Characterisation of electrochemical detection of oestrogen by using yeast oestrogen binding protein

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Abstract

Oestrogens are female sex hormones. Both natural and synthetic oestrogens have been found in many aquatic environments. There are three naturally occurring oestrogens – oestradiol, estriol and estrone. Oestradiol (correctly known as 17β oestradiol or E2) is a naturally occurring steroid hormone and is the most potent of these three. Diethylstilbestrol, dienestrol, quinestrol etc are synthetic oestrogens. These environmental steroidal and nonsteroidal oestrogens act as endocrine disruptors (EDs). Both types of oestrogens in the environmental samples can be quantified using several laboratory methods such as high pressure liquid chromatography (HPLC), gel permeation chromatography (GPC) etc. but they often require extensive training to perform.

Arxula adeninivorans is biotechnologically significant dimorphic yeast with unusual characteristics. It can use a wide range of substrates and it is thermotolerant, osmotolerant and halotolerant. It is a non-pathogenic fungus and is therefore ideal for use in industrial settings. It is a source of many enzymes and a wide range of transformation platforms have been developed to enable the production of foreign proteins. In this project, *A.adeninivorans* was transformed with histidine-tagged synthetic oestrogen binding protein (EBP) gene based on the *Candida albicans* EBP sequence. The recombinant EBP expressed in the yeast *Arxula* is separated using HisTrap columns.

Linear sweep voltammetry was used for the detection of EBP redox responses to oestrogen in solution. A three-electrode configuration was used for all measurements [auxiliary electrode (platinum wire), reference electrode (Ag/AgCl) and working electrodes (Pt 50µm diameter micro-disc and 2 mm diameter glassy carbon)]. Electron transfer from EBP to electrodes will require the use of a mediator system and TMPD, a lipophilic mediator used in this experiment.

Screen printed electrodes (SPEs) were used to detect the interaction between EBP and oestrogen. To perform experiment with SPE, EBP was immobilized on SPE using the crosslinker glutaraldehyde. Differential pulse voltammetry (DPV) was used to detect interactions of EBP and oestrogen on SPE. Immobilion N transfer membrane was impregnated with TMPD solution and electrochemistry (DPV) was performed. The purpose of using membrane is to simulate the immobilization of TMPD on SPE along with EBP for the detection of oestrogen.

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Abbreviations

AFM	Atomic Force Microscope
Ag/AgCL	Silver/silver chloride
ALP	Alkaline phosphatise
AWACSS	Automated water analyzer computer supported system
BCA protein assay	Bicinchoninic acid protein assay
BOD	Biological oxygen demand
BPA	Bisphenol A
BSA	Bovine serum albumin
Cd	Cadmium
Cu	Copper
DES	Diethylstilbestrol
DNA	Dioxyribose nucleic acid
DPV	Differential pulse voltammetry
E^0	Standard reduction potential
E2	17β oestradiol
EBP	Oestrogen binding protein
ED	Endocrine disruptors
EDCs	Endocrine disrupting compounds
ER	Estrogen receptors
$[Fe(CN)_6]^{3-}$	Ferricyanide
GA	Glutaraldehyde
GPC	Gel permeation chromatography
Hg	Mercury
HPLC	High pressure liquid chromatography
Lac Z	Gene encoding β-galactosidase enzyme
LSV	Linear sweep voltammetry
MATLAB	Matrix laboratory
Menadione	2-methyl-1,4-naphthoquinone
NAD(P)H	Nicotinamide adenine dinucleotide phosphate-oxidase

NADH	Nicotinamide adenine dinucleotide hydrogen (reduced)
OYE	Old yellow enzyme
Pb	Lead
PB	Prussian Blue
PBS	Phosphate buffer saline
Pt	Platinum
RIANA	River analyzer
Rpm	Revolutions per minute
SPCE	Screen printed carbon electrode
SPEs	Screen Printed Electrodes
SPR	Surface plasmon resonance
TMPD	2,3,5,6- tetramethyl-1,4 – phenylenediamine
Vtg	Vitellogenin
WE	Working electrode
XylE	Catechol dioxygenase gene
Zn	Zinc
TMPD	2,3,5,6,-Tetramethyl-p-phenylenediamine

Chapter 1 Introduction

Oestrogens are female sex hormones. 17β oestradiol (E2) (natural) and synthetic oestrogens such as diethylstilbestrol, dienestrol, quinestrol have been found in many aquatic environments. Environmental oestrogens can hinder normal developmental processes and physiological functions in fish and wild life in the aquatic environment. Both natural and synthetic oestrogens are excreted in an inactive conjugated form and regain their activity in the environment when they are deconjugated by bacteria **(Pettersson et al., 2006).**

Very sensitive assays are required to detect them as some of these molecules can exert their effect at very low concentrations. These are chemicals that have the potential to disrupt normal reproduction and developmental processes which can have adverse effects on health. Usage of DES has been reported to cause abnormalities of reproductive tract and also had effects in the cardiovascular, neuroendocrine and immune system **(Burkhardt-Holm, 2010)** There is increasing concern over the endocrine disrupting properties of oestrogens which has motivated the need for rapid and accurate detection methods **(Butler & Guiltbault, 2006)**.

1.1 Aims and objectives

To meet these demands, various biosensors and bioassays are being developed. In the previous research of this group electrochemical detection of oestrogen binding protein (EBP) interaction with oestrogen has been characterised in *Candida albicans* whole cell and cell lysate **(Gurazada 2008; Chelikani 2011).**

Electrochemical detection of oestrogen using yeast oestrogen binding protein

The main goal of this research is to develop a cost effective yeast based bioassays in a true portable biosensor format for detection of oestrogen that can be used inside and outside the lab without regulatory restriction to provide rapid detection of environmental oestrogens. The current measuring techniques take a relatively long time to perform and are costly The EBP based sensor has been achieved by immobilising EBPs on disposable Screen Printed Electrodes (SPEs) for the detection of oestrogens. The EBP was produced by transforming an *A.adeninivorans* strain with a histidine-tagged synthetic EBP gene based on the *C.albicans* EBP sequence. The EBP was purified, immobilized on an electrode surface and used as a bio-recognition element for the detection of oestrogen. For environmental applications, biosensors offer many advantages over conventional analytical techniques e.g. portability,

speed, miniaturization, work onsite and ability to measure contaminants or pollutants in complex.

Investigation of electron transfer from oestrogen binding protein by using mediator impregnated membrane

TMPD Impregnated Immobilion N transfer membrane was used to simulate the immobilization of TMPD on SPE along with EBP for the detection of oestrogen.

1.2 Thesis format

The thesis consists of five chapters. Chapter 2 provides background on biosensors, immobilization techniques, mediator and screen printed electrodes. Chapter 3 and Chapter 4 present the electrochemical techniques, research methodology, experimental details and results. Finally, chapter 5 summarises with a discussion and further work.

1.3 Presentations and publications

Baronian, K.H., Vijayan, V., Mehrotra, M., Giersberg, M. and Kunze, G. (2014) *Characterisation of an electrode with immobilised recombinant protein for the rapid detection of oestrogen.* Malmo, Sweden: 15th International Conference on Electroanalysis (ESEAC 2014), 11-15 Jun 2014. (Conference Contributions - Oral presentations).

Baronian, K., Vijayan, V., Mehrotra, M. and Kunze, G. (2014) *Use of Immobilized Recombinant Protein for Rapid Electrochemical Detection Oestrogen.* Amritsar, India: 11th Indian Society for ElectroAnalytical Chemistry Discussion Meet 2014 (ISEAC-DM), 20-25 Feb 2014. (Conference Contributions - Oral presentations).

Use of recombinant oestrogen binding protein for the electrochemical detection of oestrogen Vimal Vijayan, Martin Giersberg, Alexandre Chamas, Mamta Mehrotra, Venkata Chelikani Gotthard Kunze, Keith Baronian (2014). *Biosensors and Bioelectronics* (Submitted).

Chapter 2 Background

2.1 Oestrogens

Oestrogens are female sex hormones. There are three naturally occurring oestrogens – oestradiol, estriol and estrone. Oestradiol (correctly known as 17β oestradiol or E2) is a naturally occurring steroid hormone and is the most potent of these three. It is synthesized from cholesterol in the human body and is vital for the development and maintenance of female sexual characteristics **(Butler & Guiltbault, 2006)**. The type of oestrogen produced at each tissue site varies for example ovary synthesizes primarily 17β oestradiol directly, adipose tissue synthesizes estrone and the placenta synthesizes estriol. **(Ackerman & Carr 2002).** Oestrogens play an important role in the maturation of the female reproductive tract and are responsible for proper endometrial development, sperm transport and tubal transport mechanisms **(Carr 1998).** To accomplish this oestrogen must bind and activate a group of specific gene regulatory molecule called receptors. The free (vs. bound) oestrogen diffuses into cells and bind with two specific receptors called α and β in those cells where they will exert an action. These oestrogen receptors (ER) also called nuclear receptors are located in female specific target tissue cells such as breast, uterus and vagina. After binding of oestrogens to receptors, the oestrogen causes the receptors to undergo an allosteric change in structure which converts the receptor to an active form. The active forms of receptors have the ability to bind to regulatory elements of genes to express or suppress their function **(McAllister et al. 1989).**

Both natural and synthetic oestrogenic compounds from exogenous source are well known endocrine disrupting compounds (EDCs). These compounds interfere with the endocrine system and generate unfavourable effects **(Butler & Guiltbault, 2006)**. The presence of these compounds in the environment is a cause of concern as it is linked to an increasing incidence of some cancers **(Raloff, 1994).** These compounds are excreted in an inactive conjugated form and regain their activity in the environment after de-conjugation by bacteria **(Peterson et al., 2005**). Both endogenous and synthetic steroid hormones are excreted by humans as inactive water soluble glucuronide or sulphate conjugates in urine and faeces but are deconjugated by bacteria and reactivated in the soil and during the sewage treatment process or even before they reach the sewage treatment plant **(Baronti et al., 2000).** The natural (17β oestradiol) and synthetic oestrogen (17α ethinyoestradiol) have been detected in

their active unconjugated form in the effluent water from sewage treatment plants **(Desbrow et al., 1998; Larsson et al., 1999; Ternes et al., 1999b; Baronti et al., 2000; Kuch and Ballschmiter, 2001).**

Some of these molecules can exert their effect at very low concentrations and hence very sensitive assays are required to detect them. There are a wide range of compounds that mimic oestrogen action including natural products, pharmaceutical and industrial products **(Gurazada 2008)**. Foreign chemical substances found within an organism that the organism otherwise does no naturally produce are known as xenobiotic compounds. It has been reported that these can cause cancer, damage to reproductive systems and developmental problems in both humans and wild life **(Juberg, 2000; Colborn & Clement 1992).** Xenobiotic compounds are now found in humans due to the fabrication of large variety and quantity of chemical products. Xenoestrogens have hormone like properties and imitate oestrogen hence exhibit an affinity for the human oestrogen receptor (ER) **(Zayda, Jordi, and Rius 2009).).** Xenoestrogens are also present in contraceptive pills.

Diethylstilbestrol (DES) is a synthetic oestrogen. It was initially incorrectly believed to reduce the risk of miscarriage. As a result of this belief, its healthful benefits were so widespread, that it was eventually recommended as a routine prophylactic for all pregnant women, regardless of miscarriage history **(Herbst, Ulfelder & Poskanzer 1971).** Later on it was established that DES exposure in utero can also result in vaginal dysplasia, vaginal and or cervical adenosis, malformations of the uterus, cervix and vagina, increased risk of testicular cancer, lower sperm count, undescended testes, infertility, late spontaneous abortion, premature delivery, and other pregnancy complications **(Patisaul & Jefferson 2010).**

The main reason for the increase in diseases is similarity between xenoestrogens and oestrogens. Bisphenol A (BPA) is a type of xenoestrogen. It is widely used as a primary monomer in plastics or resins, as an antioxidant in plasticizers and as a polymerization inhibitor in polyvinyl chloride (PVL). BPA has been shown to acquire strong oestrogenic properties and exhibits both agonistic and antagonistic actions for ER α while only exhibits agonistic actions for ER β **(Zayda, Jordi, and Rius 2009).**

Phytoestogens are another type of oestrogenic compounds. They are naturally occurring plant derived compounds and are structurally and or functionally similar to mammalian oestrogens

and their active metabolites. They are found in a wide variety of foods, most notably soy. Most phytoestrogens are phenolic compounds. Among these isoflavones and coumestans are the most widely researched groups. While most phytoestrogens bind both ERα and ERβ, in vitro assays have found that they exhibit a higher binding affinity towards ERβ than ERα.

In the 1940's, the endocrine disrupting properties of phytoestrogens were recognised when ewes grazing on clover rich pastures in Australia were observed to have abnormally high rates of infertility, abortion, and reproductive abnormalities in their offspring. It was in the end determined that phytoestrogens were principally responsible for the observed effects.

According to the Environmental Protection Agency's definition, isoflavones are endocrine disruptors which alter the structure or functions of the endocrine system and cause adverse effects which includes disruption of lactation, the timing of puberty, the ability to produce viable fertile offspring, sex specific behaviour, premature reproductive senescence and compromised fertility. Most isoflavones bind and activate both ERα and ERβ more readily than synthetic endocrine disrupting compounds including BPA. Exposure to phytoestrogen prior to puberty and possibly before birth, poses a large lifetime risk. Isoflavones can pass through placenta from mother to fetus. Level of isoflavones found in human umbilical cord blood and amniotic fluid is comparable to concentrations seen in maternal plasma, indicates that fetal exposure is possible. **(Patisaul & Jefferson 2010)**

These endocrine disrupting compounds are excreted in either activated or inactivated form and enter the aquatic environment via wastewater. The issue of oestrogenic contamination of aquatic environment was first highlighted by finding of hermaphrodite fish in British rivers **(Purdom et al. 1994)**. This was a direct consequence of the exposure of endocrine disrupting chemicals to aquatic animals.

In an experiment, caged fish were directly placed in the effluent of a sewage treatment plant and it was found that levels of vitellogenin (Vtg) markedly increased in male fish **(Butler & Guiltbault, 2006).** Vtg is a precursor protein for the production of yolk in all oviparous vertebrates. 17β oestradiol is the natural inducer of hepatic Vtg synthesis. However, Vtg synthesis is also induced by many environmental oestrogens in both males and females **(Jobling et al., 1996; Lindholst et al., 2000; & Thorpe et al., 2001).** Therefore, the content of Vtg in blood plasma of male or juvenile fish is a sensitive and specific biomarker for the detection of oestrogenic effects in fish **(Sumpter & Jobling 1995).** An association of

vitellogenesis with these intersex fish suggested that endocrine disrupting chemicals were the cause **(Sumpter 1998).**

Jobling et al. reported on the presence of intersexuality in wild roach populations in both the vicinity of sewage treatment plants and control sites **(Jobling et al. 1998).**

The above examples are only some of many that show why there is an increasing concern over the endocrine disrupting properties of environmental oestrogens. These provide motivation for research and development of rapid and accurate methods for the detection of oestrogen in the environment.

To meet these demands, various biosensors and bioassays are being developed. With the help of this project we have tried to develop a cost effective yeast based bioassays in portable biosensor format for the detection of oestrogen that can be used inside and outside the lab without regulatory restriction for the rapid detection of oestrogen. Current detection techniques are restricted to laboratories because of complex apparatus, are time consuming and are more expensive.

In this research direct immobilization of EBP has been carried out on the disposable SPEs to reduce time and cost.

C.albicans is one of the most common fungal pathogens found in humans; the EBP contained in it has a very high level of chemical interaction and affinity towards oestrogens of the mammalian family **(Madani, Malloy, Rodriguez-Pombo, Krishnan & Feldman 1994).** Experiments have proved that EBP found in the principal organism *(C.albicans*) works perfectly and safely when genetically transferred to a non-pathogenic organism *(A.adeninivorans*). Due to pathogenicity of *C.albicans*, in this research, *A.adeninivorans* was transformed with histidine-tagged synthetic oestrogen binding protein gene based on the *C.albicans* EBP sequence for the safe production of the protein. EBP was then purified and immobilised on an electrode surface and used as a bio-recognition element for the detection of oestrogen.

Various studies suggest that when oestrogens were present in the growth medium, *C.albicans* undergoes changes in its morphology and growth rate in response to oestrogens **(Kinsman & Collard 1986, Kinsman, Pitblado & Coulson 1988, Gujjar, Finucane & Larsen 1997).** According to epidemiological studies, the pathogenicity of the fungus

increases due to changes in its morphology, this also helps the organism in combating the host immune system **(Odds 1988)**. The EBP present in C.albicans has a high affinity towards 17β oestradiol which is the only significant binding activity found in the organism so far **(Powell, Frey & Drutz 1984)**.

It has been observed by the sequence analysis that the EBP is not similar to any other members of steroid receptor superfamily but has about 46% similarity with a NADPH oxidoreductase present in *Saccharomyces cerevisiae*, old yellow enzyme (OYE) **(Madani, Malloy, Rodriguez-Pombo, Krishnan & Feldman 1994).**

The first ever identified flavoprotein, OYE is the most common model in studying its redox-active FMN cofactor which imparts it the characteristic yellow colour**. (Niino, Chakraborty, Brown & Massey 1995).**

Oxidoreductase sites that are present on EBP can be inhibited by oestrogen **(Madani et al., 1994).** In addition to 46% identity of EBP to OYE it has been shown that OYE binds to a number of phenolic compounds including 17β oestradiol but it binds to oestrogen with lower affinity compared to EBP and shows copious FMN dependent oxidoreductase activity. OYE and other homologues of EBP can only oxidize 19-nortestosterone whereas EBP not only oxidizes but also reduces it, which is the most significant difference between EBP and OYE **(Buckman &Miller 1998).**

Electron microscopy was used to immunolocalise the protein and it was found that the EBP of *C.albicans* was located near the inner surface of the tonoplast membrane of the vacuole in *C.albicans* **(Zhao, et al. 1995)**.

EBP has the special property of binding with vertebrate oestrogens only. The approximate molecular weight of the EBP is 46,000 Da. Various binding experiments showed very high specificity and steroselectivity of EBP in following order of potency in displacing [3H] oestradiol: 17β oestradiol > estrone > estriol > 17α oestradiol. Insignificant competitive potency was measured for other mammalian steroid hormones, diethylstillesbestrol, tamoxifen, and fungal hormones **(Skowronski et al; 1989).**

Oligonucleotide primers of EBP for PCR have been designed from aminoacid sequences obtained from cyanogen bromide fragments of purified EBP. An 800-bp product has been amplified and then used to identify a λ phage containing an intact EBP gene from C.albicans

genomic library. This gene can encode the protein of molecular mass 46,073 Da which is the estimated size of the EBP. The gene has been cloned and expressed in *Escherichia coli* as a lac*Z* fusion protein and this protein displays high-affinity binding for oestradiol comparable to the wild type EBP **(Madani, et al. 1994).** Full gene sequence of EBP was deposited in NCBI by Madani et al. (1994), with accession no. L25759.

A.adeninivorans is relatively unknown, non-pathogenic, dimorphic, haploid, ascomycetous yeast. As sole source of carbon and/ or nitrogen *Arxula* is able to assimilate and ferment many compounds, and it can also utilise n-alkanes and degrade starch efficiently. It is a thermotolerant, halotolerant, osmotolerant yeast. It can grow in media containing up to 20% NaCl, and cultivation temperature of up to 48^0C. Due to unusual dimorphic characteristics of the yeast, *Arxula* grows up to 42^0C as budding cells and turn into mycelia at higher temperatures. This dimorphism is reversible and budding can be re-established when the cultivation temperature is decreased below 42^0C. These extraordinary properties of *A.adeninivorans* are useful for heterologous gene expression and as a gene donor to construct more suitable yeasts for biotechnology and have been successfully expressed in *S.cerevisiae* and *Kluyveromyces lactis*. Both the transformed yeasts assimilate and ferment starch as a carbon source. *LacZ* gene from *E.coli* as well as *XylE* gene from *Pseudomonas putida* has been expressed in *A.adeninivorans* which indicates that *A.adeninivorans* can act as a host for heterologous gene expression **(Watermann & Kunze, 2000).**

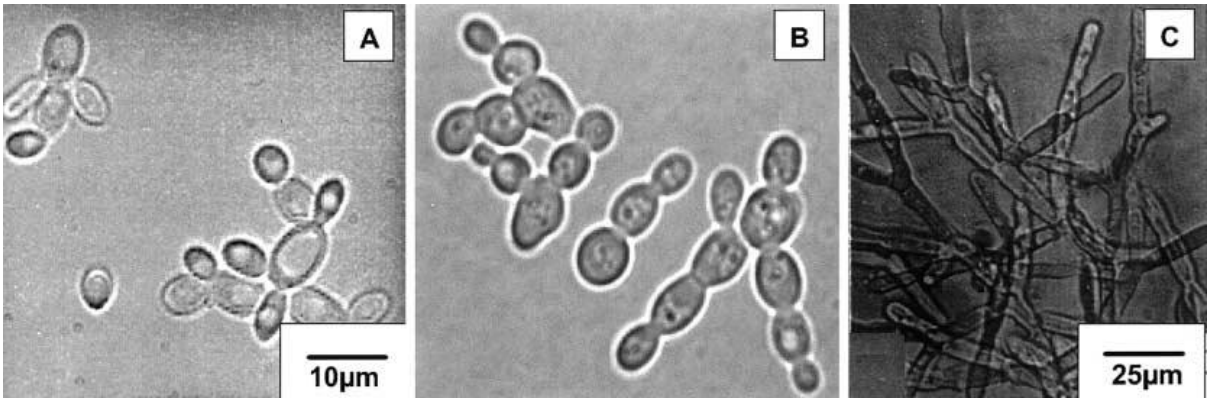


Figure 2-1 - Micrographs of *Arxula adeninivorans* grown at 30^0C (A), 42^0C (B), and 45^0C. The cells are cultured in YEPD medium for 18 hours (**From -Watermann & Kunze 2000**)

Figure 2-1 illustrates that *A.adeninivorans* is a dimorphic fungi which turns into mycelia at higher temperature and can also grow at various temperatures.

