Name	Some Sources
Alkyl-phenols (surfactants)	Nonylphenol, pentylphenol, octylphenol, nonylphenol mono and diethoxylates	Industrial and municipal effluents
Bisphenolic compounds	Bisphenol A	Used in the synthesis of polycarbonate plastic and epoxy resins
Natural hormones/ Synthetic steroids	17-β-estradiol, estrone, testosterone and ethynyl estradiol	Municipal effluent and agricultural runoff
Organochlorine pesticides	DDT, dieldrin and lindane	Agricultural runoff and atmospheric transport
Organotins	Tributyltin	Shipping harbors used in wood preservative and antibiofouling agents
Pesticides	Atrazine, trifluralin and permethrin	Agricultural runoff
Phthalates	Dibutyl phthalate, butylbenzyl phthalate and phthalate esters	Industrial effluent
Phytoestrogens	Isoflavones, ligans and coumestans	Pulp mill effluents
Polybrominated compounds	Polybrominated diphenyl ethers	Flame retardants
Polychlorinated compounds	Polychlorinated dioxins and polychlorinated biphenyls	Landfills used in coolants, Insulating fluids and PVC pipes etc.

(*http://environmentalchristian.wordpress.com/2008/02/10/endocrine-disrupting-compounds-bisphenol-a-phthalate-esters-dioxins-and-lots-of-others/*)

EDCs differ in origin, size, potency, chemical life cycle, amount, and effects. Many of them are chemicals produced for specific purposes and are used in pesticides, plastics, cosmetics, electrical transformers and other products. Other substances are generated as byproduct during manufacturing or are breakdown products of some other chemical. Some, like diethylstilbestrol (DES) and ethinylestradiol, are synthetic drugs, while others are natural plant compounds called 3-2 phytoestrogens. Concerns have been raised in particular about bisphenol A which is considered to be potentially harmful to human health and the environment due to high rate of leaching. Bisphenol A (4,4'-isopropylidenediphenol) is a typical product of the industrial society produced in large quantities worldwide, 90% or more being used as a monomer for the production of polycarbonate, epoxy resins, and unsaturated polyester-styrene resins. The final products of BPA are used as coatings on cans, as powder paints, as additives in thermal paper, in dental fillings, and as antioxidants in plastics. BPA is known to leach from these materials due to incomplete polymerization and to degradation of the polymers by exposure to high temperatures, occurring under normal conditions of use.

2.1.2 Human exposure to bisphenol A

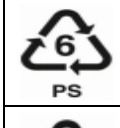
Bisphenol A is a plastic monomer produced in highest volume chemical worldwide, approximately six billion pounds or more is produced each year .Although it is known to have hazardous adverse effects, 90% or more is being used as a monomer for the production of polycarbonate, epoxy resins, and unsaturated polyester-styrene resins (Qiu *et al.* 2010). It is basically a primarily building block of polycarbonate plastic, which is a lightweight, high-performance plastic that possesses a unique balance of toughness, dimensional stability, optical

clarity, high heat resistance and excellent electrical resistance. These qualities make it an ideal material used in a wide variety of common products including digital media (e.g. CDs, DVDs), electronic equipment, automobiles, construction glazing, sports safety equipment and medical devices. The durability, shatter-resistance and heat-resistance of polycarbonate also make it an ideal choice for tableware as well as reusable bottles and food storage containers that can be conveniently used in the refrigerator and microwave (APME). In the past years several researchers in academics and industries worldwide have studied the potential of low levels of BPA to migrate from polycarbonate products into food and beverages. Under the conditions typical for the use of polycarbonate products these studies consistently show that the potential migration of BPA into food is extremely low, generally less than 5 parts per billion. The estimated dietary intake of BPA from polycarbonate is less than 0.0000125 milligram per kilogram body weight per day. When epoxy resins is used as interior coating of metal cans it protects the integrity and quality of our food supply by preventing rusting and corrosion of canned foods and beverages from metal.

Many of these resins are synthesized by the condensation of BPA with epichlorhydrin to create BADGE. When incomplete polymerization occurs, residual BPA may leach from the epoxy resin and has the potential to contaminate stored foods; quantity of BPA leaching estimated was at a range of 4-23 µg per can (Kang *et al*. 2000). Several studies were done to find conditions that support or enhance BPA migration from the coating of cans. It was obtained that cans from manufacturers and performed carefully controlled studies on the influence of heating time, heating temperature, storage time, storage temperature, and other factors on the level of BPA migration. BPA coated in the lining of cans is known to leach into the infant formula due to

heating temperatures. There was very low BPA level found in water stored in unheated cans supporting the fact that heating temperature has great influence in BPA migration than heating time. It is also known to enter the environment through leaching from final products and during manufacturing processes in a number of lager industries including industries of resins and plastic and wood preservation, petroleum refining, dye chemicals and textiles. It's commonly released into streams & rivers mainly through discharges of sewage treatment plants. BPA has also been used as an inert ingredient in pesticides, as a fungicide, antioxidant, as flame retardant, rubber chemical and polyvinyl chloride stabilizer (Rodriguez-Mozaz *et al.* 2005). Human get exposed to BPA when it's released from these materials increasing concern due to its endocrine disrupting potential. It has been proven to be one of the first chemicals discovered to mimic estrogens and it motivate the growth of the rodent uterus which is a sign of estrogenic action since 1936 (Rodriguez-Mozaz *et al.* 2005). Previous studies proved that children in daycares and pre-school are mostly likely exposed to BPA due to high level of this chemical detected in liquid and solid food, floor dust and play area soil in both locations at similar levels. It was estimated that the average BPA exposure level for young children is 42.98 ng/kg/day based on these environmental levels (Vandenberg *et al.* 2007). The table below shows the BPA containing products and BPA free.

Table 2.1.2.1: BPA containing products and BPA free

Number	Code	Name	Examples	BPAs
	PETE	Polyethylene terephthalate (PET)	Soda and water containers, some waterproof packaging.	No
	HDPE	High-density polyethylene	Milk, detergent, oil bottles. Toys and plastic bags.	No
	V	Vinyl/polyvinyl chloride (PVC)	Food wraps, vegetable oil bottles and blister packages.	Yes
	LDPE	Low-density polyethylene	Many plastic bags, shrink wrap, and garment bags.	No
	PP	Polypropylene	Refrigerated containers, some bags and most bottle tops,	No
	PS	Polystyrene	Throw away utensils, meat packing and protective packing.	Yes
	Other	Usually layered or mixed plastic.	Acrylic, polycarbonate, polylactic acid, nylon and fiberglass.	Maybe

(www.shahine.com/omar/BisphenolA.aspx)

2.1.3 Health effects from bisphenol A

BPA is a well known endocrine disrupting compounds chemicals known to have potential to reduce negative effects on endocrine systems of humans and wildlife. It is one of the polluting chemicals, easily absorbed by animals and human through skin and mucous membranes. Their toxicity is directed greatly on variety of organs and tissues, primarily lungs, kidneys and genitor-urinary system. They are known for elimination of natural hormones in the body that are

responsible for the maintenance of homeostasis, reproduction, development, and/or behavior (USEPA, 1997). They mimic or inhibit the behavior of natural hormones in human beings and wildlife. During manufacturing of some products BPA released into the environment to contaminate rivers and human and wildlife gets exposed to BPA may cause health effects such as cancer and disruption of endocrine system. Absorption of BPA through the human skin has been shown to produce extensive damage to the kidneys, liver, spleen, pancreas, and lung. Several studies have shown that very low levels of BPA occurs in blood and urine samples due to an efficient metabolism of BPA in humans. It is necessary to determine BPA in the environment due to its potential risk to cause reproduction and development effects, neurochemical effects and behavioral effects (Yin *et al.* 2011).

Previous studies have determined that BPA can be measured in humans in serum, urine, amniotic fluid, follicular fluid, placental tissue, and umbilical cord blood (Yin *et al.* 2011). The levels of total BPA (free and conjugated) in human blood and other fluids are higher than the concentrations that have been reported to stimulate a number of molecular endpoints in cell culture in vitro, and appear to be within an order of magnitude of the levels of BPA in animal studies. Due to large availability of BPA in the environment, the estrogenic activity in specific responses in *vitro* and in *vivo*, unfavorable effects of BPA exposure on human health are possible (Qiu *et al.* 2010). BPA level in the blood of women has been associated with a variety of conditions including obesity, endometrial hyperplasia, recurrent miscarriages, abnormal karyotypes and polycystic ovarian syndrome. Over the last 50 years European and US human populations hypothesized that exposure during early development to xenoestrogens such as BPA

may be the essential cause of the increased incidence of infertility, genital tract abnormalities and breast cancer was observed.

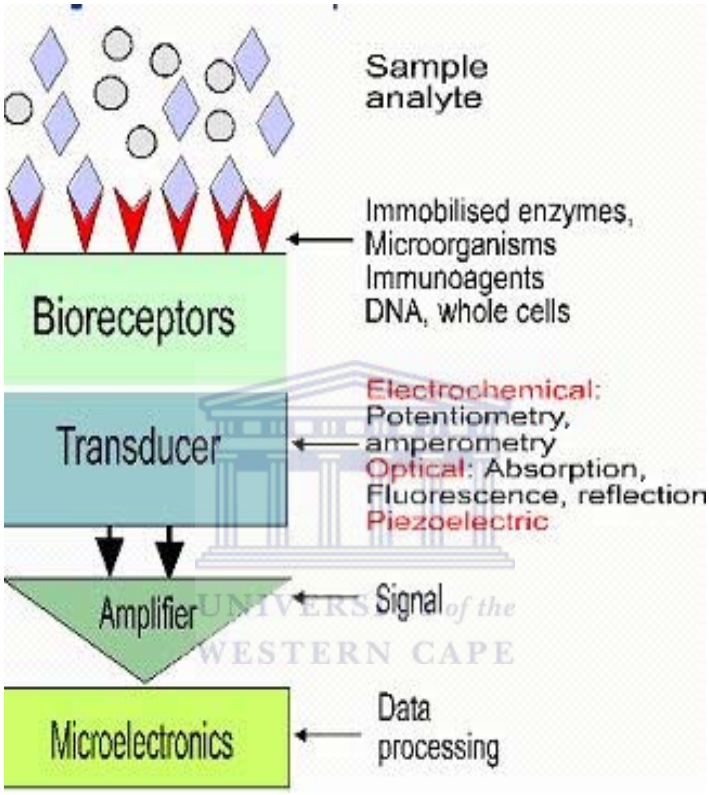
2.1.3 Residue of bisphenol A in food and water

Bisphenol A one of the highest volume industrial chemicals has been widely used as the monomer for the manufacture of polycarbonate, epoxy resin and polyphenylene oxide resin and as an additive in other plastics. Food packaging, coatings for PVC water pipe walls, plastic bottles for water, baby bottles, dental fillings and sealants are used as final products (Qiu *et al.* 2010). BPA is not normally present in PVC organosol coatings. However, if BPA diglycidyl ether (BADGE) was used as an additive to scavenge hydrogen chloride in these coatings residues of BPA, as unreacted starting material in the BADGE may be present. Because of its widespread use and ubiquitous presence in the environment, the possibility of human exposure to BPA is high. The estrogenic action of BPA has been confirmed both in *vivo* and in *vitro* experiments. Additionally, BPA has been discovered to possibly cause cancer and an association between BPA and breast cancer (Qiu *et al.* 2010). Hence, BPA has emerged as a major public health issue and received much attention.

2.2 Biosensors

Biosensors (Scheme 2.2.1) are analytical devices incorporating biological recognition elements integrated within transducing micro-systems**.** The biosensors have been developed for several decentralized analytical applications and they are quickly becoming useful tool in medicine, biotechnology, genetic engineering, food quality control, military, agriculture, environmental monitoring and other practical fields (Rodriguez-Mozaz *et al.* 2005). Zhang et al. reported five

features of systematic description of a biosensor which includes the detected or measured parameter, the working principle of the transducer, the physical and chemical/biochemical model, the application and the technology and materials for sensor fabrication (Zhang *et al.* 2000).



Scheme 2.2.1: Schematic representation of biosensors.

(http://dipcia.unica.it/superf/researchsensors_0.html)

For characterization of a biosensor many parameters have been recommended, such as sensitivity, stability and response time; while other parameters are related to the application rather than to sensor function, for example the biocompatibility of sensors for clinical monitoring. Some are commonly used to evaluate the functional properties and quality of the sensor, for example the biocompatibility of sensors for clinical monitoring. An electrochemical

biosensor usually consists of a transducer such as a pair of electrodes or FET, an interface layer incorporating the biological recognition molecules and a protective coating. Sensor design, including materials, size and shape and methods of construction, are largely dependent upon the principle of operation of the transducer, the parameters to be detected and the working environment. Traditional electrode systems for measurements of the concentrations of ions in liquids and dissolved gas partial pressures contain only a working electrode (usually a noble metal wire) and an electrically stable reference electrode, such as Ag/AgCl, though a counter electrode is sometimes included. A simple electrical, or chemical, modification may sometimes improve specific electrode properties (Zhang *et al.* 2000). The biological materials can be enzymes, antibody, receptor, DNA, micro-organisms. The signal transducing element (electrode, optical, piezo crystal etc) converts biochemical response into optical and optic signals which are amplified, measured and decoded by an appropriate electronic unit. Basic characteristics of biosensors include linearity meaning the maximum linear value of sensor calibration curve, sensitivity meaning the value of electrode response per substrate concentration, selectivity meaning the interference of chemicals must be minimized to obtain correct results and time response the necessary time for having 95% of the response. In this study the enzyme tyrosinase was used as the biological recognition element while the electrosynthesized conducting polymer, poly(2,5-dimethoxyaniline) doped with poly(4-styrenesulfonic acid) was used as a redox mediator. The mediator is known to shuttle electrons between the active site of the enzyme and the surface of the electrode. Glassy carbon electrode was used as the signal transducing element. The biosensor was completed by immobilizing tyrosinase enzyme by drop coating method into a PDMA-PSS modified electrode and cross linked by 2.5% glutaraldehyde to bind the enzyme to the polymer.

2.2.1 Transducer

A transducer is known to converts the biochemical signal to an electronic signal and it sends it to the biosensor selectively or specifically. The transducer of an electrical device responds in a way that a signal can be electronically amplified, stored and displayed. Suitable transducing system can be adapted in a sensor assembly depending on the nature of the biochemical interaction with the species of interest. The physical transducers differ from electrochemical, spectroscopic, thermal, piezoelectric and surface acoustic wave technology. Electrochemical transducers that are commonly being utilized are amperometric and potentiometric.

2.2.2 Bio-components

Bio-components also known as biological recognition elements function as biochemical transducers and can be enzymes, tissues, bacteria, yeast, antibodies/antigens, liposome's, organelles. When recognition biomolecules are incorporated within a biosensor they acquire an exquisite level of selectivity but weak to excessive conditions such as temperature, pH and ionic strength. Enzymes, receptors, antibodies, cells etc. are examples of biological molecules which have very short lifetime in solution phase; hence they have to be fixed in a suitable matrix. The decreased in enzyme activity can be result from immobilization of the biological component against the environmental conditions. The essential factors that activity of immobilized molecules depend on include surface area, porosity, hydrophilic character of immobilizing matrix, reaction conditions and the methodology chosen for immobilization. There are a number of techniques used for immobilization of biological molecules in carrier materials such as physical adsorption, cross-linking, gel entrapment, covalent coupling etc. For immobilization of enzymes diverse matrices have been used such as membranes, gels, carbon, graphite, silica,

polymeric films etc. It is therefore a huge necessity to design the electrodes that are well-matched with the biological component that can lead to rapid electron transfer at the electrode surface. Conducting polymers are attractive as possible materials for such applications.

2.3 Types of biosensors

2.3.1 Electrochemical biosensors

The fundamental principle of biosensors class is that it has many chemical reactions that produce or consume ions or electrons which in sequence cause some changes in electrical properties of the reaction solution, this change can be sensed out and used as a measuring parameter. Depending on the electrochemical parameter measured, electrochemical biosensors can further be classified into amperometric, potentiometric, and conductimetric or impedimetric biosensors.

2.3.2 Amperometric biosensor

An amperometric biosensor is used to measures the current produced during the oxidation or reduction of a product or reactant usually at a constant applied potential. Such sensors are noticed to have fast response times and good sensitivity. However, the excellent specificity of the biological component can be compromised by the partial selectivity of the electrode. This lack of specificity requires sample preparation, separation or some compensation for interfacing signals.

2.3.3 Potentiometric biosensor

Potentiometric biosensors use an ion-selective electrode or a gas sensing electrode as the physical transducer to relate electrical potentials to the concentration of analyte. These have large

dynamic ranges, are selective and nondestructive. Potentiometry is hardly ever used for detection method in biosensor where enzymes are immobilized in an electrodeposited polymer layer. However, some experiments have been demonstrated using amperometric detection by PPy based electrode immobilized GOX were successful (Gerard *et al.* 2002). Difficulties encountered regarding the immobilization of enzymes in the electrodeposited polymer layer or the mechanism of the entrapment and dynamics effects on biosensors have been widely discussed and till now no conclusive reports on the comparison of different polymer matrices for immobilization of the same enzyme have been related. Designed biosensors having very slow response, the rate of potential change rather than steady state potential values, should be considered as the analytical signal for quantification of the substrate.

2.3.4 Conductimetric biosensors

Conductimetric biosensors focus on measure the changes in the conductance of the biological component occurring between a pair of metal electrodes. During electrochemical reactions, ions or electrons are either produced or consumed and this causes an overall change in the conductivity of the solution. This change is measured and calibrated to a proper scale. Conductimetric biosensors present a number of advantages for the direct analyses of many enzymes and their substrates: Simple and quite cheap planar conductimetric electrodes which suits for miniaturization and large scale production, and hence promising for practical use, they do not need a reference electrode, the applied voltage can be effectively small to reduce substantially the sensor's power consumption, large spectrum of analyte of different nature can be determined on the basis of various reactions and mechanisms (Wang *et al.* 2006) .

2.4 Biosensors for health care

In health care biosensor is used for selective determination of various blood analytes such as glucose, urea, lactate, uric acid, cholesterol etc. it is of extreme importance for the screening and treatment of a number of diseases.

2.4.1 Glucose biosensors

Glucose biosensor is a most commonly used method for glucose detection called diabetes in human blood due to its advantages, such as rapidness, simplicity and portable. Blood glucose is a very important tool to avoid the long term undesirable consequences of elevated blood glucose, including neuropathies, blindness and other sequel. This biosensor has been developed as useful tool in the management of diabetes and maintaining normal blood glucose levels in human. Glucose biosensor technology includes point of care devices, continuous glucose monitoring systems and noninvasive glucose monitoring systems has been improved drastically during the last 50 years.

2.4.2 Immunosensors

This type of sensor has brought about effective combination of immunochemistry and electrochemistry in an analytical device. It may perhaps present basis of direct electrical detection of a wide range of analytes with great sensitivity and specificity. Quite a number of immunosensors have been developed based on conducting polymers. The use of conducting polymer for reversible immunosensors is a unique approach based on pulsed electrochemical detection developed by Sargent's group. This approach has been employed for the detection of

organochlorine pesticides including PCBs, atrazines and chlorinated phenols. Porter and colleagues investigated electroplated conducting polymers as antibody receptor in Immunosensors (Porter *et al*. 2000). Immunosensors are portable instrument and permits a total test time of less than 15 minutes when used for atrazine estimation and can be used to detect pesticide at the level of parts per billion.

2.4.3 DNA biosensors

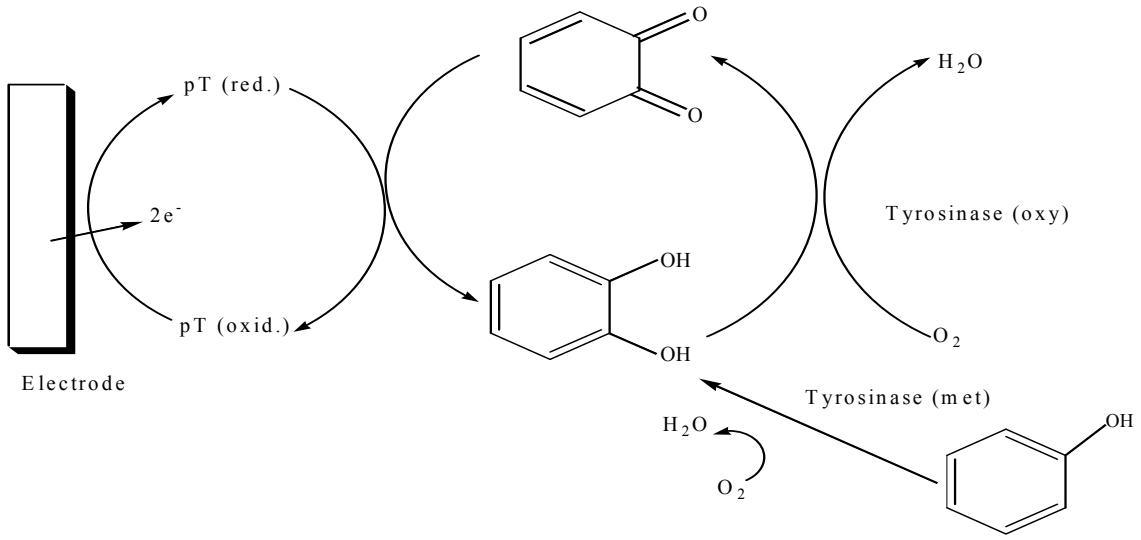
DNA biosensors are very popular and they have massive applications in clinical diagnostics of inherited diseases, rapid detection of pathogenic infections, and screening of cDNA colonies required in molecular biology. Currently, methods require genetic analysis for pre- and post-treatments to modify DNAs with probes as proteins. Conducting polymers are used for this biosensor development because of its potential to presents intelligent material which possess superior intelligent material properties of self-assembly, self-multiplication, self-repair, self-degradation, redundancy, and self-diagnosis. Immobilization of DNA on a conducting polymer matrix facilitates the detection of a signal (amperometric or potentiometric) generated as a result of interaction of proteins or drugs with DNA.

2.5 Enzyme biosensors

Electrochemical methods based on enzymes have been commonly used for the measuring of phenolic compounds because of their advantages of good selectivity, long-term stability and potential for efficiency and automation. An enzyme biosensor is derived from a combination of a transducer with a thin enzymatic layer, which normally measures the concentration of a substrate. The enzymatic reaction transforms the substrate into a reaction product that is

detectable by the electrode. The concentration of any substance can be measured provided that its presence affects the rate of an enzymatic reaction which is especially true for enzyme inhibitors. The signal (current or potential) measured is proportional to the rate-limiting step in the overall reaction. Immobilization of an enzyme provides multiple and repetitive use by increasing stability of enzyme. The usage of free tyrosinase is limited because of its instability and rapid inactivation. Comparing free enzyme in solution, the immobilized enzyme is more stable and resistant to various environmental changes. Some authors have reported immobilization via entrapment of redox proteins or enzymes, including tyrosinase in membranes consisting of poly(vinyl pyridine), poly(vinyl imiazol), poly(acrylic acid) or poly(allyl amine).

The immobilization of enzymes is a crucial step in the biosensor fabrication. Immobilization of enzyme in a conducting polymer matrix employing electropolymerization is simple; one-step procedure. It prevents contamination and protects enzyme against changes in pH, temperature and ionic strength in the bulk solvent. For this study tyrosinase enzyme is used as the bio-component and conducting polymers as the matrix having many advantages in biosensors fabrication. Tyrosinase based biosensor is developed for simple and affective analytical methods for determination of phenolic compounds. It has been shown to be a very simple and convenient tool for phenol analysis due to its high sensitivity, effectiveness, and simplicity. The schematic diagram below illustrates proposed processes that take place at biosensor layers, where monophenol is oxidized to diphenol process catalyzed by tyrosinase enzyme with the presence of oxygen molecule.



