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INVESTIGATIONS INTO THE SENSING CAPABILITIES OF AN ELECTROCHEMICAL IMMUNOSENSOR EMPLOYING POLYANILINE AS A MEDIATOR SPECIES

by

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Abstract

The aim of this thesis was to expand the sensing capabilities of a conducting polymer-based sensor platform developed in our laboratory. The platform itself is composed of a polyaniline – poly(vinylsulphonate) (PANI/PVS) modified screen-printed carbon paste electrode used as a substrate for the immobilisation of antibody, and the subsequent competition assays of protein analytes. Chapter 1 is a literature review encompassing the field of electrochemical immunosensing. Chapter 3 outlines work towards developing real-time immunosensors to oestradiol and p60. This work involved the synthesis of horseradish peroxidase (HRP) conjugates with oestradiol, and p60 for use in a competitive immunosensor, as well as immunoassay characterisation of the proteins, and their application to the biosensor platform. Chapter 4 outlines efforts to address sub-monolayer protein immobilisation issues to the PANI/PVS matrix by optimising experimental parameters governing the electrostatic immobilisation process. It also details experiments probing microperoxidase-11 as an alternative labeling enzyme to HRP. Chapter 5 deals with the investigation of a novel surfactant-based strategy to reduce non-specific interactions between species present in the bulk solution, and the PANI/PVS matrix. This research highlights the need for developing the technology for real-time electrochemical immunosensing, and examines some of the critical issues involved.

Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of masters (insert title of degree for which registered) is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work. Signed: Padraic Walsh (Candidate) ID No.: 53154631 Date: 4/10/06

Acknowledgements

I wish to acknowledge the guidance, and assistance of Professor Malcolm Smyth, Doctor Anthony Killard, and Doctor Aoife Morrin in the completion of this work. Their experience and counsel have been invaluable.

Dedication

This work is dedicated to my parents without whose ongoing support and encouragement this would not have been possible.

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Abbreviations

ADA	Adenine deaminase
AP	Alkaline phosphatase
Ag/AgCl	Saturated silver/silver chloride electrode
BCA	Bicinchoninic acid assay
BOD	Biological oxygen demand
BSA	Bovine serum albumin
CDH	Cellobiose dehydrogenase
DET	Direct electron transfer
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
EDTA	Ethylendiaminetetraacetic acid
EO	Ethenyl oestradiol
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment, antibody-binding
FDA	Federal drug administration
FOH	Formaldehyde dehyrdrogenase
GOD	Glucose oxidase
hCG	Human chorionic gonadotropin
HRP	Horseradish peroxidase
IgG	Immunogammaglobulin
ISFET	Ion selective field effect transistor
LDH	Lactate dehydrogenase
MA	Methamphetamine
MP-11	Microperoxidase-11
NAD	Nicotinamide adenine dinucleotide
NTA	Nitrilotriacetic acid
O-HRP	Oestradiol-HRP
PAN	Polyaniline–polyacrylonitrile
PANI	Polyaniline
PBS	Phosphate buffered saline
PPY	Polypyrrole
PET	Polyethylene terephthalate
PVS	Poly(vinylsulphonic acid)
RNA	Ribonucleic acid
SAM	Self-assembled monolayer

SCE	Saturated calomel electrode
SCFV	Single chain variable fragment
SDS	Sodium dodecyl sulphate
SPE	Screen-printed electrode
TCNQ	7,7,8,8-tetracyanoquinodimethane
ThO	Theophylline oxidase
TTF	Tetrathiafulvalene
UV	Ultra violet

Chapter 1

Electrochemical biosensors: A literature review

1.1 INTRODUCTION

To date, effective chemical and clinical analysis relies on complex, and time-consuming assay techniques, requiring special training, and expensive equipment. Clinical diagnosis relies largely on successful culture of suspect microorganisms, which can take days to achieve, with the possibility of false negative results. With the exception of devices for the monitoring of glucose, and a select few other targets, real-time assessment of the clinically relevant biological targets is virtually non-existent. Continual observation requires patients to commute in and out of hospitals for regular testing to monitor their conditions. At that, the results obtained are limited by the lag between sampling, and results, as well as a myriad of problems stemming from cross-reactivity, background noise, and matrix contamination.

Substantial research has gone into the development of novel methods of sample analysis (Wu, 2006). Transduction technologies such as optical, piezoelectric, thermal, magnetic, and electrochemical systems have been introduced, and successfully integrated into sensing devices. Many problems afflicting previous generations of sensing technologies have been eliminated, or at least alleviated. However, obstacles still exist in the form of time lag between sample collection, and result generation. New technologies require particular expertise, and equipment, that is as expensive as it is sophisticated. These requirements limit the application of new techniques to laboratories, by individuals who have undergone the necessary training.

Recently, the concept of biosensors as a solution to these problems has come under new scrutiny (D'orazio, 2003). The first successful biosensor was the glucose electrode, based on electrochemical sensing of oxygen generation at the electrode surface from the activity of immobilised glucose oxidase enzyme on glucose found in biological samples (Clarke and Lyons, 1962). Since its creation, this biosensor has undergone several iterations, all producing portable, hand-held devices, which can be used by individuals without any special training on a continuous basis to monitor blood glucose levels, in real-time. If such a design could be expanded to detect additional molecules, it would be possible for real-time analysis of virtually any target protein without special

training. The ramifications in terms of clinical diagnosis, and monitoring are obvious. Early diagnosis of any condition by GPs during routine check-ups would cause significant reduction in the prevalence, and mortality of numerous conditions. Quality of life would be much improved for those afflicted with chronic ailments. Financial savings from reduced hospital visits would be substantial. Successful implementation of the biosensor promise is very much a present-day philosopher's stone.

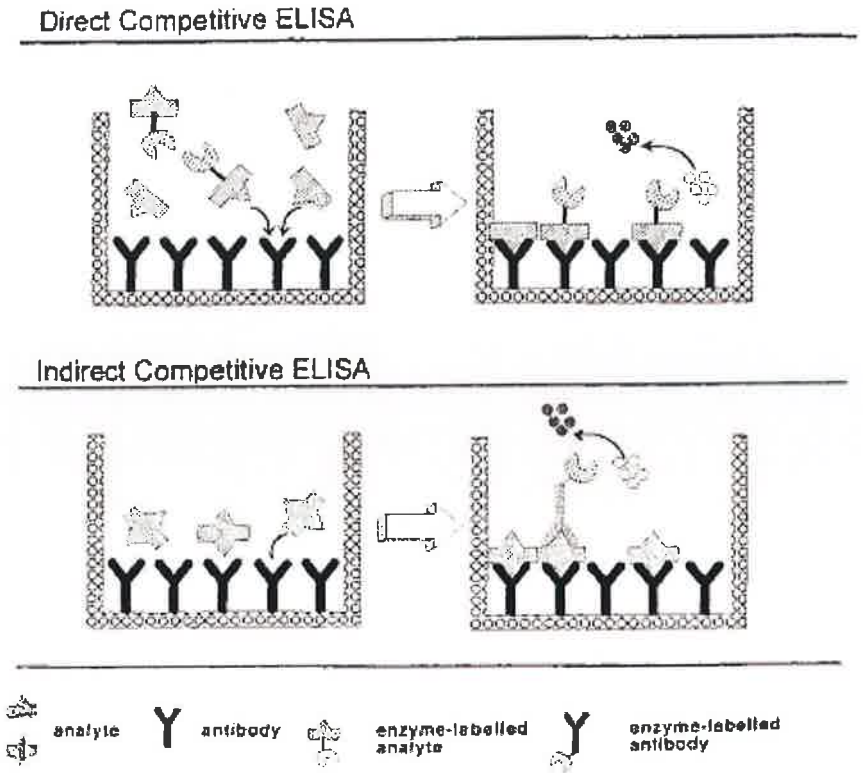
With the exception of the glucose biosensors developed for diabetics, there are very few effective biosensors, or immunosensors in operation outside of experimental settings. Based on use of antibodies, (or antibody-derived molecules), as the sensing element, married to an electrochemical transduction system, electrochemical immunosensors offer the best chance of realising the ultimate goal of biosensors (Killard *et al.*, 1995). Electronic technologies are readily miniaturised allowing the production of small portable devices to house biosensing apparatus, as well as being commonplace in many household appliances for decades making it instantly familiar to potential users without any special expertise or training. In contrast the technology required by alternative transduction methods such as optical, and piezoelectric technologies cannot as readily be miniaturised to such a degree, and the use of these technologies can often require very particular expertise. Of the biological elements available enzymes, and antibodies are the most frequent choices, and of these antibodies are potentially far more effective than enzymes due to the greater diversity of specificity, the greater avidity of this specificity, and our ability to synthesise antibodies with the desired selectivity in instances where a naturally occurring antibody with this selectivity is not readily available.

Since the research work contained in the accompanying thesis was conducted on electrochemical immunosensors, this literature survey will be concerned with that category of biosensor work.

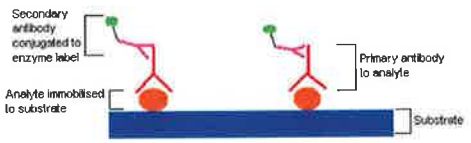
1.2 IMMUNOASSAYS

Immunoassays combine the specific binding capacity of antibodies, with the signaling capabilities of various systems, (Yagiuda *et al.* 1996). Since the binding event cannot be assessed directly, labels are introduced into the system. These take the form of enzymes, radioactive isotopes, (although these are less common), and fluorescent species which produce a measurable signal. These label species are attached to analytes, and following binding by an antibody, the signals they generate may be measured.

Effective measurement of analytes is dependent on the format of the immunoassay. Two main formats exist; namely competitive and non-competitive immunoassays, (Yagiuda *et al.*, 1996). The competitive immunoassays can be further divided into direct, and indirect formats. Competitive immunoassays employ a limited concentration of antibody immobilised to a suitable substrate. In order to assess the unknown concentration of analyte in a sample, a known amount of labeled analyte is incubated with the test sample. Both free, and labeled analyte will compete for the limited antibody binding sites available. Once the incubation period is complete, free/labeled analyte, which has not been bound, is removed. Substrate is then added, and the signal generated by the remaining labeled (antibody-bound) analyte is then measured. From this, the unknown sample concentration can be extrapolated using data for standard controls. The indirect format retains a limited concentration of antibody; however, it is the analyte that is immobilised to the solid phase. The non-competitive immunoassays employ antibody in excess (Yagiuda *et al.*, 1996). This binds all free analyte in test samples. A secondary antibody (to the same analyte) is then added to the assay vessel, which binds to the (antibody-immobilised) analyte. This secondary antibody is attached to a suitable label species, generating a signal, which is then measured, and used to calculate the unknown concentration in the sample. Figure 1.1 illustrates the principles of direct, and indirect ELISAs.



(a)



(b)

Fig. 1.1 (a) Schematic representation of direct and indirect ELISA formats, both direct and indirect competitive formats involve the immobilisation of antibody molecules, however the indirect format binds a larger proportion of analyte from the sample, reproduced from Hennion and Barcelo, 1998, (b) schematic representation of a non-competitive ELISA, here conjugate is immobilised to a solid phase, which is then treated with a primary antibody which binds the analyte, this is in turn bound by an enzyme-labeled secondary antibody, the system is then treated with enzyme substrate.

1.2.1 ANTIBODIES

Antibodies are immunoglobulin proteins, composed of two heavy (450 amino acid

residues) and two light chains (212 amino acid residues) (Fitzpatrick *et al.*, 2003). Both chains have constant and variable regions, with the constant region accounting for 90 % of total mass. The extremely high amino acid variability of the variable regions determines the specificity of antibodies. The formation of multiple non-covalent interactions is necessary for binding between antibody and analyte.

Polyclonal and monoclonal antibody formats are available, and the advent of genetic engineering in recent years has led to the production of antibody fragments, (Fitzpatrick *et al.*, 2003). A polyclonal antibody preparation contains antibodies with specificities to multiple epitopes of a protein target, increasing the sensitivity of the immunoassay, but at the cost of lower specificity. Monoclonal antibodies will bind to a single epitope of the target protein only, giving them much higher specificity, but reduced sensitivity. Production of both antibody types involves inoculation of a suitable host species with a preparation of the target protein. Following several rounds of booster shots, the host animal is sacrificed, the spleen is harvested, and antibody-containing serum is extracted. This supernatant is a polyclonal antibody mixture. If monoclonal antibodies are desired, antibodies are segregated according to their specificity for particular epitopes. These are then fused with tumorigenic B-lymphocytes creating immortal hybridoma cells, which produce just one type of antibody. These cells can be cultured indefinitely to produce antibodies against one protein epitope. Figure 1.2 illustrates the production of monoclonal antibodies in this way.

By isolating the antibody fragments containing the variable region, smaller binding proteins can be produced which give better surface coverage, and more binding sites. Nowadays recombinant genetic engineering techniques are used to produce these fragments, (Fitzpatrick *et al.*, 2003, Chames and Batty, 2000, Longstaff *et al.*, 1998). Single chain variable fragments, (ScFv), are the fragments most often employed. These consist of variable light chain, and variable heavy chain fragments.

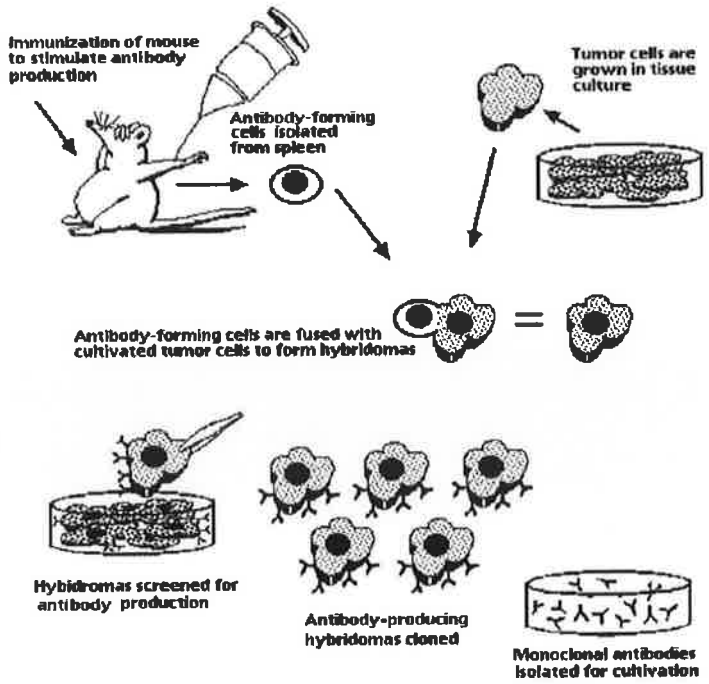


Fig 1.2 Production of murine monoclonal antibodies, reproduced from Wu, 2006, a suitable host species is first inoculated with a preparation of the analyte to which an antibody is desired, following a complete immune response by the host animal the spleen is harvested and both antibody, and antibody-producing B cells are isolated, these cells when combined with immortal tumor cells can be cultured indefinitely to produce monoclonal antibodies.

1.3 BIOSENSORS

A biosensor is defined as a device consisting of a biological element, integrated with a suitable transduction technology to detect analytes of interest (Andreescu and Marty, 2006, Albareda-Sirvent *et al.*, 2000). Transduction techniques available include optical, piezoelectric, and electrochemical detection systems. The biological elements most commonly employed are enzymes, or antibodies; however, recent work has begun to use nucleic acids.

The biosensor developed by Smyth's group consists of an antibody layer immobilised to a layer of polyaniline on a screen-printed carbon paste electrode (Andreescu, Barthelmebs, and Marty, 2002). Binding of conjugate species by antibody brings the enzyme molecule of the conjugate proteins into sufficient proximity with the electrode surface to allow electrical communication with the carbon paste electrode, via the PANI layer (Sotiropoulou and Chaniotakis, 2005). Following oxidation, electrons are required to regenerate (by re-oxidation) the enzyme species. Exploiting electron delocalisation over the PANI matrix, electrons are passed from the electrode, to the enzyme, re-oxidising it, and generating a current change which is related to the mass of conjugate bound by antibody.

PANI is a conducting polymer with potential for application in areas such as energy storage, catalysis, indicators, and sensors (Turner *et al.*, 1987, Castillo *et al.*, 2004). Leucoemeraldine, and pernigraniline are the fully reduced, and fully oxidised forms. Growth of PANI films using potentiodynamic techniques gives greater control over the morphology of the film. The generally accepted mechanism for the polymerisation of aniline is as follows: the radical cations of aniline are formed by oxidation at the surface of the electrode; these radical cations then dimerize. The resultant dimers are oxidised with additional radical cations, resulting in propagation of the chain, (Turner *et al.*, 1987, Castillo *et al.*, 2004). Figure 1.3 illustrates the proposed mechanism for aniline polymerisation.

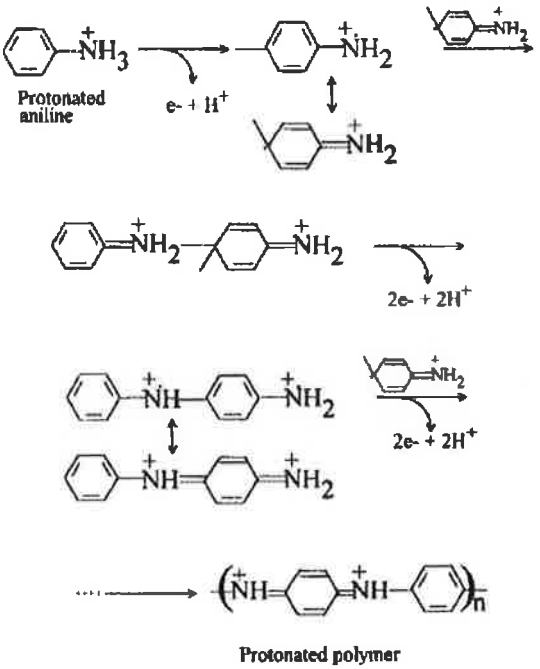


Fig. 1.3 Schematic of aniline polymerisation mechanism, protonation of aniline leads to dimmer formation, dimers then combine to form longer chains, this process continues to form polymer chains, reproduced from Iwouha *et al.*, 1997.

The crystalline structure of carbon gives it electrochemical properties, which are very amenable to sensing applications. Biosensor work on glucose has involved the use of a screen-printed carbon paste electrode (Luppa *et al.*, (2001), Castillo *et al.*, 2004). This was based on the use of a thick film printing technology to print various inks onto a suitable substrate, producing a working electrode. For biosensing work within Smyth's group, screen-printed electrodes were produced with three ink layers, a silver conducting track, a conducting carbon paste spot at the end of this track, and finally an insulation layer, which defined the working electrode area. The inks were applied to a pre-shrunk polyethylene terephthalate, (PET), substrate.

