Use of *Candida albicans* and its oestrogen binding protein as a bio-recognition element for detection of oestrogen

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Compared with bacteria, yeast have rarely been studied for use as a biocomponent for biosensors. Yeast are easy to culture and are eukaryotes, which means their biochemistry in many respects is similar to that of higher organisms. *C. albicans* and some other yeast species are known to have an oestrogen binding protein (EBP), which oxidises NAD(P)H to $NAD(P)^+$ Oestrogen, when present, binds to the NAD(P)H oxidation site which leads to an accumulation of NAD(P)H (Madani, et al. 1994). Previous research has shown that oestrogen can be quantified using *S. cerevisiae* whole cells as the detection element and measuring NAD(P)H with a double mediator electrochemical system.

This thesis employs the mediated electrochemical systems to investigate the influence of growth phase on EBP production in *C. albicans* and the response of cells of different ages to different concentrations of oestrogen. A cell not known to possess EBP (*Arxula adeninivorans*) was also investigated for its response to 17β-oestradiol. As expected, *A. adeninivorans* did not show a detectable response to 17β-oestradiol but surprisingly, its catabolism was inhibited.

By using *C. albicans* cell lysate in the oestrogen detecting assay, utility was systematically increased and the complexity of the whole cell assay was decreased. In this assay, only a

hydrophilic mediator was used, removing the need for a lipophilic mediator. The assay was used successfully in a complex medium, the upper detection limit was raised to 100 nM of 17β-oestradiol, and the assay period was reduced to 20 min. The electrodes were modified to directly detect NAD(P)H in cell lysate at a lower potential to avoid interference by oxidants such as ascorbic acid.

Furthermore, EBP was purified using 17β-oestradiol affinity chromatography, and the protein was used with NADH in an oestrogen bioassay. In this assay NADH was electrochemically detected directly and could differentiate 'with' and 'without oestrogen' samples. This research also showed that a mediator can interact directly with EBP, i.e. without the use of NADH and further, direct electron transfer from EBP to both glassy carbon and pyrolyzed photoresist film (PPF) electrodes were demonstrated (i.e. without the use of a mediator). This research has further simplified the assay and will facilitate the development of rapid oestrogen detecting protein biosensor.

Keywords: Yeast Oestrogen Binding Protein, EBP production with cell growth phase, oestrogen inhibition of catabolism, oestrogen assay, NADH detection, electrochemical detection, mediated electrochemical detection, voltammetry, mediated electron transfer, direct electron transfer.

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Chapter 1
Introduction

1.1. Oestrogens

Oestrogens are female sex hormones. Both natural and synthetic oestrogens are discharged in inactive conjugated form but are de-conjugated in the environment and regain their oestrogenic activity (Pettersson, et al. 2006). In recent years, there has been increased interest in chemical compounds that can alter the endocrine system. These chemicals have the potential to disrupt normal reproduction, and cause other adverse effects including breast and testicular cancer. Oestrogen molecules are characterised by their ability to bind and activate the oestrogen receptor, a transcriptional factor belonging to steroid receptor family. Some compounds can disrupt endocrine system without directly interacting with the receptor (Hilscherova, et al. 2000). These molecules have an effect at very low concentrations and therefore very sensitive assays are required for detection of physiological concentrations (Gurazada 2008).

1.2. Oestrogen bioassays

Currently, there are a wide variety of *in vitro* and *in vivo* bioassays to determine the oestrogenic activity in environmental samples and other compounds. *In vitro* assays like E-Screen (Soto, et al. 1995), YES assay (Routledge and Sumpter, 1996) are gaining popularity since i*n vivo* assays are expensive and time consuming. Most of the assays currently available are based on mammalian cell lines. These can be more sensitive than yeast based assays (Legler, et al. 2002) and are considered more relevant for human health but the responsiveness of bioassays vary between the different types of mammalian cell lines as the cellular environment and receptor expression alter. This complicates the interpretation of results of bioassays using mammalian cell lines, reducing their predictive value (Gutendorf and Westendorf 2001; Zacharewski 1997).

Yeast oestrogen assays can be very useful compared to assays using mammalian cell lines in the first stage of screening as these are inexpensive and avoids the need for costly cell culture. Another major advantage is that yeast cells are more resistant to environmental contaminants than mammalian cells, which is advantageous when screening complex samples. The assay developed in this study is based on wild type *C. albicans* and its oestrogen binding protein (EBP) for detecting oestrogen.

1.3. Aims and objectives

There is an immediate need for more sensitive, faster and less expensive detection methods to identify and quantify a wide range of important organic molecules. The aim of this research was to develop a bioassay using EBP from *C. albicans* to detect oestrogens.

Previous research on *C. albicans* and its EBP has concentrated mostly on its pathogenesis and the disease Candidiasis. The EBP of *C. albicans* interacts with oestrogen to transform the non-pathogenic yeast to mycelial growth which is believed to be associated with the organism's virulence. This research will demonstrate for the first time that the responses of *C. albicans* and its EBP can be used to quantify oestrogen molecules in complex matrices.

- **Investigate yeast responses to oestrogen by electrochemical detection.**

The first objective was to establish *C. albicans* and its EBP as a bio-recognition element to detect oestrogen with optimal growth period for maximal EBP production. Also to explore a non-pathogenic yeast which is not known to have EBP like *Arxula adeninivorans* for responses to oestrogen by electrochemical detection (Chapter 3).

- **Electrochemical detection of oestrogen binding protein interaction with oestrogen in *C. albicans* cell lysate.**

The objective of this investigation was to simplify the use of mediators in the detection of oestrogen, increase the dynamic range of the assay, and to reduce the duration of the assay (Chapter 4).

- **Investigate electron transfer from oestrogen binding protein by electrochemical detection.**

The objective of this investigation was to purify EBP from *C. albicans* and use this protein as a bio-recognition element for detecting oestrogen to further simplify the assay. Explore EBP for any electron transfer (Chapter 5).

1.4. Hypothesis.

- ***C. albicans* and its EBP can be used to detect oestrogen.**

Based on preliminary results from Baronian et al. (2007), it is hypothesized that *C. albicans* and its EBP can be used in oestrogen detecting assay whereas yeast cells that do not posses an EBP cannot be used to detect oestrogen.

- **Oestrogen assay based on *C. albicans* can be improved.**

By lysing the *C. albicans* cells to remove transport constraints, it is hypothesized that oestrogen can be detected with a single hydrophilic mediator thus avoiding the need for a lipophilic mediator, and also the incubation period can be reduced.

- **EBP can be purified from *C. albicans* and used for detecting oestrogen**

Based on other studies (Madani, et al. 1994; Skowronski and Feldman 1989), it is hypothesized that EBP can be purified from *C. albicans* and be used to detect oestrogen. Based on studies on other flavoenzymes (Hasebe, et al. 1995), it is hypothesized that electron transfer from EBP can be detected.

1.5. Thesis format

The chapters in this thesis are organized as stand-alone scientific papers. This has led to some overlap in material and methods section (particularly between chapters 3, 4, and 5). Some material has been intentionally left out of the general introduction and literature review to

avoid repetition in the introductions to data chapters. The specific discussions in each data chapter (chapters 3 to 5) contain most of the discussion material, while a more concise general conclusion at the end is aimed to raise synergies between the different chapters and to show the coherence of different chapters to the overall purpose of the research. That too has been intentionally kept short to avoid repetition.

Chapter 2
Literature Review

2.1 Biosensors

A biosensor is an analytical device which converts a biological response into a measurable signal. In these devices, a biological component such as an enzyme or protein, antibody, nucleic acid or microorganism (whole cell) interacts with a target analyte, which is then detected by the electronic component (transducer) and transmitted into a measurable signal. The most popular biosensors are based on electrochemical detection.

2.2 Yeast as a sensing element in biosensors

Bacterial cells are the most commonly used detector in whole cell biosensors. There are some disadvantages using bacterial cells. For example, they are relatively fragile in the assay environment and the limited pH, osmotic and temperature tolerance of individual bacterial species mean that the operating assay parameters are limited. In addition, the bacterial cellular responses to some chemical molecules are different from eukaryotes. All these effects can lead to a limitation of their use in toxicity evaluation and drug screening for higher organisms.

Yeast have three major advantages over bacteria when used as biosensors. They are:

1) Rapid growth of yeast and a wider pH, temperature and ionic tolerance (Buckman and Miller, 1998).

2) Ease of manipulation and growth on a broad range of substrates

3) Yeast are eukaryotes and can provide information which are relevant to other eukaryotes (Ghisla, et al. 1974).

Despite these advantages, the number of yeast used in the biosensor applications to date is limited. The total number of yeast likely to exist and the environments they are known to

inhabit makes the wild type yeast an extensive biosensor resource. Yeast can be immobilized on membranes, in hydrogels and pastes, and can be stored for long-term by drying but may be not as long as required for commercial applications (Obayashi, et al. 2004)

Yeast biosensors have been constructed for a range of specific molecules. Most yeast biosensors detect common catabolic substrates such as ethanol and glucose and are able to discriminate the target molecule in a mixture of substrates. However in some instances, interference from the other substrates can pose a problem in whole cell biosensors, for example, when *Hydrangea anomala* was immobolized on the surface of a pH probe, a fall in pH occurred on exposure to glucose substrate (Massey, et al. 1969). Although the response to glucose was greatest, a significant interference was observed when mannose, fructose and maltose were also present. A similar problem was reported with *Saccharomyces*-based biosensor (Mascini and Memoli, 1986). Racek and Musil 1987, described a *H. anomala* sensor designed to detect lactose in blood; responses to blood glucose was completely blocked when sodium fluoride was added (Racek and Musil 1987). Lobanov et al., 2001 reported the use of chemo-metric analysis of the responses of the bacterium *Gluconobacter oxydans* and the yeast *Pichia methanolica* to discriminate accurately between ethanol and glucose in a mixture of various concentrations between 1 and 8 mM. (Lobanov, et al. 2001) When the results were processed by neural network analysis (neural network analysis is an artificial intelligence (AI) approach to mathematical modelling), the upper limit was increased to 10 mM. These results indicate that it is possible to increase the specificity of the cellular response by blocking responses to specific interfering molecules or by using more than one metabolic type of cell and applying appropriate data processing.

Yeast biosensors are able to provide information that biological molecules or a collection of molecules are unable to do, for example toxicity, and genotoxicity**.** The most common way of detecting the cellular response is by detecting the oxygen (O_2) consumption by the catabolic cells (Baronian 2004). Recently, O_2 has been replaced by redox mediators to monitor bacterial

catabolism. The oxidised redox mediators capture electrons from redox molecules in the electron transport chain (Pasco, et al. 2000; Ramsay and Turner 1988; Richardson, et al. 1991) and undergoes reduction and this is then quantified by electrochemical methods such as amperometry, voltammetry or couloumetry (Baronian, et al. 2002).

2.2.1. Proteins or enzymes as sensing element in biosensors

Purified enzymes can be used as biocomponents in biosensors as they exhibit high substrate specificity, sensitivity and quick response. The most popular biosensor is blood glucose biosensor which is based on enzyme glucose oxidase (Wang 2008). The disadvantage of enzymes in biosensors is they can be expensive and they can easily get denatured. The enzymes involved in electron transport chain are known to be comparatively more stable and are used in biosensors (Bistolas, et al. 2005). Other popular enzymes used as sensing element in biosensors are microperoxidase, peroxidases, and PQQ-enzymes (Gorton, et al. 1999).

2.2.2. Trans-plasma electron transport (tPMET) in prokaryotes and eukaryotes

Mammalian, yeast and plant cells contains tPMETs in which NAD(P) H oxidase is considered the key enzyme (Crane and Löw 1976; Gurazada 2008). Impermeable ferricyanide was effectively reduced in cell suspensions of yeast. Other enzymes reducing ferricyanide in the yeast tPMET are ferrireductases. The ferrireductases are enzymes involved in the reductive system of iron uptake (Gurazada 2008; Yamashoji, et al. 1989; Yamashoji and Kajimoto 1986). *Shewanella. putreficans* consists majority of its membrane-bound cytochromes on its outer membrane (Myers and Myers, 1992) and is electrochemically active when grown under anerobic conditions (Kim, et al. 2002).

2.3 Mediators

Mediators are small molecules that can undergo redox reactions with cellular component(s). Hydrophilic mediators such as hexacyanoferrate (III) (ferricyanide) are soluble in the aqueous environment but do not cross the cell membrane. In contrast, lipophilic mediators are soluble in the cell membrane and can enter the cytoplasm to interact with the electron transport chain of eukaryotes. Some examples of lipophilic mediators are, 2-methyl-1, 4-napthoquinone (menadione), benzoquinone and benzoamines, such as 2,3,5,6-tetra methyl-1,4-phenylenediamine (TMPD).

Lipophilic mediators can detect catabolic responses in both prokaryotes and eukaryotes (Rabinowitz et al., 1998) whereas hydrophilic mediators can detect such responses only in prokaryotes. Hydrophilic mediators can thus only be used in the prokaryotes that have respiratory electron transport redox proteins located in the cell membrane. Double mediated systems comprising both liphophilic and hydrophilic mediators have been used for electrochemical detection of intracellular redox activity in mammalian cells (Rabinowitz et al., 1998). For example, the lipophlic mediator menadione was used to transfer the electrons from intracellular redox sites to the cell surface to react with the hydrophilic mediator ferricyanide.

2.3.1. Interactions of mediators

Catabolism in prokaryotes detected by mediated electrochemical detection is well documented but the application of this technique to eukaryotic cells has received less attention. Baronian et al., 2007 used yeast as biocomponent for mediated electrochemical biosensors. As yeasts are eukaryotes, a lipophilic mediator is necessary to interact with the intracellular redox molecules associated with the catabolism. In this double mediator detection system, Baronian et al.,2007 used a lipophilic mediator, TMPD to cross the cell membrane and oxidise reduced catabolic molecules such as NADH and NADPH, and a hydrophilic mediator that oxidised the reduced lipophilic mediator at the cell surface

(Baronian, et al. 2002). The reduced hydrophilic mediator could then be electrochemically oxidised to indirectly quantify reduced intracellular molecules. In S. *cerevisiae,* catabolic responses were observed in both single and double mediator trials but signals from the double mediator system was three orders of magnitude larger than from the single mediator system.

In a recent study, Baronian et al., 2007 concluded that the double mediated electrochemical system can be used for *in vivo* study of behaviour of EBPs under controlled conditions and the use of EBP was developed as a rapid assay to detect environmental estrogens (Baronian and Gurazada 2007). The binding affinities for major estrogens in different species were similar including the human oestrogen receptor. The time taken for this assay was just five hours.

2.4 Electrochemical detection

Voltammetry, coulometery and amperometry are three electrochemical techniques used to quantify redox species in the solution.

2.4.1 Voltammetry

Linear sweep voltammetry and chronoamperometry are the most convenient procedures to detect the amounts of oxidised and reduced forms of an electro active species in solution. The magnitude of the current on the Y-axis gives an immediate indication of the proportion of each oxidation state, and anodic and cathodic plateau currents allow quantification of each redox form (Baronian, et al. 2002). Malfunctions can be easily detected using this technique by noting the shift of the recorded full voltammogram than with the techniques that give partial data. This technique also offers quick detection of the surface fouling and corresponds loss of electrochemical response due to the surface absorption of highly reactive intermediates produced over the course of mediator (ferricyanide) oxidation.

2.4.2 Coulometry

Coulometry is used as an analytical method to determine the amount of the matter transferred during electrolysis by measuring amount of electricity consumed or produced. In this method,

a measured amount of charge is delivered using a constant voltage. It is difficult to oxidise or reduce a chemical species completely at the electrode. Hence coulometry is usually performed with an intermediate reagent such as redox mediator that quantitatively reacts with the analyte to avoid concentration polarisation. This mediated cell reaction is carried out without regeneration of the mediator at an electrode. So, the reduced mediators will gradually accumulate and this reduced mediator is oxidised at the electrode and quantified. This quantity is directly related to the cell-substrate interaction.

2.4.3 Amperometry

Amperometry is performed at a fixed potential at the working electrode with respect to the reference electrode. The reduction or oxidation of a redox species at the surface of working electrode generates a current and this current is measured.

2.5. *Candida albicans*

C. albicans is a diploid fungus capable of sexual reproduction and is normally present on the skin, vagina, mouth, and rectum mucous membranes. Most of the time, Candida infections of the mouth, skin, or vagina occur for no apparent reason. *C. albicans* causes disease more frequently in women than men. A common cause of infection may be the overuse of antibiotics that destroy both beneficial and harmful microorganisms in the body, permitting Candida to multiply in their place. The fungus can also be transmitted through the blood stream and affect the throat, intestines, and heart valves. The resulting condition is known as Candidiasis or Moniliasis, or thrush, or a "yeast" infection.

Microbial systems have been identified that show the presence of genetic master switches which sense environmental signals and generate an intracellular signal that activates the genes that adapt the organism to its altered environment. The role of gene regulation of virulence is mainly to transform the microorganism from the saprophytic growth to pathogenicity. Various microorganisms are known to increase their virulence in response to chemical signals such as

temperature changes, iron concentration and osmotic stimuli but no such signals have been identified for the *C. albicans* (White and Larsen 1997).