Scheme 2.5.1: Proposed surface processes at biosensor layer redrawn from (Dempsey et al. 2004)

2.5.1 Enzyme immobilization techniques

The majority applications of tyrosinase normally require immobilization to allow the enzyme to be utilized repeatedly. It is advantageous for immobilized preparation to have high stability against chemical denaturants, pH change and high temperature. The immobilization of enzyme is identified by many authors as a crucial step in fabrication of enzyme based biosensors because it ensures the intimate contact between the enzyme and the underlying transducer and it also prevents the enzyme from being washed off the electrode when analysis are performed in aqueous samples (Faria *et al.* 2007). The immobilization methods reported in literature includes polymer entrapment (Chen *et al.* 2003), electro-polymerization (Arslan *et al.* 2005), drop-coating (Zhuo *et al.* 2006; Wang *et al.* 2009; Alarcon *et al.* 2010) etc and covalent linking (Biegunski *et al.* 2006). However, some of these immobilizations are relatively complex and result in poor stability and bioactivity. Activity of an enzyme can be decreased by immobilization of biological components and environmental conditions (Alarcon *et al.* 2010). The methodology selected for

immobilization, surface area, porosity, hydrophilic character of immobilizing matrix and reaction conditions are very critical for the activity of immobilized molecule due to its sensitivity. The spatial distribution of the immobilized enzymes, the film thickness and changes in enzyme activity can be controlled by changing the state of the polymer. The good spreading of electrons from the surface of the electrode to the enzyme active site brings to mind the concept electrical wiring. Conducting polymers are expected to supply a 3-dimensional electrically conducting structure for this purpose. As for phenol biosensors and wastewater treatment processes enzyme has been immobilized in different supports such as, a glassy carbon modified with electrodeposited gold nanoparticles (Carralero Sanz *et al.* 2005), a boron-doped diamond electrode (Notsu *et al.* 2002), a chitosan (Abdullah *et al.* 2006), a carbon fiber electrode (Kuramitz *et al.* 2001), an electrode modified with single walled nanotubes (Zhao *et al.* 2005) and electrode modified with poly(thionine) film (Dempsey *et al.* 2004). However, some of these immobilization methods are somewhat difficult and do not present an excellent enzyme stability. Studies have attracted and highlighted that conducting polymers as suitable material for the entrapment of enzyme and they reveal conducting polymer material serves as promising matrix for enzyme immobilization (Gerard *et al.* 2002). Electrochemical synthesized conducting polymers permits direct deposition of the polymer film on the electrode surface, at the same time trapping the protein molecules. Conducting polymers have ability to transfer electric charge created by the biochemical reaction in the electronic circuit. They present excellent detestability and fast response as the redox reaction of the substrate, catalyzed by an appropriate enzyme, takes place in the bulk of the polymer layer. The electronic conductivity of conducting polymers can be affected by quite a few orders of magnitude in response to changes in pH and redox potential on their environment. The method of incorporating enzymes into electro-polymerized

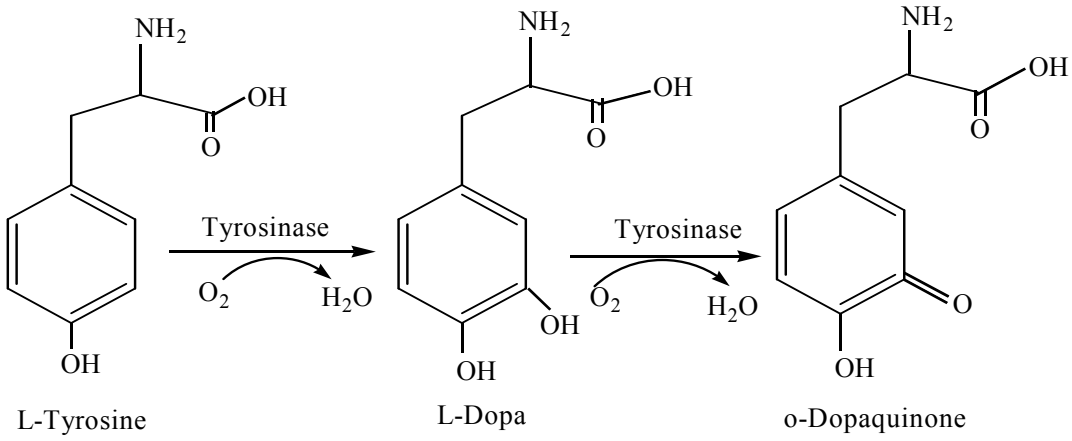
polymeric films allows delocalization of biological active molecules onto the electrode surface of any size of geometry. This technique is mostly suitable for the fabrication of multi-analyte micro-amperometric biosensor.

2.6 Tyrosinase enzyme

2.6.1 Applications of tyrosinase enzyme

Tyrosinase or polyphenol oxidize (EC 1.14.18.1) is a multifunctional copper containing enzyme and is widely distributed in microorganisms, plants and animals It is commonly found in yeast, mushroom, apples, and potatoes, although it's considered to be poisonous to the color quality of plants-derived foods and beverages (Matoba *et al.* 2006). Tyrosinase catalytic cycle consists of three different enzymatic forms involved in its reactions. Throughout the catalytic reaction, the type three copper centers of tyrosinase exist in three redox forms (1). The deoxy form [Cu (I)-Cu (I)] is a reduced species, it binds with oxygen to give oxy form [Cu (II)-O_2^{2-}-Cu (II)]. Within the oxy form, molecular oxygen is bound as peroxide in a μ-η^2:η^2 side-on bridging mode, which weakens the O–O bond and makes it active. While met form [Cu (II)-Cu (II)] is understood as an inactive enzymatic form, where Cu (II) ions are normally bridged to a small ligands, such as a water molecule or hydroxide ion (Matoba *et al.* 2006). Tyrosinase is used to catalyze the synthesis of melanin through the hydroxylation of L-tyrosine to dihydroxy L-phenylalanine (L-DOPA) and followed by oxidation of L-DOPA to dopaquinone mechanism (Rani *et al.* 2007) shown in Scheme 2.6.1. However, the produced dopaquinone is unstable so it polymerizes and precipitates into melanin. The cresolase activity of tyrosinase in particular is very important because it synthesizes L-DOPA. Tyrosinase enzyme is mainly employed for biosynthesis of the large biological pigment, melanin responsible for skin, eye, inner ear and hair melanization, and

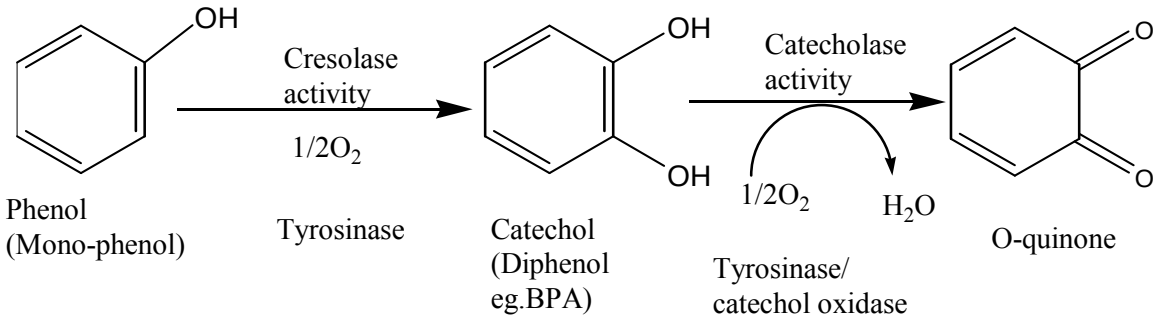
browning in fruits and vegetables. Melanin's pigments are one of many illness conditions caused by adjustments of melanin biosynthesis found in the mammalian brain. Tyrosinase has widespread applications as well in medical and industrial fields. It may play a role in neuromelanin formation in the human brain, could be central to dopamine neurotoxicity and contributes to the neuro-degeneration associated with Parkinson's disease. Melanoma-specific anticarcinogenic activity is also known to be linked with tyrosinase activity. Patients who suffer from Parkinson's disease shows major decrease in the concentration of dopamine found in the large area of the brain (Rani *et al.* 2007). A neurological disorder is a disease which normally affects old people and manifest in forms of tremor, rigidity, slowness of speech and lastly dementia caused by shortage of a neurotransmitter dopamine (Munjal *et al.* 2002). Also, the production of epidermal hyper pigmentation of melanin causes some dermatological disorders such as melasma, freckles, ephelide, senile, lentignes, etc. L-dopa has been approved as a drug for curing Parkinson's disease and the mentioned disorders in the market since 1967 (Faria *et al.* 2007). Although the chemical synthesis of DOPA is fast, the resultant racemic DL-mixture is inactive and it was very hard to further separate the enantiomerically pure L-DOPA from this mixture. D-DOPA interferes with the activity of DOPA decarboxylase, the enzyme involved in the production of dopamine in the brain. Therefore, a one-step economical bioconversion of L-tyrosine into L-DOPA is highly important. Once more tyrosinase inhibitors are clinically helpful in the treatment of above mentioned dermatological disorders associated with melanin. It has been used as part of an enzyme electrode system to detect catechols and assess catecholamines in the urine of patients with neural crest tumors. Scheme 2.6.1 shows a mechanism representation of oxidation of L-tyrosine to L-dopa catalyzed by tyrosinase and molecular oxygen.



Scheme 2.6.1: L-tyrosine oxidation to o-dopaquinone mechanism (Faria et al. 2007)

Tyrosinase is not only responsible for melanization in animals but also browning in plants. This unfavorable darkening is made possible by enzymatic oxidation of phenols generally results in a loss of nutritional value and has been of great concern. In industrial part, tyrosinase is utilized in determination of phenols and its derivative, especially for cleaning surface water and the effluent in industrial discharges (Fenoll *et al.* 2001). Some of the industrial sources of phenol discharge include oil refineries, coke and coal conversion plants, plastics and petrochemical companies, dyes, textiles, timber, mining, and the pulp and paper industries. Practically all phenols are very toxic. Moreover, they have a high oxygen demand and can deplete oxygen. Therefore, this possibly might affect the ecosystem of water sources where phenols are discharged. Tyrosinase influences the precipitation of phenols, which then make it possible to filterer it out from surface waters and industrial discharge sources. The enzyme has also been used as a sensor to detect the concentration of phenols in waste water. Tyrosinase catalyzes two opposite reactions of melanin biosynthesis and the *ortho*-hydroxylation of monophenol to *o*-diphenol using molecular oxygen (O_2) and finally the oxidation of the diphenol to *o*-quinone. This enzyme utilizes molecular oxygen to catalyze oxidation process of monophenol. The phenolic compound mainly converts

into quinone, and the product is electrochemically active, can be reduced on the electrode. The quinone generated is reduced electrochemically at appropriate potential therefore reduction current obtained serve as analytical signal which is proportional to the concentration of phenol or phenolic compound in solution. Tyrosinase based biosensor utilizes reduction of quinone at negative potential to prevent phenol polymerization as well as interferences from other oxidizable species. The mechanism below represents the oxidation phenolic substrate by polyphenol oxidize (tyrosinase) produces a dihydroxybenzene (catechols) which is further oxidized to *o*-quinone in the presence of molecular oxygen. *O*-quinone is unstable normally so it undergoes a non-enzymatic polymerization to form pigments called melanin's (Matoba *et al.* 2006). The X-ray instrument confirmed two enzymes similar to tyrosinase. Siegbahn reports, all three enzymes are different in a sense that hemocyanin is an oxygen transport protein without phenolase and diphenolase activity and the catechols oxidize mainly has diphenolase activity but monophenolase activity can also be demonstrated. However, tyrosinase enzyme has both phenolase and monophenolase activities.



Scheme 2.6.2: Tyrosinase mechanism mediated phenol oxidation. (Khan 2007; Ramsden et al. 2010)

Chemical and spectroscopic studies proposed that tyrosinase has five histidine ligands while hemocyanin has three histidine ligands for each copper and catechols oxidize has six histidine ligands, but one of them is covalently linked to cysteine (Siegbahn 2003). The crystal structures of copper-bound and metal-free tyrosinase were determined at high resolution in a complex with ORF378 designated as a "caddie" protein since it helps with transportation of two Cu (II) ions into the catalytic center of tyrosinase. By appearance at these structures Matoba suggested that the caddie protein covers the hydrophobic molecular surface of tyrosinase and interferes with the binding of a substrate tyrosine to the catalytic site of tyrosinase (Matoba *et al.* 2006). Figures below illustrate the structures of tyrosinase enzyme clearly showing two copper ions active site bonded together.

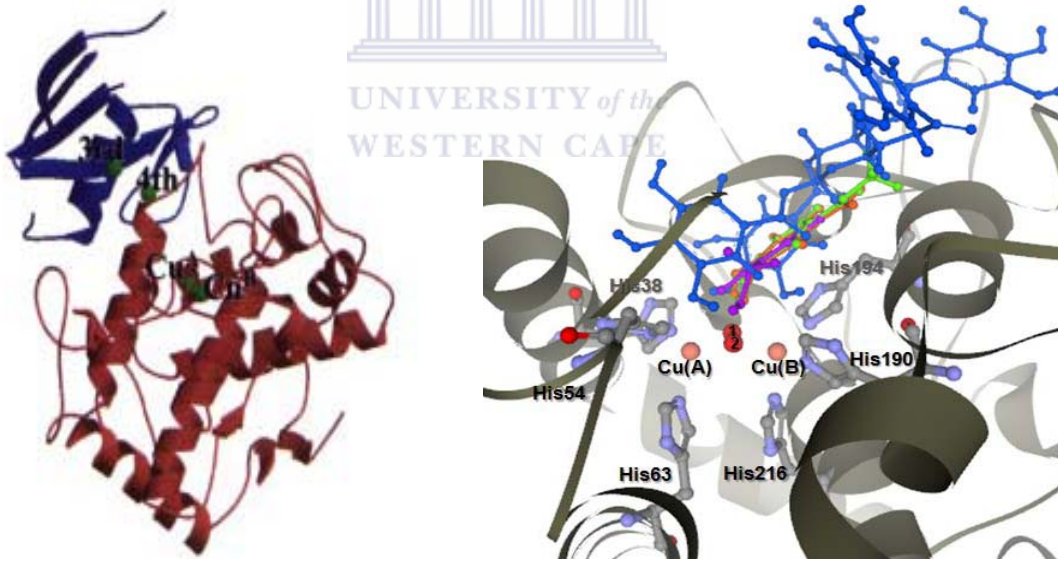
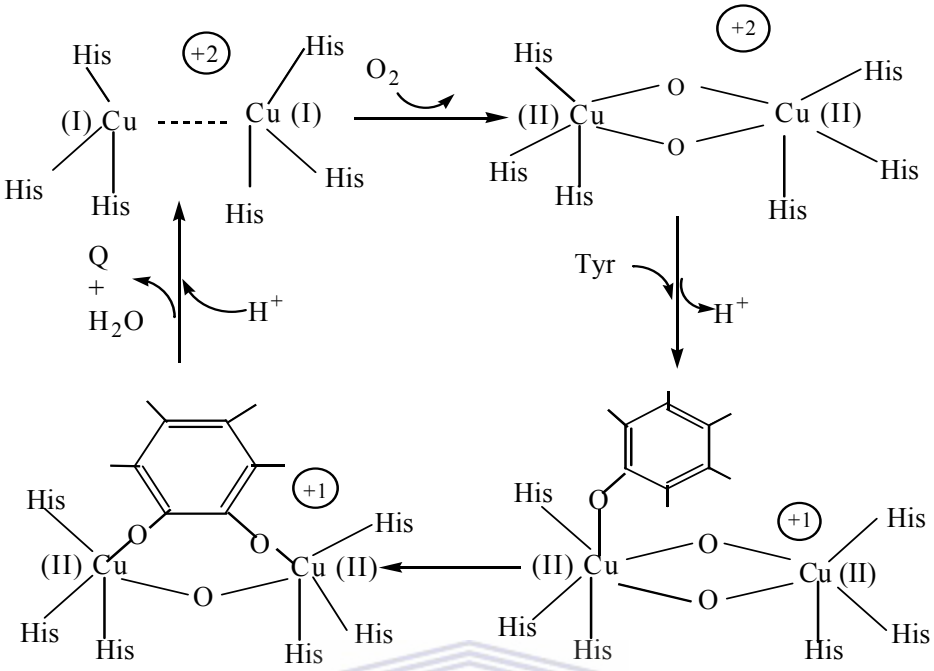


Figure 2.6.1: Structure tyrosinase enzyme showing bio-nuclear copper (Cu) active site.
(Matoba *et al.* 2006)

2.6.2 Tyrosinase kinetics

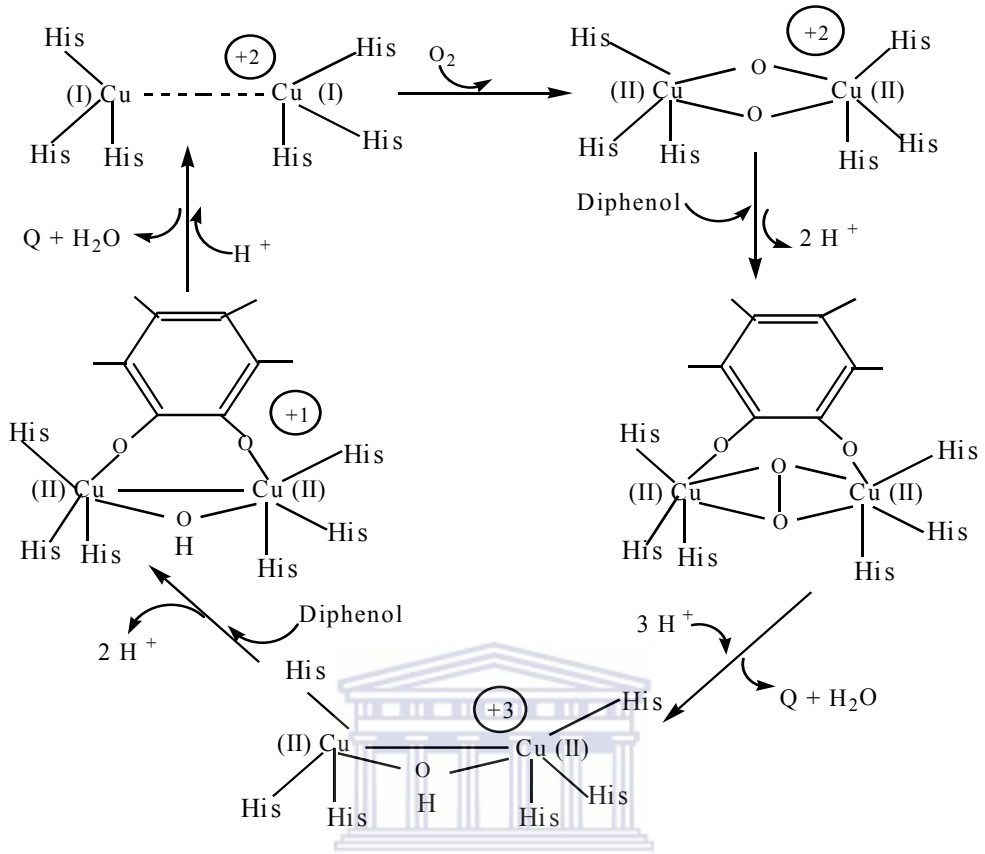
Tyrosinase, phenol oxidize and catechols oxidize share very similar active site and they belong to copper protein family although; they have different functions, structure and sequence (Matoba *et al.* 2006). This is supposed to result from a difference in the substrate-binding pocket or the accessibility of the substrate to the active site. Two different enzymatic reactions are catalyzed by tyrosinase using molecular oxygen such as *ortho*-hydroxylation of monophenol to *o*-diphenol (monophenolase cresolase activity) and oxidation of *o*-diphenol to *o*-quinone (diphenolase catecholase activity) (Zhao *et al.* 2005; Akyilmaz *et al.* 2010). Tyrosinase can catalyze both reactions while catechols oxidize performs only the final reaction. These reactions take place in the active site of tyrosinase containing two copper ions binding sites Cu_A and Cu_B, in which interact with two molecular oxygen O_2 and histidine residues. The active site exists in three intermediate states such as deoxy (Cu^{+}–Cu^{+}), oxy (Cu^{2+}–O_2–Cu^{2+}) and met (Cu^{2+}–Cu^{2+}) depending on the copper-ion valence and the linking with molecular oxygen (Fenoll *et al.* 2001). These three states determine the ability of tyrosinase to bind to its substrates and therefore determine the reaction kinetics. Siegbahn suggested mechanism for tyrosinase active site reacting with phenol demonstrating two catalytic cycles such as diphenolase and monophenolase cycles. He speculates that these cycles are interconnected because diphenol is first produced in monophenolase cycle (cresolase activity) and again used in diphenolase cycle (catecholase reaction) even though there is no convincing confirmation currently to prove that the reaction occurs this way (Siegbahn 2003). For both catalytic cycles an interesting information was discovered ,there strong flow of protons in and out of the complex while the overall reactions does not need any protons from outside reaction though diphenolase requires three protons coming in at the stage of O-O bond cleavage. This stage is a very important because it requires

very efficient proton and unusually high flexibility in changing the charge of the system. Both catalytic cycles do not give more information as to how the O-O bond is supposed to be cleaved and this step is expected to be the most critical step of the entire mechanism. But crystal structure of hemocyanin or catechols oxidize does not require this. As shown in Scheme 2.6.3, the monophenolase activity starts with the binding of the substrate monophenol to one of the Cu atoms of the oxygenated form (oxy-tyrosinase), Oxy-T is formed. Followed by, *o*-hydroxylation of the monophenol by the bound peroxide take place, and an enzyme-coordinated *o*-diphenol structure (Met-D) is formed. Subsequently, Met-D is oxidized further to produce *o*-quinone and the enzyme is changed to deoxy form (deoxy-tyrosinase). Deoxy form is transformed to oxygenated form by oxygen molecular binding. Alternately, *o*-diphenol is released, and the enzyme is converted to the met form (met-tyrosinase), which will be involved in the diphenolase cycle. It is important to keep in mind that monophenol can react with oxy-tyrosinase, but not with met-tyrosinase, to form the product *o*-quinone. Monophenolase activity shows a characteristic lag period. This may be due to the fact that tyrosinase in the resting form contains 15% oxy sites, which is the only form that can react with monophenol substrates (Likhitwitayawuid 2008).



Scheme 2.6.3: Monophenolase cycle suggested on the basis of experiments. Q stands for ortho-quinone. Mechanism redrawn from (Siegbahn 2003).

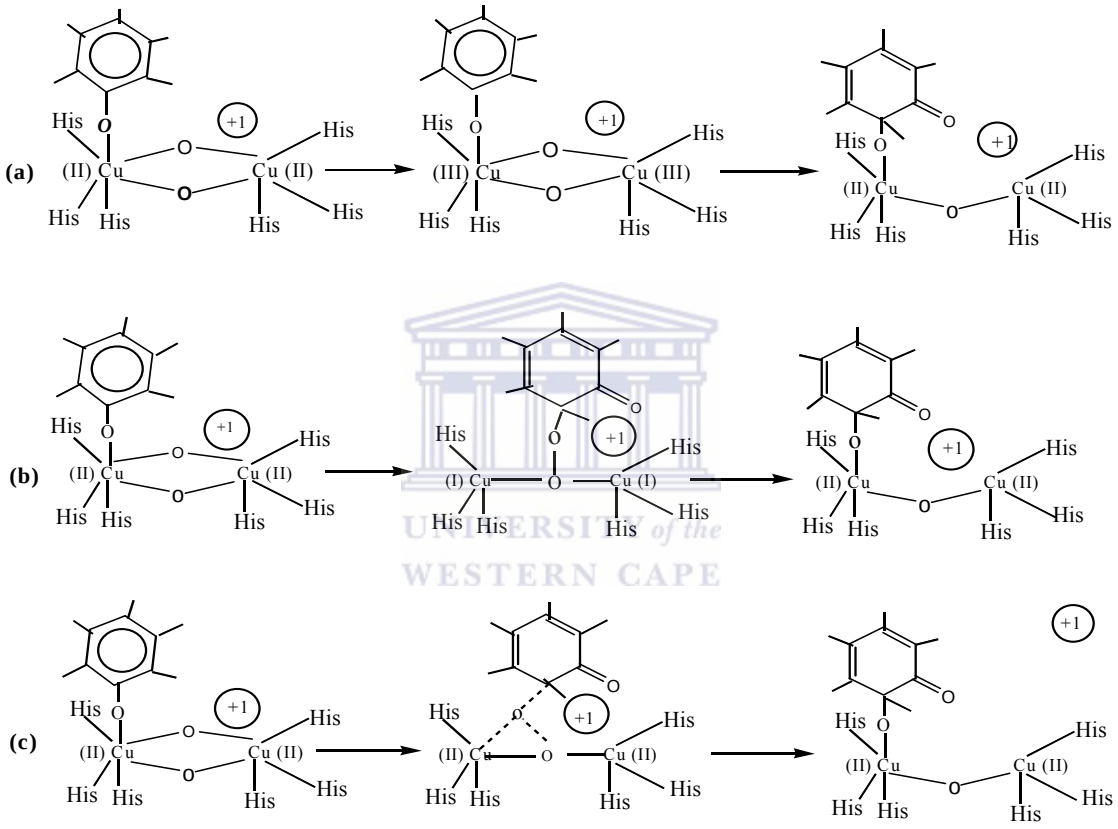
In the diphenolase cycle shown in Scheme 2.6.4, the o-diphenol can react with both the oxy and the met forms to produce *o*-quinone. Literature reports reaction rate the diphenolase activity as (k =107 per s) occurring at a faster rate than the monophenolase which is (k =103 per s) regardless of the substrates. The reaction of diphenol with met-tyrosinase changes the enzyme to the deoxy form, bringing it into the monophenolase cycle.