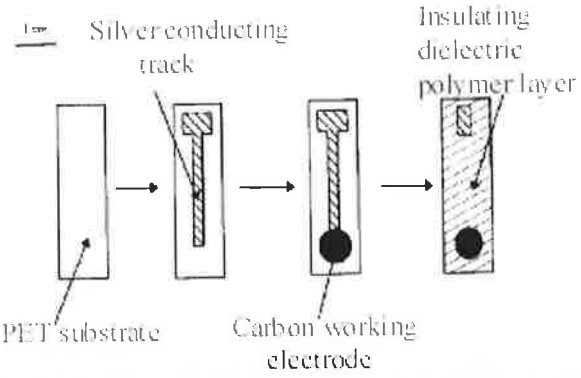


Fig. 1.4 Printing process for production of screen-printed carbon paste electrodes, inks are applied to the PET substrate through a mesh which defines the area, and shape of each layer, Reproduced from Cagnini *et al.*, 1995.

1.3.1 WHOLE CELL BIOSENSORS

Biosensors have been constructed which use whole microbial cells as their biological elements. Such whole-cell biosensors are more robust to variations in pH and temperature, as well as being less sensitive to inhibition by other non-target compounds, which may be present in biological samples (Arikawa *et al.*, 1998). Also, because these sensors employ whole, living cells, regeneration is much easier to achieve by simply soaking the sensor in a nutrient bath. However, these types of biosensors also have a slower rate of activity compared to biosensors employing isolated enzyme proteins. They also exhibit a lower specificity as a result of the myriad of metabolic activities, which take place within the living cells themselves. To date, whole cell biosensors are most effectively employed in the measurement of biological oxygen demand (BOD) (Mello and Kabuto, 2002).

Microbial biosensors have been developed for other substrates. Akyilmaz and Dinckaya have reported an amperometric biosensor for the determination of ethanol using the yeast species *Candida tropicalis* (Akyilmaz *et al.*, 2006). Cells were immobilised to a Teflon membrane on an oxygen probe. The ethanol content of samples was measured by following changes in the dissolved oxygen concentration. These changes were the result of increased respiration of cells following exposure to ethanol. This biosensor was able to detect ethanol within the limits of 0.5 and 7.5 mM. Aykilmaz was also involved in the development of a microbial biosensor capable of determining the concentration of L-

lysine using cells of *Saccharomyces cerevisae* (Liu and Mattiasson 2002). Using a similar procedure to their ethanol biosensor, cells were immobilised to a Teflon membrane above an oxygen probe. Exposure to L-lysine induced changes in the respiration rate of the immobilised cells allowing determination of L-lysine between 1 and 10 μM.

Conventionally, to determine BOD, the oxygen content of a water sample is determined by titration, then again after a period of 5 days at 20°C in the dark (Tan and Wu, 1999). The BOD of the sample is then calculated by finding the difference in these two measurements. This is a slow method, and a means of real-time BOD analysis would be preferred. To this end, there has been considerable work with microbial biosensors to determine the BOD of water samples in real-time. Microbial biosensors of this type can use a single strain; however, in this situation the sensor is limited by the narrow substrate spectrum of a single bacterial species. In contrast, biosensors exploiting a complex bacterial cell population have a superior detection capacity. However, the larger population of bacterial species results in lower sensor stability as the composition of the immobilised bacterial population changes over time. The more complex the bacterial population used, the better it reflects the normal microbial population of natural samples such as wastewater, or sludge. To benefit from heterogeneous populations, without losing sensor stability, microbial BOD biosensors often employ two different bacterial strains for BOD determination (Tan and Wu, 1999, Heim *et al.*, 1999, Tan *et al.*, 1992). Cells are most commonly immobilised by simple adsorption, or entrapment on the surface of a porous matrix membrane using an aqueous solution of polyvinyl alcohol (Tan *et al.*, 1992) or poly (-carbomyl) sulphonate (Chan *et al.*, 1999, Lehman *et al.*, 1999). Measurement of dissolved oxygen is achieved by monitoring changes in oxygen concentration in the vicinity of the electrode. As dissolved oxygen diffuses toward the immobilised bacterial cells, it is consumed during respiration. Unconsumed oxygen is detected by an oxygen electrode establishing a steady-state current. Addition of water samples causes increased oxygen consumption, resulting in a fall in the concentration of dissolved oxygen, and a change in the current response of the oxygen electrode. The process is controlled by substrate diffusion to the immobilised bacterial cells, meaning the sensor signal is proportional to the concentration of biodegradable organic substrates in the sample (Liu and Mattiasson 2002). Such microbial biosensors for determination of

BOD have been developed using the species *Trichosporon cutaneum* (Praet *et al.*, 1995) *Pseudomonas putida* (Chee *et al.*, 1999) *Bacillus subtilis* (Riedel *et al.*, 1988) and many other bacterial species. In the case of BOD biosensors it is the diversity of organic compound metabolism that contributes to the suitability of microbial biosensors for this task. However, BOD measurement is not typical of the requirements of most analytical, or diagnostic settings. Usually, analysis of a single analyte is required. This is not possible with whole-cell microbial biosensors because the cells will metabolise many species within the biological sample, in addition to the specific analyte under examination. The resulting signal cannot be taken as an accurate reflection of the analyte presence in the sample.

1.3.2 ENZYME-BASED BIOSENSORS

Enzymes were amongst the first biologically active molecules to be used in biosensor systems (Rogers, 2006). The availability of a stable source of material (primarily through biorenewable sources) is a contributing factor to the widespread application of enzyme species in biosensor work. Current genetic engineering techniques permit modification of the catalytic properties, and substrate specificity of enzyme molecules. It is also possible to amplify the biosensor response by modulating the enzymatic activity with respect to the target analyte. A difficulty in the use of enzymes for biosensing is that many enzymes are cross-reactive with several members of some protein groups, (e.g. Groups of steroid hormones which are similar in structure). There is also a limited diversity of enzymes, and current technology is not capable of synthesizing enzyme molecules with addition specificities. The oxidases are one of the most frequently employed classes of enzyme. In biosensor work these, and other enzyme classes, are immobilised to a suitable transducer, the catalytic activity of the enzyme producing some measurable signal, which can be assayed to determine the concentration of biological components (Mello and Kubota, 2002, Rogers, 2006, Luppa *et al.*, 2001).

Glucose, cholesterol, lactate, and urea are amongst the most important clinically relevant metabolites, which have been targeted for enzyme-based biosensor development. The availability of enzymes specific for each of these substrates is a significant factor in the construction of these biosensors. Glucose biosensors have been developed for on-line

(Rhemrev-Boom *et al.*, 2001) off-line (Fei *et al.*, 2003) and *in vivo* (Atanasov *et al.*, 1997) analysis. All of these biosensors used the enzyme glucose oxidase to metabolise glucose present in biological samples, achieving a linear range up to 25 mM (30 mM for off-line monitoring). Determinations were performed on whole blood samples for off-line monitoring, while *in vivo* biosensing was achieved using a biosensor implanted sub-cutaneously in dogs. Biosensors for the on-line (Yao *et al.*, 2004) and off-line (Guilbault *et al.*, 1995) determination of lactate using lactate oxidase have also been reported with a linear range of 0.2 – 10 mM for on-line analysis of rat brain and human serum control samples. Urea, and cholesterol biosensors exploiting cholesterol oxidase (Brahim *et al.*, 2001) and urease (Pizzariello *et al.*, 2001) for off-line monitoring of biological samples have also been reported. Other biosensors have been developed for use in the food and drink industries. Several biosensors have been reported for the determination of sugars such as glucose (Zhu *et al.*, 2002) fructose (Campuzano *et al.*, 2003) and lactose (Jenkins and Delwiche 2003) in soft drinks, wines, beers, and milk. These biosensors again employ either oxidase or dehydrogenase enzymes to produce electroactive substances by metabolism of target analytes in samples. Similarly, biosensors for the detection of heavy metals in the environment based on the inhibition of enzyme activity have also been developed. Some of the enzymes employed in that work are urease (Preininger and Wolfbeis 1996, Zhylyak *et al.*, 1995) and cholinesterase (Budnikov *et al.*, 1991).

In all cases of biosensor work employing enzymes as the recognition element the same major drawbacks exist. The construction of a biosensor to a particular analyte is dependent on the availability of an enzyme specific for that analyte. Multi-enzyme formats have been employed in some instances using one enzyme specific for the target analyte to produce a substrate, which a second enzyme may metabolize to some electroactive species. However, in such cases losses of sensitivity, and selectivity are inevitable due to cross-reactivity of enzyme species with non-target reagents within the sample to be tested.

1.3.3 BIOAFFINITY SENSORS

Technically this class of sensors may include chemoreceptors, and nucleic acids,

but for the most part it refers to antibody-based biosensors, or immunosensors, as they are normally termed. Affinity-based biosensors exploit selective reactivity with different analytes, which confers specificity to any biosensor device, which incorporates them. The major advantages with regards to immunosensors are that antibody species can be developed to virtually any analyte necessary, which permits a vast diversity of species to be assayed using immunosensor techniques (Killard *et al.*, 1995). Using either polyclonal, or monoclonal antibody formats, it is also possible to specify the sensitivity, and specificity of the antibody species (Killard *et al.*, 1995). The limitation of antibodies used in immunosensors is that the actual binding event between antibody and antigen generates a physicochemical change, which is very difficult to measure. In order to probe the binding event another species such as enzymes, fluorescent compounds, or radionuclides are often introduced to generate a measurable response (Rogers, 2006, Luppa *et al.*, 2001). A further limitation of immunosensors is that the binding event between antibody and antigen is essentially irreversible (Hock, 1997). Immunosensors are often designed as disposable, single-use set-ups. Alternatively, regeneration has been attempted by modulation of the electrode potential in an effort to disrupt the non-covalent interactions maintaining the bound state between antibody and antigen (Gooding *et al.*, 2004).

The most effective solution to the limitations of antibodies used in immunosensors to date is the incorporation of either a second antibody conjugated to an enzyme species, or using a competitive approach where free analyte, and enzyme-conjugated analyte compete for limited numbers of antibody binding sites. The latter format has been used to develop immunosensors against complement factor 3 (Killard *et al.*, 1995) and *Schistosoma japonicum* (Zhou *et al.*, 2003). Secondary enzyme conjugated antibodies have been reported for the determination of hormones in human serum samples (Petrou *et al.*, 2002), as well as the assay of *Mycobacterium tuberculosis* (Diaz-Gonzalez and Gonzalez-Garcia, 2005), and the detection of mycology in whole blood samples (O' Regan *et al.*, 2002).

1.3.4 NUCLEIC ACID-BASED BIOSENSORS

Nucleic acids such as deoxyribonucleic acid (DNA), and ribonucleic acid (RNA)

are composed of the four nucleotide bases adenine, thymine, guanine, and cytosine. A persistent pairing scheme exists between adenine and thymine, (maintained by 2 hydrogen bonds) and between cytosine and guanine (maintained by 3 hydrogen bonds). A single strand of either RNA, or DNA is capable of annealing to a second strand containing the complementary base pair sequence. For the purposes of detection using biosensors, short RNA/DNA probes are immobilised to appropriate solid supports, and then exposed to samples of unknown genetic material (Rogers, 2006, Luppa *et al.*, 2001). Where an unknown fragment of nucleic acid has a sequence that is complementary to that of an immobilised probe the two sequences will associate (hybridise) forming a double-stranded molecule. The sequence of the immobilised probes is known to researchers, which allows them to determine the sequence of unknown strands where hybridisation occurs with the immobilised probes. In this way biological samples can be assayed for genetic material of pathogenic origin.

The primary limitations of nucleic acids employed in biosensors construction are the availability of probe sequences, and the complicated pre-treatment procedures necessary before effective biosensing of a nucleic acid sample can occur (Junhui *et al.*, 1997). In the last decade there has been a surge in the amount of sequence information available for different species. This has led to an increase in the number of species-specific probes, which can be used to identify organisms. However, analysis of this kind is impossible for biological, or chemical substances of clinical, pharmaceutical, and industrial significance. A nucleic acid-based biosensor cannot be constructed for the determination of glucose found in a diabetic patient for example, since glucose molecules have an entirely different structure to that of nucleic acids. For this reason biosensors using nucleic acid as probes are limited to the identification of specific gene sequences in biological samples. In order for analysis of nucleic acid content to be possible, cells must first be subjected to considerable treatments. The purpose of this is to free the nuclear material form the cell, and also to digest it down to smaller fragments, which may interact with the probe sequence of a biosensor. Despite these limitations, biosensors using DNA/RNA sequences have been used to detect genetically modified microorganisms (Mannelli *et al.*, 2003) bacteria (Baeumner *et al.*, 2003) and toxic substances (Pope *et al.*, 2001).

1.4 IMMUNOSENSORS

Immunosensors are defined as biosensors, which monitor the interaction between antibodies, and antigens (North, 1985). One or other of the antigen, or antibody are immobilised to a solid phase. The immobilised component can then take part in a biospecific reaction with the free component; this interaction generates a measurable signal, which can then be measured by the choice of a suitable transduction technology. The physical quantity modified by the antigen-antibody reaction is assessed using a measuring device; an example is the potentiostat, which can be used to measure signals generated by electrochemical immunosensors. Electrochemical transduction is exploited in amperometric, potentiometric, and conductimetric immunosensors. Generally immunosensors can be classed as direct (homogenous), or indirect (heterogeneous) immunosensors. Direct immunosensors probe a change in some physical property, which is a direct result of the antibody-antigen binding event. Indirect immunosensors involve the addition of a suitable label to one of the immunoreagents. Indirect immunosensors often require a separation step, and a secondary reaction, (such as the addition of a substrate) to produce a measurable change in the system (Luppa *et al.*, 2001). In order to achieve appreciable detection limits, antibody proteins with high affinity binding constants must be used (Morgan *et al.*, 1996).

1.4.1 AMPEROMETRIC IMMUNOSENSORS

Amperometric immunosensors exploit the fact that certain substances will be either oxidised or reduced at inert electrodes maintained at a constant potential (D'Orazio, 2003). The cell is maintained at a fixed potential relative to a suitable reference electrode (often an Ag/AgCl electrode). Depending on whether the working electrode is maintained at a positive, or negative potential relative to the species of interest, an oxidation, or reduction, reaction will be supported. In this way the working electrode monitors the formation, or consumption of electroactive species. Most analytes of interest will not produce electroactive substances, which necessitates the introduction of an appropriate label species. Enzymes are commonly conjugated to antibodies, or analytes for use in immunosensor devices. Catalase is an example of an enzyme used in immunosensing

applications. This is due to its high enzymatic rate constant of 10^5 s^{-1} catalysing the decomposition of H_2O_2 (Aizawa *et al.*, 1979). Monoclonal human antibody to human chorionic gonadotropin (hCG) was immobilised to an amperometric oxygen electrode. Catalase-bound hCG and free hCG were then added to the reaction vessel and competed for the antibody binding sites. After a washing step to remove unbound hCG solution containing H_2O_2 is added. Catalase molecules conjugated to antibody-bound hCG generated oxygen from H_2O_2, which could be detected by an oxygen electrode. Alkaline phosphatase has also been used as an enzyme label in amperometric immunosensors with a catalytic rate constant of 10^3 s^{-1}.

Competitive, and sandwich immunoassay formats are often employed in amperometric immunosensors of this type. Following substrate addition to the system, an electroactive species is generated by antibody-bound labeled analyte. This species can be measured as an indirect probe of the concentration of labeled analyte in the system. The amount of labeled analyte bound by antibody is inversely proportional to the concentration of free analyte in the biological sample. Knowledge of this relationship allows the concentration of free analyte to be calculated when the concentration of labeled analyte is known. Non-competitive immunoassays are also exploited in amperometric immunosensor where the secondary antibody is attached to an enzyme species, the activity of which produces an electroactive species, which can be measured. The resultant signal is a direct measure of the analyte under study.

1.4.2 POTENTIOMETRIC IMMUNOSENSORS

Antibodies and antigens have a net charge, when an immunocomplex between antibody and antigen is formed. That complex should have its own unique charge distinct from that of either antibody, or antigen alone (Janata, 1975). This principle is exploited in potentiometric sensing by immobilising one of the two species, and monitoring the sensing surface for any changes in the surface charge density following formation of the antigen-antibody immunocomplex. Formation of the immunocomplex is an equilibrium process; thus the signal should be proportional to the concentration of immunoreagent in solution. Potentiometric immunosensors have been difficult to realise due to the high

interfacial charge resistance required, and the susceptibility of the immunosensor to other reagents present in biological matrixes (Janata and Blackburn, 1984). A potentiometric immunosensor to cardiolipin antigen has been demonstrated previously (Aizawa *et al.*, 1977). Antigen was immobilised to a triacetyl-cellulose membrane at the surface of a carbon electrode. The immobilised antigen reacted with antibody present in solution eliciting a potential change, which was found to be proportional to the concentration of antibody. The potential change, which was recorded, was attributed to a change in the charge density of the membrane following the formation of the immunocomplex. However, this sensor was also sensitive to changes in the concentration of organic ions found in the sample.

Variations on the principle of potentiometric immunosensors have also been developed using ion-selective, and gas-sensing electrodes. In one such effort, an ion carrier dibenzo-18-crown-6 was conjugated to model antigens such as BSA, or the hapten dinitrophenol. These conjugates were incorporated into ion-selective membranes of polyvinyl chloride, or triacetylcellulose, and potentiometric response to the antibody was monitored following addition of antiserum to background solutions of fixed ionic content (Solsky and Rechnitz, 1979). Another group incorporated an ionophore-antigen conjugate into a polyvinyl chloride membrane. This potentiometric sensor was then exposed to a constant activity of the ion, which the ionophore was selective for. Antibody binding to the antigenic portion of the ionophore-conjugate resulted in a change in the activity of the ionophore, which caused a change in potentiometric signal, which was proportional to antibody concentration (Keating and, Rechnitz, 1984).

A further example of potentiometric immunosensing exploited an enzyme-channeling approach to produce a pseudo-homogeneous sensor (Brown and Meyerhoff, 2001). IgG and adenosine deaminase (ADA) were immobilised to the surface of an ammonia-selective membrane. A conjugate of alkaline phosphatase (AP), and protein A was also synthesised. Following addition of adenosine monophosphate to a test solution, sequential activity of AP, and ADA produced ammonium ions. Where IgG was present in sample solution the protein A-AP conjugate did not associate with the membrane surface. Rather the enzyme intermediate had to diffuse toward the membrane surface, which

reduced the rate of ammonium ion production, and detection by the membrane. In this way the potentiometric immunosensor could be used to test for IgG proteins.