2.6. Cellular and molecular responses of oestrogen binding protein in *Candida albicans*.

C. albicans possesses an EBP that binds to oestrogen with high affinity and specificity (Skowronski and Feldman 1989) and oestrogen has been identified as a chemical signal which up-regulates the organism's virulence, by causing transformation from yeast to hyphal growth. Evidences indicate that estrogens increase susceptibility to Candida infections and oestradiol has been shown to stimulate yeast to form mycelia (Zhao, et al. 1995) which increases the organism's virulence but there is some uncertainty as to whether yeast to mycelia transition is a true virulence factor. The mycelial formation probably indirectly promotes virulence through cell surface hydrophobicity, distribution of surface antigens or by its interaction with the endogenous and exogenous fungal agents (White and Larsen 1997). The fate of oestrogen is unknown after binding to EBP.

The addition of 17-β-estradiol or ethinyl estradiol to *C.albicans* cells increases germ tube length and is dose and strain dependent. There is increase in germ tube formation when 10^{-5} M 17-β-estrodiol is added to SC5314 cultures of *C. albicans* (Cheng, et al. 2006). Even a slight increase in the formation and length of germ tubes will help the organism withstand sloughing of the vaginal epithelium and maintain the host colonization. Microarray technology has been used to gain a better understanding of the effects of *C. albicans* to oestrogen exposure (Cheng, et al. 2006).

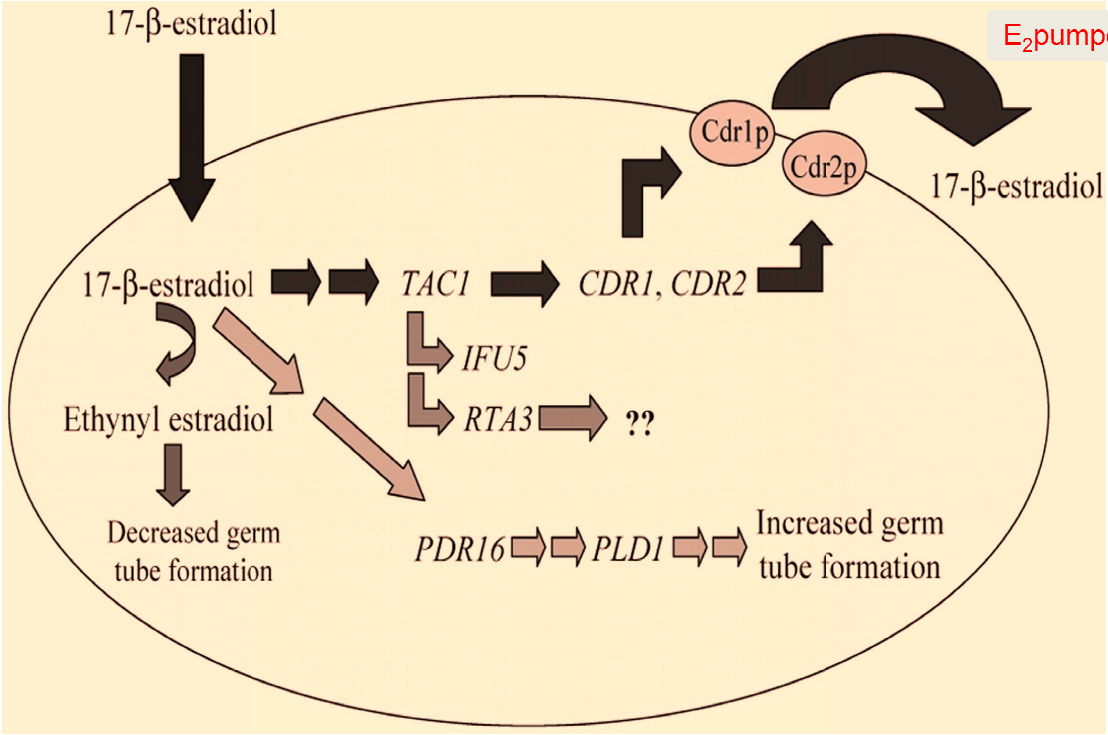

Figure 2.1. Effects on cell biology on exposure of oestrogen on *C. albicans* was determined using microarray observation of gene expression patterns (Cheng, et al. 2006).

In the presence of supra physiological levels of 17-β-estradiol, *C. albicans* up-regulates the CDR1 and CDR2 coding sequences to pump the oestrogen from the cell that results in low intracellular levels of 17-β-estradiol despite the supra physiological levels of oestrogen in growth medium (Cheng, et al. 2006). The addition of 17-β-estradiol to *C. albicans* cells also up-regulates the expression of gene PDR16 resulting in increased germ tube formation. The potential for 17-β-estradiol to be converted to another biologically active compound such as ethinyl oestradiol is far less favoured than the pathways mentioned above.

If the efflux pumps were slower to remove intracellular ethinyl oestradiol, then the higher intracellular concentrations will favour the pathway that leads to a decrease in germ tube formation. The efflux pumps can reduce the intracellular concentration by up-regulating the CDR1 and CDR2 genes within a short period. This model shown in Fig.2.1 is taken from Cheng et al. 2006 with minor modifications describes the effect of oestrogen on germ tube formation and length as a balance between competing reactions and interactions.

It was found that when *C. albicans* strain 158 was grown in Sabouraud-dextrose medium, elevated levels of EBP were expressed in the early growth phase which decreased rapidly as cell density increased and reached the stationary growth phase (Zhao, et al. 1995). However, the EBP levels vary among the different strains of *C. albicans* and growth stages. For example, the *C. albicans* strain 422, showed a 17-fold higher abundance of EBP compared to strain 158. In the strain 158 of *C. albicans,* the EBP levels were related to the growth phase and were highest at the beginning of the cell growth and declined to low levels as cell approached the stationary growth phase. The EBP levels were 9090 fmol (mg protein^{-1}) after 2.5 h of inoculation and declined to 909 fmol (mg protein)$^{-1}$ after 24 h of growth, whereas the *C. albicans* strain 422 showed a higher EBP abundance of 18455 fmol (mg protein)$^{-1}$ and remained relatively constant throughout the growth phase. The EBP levels are thus expressed in high levels and continuously in the strain 422 of *C. albicans* compared to the strain 158 (Zhao, et al. 1995).

2.6.1. Similarities and differences between oestrogen binding protein and old yellow enzyme

The cloned and sequenced EBP gene shows that the gene contains highly conserved amino acid sequence similar to the several flavin-containing enzymes. In addition, the EBP display 45% amino acid identity to the old yellow enzyme (OYE), an oxidoreductase present in *S. cerevisiae* and other yeasts (Niino, et al. 1995). EBP also has oxidoreductase activity (Madani et al., 1994) and can be inhibited by oestrogen. In addition to 45% identity of EBP to OYE, there are two functional features that are interesting. OYE has been shown to bind to a number of phenolic compounds, including 17-β-estradiol but it binds to oestradiol with much lower affinity than EBP and shows abundant FMN (flavin mononucleotide) dependent oxidoreductase activity. The most significant difference between EBP and OYE is their reactivity with the 19-nortestosterone. The OYE and other homologues of EBP like morphinone reductase and OPDA reductase can only oxidise 19 nortestosterone where as the

EBP not only oxidizes 19-nortestosterone, but also reduces it (Buckman and Miller, 1998). It was also found that EBP prefers NADPH to NADH (Buckman and Miller, 1998). Antibodies against EBP have been developed. Immunolocalization of the protein was carried out by electron microscopy and EBP was found located near the inner surface of the tonoplast membrane of the vacuole in *C. albicans* (Zhao, et al. 1995).

2.6.2. Cloning and complete nucleotide sequence of a gene encoding a *C. albicans* oestrogen binding protein

Oligonucleotide primers of EBP for PCR were designed using amino acid sequences obtained from the cyanogen bromide fragments of purified EBP. An 800-bp product was amplified and used to identify a λ phage containing an intact EBP gene from *C. albicans* genomic library. This gene was capable of encoding the protein having a molecular mass of 46,073 Da, the estimated size of the EBP. The gene was cloned and expressed in *Escherichia coli* as a lacZ fusion protein and this protein showed high-affinity binding for oestradiol comparable to the wild-type EBP.

Previously, it was hypothesized that the steroid-binding proteins in microorganisms may represent the early ancestors to the mammalian steroid-receptor system but computer searches of the database of related genes demonstrated that *C. albicans* EBP is unrelated to the mammalian steroid-receptor system. The data search also identified a section of EBP amino acid sequence (residues 183-233) that is highly conserved among a number of flavoproteins and that this may be the domain essential for functioning of these enzymes although the only cysteine residue (Cys-225) present in EBP1 is located in the conserved region which is lacking at the corresponding positions in the other related proteins. The function of this cysteine residue is unknown but the treatment of this residue with sulphydryl-active agents decreases the binding of oestradiol by this protein suggesting that this residue is critical for interaction with the steroid (Madani, et al. 1994). Full gene sequence of EBP was deposited in NCBI by Madani et al. (1994), with accession no. L25759.

2.6.3. Some physical and chemical characteristics of oestrogen binding protein in *C. albicans*, important for the work presented in this thesis

- Binding of EBP with oestradiol was found to be optimal at pH 7.4 in the presence 0.3M KCl and was related to the EBP concentration.
- Binding with the oestradiol is very rapid and reaches a maximum in 30 min and is reversible with a dissociation constant of $13.2 \pm 1.7 * 10\ sec^{-1}$.
- Binding competition experiments showed specificity and sterioselectivity of EBP. The binding specificity of EBP to 17-β-estradiol is > estrone > estriol > 17-α-estradiol.
- The molecular size of EBP measured by Sephacryl S-200 gel exclusion chromatography, yielded a stokes radius of approximately $29A^{o}$ and sucrose density gradient sedimentation showed a sedimentation coefficient of ≈4 S
- The approximate molecular weight of EBP is 46,000 Da with an axial ratio of 1 an indication of the symmetrical shape of the molecule (Skowronski and Feldman 1989).

2.6.4. Oestrogen binding protein mediated oxidoreductase activity.

Oestrogen binding protein has NAD(P)H oxidase activity that mediates the oxidation of NAD(P)H to provide $NAD(P)^{+}$ for the cells catabolic processes, which is inhibited by the presence of 17β-oestradiol. Oestrogens have shown to increase hyphal formation in *C. albicans.* In yeast species of *C. albicans* and *P. brasiliensis*, EBP mediated oxido-reductase activity is involved in pathogencity of these organisms. In case of *P. brasiliensis* infection is more prevalent in males than females. In *P. brasiliensis*, low level of oestradiol promotes the predominance of the yeast form (Clemons, et al. 1989). This suggests that EBP binding to oestrogen is mediating the regulation of protein expression which is analogous to mammalian steroid receptor (Skowronski and Feldman 1989).

Based on the literature there is a need to develop sensitive and cost effective biosensor for detection of oestrogen in complex matrices. Development of bioassay using wild type *C. albicans* and its EBP has advantages over genetically modified yeast assays, human cell lines, and other mammalian cell lines since these are expensive, time consuming, and require special facilities.

Chapter 3
Investigating yeast cell responses to oestrogen by electrochemical detection

3.1. Abstract

C. albicans and some other yeast species are known to have an oestrogen binding protein (EBP), which oxidises NAD(P)H to $NAD(P)^+$. 17β-oestradiol, when present, binds to the NAD(P)H oxidation site which leads to an accumulation of NAD(P)H. Our previous research has shown that oestrogens can be quantified using *S. cerevisiae* cells as the detection element and a double mediator electrochemical system to measure NAD(P)H. In a modification to the whole cell method, *C. albicans* cell lysate and a single hydrophilic mediator was used to quantify 17β-oestradiol. This paper employs the mediated electrochemical systems to investigate the influence of growth phase on EBP production in *C. albicans* and the response of cells of different ages to different concentrations of oestrogen. A cell not known to possess EBP (*Arxula adeninivorans*) was also investigated for its response to 17β-oestradiol. As expected, *A. adeninivorans* did not show a detectable response to 17β-oestradiol, but surprisingly, its catabolism was inhibited.

Keywords: Yeast Oestrogen Binding Protein, EBP production with cell growth phase, oestrogen inhibition of catabolism, mediated electrochemical detection.

3.2. Introduction

A number of yeast are known to have steroid binding proteins. For example *Candida albicans, Paracoccidioides brasiliensis* and *Saccharomyces cerevisiae,* have an oestrogen binding protein (EBP) (Feldman, et al. 1982; Restrepo, et al. 1984; Skowronski and Feldman

1989). In these fungi, oestrogens stimulate and/or regulate growth e.g. the interaction of oestrogen with EBP induces the conversion of *C. albicans* and *P. brasiliensis* from the yeast form to the mycelial form (Clemons, et al. 1989). *C. albicans and P. brasiliensis* are pathogens and EBP is reported to have a role in their pathogenicity (Clemons, et al. 1989). That *Saccharomyces,* a non-pathogen, has an EBP is not so surprising when it is considered that *Candida* and *Saccharomyces* evolved from a common ancestor approximately 2 million years ago and EBP continues to have a growth regulation function in the latter (Berman and Sudbery 2002; Cheng, et al. 2006; Madani, et al. 1994; Williams and Bruce 2002; Zhao, et al. 1995). Other yeast species reported to have steroid binding proteins include *Rhizopus nigricans*, *Aspergillus fumigatus*, *Coccidioides*, and the dermatophytic fungi (Clemons, et al. 2010).

Yeast steroid binding proteins are unrelated to the vertebrate steroid receptor super family and belong to the flavoprotein family of oxido-reducatase enzymes e.g. have some similarity to cytochrome c (Madani, et al. 1994). *S. cerevisiae* old yellow enzyme (OYE) has 48% similarity to *C. albicans* EBP. Oestrogen binds to OYE weakly and Karplus et al. have shown that oestrogen binds across part of the OYE NAD(P)H oxido-reductase site to prevent the oxidation of NAD(P)H (Karplus, et al. 1995).

Compared with bacteria, yeast are rarely studied as a recognition element for use in bioassays. However, yeast are easy to culture and are eukaryotes, which means their biochemistry in many respects is similar to that of higher organisms. We have previously described the use of *S. cerevisiae* whole cells (Baronian and Gurazada, 2007) and *C. albicans* cell lysate as the detection element in an oestrogen bioassay in the next chapter (Chelikani, et al. 2011). The electrochemical detection of the oestrogen interaction with EBP in whole cells involved incubation with a double mediator system and analysis by linear sweep voltammetry. In this double mediator detection system, a lipophilic mediator, 2, 3, 5, 6-tetra methyl-1, 4-phenylenediamine (TMPD) crosses the cell membrane, oxidises reduced molecules such as

NADH and NADPH, and a hydrophilic mediator oxidises the resulting reduced lipophilic mediator at the cell surface. Trans membrane electron transport proteins (tPMETs) are also oxidised by the hydrophilic mediator. The reduced hydrophilic mediator can then be electrochemically oxidised to indirectly quantify reduced intracellular molecules and tPMETs. The system also detects catabolism in whole cells, which means that in a whole cell oestrogen assay, there are two signals that need to be separated i.e. the catabolic response and the oestrogen response. The use of cell lysate as the sensing element removes the contribution of the cells' catabolic response and simplifies the assay. Because, lysing the cells destroys the cell membrane, the need for the lipophilic mediator is removed and a single hydrophilic mediator can be used to detect the cell lysate/oestrogen interaction (discussed in detail in next chapter)

We have developed analytical methods for oestrogen measurement based on use of whole cells and cell lysate with mediated electrochemical detection (chapter 4). Here the mediated electrochemical method is used to gain fundamental insight into the interaction of oestrogen with yeast. We explore the production of EBP by *C. albicans* and we investigate the effect of oestrogen on *Arxula adeninivorans* LS3, a non-pathogenic yeast not known to produce EBP.

3.3. Materials and Methods

3.3.1. Cells

C. albicans CDCS S-24 was purchased from ESR, Porirua New Zealand and *Arxula adeninivorans* LS3 was from IPK Gatersleben, Germany. Both were maintained on YEPD agar plates at 4°C.

3.3.2. Cultivation of cells

C. albicans and *A. adeninivorans* were cultivated in YEPD broth, a general-purpose fungal selective medium (yeast extract 10 g Merck, peptone 110 20 g GibcoBRL, dextrose 20 g BDH Analar per liter). Cells were incubated at 37°C for 16 h unless stated otherwise, in

indented flasks shaken at 180 rpm, and harvested by centrifugation (2604 × g, 8 min, 10°C), washed twice in phosphate buffered saline (PBS) pH 7 and then re-suspended in PBS pH 7 at OD_{600} = 3.0. Cell counts were made by haemocytometer.