Scheme 2.6.4: The diphenolase cycle suggested on the basis of experiments. Q stands for the ortho-quinone. Mechanism redrawn from (Siegbahn 2003)

The mechanisms below shows the alternatives used in order to connect phenol in the copper active site. The copper active site gets crowded after oxygen molecule interaction so for phenol to incorporate in an active site should be created. This can be achieved by O-O bond cleavage, which is very critical step of the entire mechanism. Siegbahn studies, he suggested other three possible alternatives of O-O bond cleavage, the first one was the O-O bond is homolytically cleaved forming highly oxidized Cu_2 (III,III) intermediates this is supported by synthetic copper dimmer complexes (Siegbahn 2003), second alternative involve oxidation of Cu_2 (II,II) state oxidized to Cu_2 (I,I) state and back, this reaction occurs when two oxygen molecules attacks the phenolic ring forming peroxide bridge and thereafter O-O bond is cleaved. The final possibility

occurs when O-O cleavage and peroxide attack are combined in single step and the copper complex remains Cu_2 (II,II) state throughout the reaction. The last two possibilities of O-O bond cleavage mechanism have been proven experimentally. The tyrosinase mechanism for O-O activation by copper has been of interest in the previous theoretical studies (Matoba *et al.* 2006).



Scheme 2.6.5: Three different possibilities to cleave the O-O bond in tyrosinase (a) O-O bond cleavage leading to Cu_2 (III,III). (b) O-O bond cleavage followed by initial formation of a peroxide bond to tyrosinase. (c) Concerted O-O bond cleavage with peroxide formation. Mechanism redrawn from(Siegbahn 2003)

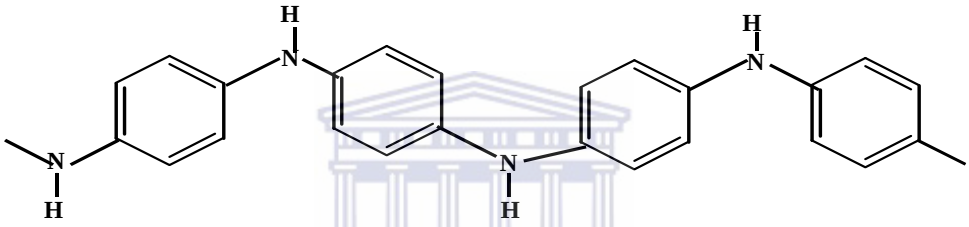
2.7 Background history of conducting polymers

2.7.1 Conducting polymers

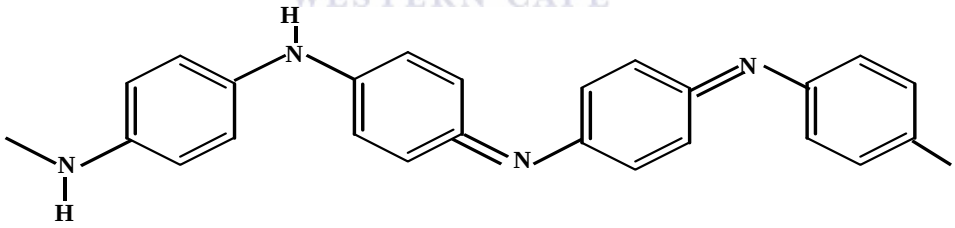
Conducting polymers are primarily organic compounds that have extended π-orbital systems which are responsible for electron movement from one end of the polymer to the other. For adjustment of electronic and mechanical properties of conducting polymer chemical modeling and synthesis required. Conducting polymers have been used in different biosensors fabrication in various fields such as health care which includes medical diagnosis, for example glucose, fructose, lactate, ethanol, cholesterol, urea etc. Immunosensor is another sensor used in medical diagnostics and environmental sensors, the DNA sensors used for detection of various genetic disorders and lastly environmental monitoring used to control of pollution and detection of hazardous chemicals in biosensors (polyphenol, sulfites, peroxides, formaldehyde etc.). Common classes of organic conductive polymers includes poly(acetylene), poly(pyrrole), poly(thiophene), poly(terthiophene), poly(aniline), poly(fluorine) etc. Conducting polymers have come out as potential candidates for electrochemical sensors. Due to their straightforward preparation methods, unique properties, and stability in air, conducting polymers have been applied as energy storage devices, electrochemical devices, chemical sensors and electro-catalysts in biosensors. They are a promising group of compounds, which are widely applied in chemical sensors and a variety of other applications. Conducting polymers contain π-electron backbone responsible for their unusual electronic properties such as electrical conductivity, low energy optical transitions, low ionization potential and high electron affinity. The extended π-conjugated systems of the conducting polymers have single and double bonds alternating along the polymer chain (Gerard *et al.* 2002). They show almost no conductivity in the neutral (uncharged) state but

become electrically conductive upon partial oxidation or reduction, a process commonly referred to as ‘doping’. By chemical modeling and synthesis, it is possible to change the required electronic and mechanical properties of conducting polymers. They can be also doped or undoped between their conducting and insulating states. Conducting polymers can be synthesized either electrochemically (Sulak *et al.* 2010) and chemically oxidative polymerization methods (Storrier *et al.* 1994). The electrochemical synthesis of polymers has the advantage of being more pure, the chemical synthesis is favored because it can be synthesized in large quantities which are attractive from a practical point of view. Chemical synthesis is known to permit the scale-up of the polymers, which is currently not possible with electrochemical synthesis. However, electrochemical polymerization is the most preferred general method for preparing conducting polymers because of its relatively straightforward synthetic procedure, simplicity and reproducibility. The common technique used is the oxidative coupling involving the oxidation of monomers to form a cation radical followed by coupling to form di-cations and the repetition leads to the polymer formation. Generally, electrochemical polymerization can be carried out galvanostatically (constant current), potentiostatically (constant potential) or by potential scanning/cycling or sweeping methods. By varying either the potential or current with time, the thickness of the film can be controlled. In this procedure, the monomers at the working electrode surface undergo oxidation to form radical cations that react with other monomers or radical cations, forming insoluble polymer chains on the electrode surface. Among conducting polymer, polyaniline and polypyrrole are mostly used conducting polymers worldwide because of their desirable electrical conductivity, environmental stability, low cost of production, ease of synthesis and favorable physiochemical properties. These polymers preserve extensive interest because of several technological applications in different fields such as sensors and biosensor,

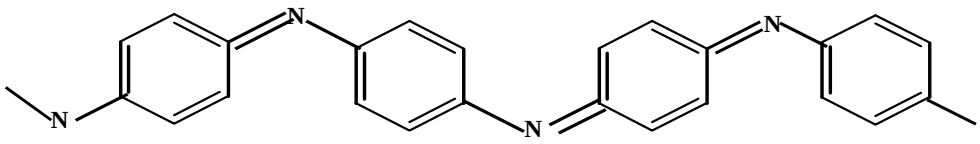
rechargeable batteries, microelectronics devices and corrosion protection (Nabid *et al.* 2008). Amongst organic conducting polymers PANI is unique due to its electrical properties which are controlled by both oxidation state and protonation. However, these polymers are mechanically weak, low processable and also its application is limited due to its poor solvent solubility (Conklin *et al.* 1995; Mavundla *et al.* 2008). Conducting polymers exist in three forms such as fully reduced leucoemeraldine; half oxidized emeraldine and fully oxidized pernigraniline as shown below.



Luecoemaraldine base (fully reduced)



Emaraldine base (partially oxidized)



Pernigraniline (fully oxidized)

Scheme 2.7.1: Different forms of polyaniline

Several methods have been reported to beat this problem, one of them is to modify the redox reaction of conducting polymers by incorporation of polymeric electrolyte such as p-toluene-sulfonic acid, dodecyl-benzene-sulfonic, camphor sulfonic, *m*-sulfonic acid and poly(styrenesulfonic acid),which are functionalized protonic acids and widely used by polypyrrole. These acids improve the solubility which attributes to the presence of polar substituent which promotes compatibility between polymer and the solvent. Polymeric electrolyte long chain lengths are trapped inside the conducting polymer film leading to the formation of polymer composite. The sulfonate present in polyelectrolyte given out anions to maintain electroneutrality of the doped-undoped polymer, cations of a smaller size are usually exchanged during doping-dedoping processes of conducting composites and there polymer composites have been regarded as being cation selective. Method used to recover the problem of PANI insolubility is the use of substituted polyaniline, for instance aniline substituted with methoxy group has been reported to produce very soluble polymer, poly(2,5-dimethoxyaniline), poly(2-methoxyaniline), poly(methylaniline) etc with similar conductivity like that of PANI. It is supposed that the substituent's on these polymer chains decreases the stiffness of the polymers making it easier to dissolve. Unlike the mono-substituted polyaniline which has been reported to have low conductivity, disubstituted polyaniline like poly(2,5-dimethoxyaniline) has been reported to have conductivity similar to that of PANI and are soluble in most organic solvents (Li *et al.* 2006). Studies reported that PDMA is a well known polymer for its potential to further elaboration via cleavage of the methoxy groups. PDMA is less conductive than PANI due to an electron donating ($-OCH_3$) group substituted on phenyl ring. This bulky substituent ($-OCH_3$) causes steric effect that can provide torsional twists in the polymer backbone, reducing the co-planarity and average electron delocalization length. The presence of $–OCH_3$ group in DMA unit

induced easy oxidation and enhanced solubility. Hence in this study, we report polymeric nanobiosensor attained by electrochemical synthesis of poly(2,5-dimethoxyaniline) Figure 2.7.1 doped with poly(4-styrenesulfonic acid) by scanning the potential repeatedly within a potential range on glassy carbon electrode. The green film was formed uniformly of GCE surface, used as a mediator for electron transfer between electrode surface and tyrosinase enzyme immobilized on top of the polymer composite.

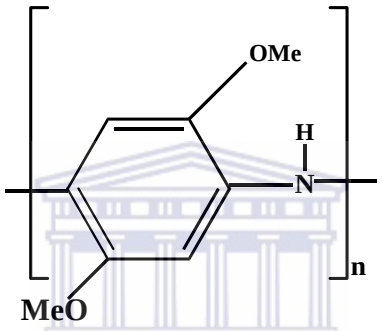
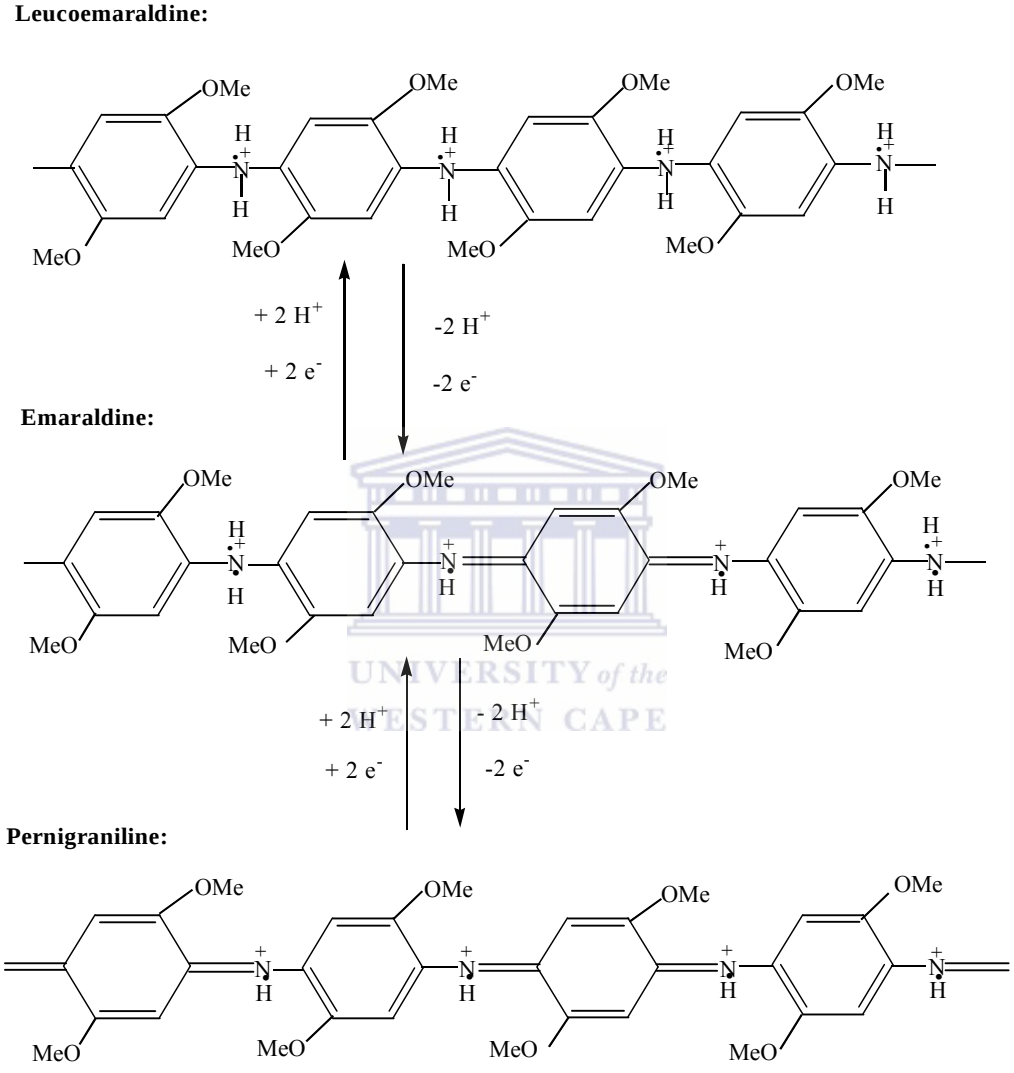


Figure 2.7.1: Structure of poly(2,5-dimethoxyaniline)

2.7.2 Electrochemical synthesis of DMA

Chemical and electrochemical synthesis of both polyaniline (PANI) and poly(2,5-dimethoxyaniline) (PDMA) has similar oxidation transitions, redox transitions responsible for leucoemeraldine to emeraldine forms and emeraldine to higher oxidation forms was observed. When PDMA cyclic voltammogram is compared to that of PANI, the first oxidation takes place close to PANI, while the second oxidation is different made possible by presence of electron donating $–OCH_3$ groups. The mechanism below shows oxidation transition that occurs during electrochemical synthesis of 2,5-dimethoxyaniline (DMA) and the electron transfer during the

reversible redox processes to form poly(2,5-dimethoxyaniline) (PDMA) a dark green polymer film onto the electrode surface, most probably due to the formation of soluble oligomer.



Scheme 2.7.2: Scheme for PDMA redox processes. Schematic redrawn from (Storrier et al. 1994)

2.7.3 Conductivity and doping of polyaniline

Polyaniline is known as electro-conducting polymer containing a system of conjugated double bonds. Most researchers use PANI for developing lightweight batteries, electrochromic display devices, microelectronic devices and anticorrosion coating due to its stability and electrochemical properties. These applications are based on PANI electrochemical properties therefore it is essential to improve its quality through a variety of modifications. PANI can be doped to highly conducting state by protonic acid or by electrochemical oxidation. It shows moderate conductivity upon doping with protonic acids and has excellent stability under ambient condition. The best way to modify the redox reaction of conducting polymers is the incorporation of polymeric electrolytes such as poly(4-styresulfonate) (PSS) and poly(vinyl sulfonate) (PPy). The use of PSS dopant structure shown in Figure 3 (b) has become relevant due to their excellent conductivity, including the improvement of electron transfer between enzymes and electrode surfaces and at the same time provides a very good matrix for enzyme immobilization. Due to the long chain lengths of the polymeric electrolytes, they are trapped inside the conducting polymer film, leading to formation of polymer composites. During oxidation of conducting polymers the positive charge is introduced into the polymer matrix to make the polymer to be more electrically conductive for example PPy, are usually compensated by the anions and sulfonate, present in the polyelectrolyte. PSS dopant introduces a negative charge by bonding with amine producing a neutralize polymer chain. This can be done by oxidation or reduction reactions, commonly called ‘doping’ and ‘dedoping’, respectively, which can be performed by different chemical or electrochemical processing. Chemical doping-dedoping occurs when the polymer is exposed to an oxidizing or reducing agent, whereas electrochemical doping-dedoping can be obtained by anodic and cathodic polymerization in

suitable electrolyte. To maintain electroneutrality of the doped-dedoped polymer cations of small molecular sizes are usually exchanged during doping-dedoping process of the polymer composite therefore polymer composites have been regarded as being cation selective. For this reason these composites, as well as polymer composites obtained from monomer having sulfonic and carboxylic acid group such as poly(aniline-2-sulfonic acids) are self-doped (Martins *et al.* 2003) Substituted monomer dimethoxyaniline has higher oxidizable compatibility due to the presence of electron donating group $–OCH_3$ group in the phenyl group. The substitution of groups in phenyl ring or N-position of polyaniline units results in decrease of conductivity. The substituent present in unit polymer caused decrease in the stiffness and lead to more dissolution in solvent. However, 2,5-dimethoxyaniline (DMA) has been reported to produce soluble polymer, poly(2,5-dimethoxyaniline) (PDMA) with conductivity close to polyaniline (Huang *et al.* 2003). In literature poly(4-styrenesulfonic acid) is identified as the promising dopant to enhance electrical, stability, reproducibility and sensitivity of polymer.

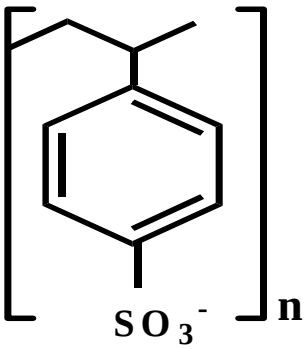
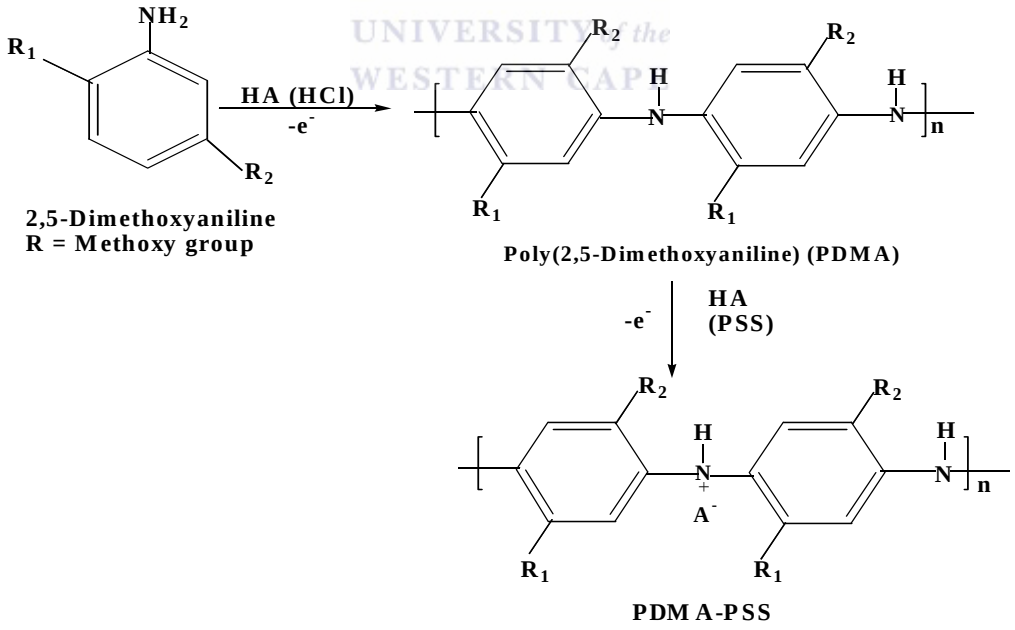


Figure 2.7.2: Structure of poly(4-styrenesulfonic acid)

The mechanism below illustrates electrochemical synthesis of DMA in acidic medium (1.0 M HCl) obtained by scanning the potential repeatedly within a potential range onto the surface of glassy carbon electrode and PDMA polymer was electrodeposited uniformly on the surface. The drawback with this polymer is that it's not conductive enough and it's positively charged because of the amide group present on this polymer. To enhance PDMA conductivity, the polymer is doped with PSS. This protonic acid is incorporated into poly(2,5-dimethoxyaniline) introducing sulfonate group (SO_4^{2-}) that has a potential to react with amine group (NH_2^+) to neutralize the polymer chain and makes it more conductive suitable for the purpose. Their function is to improve electrochemical properties of polymers by introducing functional groups into the polymer chain. This process assists in an incorporation of enzyme into the polymer composites bonding.



Scheme 2.7.3: Mechanism for DMA doped with PSS redrawn from (Martins et al. 2003; Paradee et al. 2009)

2.8 Conclusion

BPA is generally known endocrine disrupting compounds which are chemicals with the potential to bring forth negative effects on endocrine systems of humans and wildlife. They are a group of polluting chemicals, easily absorbed by animals and human through skin and mucous membranes. BPA is basically a primarily building block of polycarbonate plastic, which is a light weight, high-performance plastic that possesses a unique balance of toughness and excellent electrical resistance. During manufacturing of some products BPA released into the environment to contaminate rivers and human and wildlife gets exposed to BPA may cause health effects such as cancer and disruption of endocrine system. The analysis shows that biosensors can be suitable tool for continuous environmental monitoring of these bisphenol A in waste water to replace or complement the alternating analytical techniques because to their low cost of analysis, ease of operation, high sensitivity, timesaving and minimization of sample pre-treatment. Over the last few years biosensors have undergone quick development due to the combination of new bio-receptors with the ever-growing number of transducers and transducer modification materials. Development of enhanced performance biosensor is made possible by using combination of electronically conducting polymers, making use of the particular properties of conducting polymers with immobilized enzymes. Enormous progress has been shown in this field of biosensor support that electrode surface modification using proper matrices gives a favorable micro-environment for the enzyme to exchange its electrons directly with the underlying electrode and therefore afford a new opportunity for the detailed study of the enzyme electrochemistry and its direct electron transfer. There is still many unresolved technical problems enquired even though many studies are performed on biosensors and a number of factors such as sensitivity, selectivity, efficiency for in-vivo applications, incorporation of signal

processing steps on a chip, building of arrays for more complex pattern recognition analysis, biocompatibility, stability and response time still require investigation. Presently, the research is paying attention on improving biosensor sensitivity through the use of nanostructured materials as mediators. Nanostructured PDMA-PSS is one of the most promising candidates for the development of nanobiosensor due to their high surface and electric properties.