1.4.3 CONDUCTIMETRIC IMMUNOSENSORS

Conductimetric immunosensors measure changes in conductivity caused by the consumption or generation of ions. The reactive biological element is immobilised to a pair of noble metal electrodes, which allows changes in the capacitance of the electrochemical system to be measured (Luppa *et al.*, 2001, Mello and, Kubota, 2002). The major difficulty with conductimetric systems is their sensitivity to the high ionic strength biological matrix which analytes of interest are contained in. This makes it difficult to accurately record the small conductivity changes that occur following the signaling reaction (Berney *et al.*, 1998). One means of circumventing this difficulty is amplification of the change in conductivity. This has been reported by constructing a network of molecular ion channels, composed of linked gramicidin A molecules aligned across a lipid bilayer membrane (Cornell *et al.*, 1997). The conductance of this network was demonstrated to change in response to an antigen-antibody binding event, producing a net change in conductivity, which was large enough to be accurately measured. Another alternative is to measure the change in surface conductivity. One group has demonstrated this for the detection of methamphetamine (MA) in urine samples (Yagiunda *et al.*, 1996). Monoclonal antibody to MA was immobilised to the surface of a pair of platinum electrodes, and exposed to biological samples. Following antibody binding of MA, a decrease in conductivity could be measured between the two electrodes.

1.5 ELECTRODE FABRICATION

An electrochemical biosensor comprises a suitable transducer such as a pair of electrodes, and an interface or sensing layer, which incorporates the biological species of the biosensor. Some formats also include a coating designed to prevent non-specific interactions at the sensing surface. Traditional electrode systems constructed to measure the concentrations of ions in liquids contain a working electrode, and a reference electrode. However, such a simple set-up is insufficient to the increasingly complex requirements of contemporary biosensor systems. Changes to the electrode, both complex, and simple, can elicit significant, and advantageous changes in the behaviour of electrodes. Screen-printing is an example of a thick film technology, which has been adapted from its conventional use to the production of electrodes for biosensing applications.

1.5.1 SCREEN-PRINTING

Screen-printing is a thick film technology that has been in use within the textiles industry for some time. A suitable paste, or ink of the desired electrochemical properties is applied to an appropriate substrate material through a mask-net which has been imprinted with a specific pattern (Zhang *et al.*, 2000). In this way layers of inks can be applied to the substrate, producing electrodes with the desired conductive properties. Within our own group screen-printing is used to produce electrodes for biosensing. A layer of conducting silver ink is first applied, followed by a conducting carbon layer, and finally an insulation layer is printed which defines the working electrode area (Killard *et al.*, 1999). Screen-printed electrodes are now used for a great deal of biosensor work.

Wang *et al.* used screen printed electrodes loaded with tyrosinase enzymes to produce an inhibition based biosensor device for the determination of enzyme inhibition caused by substances such as herbicides, and pesticides (Wang *et al.*, 1996). The flexibility of electrode design inherent to screen printing has been exploited by Silber *et al.* to develop a seven channel electrode which could be used for the analysis of blood serum electrolyte (K^+, Li^+) and metabolite (glucose, urea, lactate) status (Silber *et al.*,

1996). Dock *et al.* have recently reported an electrochemical cell designed for the amperometric, steady-state, and flow-injection analysis of screen-printed eight-electrode arrays (Dock *et al.*, 2005). Khan *et al.* developed a "biosensor paste" which exploited screen-printing to produce an entirely printable biosensor (Khan, 1996). TCNQ powder and TTF solution were mixed together to form charge transfer complexes, which were then coated with a monolayer of glucose oxidase by adsorption. The resultant complex was the mixed with an appropriate binder, and a solvent producing a printable paste, which could be employed to produce a functional biosensor by screen-printing. Turner *et al.* further enhanced screen-printing as a means of producing electrodes by using solvent resistant materials, heat stabilised polyester sheet, carbon base tracks, and an epoxy-based polymer (Kroger and Turner 1997). The electrodes produced using these modifications were suitable for applications involving water-miscible organic solvents.

Screen-printed electrodes are routinely employed in the development of electrochemical biosensors nowadays. Our own group has used them for the development of biosensors for the detection of HRP (Iwouha *et al.*, 1997) biotin (Killard *et al.*, 1999, Killard *et al.*, 2001) and the herbicide atrazine (Grennan *et al.*, 2003). Ruthenium dioxide has been applied to the production of a planar, screen-printed ascorbic acid biosensor by Wu *et al.* (Wu *et al.*, 2000). Using cyclic voltammetry, the oxidation of ascorbic acid, uric acid, and H_2O_2 was monitored using this biosensor. Karube *et al.* have reported a glucose sensor using screen printing to coat a gold electrode with a paste containing glucose oxidase covalently attached to ferrocenecarboxylic acid (Karube *et al.*, 1995). The sensors produced were demonstrated to be reproducible, and applied to the measurement of glucose in samples.

1.6 MEDIATORS

During a bio-interaction process, electrochemical species such as electrons are consumed or generated producing an electrochemical signal, which can in turn be measured by an electrochemical detector. Thus, electrochemical biosensors are based on the mediated, or unmediated electrochemistry for electron transfer. Amperometric biosensors measure changes in the current on the working electrode due to direct oxidation of the products of a biochemical reaction. The measurement can be based on a direct, or an indirect format. The indirect amperometric format uses conventional detectors to measure the products or substrates of biochemical reactions. However, direct amperometric biosensing involves a biological redox reaction where an electrode is modified with an electron acceptor in place of the natural electron donor. The electron transfer which drives amperometric measurements is thought to be facilitated by small redox species (termed mediators).

1.6.1 FIRST GENERATION BIOSENSORS

Oxygen is a natural co-substrate of the oxidase enzymes, which is transformed into H_2O_2 during reaction with the substrate. The activity of oxidase enzymes at the electrode surface causes a substrate-dependent change in the oxygen, and H_2O_2 concentrations. First generation biosensors have a biorecognition element immobilised to a suitable transducer electrode. The electrode is set to a potential, which either reduces the oxygen molecules, or oxidises the H_2O_2 molecules. This generates a current response, which is proportional to the concentration of the substrate. Low concentrations of oxygen in the proximity of the electrode can limit the response of first generation biosensors. Also, at the potentials necessary for redox activity to take place, a large interference can occur due to the oxidation/reduction of other species at the electrode surface. This results in a low selectivity for first generation biosensors.

The best-known example of a first generation biosensor is the glucose biosensor developed by Clark and Lyons (Clark and Lyons, 1962). An oxygen electrode coated with glucose oxidase (GOD) was used as the cathode in this biosensor. The conversion of glucose to glucolactone by the catalytic activity of GOD consumed oxygen at the electrode surface according to the equation,

$$\text{Glucose} + O_2 \rightarrow \text{Glucolactone} + H_2O_2$$

The current response of this biosensor was directly proportional to the concentration of glucose in the biological sample under study. Thus by measuring the current, the unknown concentration of glucose could be determined. The response of this electrode was dependent on the concentration of dissolved oxygen in the sample. If the latter concentration was below a certain threshold level the current response was no longer proportional to the concentration of glucose, but to the concentration of dissolved oxygen. Subsequent glucose biosensors relied on the consumption of H_2O_2 produced by the GOD reaction (Guilbault and Lubrano, 1973). H_2O_2 was consumed at the anode according to the equation,

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^-$$

The anode was maintained at a potential of 0.7 V vs. Ag/AgCl. When GOD was immobilised to a suitable electrode under these conditions the current measured was proportional to the concentration of glucose in the biological sample. However, as with glucose biosensors responding to the consumption of oxygen, the reaction rate was still dependent on the concentration of dissolved oxygen, rather than the glucose concentration. The second major difficulty encountered with this biosensor was the oxidation/reduction of other species present in bulk solution at an electrode potential of 0.7 V. This potential is essential to ensure the consumption of H_2O_2 but at this potential other biological species will also react at the anode, in particular ascorbic acid (Harwood and Pouton, 1996).

1.6.2 SECOND GENERATION BIOSENSORS

Second-generation biosensors replace the co-substrate with an artificial redox mediator. The formal potential of these redox mediators facilitates regeneration of the redox center of the enzyme. The current response that results is directly related to the level of enzymatic activity, giving a more accurate measure of analyte concentration in

-100 mV to 0 mV vs. SCE, which minimizes the oxidation of electrochemical interferants. For second-generation biosensors, the redox mediators used have a fast reaction rate for electron transfer between both the redox center of the enzyme, and the electrode surface (Turner *et al.*, 1987). Most of the mediators used are either inorganic or organometallic derivatives of iron (II) (ferrocenes), or complexes of osmium (II) and ruthenium (II). One of the difficulties of second-generation biosensors occurs when samples with complex biological matrices are being analysed, where it is essential to avoid contamination of the sample matrix with any artificial mediator. Generally, two formats of second-generation biosensors exist; namely homogenous, or heterogeneous. In homogenous systems the mediator and the enzyme freely diffuse in solution. At the electrode the mediator becomes oxidised or reduced, then diffuses away from the electrode toward the enzyme, which it reacts with. This converts the enzyme back into the active format, and re-oxidises/reduces the mediator. In heterogeneous mediation the mediator species can either be added to the bulk solution, or can be present in the electrode. Where the mediator is present in the electrode it diffuses into the bulk solution during measurements. A further difficulty with these second generation biosensors is that leaching of the mediator species from the electrode surface can significantly reduce the lifetime, and operational stability of the biosensors. Figure 1.5 provides a schematic for the proposed mechanism of mediator transduction.

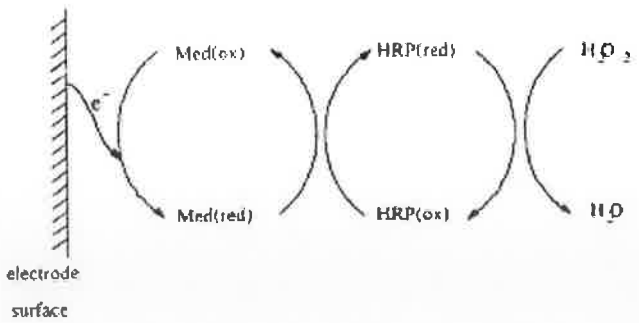


Fig. 1.5 The proposed mechanism of mediator transduction of electrochemical activity, transfer of electrons via mediator species allows the re-oxidation/reduction of electroactive species within the biosensor, reproduced from Killard *et al.*, 1995.

Ferrocenes were amongst the first transition-metal containing mediators reported and are still widely used today (Chaubey and Malhotra, 2002). Kajiya *et al.* have

developed a biosensor to cholesterol, which used cholesterol oxidase, and ferrocenecarboxylate ions incorporated into polypyrrole films by electro-polymerization of pyrrole in an aqueous solution containing these substances (Kajiya *et al.*, 1991). Tkac *et al.* have reported the use of ferrocene in the construction of a fructose biosensor by embedding ferrocene in an acetate membrane (Tkac *et al.*, 2001). Mediator species used by other groups include tetracyanoquinodimethane (TCNQ) and tetrathiafulvalene (TTF). Kataky *et al.* have reported a disposable screen-printed biosensor for monitoring formaldehyde using tetrathiafulvalene–tetracyanoquinodimethane (TTF-TCNQ) and the enzyme formaldehyde dehydrogenase (FOH) with the co-factor β-NAD^+, capable of generating a linear response between 10^{-3} and 10^{-4} mol dm^{-3} (Kataky et al., 2001). Llopis *et al.* have reported the use of a TTF·TCNQ conducting organic salt within a non-conducting epoxy resin with glucose oxidase for the electrochemical determination of glucose (Llopis *et al.*, 2005).

A subset of second generation biosensors involves the co-immobilisation of biorecognition element, and the redox mediator species. By using the same methods to immobilise both the biorecognition element, and the redox mediator an electron transfer pathway is established between the redox active center of the enzyme and the electrode surface. Several redox polymer species have been employed for this purpose including poly (pyrrole), poly (aniline), and osmium poly (vinyl pyridine). This co-immobilisation approach further enhances the transfer of electrons, which results in increased accuracy of measurements, and more efficient electrochemical communication between enzyme, and electrode. This brings biosensing devices into the realm of effective diagnostic, and analytical devices.

Polypyrrole (PPY) and polyaniline (PANI) are two of the most popular conducting polymers used for biosensing applications. PPY is typically synthesised at neutral pH, which also allows biomolecules to be incorporated into the sensing surface during the polymer deposition; however, limited control over the amount of biomolecule entrapped in the polymer, and the occurrence of non-specific interactions with the polymer limit its application (Barisci *et al.*, 1998). In spite of these difficulties PPY has still been employed in the development of biosensors. Razola *et al.* have reported an amperometric biosensor

in the development of biosensors. Razola *et al.* have reported an amperometric biosensor based on the entrapment of HRP within a PPY layer electro polymerized onto a platinum electrode. The biosensor thus produced was capable of detecting hydrogen peroxide within the limits 4.9 x 10^{-7} and 6.3 x 10^{-4} M. (Razola *et al.*, 2001). Brahim *et al.* have reported a biosensor for the amperometric detection of cholesterol in serum by entrapment of cholesterol oxidase within a composite poly (2-hydroxyethyl methacrylate) (p (HEMA))/polypyrrole (p(pyrrole)) membrane (Brahim *et al.*, 2001).

PANI is most effectively employed when grown electrochemically as this gives greater control over the thickness and morphology of the film (Trivedi, 1997). Doping of the PANI layer with anions such as poly(vinylsulphonate) is necessary to maintain the electro neutrality of the resulting film, allowing conductivity to take place at neutral pH. PANI matrixes produced in this fashion have been exploited by Smyth's group and others for the development of electrochemical biosensors. Qu *et al.* have developed a biosensor for choline using a functionalized carbon nanotube and polyaniline multilayer film (Qu *et al.*, 2005). The biosensor had a response time of just 3 s, and the linear response range was between 1 x 10^{-6} and 2 x 10^{-3} M. Xu *et al.* reported a biosensor for glucose constructed by the electropolymerisation of aniline into micro porous polyacrylonitrile-coated platinum electrode in the presence of glucose oxidase (Xue *et al.*, 2005). The biosensor demonstrated no significant loss in activity after 100 consecutive measurements and over 100 days when stored in a phosphate buffer at 4°C. The same group has also reported a biosensor for phenol determination constructed by the immobilisation of polyphenol oxidase into a polyaniline–polyacrylonitrile (PAN) composite matrix (Xue and Shen, 2002). This resulted in intercalation of enzyme into the conducting PAN matrix permitting the analysis of phenol, *p*-cresol, *m*-cresol and catechol with sensitivities of 0.96, 1.38, 1.5 and 2.03 AM^{-1} cm^{-2} respectively.

1.6.3 THIRD GENERATION BIOSENSORS

Third generation biosensors involve the immobilisation of enzyme species to the electrode surface, with the electro activity of the enzyme species communicated directly to the underlying electrode without the use of a mediator species, a phenomenon referred

to as direct electron transfer (DET) (Shleev *et al.*, 2005). DET has been reported for the peroxidase family of enzymes and is proposed to be driven by the mechanism present in Figure 1.6 (Ruzgas *et al.*, 1996, Shleev *et al.*, 2005). It was found that if an electrode coated with peroxidase was placed into a sample at a potential more negative than 0.6 V vs, SCE reduction current observed was proportional to the peroxide concentration (Ruzgas *et al.*, 1996). The proposed mechanism for this reduction activity involves the electrochemical reduction of compound I to compound II. This mechanism is presented in Figure 1.6 below. Further evidence in favour of this mechanism was that that reduction of peroxide began at potentials close to the formal potential of compound-I/-II and compound-II/HRP(Fe^{3+}).

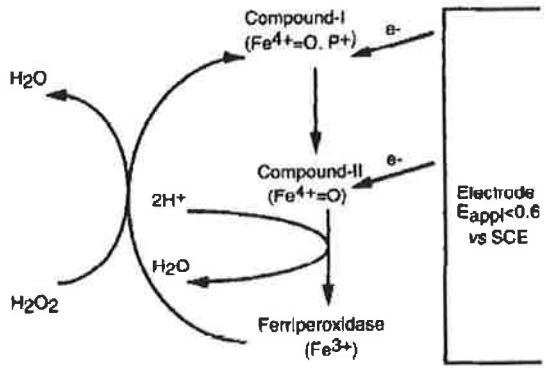


Fig. 1.6 Mechanism for the direct bioelectrocatalytic reduction of hydrogen peroxide at peroxidase modified electrodes. P' is a cation radical localized on porphyrin ring or polypeptide chain, reproduced from Ruzgas *et al.*, 1996.

The use of peroxidase electrodes without any mediating species involves the immobilisation of either an antibody or and antigen to a solid phase which is used as a probe for target analyte presence in biological samples. Analyte in sample will bind to the immobilised affinity protein at which point an anti-analyte antibody labeled with peroxidase enzyme is introduced. This antibody will form an immunocomplex with the affinity-immobilised analyte molecules which allows the enzyme molecule to produce either hydrogen peroxide or some highly reactive molecule which reduces oxygen to hydrogen peroxide. This species can then be registered without the need for mediation at the peroxidase electrode.

Pita *et al.* have reported DET between gold electrodes and fungal laccases from basidiomycetes *Trametes hirsute*. It was shown that hydrogen peroxide was produced by the electro reduction of oxygen on gold electrodes modified with this laccase (Pita *et al.*, 2006). Stoica *et al.* investigated DET between cellobiose dehydrogenase (CDH) from *Trametes villosa* and *Phanerochaete sordida* and *Myriococcum thermophilum* and alkanethiol-modified Au electrodes (Stoica *et al.*, 2005). Christenson *et al.* report DET between the heme containing enzyme theophylline oxidase (ThO) and the surface of both graphite and gold electrodes, establishing a detection limit of 0.2 mM (Christenson *et al.*, 2004).

1.7 IMMOBILISATION

By definition, all biosensing devices possess a biological element. Depending on the identity of the biological species it can simply confer selectivity on the biosensing device, or it can generate a measurable response to the target analyte, or as is often the case with enzymes it can serve both these functions. However, in order to serve any function it is necessary that the biological element be localised such that it is free to interact with analyte molecules in samples, whilst also optimising any mechanical or kinetic parameters that dictate the efficiency of catalytic signal transduction. The latter is particularly relevant to electrochemical transduction. Additional considerations when immobilising biological molecules are retention of the native activity, and conformation of the species in question, as well as achieving a functional orientation of immobilised molecules. Many covalent methods of immobilisation allow a high degree of control over the orientation of biological species, but require modification or addition of functional groups to them, which can be detrimental to their activity. Where enzymes are the biological element, any structural changes may result in a loss of specificity, or reduced activity; with antibodies similar changes in structure can be deleterious to the binding capacity of species. Ideally, the substrate to which the protein is being immobilised should be biocompatible, stable, and possess functional groups, which facilitate the immobilisation process (Andreescu and Marty, 2006). Most often the materials chosen must be modified to satisfy these requirements.