3.3.3. Reagents

Potassium hexacyanoferrate (III) ($[Fe(CN)_6]^{3-}$ Merck pro analysis), 0.5 M, was prepared in distilled water; 2, 3, 5, 6-tetramethyl-1, 4-phenylenediamine (TMPD, Aldrich), 20 mM, was dissolved in 96% ethanol; and 17β-oestradiol, (Sigma),1.1μM (300 μgL^{-1}),was prepared in 96% ethanol. Lyticase from *Arthrobacter luteus*, chitinase from *Trichoderma viride*, β-glucuronidase type H2 from *Helix pomatia* and DL-dithiothreitol were purchased from Sigma. All solutions were sterilized by filtration (0.45 μm Millipore) and stored at 4°C.

3.3.4. Production of cell lysate

A cell suspension, OD_{600} = 3, was pre-incubated for 15 min with 2 mM dithiothreitol and then incubated with 50 mL of lyticase (100 units mL^{-1}), 16 units of chitinase, and 200,000 units of glucuronidase and protease inhibitor cocktail (leupeptin, pepstatin, chymostatin, benzamidine and trypsin inhibitor) for 3 h at 30ºC at 50 rpm. Treated cells were harvested by centrifugation for 15 min at 18514 ×g to remove cell debris. The supernatant containing the sphaeroplasts was removed, shaken vigorously for 2 min and passed through a 0.45 μm filter (Millipore) to lyse the sphaeroplasts. It was stored at 4°C until use.

3.3.5. Experimental procedure

The cell incubation and electrochemical detection methods have been described previously (Baronian, et al. 2005). The electrochemical analyser was an eDaq potentiostat coupled to a Power Lab 2/20 controlled by eDaq Echem software. Linear sweep measurements were made with a 50 μm diameter Pt microdisc working electrode, a Ag/AgCl reference electrode and a Pt wire auxiliary electrode, all G-Glass, Australia.

Experimental samples comprised: either 4065 μL whole cell suspension or cell lysate, 800 μL ferricyanide solution (incubation concentration 80 mM), 125 μL TMPD solution (incubation

concentration 0.5 mM) or 125 μL PBS in the cell lysate experiments, 100 μL oestrogen solution (incubation concentrations of 1 nM to 50 nM) and ethanol (100 μL or less in endogenous control groups and oestrogen groups where required). Note the cell lysate of 4065 μL is extracted from an equivalent number of cells that are present in 4065 μL of whole cell sample.

The experimental samples were incubated at 37°C with oxygen-free nitrogen sparging for 5 h for cell samples and lysate samples. At the completion of incubation, the cells were pelleted by centrifugation (4629 ×g, 20°C for 10 min) and the supernatant removed for voltammetric analysis. All trials were run in duplicate or triplicate. All experiments included a negative control to determine the base level endogenous catabolic activity (cell trials) or redox activity (lysate trials).

The supernatant of each sample was analysed in duplicate or triplicate using steady state voltammetry. The steady-state anodic plateau current measured at E = 425 mV was used as a relative measure of the amount of ferrocyanide produced, and hence the number of cell redox molecules oxidized by TMPD, either in relation to yeast catabolism (Baronian, et al. 2002) and/or the EPB/oestrogen/NADH oxidase interaction. Error bars are ±1 standard deviation.

3.4. Results and Discussion

3.4.1. Schematic representation of the detection of the EBP response to oestrogen

Fig.1 A is the schematic representation of the function of EBP in *C. albicans* cells. EBP is an oxido-reductase enzyme, which converts NAD(P)H to $NAD(P)^+$. EBP accepts two electrons and transfers it to βunsaturated carbonylmolecules such as cyclohexanone (Fox and Karplus 1994; Khan, et al. 2002). Fig.2 B is the schematic representation of the proposed behaviour of EBP in the presence of oestrogen. Oestrogen binds to the oxido-reductase site of EBP and blocks the conversion of NADH to NAD^+, causing NADH to accumulate and be available to

reduce the lipophilic mediator TMPD which then reduces the hydrophilic mediator $[Fe(CN)_6]^{3-}$. Thus by measuring NADH in the cells, oestrogen can be quantified (Baronian and Gurazada, 2007; Madani, et al. 1994; Skowronski and Feldman, 1989).

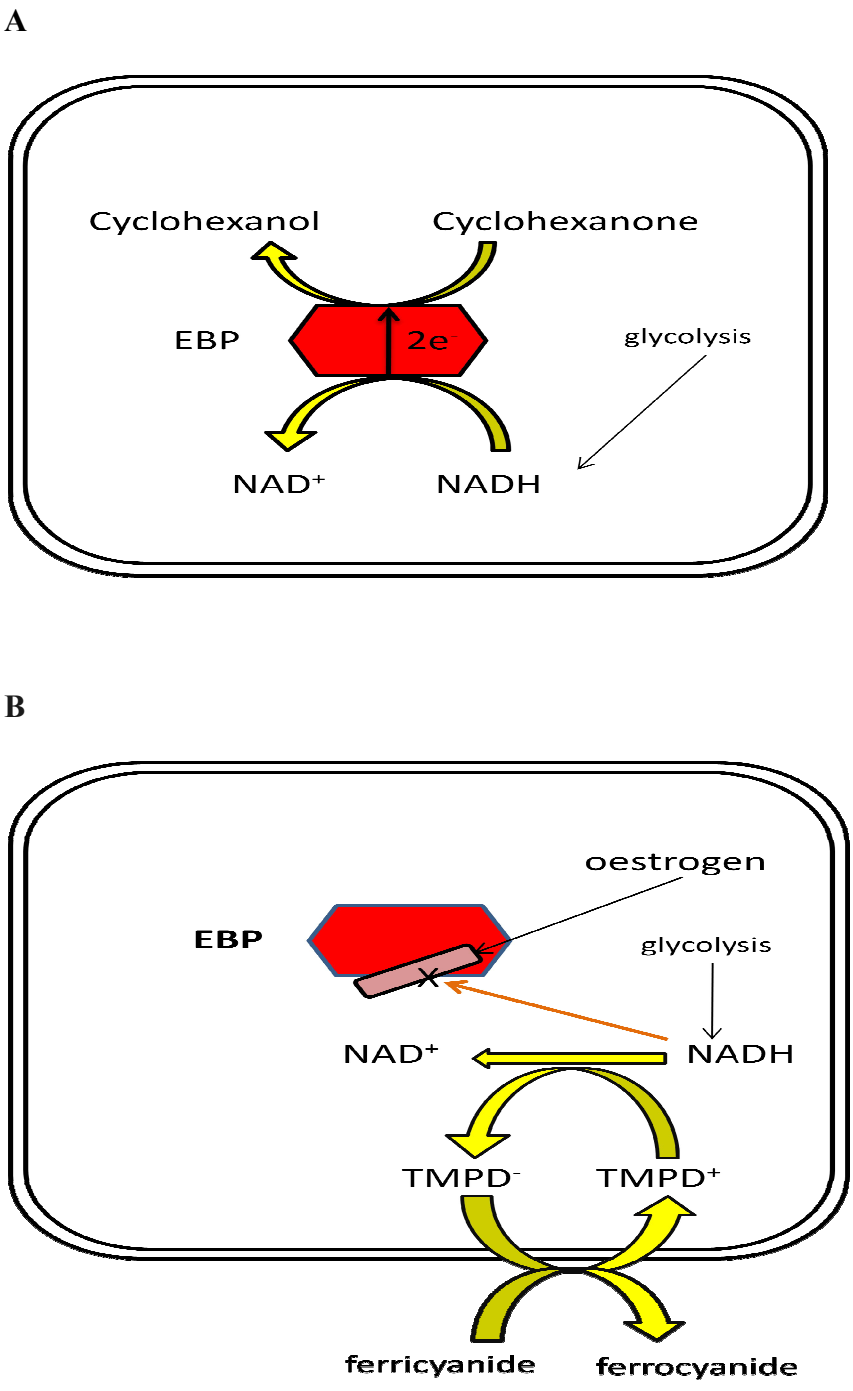

Figure 3.1. Schematic representation of: A. Function of EBP in the absence of oestrogen and B. In the presence of oestrogen and TMPD. Oestrogen blocks the oxidation of NADH, which is then available to reduce TMPD

3.4.2. Linear sweep voltammery

Typical linear sweep voltammograms of the solutions used to investigate cell responses to 17β-oestradiol are shown in Fig.2. Considering the scans from bottom to top in Fig. 2, (1) $[Fe(CN)_6]^{3-}$ only. $[Fe(CN)_6]^{3-}$ is in the oxidised state and there is therefore no oxidation current. (2) $[Fe(CN)_6]^{3-}$ and TMPD. TMPD is received in the reduced state resulting in a small proportion of the $[Fe(CN)_6]^{3-}$ being reduced when they are first mixed. $[Fe(CN)_6]^{3-}$ is 160 times more concentrated than TMPD i.e. is in over supply and the small proportion of $[Fe(CN)_6]^{3-}$ that is reduced does not prevent the double mediator system from responding fully to the cells' redox processes in the period of the assay. (3) $[Fe(CN)_6]^{3-}$ and cells. tPMETs on the surface of the cell membrane reduce a fraction of the $[Fe(CN)_6]^{3-}$ (4) $[Fe(CN)_6]^{3-}$, TMPD and cells. TMPD is able to cross the cell membrane and oxidise internal redox sites. It diffuses out of the cell and reduces $[Fe(CN)_6]^{3-}$. tPMETs also reduce $[Fe(CN)_6]^{3-}$ so that the signal observed is a composite of internal reduction and tPMET reduction of $[Fe(CN)_6]^{3-}$.(5) $[Fe(CN)_6]^{3-}$, TMPD, cells and 17β-oestradiol. Blocking of the EBP oxido-reductase site by 17β-oestradiol causes an accumulation of NAD(P)H in the cell resulting in increased reduction of TMPD and hence of $[Fe(CN)_6]^{3-}$(Baronian and Gurazada 2007; Chelikani, et al. 2011). The amount of $[Fe(CN)_6]^{4-}$ produced, measured by its oxidation current at E = 425 mV, and corrected for the control thus quantifies the cells' interaction with 17β-oestradiol.

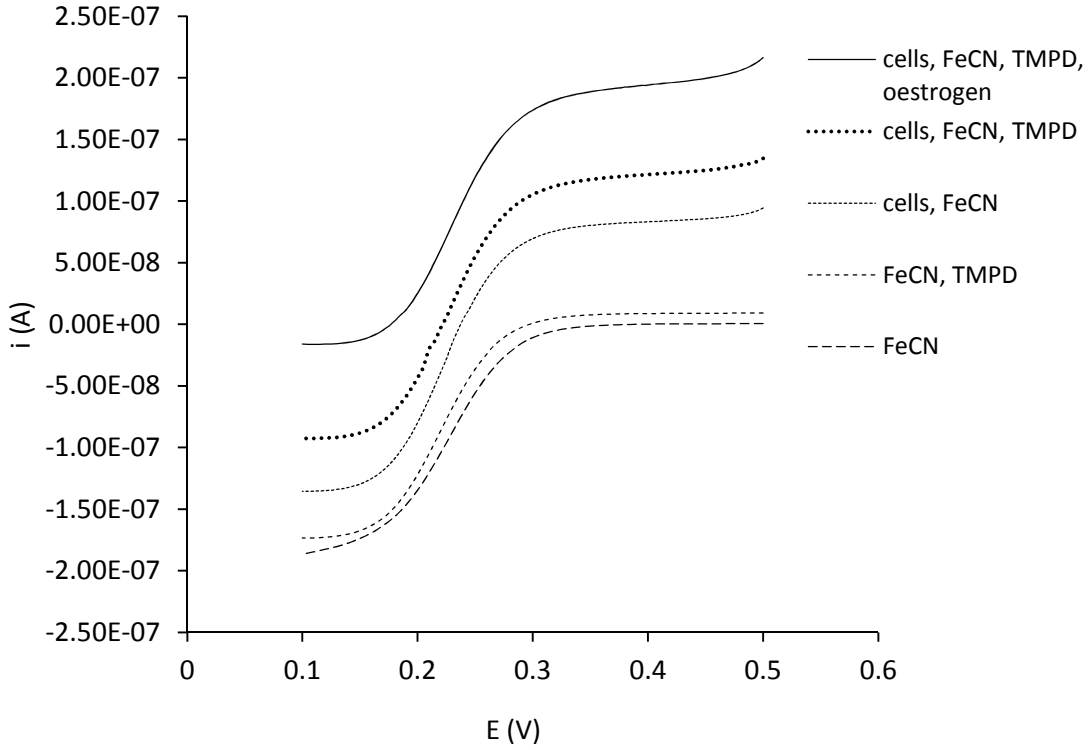

Figure 3.2. LSV of *C.albicans* whole cell responses in the presence $K_3[Fe(CN)_6]^{3-}$, $K_3[Fe(CN)_6]$ + TMPD and $K_3[Fe(CN)_6]$ + TMPD in the presence of 17β-oestradiol 10 nM. This Fig. also includes LSVs of Ferricyanide and Ferricyanide+ TMPD as reference scans. The data points are the steady state currents obtained by LSV after 8 h incubation.

3.4.3. Responses at different cell ages

In my work using *C. albicans* (as whole cells and as cell lysate) to measure 17β-oestradiol concentrations, cells were grown overnight before use (Chapter 4). However, the effect of cell age on response was not investigated and thus in an attempt to optimise the method, this parameter was investigated. A growth curve for *C. albicans* was established by incubating in YEPD broth at 37 ºC with rotation at 180 rpm. Cells were counted every hour using a haemocytometer. The logrithmic phase began at 3 h and ended in the 15th h. Hence, in order to investigate cell responses to 17β-oestradiol at early, mid and late log phase and stationary phase, *C. albicans* cells were harvested at 4, 7, 10, 13 and 17 h. The cells were adjusted to OD_{600} = 3 and then incubated for 8 h with 5 nM or 15 nM 17β-oestradiol and mediators

$[Fe(CN)_6]^{3-}$ and TMPD, i.e. a double mediator system. Fig 3. shows that the magnitude of the response to 17ß-oestradiol depends on cell age and that there is a difference between the patterns of responses to 5 and 15 nM 17β-oestradiol. Responses to 5 nM 17β-oestradiol increased with cell ageto reach a maximum in the early stationary phase. We assume that the increase in response is due to an increase in the number of EBP molecules with cell age. When the concentration of 17β-oestradiol was 15 nM, the responses of cells increased with cell age up to 10 h, and, at each age, were the same as the responses to 5 nM 17β-oestradiol. This suggests that the response is limited by the number of EPB molecules in these cells. However, as cell age increased further, there was no increase in response to 15 nM 17β-oestradiol.

The data in Fig.3.3 illustrate several important findings. First, the highest sensitivity to 17β-oestradiol at a low concentration was achieved with cells harvested after 17 h of growth. Hence harvesting cells after incubation overnight (17 h) was adopted as the standard procedure. Second, the increase in response to 5 nM 17β-oestradiol with cell age contrasts with the observations of Skowronski et al., 1989 who studied the effect of cell age on the abundance of EBP, as determined by 17β-oestradiol binding studies in *C. albicans* Stn-1 (Skowronski and Feldman 1989). That works s howed that levels of EBP were highest during the earliest stages of logarithmic-phase growth, declined rapidly as the cells continued to proliferate, and were lowest as the cells enter the stationary-growth phase. Assuming that there is equivalence in the two methods, our results suggest that responses to oestrogen in different phases of growth vary with strain. The third interesting aspect of the data in Fig.3.3 is the different behaviour of cells to high and low concentrations of 17β-oestradiol. We have previously observed similar behaviour with *S. cerevisiae* which showed a maximum response at 11 nM oestrogen and a dramatic decrease in response when the concentration of oestrogen was increased to 20 nM (Baronian and Gurazada 2007). Microarray studies by Cheng et al. have shown that the pleiotropic drug resistance (PDR) efflux protein system expels oestrogen

from *C. albicans* cells (Cheng, et al. 2006). I assume that this mechanism accounts for the decrease in response of both *C. albicans* and *S. cerevisiae* to high concentrations of 17β-oestradiol. This result in Fig. 3.3 suggest that efflux doesn't occur in early to mid log growth phase cells because the responses to 5 nM and 15 nM are the same over that growth period, indicating that it is the number of EBP molecules that is the limiting factor. However efflux is clearly evident in late log phase with elevated oestrogen concentrations.