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CHAPTER 3

EXPERIMENTAL SECTION

3.1 Reagents and materials

The reagents hydrochloric acid, bisphenol A (BPA), glutaraldehyde 2.5% solution, disodium hydrogen phosphate ($Na_2HPO_4.7H_2O$), potassium dihydrogen phosphate ($KH_2PO_4.7H_2O$), N,N-dimethylformamide (DMF) and poly(4-styrenesulfonic acid) (PSS) were all purchased from Sigma-Aldrich, South Africa. Tyrosinase (EC 1.14.18.1; 3566 U/mg from mushroom), bovine serum albumin (BSA) and 2,5-dimethoxyaniline (DMA) were purchased from Fluka, South Africa. All reagents were of analytical grade and were used as obtained. Deionized water purified by a Milli-Q^{TM} system (Millipore) was used as reagent water for aqueous solution preparations.

3.2 Preparation of working solutions

3.2.1 Preparation of phosphate buffer solution

Phosphate buffer solution of 0.1 M was prepared by dissolving 3.549 g of disodium hydrogen phosphate ($Na_2HPO_4.7H_2O$) and 3.4023 g of potassium dihydrogen phosphate ($KH_2PO_4.7H_2O$) separately in 250 mL deionized water, then mixing the salt solutions according to Henderson-

Hasselbalch equation to obtain the required pH 4.2 to 9.2. The pH meter was then calibrated with standard solutions and phosphate buffer was then measured at range of pH 4.2 to 9.2. The pH was controlled hydrochloric acid and Sodium hydroxide. The phosphate buffer solution was used and stored in refrigerator at 4ºC for not more than 2 weeks.

3.2.2 Preparation of 0.01M bisphenol A stock solution

Stock solution of 0.01 M bisphenol A was prepared by weighing out 0.1141 g bisphenol A and dissolving in 50 mL ethanol-water mixture (2:3) and sonicating for a few minutes. Blank solution was also prepared by mixing ethanol and water (2:3) and sonication for a few minutes.

3.2.3 Preparation of tyrosinase enzyme

Tyrosinase (EC 1.14.18.1; 3566 U/mg from mushroom) this enzyme was purchased from Fluka. Tyrosinase was prepared by weighing out 0.0014 g and dissolving it in 1 mL pH 7.2 phosphate buffers in a vial. Bovine serum albumin (BSA) purchased from Fluka was prepared by weighing out 0.004 g and dissolving it in 1 mL phosphate buffer pH 7.2 in a vail.100 µL of tyrosinase solution was transferred to a clean vial and 50 µL of BSA was added to the same vial the solution was mixed well and. About 5 µL from that stock solution was immobilized into the surface of bare glassy carbon and PDMA doped with PSS modified electrode by drop-coating method.

3.2.4 Measurement and instrumentation

Electrochemical measurements were performed using the potentiostat BAS-100B electrochemical analyzer from Bioanalytical Systems, Inc. (West Lafayette, IN) with a

conventional three-electrode system consisting of platinum wire as the counter electrode, Ag/AgCl (saturated 3.0 M NaCl) as the reference electrode and glassy carbon (bare GCE, Tyr/GCE, PDMA/GCE, PDMA-PSS/GCE and Tyr/PDMA-PSS/GCE) as the working electrode. Electrochemical impedance measurements (EIS) were carried out with Zahner IM6ex Germany at perturbation amplitude of 10 mV within frequency range of 100 kHz –100 mHz. Prior to experiments, the bare glassy carbon electrode was polished with aqueous slurries of 1.0, 0.3 and 0.05 μm alumina powder, rinsing with distilled water after polishing with each grade of alumina. The polished electrode was then sonicated in water and absolute ethanol. The auxiliary electrode was cleaned by burning in a flame for several minutes and the Ag/AgCl electrode was cleaned by rinsing with copious amounts of distilled water. All potentials were quoted with respect to Ag/AgCl. The potentiostat was computer-controlled therefore the experimental modes were selected from the software and specified during its operation. The experiments were carried out at controlled room temperature of 20 °C. All electrochemical measurements were carried out in phosphate buffer solution pH (4.2 to 9.2). The experiments were carried out without purging with argon because the enzyme requires oxygen during the oxidation of phenols and purged solution was also used for comparison.

3.3 Classification of electrochemical techniques

3.3.1 Cyclic voltammetry

Cyclic voltammetry (CV) is an electrolytic method that utilizes microelectrodes and an unstirred solution so that measured current is limited by analyte diffusion at the electrode surface. In many areas of chemistry this electrochemical technique has become an important and widely used method. It has wide applications in the study of redox processes, electrochemical properties of

analytes in solution, and for understanding reaction intermediates. This technique utilizes three or two electrode electrochemical cell systems is the measurement of current resulting from application of potential. The potential is usually measured between the reference electrode and the working electrode and the current is measured between the working electrode and the auxiliary electrode, also known as the counter electrode. The technique works by varying some applied potential at a working electrode at some scan rate in both forward and reverse directions while monitoring the current. The first scan can, for example can be negative to the switching potential, in which case if the scan is reversed; it runs in the positive direction. A full cycle, partial cycle or a series of cycles can be performed depending on the analysis targeted. Important parameters are usually obtained from cyclic voltammograms for analysis of redox properties and properties of an electroactive sample. These parameters include anodic and cathodic peak potentials ($E_{p,a}$ and $E_{p,c}$ respectively) as well as anodic and cathodic peak currents ($i_{p,a}$ and $i_{p,c}$ respectively). Important information about the sample under investigation can be obtained from the peak parameters. This includes whether the electrochemical process showed by the sample is reversible, irreversible or quasi-reversible. A typical cyclic voltammogram illustrating these parameters is shown in figure 3.3.1 below.

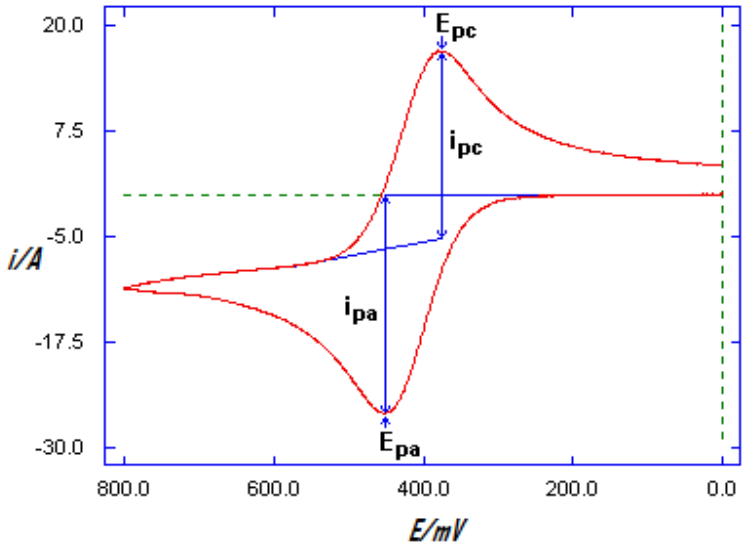

Figure 3.3.1: A typical cyclic voltammogram showing the basic peak parameters, $E_{p,a}$ $E_{p,c}$,$i_{p,a}$ and $i_{p,c}$

It also gives an insight into how fast the electron transfer process is, relative to other processes such as diffusion. For instance, if the electron transfer is fast relative to the diffusion of electroactive species from the bulk solution of the surface of the electrode, the reaction is said to be electrochemically reversible and the peak separation (ΔEp) is given by equation 1 below.

$$\Delta E_p = \left| E_{p,a} - E_{p,c} \right| = 2.230 \frac{RT}{nF} \qquad \textit{Equation (1)}$$

Where ΔE_p is the peak separation (V), $E_{p,a}$ is the anodic peak potential (V), $E_{p,c}$ is the cathodic peak potential (V), n is the number of electrons transferred, F is the Faraday constant (96,584 C mol^{-1}), R is the gas constant (8.314 J $mol^{-1}K^{-1}$). The number of electrons (n) involved in the electrochemical process can also be estimated from the equation above. For a reversible redox reaction at 25 °C with n electrons ΔE_p should be 0.0592/n V or about 60 mV for one electron. In

practice this value is difficult to attain because of such factors as cell resistance. Irreversibility due to a slow electron transfer rate results in $\Delta E_p > 0.0592/n$ V, greater, say, than 70 mV for a one-electron reaction. In this study, CV was employed for the synthesis of the polymer and to investigate the redox processes and the electrochemical properties of analytes in solution.

3.3.2 Square wave voltammetry

Square wave voltammetry is a technique used in analytical applications and fundamental studies of electrode mechanisms. Three electrodes system is used such as (working, auxiliary and reference). To gain knowledge from this technique it is essential to have a concrete understanding of signal generation, thermodynamics, and kinetics. SWV technique is one of the most sensitive ways, for the direct evaluation of concentrations. It is widely used for several analyses, especially on pharmaceutical compounds. This method is the cause of a fair amount of confusion. The problem starts after the number of waveforms are employed, which are frequently described as simply square wave voltammetry.

3.3.3 Deferential pulse voltammetry

This technique is comparable to normal pulse voltammetry (NPV) in that the potential is also scanned with a series of pulses. However, it differs from NPV because each potential pulse is fixed, of small amplitude (10 to 100 mV), and is superimposed on a slowly changing base potential. Three electrodes system is used such as (working, auxiliary and reference). Current is measured at two points for each pulse, the first point (1) just before the application of the pulse and the second (2) at the end of the pulse. These sampling points are selected to allow for the

decay of the charging current. The difference between current measurements at these points for each pulse is determined and plotted against the base potential.

3.3.4 Steady state amperometry

In steady state amperometry uniform potential is applied and the charge in current is monitored as function of time. The determination of the concentration of some sample amperometric technique is simple, effective and fast and it is measured by current-time plot for the biosensor in response to successive additions of aliquots of an analyte into a three electrode under stirring at 400 rotations per minute at a fixed potential.

3.3.5 Electrochemical impedance spectroscopy

The electrochemical impedance spectroscopy technique is a well known method used to investigate the surfaces of unmodified and modified electrode, studying the features of surface modified electrodes. It is employed to analyze the detailed electrochemical response of the modified electrode while using individual or mixed components. The impedance features are discussed in terms of Nyquist plots so as to analyze the contributions of different components of the cell. In Nyquist plots the electrode impedance is presented as a dependence plot between two components: the imaginary $Z'' (\omega)$ versus real $Z' (\omega)$ components that originate mainly from the capacitance and the resistance of the cell. From the shape of the impedance spectrum, the electron transfer kinetics and diffusion characteristics can be extracted. The particular semicircles of the curves correspond to the electron-transfer resistance (R) and the double layer capacity (C) nature of the modified electrode.

3.4 Other techniques

3.4.1 Ultra violet-visible (UV-Vis) spectroscopy

The UV-Vis spectra were recorded on a Nicolet Evolution 100 Spectrometer (Thermo Electron Corporation, UK). After electrodeposition of doped or undoped PDMA on glassy carbon electrode (30 cycles three times) the polymer was dissolved in 4 mL N,N-dimethylformamide (DMF) solution. The DMF solution turned into dark-green color in the case of PDMA while it turned blue when PDMA-PSS was dissolved, the color change represented the switching between reduced and oxidized sates through application of potentials their UV-Vis spectra recorded for comparison. The spectra were recorded in the region of 300-1000 nm.

3.4.2 Scanning electron microscopy

Scanning electron microscopy was used to characterize the morphology of PDMA, PDMA-PSS and that of Tyrosinase immobilized on the PDMA-PSS film. The images were acquired using either a Gemini LEO 1525 Model or a Hitachi X-650 analyzer employing the secondary electron (SE) mode with interchangeable accelerating voltages of 25 kV. Screen-printed carbon electrodes were used for electrodeposition of samples for SEM analysis. After electrodeposition of samples, the electrodes were rinsed with deionized water and left to dry at room temperature for about 30 minutes. The same samples were subjected to further analysis by EDX to determine their percentage atomic compositions.

3.4.3 Transmission electron microscopy

Transmission electron microscopy image was acquired using a Hitachi H-800 electron microscope operated at acceleration voltages of 175 keV. It was used to characterize the morphology of PDMA-PSS. The specimen was prepared by dispersing the particles of the electrosynthesized PDMA-PSS in N,N dimethylformamide, placing a few drops of the particle solution onto carbon films, then leaving the films to dry at room temperature for about 30 minutes.

3.5 Electrochemical synthesis of PDMA and PDMA-PSS

2,5 dimethoxyaniline (DMA) is the substituted aniline with a molecular weight of 153.18 g/mol and a melting point 78-80°C. The 1.0 M HCl (37% purity, density 1.19 kg/L) solution used to dissolve DMA was prepared by measuring 20.0722 mL HCl from the bottle, transferring into 250 mL volumetric flask and toping up to mark with deionized water. 0.15318 g of DMA monomer was accurately weighed in small beaker and dissolved in 10 mL of 1.0 M HCl to make up 0.1 M DMA solution. The solution was then allowed to completely dissolve by sonicating for a few minutes. The solution was transferred into a 20 mL cell, the three electrodes connected and the solution purged for 10 minutes to remove oxygen gas prior to polymerization. For electrochemical synthesis of undoped poly(2,5-dimethoxyaniline) (PDMA), the potential was cycled repeatedly from -0.2 to +0.8 V, at a potential scan rate of 40 mV s^{-1}. Electropolymerization was stopped after 10 voltammetric cycles. The electrochemical behavior of PDMA/GCE was then investigated in 1.0 M HCl solution by cyclic voltammetry in the potential range between -0.2 to +0.8 V,-1.2 to +1.2 V and at varying scan rates (2-70 mV s^{-1}) as well as in phosphate buffer pH ranging from 4.2 to 9.2. The poly(2,5-dimethoxyaniline) doped

with poly(4-styrenesulfonic acid) (PDMA-PSS composite film) was prepared by electrochemical polymerization of 2,5-dimethoxyaniline (DMA) in 1.0 M HCl in the presence of 500 μL PSS dopant (DMA-PSS ratio of 2:1) and electrodeposition on GCE surface. The potential was cycled repeatedly from -0.2 to +0.8 V, at a potential scan rate of 40 mV s^{-1}. Electropolymerization was stopped after 10 voltammetric cycles. PSS was used for doping the PDMA to enhance the electron transfer on the electrode surface. The electrochemical behavior of PDMA-PSS/GCE was then investigated in 1.0 M HCl solution by CV in the potential range between -0.2 to +0.8 V,-1.2 to +1.2 V and at varying scan rates (2-70 mV s^{-1}) as well as in 0.1 M phosphate buffer pH ranging from 4.2 to 9.2.

3.6 Biosensor construction

The electrode was first modified with PDMA-PSS, after electropolymerization the electrode was washed with deionized water on the sides to remove the excess monomer from the electrode surface. 100 μL of 4.0 mg/mL phosphate buffer solution of tyrosinase was mixed with 50 μL bovine serum albumin (BSA) in a vial then 5 μL of this mixture drop-coated on the surface of GCE modified with PDMA-PSS. 2 μL of 2.5% glutaraldehyde was then dropped into the mixture and the electrode was allowed to dry at room temperature overnight while covered to prevent contamination from dust. Tyr/PDMA-PSS/GCE (biosensor) was then characterized in 0.1 M phosphate buffer, pH 7.2 by cyclic voltammetry and differential pulse voltammetry in the potential range between -1.2 to +1.2 V. The biosensor was stored in 0.1 M phosphate buffer at 4°C when not in use.

3.7 Detection and investigation of the electrochemical behavior of bisphenol A

The electrochemical behavior of bisphenol A was investigated on bare GCE, Tyr/GCE, PDMA/GCE, PDMA-PSS/GCE and Tyr/PDMA-PSS/GCE in 0.1 M phosphate buffer at pH ranging 4.2 to 9.2 using CV and DPV within a potential range of -1.2 to +1.2 V in the presence and absence of oxygen. Aliquots of the 0.01 M stock solution of BPA were injected into phosphate buffer in increasing concentrations and their voltammograms recorded. The CV, SWV and DPV experiments were performed in unstirred solutions. Amperometric biosensor measurements were performed at an applied potential of -0.1 V and -0.61 V by successive additions of 5-100 μL of 0.01 M BPA. The experiments were performed in phosphate buffer (pH 4.2 -9.2) under magnetic stirring at 400 rpm.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Introduction

Electrochemical polymerization of monomer is achieved by employing cyclic voltammetry technique, the potential window used is dependent on the type of monomer used in particular study whether it's substituted or not and also on the type of electrode used as well as solvent. The scan rate at which the polymerization process performed under is very important because it determines the compatibility and stability of the film on the surface of the electrode. To confirm polymerization, the same peak characteristics observed during polymerization should be present during characterization in absence of the monomer and this was performed at different scan rates where the peak current is expected to increase with the increase in scan rate. This chapter discusses various techniques to characterize the polymer film onto the surface of the electrode, techniques such as cyclic voltammetry, ultra-visible spectrometry, transmission electron microscopy, and scanning electron microscopy. The techniques above are employed to confirm successful polymerization and that the polymer film is successfully deposited onto the surface of glassy carbon electrode and also proves the success of dopant employed by looking at the potential range where the redox peaks are observed in absence and presence of the dopant. Cyclic voltammetry was once again used to provide evidence that BPA has a potential to polymerization onto the surface of glassy carbon electrode leading to shielding the electrode

surface and preventing electron transfer from the electrode surface to the electrolyte, this causes electrode fouling. This fouling problem is controlled by modifying the electrode surface before detection takes place. Clearly labeled graphs are shown to represent the results obtained from the characterization study.

4.2Electrochemical synthesis and characterization of PDMA and PDMA-PSS

Electrochemical polymerization of the monomer 2,5-dimethoxyaniline on GCE surface in absence and presence of the dopant poly(4-styrenesulfonic acid) was achieved by cycling the potential repeatedly between -0.2 and +0.8 V at a scan rate of 40 mV s^{-1} for 10 voltammetric cycles. The color of the polymer film formed on GCE surface was dark green. The cyclic voltammograms for the electrodeposition of PDMA and PDMA-PSS films on GCE surfaces are shown in Figure 4.2.1. Two pair of redox peaks centered at approximately 0.2 V (a/a') and 0.55 V (c/c'), corresponding to the transition from leucoemeraldine to emeraldine and emeraldine to pernigraniline states of aniline respectively were observed for both undoped PDMA and doped PDMA-PSS. Electrodeposition of PDMA-PSS films onto GCE surface proceeds via radical cation mechanism. In this procedure, the monomers at the working electrode surface undergo oxidation to form radical cations that react with other monomers or radical cations, forming insoluble polymer chains on electrode surface. For electropolymerization, the potential is cycled repeatedly to allow uniform distribution of the film on surface electrode. The thinner the film on GCE surface, the better is the electron transfer. Multi scan voltammetry resulted in an increase in the redox peaks which indicates the formation of conducting polymer on GCE surface. It also provides further evidence that the GCE surface is conductive, elimination of electrode fouling and well behaved electrochemistry. The origin of another pair of redox peaks observed at

approximately 0.4 V (b/b') for both PDMA and PDMA-PSS is much more complex and can be attributed to many different intermediates and degradation products (cross-linked polymer, benzoquinone, etc.) (Songa *et al.* 2009).

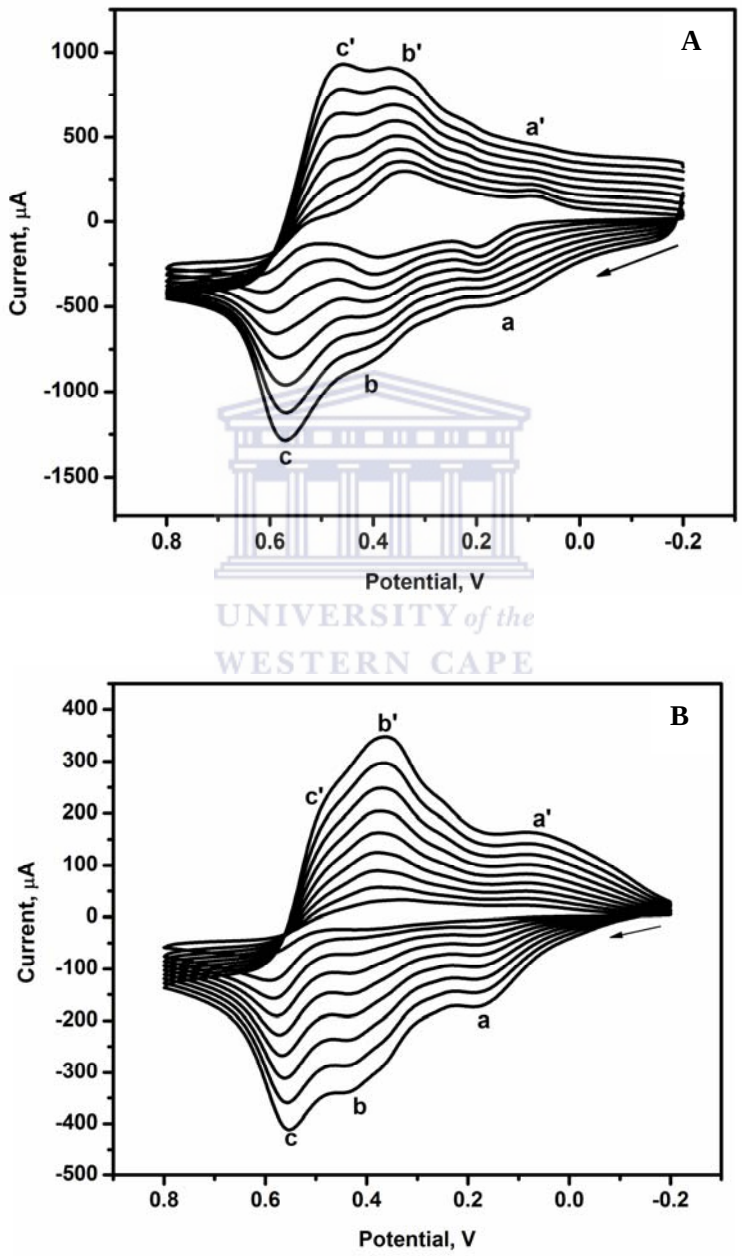

Figure 4.2.1: Electrochemical synthesis of (a) PDMA and (b) PDMA-PSS in 1.0 M HCl at scan rate 40 mV s^{-1}.

4.3Characterization of PDMA and PDMA-PSS by cyclic voltammetry

Electrodeposited PDMA and PDMA-PSS film was further subjected to characterization by cyclic voltammetry in 1.0 M hydrochloric acid at various scan rates. Cyclic voltammetric characterization of polymer films in 1.0 M HCl showed three distinct redox pair, which proved the PDMA and PDMA-PSS films are electroactive and showed fast reversible electrochemistry and the results are shown in Figure 4.3.1. Three pair of redox peaks centered at approximately 0.2 V (a/a', corresponding to the transformation of leucoemeraldine base to emeraldine salt), 0.4 V (b/b', as a result of degradation products) and 0.5 V (c/c', corresponding to the transformation of emeraldine salt to pernigraniline salt) can be observed for both undoped PDMA (Figure 4.3.1 a) and PDMA-PSS (Figure 4.3.1 b) modified electrodes in 1.0 M HCl solution (Songa *et al.* 2009). It was observed that the peak currents increased with increase in scan rates for both PDMA and PDMA-PSS, which confirmed that the polymer was electroactive and diffusion of electrons was occurring along the polymer chain. The anodic peak currents showed a linear dependence on the scan rate indicating that a thin film of surface bound conducting electroactive polymer was observed, undertaking fast reversible electron transfer reaction (Mathebe *et al.* 2004). Characterization of the polymer was performed to study the position of the redox peak couples in order to investigate if polymerization took place. To confirm polymerization, the same peak characteristics observed during polymerization should be present during characterization of the polymer. The results of characterization obtained in this study confirmed successful polymerization and that PDMA and PDMA-PSS were successfully attached onto the glassy carbon electrode surface and also proves the success of the dopant used by looking at the potential range where the redox peaks are observed with the absence and presence of the dopant.