2.2 Biosensors

Biosensors result from the pioneering work of Clark **(Clark and Lyons, 1962)** and have found extensive use in diverse applications where continuous measurements in biological media are required **(Wilson & Raeann 2005).**

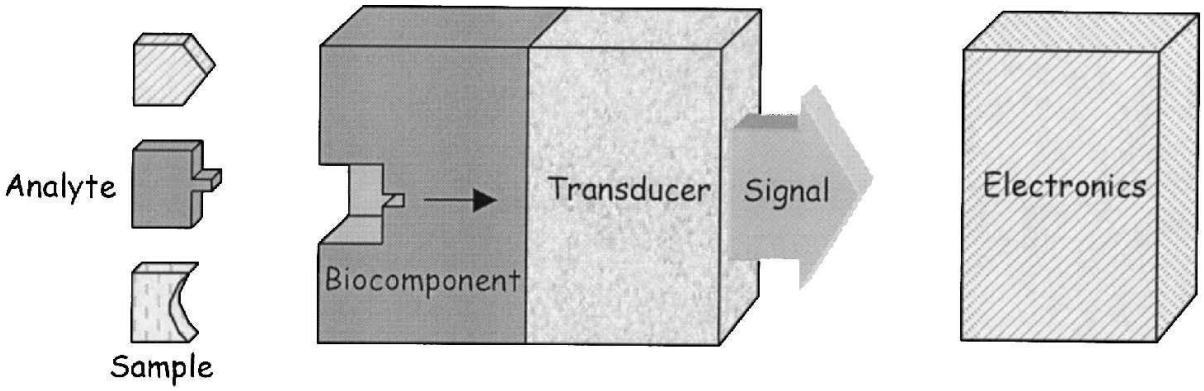


Figure 2-2 - A generic biosensor schematic (From-Pasco et al. 2001)

An electrochemical biosensor is a device which can detect, transmit and record information about a physiological or biochemical change. It combines a biological component with an electronic or other transducer, which transforms a biochemical information bearing signal into a measureable electrical response. The biosensor response is dependent on the biochemical activity of the biological sample. The important considerations in selecting the biological samples are governed by factors such as operational and environmental stability, specificity, storage conditions, the type of analyte to be detected that covers a broad range such as chemical compounds, antigens, microbes, hormones and nucleic acids. Types of biological material used as sensing elements are enzymes, antibodies, DNA, receptors, organelles, microorganisms, animal and plant cells or tissues. Major advantages offered by a good biosensing system are its specificity, sensitivity, reliability, portability, ability to function even in optically opaque solutions, real-time analysis and simplicity of operation. **(D'Souza 2001)**

At present, water quality monitoring has relied on the collection of water samples followed by extraction and laboratory based instrumental analysis. This technique fails to provide more realistic information due to variations in water characteristics depending on time and place of

collection of the samples. Biosensors can be very useful for the continuous monitoring of a contaminated area. Biosensors also offer the possibility of determining specific chemicals and their biological effects, such as toxicity, cytotoxicity, genotoxicity or endocrine-disrupting effects. It can also be used as environmental quality monitoring tools or for the chemical monitoring of both inorganic and organic pollutants **(Rodriguez-Mozaz, Lopez de Alda and Barcelo 2006).**

The number of organisations involved in the analysis of contaminants spans a broad spectrum, some of the most notable being the industrial sector comprising agricultural food, water and healthcare, the regulatory authorities and the food control and environmental agencies. Biosensors make important tools and instrumentation and kits are already becoming available for use. It is obvious that biosensors are becoming part of main stream real-time analytical tools. A number of instruments are already commercially available. In a biosensor, the transducer element and biological sensing elements both will form the basis of the future sensors released onto the market. Polyclonal and monoclonal antibodies form a major part of current immunosensors. However, new biological elements, including abzymes (antibody possessing enzymic activity), synthetic peptides and protein nucleic acids are also likely to be used **(Patel 2002).** The development of glucose enzyme based biosensors which are the most successful enzyme based biosensors began in 1962. First enzyme based biosensor was designed by Clark and Lyons using glucose oxidase enzyme **(Wang. 2008)**

Biosensors can be classified into various groups, according either to signal transduction element or biorecognition element **(Rodriguez-Mozaz, et al., 2006)**. A transducer is basically an analytical device for which the output signal magnitude is a known function of the input signal magnitude (**Monosik, et al., 2012).**

Biosensors can also be categorised on the basis of their transducing elements such as electrochemical, optical, piezoelectric and thermal sensors. Biorecognition element based biosensors are immunosensors, enzyme based biosensors, receptor based biosensors, and cell based biosensors. **(Rodriguez-Mozaz, Lopez de Alda and Barcelo 2006).** Figure 2-2 **s**hows a general diagram of a biosensor.

2.2.1 Transduction based biosensors

2.2.1.1 Electrochemical biosensors

Basic principle of these biosensors is that chemical interactions between immobilized biomolecule and target analyte result in loss or gain of ions/electrons which are measured by changes in current or potential. Such electrochemical biosensors are classified into amperometric and potentiometric.

2.2.1.1.1 Amperometric biosensors

These are very sensitive and most widely used biosensors and are more suitable for mass production than the potentiometric biosensors. Usually a noble metal or screen printed layer acts as the working electrode that is covered by the bioelement. Most Amperometric biosensors use biochemical reaction mediators for shuttling electrons between the substrate and the enzyme. New generation or third generation biosensors do not require mediator and transfer of electrons takes place directly from analyte to electrodes (Wang 2008).

2.2.1.1.2 Potentiometric biosensors

The basic principle of these biosensors is to measure the difference in potential (almost at zero current) that is generated across the ion-selective membrane separating two solutions. Modern silicon or thick film technologies make possible the mass production of these biosensors in miniaturised form.

2.2.1.2 Optical biosensors

These types of biosensors measure light as an output transduced signal. For example, Surface Plasmon Resonance (SPR) biosensors are optical based sensors using special electromagnetic waves known as surface plasmon polaritons. Interactions between an analyte in solution and a bioelement immobilized on the SPR surface result in mass concentration changes that are measured as a shift in the SPR angle. Their applications include detection of biological analytes, analysis of biomolecular interactions in real time (**Monosik, et al., 2012)**.

The SPR biosensor can be used with the human oestrogen receptor for the detection of oestrogens, xenoestrogens, and for studying binding properties of target compounds. The oestrogenic activity in water samples is detected by optical biosensors based on recombinant cells to co-express human ER **(Rodriguez-Mozaz, et al., 2006).**

2.2.1.3 Piezoelectric biosensor

This type of biosensor uses the coupling mechanism between the bioelement and a piezoelectric component such as quartz-crystal coated with gold electrodes. Types of materials used for these biosensors are quartz, tourmaline, lithium niobate or tantalite, oriented zinc oxide or aluminium nitride and all of these materials exhibit piezoelectric effect (**Monosik, et al., 2012).** Piezoelectric biosensors are used for the measurement of ammonia, nitrous oxide, carbon monoxide, hydrogen, methane and certain organophosphorous compounds **(Abad et al., 1998, Minunni et al., 1994).**

2.2.1.4 Thermal biosensors

Biomolecules are immobilized on temperature sensors in thermometric or calorimetric biosensors and the heat generated due to the reaction of the enzyme and substrate is measured and is proportional to the substrate concentration (**Monosik, Stredansky and Sturdik 2012).** Enzyme modified integrated circuit temperature sensitive structures have been used to determine substrates, enzymes, vitamins and antigens **(Chaubey & Malhotra 2002).**

Certain practical issues prohibit the wide use of these biosensors, for example, optical biosensors are very sensitive but cannot be used in turbid media while thermal biosensors cannot be used with systems with very little heat change. For these reasons the electrochemical biosensors have emerged as the most commonly used ones and have overcome all the hurdles to become an off the shelf sensor **(Chaubey & Malhotra 2002).**

Another class distinction recognizes biosensors on the basis of the biorecognition element, for example, they can be classified as immunological, enzymatic, non-enzymatic receptor, whole-cell and DNA biosensors. In the immunological biosensors, antibodies can be produced to bind a variety of structurally different compounds and this feature makes them very selective, sensitive and versatile. There are wide variety of enzymes available as biorecognition elements and quite often their catalytic properties or substrate specificity can be tailored by means of genetic engineering.

Some natural receptors that are proteins of non-catalytic or non-immunogenic origin can be integrated within a membrane and then paired to a transducer to realise a biosensor. Whole cell based biosensing elements are of living organisms, such as bacterial, yeast, fungi, plant and animal cells or even tissue slices, they can estimate the toxicity of some compounds to

the specific cells as well as measure the biological oxygen demand (BOD). Most often genetically engineered bacteria are used in cell based biosensors.

DNA based biosensors are used to detect pollutants by the hybridisation detection of nucleic acid sequences from infectious microorganisms. Another method is to monitor small pollutants interacting with immobilized DNA layer (drugs, mutagenic pollutants, etc.) **(Rodriguez-Mozaz, Lopez de Alda, Barcelo 2006).**

2.2.2 Biorecognition element based biosensors

2.2.2.1 Immunosensors

Immunological biosensors use antibodies as the biological element which binds specifically with a wide range of affinities to individual compounds or groups of structurally related compounds.

The limitations of these biosensors for environmental monitoring applications are complexity of assay formats, use of different specific reagents (e.g. antibodies, antigens etc.) that have to be developed and characterized for each compound. Only limited number of compounds can be determined in each assay as compared to multiple compounds that contaminate environmental samples **(Rogers 2006).** Recent advancement in this biosensor is use of microchip format, six toxins detected at ng/ml levels **(Rubina et al., 2005).** An automated water analyser computer supported system (AWACSS) analyses 20 analytes in surface, ground, drinking and wastewater. These are pesticides, endocrine disruptors, and industrial pollutants **(Tschmelak et al., 2005).** One of the main advantages of the antibody based biosensors is they have great affinity towards target analytes which helps in very low detection limits for immunosensor assays. The disadvantage of these biosensors is that the antigen is not easily released from the antibody after measurement has been made. The solution to this problem includes the use of disposable sensors or sensing materials (e.g. glass or polystyrene beads) that can be detached from the detection instrument (**Fahorich, Pravda & Guilbault 2003).** Regeneration of the biosensor surface is another approach to detach tightly bound antibodies and antigens in non-disposable surfaces **(Kroger, Jung, Reder & Gauglitz 2002)**. A number of antibody-based biosensors have been developed recently for the detection of oestrogenic compounds such as SPR based immunosensors for the measurement of bisphenol A **(Marchesini, Meulenberg, Haasnoot, & Irth 2005).** One more fully automated optical biosensor called the River Analyzer (RIANA) is used for multi-

analyte analysis (**Tschmelak, Proll & Gauglitz 2004**). RIANA and AWACSS continuous in-line water monitoring biosensors have also been utilised for the measurement of oestrogenic and endocrine disrupting compounds **(Andreescu et al., 2004, Dempsey et al., 2004, Tschmelak et al., 2005).**

2.2.2.2 Enzyme based biosensors

Enzymes were the first molecular recognition elements included in biosensors and there are several advantages in using enzyme based biosensors and the first one is the stable source of biorecognition element. The enzyme can be genetically modified to optimise the substrate-enzyme reaction kinetics.

Environmental applications of enzyme based biosensors are limited by the number of substrates for which enzymes have been evolved. The interactions between specific enzymes and environmental pollutants also are few in number. Lack of specificity in differentiating compounds of similar classes and lack of versatility are the other limitations associated with enzyme based biosensors. Genetic modification of enzymes to increase assay sensitivity, stability and shelf life, improved electrochemical interfaces and use of mediators, immobilization of bio recognition element to the sensor surface are the advancements in this technology **(Rogers 2006)**.

2.2.2.3 Receptor-based biosensors

The receptor based biosensors can be used to measure any harmful environmental pollutant by binding to the receptor at physiologically relevant concentrations and a broad spectrum of pollutants that are structurally different with a similar toxicity mechanism are identifiable. Due to concerns regarding the adverse effects of endocrine disruptors on human health, recently the advancement in receptor based biosensors has been directed towards the human oestrogen receptor –α and based on this endocrine receptor, an assay has been developed using the BIAcore Surface Plasmon Resonance sensor platform **(Seifert, Haindl &Hock 1999)**. The oestrogen receptor is amalgamated with an electrochemical biosensor and based on impedance measurements it can be used to sense both oestrogenic and non-oestrogenic compounds **(Granek & Rishpon 2002).**

2.2.2.4 Cell-based biosensors

These biosensors can be organised according to cell type for environmental applications. For example: bacteria, yeast, algae, and tissue culture cells **(Rogers 2006).** For detecting

environmental pollutants, these biosensors have been developed using microorganisms. An insight into the physiological effects of an analyte can be gained when cell based biosensors' response is analysed **(Rainina, Efremenco, Varfolomeyev, Simonian & Wild 1996).** Cell based biosensors are mostly based on genetically engineered bacteria, as an example, gene fusions have been performed by genetically engineered bacteria made up of a native promoter gene linked to reporter genes **(Rogers 2006).** One more example of a genetically engineered bacterium is that of a bioluminescent gene which is fused to the reporter sequence of a gene of applicable pathway. Genetically modified bacteria are basically the complete cell sensor elements and can sense naphthalene and its metabolic product salicylate **(Heitzer et al., 1994, King et al., 1990).** They can also detect benzene, toluene **(Applegate et al., 1998, Burlage et al., 1994)** as well as mercury (Hg) and middle chain alkanes like octane (**Sticher et al., 1997**).

There are various applications of cell-based biosensors from pharmaceutical screening to environmental monitoring. **(Pancrazio, Whelan, Borkholder, Ma & Stenger 1999).**

Food quality control and monitoring is a large industry where biosensors based on microorganisms are utilized. The field of food monitoring includes milk quality monitoring, detection of amino-acids, vitamins (vitamin B-12, B-6, and ascorbic acid) and determination of sulphites in foods. Other significant areas where they are used are detection of pesticides, insecticides, heavy metals, chemical contaminants in agricultural products or processed foods, in medicine and pharmaceuticals for estimation of steroids such as cholesterol, androstendione, testosterone, antibiotics such as nystatin, and compounds such as gonadotropin releasing hormone, urea, uric acid, creatinine and iron. Heavy metals such as Hg, chromate-copper arsenate, nickel (Ni), and chromate, antimonite and arsenite, cadmium (Cd), lead (Pb) can be detected using bioluminescent microbial biosensors **(D'Souza 2001).** Heavy metals such as zinc (Zn), copper (Cu), and Cd can be tested for toxicity levels in combination as well as in isolation by means of two luminescent microbial biosensors *Escherichia coli* and *Pseudomonas fluorescence* **(Preston et al., 2000).** There is a lot of scope in applications of microbial sensors in harsh realistic environments for microorganisms, for example using thermophiles, alkalophiles, halophiles, psychrophiles, metallophiles, and ormophiles **(Ogawa & Shimizu 1999).** The information provided by using fungal cells can be more relevant to other eukaryotic organisms, though, most of the whole cells based biosensors are bacterial cells based **(Baronian 2004).**

Very encouraging results have been reported for BOD measurements even in salt water for a salt tolerant dimorphic (budding and mycelial) yeast *A. adeninivorans* LS3 **(Riedel et al., 1998; Tag et al., 1999; Chan et al., 1999).**

- **Advantages of yeast based microbial biosensors compared to bacterial based biosensors**

One commonly used biomaterial in whole cell biosensors are bacterial cells even though there are disadvantages like fragility, limited pH operation and osmotic and temperature tolerance of few bacterial species. Different bacterial cellular response to some chemical molecules than seen in eukaryotes along with the above mentioned problems limits their use in toxicity evaluation and drug screening for higher organisms (**Parry 1999)**

A lot of care has to be taken in using selectable markers in modification strategies as bacteria like *Escherichia coli* and *Salmonella sp*. used in few biosensors are human pathogens. On the other hand, the yeast in human food like breads, beverages and food additives are non-pathogenic (infectious to immunocompromised people only) and so their use in biosensors carries very low risk **(Walmsley & Keenan 2000).** For detection and evaluation of carcinogens the use of yeast species compared to bacterial cells is much better i.e. higher pH tolerance as well as higher temperature and osmolarity/ionic strength tolerance **(Gurazada 2008).** For an oestrogen assay, the detectors utilized are *Saccharomyces cerevisiae* whole cells and *Candida albicans* cell lysate **(Chelikani et al., 2011).** At a storage temperature of 4 ^{0}C a yeast biosensor has reportedly performed well for one year **(Preininger et al., 1994).**

The two major and distinct advantages of yeasts over bacteria when used as a biosensor are robustness and the fact that they are eukaryotes. Being robust they can grow in a broad range of substrates, have a broad physiochemical tolerance and tough cell walls. Being eukaryotes the information they provide is in context of other eukaryotes such as humans in genotoxicity tests, this information is not decipherable from prokaryote cells **(Walmsley & Keenan 2000).** However, there is limited use of yeast-based biosensors in the industry despite the above mentioned advantages **(Chelikani 2011).** Common catabolic substances like ethanol and glucose can be detected by yeast biosensors and they can also differentiate target analyte from a mixture of substrates. A potentiometric biosensor using *Hansenula anomala* immobilised at the electrode surface by Calcium alginate gel developed by Racek has been used successfully to measure glucose in urine and this biosensor had a 2 months working life

(Racek, 1991). By incorporating *H anomala* into a carbon paste electrode it was effective in estimating the concentration of L-lactate **(Kulys, Wang, & Razumas 1992).** In order to find the sucrose level in dairy products Corton and Locascio have reported using *Saccharomyces cerevisiae* in a carbon dioxide electrode **(Corton, & Locascio 1998).** Biosensors have been designed that use immobilized yeasts to detect formaldehyde and measure toxicity of cholanic acids **(Pancrazio, Whelan, Borkholder, Ma and Stenger 1999).** Salt tolerant dimorphic yeast *A. adeninivorans* was used in assessing BOD (Biological Oxygen Demand) by Lehman and co-workers and got better correlation of their results with commercial biosensor **(Lehmann et al., 1999).**

In whole cell biosensors interference from other substrates may be an issue. In *H anomala* based biosensors for detecting lactose in blood, when sodium fluoride was added then the response to blood glucose was totally blocked **(Racek and Musil 1987).**

To differentiate precisely between ethanol and glucose in a mixture of various concentrations between 1 mM and 8 mM, Lobanov et al., 2001 have reported using chemo-metric analysis of the responses of the bacterium *Gluconobacer oxydans* and the yeast *Pichia methanolica*. The upper limit was advanced to 10mM when an AI tool called neural network analysis was used for processing results. They concluded that by blocking responses to interfering molecules or by using more than one metabolic type of cell and applying appropriate data processing it was possible to increase the specificity of the cellular response **(Lobanov, et al. 2001).**

2.2.2.5 DNA-based biosensors

Due to their broad spectrum of physical, chemical, and biological actions the nucleic acids have been used in a variety of biosensors and bioanalytical assays. They have the capability to adapt to environmental applications and have been used to measure Pb^{2+} due to DNA's catalytic activity **(Rogers 2006).**

They rely on the hybridization of known molecular DNA probes or sequence with complementary strands in a test sample and provide a high degree of certainty even though a lot of sample preparation like amplification, hybridization and detection is needed **(Pancrazio, Whelan, Borkholder and Stenger 1999)**.

Using DNA hybridization microarrays have also been suggested for detection of multiple pathogenic microorganisms relevant to biodefense, environmental contamination, and for the detection of food borne pathogens and their virulence factors. The detection and identification of multiple species of *Campylobacter* and *Listeria*, enterotoxins produced by *Staphylococcal* species and toxins produced by *Clostridium perfringens* were made possible using this technique. Other advances along these lines are visual DNA chips for the detection of hepatitis virus, hybridization using labeled oligonucleotides detected by anodic stripping voltammetry, optical detection of hybridization using gold nanoparticles, and electrochemical detection of DNA hybridization using silver precipitation on gold nanoparticle-labeled oligonucleotides. **(Rogers 2006).**

2.3 Immobilization

Immobilisation refers to the proper binding of biological element to the transducer in order to make a useful biosensor **(Eggins 1996).** Physiochemical changes are brought about by biological material in close proximity of the transducer and immobilisation forms this proximity as well as helps in stabilization of biomaterial for reuse. The biomaterial is immobilized either directly on the transducer or in membranes, which can be placed on the transducer. The process used for immobilization can be adsorption, covalent binding, entrapment, encapsulation, cross-linking or a combination of these techniques. The type of the biomaterial, the substrate used and transducer configuration are key factors in deciding technique as well as the type of support used **(D'Souza 2001).** The advantages and disadvantages of five major immobilization methods used for enzymes/cells are listed. These are adsorption, covalent bonding, entrapment, encapsulation, cross-linking **(Bickerstaff 1997).**

2.3.1 Adsorption

This requires minimal preparation, is the simplest method **(Eggins 1996)** and most useful when viable cells are used **(Bickerstaff 1997).** Reversible surface interactions between enzyme/cell and support material are involved, these forces are Van der Waals forces, ionic and hydrogen bonding interactions and are weak but large enough in numbers to enable binding. As an example, use of a positively charged support material will enable immobilization for yeast cells as they cells have a surface chemistry that is substantially negatively charged. The biological component is adhered to a support material with

adsorption properties under suitable conditions of pH, ionic strength etc. for an incubation duration. This is followed by collection of the immobilized material and extensive washing to remove unbound biological components **(Bickerstaff 1997).** Many materials can adsorb enzymes on their surfaces e.g. alumina, charcoal, clay, cellulose, kaolin, silica gel, and glass and adsorption can be partitioned into two classes: physical (physisorption) and chemical (chemisorption) adsorption. While physisorption is weak and occurs via van der Waals bonds, sometimes with hydrogen bonds or ionic forces (charge transfer forces), chemisorptions is strong with covalent bonds and the adsorbed biomaterial may be very sensitive to changes in pH, temperature, ionic strength and the substrate **(Eggins 1996).** The advantages and disadvantages of adsorption are:

Advantages include:

- Almost no damage to enzyme/cells.
- Simple, inexpensive and rapid immobilization process
- Support or enzyme/cells don't have any chemical changes.
- Reversible: Allows regeneration with fresh enzymes/cells.

Disadvantages include:

- Leakage of enzymes/cells from the support/concentration of product.
- Non-specific binding.
- Support overload.
- Steric hindrance by the support.

Leakage of biocatalyst from the support is a significant disadvantage and desorption can occur under conditions like environmental changes in pH, temperature, and ionic strength. Even when a cell/enzyme is a firmly adsorbed desorption can take place due to substrate binding, binding of contaminants in the substrate, product production, or conditions causing change in protein conformation. Flow rate, bubble agitation, particle-particle abrasion, and scouring effect of particulate materials on vessel walls are the physical factors that can result in desorption. If support regeneration is incorporated in the functionality to allow rapid expulsion of exhausted biocatalyst and replacement with fresh biocatalyst then desorption turns out to be advantageous but, if substrate, product or residual contaminants are charged and interact with the support then non-specific binding is a problem. Support overload

without cautious control will reduce catalytic activity while steric hindrance issue results when a suitable spacer is not there between enzyme and support **(Bickerstaff 1997).**

2.3.2 Covalent bonding

Covalent bonds form between a functional group in the biomaterial and the support matrix (**Eggins 1996)** and mostly functional groups are the amino group (NH_2) of lysine or arginine, the carboxyl group (CO_2H) of aspartic acid or glutamic acid, the hydroxyl group (OH) of serine or threonine and the sulfhydryl group of cysteine.

To maintain enzyme activity in a support environment, hydrophilicity is very important. For example, (hydrophilic) polysaccharide polymers are a popular choice for support materials for enzyme immobilization. The sugar residues of some polymers like cellulose, dextran (Sephadex), starch and agarose (Sepharose) are used for enzyme immobilisation; these contain hydroxyl groups which are ideal for chemical activation to form covalent bonds. They form hydrogen bonds with water molecules and create aqueous (hydrophilic) conditions in the support. Silica and glass (both porous) are popular support materials for enzyme immobilisation, polysaccharide supports disintegrate due to microbial/fungal presence while organic solvents shrink the gels.