1.7.1 PHYSICAL ADSORPTION

Physical adsorption of protein exploits the formation of large numbers of individually weak interactions such as Van der Waals, hydrogen, and electrostatic interactions (Albareda-Sirvent *et al.*, 2000). No modification of the substrate or of the protein itself is required thus there is often detrimental change to either. However, physical adsorption gives no control over the immobilisation process. The orientation of protein species is completely random, as is the distribution, and density of the resulting coverage. Furthermore, sensors produced using physical adsorption exhibit poor operation and storage stability (Andreescu and Marty, 2006). Depending on changes in the physical properties of the biosensors, and buffer solutions employed such as pH, temperature, and

ionic strength protein can be leached from the surface (Albareda-Sirvent *et al.*, 2000) when immobilised using physical adsorption.

Bonnet *et al.* have reported an amperometric biosensor based on acetyl cholinesterase was constructed by simple adsorption of the enzyme on screen-printed electrodes (SPE) (Bonnet *et al.*, 2003). The purpose of this biosensor was to investigate the inhibitory effects of organophosphorus and carbamate insecticides on acetyl cholinesterase. Work has also been reported involving the physical adsorption of acetyl cholinesterase and choline oxidase onto the surface of carbon-nanotube modified screen-printed electrodes (Joshi *et al.*, 2005). Palchetti *et al.* have constructed a biosensor based on an electrode coupled with choline oxidase immobilized by adsorption (Palchetti *et al.*, 1997) capable of detecting pesticides at levels as low as 1 nM. Sprules *et al.* have developed a disposable amperometric biosensor for lactic acid based on the immobilisation of the enzyme L-lactate dehydrogenase (LDH) and its cofactor nicotinamide adenine dinucleotide (NAD) (Sprules *et al.*, 1995). The same group has also reported a screen-printed amperometric biosensor for the determination of alcohol in beverages using alcohol dehydrogenase immobilised to a PVS substrate (Sprules *et al.*, 1996). Zhang *et al.* have reported a bi-enzyme biosensor for the simultaneous determination of glucose, and maltose in a sample using the immobilised enzymes glucose oxidase, and amyloglucosidase (Zhang *et al.*, 1998).

1.7.2 COVALENT IMMOBILISATION

Covalent immobilisation of biomolecules requires the use of cross-linkers binding to the solid substrate on one side, and to the biological molecule on the other side. Glutaraldehyde, aminopropyltriethoxysilane, and carbodiimide/succinimide are three of the cross-linking agents most commonly employed. The main advantage of covalent immobilisation is the increased stability of the immobilised biomolecule. However, a large amount of bioreagent is required to achieve covalent immobilisation, and the resulting protein layer has a poor reproducibility (Andreescu *et al.*, 2002a). As well, covalent immobilisation induces chemical changes to the biomolecules involved, which can change their binding, and activity characteristics (Albareda-Sirvent *et al.*, 2000).

Glutaraldehyde cross-linking was employed by Skladal *et al.* in the development of amperometric biosensors to pesticides (Skladal *et al.*, 1997) by immobilising choline esterase to a platinum-paste based working electrode. Kulys and D'Costa reported an amperometric biosensor used for the detection of organophosphates between 0.06 and 8 ppm based on the immobilisation of butrylcholinesterase (Kulys & D'Costa, 1991) to a PBS substrate following the printing of ink prepared with TCNQ. Hartley and Hart developed an amperometric biosensor for the determination of organophosphate pesticides using glutaraldehyde cross-linking to immobilise acetyl cholinesterase to a screen-printed carbon paste electrode (Hartley & Hart, 1994). Carsol *et al.* used glutaraldehyde cross-linking to immobilise the enzymes hypoxanthine, and xanthine oxidase to a screen-printed carbon paste electrode for the determination of fish freshness (Carsol *et al.*, 1997).

1.7.3 SELF-ASSEMBLED MONOLAYER

The attachment of protein via self-assembled monolayer (SAM) is possible where noble metal surfaces are the solid substrate. Self-assemblies using alkenesilanes, disulfides, sulfides, and thiols are amongst the most studied. Where a hydrocarbon chain of 12 carbons is employed a highly ordered SAM can be produced. Shorter hydrocarbon chains result in less ordered structures, with a linear relationship between the length of the chain and the stability of the SAM (Andreescu & Marty, 2006, Chaki & Vijayamohanan, 2002). SAMs ensure orientation and spatial control of immobilised biomolecules; however, the reproducible layering of biomolecules remains a major limitation. With regards to the immobilisation of biomolecules, SAMs are particularly attractive because of the existence of a membrane-like microenvironment at the SAM surface, which is suitable for biomolecule attachment. By modifying the headgroups of SAM subunits a high degree of control over the nature of binding interactions is possible simply by applying the appropriate functional groups to enhance hydrophilic, or hydrophobic interactions as necessary. SAMs also exhibit good stability for extended periods, allowing multiple measurements to be performed. Limitations with the use of SAMs are due to the ready oxidation of SAM layers in the course of chemical investigations. This can also

result in changes to other physico-chemical properties in the vicinity of the SAM, which can be detrimental to the activity of immobilised biomolecules. A further difficulty in the use of SAMs is the occurrence of non-specific adsorption as a result of the high surface energy. Hydrophobic SAM surfaces can also experience accumulation of contaminants, and unwanted impurities, which can destroy the specificity of a biosensing device.

Collinson *et al.* reported an amperometric biosensor where cytochrome C was immobilised to a carboxylic acid-terminated SAM surface using a carbodiimide reaction (Collinson *et al.*, 1992). Shoham *et al.* have reported the amperometric detection of bilirubin using a bilirubin oxidase multilayer electrode by the covalent attachment of bilirubin oxidase layers to a self-assembled monolayer of 3-mercaptopropionate ester associated with a gold surface (Shoham *et al.*, 1995). Ruan *et al.* reported a reagentless hydrogen peroxide biosensor constructed by the immobilisation of thionine and horseradish peroxidase to a cystamine-assembled gold electrode using glutaraldehyde as a bifunctional reagent (Ruan *et al.*, 1998).

1.7.4 PHYSICAL ENTRAPMENT

The generation of a protein-containing matrix can be achieved using several different methods such as sol-gel construction, electrodeposition of various conducting polymers, and photopolymerisation. Entrapment of biomolecules by photopolymerisation is achieved simply by mixing the protein species with the photopolymer under a neon light (Andreescu *et al.*, 2002a). Protein-loaded Sol-gel matrices are produced typically by formation of a sol-gel network around the protein, followed by a chemical condensation reaction. During the electrodeposition of conducting polymers it is possible to entrap biomolecules. This particular method has the added advantage of control over the thickness of the polymer/protein layer (Cosnier, 1999). All of these methods of entrapment exhibit improved properties of stability and storage compared to other immobilisation methods. The absence of any covalent modification of biomolecules means that the native activity is maintained. However, these methods are largely limited to the immobilisation of enzyme molecules due to the diffusion barriers which may occur. In addition leaking of biomolecules may occur from the entrapment matrix (Andreescu *et*

al., 2006b).

Early work on the immobilisation of biomolecules using entrapment procedures involved the incorporation of enzyme molecules into growing PPY layers (Umana & Waller, 1986). A working electrode was soaked in an aqueous solution containing pyrrole monomers and enzyme molecules, an appropriate potential was then applied to the working electrode, which caused growth of a PPY layer. As the PPY layer grew, enzyme molecules became trapped within it. This method gave researchers control over the thickness of the polymer/enzyme layer by monitoring the electrical charge passed during the electrochemical polymerization. Also, because there is no chemical modification of the enzyme molecules, there is no loss of catalytic activity. This kind of entrapment procedure is generally used with conducting polymers such as polyacetylene, polythiophene, polyaniline, polyindole and polypyrrole (Shinohara *et al.*, 1988, Pandey, 1988). Most work using immobilising biomolecules by entrapment has been conducted using enzyme species. Mu *et al.* have reported a method for intercalation of enzyme molecules into a polyaniline matrix by electrochemical doping. During the oxidation or reduction of the PANI film, negatively or positively charged proteins can be intercalated into the PANI matrix respectively (Shaolin, 1994, Shaolin *et al.*, 1991, Yang & Shaolin, 1997). The PANI fibril has a reported length of 200 nm (2000 Å), which is sufficient for the intercalation of enzymes such as glucose oxidase (Shaolin *et al.*, 1991) galactosidase oxidase (Jinqing *et al.*, 1997) and peroxidase (Yang & Shaolin, 1997) into the PANI film.

Sol-gel derived glasses have emerged in recent years as an appealing method of immobilisation. These are highly applicable to the immobilisation of biomolecules due to their physical rigidity, chemical inertness, high photochemical and thermal stability, excellent optical transparency, and experiences negligible swelling in aqueous and organic solvents. Glucose oxidase has been immobilized in sol-gel/copolymer matrix to develop an amperometric glucose biosensor (Wang & Lu, 1998). The sensor exhibited a rapid response time of just 11 s due to the fast diffusion of the substrate molecule in the thin sol-gel/co-polymer film. Sol–gel-derived metal oxide/Nafion composite films have been used for the immobilisation of glucose to produce an amperometric glucose biosensor (Choi *et al.*, 2005), which exhibited a response time of just 2 s. This was

been used for the immobilisation of glucose to produce an amperometric glucose biosensor (Choi *et al.*, 2005), which exhibited a response time of just 2 s. This was attributed to the large pore size of the composite film. The use of sol-gel immobilisation in the development of a biosensor to H_2O_2 has also been reported (Tian *et al.*, 2001).

1.8 SCREEN-PRINTED PANI-BASED BIOSENSOR PLATFORM

The biosensor developed by Smyth's group consists of antibody immobilised to a layer of PANI on a screen-printed carbon paste electrode (Killard *et al.*, 2001, Grennan *et al.*, 2003). Antibody molecules are doped onto the surface of the polymer by electrostatic interactions with the polymer backbone (Shaolin *et al.*, 1991). This means of immobilisation is thought to avoid diffusion of substrates and biorecognition molecules throughout the polymer matrix as is required with other polymer entrapment systems (Arai *et al.*, 1998). Immobilised in this way, the antibodies can take part in immunochemical reactions with enzyme-labeled haptens (Killard *et al.*, 1999). Antibody binding of conjugate species brings the enzyme molecule of the conjugate proteins into sufficient proximity with the electrode surface to allow electrical communication with the carbon paste electrode, via the PANI layer (Lewis *et al.*, 1999). Figure 1.7 illustrates the operation of this biosensor.

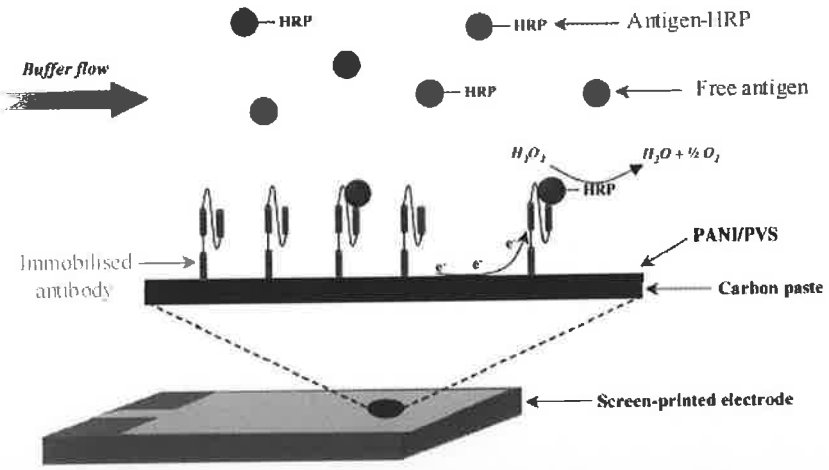


Fig. 1.7 Competition assay at the PANI/PVS interface, competitive binding of free analyte protein inhibits signal response of HRP-labeled analyte reproduced from Grennan *et al.*, 2003.

PANI is a conducting polymer with potential for application in areas such as energy storage, catalysis, indicators, and sensors, (Gospodinova and Terlemezyan, 1998, Kroger *et al.*, 1998). Leucoemeraldine, and pernigraniline are the fully reduced, and fully

oxidised forms. Growth of PANI films using potentiodynamic techniques gives greater control over the morphology and thickness of the PANI film and was the method employed by Smyth's group (Trivedi, 1997). The PANI matrix provides a means of direct electrical communication between the redox center of enzyme molecules and the electrode surface (Lewis *et al.*, 1999). This is achieved by delocalisation of redox charges over conducting polymer groups which results in the "electrical wiring" of the enzyme species to the electrode (Lu *et al.*, 1998). The main advantage of this set-up is that it removes the need for additional diffusional mediators within the sensing system to facilitate electron transfer. The mechanism for the polymerisation of aniline involves the formation of radical cations of aniline by oxidation at the surface of the electrode, these radical cations then dimerize and the resultant dimers are oxidised with additional radical cations, resulting in propagation of the chain (Gospodinova & Terlemezyan, 1998, Kroger *et al.*, 1998). The electropolymerisation of PANI was conducted in the presence of the poly(vinylsulphonic acid) (PVS) ion to ensure that electrical neutrality of the oxidised PANI layer was maintained. PVS inclusion also increases the structural stability and conductivity of PANI at a broader range of pH values (Michaelson *et al.*, 1993).

Screen-printed carbon paste electrodes were used in the construction of this biosensor. The advent of screen printing as a way to produce electrodes for use in biosensing provides a low cost means of electrode production which is also flexible in terms of electrode design with a very short time between design and manufacture of electrodes (Grennan *et al.*, 2006). Electrodes are produced using a thick film printing technology to print inks onto a suitable substrate producing a working electrode. For biosensing work within Smyth's group screen-printed electrodes were produced with three ink layers, a silver conducting track, a conducting carbon paste spot at the end of this track, and finally an insulation layer (Killard *et al.*, 1999), which defined the working electrode area. The inks were applied to a pre-shrunk PET substrate. Poor reproducibility remains the major obstacle to the use of screen printed electrodes. In spite of this difficulty screen printed electrodes have been employed effectively by Smyth's group and others in the development of biosensors (Killard *et al.*, 2001, Grennan *et al.*, 2003, Hart & Wring, 1994).

This biosensor has been used to develop a separation-free immunoassay system for the real-time monitoring of biospecific interactions biospecific interactions. HRP enzyme is conjugated to the analyte under study. Samples containing both free analyte, and HRP-conjugated analyte are then applied to the sensing surface resulting in competition between HRP-labeled analyte, and free analyte. Antibody-binding of HRP-analyte conjugate results in the transduction of H_2O_2 reduction by antibody-bound HRP-analyte conjugate. Only catalytic activity of antibody-bound species is coupled to the electrode surface which means that any catalytic current response is limited to protein bound by antibody and free from any electro activity taking place in the bulk solution. This format has been used in the development of biosensors for the determination of HRP (Iwouha *et al.*, 1997) the hapten biotin (Killard *et al.*, 1999, Killard *et al.*, 2001) and the herbicide atrazine (Grennan *et al.*, 2003) using both batch and flow cell analysis for quasi-equilibrium, and real-time analysis of samples.

Through the course of this thesis work will be presented detailing efforts to expand the bisosensing capabilities of the biosensor platform described above to detect the hormone oestradiol, and the surface protein p60. Efforts to improve the reproducibility, and magnitude of catalytic signal will also be details, focusing on optimizing the pH of buffer solution, and the concentration of antibody protein used during protein immobilization, as well as investigating an alternative enzyme label to HRP. Finally surfactant molecules will be probed as a potential means of reducing non-specific protein binding within the system.

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Chapter 2

Materials and methods

2.1 *Reagents*

Original stocks of oestradiol, p60 conjugates, polyclonal oestradiol antibody, and polyclonal p60 antibody with a hexa-histidine tag were donated by Prof. Richard O' Kennedy, School of Biotechnology, Dublin City University. Phosphate buffer tablets (P4417), poly(vinylsulphonate) (2784-4), sodium hydrogen carbonate (S6297), sodium periodate (3144-8), sodium borohydride (452882882), research grade HRP (P6782), oestradiol (E8875), rabbit IgG (I5006), anti-rabbit-HRP (A6154), dimethylformamide (D4551), butyl-chloroformate (184462), and methyl-morpholine (407704), rabbit IgG (I5006), and anti-rabbit-HRP (A6154), microperoxidase-11 (M6757), PVS, SDS, NTA, $NiSO_4$, Tween-20 (P1379), Triton-X-100 (T9284), Triton-X-405 (234737), butanol (B7906), and octanol (O4500), were all ordered from Sigma-Aldrich. Aniline was purchased from Sigma-Aldrich (13,293-4), vacuum distilled and stored frozen under nitrogen. Potassium hydrogen phosphate (04248) was ordered from Reidel de Haan. Hydrogen peroxide (K30615197221) 30% w/v was purchased from Merck. Carbon paste (C10903D14) was obtained from Gwent Electronic Materials Ltd. (Gwent, UK). Silver conductive ink (Electrodag® PF-410) and dielectric polymer ink (Electrodag® 452 SS BLUE) were purchased from Acheson. Poly(ethylene) terephthalate substrates were Melinex® (pre-shrunk) films obtained from HiFi Industrial Film Ltd. (Dublin 6, Ireland).

2.2 *Buffers and solutions*

All solutions were made up in phosphate buffer saline (PBS, 0.1 M Na_2HPO_4, 0.137 M NaCl and 2.7 mM KCl and 0.1 M KH_2PO_4, 0.137 M NaCl and 2.7 mM KCl, pH 6.8). Conjugation reactions were carried out in 0.1 M $NaHCO_3$, pH 8.1, 8 mM $NaIO_4$ and 100 mM $NaBH_4$.

2.3 *Instrumentation*

Electrode modification and protein immobilisation were performed on a CHI1000 potentiostat using CH1000 software. A platinum mesh electrode was employed as the auxiliary electrode in all experiments. An Ag/AgCl electrode was used as the reference electrode in all experiments. Electrochemical batch, and flow cells were constructed according to Killard *et al.* (1999). Cells were made using polycarbonate, and designed to house screen-printed electrodes. Internal Ag/AgCl reference, and platinum auxiliary electrodes facilitated electrochemical measurements. The batch cell and flow cell had volumes of 2 ml and 25 µl respectively.

Screen-printing of in-house electrodes was performed using either a semi-automated DEK 247, or a DEK248 printing machine, (Weymouth, UK). Polyester screens with a mesh thickness of 7 were used, and mounted at 45° to the print stroke. Blade rubber squeegees were employed, and a flood blade was incorporated into the operation of the DEK 248. All inks were cured in a conventional oven, apart from the insulation layer, which was cured with the UV lamp curing system from UV process supply, Inc. (Cortland, Chicago, IL, USA).

2.4 *Electrode pre-treatment procedure*

Pre-treatment of electrodes was performed to oxidise any unwanted species present on the electrode surface. A single voltammetric cycle between –1.2 and 1.5 V vs. Ag/AgCl was carried out for this purpose in 10 ml of 0.2 M H_2SO_4, at a scan rate of 100mV/s, and a sensitivity of 1×10^{-3} A.