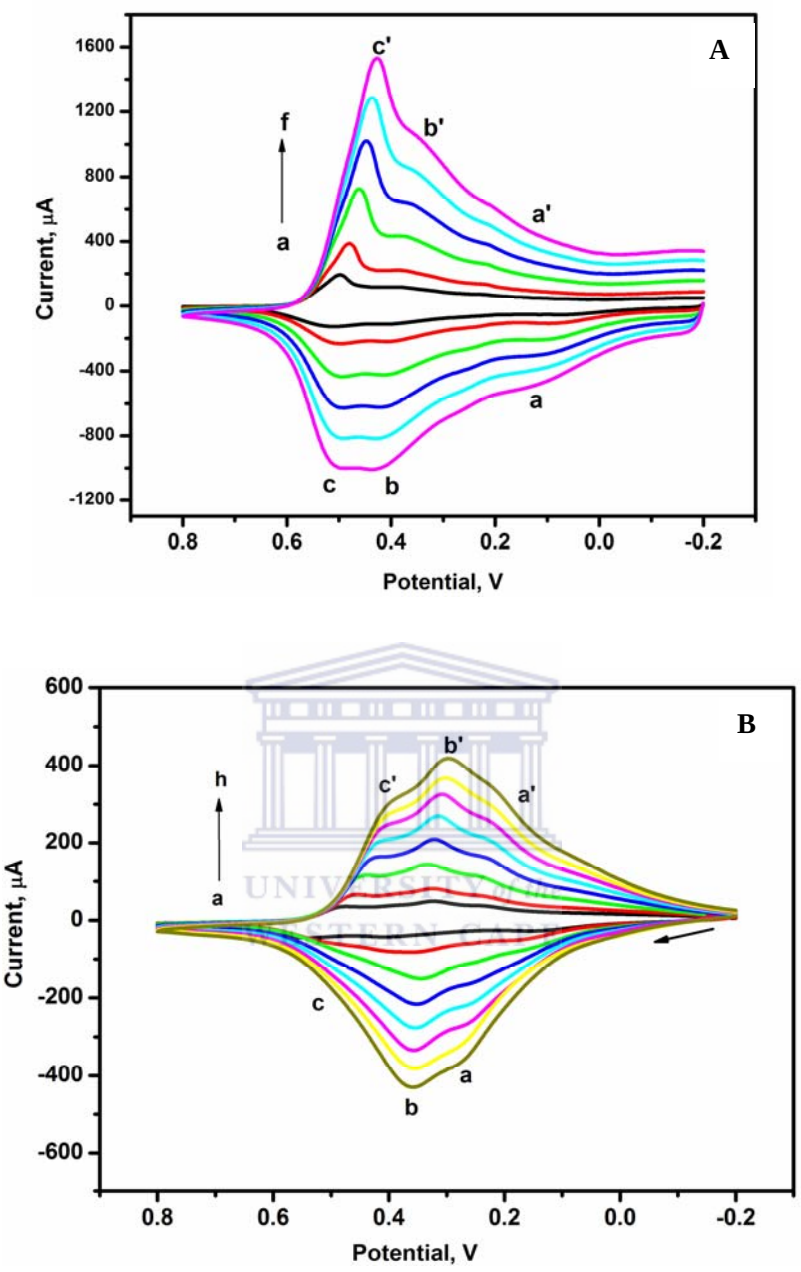

Figure 4.3.1: .Cyclic voltammograms for electrochemical characterization of (a) PDMA at scan rates 5, 10, 20, 30, 40 and 50 mV s^{-1} (a-f) and (b) PDMA-PSS at scan rates 5, 10, 20, 30, 40, 50, 60 and 70 mV s^{-1} (a-h) in 1.0 M HCl solution.

4.4Characterization of PDMA and PDMA-PSS films

Electrodeposited PDMA and PDMA-PSS film was further subjected to characterization by cyclic voltammetry in 0.1 M phosphate buffer pH ranging from 4.2 to 9.2 at potential range -0.2 to + 0.8 V at different scan rates to prove that 1.0 M HCl was the suitable solution to characterize the polymers due to its lower pH. The results are shown in Figure 4.4.1 but only one 10 mV s^{-1} scan rate shown compared with 1.0 M HCl results. For pH 4.2 to 5.2 two pair of redox peaks centered at approximately +0.1 V (a/a',) and -0.1 V (b/b$^{'}$) was observed for both undoped PDMA in 0.1 M phosphate buffer pH 4.2, which confirmed that the polymer was electro-inactive and no diffusion of electrons were occurring along the polymer chain (Figure 4.4.1 a) and PDMA in 1.0 M HCl (Figure 4.4.1 b) modified electrodes at potential range -0.2 to +0.8 V and scan rate 10 mV s^{-1}. Then pH 6.2 to 9.2 showed only one peak in this case the polymer was completely electro-inactive. It was observed that the peak currents increased with increase in scan rates for both PDMA and PDMA-PSS. Characterization of the polymer is performed to study the position of the redox peak couples in order to investigate if polymerization took place. In confirming polymerization, the same peak characteristics observed in polymerization are expected to be present during characterization of the polymer. This behavior was caused by lack of H^{+} involved in phosphate buffer pH 4.2 to 9.2 and polymer is known to denatures at higher pH (Kuramitz *et al.* 2001). The results obtained for characterization in this study did not confirm successful polymerization because phosphate buffer pH 4.2 to 9.2 was a poor method to confirm PDMA and PDMA-PSS successfully deposition onto the surface of glassy carbon electrode due to pH difference. 1.0 M HCl pH was checked on pH meter to be around pH 0.4. These results proved that 1.0 M HCl is the best solution for polymer characterization.

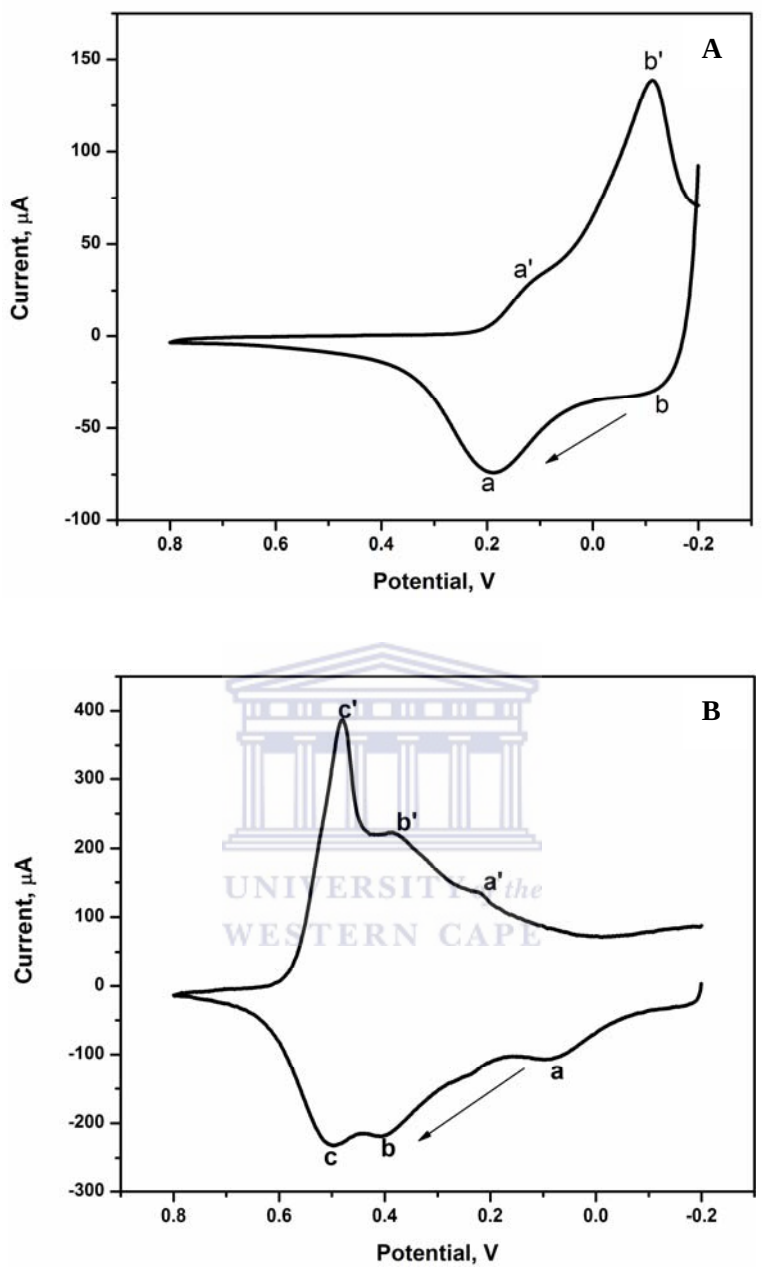

Figure 4.4.1: Characterization of (a) PDMA/GCE in purged phosphate buffer pH 4.2, (b) PDMA/GCE in 1.0 M HCl at 10 mV s^{-1}.

4.5Electrochemical polymerization of BPA on glassy carbon electrode surface

The electrochemical polymerization of BPA on glassy carbon electrode was investigated by addition of about 30 µL (6.0 x 10^{-6} M) of 0.001 M BPA in 5 mL phosphate buffer pH 4.2 to 9.2 this was achieved by cycling the potential repeatedly between -0.2 and +0.8 V at a scan rate of 40 mV s^{-1} for 5 voltammetric cycles. Only one oxidation peak was observed at potential ca +0.56 V. The cycles were observed to decrease in peak current as the number of cycles increased. This behavior confirmed the polymerization of BPA that leads to electron transfer blockage causing electrode fouling (Kuramitz *et al.* 2001). The same behavior was observed for all pH's used ranging from pH 4.2 to 9.2. This study was done to give evidence for the need of electrode modification, which is used to reduce fouling problem.

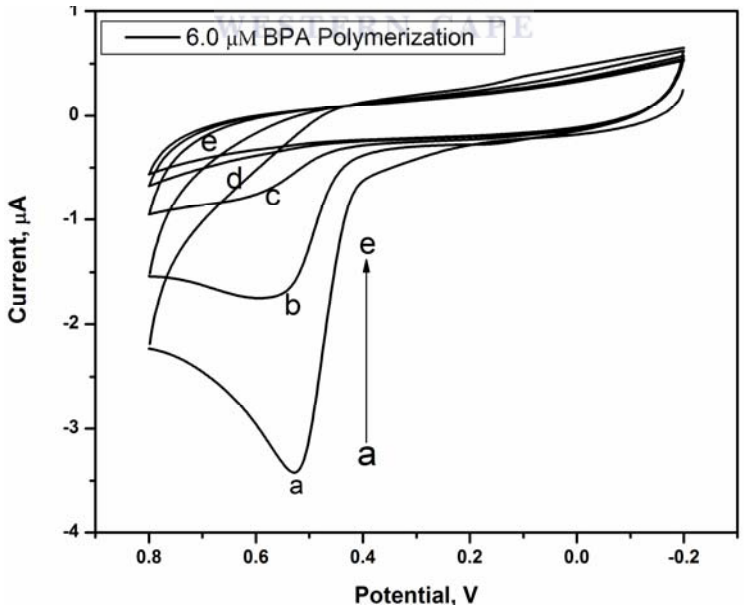

Figure 4.5.1: Electropolymerization of 6.0 µM BPA in 5 mL phosphate buffer pH 7.2 on bare glassy carbon electrode.

4.6Characterization of PDMA and PDMA-PSS films by UV-Vis spectroscopy

UV-Vis spectroscopy is a commonly used technique to give qualitative indication of the intrinsic redox states of conducting polymers. In this study, UV-Vis spectroscopy has been used to give an indication of the redox state of PDMA-PSS and to study the changes that occur as a result of doping (Songa *et al.* 2009). The electropolymerized PDMA and PDMA-PSS films were dissolved in DMF and subjected to UV-Vis analysis. The UV-Vis spectra results are shown in Figure 4.6.1 below. Differences in the positions and size of absorption bands are observed when UV-Vis spectrum of undoped PDMA is compared with that of PDMA-PSS. The UV-Vis spectrum of PDMA-PSS film shows a broad peak in the region ca. 600 nm and a small peak in the region ca. 910 nm. The appearance of the quinoid π-π* transition band at ca. 600 nm in the spectra for doped PDMA (PDMA-PSS) is indicative of incomplete doping of PDMA during electropolymerization (Songa *et al.* 2009). The undoped PDMA on the other hand displays a small peak at ca. 360 nm, a strong band at ca. 600 nm that is characteristic of emeraldine base and a small band at 910 nm. No peak was however observed at ca. 360 nm in doped PDMA spectra. The features observed for PDMA and doped PDMA were found to be similar to those of polyaniline and its derivatives indicating the immobilization of the polymer on the electrode surface.

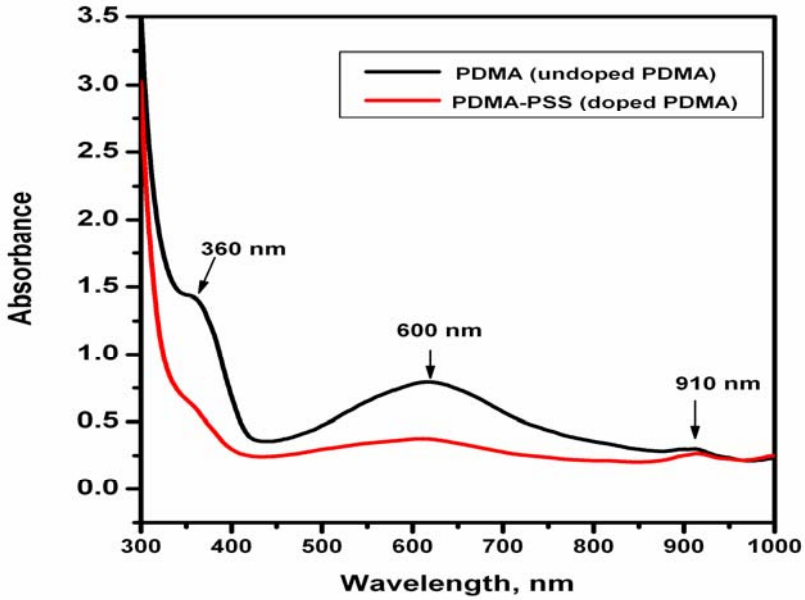

Figure 4.6.1 : UV-Vis spectroscopy of PDMA and PDMA-PSS.

4.7 Characterization of PDMA and PDMA-PSS films by scanning electrode microscopy and transmission electrode microscopy

The morphologies of PDMA and PDMA-PSS on carbon screen printed electrode were characterized by scanning electron microscopy (SEM, Philips, and XL30 SFEG). The scanning electron microscopy and transmission electron microscopy images of the electrosynthesized PDMA and PDMA-PSS films are shown in (Figure 4.7.1.a, b, and c). The SEM image of the synthesized PDMA film by the electrochemical "no-template" method i.e. without employing any template is shown in Figure 4.7.1 (a). A sponge-like branched, porous-structured, high-surface area polymer film of a SPE was observed. The SEM image of PDMA synthesized by the electrochemical "soft template" method (PDMA-PSS), employing the polyelectrolyte PSS to act as a structure directing molecule shows globular morphology with buds of polymer having diameters less than 100 nm. As observed, there is a change in morphology when PSS is

incorporated into PDMA (Figure 4.7.1 b) indicating that PSS played a role in directing the structure and morphology and aligning the monomers to form more ordered structures. The TEM image (Figure 4.7.1 c) PDMA-PSS synthesized by the electrochemical "soft template" method shows nanotubular structures with diameters ranging from 10-40 nm. This indicates that PSS can be used to direct the synthesis of both nanoparticles and one-dimensional nanostructured substituted PANI such as PDMA.

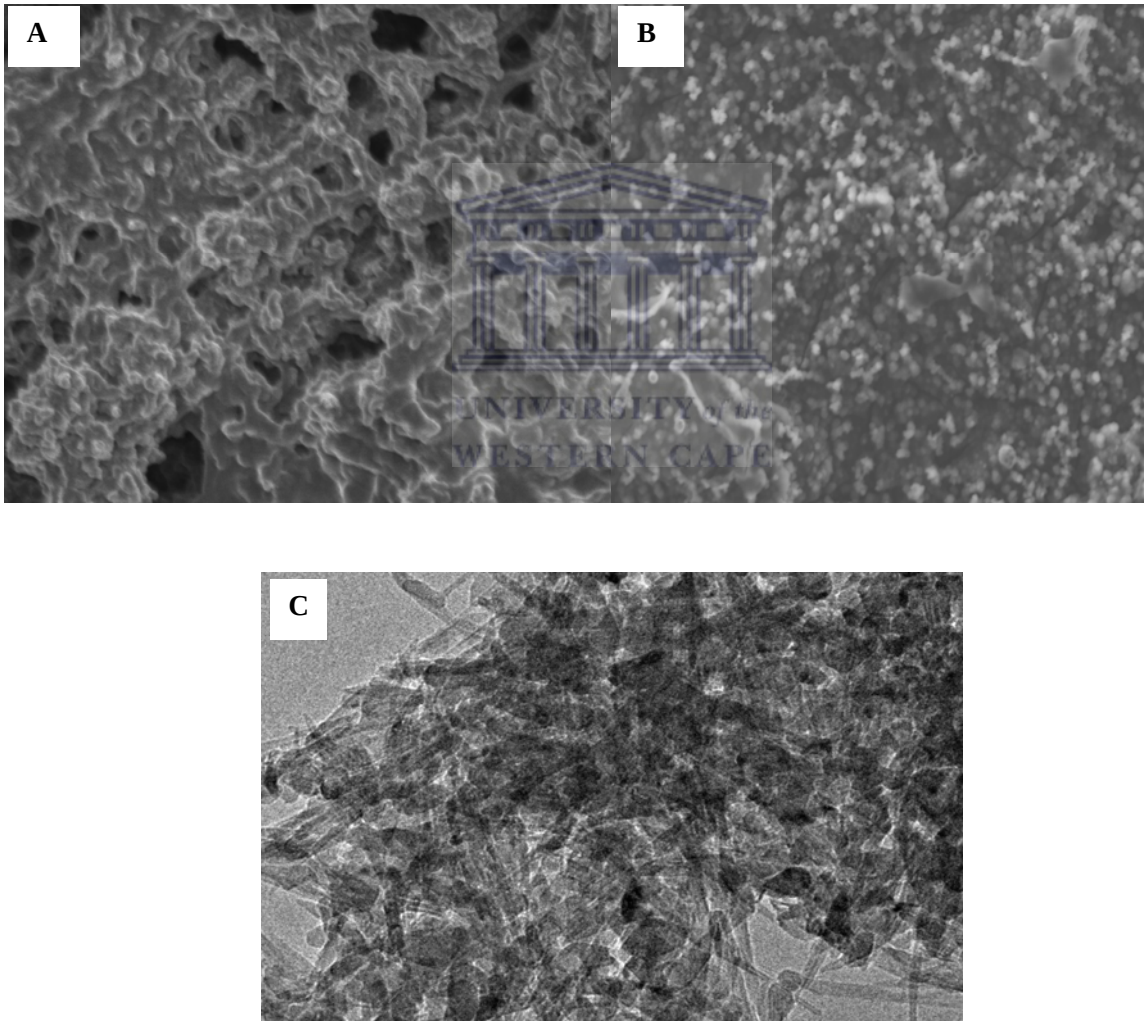

Figure 4.7.1 : Scanning electrode microscopy image of (a) PDMA microfibers and (b) PDMA-PSS and transmission electrode microscopy image of PDMA-PSS nanotubes (magnification of 100 000 x).

*4.8*Conclusion

Electrochemical synthesis of nanostructured PDMA-PSS materials were successfully prepared by means of electrochemical "soft template" method wherein PSS acted as a dopant and provided the template for the synthesis of the nonmaterial's. Redox physical transitions were clearly showed by polymerization and characterization of the polymer. The results obtained showed that when PSS is employed as a polyelectrolyte, it can direct the synthesis of both nanotubes and nanoparticles with diameters less than 100 nm. The spectroscopic techniques revealed that the PDMA-PSS materials exhibited properties similar to those of the parent doped PANI (unsubstituted) and they have backbone structures similar to that of the conventionally prepared granular doped PANI.

References

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Mathebe, N. G. R., Morrin, A. and Iwuoha, E. I. (2004). "Electrochemistry and scanning electron microscopy of polyaniline/peroxidase-based biosensor." Talanta **64**(1): 115-120.

Songa, E. A., Iwuoha, E. I. and Baker, P. G. L. (2009). "Composite poly(dimethoxyaniline) electrochemical nanobiosensor for glufosinate and glyphosate herbicides." unpublished PhD thesis , University of the Western Cape:126-129.

CHAPTER 5

BIOSENSOR CHARACTERIZATION AND APPLICATION

5.1 Introduction

Tyrosinase inhibition-based biosensor has been constructed and evaluated for the detection of BPA. After the Tyr/PDMA-PSS nanobiosensor was constructed and optimized, it was placed into a three-electrode electrochemical cell into which the analytes and samples of interest were injected and determined. The electrodes were connected to the computer-controlled potentiostat and the determination of the BPA achieved directly using steady-state amperometry, electrochemical impedance amperometry, square wave voltammetry and cyclic voltammetry. For all studies, the response of the biosensor before its contact with the BPA was measured by investigating its biocatalytic activity towards the oxidation of the substrate BPA. The response of the immobilized tyrosinase enzyme to its substrate BPA was a measure of its activity. The detection of the BPA was carried out in the presence of a fixed concentration of BPA. This chapter presents the results of analysis of bisphenol A by Tyr/PDMA-PSS biosensor employing voltammetric, amperometric and impedimetric techniques monitoring the interaction of tyrosinase and the bisphenol A.

5.2 Voltammetric characterization of the biosensor

Electrochemical characterization and optimization of biosensor response (Tyr/PDMA-PSS/GCE) was achieved by cyclic voltammetry and square wave voltammetry in 0.1 M phosphate buffer (pH 7.2) at a potential range of -1.2 to +1.2 V at scan rate of 20 mV s^{-1} and the results obtained are shown in Figure 5.1.1. Cyclic voltammograms for the biosensor (Tyr/PDMA-PSS/GCE) were recorded and compared with those of bare GCE and GCE modified with PDMA-PSS (Figure 5.1.1 a). Bare GCE showed a pair of redox peak at ca -0.22 V reduction and 0.21 V oxidation peak while two paired reduction peaks and one oxidation peak were observed for PDMA-PSS GCE at ca -0.26 and -0.67 V reduction and at ca 0.23 V oxidation peak and the Tyr/PDMA-PSS/GCE only has one reduction peak at approximately -0.65 V at lower peak current than PDMA-PSS but higher than the bare GCE. The increase in reduction peaks current of PDMA-PSS/GCE confirms successful electrodeposition of different layers onto electrode surface where as the decrease in peak current of biosensor Tyr/PDMA-PSS/GCE is influenced by the presence enzyme tyrosinase and also indicates a stronger enzyme binding and higher catalytic activity on modified GCE surface leading to improvement in electrochemical behavior (Li *et al.* 2005). This successfully incorporation of tyrosinase to the modified GCE surface is most likely related to glutaraldehyde used which acts as an enzyme cross-linker. The square wave voltammetry results obtained for the biosensor, bare GCE and PDMA-PSS/GCE are also shown in Figure 5.1.1 (b). One reduction peak was observed for bare GCE at ca -0.22 V, PDMA-PSS/GCE at ca. -0.19 V and one reduction peak was observed for Tyr/PDMA-PSS/GCE at ca. -0.15 V. A peak current for PDMA-PSS was observed to increasing up to 7.47 μA with a shift in potential to the negative potential influenced by the modified polymer when compared to bare GCE which was 6.84 μA while Tyr/PDMA-PSS GCE decreased in peak current to 5.77 μA with

a shift to more negative potential influenced by the presence of tyrosinase immobilized on modified GCE electrode. This suggests that the detection of BPA using the biosensor (Tyr/PDMA-PSS/GCE) can be achieved at much lower potentials without any interference from the immobilized PDMA-PSS.