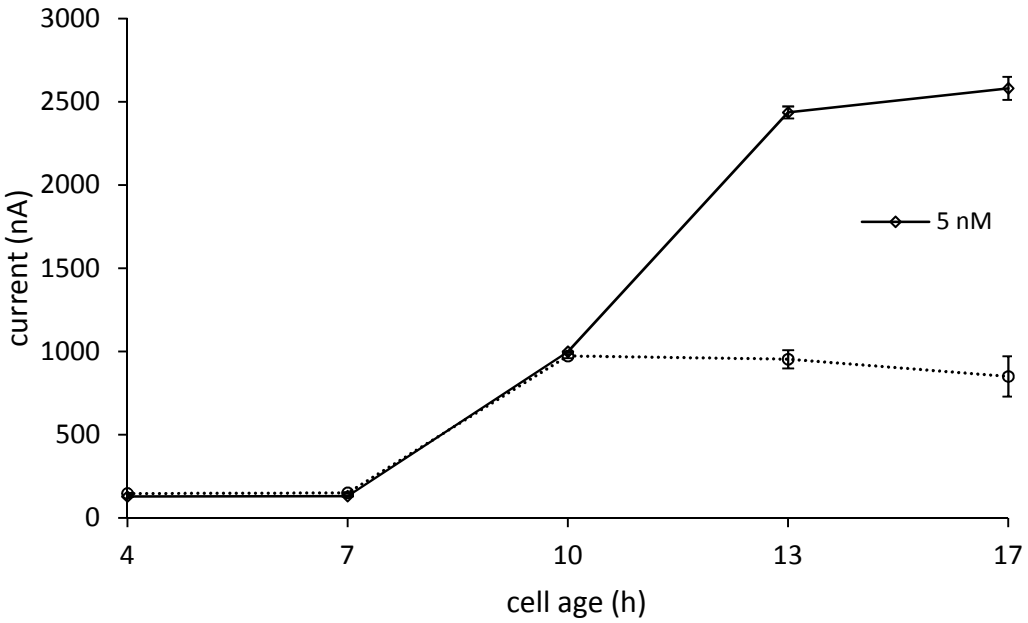

Figure 3.3. *C. albicans* whole cell responses to 17β-oestradiol (E) at 5 nM and 15 nM after 8 h incubation using a double mediator electrochemical system ($K_3[Fe(CN)_6]$ + TMPD). The data points are the magnitude of the current at 425 mV. Error bars represent ±1 SD.

I have demonstrated in the next chapter that the limited response range of the cell based assay (shown by the decrease in response to 15 nM 17ß-oestradiol, Fig.3) can be overcome by using cell lysate, in place of whole cells. Lysate from cells harvested after growth overnight, with ferricyanide as a single mediator, gave a linear response up to 100 nM 17ß-oestradiol (Chelikani, et al. 2011). Presumably this is because the efflux pumps are non-functional and are unable to remove oestrogen from the EBP environment.

3.4.4. Responses of a non-EBP yeast to oestrogen

Vertebrate steroids have been demonstrated to have profound effects on the yeast genome expression (Buckman and Miller, 1998; Clemons, et al. 2010). The question of what other electrochemically detectable effects, oestrogen may have on yeast cells was explored by identifying a yeast without the known EBP protein or the EBP sequence in its genome, and exploring its response with the double mediator electrochemical technique. *A. adeninivorans*is a non pathogenic environmental yeast (Middelhoven, et al. 1991) which probably would not produce EBP. The protein and genome sequence data is incomplete however a search by BLAST in NCIB was made. The search did not reveal an equivalent endogenous oestrogen response system and it was assumed that *Arxula* would not respond to oestrogen.

A. adeninivorans cells were incubated with and without oestrogen and the cell response was monitored using the double mediator electrochemical system. Not suprisingly there was no increase in the signal with oestrogen at various concentrations but it was found that oestrogen decreased catabolism (Fig.3.4) in a dose response manner ($P = 0.462$). The concentrations in Fig.3.4 are at the lower end of the *C. albicans* response range, however the effect at 10 nM is similar (data not shown). The results show a fundamental difference between *C. albicans* and *S. cerevisiae,* and *A. adeninivorans* in that the latter does not have an endogenous response to oestrogen and further, oestrogen appears to have an inhibitory effect on *A. Adeninivorans* at 0.3 nM oestrogen, catabolism is depressed (approximately 15%). Specific effects of oestrogen on catabolism have been reported e.g Obayashi et al.report that oestrogen controls the catabolism of branched chain fatty acids in female mice (Obayashi, et al. 2004) while more general effects are also reported e.g. Cheng et al. noted a depress effect of 17β-oestradiol on biomass dry weight in one of two strains of *C. albicans*.

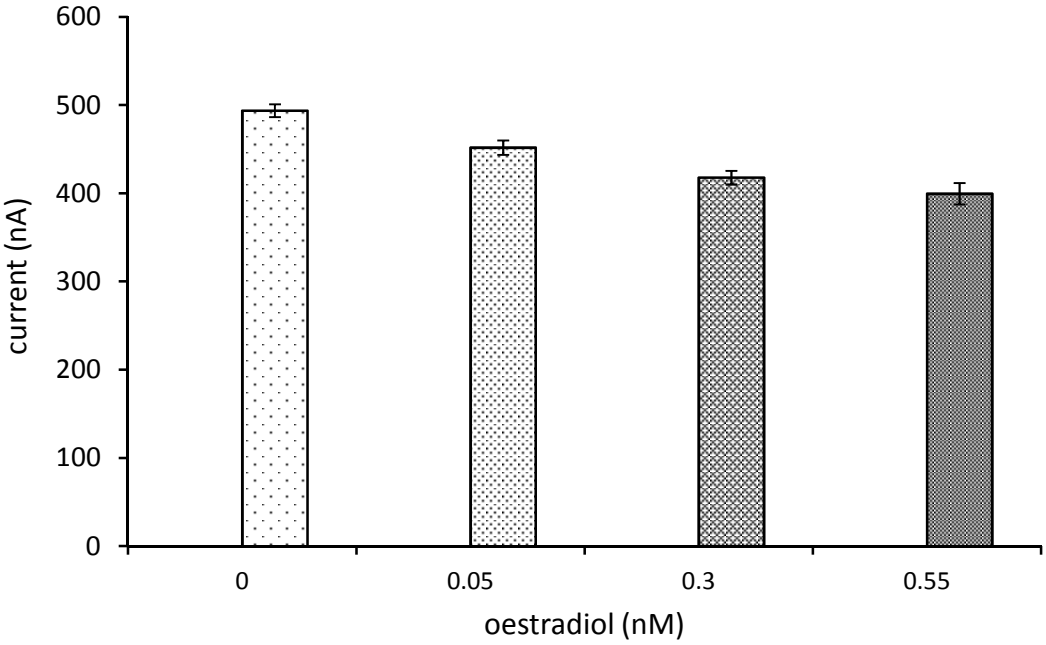

Figure 3.4. *A. adeninivorans* cell endogenous response and responses to 17β-oestradiol (0.05, 0.5, 0.55 nM) after 5 h incubation using a double mediator electrochemical system. The data points are the magnitude of the current at 425 mV. Error bars represent ±1 SD.

Both *S. cerevisiae* and *A. adeninivorans* are used as platforms for recombinant oestrogen screens (Hahn, et al. 2006; Routledge and Sumpter 1996). We have demonstrated a dose dependent positive response to oestrogen in *S. cerevisiae* (Baronian and Gurazada, 2007) but conclude that the endogenous response to oestrogen will not have an effect on the YES screen because the detection methods used in that assay cannot detect the EBP-oestrogen interaction (Hahn, et al. 2006; Routledge and Sumpter, 1996). Similarly we expect the depressing effect of oestrogen on catabolism in *A. adeninivorans* will not affect the AYES assay because the effect is small (< 15%) and because the assay functions independently of catabolism (Hahn, et al. 2006)

3.4.5. *A. adeninivorans* cell lysate response

The lack of an oestrogen response was also confirmed using *A. Adeninivorans* cell lysate (Fig. 5) where responses to 3 oestrogen concentrations (1 nM, 5 nM, 50 nM) were the same ($p = 0.234$). This result also supports notion that oestrogen has inhibitory effect on *Arxula* cells as here there is no intact cell structure or function in the lysate and no effect due to oestrogen can be detected. This finding raises the question as to whether oestrogen may be decreasing catabolism in cells that have an EBP, but the effect is masked by the EBP response to oestrogen. This is a question that will be investigated by catabolite analysis and/or genomic studies.

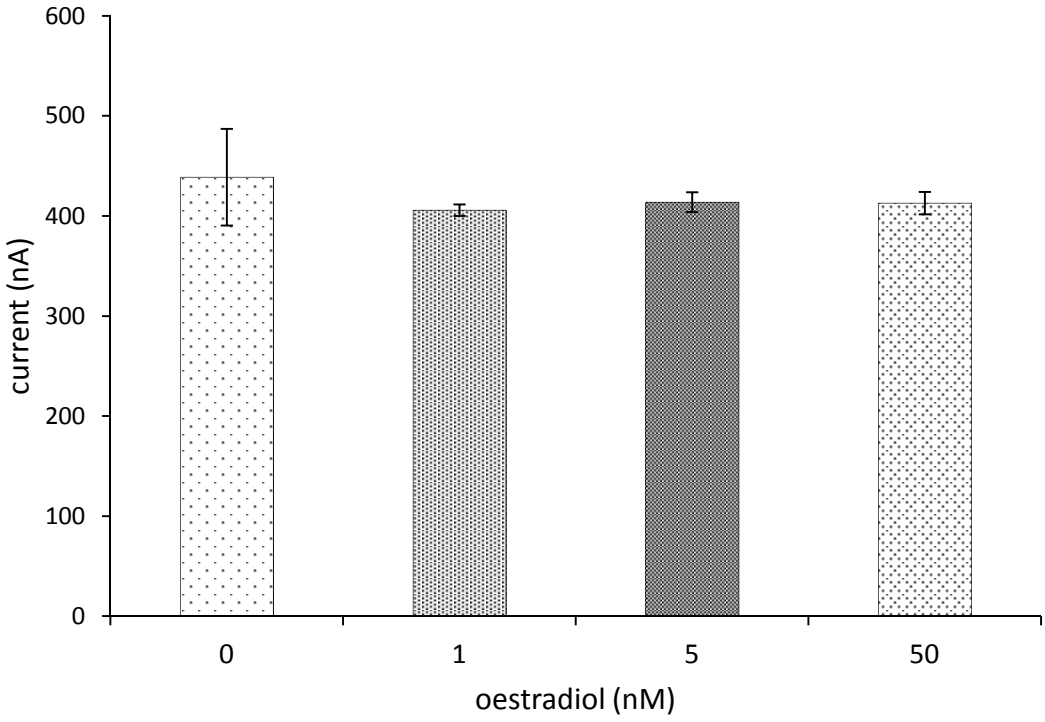

Figure 3.5. *A. adeninivorans* cell lysate endogenous response (control) and responses to 17β-oestradiol (1, 5, and 50 nM) after 5 h incubation using a double mediator electrochemical system. The data points are the magnitude of the current at 425 mV. Error bars represent ±1 SD.

3.5. Conclusions

Yeast oxido-reductase binding proteins can be used as recognition elements for assays and biosensors. Through mediated electrochemical measurements, we have shown that cell age is

a factor in the production of EBP that may vary with strain, suggesting that any work with a different *C. albicans* strain should first be trialed to find the growth phase where the production of EBP is maximal. The maximum oestrogen concentration detectable by whole cells is limited by the operation of the PDR efflux pumps and can be overcome by lysing the cell and removing efflux pump functionality. While the lack of a dose related response to oestrogen by *A. adeninivorans* was expected, the inhibition of its catabolism was not and we are now using this electrochemical method to check other yeast species for responses to oestrogens.

3.6. Co-author contributions

This chapter has been submitted to Electrochimica-acta. Besides supervisory committee members, the Co-authors of this paper are Alison Downard and Gottard Kunze. Alison and Gotthard provided technical advice and are involved in manuscript correction. Gotthard also provided *Arxula* strain for this research.

Chapter 4
Electrochemical detection of oestrogen binding protein interaction with oestrogen in *C. albicans* cell lysate

4.1. Abstract

An electrochemical method to quantitatively detect vertebrate oestrogens using wild type *S. cerevisiae* cells was previously reported. That assay required the use of a double mediator system, a five-hour incubation period and had a maximum detection limit of around 11 nM 17β-oestradiol. In the work reported here we have sought to systematically increase the utility and decrease the complexity of the whole cell assay. The steps we took to achieve this goal were in order; lysing the cells to remove transport constraints, removing the lipophilic mediator and conducting the assay with the hydrophilic mediator only and finally using semi-purified protein and directly detecting NADH so that hydrophilic mediator could be eliminated. Linear sweep voltammetry was used to investigate the interaction of mediators with NADH. The assay is now cell free, mediator-less, the linear response range upper limit has been raised to 100 nM with a calculated limit of detection (LoD) of 0.005 nM with a limit of determination of 0.014 nM and the assay period has been reduced to 20 min.

Key Words: oestrogen assay; yeast oestrogen binding protein (EBP); NADH; electrochemical detection; voltammetry

4.2. Introduction

The need to detect environmental oestrogens and oestrogen analogues has been discussed in a number of publications, for example Kavlock et al., 1996, US Environmental Protection Agency (EPA US) 1997, Diamanti-Kandarakis et al., 2009 and environmental guidelines setting contamination limits have been/are being developed by some authorities e.g. EC 1999, EPA US 1996a, EPA US 1996b and more recently Registration, Evaluation and Authorisation of Chemicals (REACH) 2006 and the (EPA US) 2006. (Diamanti-Kandarakis, et al. 2009; Kavlock, et al. 1996). In response to the establishment of these guidelines a number of bioassays that will detect oestrogen and oestrogenic molecules, such as xenoestrogens and phytoestrogens in formats that enable rapid analysis have been developed. Unlike the conventional assay systems such as GCMS and HPLC assay systems, which can identify and quantify each oestrogen in a mixture of estrogens, the bioassays report an oestrogen equivalence (EEQ), which gives the oestrogenic potency of the sample as an equivalent concentration of 17β oestradiol. Importantly, the EEQ value gives a measure of the biological activity of the sample rather than the concentration of oestrogens in the sample. In general these bioassays still require specialised laboratories and highly skilled staff. Assays using mammalian cells and tissues provide the lowest levels of detection but are complex and time consuming while assays based on competitive receptor binding are fast but their detection limits are higher. Genetically modified yeast systems containing a vertebrate oestrogen receptor or its binding region and a reporter gene have been constructed to provide simple alternative assay methods. Most of these assays use the human oestrogen receptor and provide relatively sensitive methods to detect EEQ (Schultis and Metzger, 2004).

S. cerevisiae and several other species of yeast are known to have an oestrogen binding protein (EBP) (Feldman, et al. 1982; Restrepo, et al. 1984; Skowronski and Feldman, 1989) that mediates the oxidation of NAD(P)H to provide $NAD(P)^+$ for the cells catabolic processes. Oestrogens, particularly 17β-oestradiol, can bind to the NAD(P)H oxido-reductase site and

block its function and as a result the concentration of NAD(P)H in the cell rises (Baronian and Gurazada 2007; Madani, et al. 1994).

Baronian and Gurazada, 2007 used *S. cereviseae* as the biorecognition element in a non-genetically modified yeast oestrogen assay. A double mediator electrochemical system comprising ferricyanide and 2,3,5,6-tetramethyl-1,4-phenylenediamine (TMPD) was used to detect NAD(P)H levels in the cell. As the concentration of oestrogen increased, so did the concentration of NAD(P)H and consequently, that of ferrocyanide, which was detected at the end of an incubation period by linear sweep voltammetry. The assay gave responses to other vertebrate oestrogens but not to a synthetic oestrogen. The assay was very sensitive with a linear response from 3.67 x 10^{-13} M to 3.67 x 10^{-9} M and calculated limits of detection and determination of 3.6 x 10^{-15} M and 2.1 x 10^{-14} M respectively with an EC_{50} of 6.25 x 10^{-13} M. This compares favourably with other oestrogen assays, which mostly have limits of detection (LoD) for 17β-oestradiol of between 1 x 10^{-12} M and 5 x 10^{-9} M (Kaiser, et al. 2010; Leskinen, et al. 2005). The response was linear up to 1.1 x 10^{-8} M, however at 2.0 x 10^{-8} M the response plummeted and the downward trend continued at 5.5 x 10^{-8} M. Our explanation for this phenomenon was that the ABC transporters that export oestrogen from the cell are not activated until the concentration of oestrogen rises above 11 nM. Once it has reached this level oestrogen is rapidly transported out of the cell reducing its blocking effect on EBP, an explanation that is supported by the work of Cheng et al., 2006. This limitation on the upper concentration that can be detected is a problem common to all yeast cell assays and generally requires sample dilution to remedy. While the LoDs of many oestrogen assays are suitable for analysis, raising the upper limit would improve the utility of these methods.

The minimum incubation period required for the *S. cerevisiae* assay is around 5 h. While this is very good in comparison with the incubation period required for the Yeast Estrogen Screen (YES) of three days (Routledge and Sumpter, 1996), it cannot be classified as a rapid test. Some recent yeast oestrogen assays have reported shorter incubation periods e.g. 2.5 h in

modified *S. cerevisiae* containing an estrogen receptor with firefly luciferase controlled by a hormone responsive promoter as the reporter (Leskinen, et al. 2005), 0.5 h for an automated 17β-oestradiol immunoassay system (Tanaka, et al. 2004) and 2 min with a carbon nanotube field effect transistor functionalised with oestrogen receptor α measuring bisphenol A (Sanchez-Acevedo, et al. 2009).

EBP will only bind to vertebrate oestrogens, which contrasts with oestrogen assays based on the vertebrate oestrogen receptor element and oestrogen receptor, which respond to oestrogens, xenoestrogens and phytoestrogens (Skowronski and Feldman, 1989). This gives the EBP assay a unique property that can be exploited to measure vertebrate estrogens only, or if used in conjunction with an ERE/ER system, will enable the EEQ of xenoestrogens to be quantified.