The enzyme should not be inactivated due to reaction with amino acids at active sites and thus selection of method is very important. The two steps required to form covalent bonds between enzymes and support materials are the activation of functional groups on the support material by a specific reagent and the addition of enzyme in a coupling reaction to form a covalent bond with the support material **(Bickerstaff 1997).** The advantages and disadvantages of covalent bonding are:

Advantages

- During usage enzymes are not released.
- Reaction takes place in substrate's presence to protect the active site (**Eggins 1996**).

Disadvantages

- Doesn't suit immobilization of cells.
- Covalent bonding the cells are exposed to potent reactive groups and harsh reaction conditions that affect the viability of cells.

- Loss in the structural integrity of the cell with continuous use, leading to loss of intracellular enzymes **(D'Souza 2001)**

2.3.3 Entrapment

When viable cells are used this technique is most effective **(D'Souza 2001)**, in this method the biomolecules are trapped in a polymeric gel matrix, polyacrylamide, starch, alginate, pectate, polyvinyl alcohol, polyvinyl chloride, polycarbonate, cellulose acetate and silica gel are commonly used when using entrapment (**Monosik, Stredansky and Sturdik 2012).** Immobilisation can be done in many ways:

- Ionotropic gelation of macromolecules with multivalent cations (e.g. alginate).
- Chemical/photochemical reaction results in organic polymerization (polyacrylamide).
- Precipitate from immiscible solvent (polystyrene).

An enzyme is mixed with polyionic polymer material and then the polymer is crosslinked with multivalent cations in an ion-exchange reaction to form a lattice structure that traps the enzyme/cells. This entrapment method is called ionotropic gelation. A variety of acrylic monomers are available for the formation of hydrophilic copolymers in the method of organic polymerisation by chemical-photochemical reaction, for example, acrylamide monomer is polymerized to form polyacrylamide and methylacrylate is polymerized to form polymethylacrylate. During polymerization a crosslinking agent is also added along with the monomer to from crosslinkages between polymer chains that helps to create a three-dimensional network lattice. The resulting polymer product is broken into particles of a desired size or polymerization is designed to form beads of defined size. Phase separations results in precipitation and cells/enzymes come into contact with a water-miscible organic solvent. This method is limited to highly stable enzymes or nonliving cells as most cells/enzymes are not able to tolerate these solvents.

Advantages

- Porosity of gel matrix is controlled which prevents leakage of the enzyme/cells and allows free movement of substrate and product. **(Bickerstaff 1997).**
- Advantages are due to passive trapping of cells into the pores or adhesion on cellulose surface or other synthetic membranes. These immobilised cells due to adhesion have direct contact with liquid phase that contains substrate; despite cell and liquid phase

being distinctly separate, this reduces/eliminates mass transfer issues, commonly associated with gel entrapment methods **(D'Souza 2001)**.

Disadvantages

- Diffusion of the substrate is prevented by creating large barriers, this slows the rate of reaction and so the sensor response time.
- Loss of enzyme activity through the pores in the gel **(Eggins 1996).**

2.3.4 Encapsulation

Different types of semipermeable membranes envelope biomaterials in this method which is similar to entrapment as enzymes/cells are free in solution but restricted in space. Small substrates and products can go through the semipermeable membrane but large sized proteins or enzymes cannot pass out or into the capsule. Nylon and cellulose nitrate are most popular materials for construction of capsules **(Bickerstaff 1997).** Another approach to encapsulate is the sol-gel method for the immobilization of biological molecules in ceramics, glasses, and other inorganic materials in which biomaterials trapped in a porous matrix like polymeric oxo-bridged SiO_2 network. This can be done at room temperature and protects biomaterial against denaturation (**Monosik, Stredansky and Sturdik 2012).** Encapsulation was used for the first glucose biosensor on the oxygen electrode. The advantages and disadvantages are as follows:

Advantages

- Close attachment between biological component and the transducer.
- Adaptable and reliable technique **(Eggins 1996).**
- A unique feature of this method is co-immobilization. Cells/enzymes can be immobilized in any desired combination to suit particular applications.

Disadvantages

- Diffusion linked problems are crucial and may result in rupture of the membrane if products from a reaction accumulate rapidly.
- Immobilized cell/enzyme particles may have density quite similar to the bulk solution resulting in problems with reactor configuration, flow dynamics etc. **(Bickerstaff 1997).**

2.3.5 Cross-linking

In cross-linking the biomaterial is bound to solid supports by bifunctional or multifunctional reagents like glutarladehyde, hexamethaylene di-isocyanate, 1.5-difluoro 2, 4-dinitrobenzene and bisdiazobenzidine-2, 2'-disulphonic acid, the most commonly used cross-linker for biosensors is glutaraldehyde as it couples with the lysine amino groups of enzymes (**Monosik, Stredansky and Sturdik 2012)**.

Advantages

- Useful to stabilise adsorbed enzymes (Eggins 1996).
- Enhances other methods of immobilization by reducing cell leakage in other systems **(Bickerstaff 1997).**

Disadvantages

- Damages the enzyme.
- Limits diffusion of the substrate
- Poor rigidity (mechanical strength) **(Eggins 1996).**
- Forms multilayers of enzyme, which adversely affect the activity of the immobilized layers (**Monosik, Stredansky and Sturdik 2012)**
- Toxicity of some reagents limits this method to living cells and many enzymes.

2.4 Mediators

In order to probe the chemical processes inside living cells the techniques of electrochemistry are used, especially those using electron transfer mediators. These methods enable us to observe electrochemically the biological reactions within a cell by measurement of bioelectrocatalysis **(Kalab & Skladal, 1994).**

Redox mediators interact with eukaryotic cells at different cell sites. There are two types of redox mediators, the lipophilic and the hydrophilic mediators. Hydrophilic mediators (water soluble) access the major catabolic redox molecules of prokaryotic cells and they access the cells extracellularly while lipophilic mediators cross the cell membrane and access the catabolic redox sites of eukaryotic cells in the cytoplasm and mitochondria **(Frankie et al, 2014, Ramsey & Turner (1988), Richardson, Gardner & Rawson (1991), Ertl et al, (200a; 2000b), Ertl & Mikkleson (2001), Pasco et al, (2005; 2004; 2000) and Tizzard et**

al, (2004)). The reduced electron transport chain molecules interact with and reduce the oxidised mediator. The reduced mediator is quantified by electrochemical techniques like amperometry, voltammetry, coulommetry. **Chaubey and Malhotra** classify a mediator as a redox couple that shuttles electrons from the redox centre of the enzyme to the surface of the indicator electrode and, thus participate in the redox reaction with the biological component by speeding up the electron transfer process. In one of many reaction mechanisms the mediator reacts with the reduced enzyme followed by diffusion to electrode surface for another electron transfer.

Certain desirable characteristics of the mediator are stability (oxidized/reduced forms), pH independence, rapid interaction with target molecules, fast (homogenous/heterogeneous) electron transfer, known formal potential and no interaction with the biocomponent in a way that alters its redox potential. The redox potential of the mediator should be lower than interferents (if electrochemically active) in sample and it should provide a potential gradient (or slope) for shuttling of electrons between electrode and enzymes' redox sites. Oxidative biocatalysis requires positive redox potential of mediator compared to the redox potential of enzyme active site while for reductive biocatalysis it is negative **(Chaubey et al., 2002 & Szentrimay et al., 1977).** To confirm if the mediator alters the redox potential of the biocomponent, the use of several different mediators is generally recommended. The biocomponent parameter being measured should not vary with the mediator used **(Flutz & Durst, 1982).** Most commonly used mediators are inorganic redox ions like ferricyanide **(Trivedi et al. 2009),** organometallic compound ferrocene **(Cevik et al. 2010),** or organic dyes such as methylene blue**,** toluidine blue**,** or Prussian blue **(Monosik, R., Stredansky, M., & Sturdik, E., 2012**). In recent times biosensor devices have been studied with a great interest and consequently redox mediators/ and biological molecules/ interaction and characterization has acquired a higher profile **(Cass et al., 1984).** In general there are two ways to incorporate a redox mediator into an electrochemical system. One method uses the mediator by adding in the solution and another method involves immobilization of mediator on the surface of the electrode producing modified sensors. (**Murray, Eving & Durst, 1987; Pandey, 1998**). For an immobilized redox enzyme on electrode surface interacting with a freely diffusing mediator and substrate, the catalytic response of the redox process is governed by Michaelis-Menten system of equations **(Limoges and Savent (2003).**

In order to achieve higher sensitivities, the mediator concentration and mobility need to be high and consequently immobilization of mediators is generally avoided even though it is not very convenient to use it in a solution. Sometimes preliminary study of some surface modifiers is carried out using mediators in solution. **(Priet-Simon & Fabregas, 2004).**

According to **Antiochia et al. (2004**), an electrode modified with an electrodeposited film (3,4-dihydroxybenzaldehyde-derived) offered higher sensitivity, a lower detection limit and a larger linear dynamic range compared to the bare electrode with redox mediator in solution for the study of electrocatalytic oxidation of NADH.

2.4.1 Hydrophilic mediators

Cell redox molecules which are accessible from the environment of the cells and the periplasm are the only ones for which interaction with hydrophilic mediators is possible, as an example, respiratory electron transport molecules may be oxidised by hydrophilic mediators in prokaryotic organisms. Some examples of hydrophilic mediators are potassium ferricyanide, ruthenium hexamine and carboxymethylferrocenium, potassium ferricyanide is used to couple with cellular respiration **(Hadjipetrou, Gray-Young & Lilly, 1966; Ramsey & Turner, 1988; Gaisford et al., 1991)**, ferri/ferrocyanide is used in biosensors **(Dubinin et al., 1991; Shulga et al., 1994; Katrlik et al., 1997)**. Hydrophilic mediators can be reduced by cellular mechanism like trans-plasma membrane electron transport systems (tPMET) **(Gurazada 2008).**

2.4.2 Lipophilic mediators

Lipophilic mediators cross the lipid-bi-layer membrane of cells and oxidise the intracellular reduced proteins generated during catabolism and are soluble in lipids. Examples of this type of mediators include phenazine ethosulphate, dichloroindophenol and neutral red, benzoquinones such as 2-mythyl-1,4-naphthoquinone (menadione), benzoquinone, 1,2-naphthoquinone, and benzoamines like 2,3,5,6-tetramethyl 1,4-phenylenediamine (TMPD) and N,N,N',N'-dimethyl-p-phenylenediamine **(Gurazada 2008)**. Interaction of both types of mediators with bacterial and mammalian cells has been demonstrated by using scanning electron microscopy (SEM) **(Liu et al., 2001; Cai et al., 2002)** that showed the differences in electrochemical imaging where hydrophilic mediators imaged the cell surface, while, lipophilic mediators imaged intracellular redox features. Menadione (a lipophilic mediator) interacts with all NAD(P)H dependent dehydrogenases and elements of the electron transport

chain while DT-diaphorase enzyme acts as a catalyst to transfer electrons from NADH to menadione (**Rabinowitz et al. 1998**). In a double mediator set-up both hydrophilic and lipophilic mediators may be used together, for example, first the lipophilic mediator transfers electrons to an extracellular hydrophilic mediator like potassium ferricyanide which gets reduced and is detectable by current measurements using amperometry **(Catteral et al., 2001)** and Voltammetry (**Baronian et al., 2002; Tosok, Driscoll & Luong., 2001a and b).**

2.4.3 Advantages of using mediators

- Enzyme electrode working potential determined by redox potential of mediator.
- Unwanted species' interference avoided by using mediators at low redox potentials.
- Measurements less dependent on oxygen concentration.
- Enzyme electrode relatively pH insensitive if redox reaction does not involve protons, **(Chaubey & Malhotra, 2002).**

2.5 Screen Printed Electrodes (SPEs)

These devices are constructed by printing different types of inks on plastic or ceramic substrates, a vast range of screen printed electrodes are available commercially and the chemistry of inks determines the selectivity and sensitivity needed for each analysis. The inks' chemical composition is changed by addition of substances like metals, enzymes, polymers, complexing agents and the existing electrodes can be modified by surface deposition of metal films, polymers and enzymes **(Hart et al. 2004, Eggins, B. R. 2002).** The various applications of SPEs are classified by the types of material used on the working electrode surface like unmodified SPE, film modified SPE, enzyme modified SPE and antigen/antibody-modified SPE.

2.5.1 Screen-printed carbon-based electrodes (SPCEs)

Literature on the use of unmodified SPCEs for determination of analytes is scarce **(Honeychurch & Hart 2003**). Low cost and simplified technological processing are the factors for preferring graphite based electrodes that have been compared for hydrogen peroxide detection **(Krejci 2004)**, investigated for their behaviour towards cysteine and tyrosine **(Vasjari, et al., 2005)** and used for determination of metals like Pb **(Honeychurch, et al., 2000),** Cu(II) **(Honeychurch, et al., 2002),** Ag(I) **(Dilleen, et al., 1998**).

Although SPEs are commonly built using graphite, gold and silver based inks are used to determine and analyse other elements. For example, environmentally hazardous materials like copper, mercury and Cd are determined using gold based SPEs as well as to determine Pb in wastewater and soil extracts **(Noh, et al., 2006).**

2.5.2 Film-coated SPCEs

2.5.2.1 Hg film based SPCEs

In these electrodes, thin mercury film plating is applied to the graphite surface of the working electrode **(Waang &Tian, 1992).** Metals such as Pb, Cd, and Cu were determined at ppb levels on mercury-coated carbon strip electrodes **(Wang, 1994).** Advantage of these electrodes is that they can be prepared beforehand in the laboratory for on-site use to avoid handling, transport and disposal of mercury as the coating is pre-deposited on the electrode surface **(Palchetti, et al., 2005).** Disadvantage of Hg is that it is toxic and incorporation of Hg in sensors poses environmental problems as SPEs are disposable electrodes.

Other film-modified SPCEs are bismuth (Bi) and Gold-coated SPCEs. Bi-coated electrodes have been used for the detection of Pb(II) and Cd(II) **(Kadara & Tothill, 2005).** Main purpose of gold film was to eliminate the use of toxic elements such as Hg. Gold-coated electrodes have been used for the detection of Pb and Hg at trace levels **(Wang & Tian, 1993a, Wang & Tian, 1993b, Masawat, et al., 2003).**

2.5.3 Enzyme-modified SPEs

Enzymes are proteins and they are the most commonly used biological elements, despite high cost of extraction, isolation and purification. Pioneering work of Clark and Lyons **(Clark & Lyons, 1962)** showed that an enzyme could be integrated into an electrode, hence making a biosensor for the determination of glucose. Enzymatic biosensors have been used for the detection of herbicides, pesticides **(Nunes, et al., 2004).** Due to presence of number of active groups on the graphite surface it is insufficient for direct immobilization of enzyme. There are several methods that have been employed for immobilization of enzymes.

One common method of enzyme immobilisation for construction of AChE (acetylcholine) – SPCEs is based on crosslinking using bovine serum albumin (BSA) and glutaraldehyde (GA).

This method has been reported **(Suprun, et al., 2005)** to determine alidcarb and paraoxon using Prussian Blue (PB) as the electrochemical mediator.

Enzymes are also used for the analysis of heavy metals such as Cu(II), Hg(II), and Cd(II) **(Rodriguez, et al., 2004, Rodriguez, et al., 2004)**, cholesterol **(Gilmartin & Hart, 1994)**, superoxide and hydrogen peroxide **(Ledru, et al., 2006)**, ethanol **(Azevedo, et al., 2005)**, phenolic compounds **(Avramescu, et al., 2002)**.

2.5.4 Antigen/antibody modified SPEs (Immunosensors)

SPEs based immunoassays are used for analysis of hormones and detection of oestradiol levels in biological fluids such as serum and saliva in clinical settings, for example, gynaecological endocrinological infertility status investigations, post-menopausal as well as oestradiol status post fertility treatment **(Pemberton, et al., 2005)**.

An amperometric immunosensor based on SPCE for the detection of 17-β oestradiol in water has also been reported **(Butler & Guilbault, 2006).**

Due to the use of artificial insemination techniques in cattle breeding industry a significant cost saving can be realised in herd management by predicting the oestrous onset. One way the oestrous onset can be detected is by monitoring milk's progesterone levels **(Pemberton, et al., 1998)** for which several immunosensors based on progesterone have been developed. One of the examples is progesterone labeled ALP (alkaline phosphatase) that has been used in competitive assays where SPCEs are coated with antibodies **(Pemberton, et al., 1998, Kreuzer, et al., 2004, Pemberton, et al., 1999, Pemberton, et al., 2001)**.

Due to exposure to the female sex hormone oestrogen, as well as to chemicals that mimic oestrogens, feminization of male fish has been reported. To study this endocrine disruption a biomarker Vtg for xenobiotics oestrogen has been used in a disposable immunosensor **(Darain et al., 2005).**

Widespread usage of SPEs for analytical applications can be attributed to their low cost and versatile functioning. SPEs provide rapid and accurate 'in-situ' analyses and is suitable for the development of compact and portable devices **(Renedo, Alonso-Lomillo, & Martinez, 2007)**.

2.6 Summary

It has been the goal of this chapter to provide sufficient motivation and background material to highlight the importance of developing a sensitive and cost effective portable biosensor for the detection of oestrogen. Development of bioassay using EBP has advantages over other yeast assays, human cell lines and other mammalian cell lines since these are expensive, time consuming and require special laboratory facilities.

The chapter that follows, details the construction of a simple portable biosensor for the detection of oestrogen in environmental samples by using SPEs.

Chapter 3 Research Methodology

3.1 Different electrochemical techniques to detect redox responses

Various electrochemical techniques have been used in a variety of biotechnological fields such as biosensors, bioelectrochemical synthesis and biofuel cells **(Hill, 1987).** Electrochemical techniques have been used for BOD (Biological Oxygen Demand) measurements **(Pasco, et al., 2000; Trosok, Driscoll & Luong, 2001**), yeast catabolism (**Baronian et al., 2002),** and viable cell population determination (**Perez et al., 1998).** There are large numbers of proteins that are electrochemically active at the outer cell membrane but they are inactive within the cells and due to this they are unable to facilitate the transfer of electrons to the external surface **(Kim et al., 1999**). Nevertheless, redox mediators can facilitate the transfer of electrons from internal reduced proteins to the external surface. They are small molecules that undergo redox reactions and oxidize external reduced catabolic proteins. The double mediator system containing 2,3,5,6-tetramethyl 1,4-phenylenediamine (TMPD, a lipophilic mediator) and menadione or potassium ferricyanide,(a hydrophilic mediator) has been used to probe intracellular redox activity of eukaryotes **(Zhao et al., 2005; heiskanen et al., 2004; Baronian et al., 2002; Rabinowitz et al., 1998).**

3.1.1 The Standard Oxidation and Reduction Potentials

The standard reduction potential is the tendency for a chemical species to be reduced, and is measured in volts at standard conditions. The more positive the potential is the more likely it will be reduced. The standard reduction potential is in a category known as the standard cell potentials or standard electrode potentials. The standard cell potential is the potential difference between the cathode and anode. The standard potentials are all measured at 298 K, 1 atmosphere pressure, and with 1 M solutions.

It is written in the form of a reduction half reaction. For example copper's Standard Reduction Potential is represented as:

Cu^{2+} (1M) + 2 e^{-} → Cu (s) E^{red} = +0.340 V

The standard oxidation potential is much like the standard reduction potential. It is the tendency for a species to be *oxidized* at standard conditions. It is also written in the form of a half reaction, and an example is shown below.

Copper's Standard Oxidation Potential

$$Cu(s) \rightarrow Cu^{2+} + 2\ e^-$$
$$E^{ox} = -0.340\ V$$

The standard oxidation potential and the standard reduction potential are opposite in sign to each other for the same chemical species. Standard reduction or oxidation potentials can be determined using a SHE (standard hydrogen electrode) **(Petrucci et al.,2007 & Zhumdal 2007).**

3.1.2 Electrochemical Techniques-

Electroanalytical techniques based on current measurements as a function of applied voltage are known as voltammetric techniques. Three electrodes called Reference, Working and Auxiliary (or Counter) electrodes are used in a typical set-up immersed in an analyte solution and also contains a supporting electrolyte of metal alkali salt that is utilised for its conductive properties though it doesn't react with the electrodes. The reduction or oxidation reactions take place at the working electrode and current flows between the working and auxiliary electrodes, this current is continuously measured. The potential between the working and reference electrode is monitored and an electronic potentiostat maintains this potential difference constant by changing the applied potential between the working and auxiliary electrodes. The auxiliary electrode is made up of inert material like Mercury or Platinum that doesn't take part in reduction/oxidation reactions but just completes the circuit. **(Wang, 2000)**

3.1.2.1 Linear Sweep Voltammetric Techniques

In this procedure a linearly increasing voltage waveform with respect to time is applied to the electrochemical cell and the resulting current is recorded as a function of time and thus as a function of the applied voltage. The range of linear voltage is usually between 2 to 3 Volts and the plot displays current on the Y-axis vs. voltage on the X-axis **(Thomas & Henze 2001).**

3.1.2.2 Differential Pulse Voltammetric Techniques

The excitation signal applied to the electrochemical cell in this method is the superposition of a pulse and a staircase waveform. The pulse duration is significantly shorter than the pulse

period. Two current measurements are made, one just before the application of the pulse and another after the end of the pulse. The difference in current is computed and recorded as a function of the linearly increasing excitation voltage. The advantage of this method is realised by noting that currents are recorded when the difference between the desired faradic current and interfering charging current (due to capacitance) is high. Differential pulse voltammetry based methods have higher sensitivity than other voltammetric methods **(Compton & Banks 2007).**

3.2 Cultivation of microorganisms

Microorganism *A. adeninivorans* G1212 were grown in 250 mL indented flask in YEPD broth, a general purpose fungal selective medium (yeast extract 10 g, Neutralised soya peptone 20 g, D-Glucose anhydrous 20 g) by inoculating well isolated single colony grown on YEPD agar. Cells were incubated at 37^0C for 16 h in indented flasks shaken at 180 rpm.

3.2.1 Harvesting the yeast cells

1. Yeast cells were harvested by centrifugation in eppendorf centrifuge at 6000 rpm for 10 min at 12^0C.
2. The cells were washed twice with PBS (Phosphate Buffer Saline) pH 7 and cells were kept at -80^0C for 2 hours followed by freeze drying.
3. The cells were lysed by freeze drying and grinding with mortar and pestle and re-suspended in PBS buffer to produce a cell extract.
4. Lysed cells were centrifuged at 10000 rpm for 10 min at 4^0C twice to remove debris and to produce a protein rich supernatant.

3.3 Protein purification

As it has been mentioned previously in this thesis that *A.adeninivorans* was transformed with histidine tagged synthetic oestrogen binding protein gene based on the *C.albicans* EBP sequence. The recombinant EBP expressed in the yeast *A. adeninivorans* were separated using HisTrap affinity binding columns. Figure 3-1 illustrates the HisTrap column.