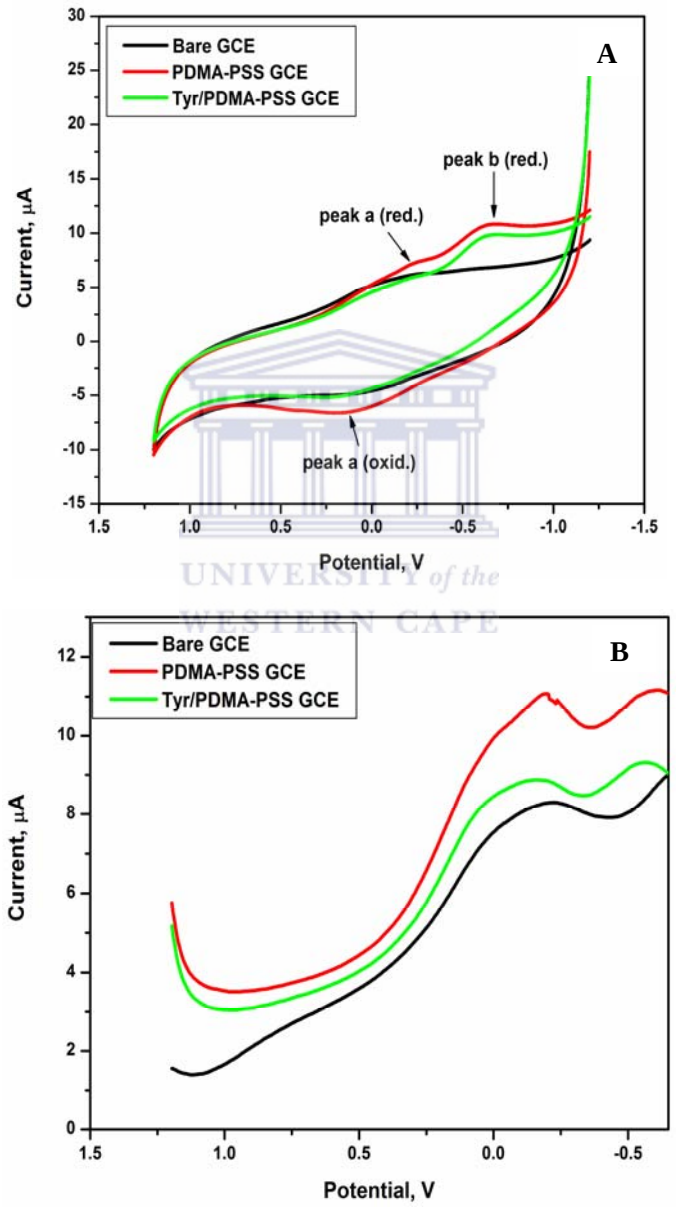


Figure 5.1.1: (a) Cyclic voltammetry, (b) Square wave voltammetry results for electrochemical characterization of bare GCE, PDMA-PSS/GCE and Tyr/PDMA-PSS/GCE in 0.1 M phosphate buffer pH 7.2.

Electrochemical characterization and optimization of biosensor response (Tyr/PDMA-PSS/GCE) was further achieved by cyclic voltammetry in 0.1 M phosphate buffer (pH 7.2) at a potential range of -1.2 to +1.2 V and the obtained results are shown in Figure 5.1.2. Cyclic voltammograms for the biosensor (Tyr/PDMA-PSS/GCE) was performed in the presence of analyte at different scan rates and the results obtained are recorded in Figure 5.1.2 cyclic voltammetry (a) and linear calibration curve (b). Two pair redox peaks are observed for Tyr/PDMA-PSS GCE at approximately -0.53 and +0.57 V reduction and at ca +0.38 and -0.43 V oxidation peak. The peak currents of the two paired redox peaks are observed to increase with increase in scan rate from 3-30 mV s^{-1}. This behavior confirms the good electron transfer between the electrode surfaces to the electrolyte, but the oxidation peaks disappears at high scan rate meaning that the BPA oxidation reaction occurs at a low rate. From the CV plot, a linear calibration curve was plotted to explore the relationship of cathodic and anodic peak current (I_{pc} and I_{pa}) against scan rate (υ), the linearity is the characteristic of surface bound or adsorbed species conducting electro-active polymer, undergoing fast reversible electron transfer reaction. The surface concentration of PDMA-PSS film on the surface of GCE, T^*_{PDMA} can be estimated from plot of I_P against v in accordance to Laviron's equation

$Ip = Wn^2F^2\Gamma^*_{PDMA-PSS}\nu / 4RT$ and $Q = nF\Gamma^*_{PDMA-PSS}A$ therefore, Laviron's equation can be described as

$$\Gamma^*_{PDMA-PSS} = \frac{Q}{nFA} \qquad \textit{Equation (2)}$$

Where n represents number of electrons (2), F is the faraday constant (96.584 C mol^{-1}), $T^*_{PDMA-PSS}$ is the surface concentration of PDMA-PSS film (mol cm^{-2}), A is the surface area of the electrode (0.017 cm^{-2}), v is the scan rate (V s^{-1}), I_p is the peak current (A) and Q is the quantity of

charge (C) calculated by A/υ, where A represents area of oxidation peak from CV for the first segment obtained from CV integration with scan rate 0.003 V s^{-1}. The surface concentration ($T^*_{PDMA\text{-}PSS}$) of the adsorbed electroactive species evaluated from the slope was estimated to be 1.0218 x 10^{-7} mol cm^{-2}. This surface concentration value obtained in this study was compared to literature and it was similar to the value reported by Mathebe et al as 1.85 x 10^{-7} mol cm^{-2} for PANI film and Songa et al with surface concentration of 1.2478 x 10^{-8} mol cm^{-2} for PANI-$FcPF_6$ (Mathebe *et al.* 2004; Songa *et al.* 2009). These results gave evidence that PDMA has indeed similar properties with PANI as reported in literature. The peak current was found to be increasing with increase in scan rate with a shift in peak potential to the positive potential and becoming broad, this indicates that the peak current is diffusion controlled. The diffusion coefficient, D_e was calculated from a CV plot of I_p against υ in Figure 5.1.2 using the Randle Sevcik equation shown in equation (2) below. The value of D_e was estimated to be 8.424 x 10^{-7} cm^2 s^{-1} and it was similar to the D_e value reported by Mathebe et al which was 8.68 x 10^{-9} cm^2 s^{-1} for PANI film and Songa et al which was 2.41 x 10^{-8} mol cm^{-2} for PDMA-PSS in gold electrode (Mathebe *et al.* 2004; Songa *et al.* 2009). The D_e value depends on the electro-deposition conditions used and the homogeneity of the film. The D_e value obtained was higher than the reported due to the presence of dopant used poly(4-styrenesulfonic acid) which increases the conductivity of PDMA film, resulting in increase in Diffusion coefficient electron value. These results confirmed high conductivity of polymeric film influenced by sulphur substituent in PDMA-PSS, thus resulting to faster electron transfer.

$$Ip/v^{1/2} = \left(2.69x10^5\right)n^{3/2}AD^{1/2}C \qquad \textit{Equation (3)}$$

Where C is the concentration of analyte in (mol cm^{-3}), n is number of electrons (2), A is the surface area of glassy carbon electrode = 0.071 cm^2, v is the scan rate (V s^{-1}), I_p is the peak

current (μA) and slope = 4.5507 x 10^{-5} obtained from the plot of I_p against $\upsilon^{1/2}$ with correlation coefficient (R^2) of 0.99641 shown in the linear calibration plot below Figure 5.1.2 (c).

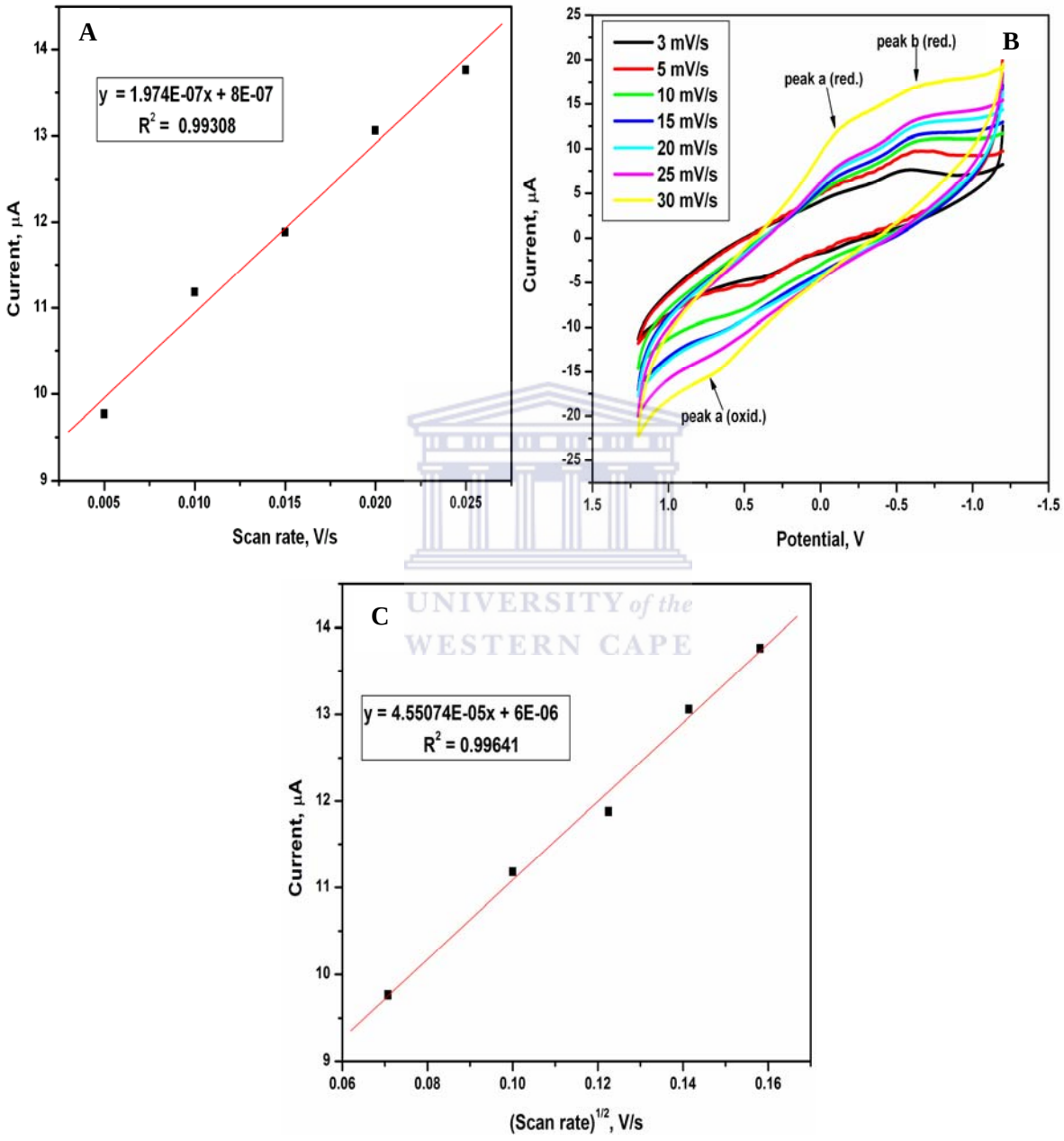


Figure 5.1.2: Cyclic voltammogram for electrochemical characterization of (c) Tyr/PDMA-PSS/GCE at scan rates 3, 5, 10,15, 20,25, 30 mV s^{-1}, (b) Linear calibration plot of current against scan rates and (c) Linear calibration plot of current against square scan rates in 0.1 M phosphate buffer solution containing 0.001 M BPA.

5.3 Impedimetric characterization of biosensor

5.3.1 Nyquist plot characterization

For further characterization of modified electrode and biosensor, the electrochemical impedance spectroscopy was used to study the features of surface modified electrodes. Charge-transfer resistance, the ion-diffusion coefficient into the polymer and the redox capacitance are important parameters of electronically conducting polymers. EIS measurements were carried out in 0.1 M phosphate buffer pH 7.2 as electrolyte at potential window -1.2 to 1.2 V investigating bare GCE, PDMA/GCE, PDMA-PSS/GCE and Tyr/PDMA-PSS/GCE. This technique is a highly effective method for probing the surface features of modified electrodes. In this study, EIS was used to investigate the change in electron transfer resistance that arose from every surface modification step. Figure 5.3.1 (a) and (b) represents the Nyquist diagrams of PDMA/GCE, PDMA-PSS/GCE and Tyr/PDMA-PSS/GCE in pH 7.2 phosphate buffer. The analysis showed a smaller semi-circle at higher frequencies for doped PDMA-PSS/GCE when compared with undoped PDMA/GCE influenced by the dopant. It indicated that the electron resistance decreases when GCE was modified with doped PDMA and the decrease in the interface impedance influenced by presence of the dopant poly(4-styrenesulfonic acid) (PSS). The dopant improves conductivity of the polymer, enhancing the electron exchange between polymer and the electrode surface. EIS of PDMA/GCE and PDMA-PSS/GCE showed almost similar behavior related to their composition and morphology. However, a huge decrease in semi-circle was observed when enzyme tyrosinase was immobilized on the surface of modified electrode surface (Tyr/PDMA-PSS/GCE). This behavior indicates a stronger enzyme binding and higher catalytic activity on modified GCE surface leading to improvement in electrochemical behavior. The fine

incorporation of tyrosinase to the modified GCE surface is most likely related to glutaraldehyde used which acts as an enzyme cross-linker. The lesser the impedance, the more conductive the surface is so the results prove that tyrosinase biosensor was more conductive than the modified electrode and bare GCE is more conductive when compared to modified and biosensor. The high conductivity was due to direct interaction of electrons to the electrode surface.

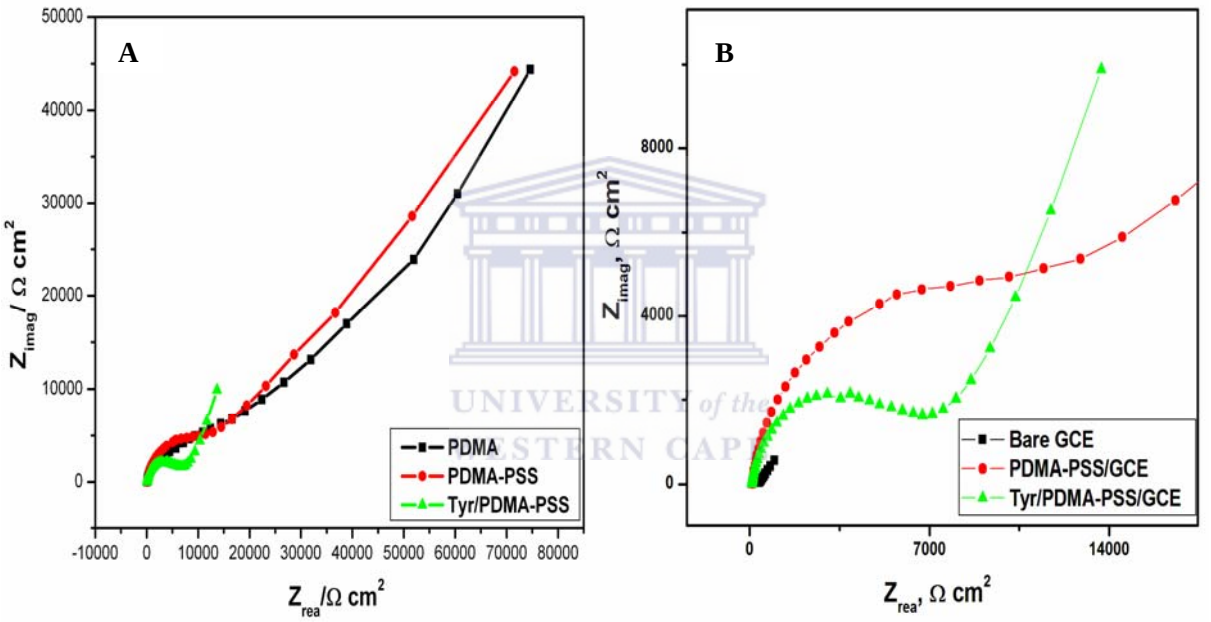


Figure 5.3.1: EIS Nyquist plot (a) represents PDMA/GCE, PDMA-PSS/GCE and Tyr/PDMA-PSS/GCE and (b) Bare GCE, PDMA-PSS/GCE and Tyr/PDMA-PSS/GCE in 0.1 M phosphate buffer.

5.3.2 Bode plot characterization

Bode plot analysis for biosensor characterization showed high conductivity in bare GCE electrode with a lower charge transfer resistance when compared to the electrode modified with PDMA-PSS and Tyr/PDMA-PSS/GCE thus allowing free flow of electron from the solution

electrolyte to the electrode surface. An increase in charge transfer resistance for bare electrode (1.218 kΩ) was due to direct interaction of electrons to the electrode surface; where as in modified undoped PDMA/GCE has a value of 9.099 kΩ, doped PDMA-PSS/GCE (9,931 kΩ) and Tyr/PDMA-PSS/GCE (5.459 kΩ) was observed. These values obtained for modified and biosensor revealed the shielding ability of the polymer thus hindering the free flow of electron from the phosphate buffer to the surface of the electrode. The exchange current value obtained for the bare electrode showed that electron transfer was faster on the bare electrode compared to the modified electrodes and biosensor. The bode plot in Figure 5.3.2 confirmed this behavior by a huge increase in impedance after the electrode was modified with the polymer with a corresponding shift in phase angle from 31° to 67.3° for the bare and polymer modified electrode respectively.

Table 5.3.1: EIS parameters attained from the circuit fitting of plots in Figure 5.3.2 for bare, modified glassy carbon electrodes and Tyr/PDMA-PSS/GCE

Element	Bare GCE	PDMA/GCE	PDMA-PSS/GCE	Tyr/PDMA-PSS/GCE
Electrolyte resistance (R_s/Ω)	2028	100.1	103.4	84.69
Charge transfer resistance (R_{ct}/kΩ)	1.218	9.099	9.931	5.459
Warburg impedance (W/kΩ)	59.85	43.66	33.93	8.803
Double layer capacity (dl/nF)	56.01	63.2	246.6	236.2

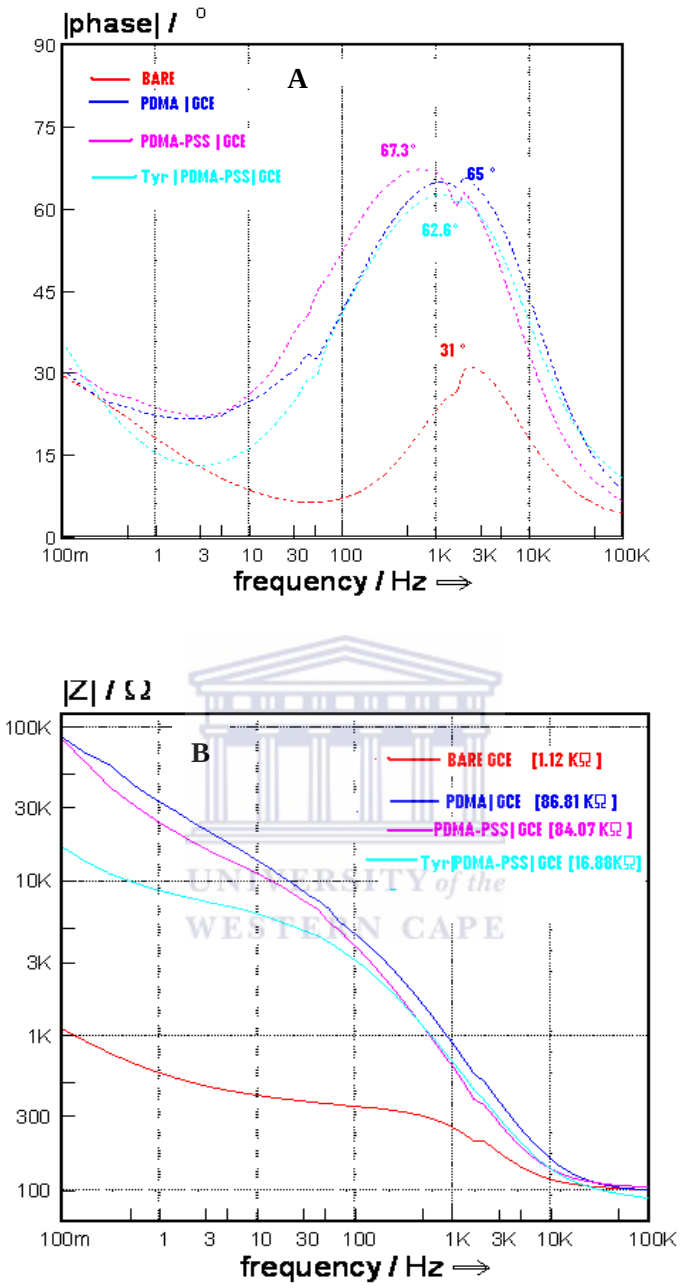


Figure 5.3.2: (a) Bode plot comparing bare GCE, PDMA/GCE, PDMA-PSS/GCE and Tyr/PDMA-PSS/GCE and (b) Plot for Frequency angles

The parameters from table 5.3.2 are used to calculate the time constant use equation 4 a and b and the exchange current by using equation 5 (Quintin *et al.* 2006; Arotiba *et al.* 2010)

Time constant = τ

$$\omega_{\max} = \frac{1}{R_{ct}C_{dl}} \qquad \textit{Equation (4 a)}$$

$$\tau = R_{ct}C_{dl} \qquad \textit{Equation (4 b)}$$

Where C_{dl} = double layer capacitance; τ = time constant; R_{ct} = charge transfer resistance. The frequency values were taken from Figure 5.3.2 b.

$\omega_{max} = 2\pi f$ *Equation (4 c)*

$$i_o = \frac{RT}{nFR_{ct}} \qquad \textit{Equation (5)}$$

$$\iota_{\circ} = nFAK_{et}C^{*} \qquad \textit{Equation (6)}$$

Where i_o = exchange current, R = gas constant, F = Faraday constant, n = number of electrons transferred, C^* = concentration of phosphate buffer pH 7.2 and K_{et} = Heterogeneous rate constant.

Table 5.3.2: Results for time constant and exchange current for bare, modified and biosensor

Element	Bare GCE	PDMA-PSS/GCE	Tyr/PDMA-PSS/GCE
Time Constant τ (s rad^{-1})	1.2079 x 10^{-4}	6.4332 x 10^{-3}	2.4615 x 10^{-3}
Exchange current i_o (A)	1.0541 x 10^{-5}	1.2928 x 10^{-6}	2.3519 x 10^{-6}
K_{et} (cm s^{-1})	7.6934 x 10^{-9}	9.4359 x 10^{-10}	1.7166 x 10^{-9}

5.4 Glassy carbon electrode

The electrochemical behavior of BPA on bare glassy carbon electrode was investigated by CV and DPV by successive additions of aliquots of 0.001 M BPA into the phosphate buffer and the results are shown in Figure 5.4.1 (CV). This study was performed at a potential range of -1.2 to 1.2 V. The diphenol BPA was used as a substrate to establish the capability of bare glassy carbon electrode optimal conditions, response time, detection limit, dynamic range and stability. The response was monitored by peak current signal, which is proportional to the BPA concentration. Cyclic voltammetry results shows two redox peaks (peaks a, b), the first reduction peak (peak a) was observed at ca. +0.05 V and the second reduction peak (peak b) was observed at ca. -0.55 V. The reduction peak currents were observed to be increasing with increase in BPA concentrations and with a slight shift in peak potentials to more negative potentials. The reduction peak currents were found to be increasing up to 20.0 μM and decreasing with further additions of BPA (the decrease data not shown on the graph) which could be due to inactivation of electrode surface which occurs once certain amounts of the substrate BPA has been oxidized. The two oxidation peaks were observed at ca. +0.38 V and ca. +0.60 V. This increase in peak currents with increase in BPA concentrations was also observed in the two oxidation peaks (peaks a, b) together with a slight shift in peak potentials to more positive potentials. Two oxidation peaks are as the results of oxidative polymerization of phenolic compounds (BPA), where peak a corresponds to oxidation monophenol to diphenol, peak b corresponds to further oxidation of diphenol to a formation of quinone and the formed quinone on electrode surface gets reduced at appropriate potential and converted into signal. Electrochemical oxidation of BPA led to deposition of electropolymerized film blocking electron transfer causing electrode fouling making bare GCE difficult to be used for detection of BPA (Notsu *et al.* 2002). The cyclic voltammetry below gave

evidence that the oxidation of BPA occurred was reversible. Bare GCE was found to have a potential in detect BPA but the problem was fouling caused by oxidative polymerization of BPA on GCE surface, a conducting polymer had to intervene because they are known to work as a mediator for shuttling of electrons from the GCE surface to substrate binder and biological element which can be enzyme, DNA and antibody etc were needed to be incorporated within the construction of the biosensor.