2.5 *Aniline polymerisation*

The aniline solution was prepared containing 7.8 ml of 1 M HCl, 2 ml of PVS, and

186 µl of aniline. This solution was degassed and polymerised to the surface of the working electrode using 20 voltammetric cycles between –0.5 and 1.1 V vs. Ag/AgCl electrode at 100 mV/s, and a sensitivity of $1x10^{-4}$ A.

2.6 Protein immobilisation

Following polymerisation of aniline to the electrode surface, the electrode was transferred to a batch cell. 2 ml of PBS was added to the cell. The polymer surface was reduced at –0.5V vs. Ag/AgCl, at a sample interval of 500 ms, over 1500 s at sensitivity of $1x10^{-4}$ A.

Once reduction of the electrode surface was complete, PBS buffer was removed from the cell and replaced with a 200 µl volume of the antibody solution (0.7 mg/ml) or 200 µl of PBS where no protein modification was required. Antibody solution was added immediately after removal of PBS to ensure that no other species were oxidized at the polymer surface. Antibody immobilisation was performed at + 0.7 V vs. Ag/AgCl for 1500 s.

2.7 Real-time amperometry (flow cell)

The modified electrode was then transferred to a flow cell, which was connected to a peristaltic pump. Phosphate buffer was flowed through the cell at a flow rate of 400 µl min−1. Time-based amperometry was run at –0.1 V vs. Ag/AgCl with a sensitivity of 1 x 10^{-4}A for 4000s. Once a steady-state current was reached, samples were passed over the electrode surface.

2.8 *Real-time amperometry (batch cell)*

Following preparation of electrode, and immobilisation of antibody against analyte at a concentration of 0.7 mg/ml, analyte-HRP conjugate solution was added to the batch cell, (25 µg/ml), and antibody binding was allowed to take place at open circuit potential for 1 hour. 2 ml of PBS, (degassed under nitrogen), was added to the batch cell, and the steady state amperometry phase was run. PBS degassing was continued throughout this phase. Once steady state current was achieved, (current < 1 µA), 16 µl of H_2O_2 solution, (1 M, final concentration in batch cell 8 mM), was added to the batch cell. Current profile was then analysed for oxidation activity of antibody-bound oestradiol-HRP conjugates.

2.9 *BCA assay*

Copper sulphate, and bicinchoninic acid were combined in a ratio of 1:50 $CuSO_4$:BCA. BSA standards covering the range from 0 to 2 mg/ml were prepared. 190 µl of the BSA standards were added to wells of a microwell plate. 190 µl of test solutions were also added to separate wells on the plate in triplicate. 10 µl of BCA solution was added to each well. The plate was covered, and incubated for 1 hr, at 50°C. Results were taken at a wavelength of 480 nm. The signal from the BSA standard wells was used to construct a standard graph, from which an equation was derived. This equation was used to find the concentration of protein in test samples.

2.10 *P60 conjugate synthesis*

1.3 mg of HRP was dissolved in 250 µl of freshly prepared 0.1 M $NaHCO_3$, pH 8.1 in a glass test tube. 250 µl of 8 mM $NaIO_4$ was then added to this, the glass tube was covered in aluminum foil. The mixture was incubated in the dark for 2 hr, at room temperature. 1 mg of p60 protein was prepared in 250 µl of PBS. This was added to the glass tube containing the HRP solution, and incubated for a further 2 hr in the dark, at room temperature. The reaction mixture was stabilised by addition of 100 µl of $NaBH_4$, (prepared in 100 mM NaOH), and this was incubated for 1 hr at 4°C. Dialysis was then carried out on the conjugation mixture.

2.11 Oestradiol conjugate synthesis

1.5 mg of oestradiol was dissolved in 100 µl of DMF, and 1 µl of methylmorpholine was added. This solution was cooled to -15°C and 1 µl of butylchloroformate was added. The solution was left to stir at -15°C for 3 min. This mixture was added slowly to a pre-cooled (0°C) solution of 11 mg of HRP in 100 µl water and 75 µl dimethylformamide (DMF). This was stirred at 15°C for 60 min, followed by 0°C for 120 min. 1 mg of $NaHCO_3$ was then added and the products were dialysed overnight against PBS.

2.12 Dialysis

Dialysis tubing with a MWCO of 12,000 MW was used for all dialysis experiments. An appropriate length of cellulose acetate dialysis tubing was cut. This was boiled for 15 min in deionised water containing 2 %, (w/v), sodium bicarbonate and 0.05% EDTA, followed by 15 min in 0.05% EDTA, and finally 15 min in deionised water. A dialysis clip was used to seal one end of the tubing, and the solution to be dialysed was added to the open end. This was then sealed using dialysis clips, and placed in 5 L of PBS buffer. Dialysis was performed for 24 hr at 4°C.

2.13 Direct ELISA

100 µl of anti-oestradiol/p60 antibody, (10 µg/ml), was immobilised to the base of a microwell ELISA plate. The plate was covered, and was incubated for 1 hr at 37°C. Washing was performed between each step a total of 6 times, 3 times with PBS 0.05% Tween-20 in PBS and 3 times with PBS. Wells were then treated with 200 µl of 1% BSA blocking solution. The plate was covered, and incubated for 2 hr at 4°. Solution was removed from wells, and washing was carried out. Wells were then treated with 100 µl of solution containing HRP conjugate, (10 µg/ml), and free oestradiol/p60, (0 – 25 µg/ml). The plate was covered, and incubated for 1 hr at 37°C. Solution was removed from wells, and washing was carried out. Finally 100 µl of o-phenylenediamine dihydrochloride substrate was added to each well. The plate was covered, and incubated for 30 min, at 37°C. The peroxidase reaction was arrested by adding 50 µl of 2 M HCl to each well.

Readings were then taken at a wavelength of 492 nm.

2.14 Indirect ELISA

100 µl of HRP conjugate, (10 µg/ml), was added to each well of an ELISA microwell plate. The plate was covered, and incubated for 1 h, at 37°C. Washing was performed between each step a total of 6 times, 3 times with 0.05% Tween-20 in PBS, and 3 times with PBS. Solution was removed from wells, and washing was carried out. 200 µl of 1% BSA blocking solution was added to each well. The plate was covered, and incubated for 2 hr at 4°C. Solution was removed from wells, and washing was carried out. 100 µl of solution containing –oestradiol/p60 antibody, and free oestradiol/p60 was then added to each well. The plate was covered, and incubated at 37°C for 1 hr. Solution was removed from wells, and washing was carried out. 100 µl of solution containing anti-rabbit antibody conjugated to alkaline phosphatase, was then added to each well. The plate was covered, and incubated for 1 hr at 37°C. Solution was removed from wells, and washing was carried out. 100 µl of p-nitrophenyl phosphate substrate was then added to each well. The plate was covered, and incubated at 37°C for 30 min. The phosphatase reaction was arrested by adding 50 µl of 3 M NaOH to each well. Readings were taken at 405 nm.

2.15 Immobilisation parameter tests (Chapter 4)

2.15.1 Optimising pH of immobilisation phase

pH was varied between 6.0 and 8.0 by the addition of HCl, or NaOH, to PBS buffer. These solutions were then used to prepare antibody at a concentration of 0.7 mg/ml. Rabbit IgG (0.7 mg/ml) was immobilised to the PANI-modified SPE according to Section 3.2.7, from PBS solution where pH of the buffer solution was varied between 6.0 and 8.0. Following immobilisation, steady state amperometry (Section 3.2.8) was used to monitor the effect of pH on immobilisation. 500 µl of anti-rabbit-HRP was passed over the electrode surface with H_2O_2 (10 mM), and catalytic signal was recorded. The pH of all protein solutions was varied by adding HCl/NaOH to the buffer solutions.

2.15.2 *Optimisation of antibody concentration for immobilisation*

P60 antibody, (0.1-1.1 mg/ml), was immobilised to the PANI-modified SPE according to Section 3.2.7, in PBS solution at a pH of 6.8. Following immobilisation of p60 antibody, steady state amperometry was carried out, (Section 3.2.9). Once steady state current was reached 500 µl of p60-HRP (10 µg/ml) or HRP (10 µg/ml) was passed with H_2O_2, (10 mM), and catalytic signal was recorded.

2.16 *Surfactant testing (chapter 5)*

2.16.1 *Real time binding*

To assess the impact of surfactant molecules on real-time association between protein, and the electrode surface electrodes were prepared with no immobilised protein. After current had stabilized during the steady-state amperometry phase 500 µl of HRP solution, (at 10 µg/ml), was passed over the surface with 1 mM H_2O_2, this was followed by 500 µl of H_2O_2 on its own at 1 mM to validate HRP signals.

2.16.2 *Activity following surfactant adsorption*

Electrodes with no immobilised protein were prepared, and current was allowed to stabilize. 500µl of PBS containing surfactant, (at concentrations ranging from 0.1-5 %), was passed over the electrode surface, followed by 500 µl of HRP solution, (10 µg/ml). 500 µl of H_2O_2 was then passed over the electrode surface to validate signals.

2.16.3 *Disruption of immobilised protein*

HRP was immobilized to the electrode surface. Current was allowed to stabilize during steady state amperometry under 1 µA. 500 µl of H_2O_2, (1 mM) was then passed over the electrode surface to establish the level of signal due to immobilized enzyme. This

was followed by 500µl of H_2O_2 containing a percentage of surfactant to be tested. Finally a third 500µl of H_2O_2 was passed over the surface to validate the resulting signal.

2.16.4 Testing surfactant in Nickel-NTA system

To polymerise nickel- Nitrilotriacetic acid (NTA) onto an electrode surface, 10 cycles of polymerization with aniline were first run on an electrode. 0-1.5 ml of a 20 % NTA solution were then added to this polyaniline solution, and a further 4 cycles of polymerization run. The electrode was then immersed in $NiSO_4.6H_2O$ for 30 min. Steady state amperometry was then run on electrodes prepared in this way. Once current had stabilized 500 µl of p60-HRP conjugate with H_2O_2, (1 mM), were passed over the electrode surface, and signal recorded. This was followed by 500 µl of H_2O_2, (1 mM), to validate signal, then another 500 µl of H_2O_2, with 0.1 % Tween-20 and signal was monitored for any rapid deterioration. Degradation of this kind would indicate that protein was being removed from the electrode surface. Several experiments were also run where 500 µl of HRP with H_2O_2 were passed over the electrode surface to analyse real-time non-specific adsorption of protein in the presence of Tween-20.

Chapter 3

Investigations into the amperometric immunosensing of oestradiol and p60

3.1 INTRODUCTION

Listeria monocytogenes is a gram-positive bacterium found in a variety of both processed, and unprocessed foodstuffs including dairy products, meat, vegetables and seafood and can grow when refrigerated. *L. monocytogenes* infection causes listeriosis (Churchill *et al.*, 2006), and the infectious dose is unknown. Lysteriolysin is a pore-forming toxin produced by *L. monocytogenes,* which is essential to infection of host organisms, and also the target of immunoassays to detect the organism. The mortality rate for listeriosis can be up to 20 %. High-risk groups are the very old, and young, immunocompromised individuals, and pregnant women. Optimal culture of the bacterium requires blood samples; however, analysis is difficult to achieve, commonly taking anything from 3 to 6 days for results on contaminated foodstuffs. Immunoassays are dependent on successful culture of the bacterium under conditions facilitating toxin production. p60 is a 60 kDa protein thought to be important in maintaining the invasive phenotype of *L. monocytogenes* cells in mouse fibroblasts (Schmid *et al.*, 2005).

Research has been conducted into the development of biosensors for the detection of *L. monocytogenes*, (Benhar *et al.*, 2001, Leonard *et al.*, 2004) using electrochemical and optical techniques. One group constructed an amperometric immunosensor, which used single chain variable fragment (scfv)-expressing phage particles to capture *Listeria monocytogenes*. The phage particles were also conjugated to peroxidase species, the activity of which generated a measurable catalytic signal (Benhar *et al.*, 2001). An optical immunosensor was also developed using an immobilised anti-Fab antibody to detect free antibody in sample solution following the removal of *Listeria monocytogenes* cells and bound antibody by means of a subtractive inhibition assay format (Leonard *et al.*, 2004). However both the amperometric, and optical immunosensors described required expertise, and equipment, which limit their application to clinical settings.

17-β Oestradiol is a member of the estrogen hormone family produced by the ovaries, the testis, and the adrenal glands. It is used to promote "marbling", (the development of fatty tissue found in muscle), in cattle populations and reduces the metabolism of ingested energy sources to fat, producing leaner meat. Within the European

Union its use has been banned outright, however the FDA in the U.S. has licensed its administration in a slow-release implantable capsule. Research has linked elevated 17-β oestradiol levels to growth problems, with a biphasic influence on osteogenesis. It has also been implicated in the risk of individuals developing breast cancer, and endometrial adenocarcinoma. Changes in oestradiol levels are associated with the onset of menopause, and it is a component of hormone replacement therapy for menopausal women (Zapantis and Santoro, 2003). There is also an association between the higher levels of oestradiol in women and the functioning of their immune system (Beagley and Gockel, 2003). Obviously any changes in serum oestradiol levels within the population at large could have detrimental effects, which makes the monitoring of oestradiol levels of critical importance.

Because of the extremely low levels of Oestradiol 17-β in serum, it is difficult to monitor any changes in serum levels, (Joosten *et al.*, 2004). Immunoassays encounter difficulties due to cross reactivity, lack of traceability, and the large turnaround time. Biosensors have been developed for oestradiol. An amperometric immunosensor to oestradiol was developed using a direct competition assay employing a monoclonal antibody labeled with alkaline phosphatase (Butler and Guilbault, 2006). Another group developed a voltammetric immunosensor to oestradiol based on the immobilisation of anti-rabbit IgG to a screen-printed electrode. This bound to a rabbit anti-oestradiol antibody, which was conjugated to alkaline phosphatase. Addition of the substrate 1-naphthylphosphate lead to the production of the electroactive substance 1-naphthol, which was measured using differential pulse voltammetry (Volpe *et al.*, 2006). A disposable immunosensor employing a competition immunoassay with immobilised rabbit anti-oestradiol has been reported which gave detection limits in the picomolar range for oestradiol detection in human serum extracts (Pemberton *et al.*, 2005).

Work presented in this chapter outlines efforts to expand the biosensing capabilities of the biosensing platform developed by Smyth's group to detect oestradiol, and p60. What follows are the results of a series of ELISA, and biosensor experiments to probing the protein species involved, and investigating their application to the biosensor

platform.

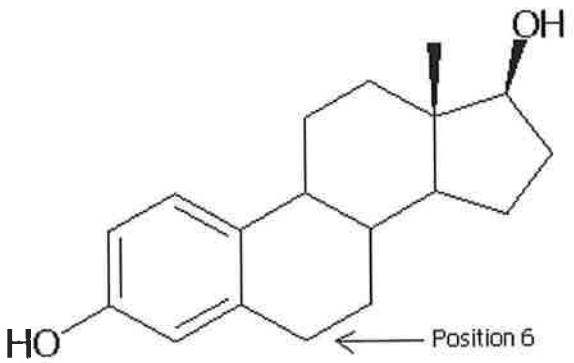


Fig. 3.1 Structure of 17-β Oestradiol, the 6th position is the most important to provoking a complete immune response necessary for the generation of antibodies.

3.2 RESULTS AND DISCUSSION

Oestradiol-HRP conjugate, and polyclonal anti-oestradiol antibody were the first set of reagents to be investigated. Previously, inhibition and competition ELISAs were developed using this polyclonal antibody (Fitzpatrick *et al.*, 2003). Both ELISAs were quick and simple to perform, with the competition assay being the faster of the two. The competition ELISA exhibited a linear response between 12 – 12,500 ng/ml, while the inhibition assay exhibited a linear response 24 – 12,500 ng/ml. An optical immunosensor was also constructed using the BIAcore 3000, which had a detection range between 12 – 100,000 ng/ml. For this work, a fresh oestradiol-HRP (O-HRP) conjugate was synthesised using a modification to a procedure described by Meyer and Hoffmann (Meyer and Hoffmann, 1987). Conjugation was performed at the 6-position of the B ring, (Figure 3.1) which allowed access by the immune system to the A and D rings of the steroid, which are the most immunogenic portions (Fitzpatrick *et al.*, 2003). Qualitative immunoassay analysis was conducted initially to verify binding, and catalytic activity of reagents. The conjugate and antibody were then applied to the biosensor platform; however, reproducible catalytic signal could not be demonstrated. The reagents themselves were then investigated using the ELISA techniques for which they were originally produced (Fitzpatrick *et al.*, 2003).

An anti-p60 antibody, and a p60-HRP conjugate were also investigated using our electrochemical platform. Earlier fundamental work using these reagents (Killard A.T., personal communication, 2004) had indicated catalytic activity, with signals in the microampere range being generated upon binding of conjugate to immobilised antibody on the PANI/PVS surface. This was significant, as it was the first instance where this biosensor platform was being used to detect large protein molecules. The work presented here outlines efforts to reproduce these findings, and fully characterise the behaviour of p60 reagents.

3.2.1 Oestradiol

3.2.1.1 Protein analysis

Qualitative enzyme immunoassays were carried out on the oestradiol reagents to probe binding and catalytic activity. Immobilisation of anti-oestradiol antibody (10 μg/ml) to the microwell plates facilitated binding of oestradiol-HRP conjugate. A blocking stage incorporated after immobilisation of anti-oestradiol using BSA (5 % w/v) prevented the non-specific adsorption of conjugate to microwells. This established a high level of confidence that the source of signal on exposure to substrate was due to the catalytic activity of HRP species of antibody-bound oestradiol-HRP. Data from these tests is presented in Figure 3.2. The signal in wells containing both antibody and conjugate showed the highest level of absorbance. This signal is approximately 2-fold higher than that observed in the control wells (i.e. where no antibody or conjugate were present) or in empty wells. Signals in control wells can be attributed to background absorbance, although it could also be the result of non-specific binding activity. The signal in wells where all reagents are present can be attributed to specific binding of conjugate to immobilised antibody. These results were taken as confirmation of antibody binding, and conjugate signaling activity.