Figure 3-1 - G E Healthcare Life sciences HisTrap FF

HisTrap columns are prepacked with the affinity medium Ni Sepharose charged with Ni^{2+}ions. Various amino acids for example histidine, form complexes with many metal ions. EBP is histidine tagged protein and presence of histidine tag increases the affinity for Ni $^{2+}$ ions. These ions are the preferred metal ions for the purification of recombinant histidine-tagged proteins. Buffers recommended for purification are binding buffer and elution buffer **(GE Healthcare, HisTrap affinity columns).**

3.3.1 Preparation of buffers-

3.3.1.1 Binding buffer (pH 7.4)-

Binding buffer was prepared by adding 20mM sodium phosphate (1.6394 g), 500 mM NaCl (14.61 g), 40 mM imidazole (1.3615 g) to 500 mL Mili Q water. The content was mixed thoroughly by stirring with a magnetic bead. The buffer was sterilized at 121^0C for 15 minutes and stored at 4^0C.

3.3.1.2 Elution buffer (pH 7.4)-

Elution buffer was prepared by adding 20mM sodium phosphate (1.6394 g), 500 mM NaCl (14.61 g), 500 mM imidazole (17.0193 g) to 500 mL Mili Q water. The content was mixed thoroughly by stirring with a magnetic bead. The buffer was sterilized at 121^0C for 15 minutes and stored at 4^0C.

3.3.2 Process of Purification of recombinant EBP by HisTrap column

1. After removing the snap-off end at the HisTrap column outlet, column was washed with the ethanol with 20 mL of distilled water.
2. Column was equilibrated with at least 25 mL of binding buffer. Protein rich supernatant was loaded with a syringe very slowly to get maximum binding. This process was repeated twice to recover maximum protein.
3. HisTrap was washed with 50 mL of binding buffer which contains low imidazole concentration of 40 mM at pH 7.4 to minimize binding of host cell proteins. 20-40mM imidazole, pH 7.4 is the optimal imidazole concentration which is suitable for many proteins.
4. In the elution step, 25 mL of elution buffer were used and the concentration of imidazole in elution buffer was 500mM which is higher than imidazole concentration in binding buffer. The imidazole concentration required for elution is protein-dependent. If concentration of imidazole is too high then it may also decrease the binding of histidine-tagged proteins. Therefore concentration of imidazole is an important factor for extracting high yield of purified protein

3.4 Desalting the sample

PD-10 Desalting Columns were used for desalting the sample after the purification step. PD-10 Desalting columns contain Sephadex G-25 medium, which helps in desalting, buffer exchange and sample clean up for e.g. separating high molecular weight substances such as proteins from low molecular weight substances like salt, free labels and other impurities. Two different protocols, gravity protocol and spin protocol can be used to separate small molecules from larger ones. In this project, spin protocol has been used. In spin protocol, columns are centrifuged to provide additional gravity force. Dilution of sample is not needed and recovery is usually in the range of 70-90%. Equilibration of the column is important since UV absorbing stabilizers are used in column packing. Figure 3-2 illustrates the desalting columns.

Figure 3-2 - PD-10 Desalting Columns

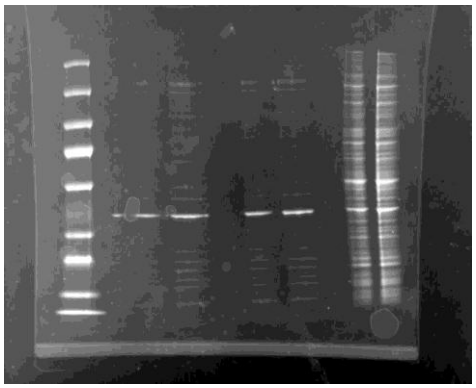
3.4.1 Spin protocol

1. For preparing PD-10 Desalting column for sample, top cap was removed and after discarding column storage solution, top filter from the column was removed using forceps.
2. Then sealed end of the column at notch was cut and PD-10 Desalting column was kept into a 50 mL collection tube by using the column adapter.
3. For equilibrating the column, it was filled with equilibration buffer (in this case PBS buffer) and buffer was allowed to enter the packed bed completely.
4. This process was repeated 4 times and discarded the flow through.
5. Fifth time column was filled with equilibration buffer (i.e. PBS) and spin down at 1000 x g for 2 minutes and buffer was discarded.
6. Sample was added (2.5 mL) slowly in the middle of the packed bed and column was kept in to a 50 mL collection tube and eluted by centrifugation at 1000 x g for 2 minutes. Whole sample was eluted in this way and elute was collected in a separate tube.

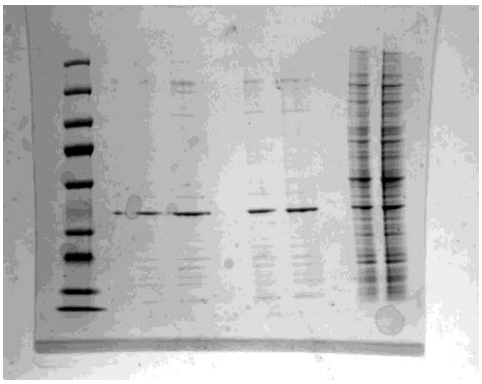
3.5 Confirmation of purification by gel electrophoresis

1. NuPAGE® Tris-Acetate Gel was used for gel electrophoresis to confirm the purification of protein.
2. To prepare buffer to run the gel, 50mL 20X NuPAGE Tris-Acetate SDS Running Buffer was added to 950mL Mili Q water.

3. Reagents were prepared in a separate eppendorf tube by mixing 25µL NuPAGE® LDS sample buffer, 75 µL Mili Q deionised water, and 10 µL NuPAGE® Reducing agent.
4. Samples were prepared by mixing 10 µL and 15 µL of sample with 5 µL of reagent mix in separate eppendorf tube.
5. Before loading on the gel, samples were heated (Benchmark Digital Heat Block) at 70^0C for 10 minutes and vortexed (SCILOGEX Vortex apparatus) for 10 sec.
6. XCell SureLock^{TM} Novex Mini-Cell gel apparatus was used for running the gel.
7. First well of the gel was loaded with 5 µL of ladder. In this project PageRuler™ Prestained Protein Ladder Plus #SM1811 from Fermentas Life Sciences has been used. It is a mixture of 9 different recombinant, highly purified coloured protein with molecular weight varies from 10 to 250 kDa. It is ready to use ladder, supplied in a loading buffer for a direct loading on SDS polyacrylamide gels.
8. Second, third, fourth, sixth and seventh wells were loaded with 5µL, 10µL, 15µL, 10 µL and 15 µL of sample mix respectively.
9. Fifth and eighth well were left blank. Ninth and tenth wells were loaded with binding buffer elute from protein purification to compare the impurities in the sample.
10. The apparent molecular weight of EBP is approximately 46 kDa. Figure 3-3 illustrates dark protein band of 46 kDa molecular weight in wells 2, 3, 4, 6 and 7.



(a)



(b)

Figure 3-3 – *A. adeninivorans* EBP (46 kDa molecular weight) in well number 2, 3, 4, 6, and 7

3.6 Determination of Protein concentration

Thermo Scientific Pierce™ BCA Protein Assay Kit was used in this research. This is a bicinchoninic acid (BCA) based assay for the colorimetric detection and quantitation of total protein. A common protein standard like bovine serum albumin (BSA) is referenced to determine the protein concentrations.

3.6.1 Preparation of Diluted Albumin (BSA) standards

1. Six eppendorf tubes containing 700 µL, 400 µL, 450 µL, 400 µL, 400 µL, and 400 µL of PBS buffer and labeled as A, B, C, D, E, F respectively.
2. In tube A 100 µL of BSA stock solution was added which is containing 700 µL of PBS buffer to make the final concentration of BSA as 250 µg/mL.
3. From tube A 400 µL was taken and added to tube B which is containing 400 µL of PBS buffer which makes the final BSA concentration of 125 µg/mL.
4. From tube B 300 µL was taken and added to tube C which is containing 450 µL of PBS buffer which makes the final BSA concentration of 50 µg/mL.
5. From tube C 400 µL was taken and added to tube D which is containing 400 µL of PBS buffer which makes the final BSA concentration of 25 µg/mL.
6. From tube D 100 µL was taken and added to tube E which is containing 400 µL of PBS buffer which makes the final BSA concentration of 5 µg/mL.
7. Tube F which is containing 400 µL of PBS buffer used as a blank.

3.6.2 Preparation of the BCA Working Reagent (WR)

Working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B.

BCA reagent A comprised of sodium carbonate, sodium bicarbonate, bicinochoninic acid and sodium tartrate in 0.1M sodium hydroxide. BCA reagent B comprised of 4% cupric sulphate.

3.6.3 Procedure for protein determination

1. Each standard and unknown sample was prepared in duplicates.
2. 100 µL of each standard and unknown sample was added to an appropriately labeled eppendorf tube.
3. 2.0 mL of WR was added to each tube and mixed well and then incubated at 37^0C for 30 minutes.
4. After 30 minutes, tubes were cooled at room temperature.
5. Spectrophotometer was calibrated and set up at 562nm.
6. Absorbance of all the samples was measured using cuvette within 10 minutes.
7. Average absorbance measurement of blank replicates at 562 nm was detected from average of each of the standard and unknown samples replicates.
8. Standard curve was plotted by the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/mL. This standard curve was used to determine the concentration of protein of each sample.

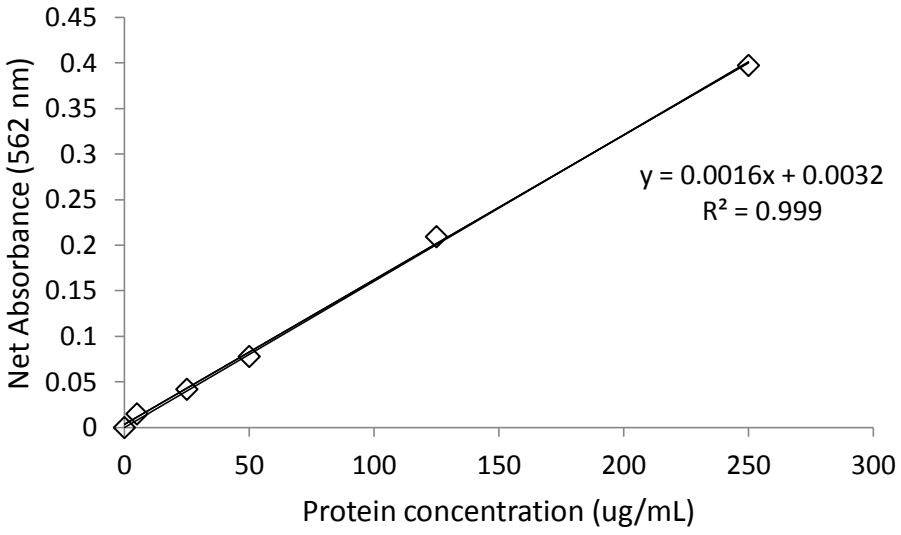


Figure 3-4 - Standard curve for BSA

Figure 3-4 illustrates the Standard curve for BSA. Protein concentration of sample was determined using above standard curve equation (where y is absorbance and x is the value of protein concentration).

3.7 Linear-sweep Voltammetry

Previously the detection of oestrogen using *C. albicans and* S. *cerevisiae* cells **(Baronian & Gurazada 2007)** and cell lysate **(Chelikani et al, 2011)** have been established.

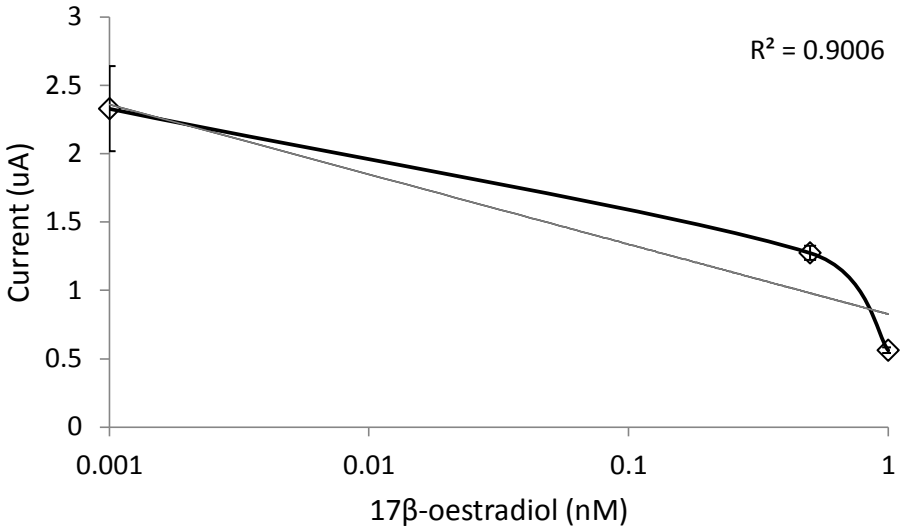
In this research linear sweep voltammetry (LSV) was used for the detection of EBP redox responses to oestrogen in solution. The electrochemical analyser was an eDaq potentiostat coupled to a Power Lab 2/20 controlled by eDaq Echem software. Linear sweep measurements were taken with a 50µm diameter Pt microdisc working electrode, an Ag/AgCl reference electrode and a Pt wire auxiliary electrode (all G-Glass Australia). During electrolysis, a concentration gradient is generated between the electrode surface and the solution. The redox reaction detection occurs at the surface of the working electrode, at the appropriate applied potential that results in the mass transport of a new material to the electrode surface and generating the current. The redox reaction at the working electrode can be facilitated by controlling the potential relative to the reference electrode. Current flows between the working electrode and auxiliary electrode.

Silver/silver Chloride (Ag/AgCl) was used as the reference electrode and its Standard reduction potential (E^0) value is +0.222 V.

Steady state was established after 20 seconds and scanning from 500 to 100 mV at a scan rate of 5mV s^{-1}. 500 mV was used as the initial potential to allow the system to stabilise as the anodic current was measured at 425 mV. The steady-state current at 425 mV was used as the sample signal and from three repeat voltammograms the mean current at this potential was calculated and used in analysis. Initially LSV experiment was done using a control sample (i.e. no estrogens used) and 2 different concentrations. This experiment was repeated several times to validate the results. Figure 3-5, Figure 3-6 and Figure 3-7 illustrate the LSV result of EBP response to different concentrations of oestrogen including the control sample.

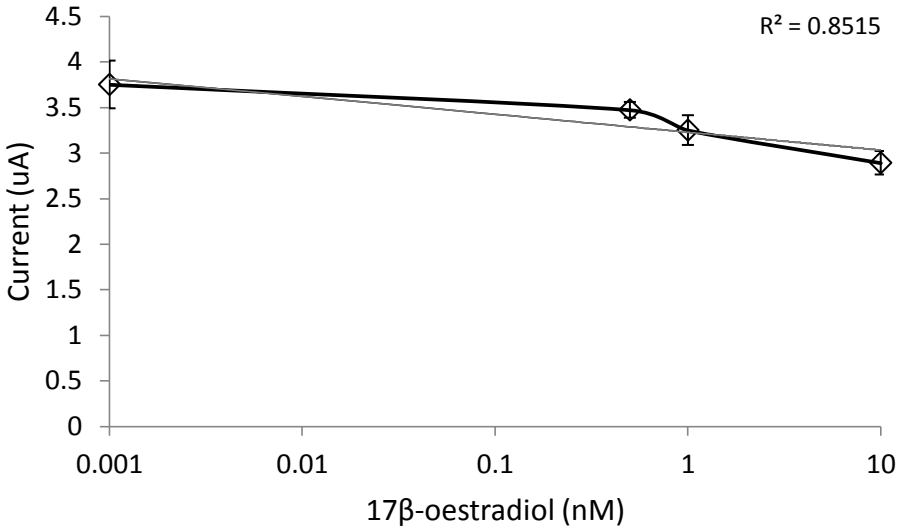
Various LSV experiments show that control sample (which includes EBP and TMPD) resulted in the highest current while after adding the oestrogen, magnitude of current got reduced. This happens because EBP molecules are adhered to the electrode surface and they are all in reduced form. In control samples when only TMPD mediator is added to the solution, large numbers of EBP molecules get oxidized but as soon as oestrogen is added to the solution, it binds to oxidoreductase site of EBP and blocks the transfer of electrons to TMPD. The rate of production of reduced mediator is measured as current by oxidation of

mediator at electrode. Hence due to less number of reduced molecules of TMPD, there will be smaller current and lower peak.



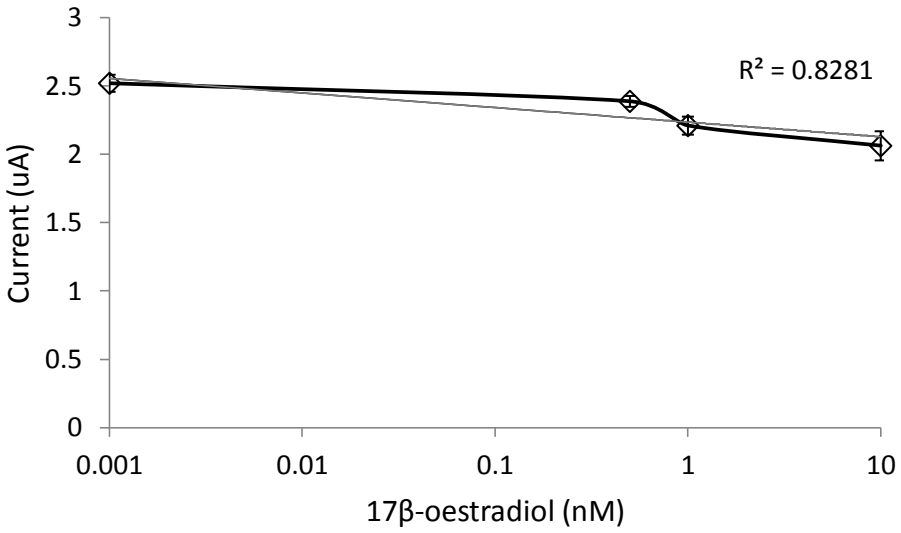
(a)

Figure 3-5 – Scatter plot of LSV result of recombinant EBP from *A. adeninivorans* G1212 strain responses to 17β oestradiol (control, 0.5 nM, 1.0 nM) using single lipophilic mediator TMPD electrochemical system. The data points are the magnitude of the current at 425mV. Error bars represent ±1 SD.



(a)

Figure 3-6- Scatter plot of LSV result of recombinant EBP from *A. adeninivorans* G1212 strain responses to 17β oestradiol (control, 0.5 nM, 1.0 nM and 10.0 nM) using single lipophilic mediator TMPD electrochemical system. The data points are the magnitude of the current at 425mV. Error bars represent ±1 SD



(b)

Figure 3-7 - Scatter plot of LSV result of recombinant EBP from A. adeninivorans G1212 strain 25 responses to 17β oestradiol (control, 0.5 nM, 1.0 nM, 10.0 nM) using single lipophilic mediator TMPD electrochemical system. The data points are the magnitude of the current at 425mV. Error bars represent ±1 SD.

3.8 Immobilization of EBP on Screen printed electrodes

In this research the protein was subsequently immobilised onto the surface of a SPCE and EBP redox responses were detected by using differential pulse voltammetry (DPV).

Immobilisation of the protein onto the surface of a disposable carbon electrode was achieved by cross linker glutaraldehyde. The protein was immobilised on carbon surface of a 'DropSens' screen-printed electrode (DropSens DRP-110 NI). These disposable Screen-Printed Carbon Electrodes are modified with Nickel (II) Oxide (NiO). These electrodes have been developed for working with micro volumes. The electrochemical cell consists of working electrode [{Nickel (II) Oxide/Carbon {4mm diameter}], counter electrode (Carbon), reference electrode (silver). Figure 3-8 illustrates the DropSens SPCEs. Few experiments were also tried out with Nickel free electrode in order to observe any deviation in performance as compared to electrodes modified with Nickel (II) Oxide (NiO).

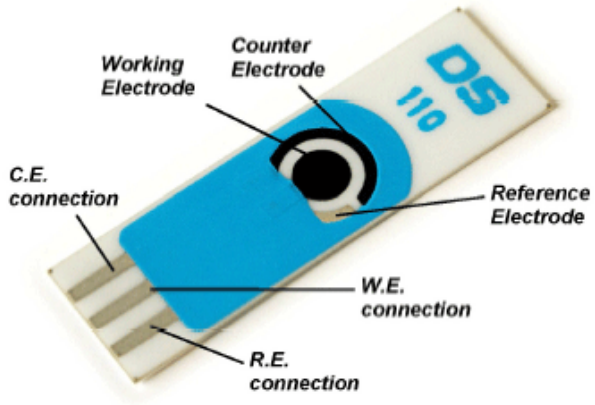


Figure 3-8 - DropSens Screen Printed Carbon Electrodes

3.8.1 Process of immobilization

1. In an eppendorf tube 1 mL 5% v/v solution of glutaraldehyde was prepared in dH_2O by mixing gently using pipette.
2. In a separate eppendorf tube, 16 µL of protein sample was mixed gently with 2 µL of glutaraldehyde solution.
3. Now using pipette, 2µL of sample was applied on the WE (working electrode) surface of the SPCEs. This should spread out on its own.
4. SPCEs were stored immediately at 75% relative humidity (this was prepared by making a slurry of NaCl in an air tight container) and at 4^0C.
5. These biosensors were ready to be used after 18 hours of preparation. After 18 hours the modified SPCEs were freeze dried and stored at 4 °C in air until use.

3.9 Differential pulse voltammetry

Differential pulse voltammogram was obtained by initial potential 0.200 V and sweep potential from 0.200 V – 0.800 V, scan rate 0.020 V s^{-1}, scan increment 0.002 V, step time 0.100 sec, pulse height 0.025 V, pulse width 0.050 sec, scan time 50 sec and current range of 1mA/V. Initially DPV was performed with 6-7 different concentrations of oestrogen on freeze-dried electrode for the validity of the experiment. Similar to LSV, DPV experiments are also showing that control sample resulted in the highest current while after adding the oestrogen, magnitude of current got reduced.

Confirmation of immobilization was achieved by carrying out DPV on several blank and immobilized electrodes. Figure 3-9, Figure 3-10, Figure 3-11, Figure 3-12 illustrate the comparison and confirmation of blank and immobilized electrodes. These images are copied directly from UiEchem software application.