Candida. albicans also expresses an EBP and this organism is because its EBP and the corresponding gene are fully characterised (Madani, et al. 1994)

The objective of these investigations was to simplify the use of mediators in the detection of oestrogen/EBP binding, increase the dynamic range of the assay and to reduce the assay period to that of a rapid test.

4.3. Materials and Methods

4.3.1. Cells

C.albicans CDC S-24 was purchased from ESR, Porirua, New Zealand and maintained on YEPD agar plates at 4°C.

4.3.2. Cultivation of cells.

C. albicans was cultivated in YEPD broth, a general-purpose fungal-selective medium (yeast extract 10 g Merck, peptone 110 20 g GibcoBRL, dextrose 20 g BDH Analar, per liter). Cells were incubated at 37°C for 16 hours in indented flasks shaken at 180 rpm, and harvested by

centrifugation (2604 x g, 8 minutes, 10°C), washed twice in phosphate buffered saline (PBS) pH 7 and then re-suspended in PBS pH 7 at OD_{600} = 3.0.

4.3.3. Reagents

Potassium hexacyanoferrate (III) (K_3FeCN_6, Merck pro analysis) was dissolved in distilled water; 0.5 M, 2, 3, 5, 6-tetramethyl-1, 4-phenylenediamine (TMPD, Aldrich) was dissolved in 96% ethanol, 20 mM, and 17β-oestradiol, (Sigma) was dissolved in 96% ethanol, 300 μgL^{-1}. Lyticase from *Arthrobacter luteus*, chitanase from *Trichoderma viride*, β-glucurinidase type H2 from *Helix pomatia and DL*-dithiothreitol were purchased from Sigma. All solutions were sterilised by filtration (0.45 μm Millipore) and stored at 4°C. OECD synthetic waste water was made by mixing160 mg peptone, 10 mg meat extract, 30 mg urea, 28 mg K_2HPO_4, 7 mg NaCl, 4 mg $CaCl_2$, 2 mg $MgSO_4.7H_2O$ in 1 L H_2O (Mean COD concentration 263 mg L^{-1}) (OECD Technical Report, 1976).

4.3.4. Production of cell lysate

A cell suspension OD_{600nm} 3 was pre-incubated for 15 min with 2 mM dithiothreitol and then incubated with 50 mL of lyticase (100 units/mL), 16 units of chitinase, and 200,000 units of glucuronidase and protease inhibitor cocktail (leupeptin (Sigma), pepstatin (Sigma), chymostatin (Sigma), benzamidine (Fluka) and trypsin inhibitor (Fluka)) for 3 h at 30ºC at 50 rpm. Treated cells were harvested by centrifugation for 15 min at 18,514 x g. The supernatant containing the disrupted cells was removed, shaken vigorously for 2 min and passed through 0.45 μm filter (Millipore) to remove any debris. It was stored at 4°C until use.

4.3.5. Experimental procedure

The cell incubation and electrochemical detection methods are those previously described Baronian and Gurazada (2007). The electrochemical analyser was an eDAQ potentiostat coupled to a Power Lab 2/20 controlled by eDAQ Echem software. The electrodes for all experiments unless stated was 100 μm diameter Pt microdisc working electrode, a Ag/AgCl reference electrodeand a Pt wire auxiliaryelectrode, all G-Glass, Australia.

Experimental samples comprising the following were prepared: either 4065 μL whole cell suspension or cell lysate, 800 μL ferricyanide solution (incubation concentration 80 mM), 125 μL TMPD solution (incubation concentration 0.5 mM), 100 μL oestrogen solution (incubation concentrations of 1 nM to 200 nM) and ethanol (100 μL or less in endogenous control groups and oestrogen groups where required). Note the cell lysate of 4065 μL is extracted from an equivalent number of cells that are present in 4065 μL of whole cell suspension.

The experimental samples were incubated at 37°C with oxygen-free nitrogen sparging for 5 h for cell samples and initial lysate samples. Subsequent lysate sample where incubated for 10-120 min. At the completion of incubation, the cells were pelleted by centrifugation (4629 x g, 20°C for 10 min) and the supernatant removed for voltammetric analysis. All trials were run in triplicate and included a negative control to determine the base level endogenous catabolic activity (cell trials) or redox activity (lysate trials) and are referred to as the control in the results.

The supernatant of each sample was analysed in triplicate using LSV. Steady-state voltammograms were obtained at a scan rate of $5mVs^{-1}$ scanning from 450 mV to 130 mV. Steady state currents were established from the LSV plateau current above the half wave potential of the $Fe^{2+/}Fe^{3+}$ couple. This anodic plateau current (quantified at E = 425 mV) was used as a relative measure of the amount of ferrocyanide produced (Baronian et al., 2002) and hence the number of cell redox molecules oxidized by TMPD, either in relation to yeast catabolism and/or the EPB/oestrogen/NADH oxidase interaction. In later lysate experiments TMPD was removed from the incubation mix and ferricyanide only was used to monitor the activity of EBP. The limit of detection was calculated by the blank-value procedure from the blank sample B, the variation of the blank sample sB, and empirical factors. Limit of detection is given by: yL=B+4.65sB and limit of determination is given by yD=B+14.1sB. Error bars are 1 standard deviation and P values were established by ANOVA. OECD wastewater incubations were prepared as follows: 3600 μL cell lysate, 800 μL ferricyanide

solution (final concentration 80mM) and 300 µL PBS. The two controls were 166 µL ethanol, 434 µL PBS, and OECD wastewater 300 µL, ethanol 166 µL 134 µL PBS. Oestrogen trials were 300 µL OECD wastewater, 166 µL ethanol with oestrogen to give final concentrations of 5, 10, 25 nM, and 134 µL PBS.

The HPLC analysis was with an Agilent 1100series HPLC (Agilent, USA) with Chemstation software. The column was a Prodigy 250*4.6 mm reverse phase column (Phenomenex, USA). Solvent A was 0.01M NaH_2Po_4 and B was methanol. The gradient used was 95% A + 5% B Detection was by UV at 260 nm and 340 nm. Injected quantity was 5 µL.

4.4. Results and Discussion

C. albicans whole cells and cell lysate were incubated with ferricyanide and TMPD for 8 h with and without 17β-oestradiol. Fig.4.1 shows that there is a detectable response to the presence of oestrogen in both whole cell and cell lysate trials. In this experiment 10 nM oestrogen was used as the analyte for both whole cells and cell lysate. Cell lysate responses are approximately two times larger than those seen in the whole cell trials.

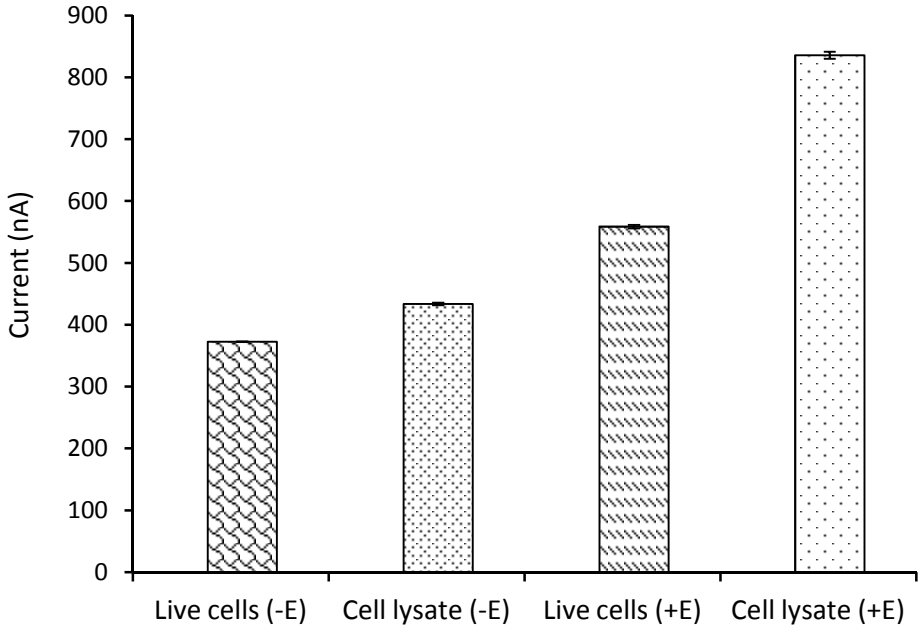

Figure 4.1. *C. albicans* whole cell and cell lysate responses in the presence and absence of 17β-oestradiol 10 nM using a double mediator system (ferricyanide and

TMPD). The data points are the steady state currents obtained by LSV after 5 h incubation. Error bars represent ±1SD.

Whole cells and cell lysate were incubated for 8 h with ferricyanide as the sole mediator. Fig.4.2 shows that there is a small and equal response detectable in whole cells both with and without oestrogen. In the cell lysate however, the responses are much larger than the whole cell responses, both with and without oestrogen, and the with-oestrogen response is larger than the without-oestrogen response.

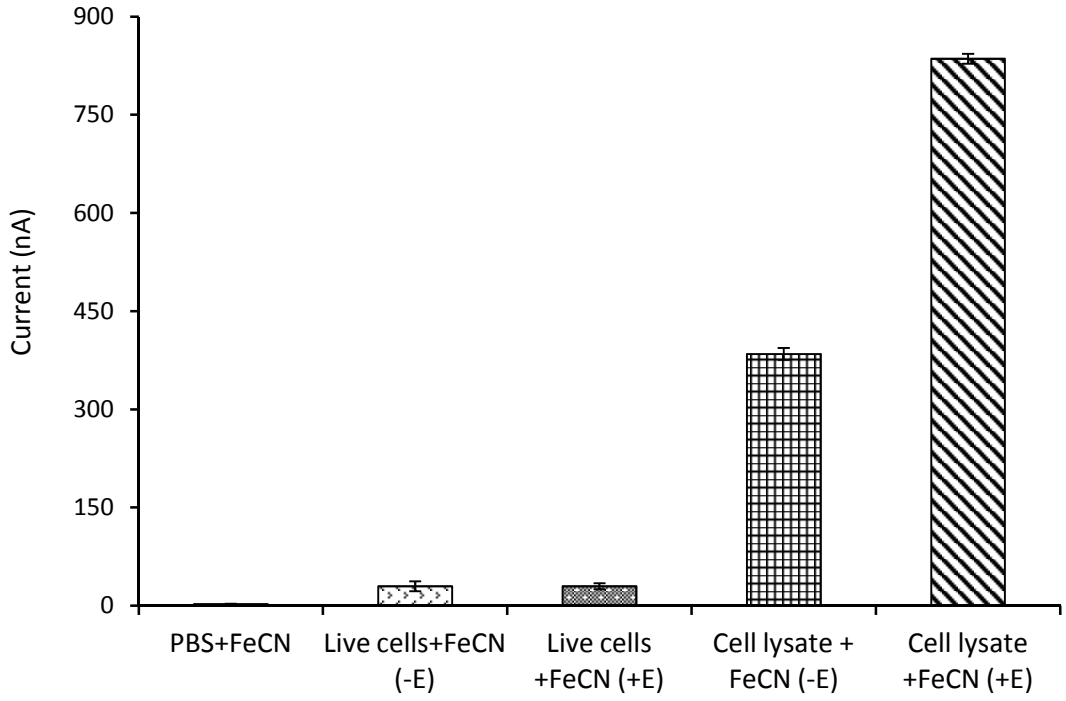

Figure 4.2. *C. albicans* whole cell and cell lysate responses to 17β-oestradiol 10 nM using a single hydrophilic mediator (ferricyanide). The data points are the steady state currents obtained by LSV after a 5 h incubation. Error bars represent ±1SD.

The response time to oestrogen in the lysate system is shown in Fig.4. 3. In the absence of cell structure the response time is reduced and the difference between endogenous catabolism (control) and responses to 10 nM 17 β-oestradiol can be detected at 20 min. In 5 h incubations the difference between the control and sample signal was greater (approximately 500 nA, Fig 1) indicating the divergence of the signals continued beyond 80 min.

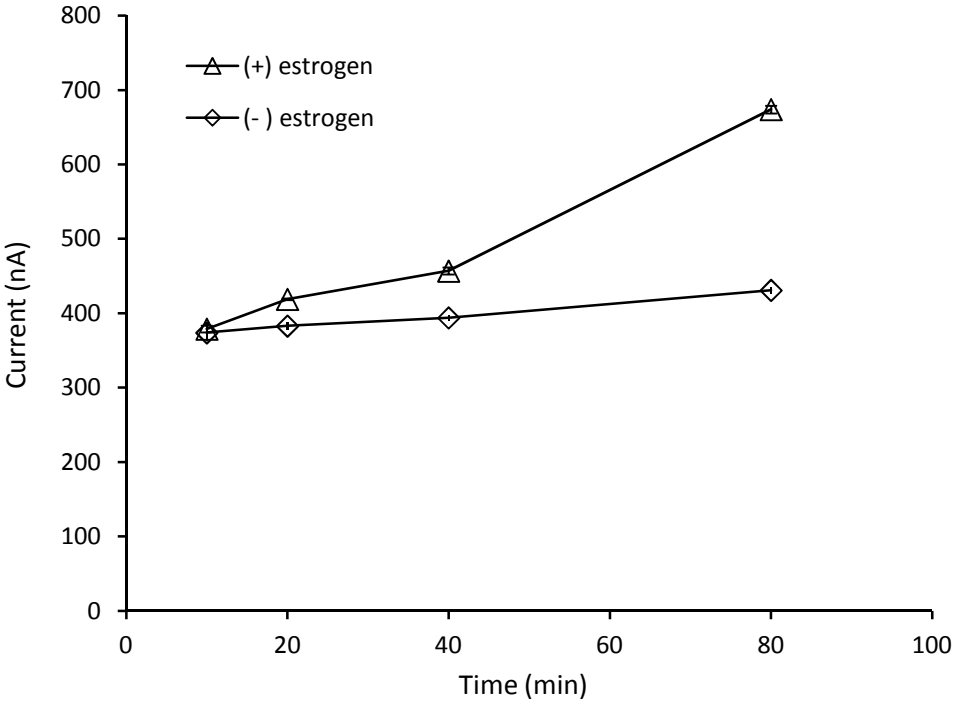

Figure 4.3. *C. albicans* lysate control (endogenous) responses and responses to 17β-oestradiol 10 nM obtained from incubations ranging from 10 to 80 min. The data points are the steady state currents obtained by LSV. Error bars represent ±1SD

HPLC analysis for NADH showed that its concentration in cell lysate with oestrogen was approximately twice that in cell lysate without oestrogen, which supports the role of oestrogen in EBP function outlined in the introduction.

The plot of the cell lysate responses to oestrogen shown in Fig. 4.4 is a composite of two experiments exploring the lower and upper ranges of the dose response curve. These ranges are at the lower limit to beyond the upper limit of the S. cereviseae whole cell response range (Baronian and Gurazada, 2007). The calculated limit of detection is 0.005nM with a limit of determination of 0.014 nM. The control responses have been subtracted from the responses due to oestrogen.

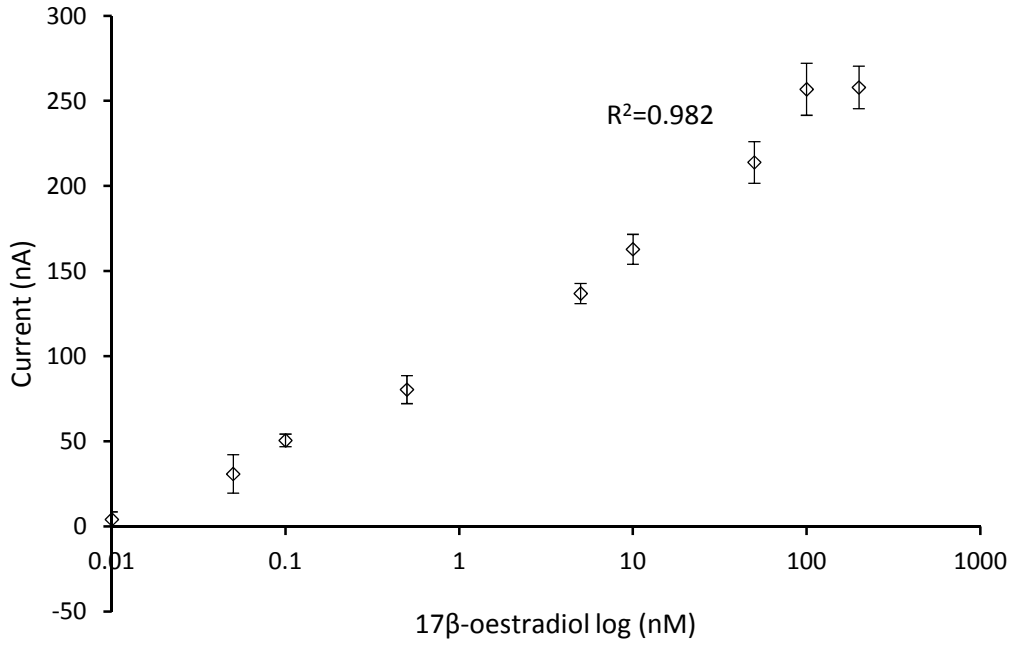

Figure 4.4. *C. albicans* cell lysate responses to 17-β oestradiol were explored in two experiments, 0.01–0.5nM and 5–200nM oestrogen. Both experiments are plotted. Responses due to the background (controls 342 nA and 315 nA, respectively) have been subtracted from the oestradiol responses. Error bars represent ±1SD.