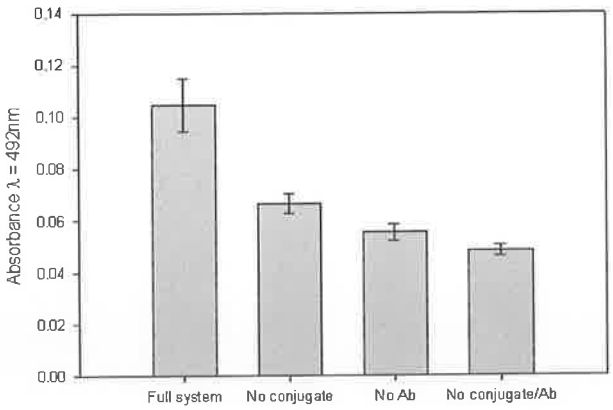
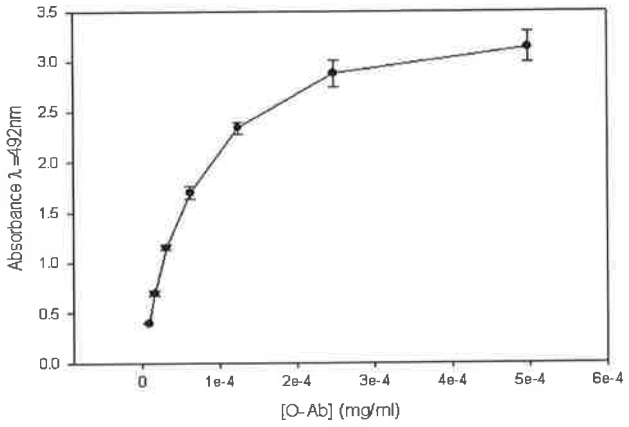
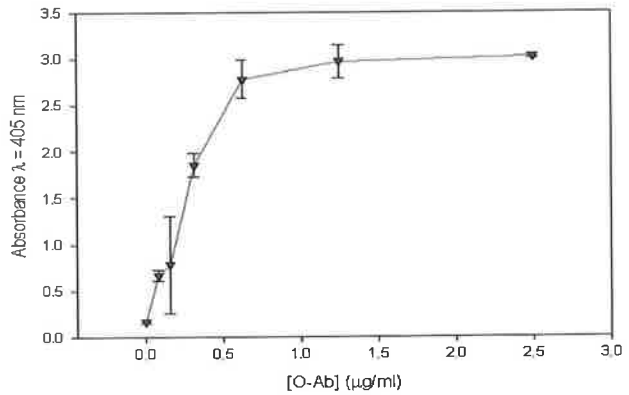


Fig. 3.2 Data collected for qualitative ELISA probing activity of anti-oestradiol antibody, and O-HRP conjugate, highest signal (measured at λ = 492 nm) was recorded in wells containing both antibody, and conjugate species, confirming binding, and catalytic activities (n = 3).

Following biosensor experiments, it was thought that biosensor difficulties might be the result of problems with the reagents themselves. Work now focused on reproducing the original findings reported using the oestradiol antibody and conjugate in order to identify any problem with the reagents. First a competitive ELISA was performed employing free oestradiol over a broad concentration range. A direct linear relationship between the concentration of free competing oestradiol and catalytic signal was observed using both direct, and indirect competitive ELISA formats. This is contrary to what would be expected according to ELISA theory. Where the higher the concentration of free antigen in the sample, the lower the signal that would be expected. Indeed, previously, an inverse relationship between free competing oestradiol and catalytic signal was demonstrated (Fitzpatrick *et al.*, 2003). To eliminate issues in this work caused by proteins employed at sub-optimal levels, reagents were optimised to determine their best operating concentrations. A new antibody titre was established using both ELISA formats (Fig. 3.3). For the direct ELISA format an optimal antibody concentration of 0.5 µg/ml was established, and for the indirect ELISA format an optimal antibody concentration of 1.5 µg/ml was established.



(a)



(b)

Fig. 3.3 Data collected for direct and indirect ELISA experiments to establish antibody titre for anti-oestradiol antibody, O-HRP was applied at a concentration of 10 µg/ml in both ELISA, antibody was varied between 0 to 0.5 µg/ml for direct ELISA (a) and 0 to 2.5 µg/ml for indirect ELISA (b) (n = 3).

The concentrations of both antibody and conjugate were then optimised using the direct ELISA format (Fig. 3.4) without free competing oestradiol. The results of these tests confirm effective functioning of both the anti-oestradiol antibody, and the O-HRP conjugate. The optimal concentration of O-HRP conjugate from this ELISA is 1.75×10^{-2} mg/ml. The change in catalytic signal with both concentration of anti-oestradiol, and O-HRP is further confirmation of antibody binding activity, and catalytic activity of O-HRP conjugate.

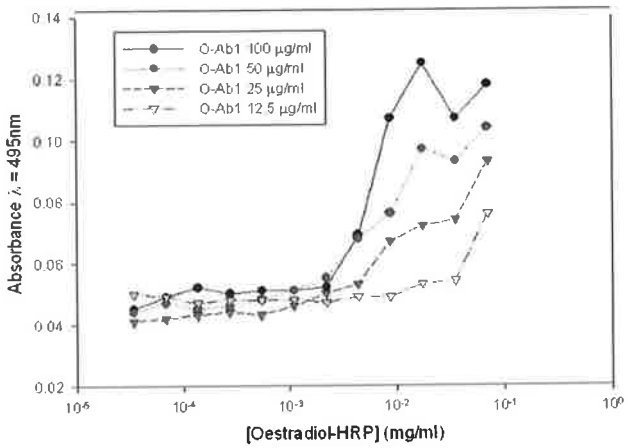
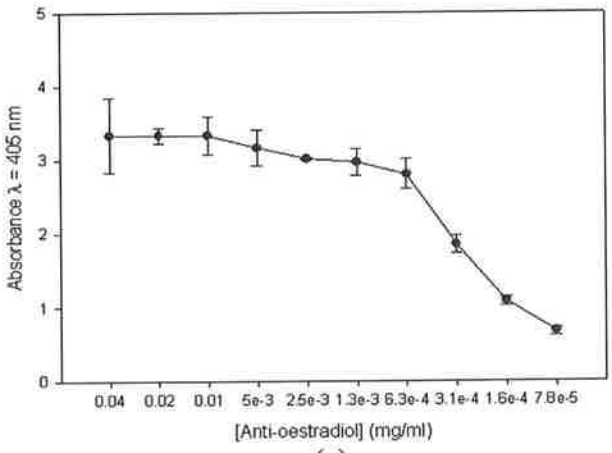
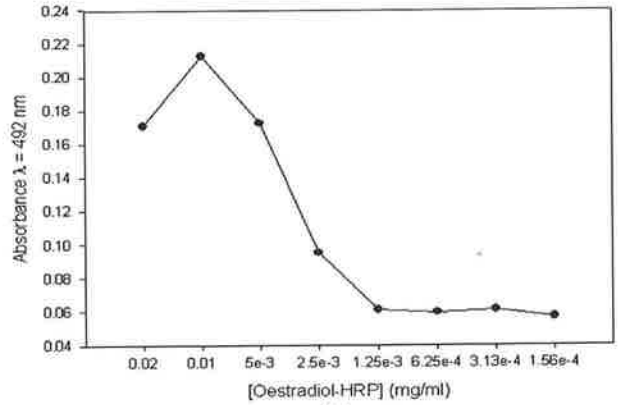


Fig 3.4 Data for a qualitative direct ELISA probing the sensitivity of signal to changes in the concentration of anti-oestradiol antibody, and oestradiol-HRP conjugate, the decrease in signal with falling concentrations of both species indicates quantitative binding/catalytic activities (n = 3).

However, despite these newly optimised conditions, a linear relationship between free competing oestradiol, and absorbance persisted. Confirmation of antibody binding of conjugate (Fig 3.2) and catalytic activity of conjugate species (Fig. 3.2) isolated the problem to the free competing oestradiol. To eliminate the possibility of issues due to the quality of reagents, fresh, analytical grade reagents were purchased, and fresh conjugates were synthesised. Antibody and conjugate were optimised again, in the absence of free competing oestradiol using direct ELISA. Binding of conjugate to antibody was demonstrated, as well as catalytic signaling of conjugate. Data for these optimisations is presented in Figure 3.5. Competitive ELISAs were carried out using these fresh reagents and the optimised conditions. However, a direct linear relationship persisted between the concentration of free oestradiol and absorbance. It is reasonable to speculate that the non-specific binding encountered initially in Figure 3.2 may be contributing to the problems with the current ELISA protocol. However, the exact nature of this complication was no elucidated.



(a)



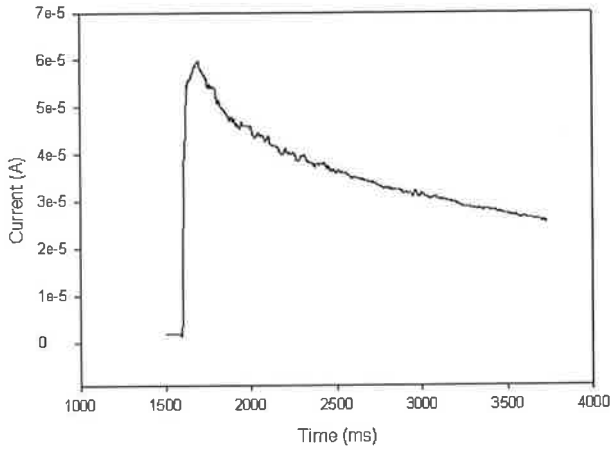
(b)

Fig. 3.5 Data collected for direct ELISA to determine optimal concentrations of anti-oestradiol antibody (a) and O-HRP conjugate(b), optimal concentration of antibody was established as 10 μg/ml, optimal concentration of antibody was established as 10 μg/ml (n = 3).

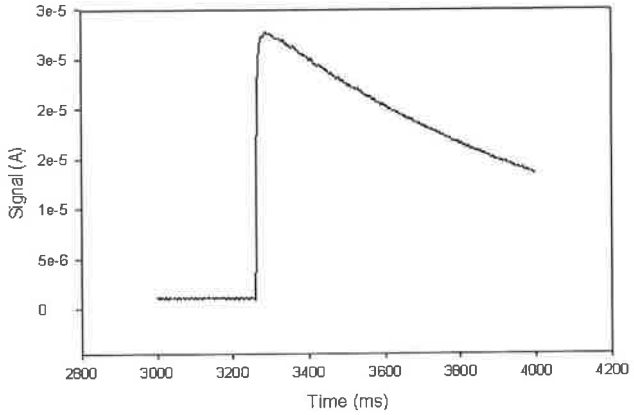
3.2.1.2 *Amperometric oestradiol immunosensor*

A non-competitive electrochemical immunosensor assay was investigated using the oestradiol antibody, and O-HRP on the PANI/PVS modified screen-printed electrodes. Experiments with oestradiol were conducted in a batch cell format allowing quasi-equilibrium analysis of samples. Anti-oestradiol (0.7 mg/ml) was immobilised to the surface of the electrode according to Section 2.6. A pre-incubation step was carried out to facilitate binding of O-HRP conjugate to immobilised antibody. This took place at open circuit potential for 1 hr to maximise conjugate occupancy of antibody binding sites. Conjugate solution was removed, and steady-state amperometry was set up at -100 mV vs Ag/AgCl in PBS, and once current had stabilised, H_2O_2 (10mM) was added to the cell (under stirred conditions).

On exposure to H_2O_2, catalytic current peaked rapidly before decaying to steady state (Fig. 3.6). The pre-incubation phase ensured that all antibody binding sites were occupied by O-HRP conjugate molecules. Following the pre-incubation step, conjugate molecules have already been bound by antibody; catalytic activity is thus limited only by the speed of substrate diffusion to the enzyme moiety of conjugate proteins. This results in a rapid increase in catalytic activity following substrate addition. Using the oestradiol reagents, catalytic activity was confirmed for immunosensor experiments; however, these signals could not be confirmed as the result of specific sensing activity.



(a)



(b)

Fig. 3.6 Catalytic signal for anti-oestradiol modified PANI/PVS electrode following pre-incubation with O-HRP conjugate at (a) 25 μg/ml and (b) 50 μg/ml.

The amperograms presented in Figure 3.6 were recorded using O-HRP concentrations of 25 and 50 μg/ml for (a) and (b), respectively of O-HRP. What is unusual about these results is that at a higher concentration of conjugate protein, a lower signal is recorded, (33 μA at 50 μg/ml compared to 65 μA at 25 μg/ml). Using the higher concentration of O-HRP, a maximum signal following antibody binding of conjugate should remain at 65 μA, (saturation of the antibody layer), or should increase, (as more antibody binding sites are occupied by conjugate molecules). What occurred was a drop in signal to 33 μA. A possible explanation of the decreased signal could be an increased

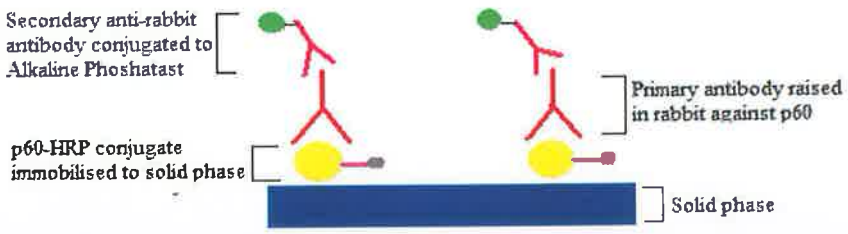
mass of protein at the PANI/PVS interface insulating the surface, and preventing efficient transduction of all catalytic activity.

It had previously been demonstrated that, at a concentration of 0.7 mg/ml, a mass of antibody sufficient to bind target protein without inhibiting transduction of catalytic activity could be immobilised (Morrin *et al.*, 2003). Using a larger concentration of O-HRP conjugate (50 μg/ml), increased occupation of antibody binding sites is anticipated, resulting in an increased catalytic signal as more redox activity is transduced. Any unbound O-HRP molecules are removed from the batch cell following the pre-incubation phase, leaving a layer of antibody bound to conjugate molecules. While it is possible that the increased electron transfer distance for O-HRP proteins, (compared to biotin-HRP, or HRP alone), may result in less efficient transduction, this would not explain the fall in signal as conjugate concentration is increased. This means that if a sufficiently large mass of protein was inhibiting signal transduction, this protein was non-specifically interacting with the PANI/PVS layer.

3.2.2 p60

3.2.2.1 Protein analysis

p60 was selected as an alternative system to interrogate on the PANI/PVS immunosensor platform. Both the antibody (anti-p60) and the antigen (p60) were donated by Prof. Richard O' Kennedy (DCU). ELISAs were initially performed in order to verify conjugation between p60 and HRP. Two sets of conjugates of p60 to HRP were synthesised according to Section 2.10. BCA assays were carried out to determine the concentration of conjugate. The concentrations of the conjugates were found to be 1.94 mg/ml and 1.50 mg/ml, for conjugates A and B, respectively. An indirect ELISA was then conducted to determine whether or not conjugation of p60 protein to HRP had been successful. p60-HRP conjugate was first immobilized to the base of the microwells. Wells were then treated with anti-p60 antibody as the primary antibody, and an anti-rabbit antibody conjugated to alkaline phosphatase as the secondary antibody. P-nitrophenyl phosphate was used as substrate, and catalytic signal was measured at a wavelength of 405 nm. Data from the non-competitive indirect ELISA is presented in Figure 3.7. A higher absorbance in the test wells compared to the controls indicated binding of antibody to immobilised p60-HRP. This method was used to determine the success of conjugation previously and the results presented here were taken as verification of conjugation between p60 and HRP. Scheme 3.1 below outlines the ELISA assay.



Scheme 3.1 Indirect ELISA assay is performed by first immobilizing p60-HRP conjugate, this is then bound by anti-p60 antibody, which is in turn bound by an anti-rabbit antibody conjugated to alkaline phosphatase.

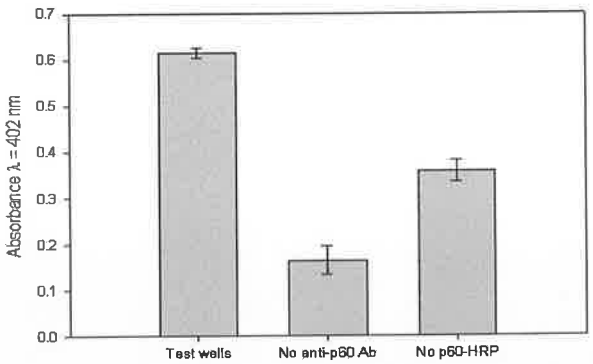
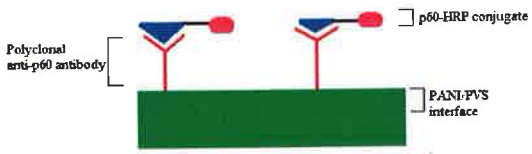


Fig. 3.7 Qualitative data for indirect p60 ELISA using p60-HRP (10 µg/ml) and anti-p60 (10 µg/ml) and anti-rabbit IgG conjugated to alkaline phosphatase (1 mg/ml) (n = 3).

3.2.2.2 *Amperometric p60 immunosensor*

Electrochemical immunosensing of p60 conjugate A using anti-p60 polyclonal antibody was carried out in a flow cell. Anti-p60 was immobilised to the PANI/PVS surface according to Section 2.6 (0.7 mg/ml). No pre-incubation step was used for this format. 4 samples of conjugate (10 μg/ml) were passed over the electrode surface with H_2O_2 (1 mM) consecutively for 20 s and the response monitored. Catalytic activity of p60-HRP protein was recorded; however, signals were 10-fold lower than had been reported previously on this platform using the same reagents (Killard A.T., personal communication, 2004) (10 – 20 μA). A second round of conjugation of p60, to HRP was performed using the same conditions as before. This fresh conjugate (B) was investigated on the immunosensor platform; however, sensor responses were equivalent to those of the original conjugate (A). Scheme 3.2 presents a schematic of the biosensor mechanism. Cumulative signals for sensors run using both conjugate species are presented in Figure 3.8.



Scheme 3.2 Binding of p60-HRP conjugate by immobilised anti-p60 antibody beings the HRP molecule into a proximity of the PANI/PVS interface which allows transduction of electrochemical activity to take place.

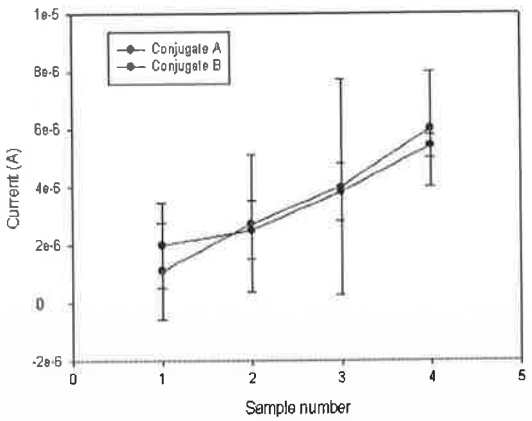


Fig. 3.8 Catalytic responses, (corrected for background activity), for both conjugate species A and B when multiple samples were passed consecutively at a fixed concentration (10 μg/ml) over the surface of electrodes modified with antibody at a concentration of 0.7 mg/ml.