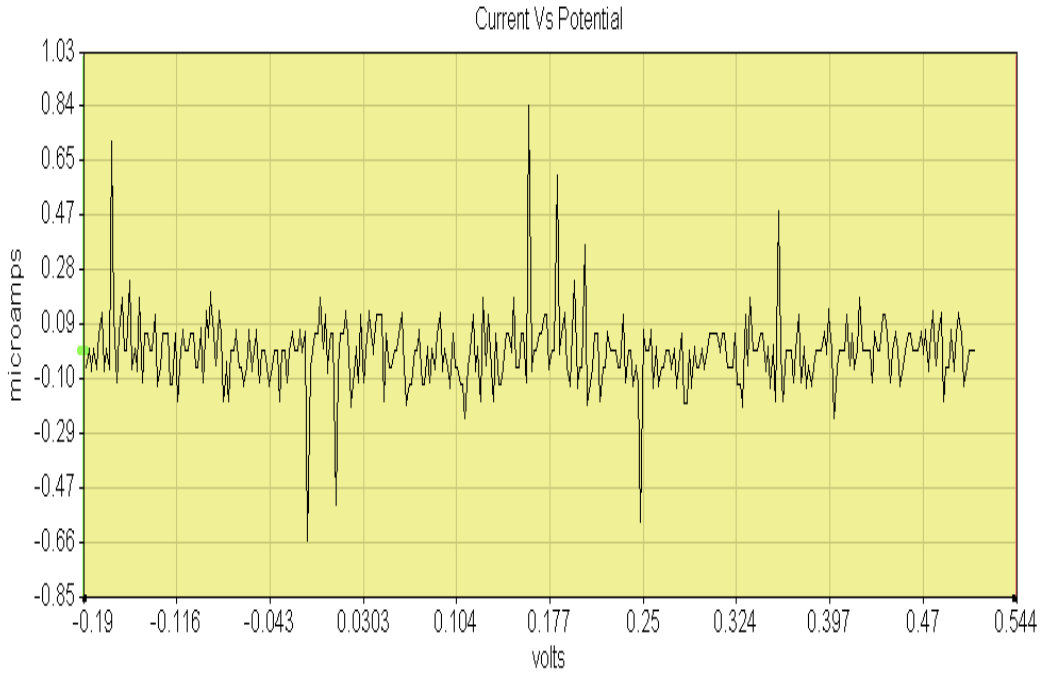


Figure 3-9 DPV showing experiment with PBS on blank electrode

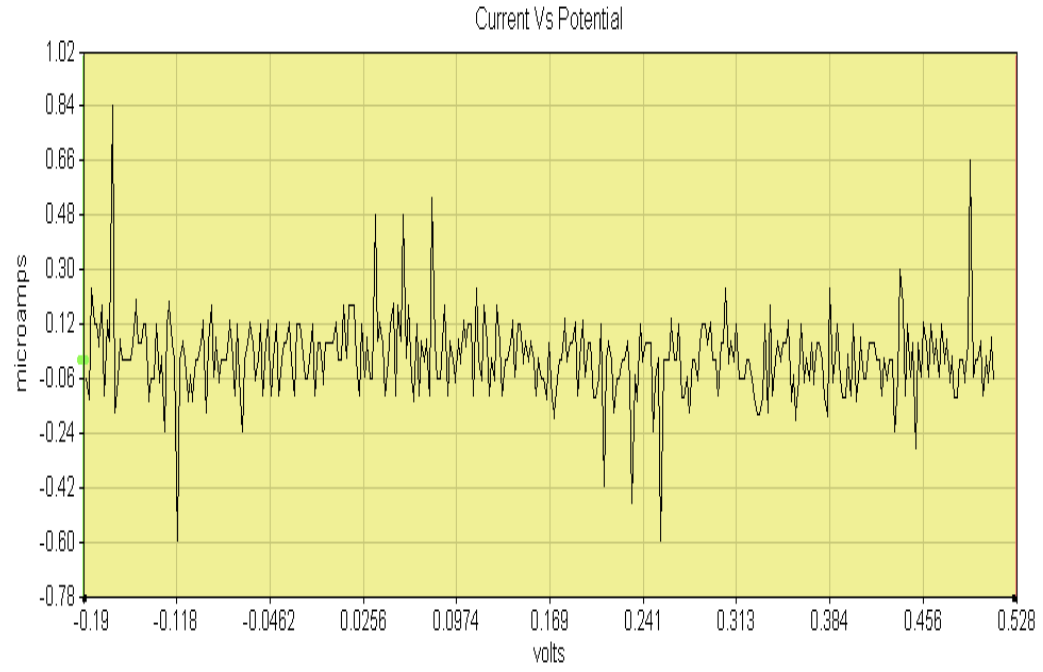


Figure 3-10 DPV showing experiment with TMPD solution on blank electrode

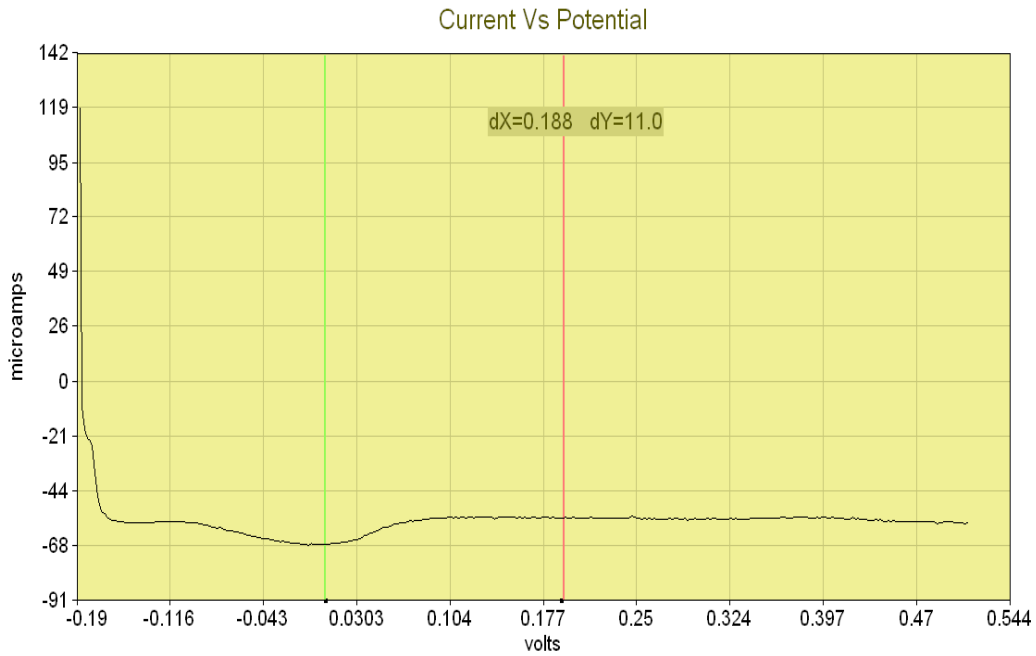


Figure 3-11 DPV showing experiment with PBS, Oestrogen and TMPD on EBP immobilized electrode

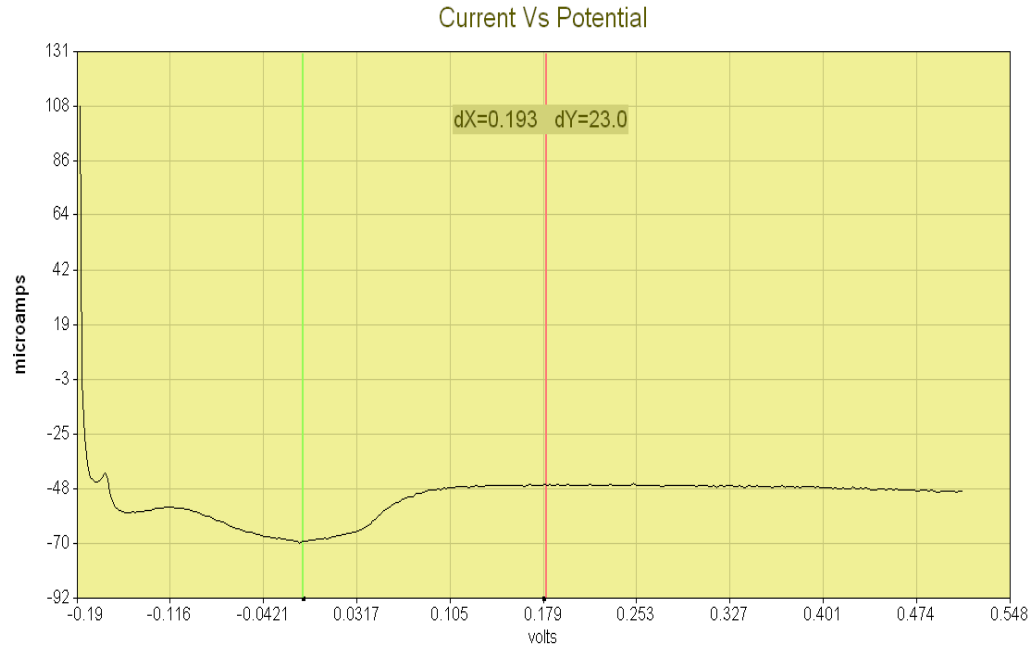


Figure 3-12 DPV showing experiment with PBS and TMPD on EBP immobilized electrode

3.9.1 Binding of oestrogens to the EBP oxidoreductase site

Oestrogen should be added first on the modified biosensor followed by TMPD so that oestrogen can bind to the oxidoreductase sites of the EBP. Figure 3-13 illustrates premixing of oestrogen with TMPD in an eppendorf tube in different concentrations and then placed on immobilised EBP on modified electrode. Experiment was also done by mixing EBP (in solution), oestrogen and TMPD in an eppendorf tube at various concentrations and then placed on a blank electrode. Figure 3-14 illustrates experiment by mixing EBP (in solution), oestrogen and TMPD in an eppendorf tube at various concentrations and then placed on a blank electrode. These experiments resulted in random variation of current with respect to oestrogen concentration and not the desired linear curve.

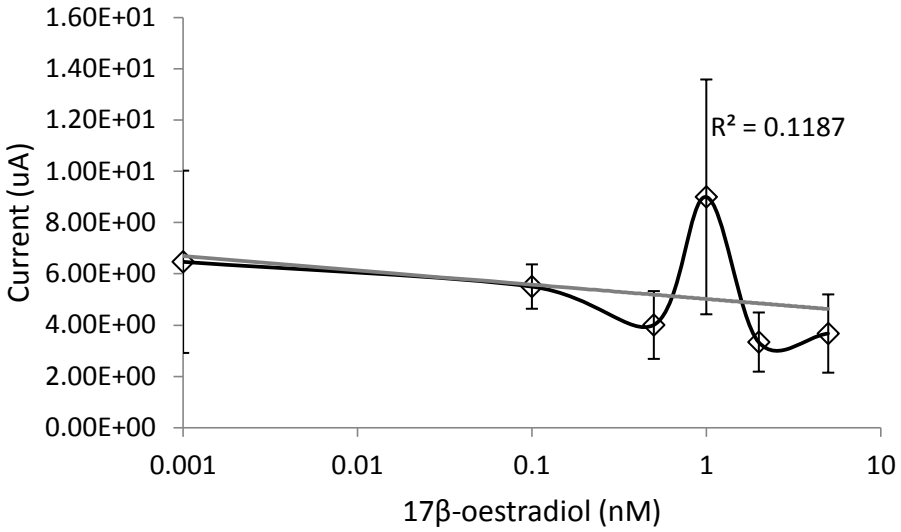


Figure 3-13 Scatter plot of DPV on immobilised electrode using premixing of oestrogen and TMPD in eppendorf tube at different concentrations (control, 0.1 nM, 0.5 nM, 1.0 nM, 2.0 nM, 5.0nM). Error bars represent ±1 SD.

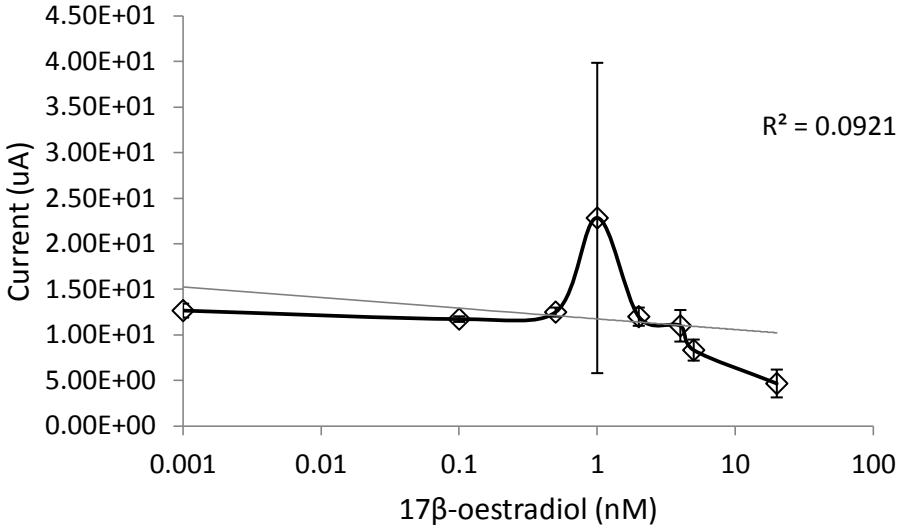


Figure 3-14 Scatter plot of DPV on Blank electrode with premixing of EBP (in solution), oestrogen and TMPD in an eppendorf tube at various concentrations and then placed on a blank electrode (control, 0.1 nM, 0.5 nM, 1.0 nM, 2.0 nM, 4.0 nM, 5.0 nM, 20.0 nM). Error bars represent ±1 SD.

In order to verify basic operation of the DPV setup 8 different concentrations of oestrogen including control were tried out using mediator TMPD and results were satisfactory as a linear response was obtained for the current as a function of oestrogen concentration.

In order to verify basic operation of the DPV setup 8 different concentrations of oestrogen including control were tried out using mediator TMPD and results were satisfactory as a linear response was obtained for the current as a function of oestrogen concentration. Figure 3-15 and Figure 3-16 illustrate the result of EBP response to different concentrations of oestrogen.

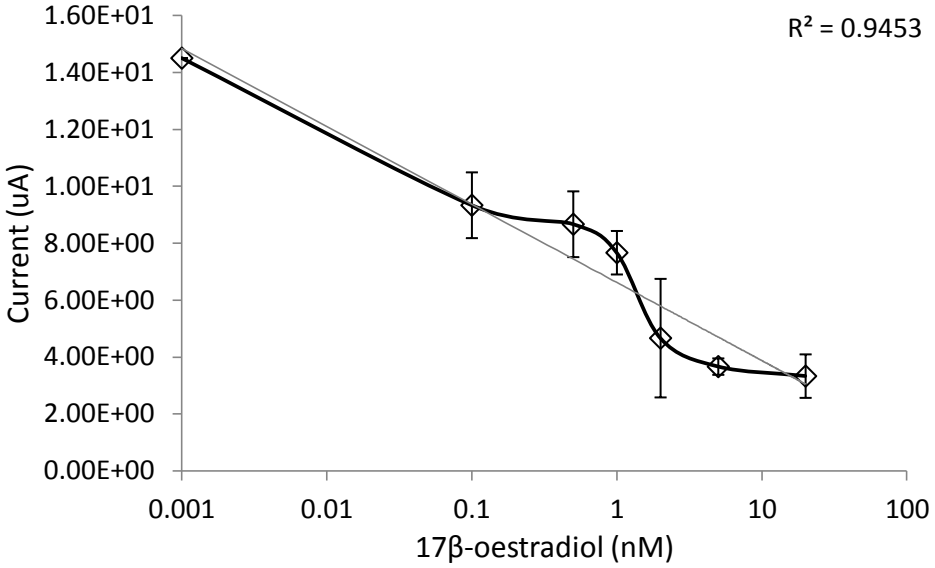


Figure 3-15 Scatter plot of DPV of recombinant EBP from *A. adeninivorans* G1212 strain responses to 17β oestradiol (0.1 nM, 0.5 nM, 1.0 nM, 2.0 nM, 10.0 nM and 20.0 nM) using single lipophilic mediator TMPD electrochemical system. Error bars represent ±1 SD.

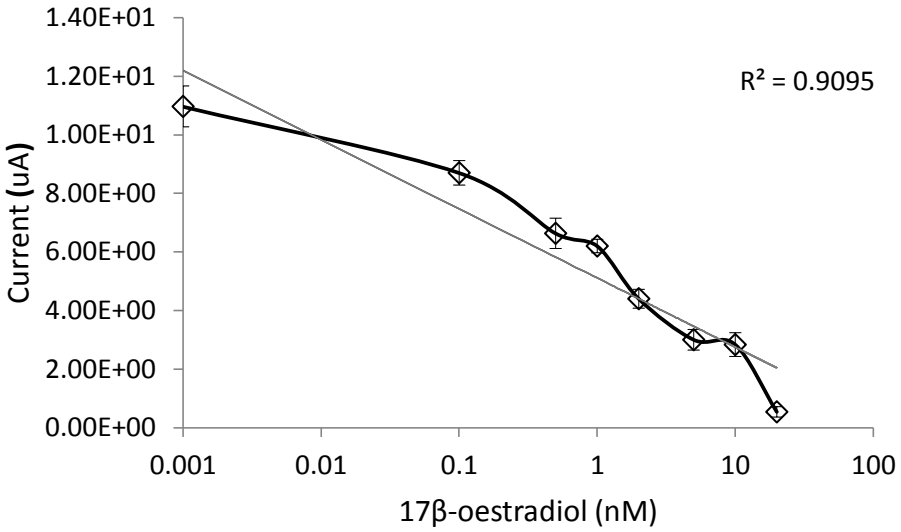


Figure 3-16 Scatter plot of DPV of EBP from A. adeninivorans G1212 strain responses to 17β oestradiol(0.1nM, 0.5nM, 1.0nM, 2.0nM, 5.0nM, 10.0nM and 20.0nM) using single lipophilic mediator TMPD electrochemical system. Error bars represent ±1 SD.

Figure 3-17 illustrates the results of experiments carried out with nickel free electrode, no specific deviation was found as compared to a nickel oxide base electrode which shows that if glutaraldehyde is used as a cross linker then there is no need to use nickel oxide based electrode.

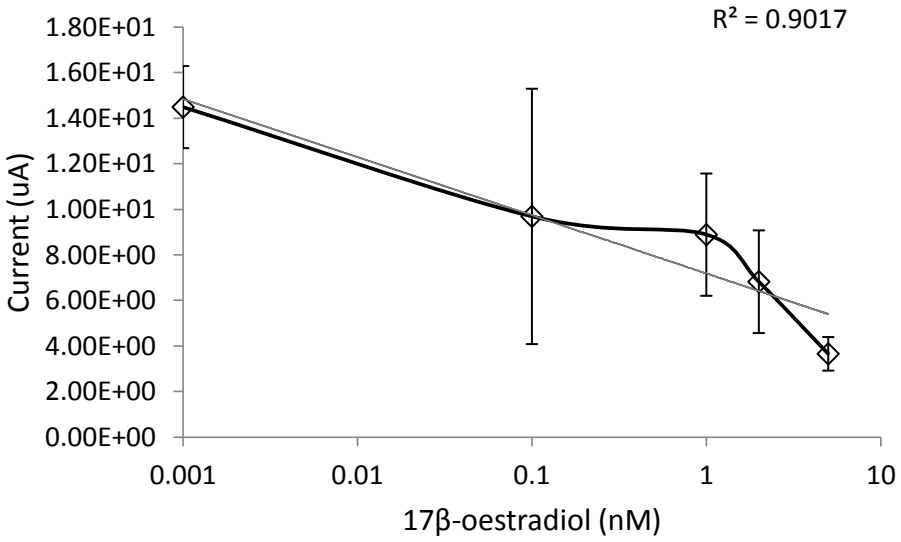


Figure 3-17 Scatter plot of DPV on nickel free electrode using different concentrations of oestrogens (control, 0.5 nM, 1.0 nM, 2.0 nM, 5.0 nM). Error bars represent ±1 SD.

3.10 Reliability, reusability and longevity of immobilized electrode

Immobilized freeze dried electrodes were tested for their reliability, reusability and longevity by various different experiments. Each month a new immobilized electrode along with electrodes from previous months were tested with 3 different concentrations. This experiment was carried out for duration of 155 days. Figure 3-18 to Figure 3-24 illustrate the DPV plots of various electrodes that were used during the course of experiments carried out for 155 days.

The reliability aspects of modified biosensors have been tested using by observing the spread in the redox potentials and currents. The biosensor output was always within the tolerable range. The difference in oxidation and reduction current for control sample (EBP, PBS and TMPD) was very close to 10 µA and difference in oxidation and reduction voltages was

approximately 0.1 volts (100 mV) in all the cases. The minor deviations observed are due to the fact that thickness of EBP on SPCEs was not very well controlled in all the immobilized electrodes. Likewise, difference in oxidation and reduction current for 0.1 nM oestrogen concentration was within 8-10 µA and difference in oxidation and reduction voltages was approximately 0.1 volts (100 mV) in all the cases. Similarly, difference in oxidation and reduction current for 5.0 nM oestrogen concentration was mostly within 5-6 µA and difference in oxidation and reduction voltages was approximately 0.1 volts (100 mV) in all the cases. As the concentration of oestrogen increases, it was observed that the difference in currents between oxidation and reduction peaks decreases.

These modified electrodes were evaluated for reusability in a semi-rigorous manner by following the same protocol every month. For each concentration of oestrogen including control sample, average of three runs of experiments were computed to plot the curve. Each electrode was run for several times and it was observed that the readings were within tolerable range. It can be concluded satisfactorily that these modified electrodes are reusable. Electrodes for first and second set of experiments were used for 45 times, electrode for third set of experiment was used for 36 times, electrode for fourth set of experiment was used for 27 times, electrode for fifth set of experiment was used for 18 times and electrode for sixth set of experiment was used for 9 times.

The longevity of the modified electrodes has been verified empirically over a period of 155 days and results were consistent.