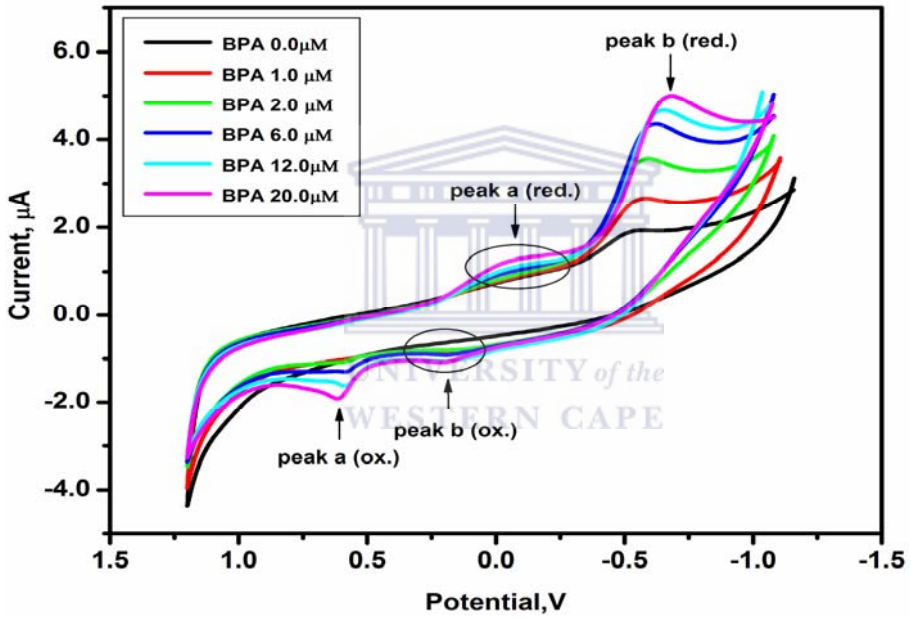
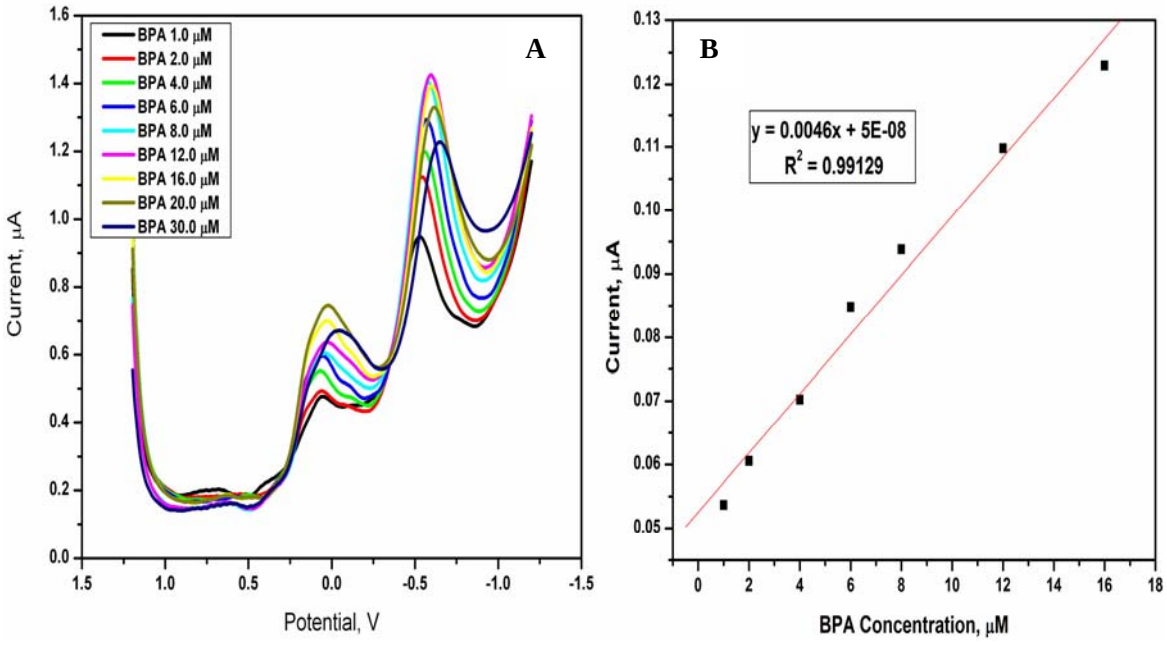


Figure 5.4.1: Cyclic voltammograms showing the electrochemical behavior of various concentrations of BPA at the surface of bare glassy carbon electrode.

Figure 5.4.2 illustrates the DPV results for the electrochemical behavior of BPA at the bare glassy carbon electrode as a result of the successive additions of aliquots of 0.001 M BPA into 0.1 M phosphate buffer. The study was performed at a potential range of -1.2 to 1.2 V. The DPV results showed two reduction peaks centered at ca. +0.05 V and ca. -0.55 V (Figure 5.4.2 a). The reduction peak currents were observed to be increasing with increase in BPA concentrations and

with slightly shift in peak potentials to more negative potentials. The peak currents for the reduction peak centered at ca. +0.05 V was observed to be increasing up 30.0 µM and decreasing with further additions of BPA. The peak currents for the reduction peak centered at ca. +0.55 V also increased with increase in BPA concentrations up to 12.0 µM then further additions of BPA led to decrease in peak currents. These could also be due to inactivation of the electrode surface which occurs once certain amounts of the substrate BPA have been oxidized. Two oxidation peaks were observed at ca. +0.38 V and ca. +0.60 V (Figure 5.4.2 c). The increase in peak currents with increase in BPA concentrations was also observed in the two oxidation peaks together with slightly shift in peak potentials to more positive potentials. The DPV results obtained were found to be similar to those obtained by CV. To conclude the inactivation of electrode surface depends on the adsorptivity of the species of bisphenol A to the electrode, which is stronger in neutral medium than that of alkaline medium (Krishnan *et al.* 1993). Figure 5.4.2 (b and d) illustrates linear calibration curve for the response of bare GCE to additions of aliquots of 0.001 M BPA representing the behavior of peak a.



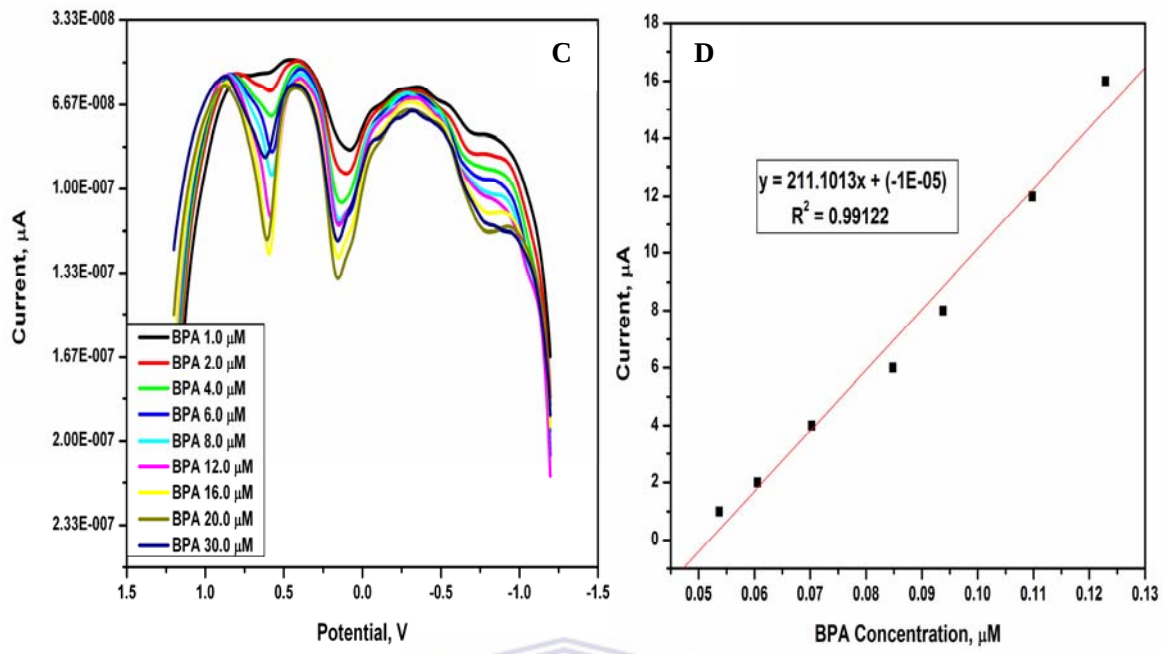


Figure 5.4.2: Differential pulse voltammograms showing the electrochemical behavior of various concentrations of BPA at the surface of bare glassy carbon electrode (a) DPV reduction, (d) DPV oxidation and (b and d) linear calibration for BPA additions representing first peak behavior.

5.5 Optimization pH study of native tyrosinase

Differential pulse voltammogram technique was employed for the optimization activation of native tyrosinase (Tyr/GCE) at potential range of -1.2 and 1.2 V. This study was performed to investigate the outcomes of native tyrosinase response to pH when immobilized on GCE surface examined in 0.1 M phosphate buffer pH range 4.2 to 9.2 with fixed BPA concentration. The pH study is performed to find the optimum activation pH of native tyrosinase. Figure 5.5.1 below illustrates DPV reduction and oxidation of Tyr/GCE comparing activation of free tyrosinase in pH ranging from 4.2 to 9.2 phosphate buffer at fixed concentration of 0.001 M BPA 3.0 µM in 10 mL cell. Previous studies reports that enzymatic activity is known to be greatly influenced by the medium pH; therefore, the Tyr/GCE response was investigated using pH ranging from 4.2 to

9.2. The cathodic peak current increased with increasing pH solution from 4.2 until it reaches 7.2. But when the pH value of the solution exceeded 7.2, the cathodic peak current decreased rapidly with each increment of pH solution. This behavior simple indicates that the tyrosinase activity increases from pH 4.2 up to 7.2 and gradually decrease response when pH values exceeds 7.2 which can be associated with a lower tyrosinase activity due to denaturing of the enzyme at high pH. It was also observed that the potential shifted with the increasing with pH, indicating that protons take part in the redox process of BPA on Tyr/PDMA-PSS modified electrode. The reason for this behavior could be probably the reduction of *o*-quinone requires H^+ in acidic medium, H^+ concentration is high enough that the nitrogen atoms of the polymer and phenol hydroxyl of BPA are protonated in the form of $–NH_3^+$ and $–OH_2^+$. This is most likely why the optimum pH shifted to the lower acidity (Kuramitz *et al.* 2001). In theory the optimum pH of free tyrosinase is near pH 6.50 and Li et al reported that this value is even more when tyrosinase is immobilized on modified surface (Li *et al.* 2005). The pH value of 7.2 was chosen as optimal and used in the following measurements. This proves that tyrosinase enzyme sustain its activation in neutral medium. In this study, the optimum pH value obtained for free tyrosinase activation was similar to the value reported by Akyilmaz et al for free tyrosinase (Akyilmaz *et al.* 2010) and Khan also reported the pH range of tyrosinase is between 4.5 and 7 (Khan, 2007). According to these data it can be assumed that the optimum pH value of free tyrosinase was not affected with the immobilization procedure used in the preparation of the biosensor.

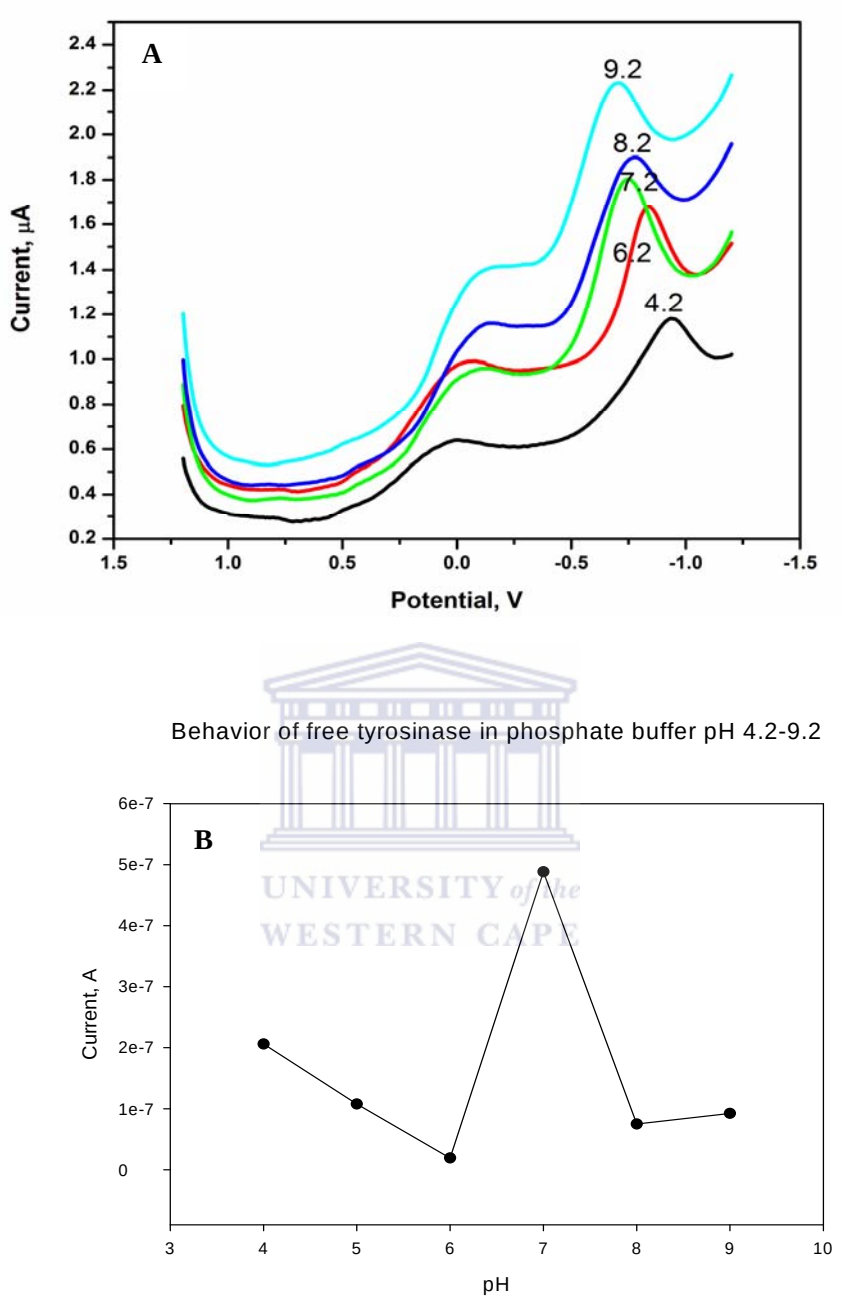
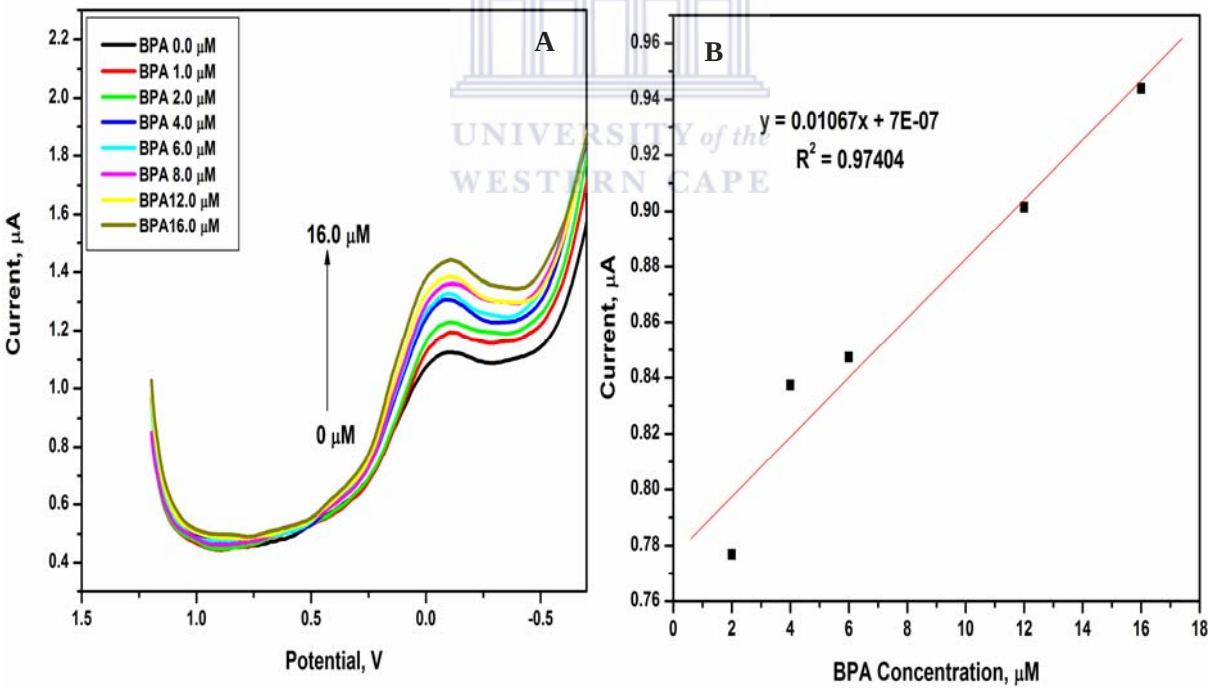


Figure 5.5.1: (a) Differential pulse voltammetry results for free tyrosinase pH optimization in Tyr/GCE in 0.1 M phosphate buffer pH 4.2-9.2 at fixed concentration of 0.001 M BPA of 3.0 µM and (b) calibration curve of DPV representing peak current against pH of phosphate buffer.

5.6 Tyrosinase modified glassy carbon electrode.

The electrochemical behavior of BPA on tyrosinase glassy carbon electrode (Tyr/GCE) was investigated by DPV by successive additions of aliquots of 0.001M BPA into the phosphate buffer and the results are shown in Figure 5.6.1 below. The study was performed at potential range of -0.7 to 1.2 V. The pH 7.2 phosphate buffer was selected as optimal and used in the following measurements. BPA was used as a substrate to establish the ability of native tyrosinase of optimal conditions, response time, detection limit, dynamic range and stability. Tyrosinase enzyme was immobilized onto the surface of bare glassy carbon and the DPV results obtained showed a reduction peak centered at ca. -0.17 V (Figure 5.6.1 a). After the first addition of BPA the reduction peaks increased with increase in BPA concentrations and with slightly shift in peak potentials to more negative potentials. The peak currents for the reduction peak centered at ca. -0.17 V was observed to be increasing up 16.0 μM and decreasing with further additions of BPA. The Tyr/GCE showed good response on BPA detection but it could not last long because tyrosinase was directly immobilized on bare GCE without the template. After few additions there was no prove if the tyrosinase was still on the GCE surface because of this there was a necessity for the polymer to work as mediator. Phenolic compounds are identified to cause inactivation for glassy carbon electrode surface since they are oxidized electrochemically, via the deposition of electropolymerized films which are created when phenoxy radical attacks an unreacted substrate (Kuramitz *et al.* 2001). This polymerized film of BPA onto the Tyr/GCE surface prevents the electron transfer between the electrode surface and the enzyme. This behavior was supported by Kuramitz on his report; inactivation of electrode surface is more easily induced in neutral and acidic medium than in alkaline medium. From all the stated reasons he speculated that inactivation of electrode surface depends on adsorptivity of species of BPA to the electrode

surface. This adsorption of BPA onto the electrode surface is identified to be stronger in neutral medium than in alkaline medium (Krishnan *et al.* 1993; Kuramitz *et al.* 2001). A defined oxidation peak was observed at ca. +0.17 V (Figure 5.6.1 b). The increase in peak current with increase in BPA concentrations was also observed in this oxidation peak together with a slight shift in peak potentials to more positive potential. The same behavior was observed in reduction peaks as well. The DPV results obtained were found to be similar to those obtained by CV but DPV was used because it's giving more defined peaks. Figure 5.6.1 (c) illustrates linear calibration curve for the response of Tyr/GCE to additions of aliquots of 0.001 M BPA. The Tyr/GCE showed promising response detection BPA, however there was no evidence proving that the enzyme was still on GCE surface.



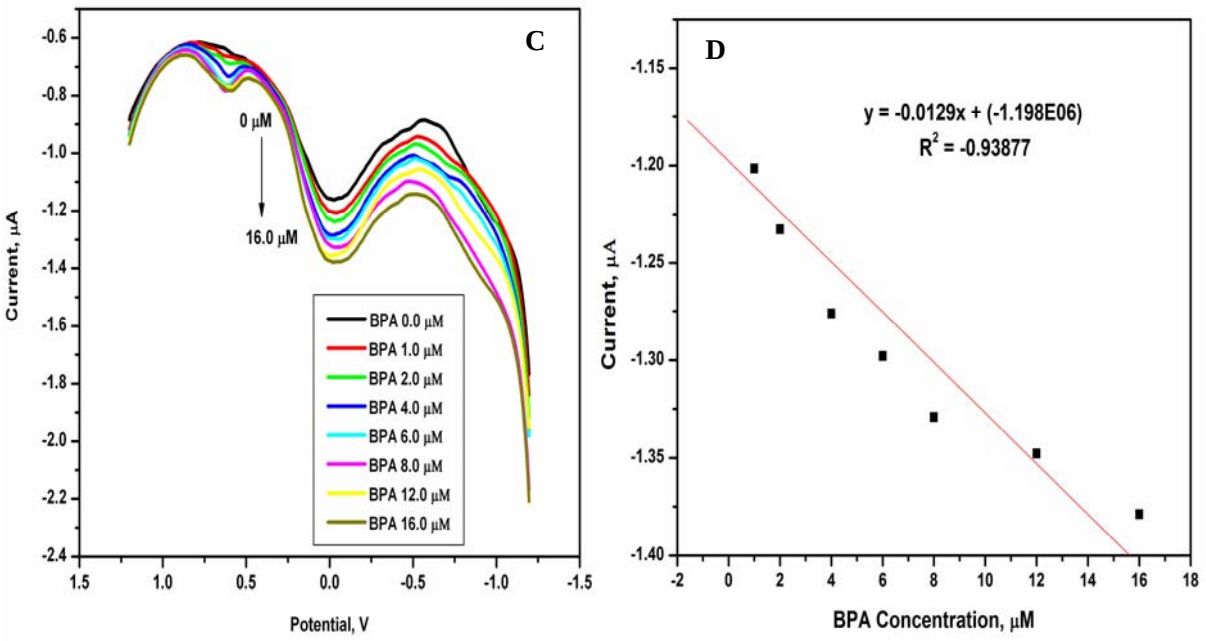


Figure 5.6.1: Differential pulse voltammograms showing the electrochemical behavior of various concentrations of BPA at the surface of tyrosinase glassy carbon electrode (Tyr/GCE) (a) DPV reduction (b) DPV oxidation and (c and d) linear calibration curve for peak.