Antibody was immobilised at a concentration of 0.2 mg/ml in previous p60 work, while the experiments presented here employed a concentration of 0.7 mg/ml. To investigate the impact of this increased antibody concentration on catalytic signal sensors were constructed using 0.7, 0.2, and 0 mg/ml antibody concentrations for immobilisation to the PANI/PVS film. The results are presented in Figure 3.9. Catalytic signals 100-fold higher were recorded for the lower antibody concentration (0.2 mg/ml). This indicated an inverse relationship between catalytic signal, and concentration of antibody immobilised to the PANI/PVS layer. Catalytic signal was higher again where an antibody concentration of 0 mg/ml was used. This result indicated that as antibody coverage on the electrode surface was reduced catalytic activity increased.

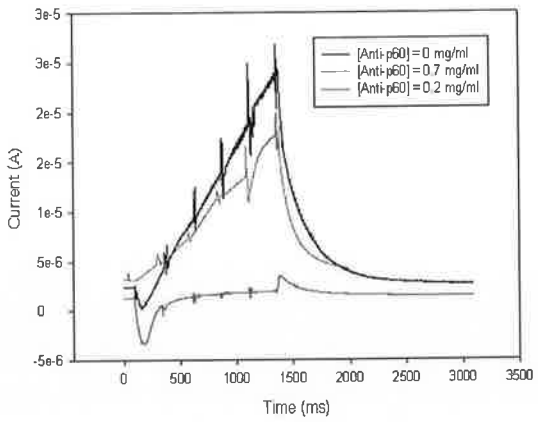


Fig. 3.9 Real-time amperometry showing catalytic responses of p60-HRP conjugate and H_2O_2 (10 mM) flowing over PANI/PVS electrodes modified with anti-p60 antibody where antibody is immobilised to the PANI/PVS surface at concentrations of 0.7, 0.2, and 0 mg/ml.

Previous work using HRP enzyme determined that the optimal concentration of protein during the immobilisation step was 0.7 mg/ml (Morrin *et al.*, 2003). At this concentration of antibody, reproducible signals were demonstrated for assay of HRP. At this concentration, a monolayer of antibody was immobilised to the PANI/PVS surface. The importance of this monolayer was two-fold. Firstly, a monolayer of antibody ensured that the maximum mass of analyte protein was bound by immobilised antibody. Secondly, a protein monolayer at the PANI/PVS surface insulated the PANI/PVS against non-specific catalytic activities. From the experimental data presented here it is clear that at an antibody concentration of 0.2 mg/ml, sub-monolayer coverage of the PANI/PVS layer was occurring. This results in transduction of non-specific bulk catalytic activity, accounting for the larger signals recorded at concentrations of 0.2, and 0 mg/ml of antibody protein compared to those recorded at 0.7 mg/ml. The non-specific activity recorded is assumed to be the result of the HRP molecules of unbound conjugate proteins associating with the PANI/PVS interface directly as samples are passed over the sensing surface. The results presented indicate this real-time non-specific association is not disrupted by flushing the surface with PBS prior to substrate addition to the flow cell. In

addition to ensuring monolayer coverage of the PANI/PVS interface with antibody by employing antibody at a concentration of at least 0.7 mg/ml, the addition of a blocking stage utilizing an inert protein may be an alternative means of reducing non-specific association. By adopting a covalent means of antibody immobilisation the inclusion of a washing step for elimination of non-specifically bound species could be realised. This would serve to reduce real-time non-specific adsorption, however, the concept of a single-step immunoassay would no longer be attainable. Covalent immobilisation of His-tagged antibody by a Nickel-NTA chelator is investigated in Chapter 5.

When a HRP monolayer is immobilised directly to the PANI/PVS layer the electron transfer distance is minimised. Biosensor detection of p60 requires immobilisation of antibody, followed by binding of p60-HRP conjugate proteins. At an antibody size of 140 KDa, and a p60 size of 60 KDa, (vs. 40 KDa for HRP) the electron transfer distance following antibody binding of conjugate molecule is considerably larger for p60 biosensors. This may result in significant losses in transduction efficiency, which may account for the lower catalytic signals recorded in this work.

3.3 CONCLUSIONS

This chapter outlines efforts to develop biosensors to the clinically relevant analytes oestradiol, and p60. Conjugate proteins were synthesised using oestradiol, and p60 conjugated to HRP enzyme molecules. Immunoassays were used to confirm successful conjugation, and to test the activity of the protein reagents. Finally antibody to the two analytes was immobilised to the PANI/PVS layer of a screen-printed carbon paste electrode, and exposed to samples containing HRP conjugates to investigate behaviour of biosensors to these analytes.

Initially, performance of oestradiol reagents was investigated using the ELISA protocols for which they had originally been produced. Binding activity of the anti-oestradiol antibody, and catalytic activity of the oestradiol-HRP conjugate were verified. The oestradiol reagents were applied to our electrochemical immunosensor platform in an attempt to design a format for real-time immunosensor analysis. This was examined in a non-competitive format, without free antigen. Catalytic signaling did occur, but was erratic. The original ELISA protocols were used to troubleshoot the oestradiol reagents. However, when a competition ELISA was carried out a linear relationship between the concentration of free oestradiol, and the catalytic signal recorded was demonstrated. According to ELISA theory an inverse relationship between free oestradiol, and catalytic signal should have been observed. Efforts were made to correct this behaviour by optimising the concentration of antibody, and conjugate proteins, and by synthesising fresh oestradiol-HRP conjugate. However, the linear relationship between free oestradiol concentration, and catalytic signal persisted. This indicated some problem with the oestradiol reagents themselves and this work was therefore discontinued.

p60 is an antigen of the bacterium *Listeria monocytogenes* and is an important analyte that requires a sensor platform capable of rapid analysis. p60-HRP conjugates were synthesised and ELISA techniques were used to verify the success of conjugation. Indirect ELISA verified the conjugation of p60 to HRP, and both p60-HRP and anti-p60 were then applied to the immunosensor platform. Catalytic signals were achieved upon exposure of the platform to conjugate with substrate. However, comparison of catalytic activity using antibody immobilisation concentrations of 0.7, 0.2, and 0 mg/ml revealed

an inverse relationship between the concentration of protein used during immobilisation, and catalytic activity. This indicated that at a concentration of 0.2 mg/ml sub-monolayer coverage of the PANI/PVS layer was achieved. Without the discrimination, and insulation provided by the antibody layer, protein is free to react directly at the PANI/PVS surface. This results in the transduction of non-specific catalytic activity. Thus at 0.2 mg/ml the catalytic signal recorded was the result of non-specific activity.

At 0.7 mg/ml monolayer coverage of the PANI/PVS layer occurs. However, compared to results for work using immobilised HRP catalytic signals obtained using p60 reagents are significantly lower. A possible explanation is the increased electron transfer distance following p60-HRP binding by anti-p60, relative to the much lower electron transfer distance for immobilised HRP. Where HRP is immobilised to the PANI/PVS layer the electron transfer distance is minimised. This produces a highly efficient transduction of any catalytic activity. Using p60 reagents, following anti-p60 immobilisation, p60-HRP conjugate is bound by antibody. The resulting electron transfer distance involves immobilised antibody, (150 KDa), p60, (60 KDa), and HRP (40 KDa) conjugated to antibody-bound p60. Under these conditions the electron transfer distance is considerably larger, which may result in a significant loss of transduction efficiency accounting for the lower catalytic signals recorded using p60 reagents.

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Chapter 4

Investigations into the effects of pH and concentration on protein immobilisation, and the amplification of label signal

4.1 INTRODUCTION

In a typical biosensor or immunosensor, enzyme or antibody needs to be immobilised efficiently to the transduction interface. In this case, the interface is a PANI/PVS-modified screen-printed carbon paste electrode, where the protein is immobilised by exploiting electrostatic interactions. The immobilisation method serves to localise the protein to the electrode without inhibiting access to the binding/active site, or catalytic activity.

Previous difficulties in incorporating p60 proteins onto our immunosensor platform indicated that sub-optimal coverage of the PANI/PVS layer was occurring. A sufficient mass of antibody needs to be immobilised so that maximum amount of analyte in samples will bind. However, if excess antibody is immobilised the resistance of the protein layer will impede transduction of electrochemical activity. Size differences between protein species may necessitate optimal antibody concentration for each individual protein, due to differences in electron transfer distances. Therefore it is imperative that the antibody concentration be optimised to ensure sufficient antibody is immobilised to allow binding of analyte, but without inhibiting transduction.

The protocol adopted by Smyth's group was originally devised by Mu *et al.*, and exploited doping of the PANI surface with protein using electrostatic interactions. The pH of the immobilisation buffer will affect the charge of the protein species; the charge a protein acquires depending on its isoelectric point and the pH of the buffer may affect it's immobilisation (Mu *et al.*, 1991). For this reason the pH of buffer solution used during immobilisation was assessed.

The folded polypeptide structure of enzymes such as HRP can inhibit exchange of electrons with the electrode as the active heme site is buried deep within the polypeptide. The result of this effect is a sub-optimal catalytic rate. MP-11 is a heme undecapeptide produced by the peptic digestion of cytochrome c, as such it lacks the large polypeptide structure of HRP. Experiments were also conducted in this work probing MP-11 as an alternative signal-generating enzyme to HRP.

4.2 RESULTS AND DISCUSSION

4.2.1 Optimisation of buffer pH during immobilisation

Work carried out by Mu *et al.* has shown that protein can be electrostatically adsorbed to the PANI surface using a doping procedure carried out by fully oxidising a PANI film in a buffered solution of the protein to be immobilised, and the method was demonstrated using glucose oxidase, xanthine oxidase, horseradish peroxidase, and sarcosine oxidase (Mu *et al.*, 1991, Mu *et al.*, 1996, Mu and Xue, 1995, Mu and Yang, 1996, Mu and Yang, 1997). This method exploits differences in charge between proteins and the PANI surface to immobilise proteins (Mu *et al.*, 1991). The pH of the buffer solution will result in charge formation on the protein related to its pI. This charge may be what facilitates the generation of electrostatic bonds between protein and the PANI/PVS surface. It was decided to re-optimise the pH of the protein immobilisation phase between the values of pH 6.0 and 8.0 to investigate the relationship between pH of immobilisation buffer and immobilisation.

The antibodies used in this biosensor work are of the IgG class. IgG proteins are positively charged (Gangopadhyay *et al.*, 1996) but heterogeneous in terms of isoelectric point (McGarry *et al.*, 1983). The isoelectric point range for rabbit IgG proteins has been reported as pH 6.7 to 7.5 (McGarry *et al.*, 1983). This makes them ideal as a model species for the immobilisation of proteins (including antibodies) with isoelectric points within this range. The pH study was conducted within the limits of 6.0, and 8.0 as these encompassed the isoelectric spectrum of IgG proteins, (the species immobilised in immunosensor work), allowing optimisation of electrostatic immobilisation of these proteins. Working within these limits also avoided any of the denaturating of proteins which occurs at higher, and lower extremes of pH. In this work, it was decided to vary the pH of protein buffer solution between 6.0 and 8.0, keeping all other factors constant, and monitoring the catalytic response of the electrodes prepared at each pH value for changes in activity. Any changes should reflect a change in the mass of antibody immobilised, or a variation of the resulting activity of the immobilised protein.

To assess the impact of pH on immobilisation, PANI/PVS electrodes were

prepared where IgG was immobilised electrostatically. Electrodes were prepared according to Sections 2.4 - 2.6, where the pH of protein solution used for immobilisation was varied within the range pH 6.0 - 8.0. 500 µl samples of anti-rabbit-HRP solution containing H_2O_2, (10 mM), were passed over the electrode surface. The resulting amperometric profile was recorded, and the current response for each pH value was identified. All pH values were tested in triplicate, and the results are presented in Figure 4.1 below. The graph shows that there does not appear to be a correlation between catalytic signal and pH of protein solution used for immobilisation. The catalytic signals had very low reproducibility. This was attributed to issues with the stability of the system related to the method of immobilisation of antibody explored in this chapter.

A T-test analysis of the collated data was performed. The results verified that the difference in response at different values of buffer pH, compared to that at a buffer pH of 6.8 (the pH used in our setup) is not statistically significant at the 5 % level. Since the difference was negligible, and all previous work had been done using a buffer pH of 6.8 during protein immobilisation this value was retained.

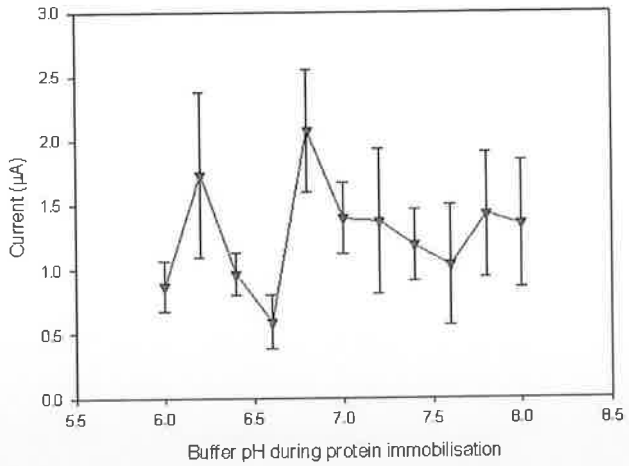


Fig. 4.1 Plot of catalytic signal generated at each pH value over the range 6.0 - 8.0. Highest catalytic response was recorded at a buffer pH of 6.8; however, signals were poorly reproducible.

4.2.2 *Optimisation of antibody concentration during protein immobilisation*

The level of coverage of the electrode surface by protein is critical to the performance of the immunosensor. Increased protein coverage on the electrode surface results in more catalytic or binding sites available, increasing biosensor sensitivity, and subsequently catalytic activity. However this theory is only true to a limiting point – at excessively high concentrations of protein, multi-layer formation may occur, and steric hindrance can become an issue, inhibiting electron transfer (Grennan *et al.*, 2006, Yang *et al.*, 2006). Ensuring optimal coverage of the electrode platform allows the minimisation of any bulk solution interferants being transduced to the electrode, whilst maximum sensitivity is achieved. The antibody concentration used for the immobilisation phase was examined in this work where the effect of concentration on catalytic signal was investigated.

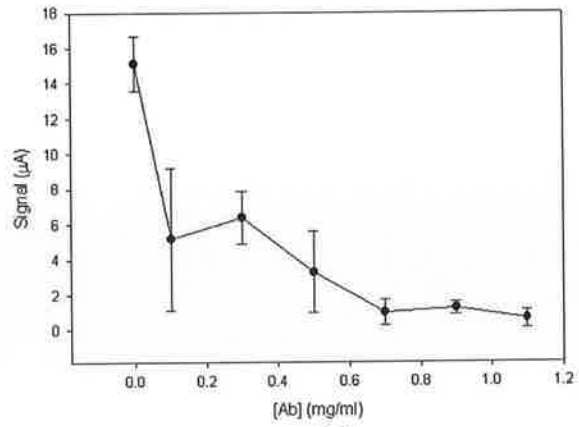
The most recent study on our PANI/PVS platform into the optimal protein concentration during immobilisation using the protein HRP had identified 0.7 mg/ml as the optimal protein concentration for the immobilisation phase (Morrin *et al.*, 2003). Mu *et al.* have demonstrated the intercalation of HRP molecules into the PANI matrix using his method of immobilisation (Mu and Yang, 1997). This means that any catalytic signal recorded following immobilisation of HRP to PANI using this technique is a reflection of catalytic activity throughout the PANI matrix, whereas work within Smyth's group has focused on monitoring activity at the surface of the PANI/PVS interface. Therefore, any optimisation data generated using HRP may not be applicable to immunosensors immobilising antibody at the PANI/PVS interface. Difficulties in producing a working immunosensor using p60 reagents indicated that the concentration of antibody used during immobilisation might be sub-optimal. Therefore, the concentration of antibody protein used during the immobilisation phase was optimised here, to ensure insulation of the PANI/PVS interface against bulk electrolyte activities, and efficient transduction of catalytic activity.

Electrodes were prepared according to Sections 3.2.5 - 3.2.9 for a range of antibody concentrations, (0 to 1.1 mg/ml). 500 µl samples of p60-HRP conjugate, or HRP

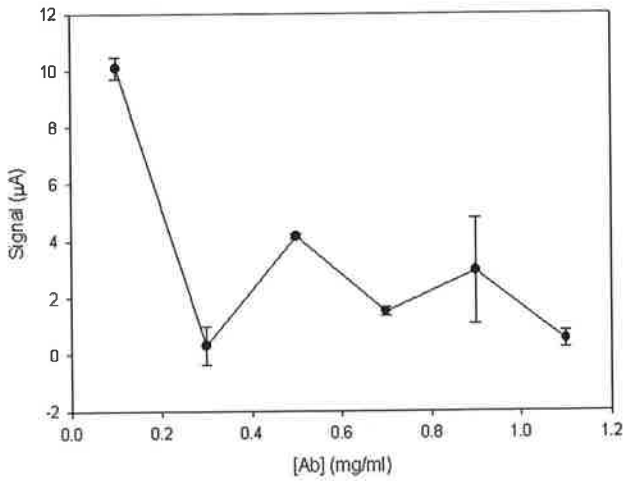
were passed over the surface of the antibody-modified electrode with H_2O_2, (10 mM). Passing HRP or p60-HRP (10 μg/ml) allowed us to monitor any changes in catalytic signal with increasing antibody concentration. The data generated where p60-HRP, and HRP were passed over the anti-p60-modified PANI/PVS surface is presented in Figures 4.2a and 4.2b, respectively.

The catalytic signals recorded following p60-HRP passage over the sensing surface indicate that at an antibody concentration of 0.7 mg/ml a monolayer of functionally orientated antibody is immobilised at the PANI/PVS interface which binds to p60-HRP. The monolayer coverage achieved at a concentration of 0.7 mg/ml is thought to prevent non-specific association between proteins and the PANI/PVS interface, indicating that any activity recorded at this concentration is the result of antibody binding action. At concentrations below 0.7 mg/ml sub-monolayer coverage of the PANI/PVS interface occurs. At these lower concentrations activity is assumed to be composed of both specific (following antibody binding of p60-HRP) and non-specific (following association of p60-HRP with the PANI/PVS interface) activity. At concentrations above 0.7 mg/ml there is no increase in signal. This may indicate that the coverage achieved at 0.7 mg/ml is also the maximum coverage possible, or it may indicate that above 0.7 mg/ml an inhibiting mass of antibody becomes immobilised which reduces the efficiency of tranduction as the distance between the PANI/PVS interface and antibody-bound HRP molecules increases.

The catalytic signal recorded following the passage of HRP over the sensing surface has a similar profile, with the largest catalytic signal recorded at the lowest concentration of antibody used, and the signal decreasing as the concentration of antibody is increased. This supports the idea that higher concentrations of antibody "block" the sensing surface against non-specific protein associations. However the catalytic signals recorded are similar in magnitude for both p60-HRP, and HRP, which indicates that there may still be a significant level of non-specific activity occurring at antibody concentrations of 0.7 mg/ml and above.