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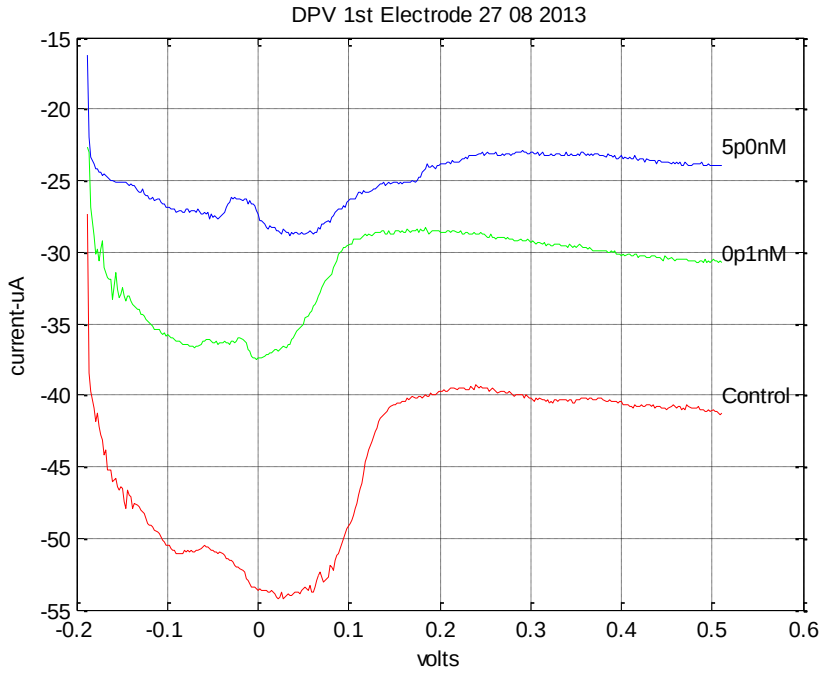
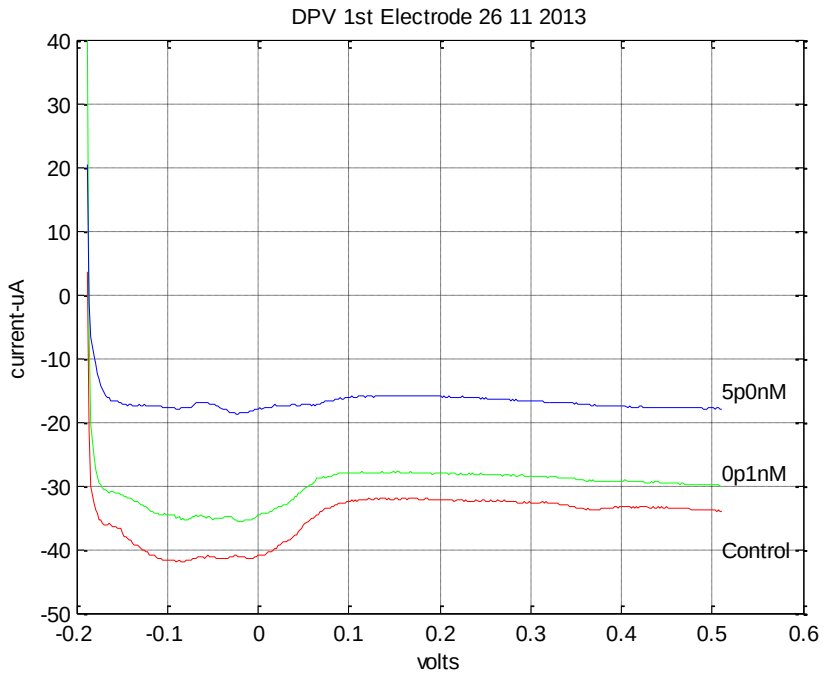

DPV 1st Electrode 27 08 2013
current-uA
volts
5p0nM
0p1nM
Control
-15
-20
-25
-30
-35
-40
-45
-50
-55
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6

(a)


DPV 1st Electrode 26 11 2013
current-uA
volts
5p0nM
0p1nM
Control
40
30
20
10
0
-10
-20
-30
-40
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-0.2
-0.1
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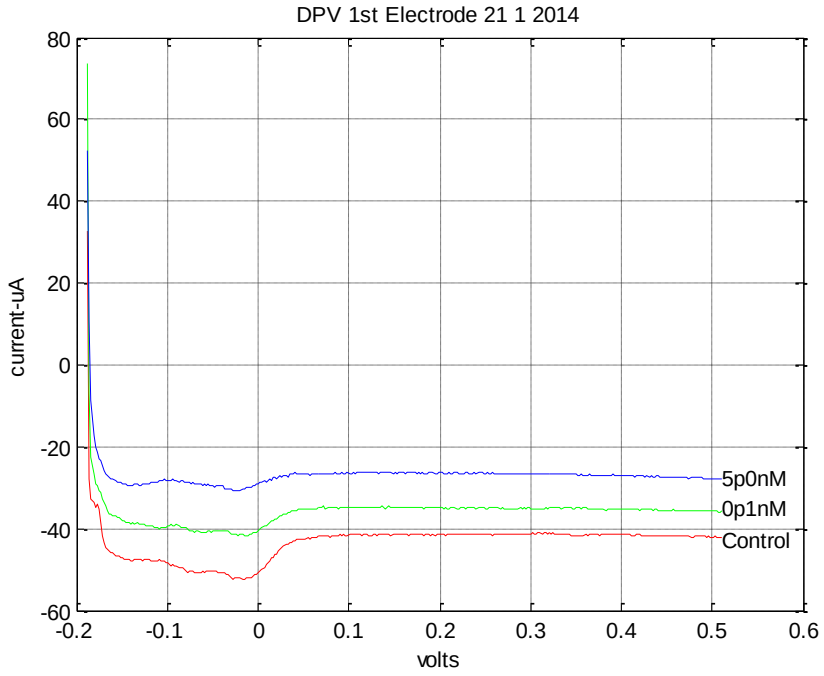
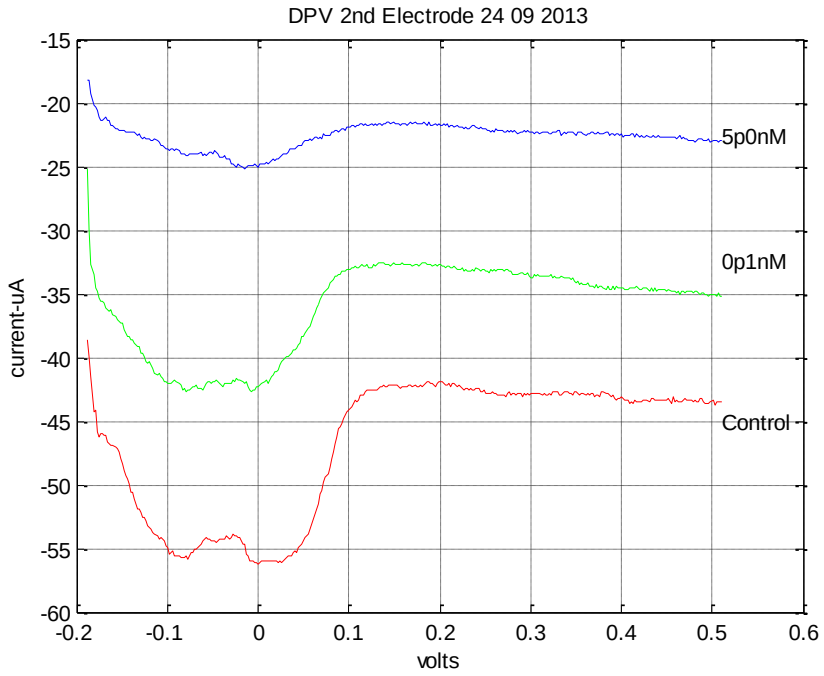
0.3
0.4
0.5
0.6

(b)

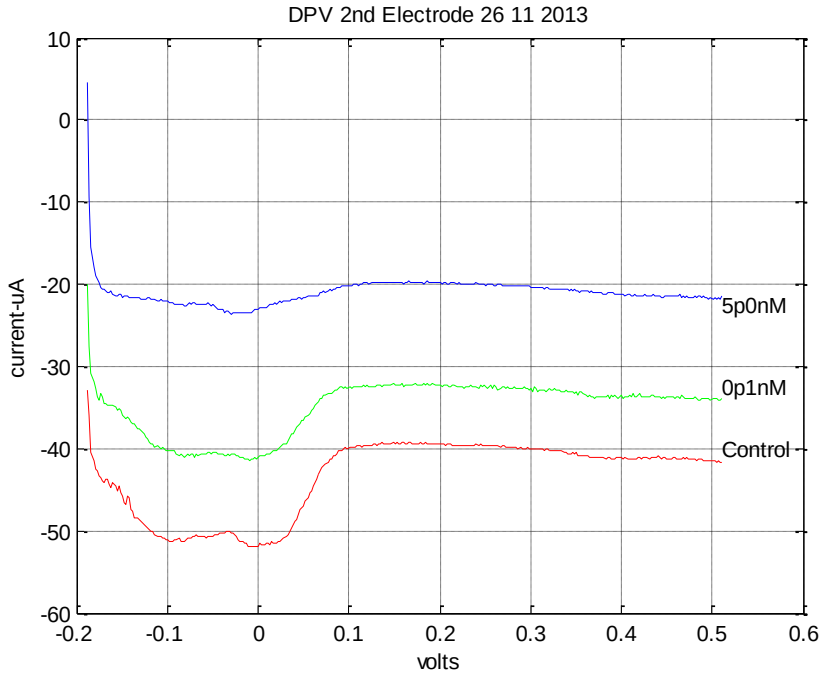


(c)

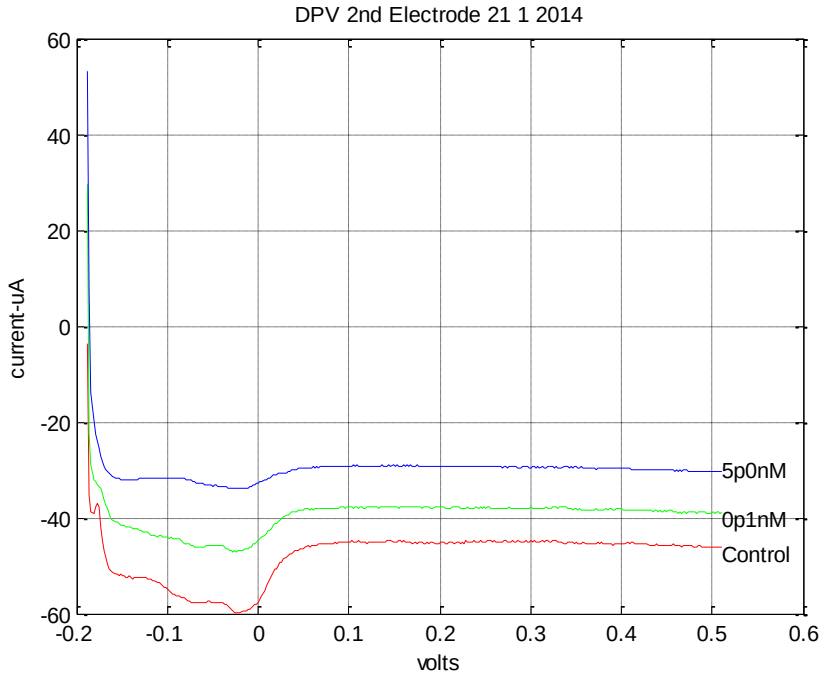
Figure 3-18 - DPV results of First electrode with 3 different concentrations in 1st (a), 4th (b), and 6th (c) month.



(a)

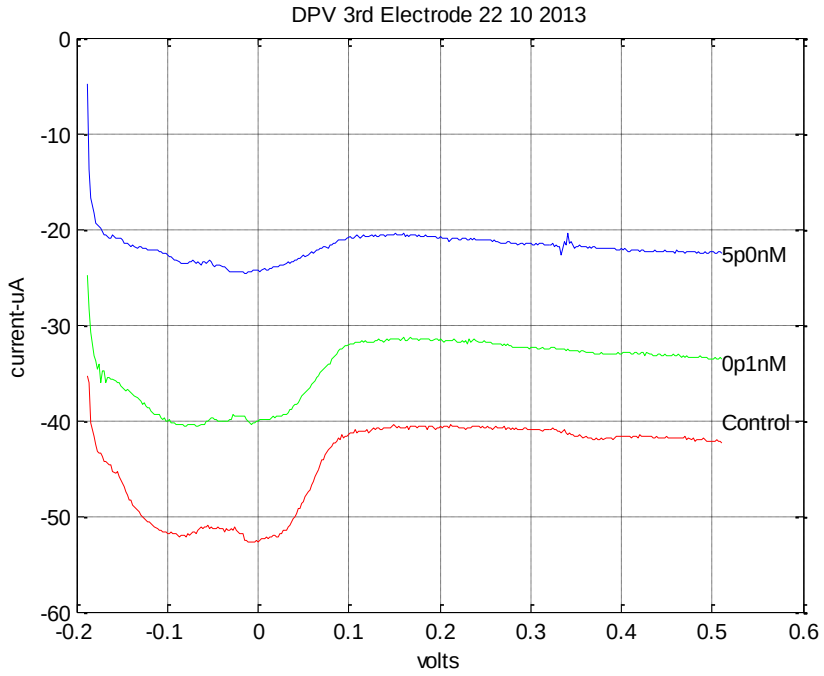


(b)

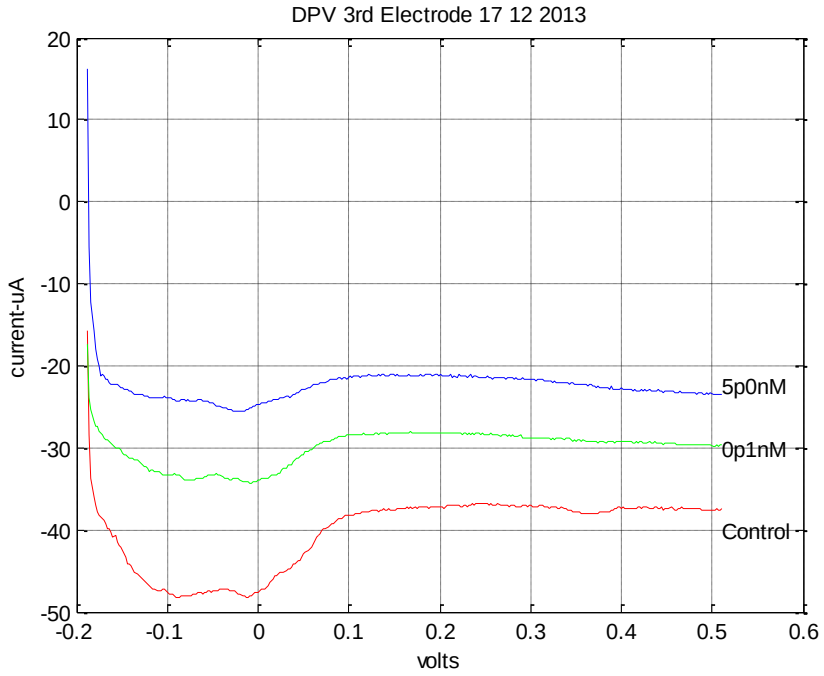


(c)

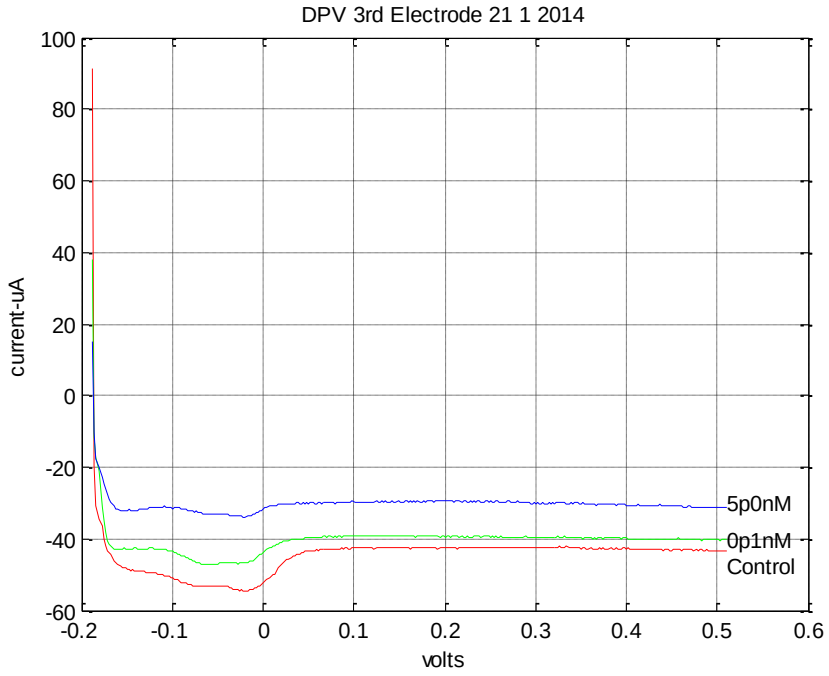
Figure 3-19 - DPV results of Second electrode with 3 different concentrations in 1st (a), 3rd (b) and 5th (c) month.


DPV 3rd Electrode 22 10 2013
current-uA
volts
5p0nM
0p1nM
Control
0
-10
-20
-30
-40
-50
-60
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6

(a)

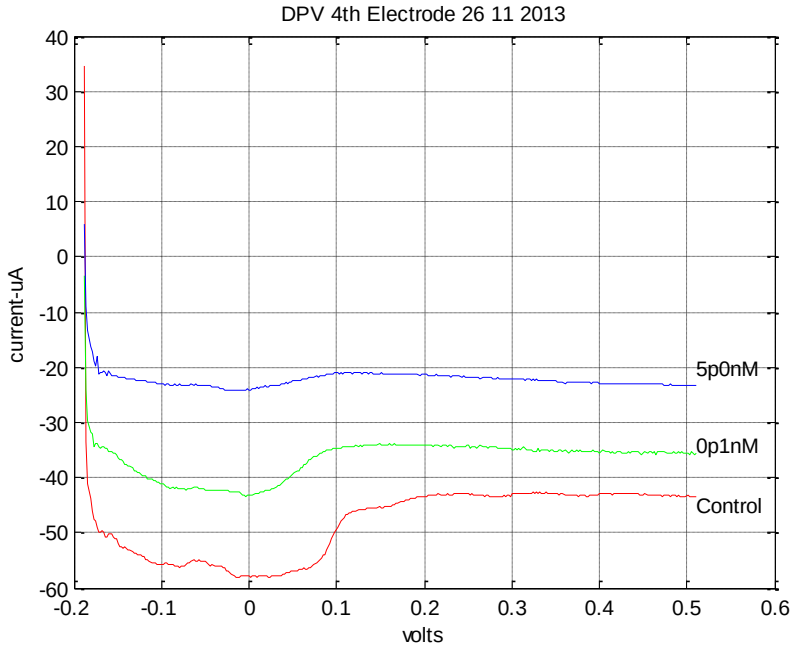

DPV 3rd Electrode 17 12 2013
current-uA
volts
5p0nM
0p1nM
Control
20
10
0
-10
-20
-30
-40
-50
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6

(b)

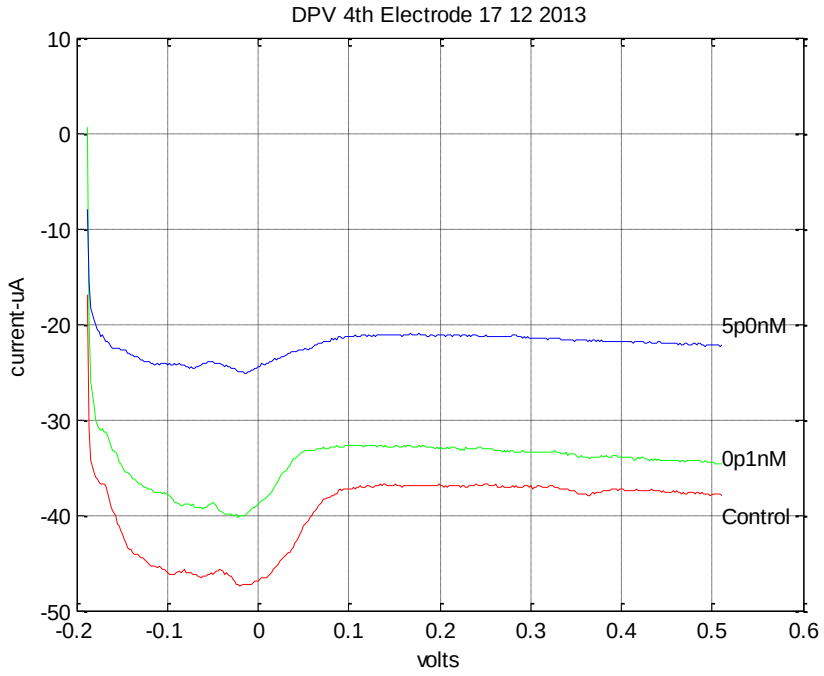


(c)

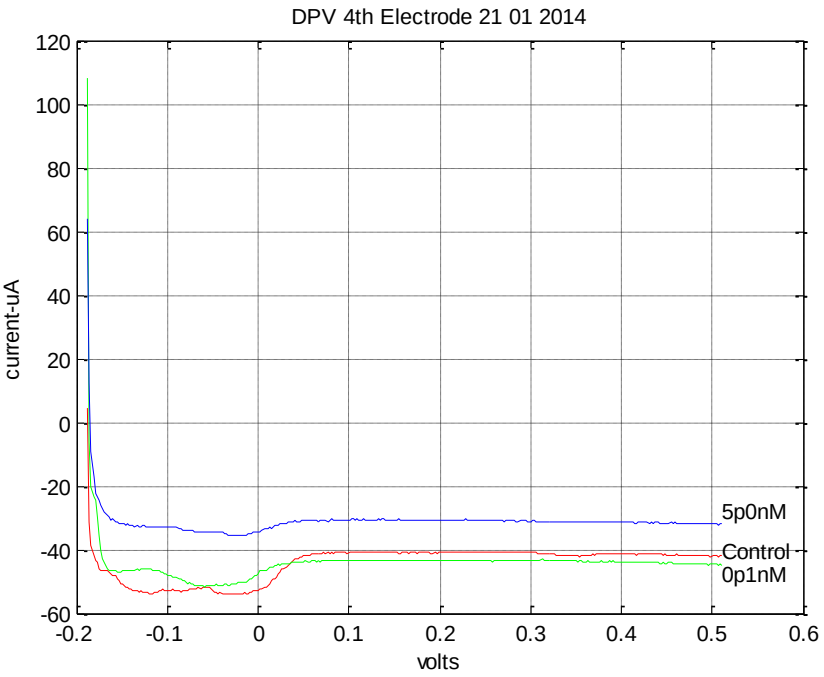
Figure 3-20 - DPV results of Third electrode with 3 different concentrations in 1st (a), 3rd (b), and 4th (c) month.



(a)

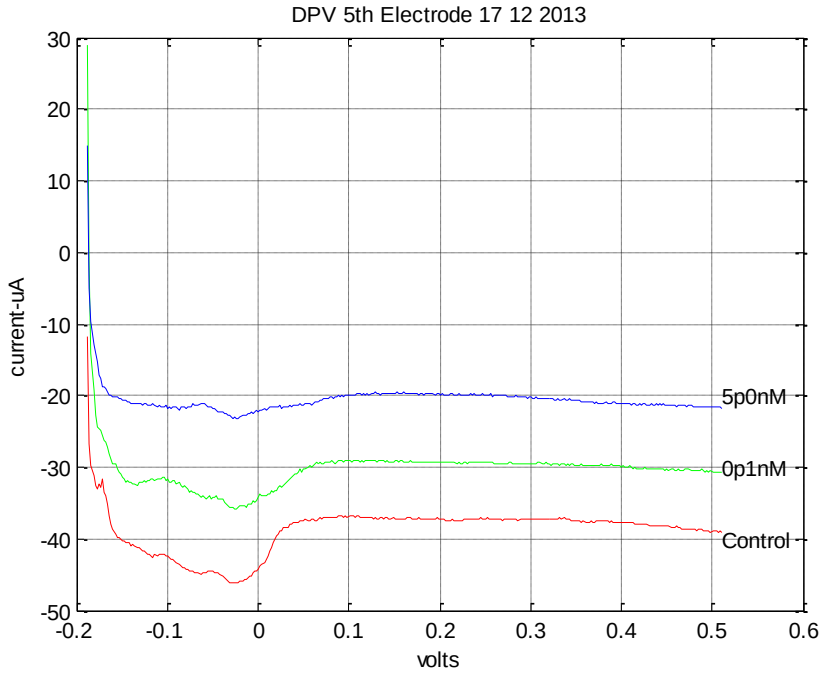


(b)

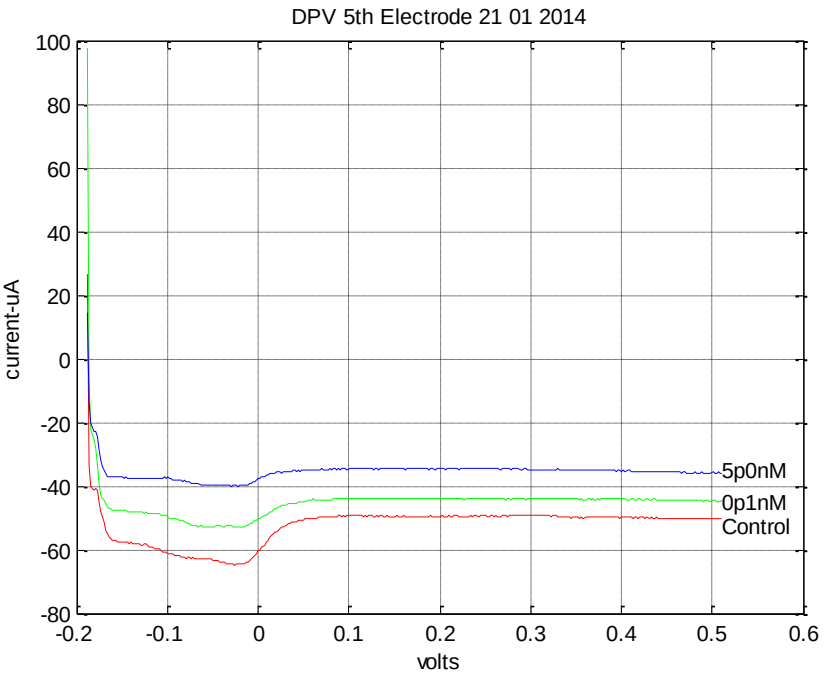


(c)

Figure 3-21 - DPV results of Fourth electrode with 3 different concentrations in 1st (a), 2nd (b), and 3rd (c) month.