Fig.4.5a shows that the response to oestrogen in both cells and cell lysate declines over 20 days. However the endogenous response of whole cells remained almost constant over the same period suggesting that the loss in response is due to the loss of activity of EBP (Fig.5.b).

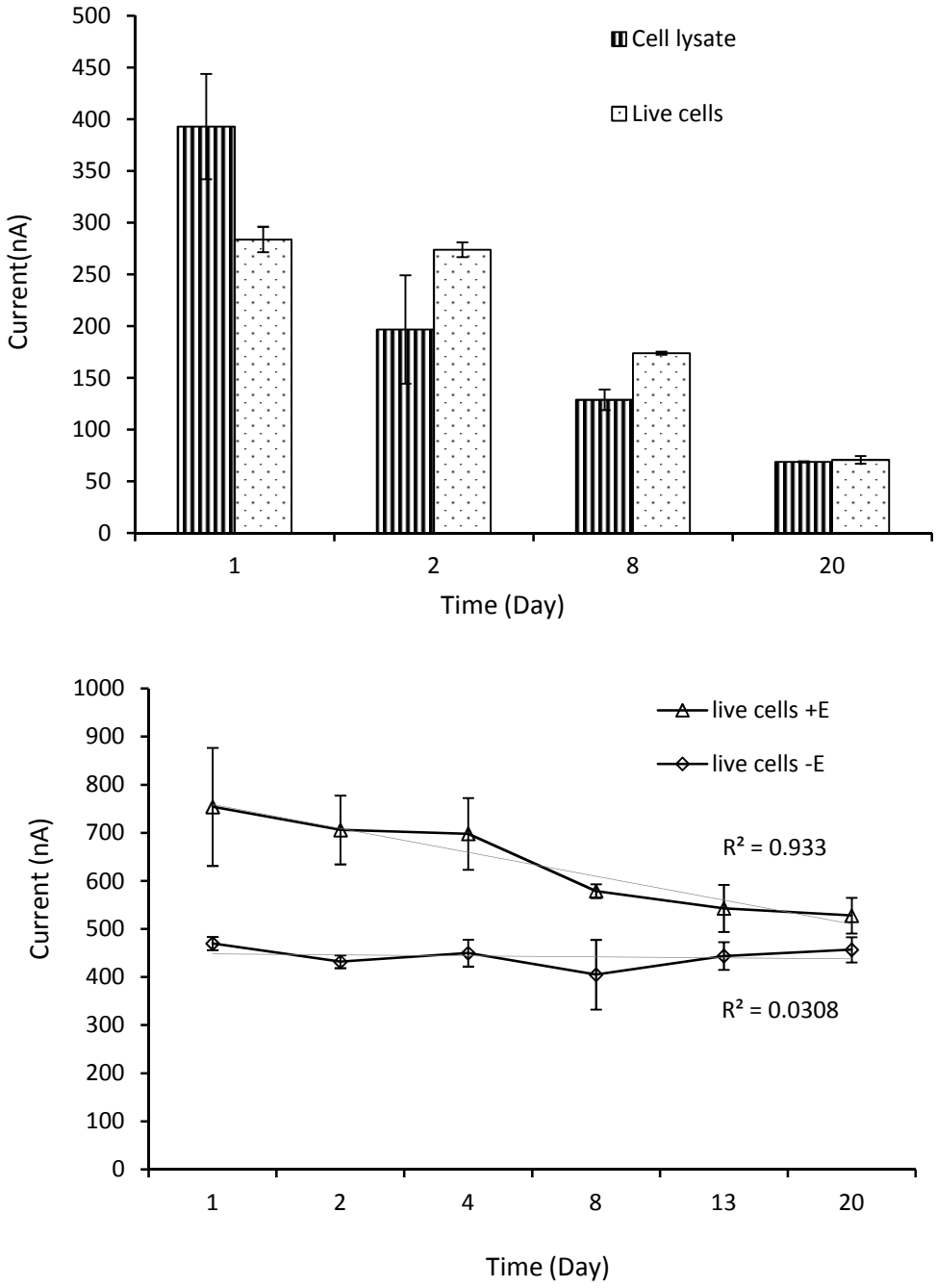

Figure 4.5. (a) *C. albicans* whole cell and cell lysate responses to 17β-oestradiol 10 nM over 20 days (b) Stability of the oestrogen response in whole cells over 20 days. The cell response is relatively constant over 20 days while the oestrogen response declines over the same period. Error bars represent ±1SD.

OECD synthetic wastewater was spiked with oestrogen and LSVs were performed after incubation with lysate. Fig.6 shows that increasing concentrations of oestrogen are detectable

in this complex matrix and that there is no detectable background response to the synthetic wastewater alone

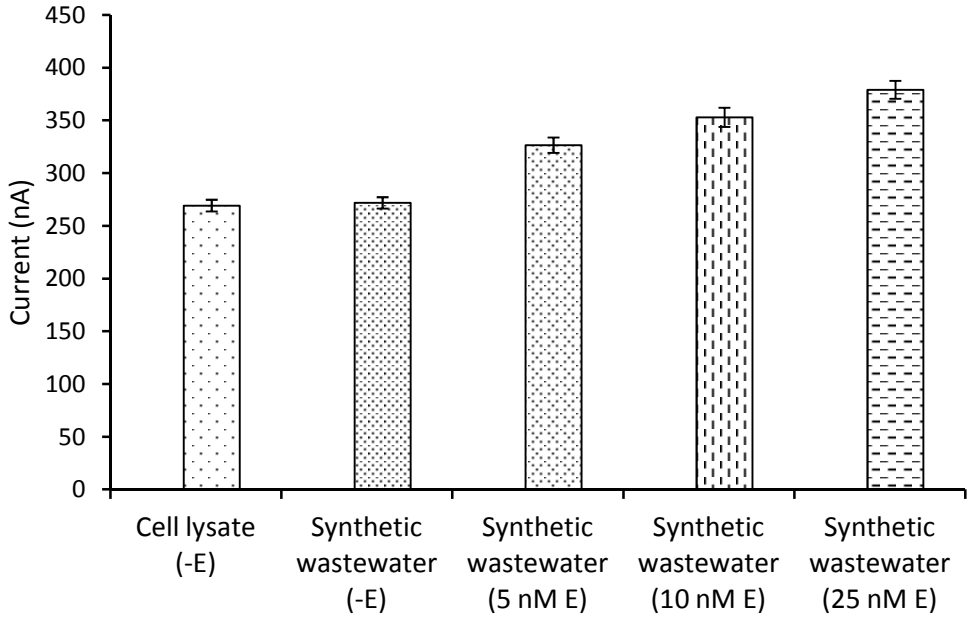

Figure 4.6. Responses to oestrogen (5, 10 and 25 nM) in an OECD synthetic waste matrix, CoD = 263 mgL^{-1}). Responses from the lysate and lysate plus OECD synthetic wastewater are the same (P = 0.6348).

Baronian and Gurazada (2007) used a double mediator system comprising ferricyanide and TMPD with *S. cereviseae* whole cells for the detection of oestrogen. TMPD is lipophilic mediator that can enter the cell and be reduced by NAD(P)H and redox molecules of the electron transport chain. Reduced TMPD exits the cell and reduces hydrophilic (and therefore extracellular) ferricyanide to ferrocyanide. The ferrocyanide is detected electrochemically with the signal directly related to the number electrons removed from the redox molecules oxidized by TMPD, either in yeast catabolism and/or the EPB/oestrogen/NAD(P)H interaction. The maximum current detected was at 11 nM oestrogen, which is a similar concentration to maximum signals reported for other whole cell oestrogen assays (Beresford, et al. 2000; Fine, et al. 2006; Leskinen, et al. 2005; Rehmann, et al. 1999). This work was repeated with *C. albicans* to establish its response parameters to oestrogens before

undertaking *C. albicans* lysate trials. The responses were observed with *C. albicans* whole cells were similar to those reported for *S. cereviseae.*

Figure 1a shows that cell lysate responses 17β-oestradiol are much higher than the response seen with whole cells. The higher signals are probably due to the absence of a cell membrane, which exerts rate limitations on the entry of oestrogen into the cells and perhaps on the export of reduced TMPD. Removal of this barrier permits the reactants to behave as molecules in solution and increases the overall rate of reaction as barriers to diffusion no longer exist.

Yeast cells have, in addition to the intracellular redox centres, transmembrane electron transport proteins (tPMETs, also known as plasma membrane oxidoreductases, PMOR), which transfer electrons from the cell interior to the external surface of the cell membrane. Common electron donors are NADH and NADPH and common extracellular acceptors are Fe^{3+} (uptake of iron), oxygen (maintenance of catabolism) and ascorbate free radical (AFR) defence systems (Ly and Lawen, 2003). Fig.4.2 shows the cell signals with ferricyanide are small and are the same with and without oestrogen. This is because the hydrophilic ferricyanide can only access the external component of tPMETs and cannot cross the cell membrane to access catabolic redox molecules. The use of ferricyanide with the lysate however produces a response equivalent to that seen in the 'with' and 'without' oestrogen double mediated whole cell trials because it is not excluded by a cell membrane. This is a simplification of the assay in that only a single hydrophilic mediator is required.

There is dramatic decrease in the signal at high 17β-oestradiol concentration in the *S. cereviseae* oestrogen bioassay (Baronian and Gurazada, 2007). This decrease is also seen in hER/ERE modified yeast cells although the decrease is less dramatic (Beresford, et al. 2000; Schultis and Metzger, 2004). The reason is not clearly understood, however Cheng et al., 2006 state that microarray studies show that increased 17β-oestradiol increases the transcription of the genes *CDR1* and *CDR2*, which seem to be related to an increase the production of efflux pumps which remove 17β-estradiol from *C.albicans* cell. Removal of the

cell membrane allows EBP/oestrogen/NADH interactions to proceed without concentration limiting cell membrane regulatory mechanisms, which increases the dynamic range of the assay to 100 nM.

These experiments have been successful in significantly reducing the incubation time to make it comparable with the most rapid oestrogen assays reported to date. Fig.4.3 suggests that it may be possible to reduce this period further by increasing the concentration of EBP, i.e. the time taken to enable the detection of the response is concentration dependant and not event dependant.

While the oestrogen response in both whole cells and cell lysate declines over twenty days (Fig.4.5a) the control response of whole cells (Fig.4.5b) and lysate remain relatively constant over the same period. This indicates that the loss of oestrogen response is due to the loss of EBP activity. Use of EBP in a long use-by-date sensor will thus require stabilisation of the molecule.

The whole cell assay referred to earlier was sensitive to background catabolic molecules and controls were required to remove this effect. In lysate experiments with OECD synthetic waste as the background there was no response to the to the waste but quantitative responses to oestrogens were clearly detectable. This is a further simplification over the whole cell assay in that the effect of the background on the oestrogen signal is reduced.

The assumptions that oestrogen blocks the reduction of NADH by EBP and that NADH is the source of electrons for both TMPD in whole cell experiments and ferricyanide in lysate experiments is supported by the HPLC analysis. NADH concentration in lysate samples is higher in lysate with oestrogen than in lysate without oestrogen (data not shown). This difference corresponds to the differences between the ‘with’ and ‘without’ oestrogen response in the double and single mediator trials shown in Fig.1a and 1b and supports the notion that NADH a source of electrons for mediator reduction.

Lysate detection of oestrogen in a complex substrate is shown in Fig 6. The oestrogen responses are clearly distinguishable from the lysate only and synthetic waste controls. There is also no difference between the two controls (P = 0.6348) indicating that disruption of the cell has prevented a catabolic response to the waste. This is a distinct advantage over the whole cell assay where the background catabolic response had to be regulated with glucose. (Baronian and Gurazada, 2007)

4.6. Conclusions

This work has successfully transferred a method for detection of oestrogens using a wild type *S. cereviseae* to a wild type *C. albicans* as the assay catalyst. The use of cell lysate from *C. albicans* has reduced the assay time to minutes and increased the dynamic range 10 fold. The assay can also be used in complex matrices without interference from catabolic substrates. Furthermore we have demonstrated that lysate stored at 4º C is sufficiently active over 8 days for use in analysis.

4.6. Co-author contributions

This chapter has been published in Biosensors and Bioelectronics. Besides supervisory committee members, the Co-authors of this paper are Frankie Rawson, Alison Downard and Gotthard Kunze. Alison, Frankie and Gotthard provided technical advice and are involved in manuscript correction.

Chapter 5
Investigating electron transfer from oestrogen binding protein

5.1. Abstract

I have previously demonstrated the use of *C. albicans* whole cells and cell lysate for detection of 17*β*-oestradiol and suggested that the molecule responsible for the electrochemically detected oestrogen dependant response was oestrogen binding protein (EBP). The shift from whole cells to cell lysate permitted the use of a single hydrophilic mediator, reduced the incubation time, expanded the dose response curve and avoided catabolic responses to sample molecules. Here I describe the purification of EBP using 17β-oestradiol affinity chromatography, and the use of the protein with NADH in an oestrogen bioassay. NADH was electrochemically detected directly and could differentiate 'with' and 'without oestrogen' samples. I have also established that a mediator can interact directly with EBP i.e. without the use of NADH. Further, we have demonstrated direct electron transfer from EBP to both glassy carbon and pyrolyzed photoresist film (PPF) electrodes.

Keywords: Estrogen Binding Protein, NADH detection, mediated electrochemical detection, direct electron transfer.

5.2. Introduction

Electrochemical properties of redox enzymes can be investigated using a number of electrochemical techniques (Bistolas, et al. 2005). Few proteins are known to exhibit direct electron transfer, for example cytochrome c, microperoxidase, peroxidises, and PQQ-enzymes (Gorton, et al. 1999). In other cases a mediator is used to transfer electrons either from the enzyme itself or from a product of the redox reaction. Both of these approaches to electron

transfer from enzyme to electrode have the potential to produce high performance sensors with excellent selectivity and sensitivity due to specific interactions between biomolecules and substrates (Freire, et al. 2003).

Iannielo et al., 1982 has demonstrated direct electron transfer between the active centres of flavoenzymes (L-amino oxidase and xanthineoxidase) to a modified electrode surface. These enzymes contain flavinadenonuclotide (FAD) as the prosthetic group, which can be reduced at a graphite electrode. L-amino oxidase is a simple flavoenzyme with one FAD moiety responsible for its enzymatic activity where-as xanthine oxidase comprises two FAD moieties which act as independent redox centres and have complex interactions with iron, sulphur etc near the active site. This enzyme gives larger reduction peaks than L-amino oxidase because of the dual FAD sites per molecule (Ianniello, et al. 1982).

Proteins tend to denature and to also passivate unmodified electrodes, however electrical contact is possible with proteins that are involved in electron transport pathways (Bistolas, et al. 2005). Although direct and mediated electron transfer from a protein is a very good approach for developing biosensors, only a few oxido-reductases have been used to construct biosensors (Freire, et al. 2003).

Old yellow enzyme (OYE) of *Saccahromyces cerevisieae* belongs to the flavoprotein family. This protein has been extensively studied for more than 60 years and its crystal structure has been determined. It binds with various efficiencies to phenolic compounds (Karplus, et al. 1995). Despite extensive studies into its properties and structure, the physiologic role of this enzyme remains unknown (Fox and Karplus 1994; Karplus, et al. 1995). Crystal structures of oxidised and reduced OYE reveal that p-hydroxybenzaldehyde, 17β-oestradiol and an NADPH analogue bind at a common site on the flavin (Fox and Karplus 1994; Karplus, et al. 1995). EBP in *C. albicans* has some similarities to OYE in that it shares NADPH oxido-reductase properties and binds to phenolic compounds.

The role of EBP in the pathogencity of *C. albicans* and *Paracoccoides brasilensis* (Clemons, et al. 1989) is well known but there are no studies on the structure of EBP. In a recent publication I proposed a mechanism of oestrogen binding protein activity (submitted) and I have successfully used *C. albicans* whole cells and cell lysate as a bio-recognition element for detecting oestrogen in double and single mediated electrochemical systems (Baronian and Gurazada, 2007; Chelikani, et al. 2011). I have designed and performed experiments to investigate mediated and direct electron transfer from the purified protein, both with and without added NADH. Assuming the NADH and oestrogen binding sites are the same, oestrogen will bind to the oxido-reductase site and prevent the oxidation of NADH to NAD^+ leading to higher level of NADH in presence of oestrogen resulting in an increase in reduced mediator and thus higher electrochemical signals. Both direct electron transfer and mediated electron transfer without NADH will result in a smaller signal in the presence of oestrogen.

The objective of this research was to further simplify the cell lysate oestrogen assay by purifying EBP and establishing a more simple robust method of detecting the electronic events that occur when oestrogen binds to its binding site.