5.7 Biosensor (Tyr/PDMA-PSS/GCE)

5.7.1 Electrochemical detection of BPA

Tyrosinase is copper-containing monophenol monooxygenase enzyme that catalyzes the hydroxylation of monophenols into *o*-diphenols (monooxygenase, cresolase, monophenolase or hydroxylase activity) and also the two-electron oxidation of diphenols (e.g. catechol, BPA etc.) to *o*-quinones (catecholase or diphenolase activity) with molecular oxygen and formation of water. The *o*-quinones generated are reduced electrochemically at appropriate potentials thus reduction currents obtained serve as analytical signals which are proportional to the concentrations of phenols or phenol derivatives e.g. BPA in solution. The active site of tyrosinase contains copper and it is where all the reactions take place thus the conversion of

phenol into *o*-quinone. In this study, the diphenol BPA was used as a substrate to establish the capability of biosensor of optimal conditions, response time, detection limit, dynamic range and stability and its conversion to *o*-quinone by tyrosinase was investigated and the signal obtained was used for quantification detection of the BPA. Figure 5.7.1 below displays SWV plots for the biosensor (Tyr/PDMA-PSS/GCE) response to successive injection of aliquots of 0.001 M and 0.01 M stock solutions of BPA into 0.1 M phosphate buffer. The SWV results showed one reduction peak centered at ca. -0.1 V. The reduction peak at ca. -0.1 V showed increase in peak currents with increase in BPA concentrations which is supported by the increase shown in the steady-state amperometry results presented in the Figure 5.7.3 in this document. The behavior observed for the peak at -0.1 V is the expected behavior in the presence of tyrosinase enzyme, which electrochemically reduces the diphenol to *o*-quinone at such a low potentials and thus it could be deduced that the peak at -0.1 V is the one due to the enzyme. The Biosensor showed good response to detection BPA, when compared to a surface without modification. Based on these results, the limit of detection for BPA (3 x standard deviation of the blank)/Sensitivity) was calculated to be 1.9 x 10^{-8} M within a concentration range of 1.0-16.0 μM BPA. Sensitivity in this case is defined as the slope of the calibration curve. The detection limit value obtained in this study was compared to literature and it was found to be similar to the value reported by Akyilmaz et al which was 9.5 x 10^{-8} M at wide range of 2.5-20.0 μM estimated for Tyr/Teflon membrane biosensor used to investigate the effect of Cu ions on activity of tyrosinase enzyme (Akyilmaz *et al.* 2010). The SWV biosensor also exhibited high sensitivity when compared to electrode and Tyr/GCE, mainly because of the good conducting polymer material deposited on bare GCE surface prior the immobilization of tyrosinase enzyme. Results obtained from Tyr/PDMA-PSS biosensor for determination of bisphenol A in water samples by the method

were compared with those of other methods, and the results are shown in Table 5.7.1. The detection limit value obtained in this study was higher but closer to those reported in literature for techniques such as Elisa, HPLC-MS, GC-MS and LC-MS. Even so application of developed polymeric tyrosinase nanobiosensor for determination of BPA compound in waste water was considered to be the best method due to short response time, cheap fabrication materials and portability while chromatographic methods are very expensive, need skilled operators, time-consuming sample pretreatment steps and long response time.

Table 5.7.1: Analytical parameters for the determination of phenols (BPA)

Technique	BPA Samples Type	Limiting detection	Retention Time	References
Elisa	Real water samples	4.4×10^{-10} M	6 minutes	(Zhao *et al.* 2002)
HPLC-MS	Water samples	2.5×10^{-9} M	15 minutes	(Jiang *et al.* 2011)
GC-MS	Water samples	2.6×10^{-9} M	8 minutes	(del Olmo *et al.* 1997)
GC	Wastewater samples	2.6×10^{-11} M	7.02 minutes	(Vílchez *et al.* 2001)
LC-MS/MS	Placenta tissue samples	8.74×10^{-10} M	10.5 minutes	(Jiménez-Díaz *et al.* 2010)
Tyr/Teflon membrane biosensor	2.5 µM +5 mM phosphate buffer	9.5×10^{-8} M	1 minute	(Akyilmaz *et al.* 2010)
Tyr-SWNTs sensor	5 µM phenol + 0.1 M phosphate buffer	2×10^{-8} M	20 seconds	(Zhao *et al.* 2005)
Tyr/PDMA-PSS biosensor	0.001 M BPA + 0.1 M phosphate buffer	1.9×10^{-8} M	12 seconds	This study

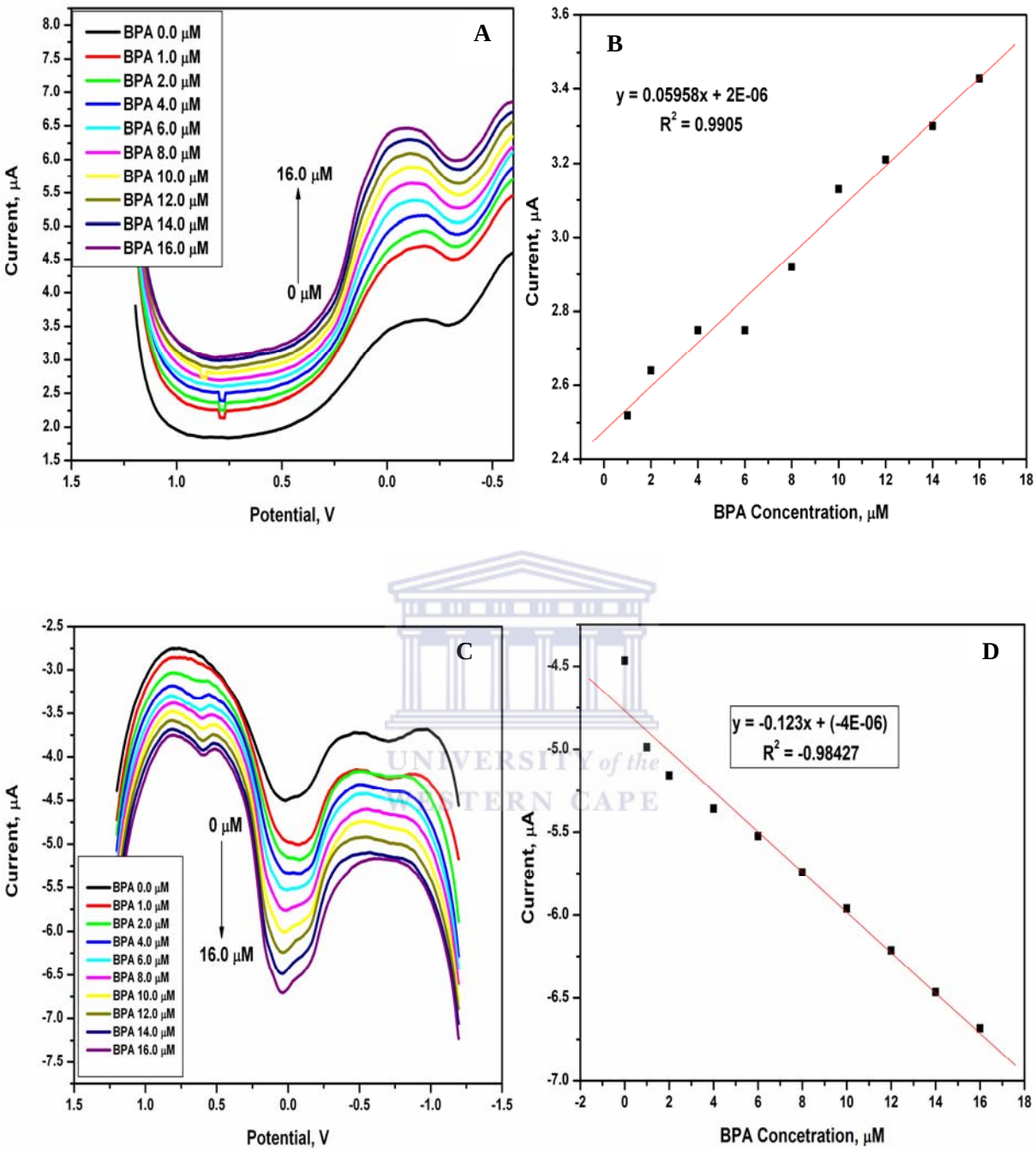


Figure 5.7.1 : Square wave voltammetry (a) SWV reduction and (b) Linear calibration curve reduction peak and (c) SWV oxidation and (d) Linear calibration curve of oxidation peak results for the biosensor in response to various concentrations of BPA.

5.7.2 Impedimetric analysis of BPA

For further investigation of BPA by biosensor Tyr/PDMA-PSS electrode, the electrochemical impedance spectroscopy was used to study the oxidation of diphenol to *o*-quinone catalyzed by tyrosinase enzyme with presence of oxygen molecules. In this study, EIS was carried out to investigate the change of electron transfer resistance that provoked from every increase in BPA concentration. Figure 5.7.2 represents the Nyquist diagram of Tyr/PDMA-PSS/GCE response to successive injection of aliquots of 0.001 M stock solution of BPA in pH 7.2 phosphate buffer. The analysis at 0.0 μM BPA concentration showed greater semi-circle at higher frequencies with charge transfer resistance (R_{ct}) value of 5.459 kΩ when compared to 10.0 μM concentration of BPA having charge transfer resistance (R_{ct}) value of 6.617 kΩ and an increase in BPA concentration resulted to decrease in charge transfer resistance R_{ct} value shown in table 5.7.1. This behavior indicates that the electron resistance decreases when BPA concentration increases and the decrease in the interface impedance can be attributed to electrostatic attraction of positive charge backbone of the polymer or the negative charge ions of the analyte and the positive charge of copper active site. The BPA improves conductivity of the biosensor, enhancing the electron exchange in the electrode surface.

Table 5.7.2: EIS parameters attained from the circuit fitting of plots in Figure 5.7.2 for Tyr/PDMA-PSS/GCE with additions of 0.001 M BPA.

Element	BPA 0.0 µM	BPA 10.0 µM	BPA 14.0 µM	BPA 18.0 µM	BPA 20.0 µM
Electrolyte resistance (R_s/Ω)	84.69	220.4	186.5	193.1	156.9
Charge transfer resistance (R_{ct}/ kΩ)	6.617	5.459	5.406	4.602	4.451
Warburg impedance (W/kΩ)	8.803	23.09	18.28	5.86	7.434
Double layer capacity (dl/nF)	236.2	659.2	562	372.1	316

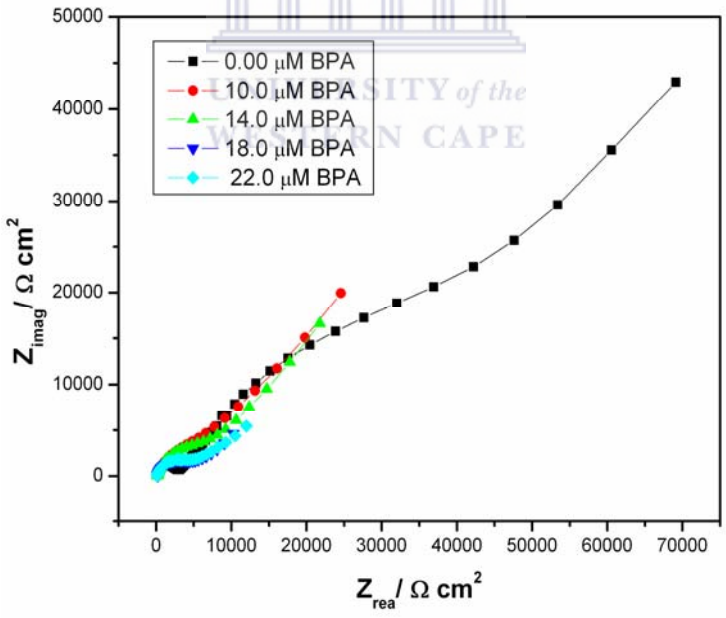


Figure 5.7.2: Nyquist plot represent impedimetric response of biosensor (Tyr/PDMA-PSS/GCE) on bisphenol A additions

5.8 Amperometric response on BPA detection

Since the cyclic voltammetry is not sensitive for low concentrations measurements, the steady state amperometry under stirred conditions or flow injection analysis with amperometric detection is employed instead of cyclic voltammetry. The tyrosinase based amperometric biosensor mechanism for phenols, is that the tyrosinase at the surface of the glassy carbon electrode is oxidized by oxygen, and then reduced by phenolic compounds. The phenolic compounds mainly convert into quinone, and the products are electrochemically active, can be reduced on the electrode. The reduction current is proportional to the concentration of phenolic compounds in solution. Detection of phenolic compounds is of great importance due to their presence in a broad range of chemical manufacturing processes and their toxicity. Amperometric sensors are simple, effective and fast, bases on the immobilized tyrosinase; amperometric sensors the detection phenolic compounds have been widely carried out. Figure 5.7.3 below illustrates a typical current-time plot for the biosensor in response to successive additions of aliquots of 0.01 M BPA into 0.1 M phosphate buffer (pH 7.2) at a fixed potential of -0.1 V and under stirring at 400 rpm. Upon additions of successive aliquots of 0.01 M BPA into 0.1 M phosphate buffer, an increase in current was observed and the current started to decrease at about 1.70 mM concentration of BPA. The response occurring immediately after the addition of BPA is attributed to the reduction of *o*-quinone as shown in tyrosinase mechanisms. Under optimized conditions, the detection limit for BPA by the developed biosensor was found to be 0.013 mM (1.3×10^{-6} M) within a concentration range of 0.02-0.28 mM. This value was closer to the detection limits reported in literature in techniques like Elisa, HPLC-MS, GC-MS and LC-MS as shown in table 5.7.1 above. The Tyr/PDMA-PSS biosensor response time obtained in this study for steady state amperometry reached 95% within 12 seconds. The response time value obtained

was nearly correspond to the value reported by Zhao et al in literature for Tyr-SWNT sensor which was 20 seconds for the detection of BPA (Zhao *et al.* 2005) and Akyilmaz et al reported 1 minute for Tyr/teflon membrane biosensor (Akyilmaz *et al.* 2010). Once more the achieved response time was compared to chromatographic techniques and the results are shown in table 5.7.1 above. The smaller response time obtained in this study makes the tyrosinase based biosensor the best when it's compared to literature and this fulfils the aim of the study, which was to develop a portable, cheap, highly sensitive and rapid response biosensor for determination of BPA. Apparent Michaelis-Menten constant K_m^{app} can be calculated from the Eadies-Hofstee form of the Michaelis Menten equation.

$$I_{ss} = \frac{I_{max} - K_{m}^{app} I_{ss}}{C_{phenol}} \qquad \textit{Equation (7)}$$

Where I_{ss} is the steady state current, I_{max} the maximum current under saturating substrate, C_{phenol} is the concentration of phenol and K_m^{app} is an apparent Michaelis-Menten constant. The K_m^{app} for phenol obtained from this work is determined to be 1.2517 mM at maximum current of 5.5457×10^{-6} A. This value is lower than 1.290 mM reported by Somerset at maximum current of 3.500×10^{-8} A for Au/MBT/PDMA-PSSA/AChE (Somerset, 2010). The K_m^{app} obtained in this work indicates that the enzymatic activity of the immobilized Tyr is high.

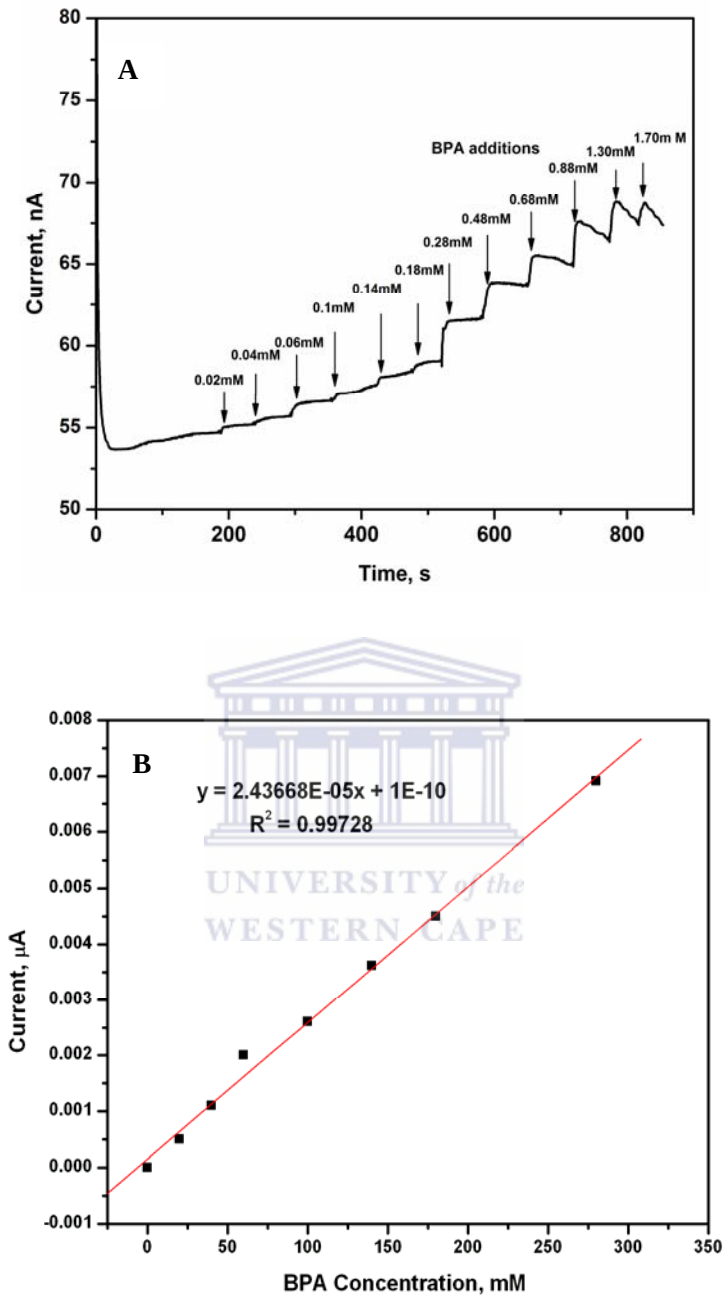


Figure 5.7.3: Amperometric responses of the biosensor (Tyr/PDMA-PSS/GCE electrode) to additions of bisphenol A. Potential -0.1 V, 400 rpm.

5.9 Conclusion

Biosensor characterization and application was performed using various methods such as cyclic voltammetry, square wave voltammetry, electrochemical impedance spectroscopy and steady state amperometry technique. The optimum activation pH for native tyrosinase enzyme was obtained at pH 7.2 proving that tyrosinase denatured at very low and high pH and bare GCE was identified to be able to detect bisphenol A but electrode fouling due to polymerization of BPA into GCE surface. Voltammetric technique confirmed electrochemical oxidation of bisphenol A as a source of electropolymerized bisphenol A film onto GCE surface. This was achieved by adding a fixed concentration of 30.0 μM bisphenol A into 0.1 M phosphate buffer solution and cycling 10 cycles of CV to immobilizing BPA onto bare GCE. Tyrosinase based biosensor Tyr/PDMA-PSS/GCE showed good bioelectrochemical response to BPA detection with the characteristics of a usual enzyme-catalyzed reaction. The biosensor was prepared by immobilization tyrosinase into poly(2,5-dimethoxyaniline) film employing electrochemical doping method, and the enzyme electrode prepared in this manner required only very small amount of enzyme. Under the optimum conditions (25°C, pH 7.2, –0.1 V), the Tyr/PDMA-PSS/GCE displayed good stability and reproducibility. EIS characterization of biosensor from Nyquist plot showed excellent response when bare GCE, PDMA/GCE, PDMA-PSS/GCE and Tyr/PDMA-PSS/GCE were compared, the charge transfer resistance of the modified electrode increased when polymer was deposited onto GCE surface confirming successful electro-polymerization of PDMA-PSS while biosensor charge transfer resistance decreased attributed with the electrostatic reaction between the negative backbone of the polymer with the positive charge in copper active site of tyrosinase. Time constant and exchange current obtained results

also supported the behavior observed from Nyquist plot. Amperometric detection of BPA performed under optimized conditions, the detection limit for BPA was found to be 0.013 mM (1.3×10^{-6} M) within a concentration range of 0.02-0.28 mM.

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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

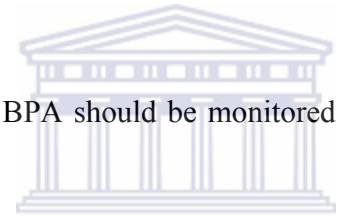
6.1 Conclusions

Electrochemical synthesis of poly(2,5-dimethoxyanilne) doped with poly(4-styrenesulfonic acid) nano-composites were successfully prepared on a glassy carbon electrode by a relatively simple and inexpensive electrochemical method. Analysis of the polymerization voltammograms of both PDMA and PDMA-PSS showed a higher current density, similar physical properties to PANI and defined peaks in PDMA-PSS than in PDMA. This was an indication that the nano-composites obtained when the dopant was incorporated had an influence on high conducting than undoped PDMA. The detection and quantification of BPA was achieved by the developed Tyr/PDMA-PSS biosensor. However bare glassy carbon electrode was identified to be able to detect BPA but the down fall was fouling meaning BPA polymerized on GCE surface blocking electron transfer and the detection potential at which it was responding was very wide. The optimum pH of native tyrosinase was studies and obtained at pH 7.2 phosphate buffers; this implied that tyrosinase activation was in neutral medium. This proved that tyrosinase enzyme sustain its activation in neutral medium than in alkaline medium. The detection using Tyr/PDMA-PSS biosensor is the most preferable due to much lower negative potentials (-0.1 V) detection of BPA than that obtained in bare electrode. This behavior is made possible by the incorporation of tyrosinase enzyme which oxidizes phenols at very low potential. EIS

characterization of Tyr/PDMA-PSS/GCE biosensor showed excellent response when comparing bare GCE, PDMA/GCE and PDMA-PSS/GCE, the increase in charge transfer resistance after electropolymerization of polymer onto GCE surface confirming the successful deposition of PDMA-PSS while biosensor charge transfer resistance decreased attributed with the electrostatic reaction between the negative backbone of the polymer with the positive charge in copper active site of tyrosinase. In this study, the detection limit obtained on steady-state amperometry biosensor as a result of 0.01 M BPA additions was 0.013 mM (1.3×10^{-6} M) within a concentration range of 0.02-0.28 mM. This value was closer to the detection limits reported in literature for techniques like Elisa, HPLC-MS, GC-MS and LC-MS. Square wave voltammetry of Tyr/PDMA-PSS biosensor on 0.001 M BPA was found to be detection limit of 1.9×10^{-8} M was also compared to the literature and the value was similar to the value reported 9.5×10^{-8} M at wide range of 2.5-20.0 μM estimated for Cu ions sensor, this implied an improved performance of biosensor in this study. The high sensitivity of the Tyr/PDMA-PSS biosensor was provided for by the nanostructured PDMA-PSS materials used for the modification of the glassy carbon electrode surface before the immobilization of tyrosinase. The PDMA-PSS film provided a suitable micro-environment for the immobilized tyrosinase and acted as a mediator enhancing the heterogeneous direct electron transfer rate of tyrosinase. Nano-structured materials can act as tiny conduction centers to facilitate electron transfer between the enzyme and the electrode surface. The high stability of the Tyr/PDMA-PSS film was due to the electrostatic attachment procedure used for the immobilization of the enzyme and the incorporation of the dopant PSS into the polymer matrix. The aim of the study was to construct a portable, highly sensitive, cheap and fast response biosensor for quantitative determination of endocrine disruptor BPA in waste water and the developed Tyr/PDMA-PSS biosensor certified the study aim. The

fabrication material cost was very low and preparation of this biosensor was less time consuming and it produced wonderful results which are reproducible. The response time for steady state amperometry reached 95% within 12 seconds. The response time value obtained was compared to literature reported for Tyr-SWNTs sensor response time was 20 seconds for the detection of BPA. The quick response time and portability of Tyr/PDMA-PSS biosensor makes it superior when compared to other methods. Working on project gave broader knowledge about the importance of the environmental health and well-informed researchers on how to control water contamination.

6.2 Recommendations



-The electrochemical behavior of BPA should be monitored at various pH including the acidic and alkaline media.

-The biosensor parameters should further be investigated and optimized in order to achieve lower detection limits.

-The applicability of the developed biosensor should be determined by analyzing real samples.

-The optimization studies Tyr/PDMA-PSS biosensor should be conducted.