(a)



(b)

Figure 3-22 - DPV results of Fifth electrode with 3 different concentrations in 1st (a) and 2nd (b) month.

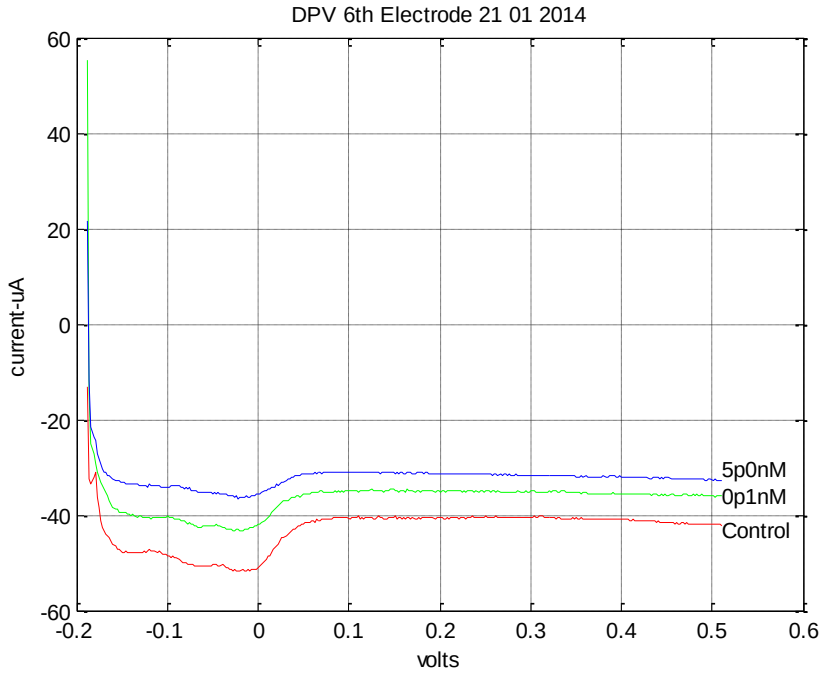
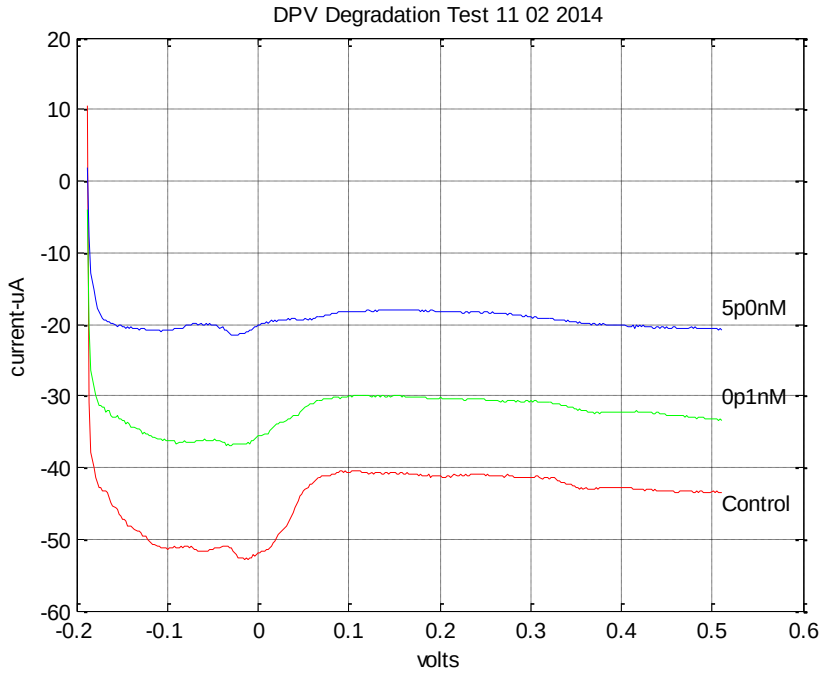
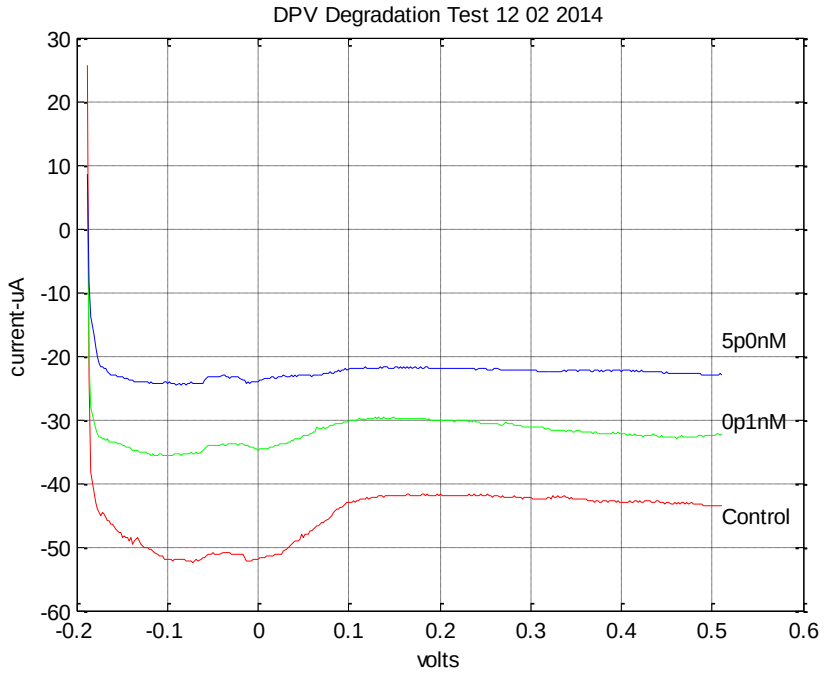


Figure 3-23 - DPV results of Sixth electrode with 3 different concentrations to check longevity of electrode.

Electrodes were also checked to see if there is any degradation in one day after running DPV.



(a)



(b)

Figure 3-24 - DPV results of an electrode with 3 different concentrations to check degradation in one day.

The average degradation of freeze-dried electrodes due to age in 155 days i.e. from 27.08.13 to 28.01.14 was calculated to be 23.33%.

Table 3-1 – Degradation of freeze-dried electrodes due to re-usability (in 155 days)

	Date	Control	0.1nM/L	5.0nM/L
Electrode 1	27.8.13-28.1.14	48%	28.34%	43.87%
Electrode 2	22.9.13-28.1.14	33.36%	16.14%	37.6%
Electrode 3	22.10.13-28.1.14	11.38%	28.14%	31.6%
Electrode 4	26.11.13-28.1.14	43.31%	22.51%	Unreliable
Electrode 5	21.12.14-28.1.14	18.3%	2.34%	26.6.%

The experimental results to determine the re-usability of electrodes are summarized in the Table 3-1 for various concentrations of oestrogen such as control (without oestrogen), 0.1nM and 5.0nM. It was noted that large degradation observed in the control sample for the 1st and 2nd electrodes correlated with the degradation observed for the same electrodes at 5.0nM concentration. The same is true for the 4th electrode; in fact, it was unreliable at 5.0 nM concentration. It was also observed that degradation was low for all electrodes at 0.1nM concentration.

3.11 Atomic Force Microscope (AFM)-

Atomic force microscopy was done to check the presence of EBP on SPEs. Figure 3-25 illustrates the scan size (of SPE) of 10.0 µm and scan rate of 0.2501 Hz. Figure 3-26 illustrates the scan size (of SPE) of 50.0 µm and scan rate of 0.2999 Hz.

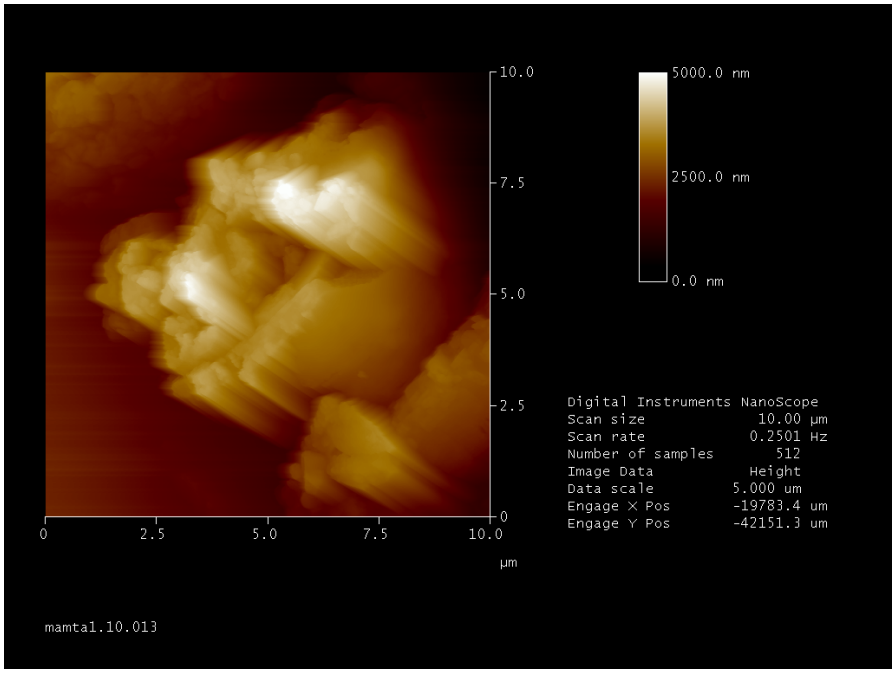


Figure 3-25 AFM picture of immobilised EBP on SPE surface.

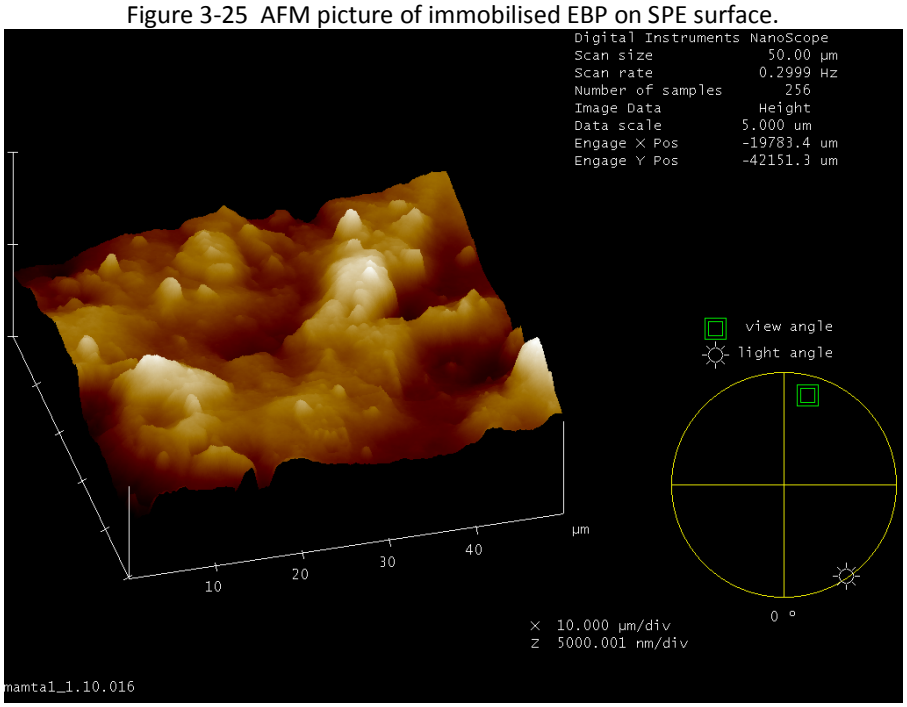


Figure 3-26 AFM picture of immobilised EBP on SPE surface.

3.12 Dektec Profilometer-

Dektec profilomenter is used to measure step heights or trench depths on a surface. Dektec profilomenter was also used in this experiment to confirm that EBP was immobilised on the SPE surface. A blank and an immobilised electrode were compared to confirm the immobilization of EBP on SPE surface. Figure 3-27 and Figure 3-28 illustrate Dektec profilometer pictures of immobilised and blank electrode. The measured height of the EBP on immobilised SPE surface is about 55.751 µm (i.e. 557510 Å).

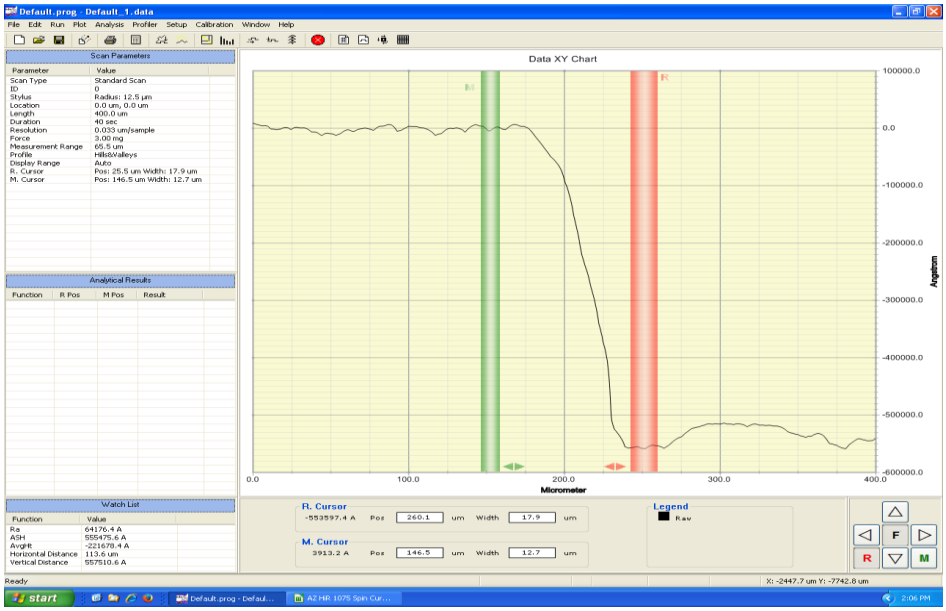


Figure 3-27 EBP immobilised on SPE surface

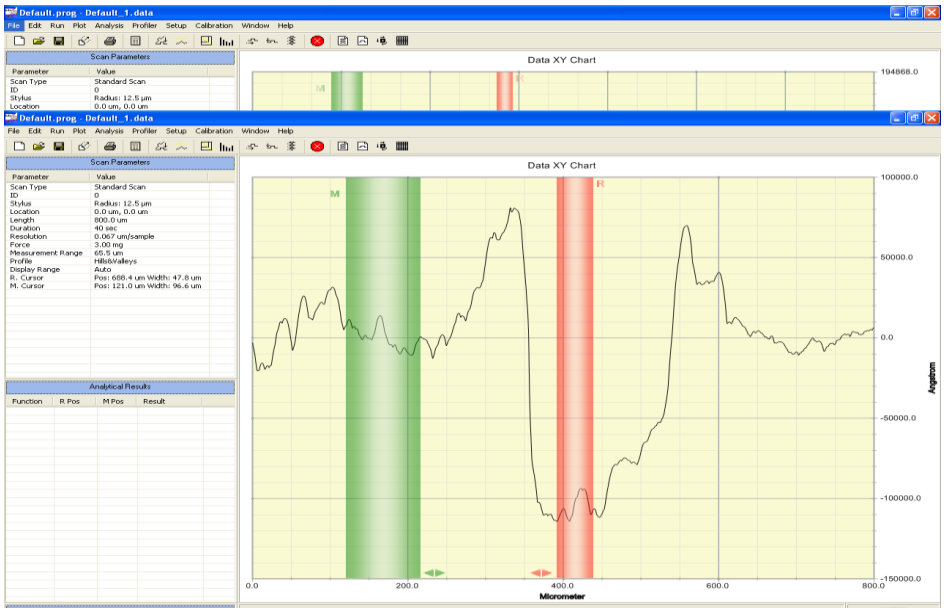


Figure 3-28 Picture of a scratched blank SPE.

3.13 Summary

Basics of electrochemistry have been reviewed in this chapter and two different types of voltammetry techniques described. Histidine tagged EBP was purified using HisTrap column. Impurities from protein were removed by using PD-10 Desalting Columns. In order to confirm that protein was purified, it was run through NuPAGE Tris-Acetate Gel and subsequently 46 kDa molecular weight proteins were determined. The Protein concentration was determined by using Thermo Scientific Pierce^{TM} BCA Protein Assay kit. Purified EBP was immobilized using glutaraldehyde as a cross linker on an electrode surface and used as a bio-recognition element for the detection of oestrogen. LSV technique was utilised for EBP in solution while DPV was used with immobilized EBP on SPEs for the determination of oestrogen. The aspects related to the reliability, reusability and longevity of the modified sensor electrodes were investigated in some detail. AFM and Dektec profilometer were also used to confirm the immobilisation of EBP on SPE surface.

Chapter 4 Experiments with different types of mediators

Different types of mediators were tried to investigate the possibility of using them in the immobilized form on the modified electrode. In a previous chapter, the desirable characteristics of mediators have already been outlined.

The various mediators used were gallocyanine, menadione, Dichlorophenolindophenol (DCPIP), Methyl red (MR), Benzoquinone, Potassium hexacyano ferrate (III), N, N, N', N'-TMPD, 2,3,5,6,-Tetramethyl-p-phenylenediamine. All were subjected to DPV on the modified immobilized SPEs. The results of the experiments are shown from Figure 4-1 to Figure 4-8. As can be observed from the figures the mediator TMPD has worked well while others have not given satisfactory result. Figure 4-8 illustrates the desired result obtained with TMPD at different oestrogen concentrations where current measured is inversely proportional to oestrogen concentration. The curve follows a linear profile with the highest current measured at control sample and lowest current at highest oestrogen concentration. TMPD works as a two electron exchange mediator and has a reversible redox reaction capability. The response of the other mediators is not so well defined in terms of current vs. oestrogen concentration which does not follow a linear profile. The current measured from N,N,N',N'-TMPD is half that from TMPD which is due to the fact that the former is one electron transfer mediator. Similarly the signal current obtained from $[Fe(CN)]^{3-}$ is one fifth of that of TMPD along with it being one electron transfer mediator. However the sizes of the current obtained from MD and TMPD are reasonably close yet the MD did not performed satisfactorily as a mediator due to low redox potential (0.03) compared to redox potential of TMPD (0.257). The currents obtained from other mediators: Gallocyanine, DCPIP, Benzoquinone, and Methyl red are small compared to that from TMPD (**Rawson et al., 2014)**. As enunciated by **Keisuke et al.,** an important consideration in a mediator's reaction kinetics with an enzyme is its chemical structure (**Keisuke et al., 2001).**

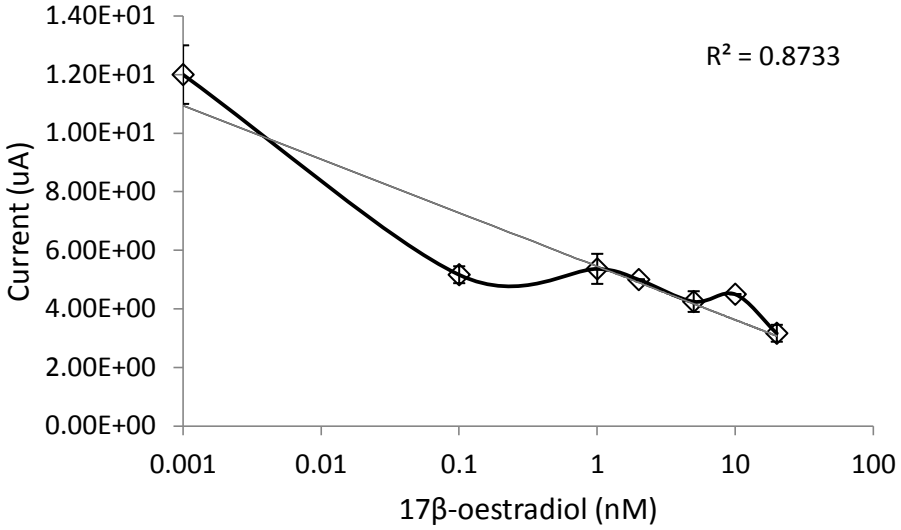


Figure 4-1 Scatter plot of current vs. oestrogen at different concentrations for DPV using Gallocyanine . Error bars represent ±1 SD.

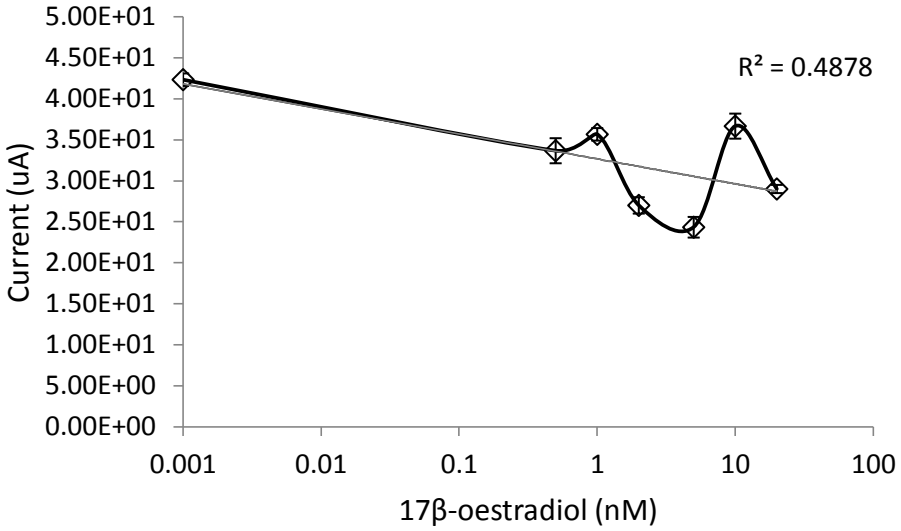


Figure 4-2 Scatter plot of current vs. oestrogen at different concentrations for DPV using Menadione. Error bars represent ±1 SD.