5.3. Materials and Methods

5.3.1. Cells

C. albicans CDC S-24 was purchased from ESR, Porirua, New Zealand and maintained on YEPD agar plates at 4 °C.

5.3.2. Cultivation of cells

C. albicans was cultivated in YEPD broth, a general-purpose fungal-selective medium (yeast extract 10 g Merck, peptone 110 20 g GibcoBRL, dextrose 20 g BDH Analar, per liter). Cells were incubated at 37 °C for 16 h in indented flasks shaken at 180 rpm, and harvested by centrifugation (2604 × *g*, 8 min, 10 °C), washed twice in phosphate buffered saline (PBS) pH 7 and then re-suspended in PBS pH 7 at OD_{600} = 3.0.

5.3.3. Reagents

Potassium hexacyanoferrate (III) (K_3FeCN_6, Merck pro analysis) was dissolved in distilled water; 0.5 M, 2-methyl-,4-naphthoquinone (menadione, Sigma) was dissolved in 96% ethanol, 20 mM, and 17β-oestradiol (Sigma) was dissolved in 96% ethanol, 300 μg L^{-1}. Lyticase from *Arthrobacter luteus*, chitanase from *Trichoderma viride*, β-glucurinidase type H2 from *Helix pomatia* and DL-dithiothreitol were purchased from Sigma. All solutions were sterilised by filtration (0.45 μm Millipore) and stored at 4 °C.

5.3.4. Production of cell lysate

Cell lysate production was as described by Chelikani et al., (2011). A cell suspension OD_{600nm} 3 was pre-incubated for 15 min with 2 mM dithiothreitol and then incubated with 50 mL of lyticase (100 units/mL), 16 units of chitinase, and 200,000 units of glucuronidase and a protease inhibitor cocktail (leupeptin (Sigma), pepstatin (Sigma), chymostatin (Sigma), benzamidine (Fluka) and trypsin inhibitor (Fluka)) for 3 h at 30 °C at 50 rpm. Treated cells were harvested by centrifugation for 15 min at 18,514 × *g*. The supernatant containing the disrupted cells was removed, shaken vigorously for 2 min and passed through 0.45 μm filter (Millipore) to remove any debris. It was stored at 4 °C until use.

5.3.5. Protein purification

Cell lysate or supernatant of cells ground using 0.5mm glass beads was passed twice through PD10 DEAE desalting columns (GE Health Science). The second (Madani, et al. 1994) eluate was collected for oestradiol affinity chromatography. A 1 x 1 cm column containing 17β-oestradiolagarose was prepared using a Pharma-Link Immobilisation Kit (Thermo-Fischer Scientific). The concentrated DEAE column eluate was applied to the columns and incubated at ambient temperature for 1 h. After incubation, the column was washed with 20 mL wash buffer from the kit to remove unbound proteins and EBP was then eluted from the resin with 6 mL of KTED buffer (10 mM Tris adjusted to pH to 7.5 with HCl, 1.5 mM EDTA, adjusted to pH to 8 with NaOH, 1 mM dithiothreitol and 0.6 M KCl).

5.3.6. Electrochemistry

The electrochemical analyser was an eDAQ potentiostat coupled to a Power Lab 2/20 controlled by eDAQ Echem software. Linear sweep measurements were made with a 50 μm diameter Pt microdisc working electrode, a Ag/AgCl reference electrode and a Pt wire auxiliary electrode, all G-Glass, Australia. Cyclic voltammetry experiments were done using a 3 mm glassy-carbon electrode working electrode from Tianjin AiDaHengsheng Technology Co., Ltd, China.

5.3.7. Glassy carbon-osmium complex electrodes.

Osmium complex modified glassy carbon electrodes were prepared by the method described by Rawson et al. (Rawson, et al. 2011).

5.3.8. Experimental procedure.

Experimental samples comprising the following were prepared for linear sweep experiments: 2032.5 μL semi-purified protein solution (0.28 μg/ml), 400 μl ferricyanide solution (incubation concentration 80 mM), 62.5 μl menadione solution (incubation concentration 0.5 mM), 50 μL oestrogen solution (incubation concentrations of 10 nM) and ethanol (50 μL or less in endogenous control groups and oestrogen groups where required). All components were added in the above order. In other experiments samples were prepared comprising of 2032.5 μL semi-purified protein solution (0.28 μg/ml) and 50 μL ethanol or oestrogen solution.

Semi-purified protein for direct detection of NADH experiments were incubated at room temperature sparged with O_2-free N_2 for 5 min. Direct detection of NADH was by cyclic voltammetry at 5 mV s^{-1} using a 3 mm glassy-carbon electrode from Tianjin AiDaHengsheng Technology Co., Ltd, China. Experiments with the semi-purified protein required the addition of NADH because it was removed during the preparation of the semi-purified protein.

5.4. Results and Discussion.

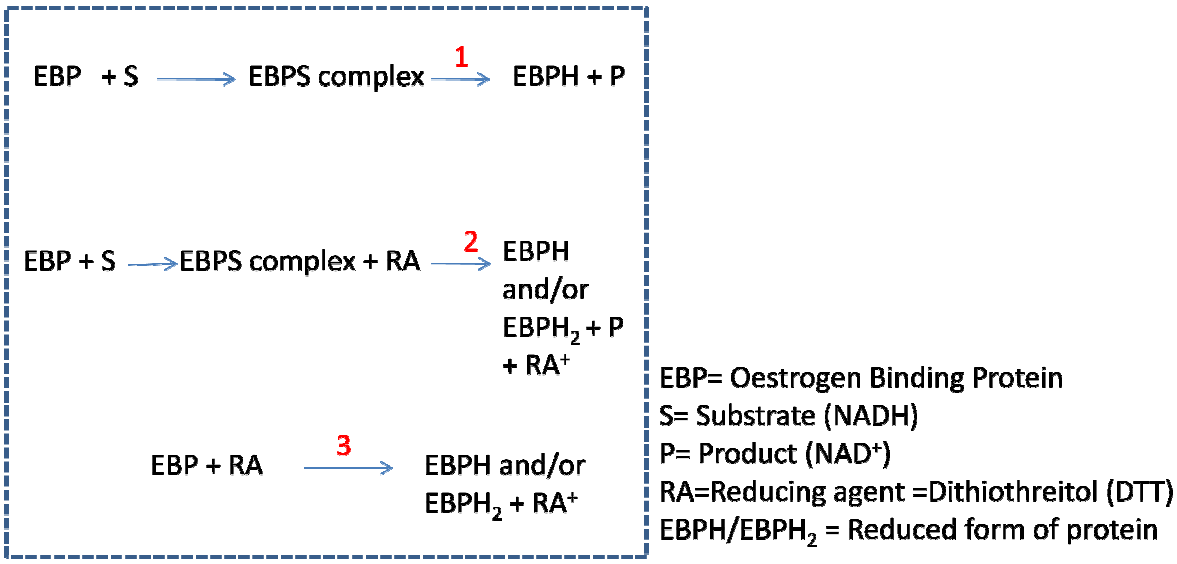

Figure 5.1. Proposed mechanism of EBP in 1) whole cells, 2) Cell lysate or protein provided with NADH and 3) semi purified protein

5.4.1. Proposed mechanism of EBP

Fig.1.shows a proposed mechanism for EBP behaviour in whole cells, cell lysate or semi purified EBP solution provided with NADH and just semi purified protein. In the whole cell system EBP is involved with conversion of NAD(P)H (substrate) to $NAD(P)^+$ (product). When oestrogen is present, the conversion is inhibited because oestrogen binds to same site as the substrate. Thus in the cell system oestrogen is indirectly quantified by detecting NAD(P)H using mediators (Baronian and Gurazada, 2007; Chelikani, et al. 2011). The same detection method was applied to cell lysate and semi purified protein provided with NADH except that NADH was detected directly, i.e. without mediators. Dithiothreitol (DTT), was used in preparation of cell lysate and semi purified protein. It is a strong reducing agent and reduces EBP. In case of protein, without any addition of NADH, DTT is acting as sole reducing agent and EBP is believed to be transferring electrons when oestrogen not present in the solution

due to reduction by DTT. Reduced EBP can be oxidised by the potential of the electrode and can be visualised by cyclic voltammetry. The reduced form of the enzyme may react with oxygen to regenerate the oxidised form of the enzyme and hydrogen peroxide. In xanthine oxidase, amplified consumption of oxygen has been observed in the presence of DTT which supports this proposition (Hasebe, et al. 1995). In these experiments the incubation mix was sparged with oxygen-free nitrogen to ensure this reaction did not occur. Whether the reduced form of the enzyme is EH or EH_2 is not clear. It is likely that EH is produced initially and is subsequently reduced to EH_2 by DTT. This mechanism is based on the mechanism proposed for xanthine oxidase by Hasebe et al. (Hasebe, et al. 1995).

The limited response range of the cell based assay was overcome using cell lysate which gave a linear response up to 100 nM (Chelikani, et al. 2011). Presumably this is because the efflux pumps are non-functional and unable to remove oestrogen from the EBP environment. The assay could be further simplified by the direct detection of NADH in the cell lysate.

The presence of NADH in the lysate was first confirmed by HPLC and then electrochemical detection was attempted. The best results obtained were with an osmium complex modified glassy carbon electrode. While the detection of NADH on the first scan was good, subsequent scans were impaired. This is probably due to electrode fouling, presumably by a component(s) of the cell lysate. Fabrication of an osmium complex modified electrode using screen printed carbon may allow the construction of a single-use electrode, albeit more expensive than single use electrodes costructed without osmium

5.4.2. Protein Purification

To avoid the problem of electrode fouling, EBP was purified using oestradiol affinity chromatography.

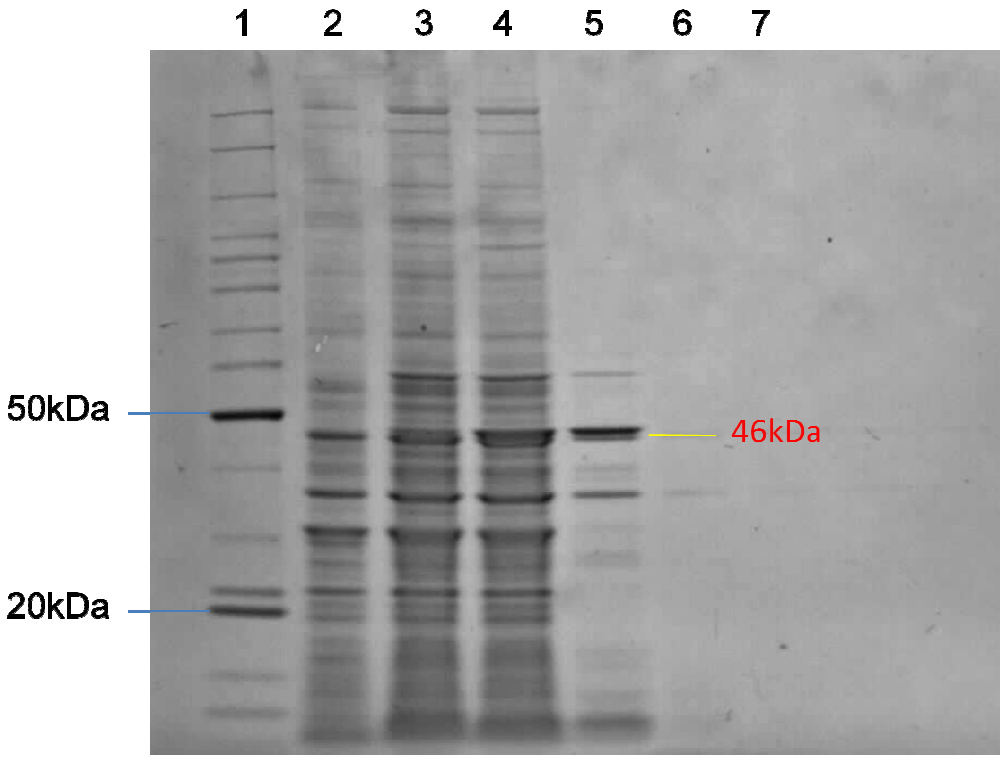

Figure 5.2. SDS-PAGE gel of the *C. albicans* EBP purification. Samples from the protein purification procedure were denatured and subjected to SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R250. Lanes: 1, Nupage benchmark Protein Ladder; 2, PD10 Column eluate after passing through oestradiol affinity column; 3&4, eluate from PD10 columns 5, first eluate from oestradiol affinity column; 6, final eluate from oestradiol affinity column; 7, control (distilled water).

After cell lysis, PD10 desalting columns and oestradiol-linked agarose affinity chromatography was used to purify the protein. The cell lysate was first passed through DEAE-Sephacel ion exchange chromatography columns to produce a flow-through fraction containing the EBP and other proteins. This fraction was then passed through an oestradiol affinity column. The EBP that was bound to the oestradiol resin was then eluted using a KTED buffer. The SDS-polyacrylamide gel of the sequential steps used in the purification scheme is shown in Fig.5.2. Multiple bands are detected using Coomassie Brilliant Blue staining in the PD10 column elutes; however, the number of bands was reduced after oestradiol affinity chromatography. The dominant band present in the sample eluted from the oestradiol affinity column (Fig.5.2, lane 5). has molecular mass of approximately 46 kDa which in agreement with the findings of Skowronski & Feldman, 1989 who identified a 46

kDa band as EBP (Madani, et al. 1994; Skowronski and Feldman, 1989). These results demonstrate that the oestradiol affinity column specifically binds EBP, which can then be isolated as pure protein.

5.4.3. Direct detection of NADH

Direct detection of NADH as an indicator of oestrogen concentration would simplify the assay; however large oxidation over-potential is required for its quantitation by cyclic voltammetry. For a discussion on problems associated with the electrochemistry of NAD(P)H see Radoi and Compagnone (2009). Despite these problems a glassy carbon (GC) electrode was used successfully to detect NADH in the semi-purified protein preparation. A sample voltammogram is shown in Fig.5.3. A difference can be seen between the sample without oestrogen where less NADH was available for electro-oxidation because EBP was also oxidising it, and the trial with oestrogen where oestrogen blocks the NADH oxido-reductase site on EBP making more NADH available for electro-oxidation. The over-potential that is optimal for the oxidation of NADH in complex matrices is considered to be between −100 and +200 mV vs. Ag/AgCl (Gorton and Domınguez 2002) and I am working to improve our electrodes to achieve this specification so that the method can be used with ‘real’ samples. This approach has the potential to further simplify this assay.

Direct detection of NADH in the semi-purified protein preparation was by cyclic voltammetry with a GC electrode Fig5.3. Although the over-potential is high, the technique clearly differentiates between the semi-purified protein with and without oestrogen. A negative control of semi-purified protein preparation only did not have any peaks and a control of NADH only produced a peak at the same potential indicating that there are no redox molecules in the semi-purified protein preparation that interfere with the NADH peak.

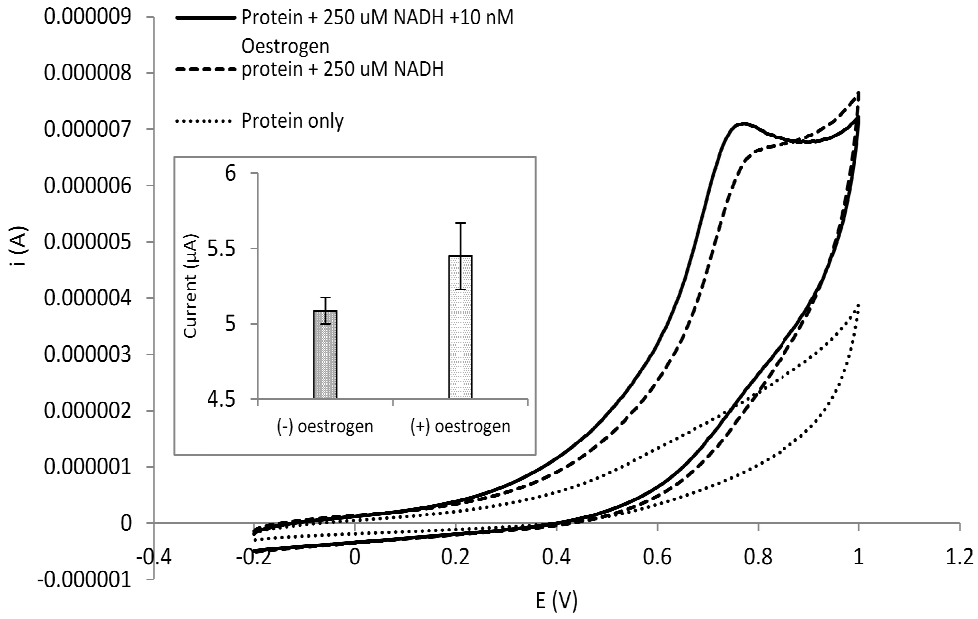

Figure 5.3. Detection of an oestrogen response in the eluate from an oestradiol affinity column by direct voltammetric detection of NADH. The scan rate is 5 $mVsec^{-1}$. The inset graph is histograms of mean peak currents measured from the extrapolated base line to the peak. The histograms show that there is a significant difference between the 'without' and 'with' oestrogen CVs. Error bars represent ±1SD, P = 0.0171. All scans were in triplicate.

5.4.4. Oxidation peaks were observed with glassy carbon and PPF (Fig.5.4a & 5.5b.) with EBP solution sparged with oxygen-free nitrogen. A peak is not observed in the protein elution buffer i.e. the control. (Fig.5.4c).

Strong reducing agents like dithionite are known to reduce proteins (Lambeth and Palmer, 1973). Interestingly Hasebe et al., 1995, observed that the combination of some oxidases such as xanthine oxidase and suitable reducing agents, for example DTT, leads to the redox recycling of the substrate i.e. enzymatically oxidized product is reduced by DTT back to the original substrate and then reoxidized. In this system the signal is amplified due to recycling of substrate. Flavin adenine dinucleotide (FAD) is a cofactor of the active site of xanthine oxidase and catalyzes oxidation of hypoxanthine and xanthine to uric acid (Hasebe, et al. 1995). Hasebe et al., 1995 also suggested that probably not only xanthine and hypoxanthine

but DTT also reduced the protein which reacts with oxygen, regenerating the protein with conversion of oxygen to hydrogen peroxide or superoxide anion (Ballou, et al. 1969; Massey, et al. 1969) (Ballou, et al. 1969; Massey, et al. 1969). Oxygen consumption was observed to increase with the addition of DTT (Hasebe, et al. 1995). We have also observed that DTT, which is component of oestrogen affinity column elution buffer, reduces EBP leaving it in reduced form (anaerobic conditions were maintained by sparging with oxygen-free nitrogen so that protein could not be oxidised by oxygen). It is also possible that the electrode is reducing the protein but reduction peaks are not detectable by non-modified electrodes such as GC and PPF. (There is evidence in literature that xanthine oxidase gives strong reduction peak at -500 v with modified graphite electrodes, suggesting that flavoenzymes can be reduced by electrode itself).

Glassy carbon electrodes modified with osmium complex were able to detect EBP oxidation and reduction peaks (Fig.5.5). Interestingly an oxidation peak at the same potential that is visible in unmodified glassy carbon and PPF electrodes (5.4a & 5.4b) is also visible with a glassy carbon electrode modified with osmium (Fig 5.5). It is possible that EBP is interacting with both the osmium complex and directly with the electrode giving the expected osmium peak and a second peak that is direct electrode oxidation of EBP. This provides evidence that EBP solution is in the reduced state (probably reduced by DTT) and transfers electrons when EBP interacts with electrode.

This is consistent and suggests that there is a direct electron transfer from EBP to electrode.

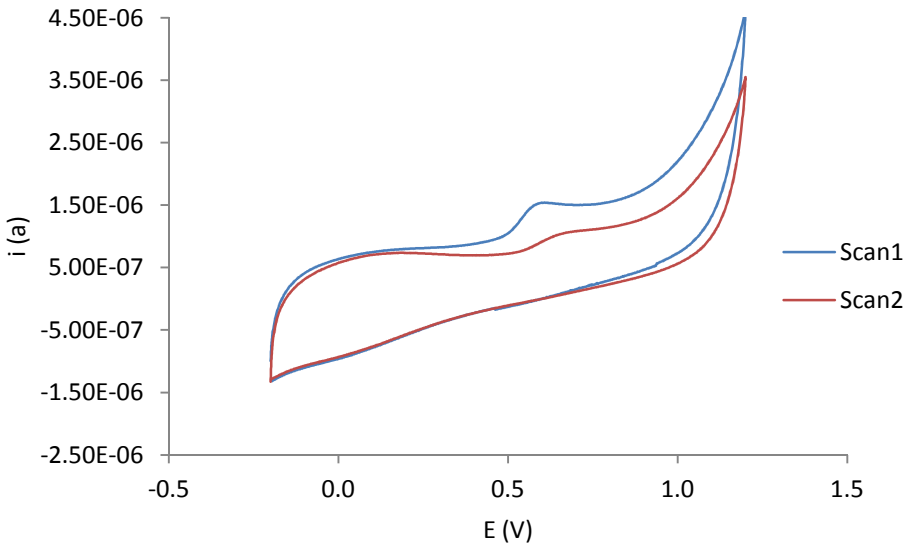

Figure 5.4a. CVs from +0.8V to -0.2V at 5 mV s^{-1} of EPB solution using glassy carbon working electrode, purged with nitrogen.

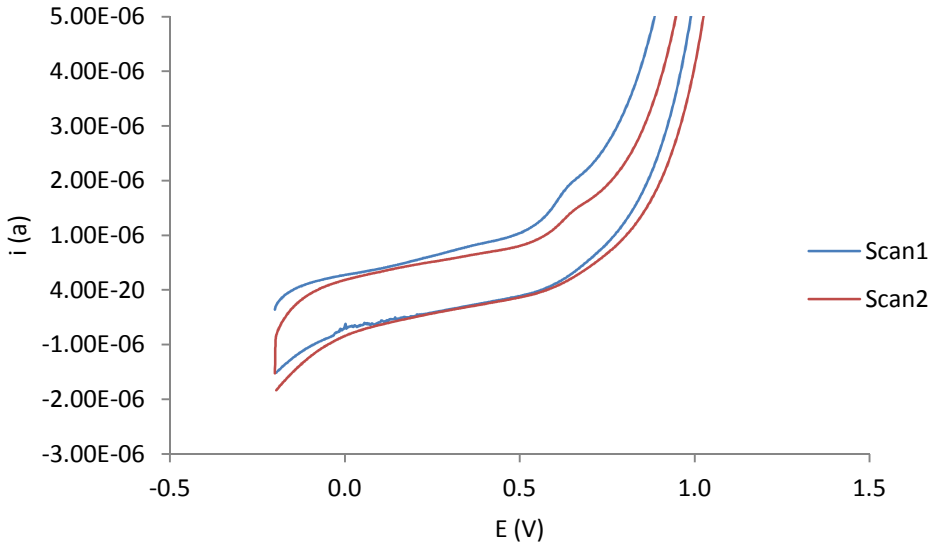

Figure 5.4b. CVs from +0.8V to -0.2V at 5 mV s^{-1} of EPB solution using PPF working electrode, purged with nitrogen.

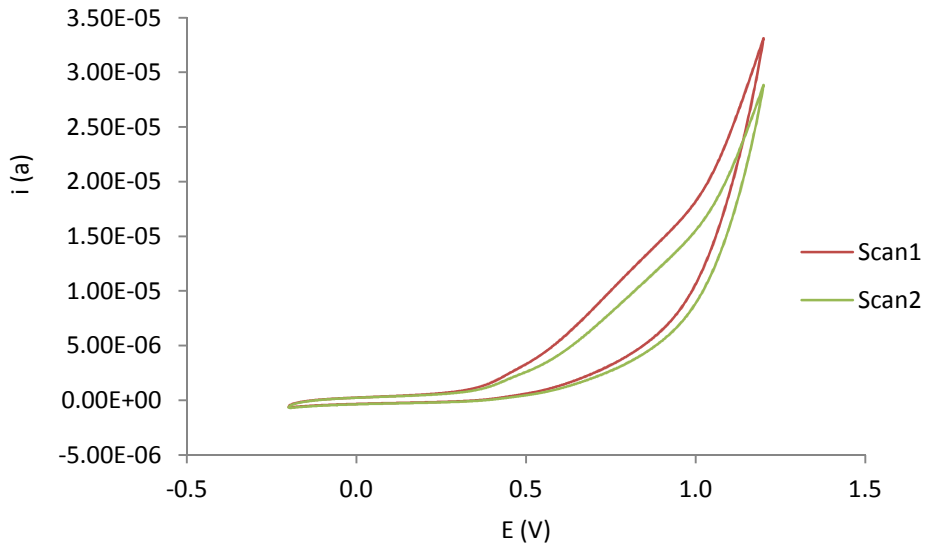

Figure 5.4c. CVs from +0.8V to -0.2V at 5 mV s^{-1} of protein elution buffer using glassy carbon working electrode, purged with nitrogen.

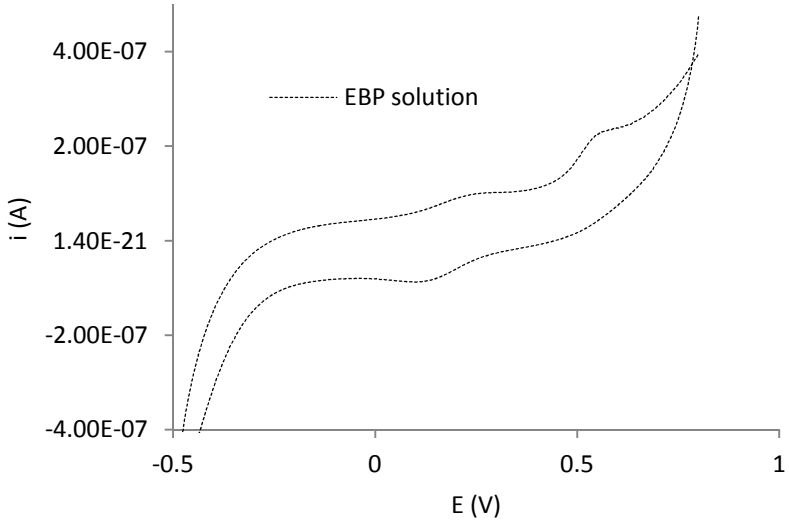

Figure 5.5. CVs from 0.8V to -0.5V at 5 mV s^{-1} of EPB solution purged with nitrogen with an Osmium complex modified glassy carbon electrode.

5.4.5. Double mediator electrochemical system

I hypothesize that there is a common active site in EBP which binds to both oestrogen and NAD(P)H. It is also capable of transfering the electrons to an electrode. Blocking the active site of EBP with oestrogen, reduces the transfer of electron from EBP to the electrode. To clearly establish this phenomenon, we used a double mediator electrochemical system using

menadione which known to directly interact with proteins and is used for detecting direct electron transfer in cytochrome P450 (Bistolas, et al. 2005). Ferricyanide, a hydrophilic mediator was used to report the reduction of menadione because its detection at an electrode is straightforward. In this double mediator electrochemical system menadione is reduced by EBP which in turn reduces ferricyanide to ferrocyanide. Ferrocyanide can be measured electrochemically. Conversely, in the presence of oestrogen, EBP is prevented from interacting with Menadione so there will be a smaller signal. It is possible however that some reduction of mediators may occur due to presence of DTT.

This contrasts with the experiments involving EBP supplied with NADH because oestrogen blocks the oxidation of NADH causing NADH concentration to increase with increasing oestrogen concentration. Thus the detected current rises as the concentration of oestrogen increases.

Our preliminary data suggests that there is an interaction of menadione with EBP which results decrease in the signal in the presence of oestrogen. This hypothesis needs to be tested in future research. The future direction of this research could also involve establishing the stability of the protein and immobilizing the protein on an electrode.

5.5. Conclusion

The incubation time for the detection of oestrogen has been further reduced; initially it was 5 h for whole cell experiments, reduced to 20 min for lysate experiments, and here to 5 min for purified protein. Direct detection of NADH as an indicator of oestrogen concentration simplified the assay. A glassy carbon electrode was successfully used to detect NADH in a semi-purified protein preparation to quantify oestrogen. The over-potential that is optimal for the oxidation of NADH in complex matrices is considered to be between −100 and +200 mV vs. Ag/AgCl (Gorton and Domınguez 2002) and I am working to improve electrodes to

achieve this specification so that the method can be used with 'real' samples. This approach has the potential to further simplify this assay

I have also explored the potential of direct electron transfer from EBP in its use as a bio-recognition element for detecting oestrogen. This research provides evidence for mediated electron transfer from EBP. The mechanism of electron transfer is not clearly understood. Interaction of mediators with this protein needs to be further investigated. This work also suggests that other fungal flavoenzymes should be explored as potential recognition elements for biosensors.

5.6. Co-author contributions

This chapter is in preparation for submission to Biosensors and Biolectronics. Besides supervisory committee members, the Co-authors of this paper are Alison Downard Gottard Kunze and Frankie Rawson. Alison and Gotthard provided technical advice and are involved in Manuscript correction. Frankie provided glassy carbon modified with osmium complex, provided technical assistance and was involved with data analysis of Fig.5.3. Special thanks to Mulinti Manohar for his technical assistance with Fig.5.2.

Chapter 6
General discussion, conclusions and future research

Yeast are eukaryotes and have some advantages over bacteria for application in biosensors such as rapid growth, wide pH, temperature and ionic tolerances (Buckman and Miller, 1998), ease of manipulation, growth on a broad range of substrates, and can provide information more relevant to other eukaryotes (Ghisla, et al. 1974). Despite these advantages yeast have rarely been studied as a biocomponent in biosensors.

Previous research in our laboratory has demonstrated a dose dependent response to oestrogen in *S. cerevisiae* (Baronian and Gurazada, 2007). This assay used a double mediator system comprising ferricyanide and TMPD with *S. cereviseae* whole cells for the detection of oestrogen. TMPD is a lipophilic mediator that can enter the cell and be reduced by NAD(P)H and redox molecules of the electron transport chain. Reduced TMPD exits the cell and reduces hydrophilic (and therefore extracellular) ferricyanide to ferrocyanide. The ferrocyanide is detected electrochemically with the signal directly related to the number electrons removed from the redox molecules oxidized by TMPD, either during yeast catabolism and/or the EPB/oestrogen/NAD(P)H interaction. The maximum current detected was at 11 nM oestrogen, which is of a similar concentration to the maximum signals reported for other whole cell oestrogen assays (Beresford et al. 1999, Rehmann et al. 1999, Fine et al. 2006, Leskinen et al. 2005).

This work was repeated with *C. albicans* to establish its response parameters to oestrogens. The responses observed with *C. albicans* whole cells were similar to those reported for *S. cereviseae.* Through mediated electrochemical measurements, we have shown that cell age is a factor in the production of EBP which may vary in different strains, suggesting that any

work with a different *C. albicans* strain should first be trialled to find the growth phase where the production of EBP is maximal.

While the lack of a dose related response to oestrogen by *A. adeninivorans* was expected, the inhibition of its catabolism was not. I am now using this electrochemical method to check other yeast species for responses to oestrogens. This method can with modifications be developed to test the effect of different chemicals on eukaryotic cells and has the potential to be used in preliminary toxic assessment of different chemicals.

There is a dramatic decrease in the response at high 17β-oestradiol concentration in the *S. cereviseae* oestrogen bioassay (Baronian and Gurazada 2007) and in *C. albicans*. The maximum oestrogen concentration detectable by whole cells is limited by the operation of the PDR efflux pumps. The reason for the decrease in response is not clearly understood. However, Cheng et al., (2006) showed through microarray studies that increased 17β-oestradiol increases the transcription of the genes *CDR1* and *CDR2*, which seem to be related to an increase in the production of efflux pumps which remove 17β-estradiol from *C. albicans* cells.

Cell lysate preparation lead to removal of the cell membrane thus removing efflux pump functionality and this allows EBP/oestrogen/NADH interactions to proceed without concentration limiting cell membrane regulatory mechanisms, which leads to an increase in the dynamic range of the assay to 100 nM.

The use of ferricyanide with the lysate produced a response equivalent to that seen in the 'with' and 'without' oestrogen double mediated whole cell trials because ferricyanide is not excluded by the cell membrane. This simplified the assay in that only a single hydrophilic mediator was now required and the incubation time in these experiments was also reduced making it comparable with the most rapid oestrogen assays reported to date.

The incubation time for the detection of oestrogen was further reduced, initially it was 5 h for whole cell experiments, which was reduced to 20 min for lysate experiments, and then to 5 min with the purified EBP. Direct detection of NADH as an indicator of oestrogen concentration also simplified the assay. NADH was successfully detected using GC electrode in a semi-purified protein preparation to quantify oestrogen. The over-potential that is optimal for the oxidation of NADH in complex matrices is considered to be between −100 and +200 mV vs. Ag/AgCl (Gorton and Domınguez, 2002). I am working now to improve our electrodes to achieve this specification so that the method can be used with 'real' samples. This approach has the potential to further simplify the assay.

I also explored the potential of direct and mediated electron transfer from EBP to an electrode. Although both of these processes were demonstrated, the mechanisms of electron transfer are not yet clearly understood.

This research has contributed a significant advancement in the development of an oestrogen detecting biosensor. It also gives insight into binding properties of EBP and suggests that other steroid binding proteins could also be investigated to increase understanding of the mechanisms of recognition in these molecules, and the potential of those proteins for detecting other steroids. It is suggested that other fungal redox enzymes such flavoenzymes should be explored as potential recognition elements for biosensors.

The future direction of this research should include testing the interaction of mediators with EBP, establishing the stability of the protein and immobilizing the protein on an electrode. Structural studies of this enzyme would also be very useful in that its interactions with oestrogen could be better understood and they may also allow development of drug(s) for treatment of Candidiasis, as EBP is known to play a major role in pathogencity of this organism.

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Appendix A

Electrochemical detection of oestrogen binding protein interaction with oestrogen in *Candida albicans* cell lysate