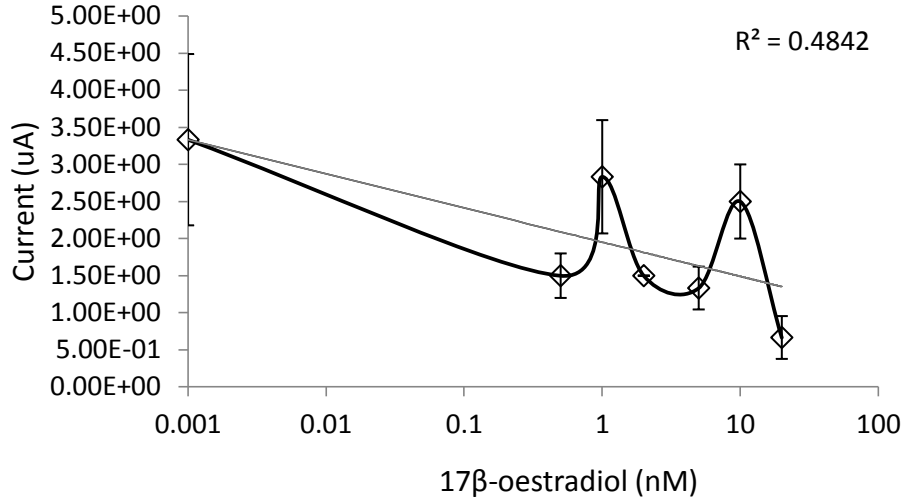


Figure 4-3 Scatter plot of current vs. oestrogen at different concentrations for DPV using DCPIP. Error bars represent ±1 SD.

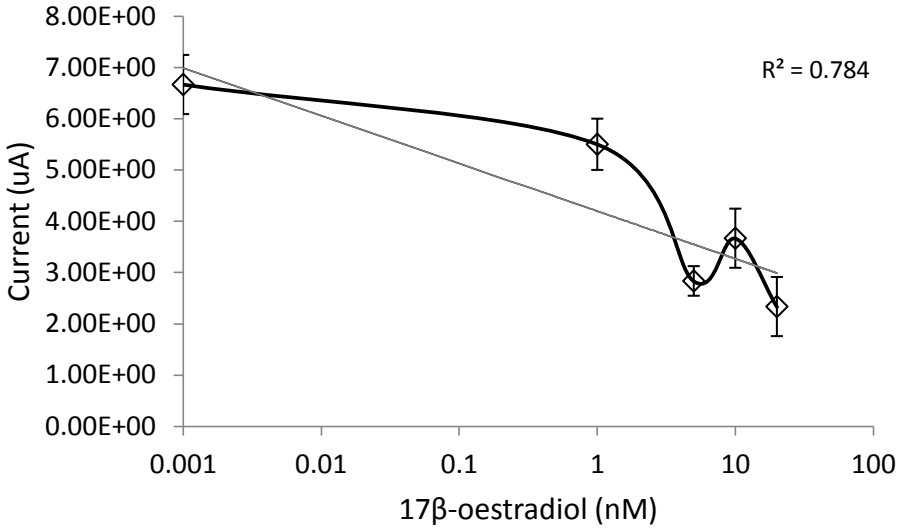


Figure 4-4 Scatter plot of current vs. oestrogen at different concentrations for DPV using Methyl red. Error bars represent ±1 SD.

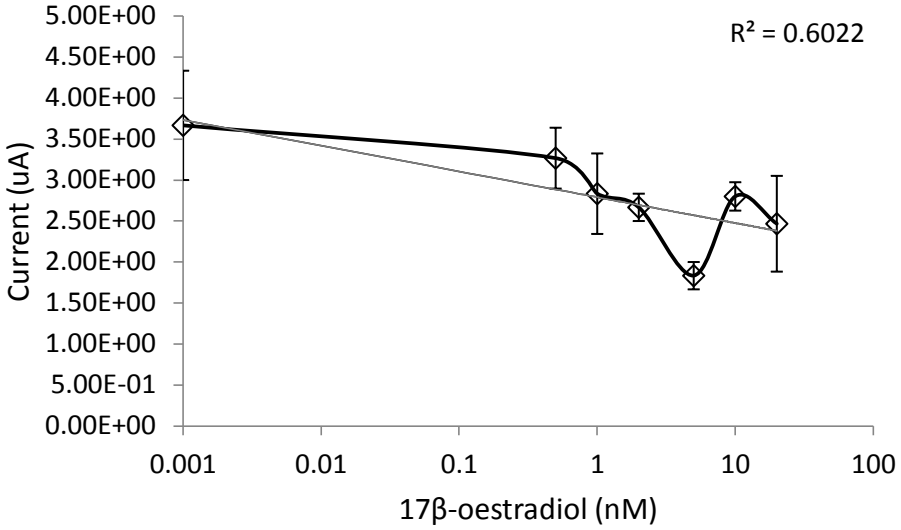


Figure 4-5 Scatter plot of current vs. oestrogen at different concentrations for DPV using Benzoquinone. Error bars represent ±1 SD.

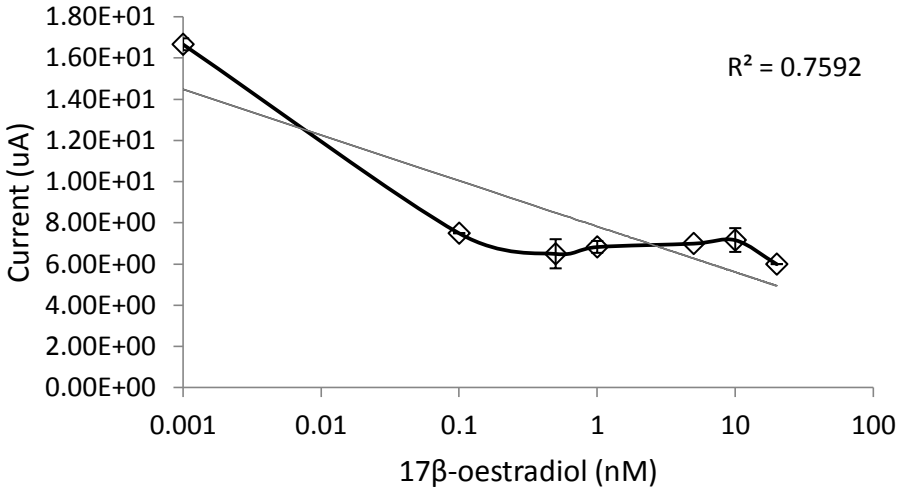


Figure 4-6 Scatter plot of current vs. oestrogen at different concentrations for DPV using Potassium hexacyanoferrate (III). Error bars represent ±1 SD.

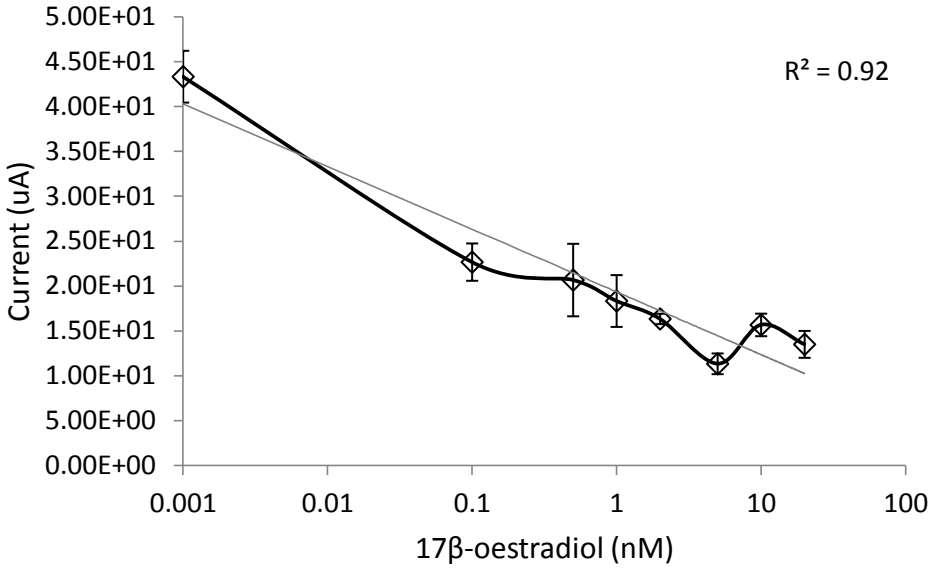


Figure 4-7 Scatter plot of current vs. oestrogen concentrations for DPV using N,N,N'N' TMPD. Error bars represent ±1 SD.

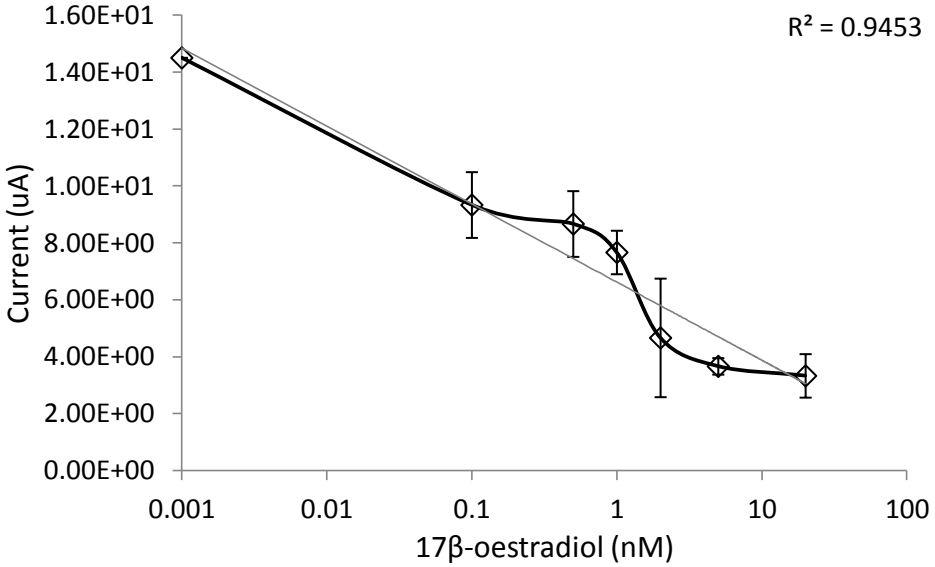


Figure 4-8 Scatter graph of current vs. oestrogen concentrations for DPV using TMPD. Error bars represent ±1 SD.

4.1 Mediator impregnated membrane

As discussed in a previous chapter one of the aims of this work was to develop a specially modified sensor electrode that enables direct electron transfer using mediator. Based on this a

new technique was tried out for the detection of oestrogen (17 β oestradiol) using 2,3,5,6 – Tetramethyl-p-phenylenediamine impregnated on Immobilon-N Transfer Membrane.

To detect if membrane is electroactive, an experiment was done using blank electrode with blank membrane and PBS. Figure 4-9 illustrates the result and shows that there is no electroactive species present on the membrane.

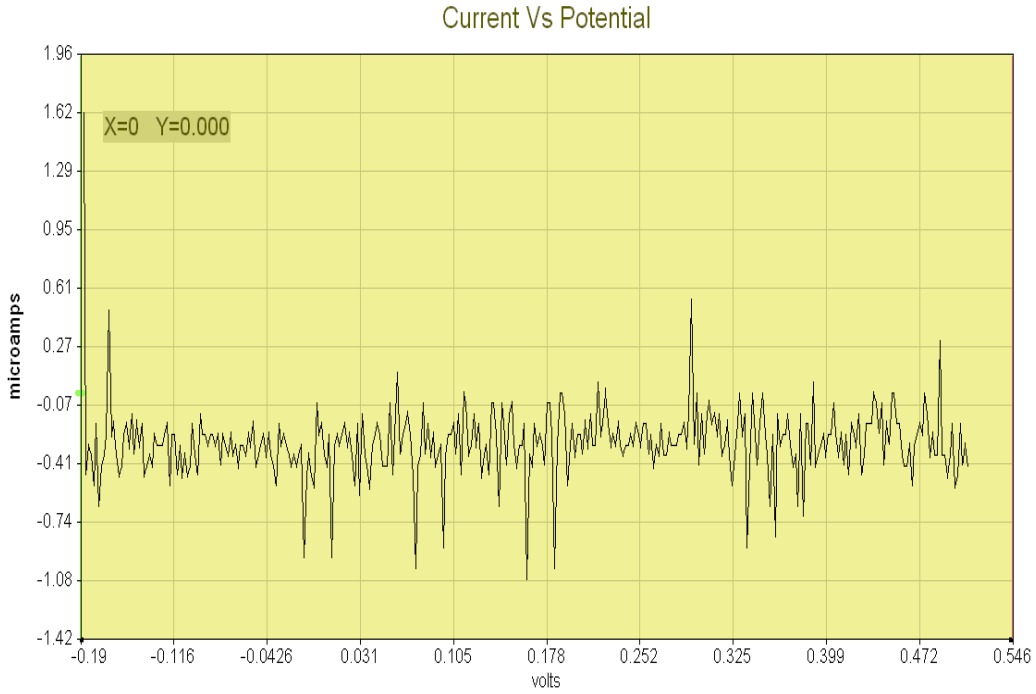


Figure 4-9 DPV using Blank electrode, blank membrane and PBS

4.1.1 Membrane Impregnation Technique-

The membrane was cut into multiple round discs, each of 6.0 mm diameter. These discs were impregnated with 10 µL of 20 mM TMPD solution and kept for 5 minutes at a temperature of 56^0C in an incubator for drying. The dried discs were then used in the experiment instead of the TMPD mediator solution.

4.1.2 Experiments with impregnated membrane

For control sample (without oestrogen), 2 µL of ethanol was dispensed on working electrode area of SPCEs and the membrane was placed on ethanol followed by 6 µL of PBS being dispensed on top of the membrane. Electrolysis was performed using DPV with an initial

potential of -0.2V, Sweep-Potential-1 of -0.2V and Sweep-Potential-2 of +0.5V. The scan time of the experiment was 50 seconds with a scan rate of 0.020 seconds, the scan increment of 0.002V and step time of 0.100 seconds. The pulse height was 0.025V with a pulse width of 0.050 seconds and the current range was 1mA/V.

In order to develop a method to detect oestrogen, the ethanol used in the previous step was replaced by samples that contained precisely known amounts of oestrogen. Many different concentrations of oestrogen samples were tried out while electrolysis was carried out using above mentioned parameters of DPV.

The various concentrations of TMPD solution such as 250 mM, 100 mM, 75 mM, 50 mM, 40 mM and 20 mM were tried for solubility in ethanol. However, at room temperature the solubility of TMPD in ethanol was observed only at 20mM and 40mM concentrations. At higher than 40mM concentrations TMPD was not soluble and so not suitable for electrolysis.

The following experimental results are presented using the mediator TMPD impregnated in the membrane. Figure 4-10 and Figure 4-11 illustrate the scatter plot of TMPD mediator at 20 mM and 40 mM concentration respectively by membrane technique.

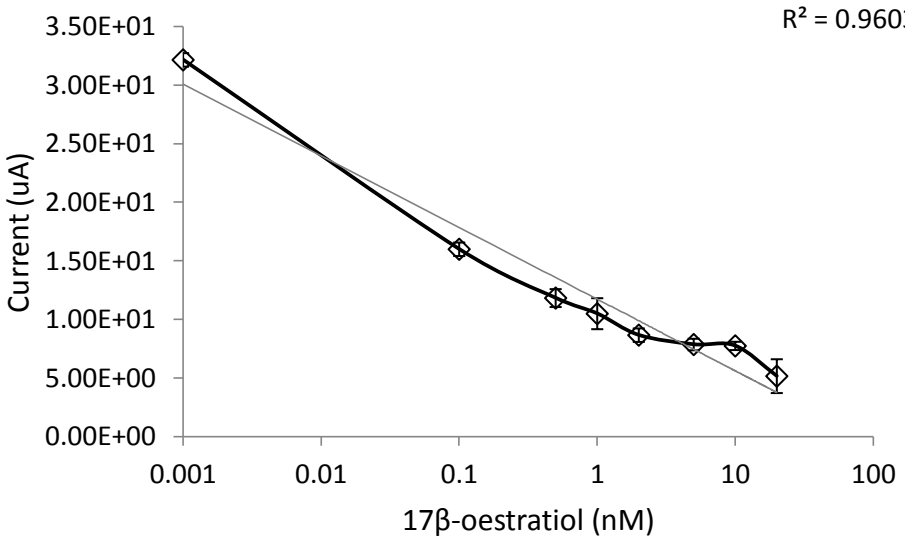


Figure 4-10 Scatter plot of current vs. oestrogen concentrations for DPV using 20mM TMPD by membrane technique. Error bars represent ±1 SD.

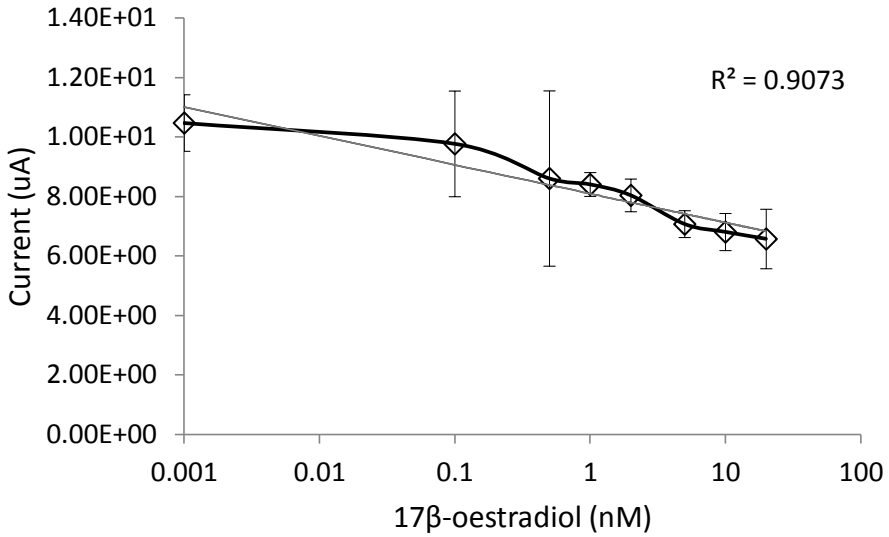


Figure 4-11 Scatter plot of current vs. oestrogen concentrations for DPV using 40mM TMPD by membrane technique. Error bars represent ±1 SD.

4.2 Summary-

Eight different types of mediators were tried out for facilitating the redox kinetics of oestrogen detection using EBP immobilised modified sensor electrodes. Only TMPD was found to work satisfactorily giving linear response. Some reasons for other mediator's non-functionality have been discussed. A new TMPD impregnated membrane technique was introduced and worked well at lower concentrations.

Chapter 5 Conclusion and Further Work

Bacterial cells are the most common material used in whole cell biosensors even though there are limitations in their usage. The use of yeast species is preferred over bacterial cells due to advantages like higher pH/temperature tolerance and better osmolarity/ionic strength tolerance and can be grown in a wide range of substrates. Being eukaryotes yeasts can provide more relevant information about other eukaryotes compared to bacterial cells **(Walmsley & Keenan 2000).** In this research work yeasts have been used as bioassays.

It has been demonstrated earlier in our laboratory that *S.cerevisiae* exhibits a dose dependent response to oestrogen **(Baronian & Gurazada 2007)**. The detection of oestrogen with *S. cerevisiae* whole cells was also demonstrated using a double mediator system composed of a hydrophilic mediator (ferricyanide) and a lipophilic mediator (TMPD) **(Chelikani 2011).**

In this research a single lipophilic mediator TMPD has been used with immobilised recombinant EBP on modified electrode surface to detect oestrogen. The rate of production of reduced mediator is measured as current by oxidation at electrode.

This research has demonstrated that recombinant oestrogen binding proteins can be used to quantify oestrogenic molecules. The aim of this research work was to develop a sensitive and cost effective portable biosensor for the detection of oestrogen. This research was completed in three different steps-

1. Purification of Oestrogen Binding Protein from *A.adeninivorans* strain G1212.
2. Use of Screen printed electrodes (SPEs) for the detection of Oestrogen with the yeast based assay. In this research for the purpose of comparison 2 different Voltammetry methods such as Linear sweep voltammetry and Differential pulse voltammetry have been used to measure the current produced during redox reactions.
3. To investigate direct electron transfer, a novel technique has been used by impregnating Immobilion N transfer membrane with TMPD (lipophilic mediator) solution. The purpose of using membrane is to show that TMPD can be immobilized on SPEs along with EBP for the detection of oestrogen to further simplify the assay.

Different experiments were performed on immobilized freeze dried electrodes to test their reliability, reusability and longevity. After the duration of 155 days it was observed that

electrodes were still reusable and reliable. AFM and Dektec profilometer were used to confirm the immobilisation of EBP on SPE surface.

The reusability aspect of modified electrodes was verified semi-rigorously by following the same protocol every month. Electrodes for first and second set of experiments were used 45 times, electrode for the third set of experiment was used for 36 times, electrode for the fourth set of experiment was used for 27 times, electrode for fifth set of experiment was used for 18 times and electrode for sixth set of experiment was used for 9 times. The longevity of the modified electrodes was verified over a period of 155 days and results were consistent.

Different types of mediators were tried to investigate the possibility of using them in the immobilized form on the modified electrode. Desired result was obtained only with TMPD at different oestrogen concentrations where current measured is inversely proportional to oestrogen concentration. The curve follows a linear profile with the highest current measured at control sample and lowest current at highest oestrogen concentration. Other mediators did not provide satisfactory result due to the fact that N, N, N', N'- TMPD, potassium hexacyano ferrate (III), work as one electron exchange mediator and currents obtained from other mediators: gallocyanine, DCPIP, benzoquinone, and methyl red are small compared to TMPD. The sizes of the current obtained from MD and TMPD are reasonably close yet the MD did not performed satisfactorily as a mediator due to low redox potential (0.03) compared to redox potential of TMPD (0.257).

A membrane technique was used by impregnating Immobilion N transfer membrane with TMPD solution. The purpose of using membrane is to simulate that the immobilization of TMPD is feasible on SPE along with EBP for the detection of oestrogen. TMPD impregnated membrane technique was introduced and worked well at lower concentrations.

Further work in this field of research may address possibilities of immobilizing TMPD on the electrode surface to improve utility direct electron transfer or use of third generation biosensors which do not require mediator and transfer of electrons takes place directly from analyte to electrodes (Wang 2008).

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