(a)



(b)

Fig. 4.2 Variation in catalytic signal with increasing concentration of anti-p60 antibody as p60-HRP (a) and HRP (b) were passed over the surface of anti-p60 modified electrode at 10 μg/ml in solution containing H_2O_2 at a concentration of 1 mM.

4.2.3 *Investigation of MP-11 as an alternative immunolabel to HRP*

According to the theory of Marcus & Sutin (Marcus and Sutin, 1985), the rate constant of direct electrochemical communication between an immobilised biocatalyst, and the electrode surface is governed by the distance between the active site of the enzyme, and the electrode surface. HRP has a molecular mass of approximately 40 kDa, (Lotzbeyer *et al.*, 1996), while MP-11 has a molecular mass of just 1.9 kDa, (Lotzbeyer *et al.*, 1996). The folded polypeptide structure of HRP hinders diffusion of substrate to the active site, reducing the turnover rate of the enzyme, (Lotzbeyer *et al.*, 1997, Lotzbeyer *et al.*, 1996). The smaller size of MP-11 makes its catalytic site more accessible, increasing the rate of electron-transfer, which increases catalytic rate, and reduces its refractory period. Work by Lotzbeyer *et al.* has demonstrated superior catalytic ability for haemin, cytochrome c, and MP-11, when covalently immobilised to the surface of an electrode as compared to that of HRP. In homogenous solution HRP has an activity of 10^{10} U/mol, MP-11 has an activity of 10^{7} U/mol (sigma-aldrich). However, when immobilised using self-assembled monolayers, HRP had a slower electron transfer rate compared to MP-11 (Lotzbeyer *et al.*, 1997, Lotzbeyer *et al.*, 1996). This faster electron transfer has been attributed to the smaller size of MP-11 resulting in a larger concentration being immobilised, and easier access to its active site as a result of the shorter distance to the electrode (compared to HRP). This access results in a faster observed catalytic rate, and a shorter refractory period for MP-11 "enzyme". An amperometric immunosensor was constructed demonstrating the electrochemical detection of the antibody-MP-11 conjugate bound via a biotinylated antigen and streptavidin immobilized on a gold electrode generating signals in the nA to μA range (Padeste *et al.*, 1998). A multi-layered biosensor for H_2O_2 was constructed which exploited self-mediation between upper and lower layers of MP-11 to achieve comparable levels of sensitivity (Tatsuma *et al.*, 2005).

4.2.3.1 *Immunosensor analysis of MP-11*

In order to investigate the suitability of MP-11 as an immunolabel in our system, experiments were conducted where both MP-11 and HRP were immobilised to the PANI/PVS surface and their catalytic activity was compared. Electrostatic immobilisation

was the immobilisation method used (Section 3.2.8). It was expected that MP-11 would yield higher catalytic signals to H_2O_2, as a result of shorter electron transfer distances, compared to HRP. However, the level of catalysis observed for MP-11 was lower than that of HRP, (Fig. 4.3). Immobilised HRP generated an average signal of 73.17 ± 8.9 μA, whereas a signal of just 2 ± 0.6 μA was generated for MP-11. The reason for this low activity of MP-11 is proposed.

Previous successes with MP-11 (Padeste *et al.*, 1998), related to the construction of a biosensor by immobilising MP-11 using a SAM. Work by Smyth's group had shown that protein could be immobilised to the PANI layer using electrostatic methods, (Killard *et al.*, 2001, Morrin *et al.*, 2003, Killard *et al.*, 1999) and signals recorded for HRP indicate that it was immobilised to the PANI/PVS layer. In contrast, the low signals generated for MP-11 indicate that negligible quantities of MP-11 protein were immobilised. The reported isoelectric point of MP-11 is 5.4, (Ehrenberg and Theorell, 1955). The reported isoelectric point for the major natural isozymes of HRP are 9.0, and 6.5-7.3 (Sigma). The difference in isoelectric point between MP-11, and HRP may account for a lower immobilisation efficiency of MP-11 as the electrostatic method of immobilisation employed relies on differences in charge between the protein to be immobilised, and PANI/PVS matrix. At a buffer pH of 6.8 MP-11 may not possess sufficient charge to immobilise in appreciable quantities.

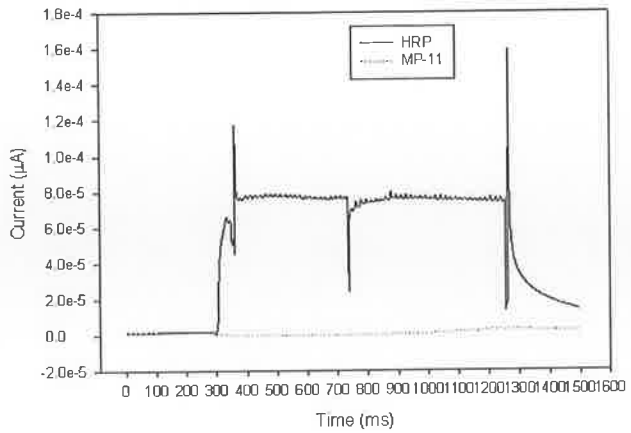


Fig. 4.3 Amperometric responses for reduction of H_2O_2 by immobilised HRP (black) or MP-11 (red) (concentration of protein used for immobilisation was 0.7 mg/ml).

4.3 CONCLUSIONS

The objective of this work was to re-optimise the parameters for the immobilisation stage of biosensor production to identify the optimal values for achieving an antibody-coverage of the PANI/PVS layer sufficient to bind the maximum amount of analyte, without inhibiting electrochemical transduction. The parameters studied were the pH of the protein buffer, and the concentration of antibody used during the immobilisation stage. MP-11 was also probed as an alternative label to HRP.

Re-optimisation of buffer pH was performed by immobilising protein to the PANI/PVS layer at pH values between 6.0, and 8.0. IgG proteins were used as these antibodies are the accepted standard in immunoassay, and biosensor work. Given the heterogeneous nature of the isoelectric points of IgG populations the results of the pH test could be extrapolated to protein species having isoelectric points within a similar range. From the data obtained it was concluded that between the limits of pH 6.0 and 8.0 there was no significant difference in the mass of IgG protein immobilised to the PANI/PVS interface.

Study of the antibody concentration used during the immobilisation phase was carried out using p60 reagents. The motivation for this study was an inverse relationship between the concentration of antibody used during immobilisation, and catalytic signals recorded in response to binding of analyte, indicating a sub-monolayer protein coverage of the PANI/PVS interface. However, the results of these experiments revealed transduction of non-specific catalytic activity masking any specific activity present. To accurately determine the specific catalytic activity a means of eliminating non-specific activity is necessary.

Based on reports of a high level of activity when covalently immobilised compared to HRP, MP-11 was investigated as an alternative immunolabel. Results indicated that MP-11 did not immobilise in appreciable quantities, this is attributed to charge differences between HRP and MP-11 arising from differences in their isoelectric point.

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Chapter 5

Analysis of surfactant activity within an immunosensor system as a means of reducing non-specific activity

5.1 INTRODUCTION

The major factors causing non-specific protein adsorption in immunosensing techniques have been identified as electrostatic interactions, and hydrogen-bonding forces (Wang *et al.* 2006, Ahluwalia *et al.*, 1995, Ahluwalia *et al.*, 1992). This kind of real-time activity has not been investigated on our biosensor platform before, as it was assumed that, at the optimal concentration of antibody used during the immobilisation phase, a monolayer of protein was immobilised to the PANI/PVS layer, which insulated it against any activities other than those of antibody-bound conjugate. However, the results of experiments to re-optimise this concentration for assaying p60 revealed a large non-specific character to the signals that were recorded. Non-specific activity reduces the accuracy and precision of assay results, and the increased mass of protein associating with the PANI/PVS surface could reduce the lifetime of electrodes designed to analyse multiple samples. The introduction of sample pre-treatment is a possible means of reducing non-specific activity; however this would limit the application of biosensors to clinical settings, and additional steps reduce the overall sensitivity of assay systems. One such pre-treatment is blocking of the PANI/PVS layer with BSA. This would prevent protein from interacting directly at the PANI/PVS interface; however, the increased mass of protein as a result of BSA blocking will increase resistance at the PANI/PVS surface, which will reduce transduction efficiency.

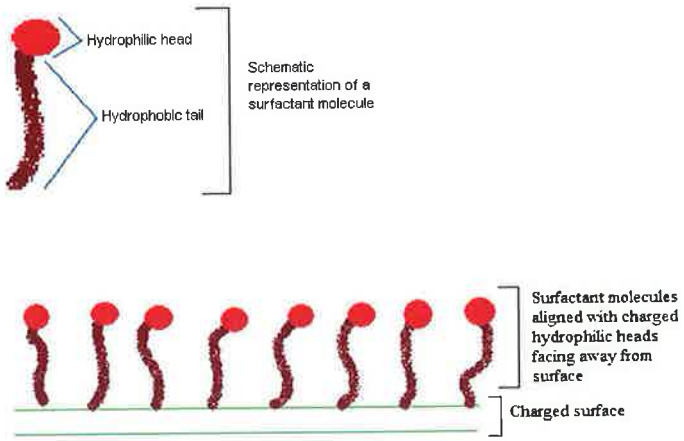
Work by other groups has investigated surfactants as a means to control this non-specific adsorption (Wang *et al.*, 2006, Wang and Jin, 2004). Incorporation of surfactants into biosensor set-ups during the covalent immobilisation of protein molecules inhibited non-specific protein immobilisation. Surfactant adsorption occurs via many of the same types on interactions involved in non-specific protein immobilisation, and surfactant molecules adsorbed in this way act as a "block" against the non-specific adsorption of protein. For example, the non-ionic surfactant Tween-20 has been used to eliminate non-specific protein binding due to hydrophobic interactions (Wang *et al.*, 2006). As a non-ionic surfactant, hydrophobic interactions are essential to the absorption of Tween-20; therefore, when added to protein buffer, Tween-20 molecules will immobilise by hydrophobic interactivity. Using covalent means, protein is also immobilised in this situation, resulting in a layer at the solid absorbate composed of protein, adjacent to

Tween-20 molecules adsorbed by hydrophobic interaction. Non-specific binding of protein by hydrophobic means is impossible at this interface, since the Tween-20 molecules have already occupied spaces, which are reactive to hydrophobic differences, and the remainder of the absorbate surface is already coated with protein.

This chapter details experiments investigating the potential of surfactants as a means to eliminate non-specific activity within our system. The immobilisation of protein within our system is based on the same electrostatic interactions, which are required for the adsorption of surfactant. For this reason it was thought that co-immobilisation of surfactant and protein might result in preferential adsorption of surfactant, with a reduction in the mass of protein immobilised. Instead, surfactants were studied to see if real-time adsorption would yield similar reductions in non-specific activity. It has also been reported that the use of surfactants has enhanced the bioaffinity interactions within several systems; this was considered to be a potential ancillary benefit (Zampieri *et al.*, 2000). Experiments were carried out examining the effects of several types of surfactants on non-specific binding of HRP. Tests were run on several different classes of surfactant, starting with Tween-20, poly(vinylsulphonate), and sodium dodecylsulphate, and then being expanded to include other groups of surfactant molecules such as the Triton-X series.

5.1.2 SURFACTANT ADSORPTION

Surfactants are surface-active species employed in a variety of industrial processes such as dispersion-flocculation, flotation (Scamehorn *et al.*, 1988), dewatering, and oil recovery (Paria *et al.*, 2004). Surfactant species can become adsorbed at different kinds of interfaces, such as the solid-liquid interface found in this sensor work. This adsorption is the result of energetically favorable interactions between the solid adsorbate, and the surfactant species; these interactions being influenced by the solid, solvent, and solute components of the system. Interactions such as electrostatic attraction, covalent bonding, hydrogen bonding, non-polar interactions between the adsorbate, and the adsorbate species, and lateral interactions between the adsorbed species as well as their desolvation can contribute to the adsorption, and desorption processes (Somasundaran & Huang, 2000). Scheme 5.1 presents a general schematic for surfactant adsorption.



Scheme 5.1 Schematic representation of surfactant adsorption, the surfactant molecules will orient themselves with their hydrophilic, or hydrophobic portions facing away from the surface depending on whether the surface itself is hydrophilic or hydrophobic. This serves to block the surface from non-specific protein binding.

Adsorption can be broadly classified into two groups. Physical adsorption is usually weak, and reversible, exploiting Van der Walls, and electrostatic forces. Physical adsorption is usually characterised by a high rate of adsorption, and the formation of multilayers (Parfitt and Rochester, 1983). Chemical adsorption is usually much stronger,

irreversible, and results in the formation of a monolayer. Chemical adsorption involves covalent interactions between the adsorbate, and surface species found on the solid. More accurately, most adsorption/desorption processes are best described as a mix of both physical and chemical adsorption, often beginning with physical adsorption which facilitates adsorbent-adsorbate interactions resulting in chemical adsorption (Somasundaran and Krishnakumar, 1997, Zangwill, 1988). Ultimately, adsorption is the net result of several activities, including electrostatic, and covalent interactions, lateral interactions due to cohesive chain-chain interaction among adsorbed long chain surfactant molecules, and interaction between the hydrocarbon chains of surfactant molecules and hydrophobic sites on the solid adsorbate.

The adsorption behaviour of ionic surfactants can be described by a four-region isotherm. Region 1 is characterised by the existence of electrostatic interactions between the ionic surfactant and the oppositely charged solid surface. Region 2 reflects an increase in adsorption resulting from the onset of surfactant aggregation at the surface through lateral interactions between the hydrocarbon chains. These interactions give rise to solloid (surface colloid) formation at the adsorbate surface in the form of hemi-micelles, admicelles, and self-assemblies (Somasundaran and Feurstenau, 1966, Somasundaran *et al.*, 1991). Region 3 shows a decrease in the rate of adsorption caused by increasing electrostatic hindrance to surfactant adsorption as a result of interfacial charge reversal following adsorption of charged species in region 3 and region 4. Region 4 corresponds to maximum surface coverage of adsorbate by surfactant molecules. Any further increase in surfactant will have no effect on the adsorption density.

The adsorption of non-ionic surfactants differs from that of ionic surfactants primarily in the absence of electrostatic interactions. The isotherm describing adsorption of non-ionic surfactants is similar to that for ionic surfactants, but the absence of electrostatic interactions results in a higher slope to the hemi-micellar region of region 2, and the absence of region 3 in a non-ionic isotherm. It has been proposed that, at lower non-ionic surfactant concentrations, hydrogen bonding is the main driving force for adsorption, while at higher concentrations hydrophobic chain-chain interactions become more significant to the adsorption process. For non-ionic surfactants the polarity of

functional groups, and the length of the alkyl chain determine the extent of hydrogen bonding, and hydrophobic interactions leading to micelle formation, and adsorption of surfactant (Somasundaran *et al.*, 1991).

5.2 RESULTS AND DISCUSSION

5.2.1 Surfactant-mediated control of non-specific activity

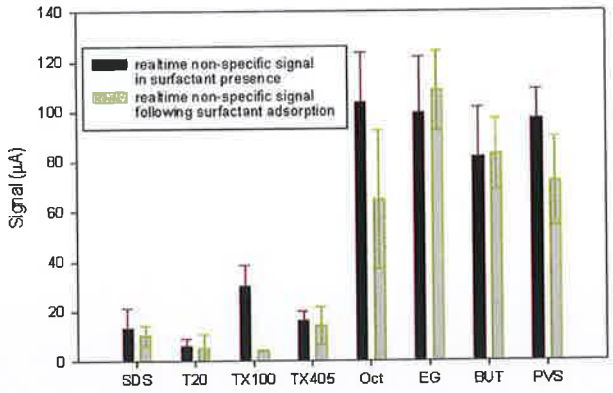
Ionic, and non-ionic surfactant species were investigated for their ability to inhibit non-specific protein adsorption at the PANI/PVS surface. Testing began with the non-ionic surfactant Tween-20, the ionic surfactants poly(vinylsulphonate), and sodium dodecylsulphonate. These three surfactants are common laboratory reagents, and characterised to a high degree. Work was then expanded to incorporate the non-ionic surfactants Triton-X 100, and Triton X-405, as well as the microsurfactants octanol, butanol, and ethylene glycol. Results indicate that adsorption of surfactant molecules took place in real-time producing a layer of surfactant at the PANI/PVS interface which prevented non-specific protein interaction. However, surfactant adsorption also resulted in reduced catalytic signal when passed over a surface to which protein had previously been immobilised. The exact cause of this loss in signal is unknown. A possible explanation is that surfactant adsorption caused a disruption in the electrostatic interactions used to immobilise adjacent protein molecules, resulting in a loss of protein from the surface, and the subsequent fall in catalytic signal.

Initial results with Tween-20 were encouraging. Signal due to non-specific adsorption of protein was reduced significantly. However, experiments probing real-time non-specific adsorption in the presence of an immobilised protein layer exhibited a falling catalytic response. From the results obtained, it appeared that surfactant molecules were being adsorbed in preference to protein, with the result that any non-specific real-time adsorption was being inhibited, but any protein, which had been immobilised previously, was also being removed from the PANI/PVS surface as surfactant was adsorbed in its place. Similar behaviour was recorded for the ionic surfactants sodium dodecyl sulphate (SDS), and PVS, and the smaller non-ionic surfactants Triton-X-100, and Triton-X 405. The microsurfactants did not exhibit the same level of disruption to immobilised protein, but they also did not inhibit real-time non-specific protein adsorption.

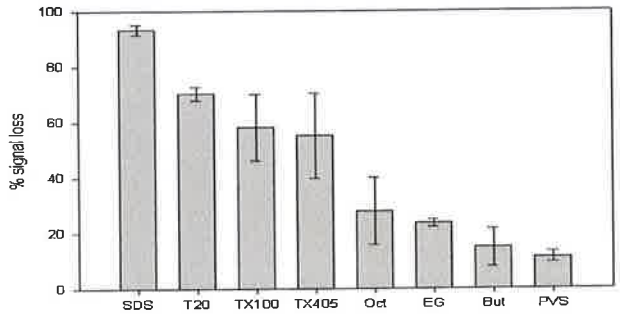
Incorporation of the Nickel-NTA chelate into the PANI/PVS matrix has previously been investigated as an alternative means of immobilising protein (Lori et al

(2006)). The tetradentate nickel-NTA forms a hexagonal complex with divalent metal ions occupying 4 of the 6 available binding sites. The remaining two sites can be used to immobilise proteins which have a hexa-histidine label engineered into them. This method of immobilisation was thought to exploit stronger interactions than the electrostatic forces exploited as a conventional means of immobilising protein. This system was tested with Tween-20, and a polyclonal anti-p60 antibody synthesised with a hexa-histidine label to determine whether this stronger means of immobilisation could be combined with the use of a surfactant molecule to eliminate non-specific activity.

Collated data for the surfactants is presented in Figure 5.2. It is clear that the larger surfactants SDS, Tween-20, Triton-X100, and triton-X405 effected the largest reduction in non-specific real-time protein adsorption, (Figure 4.1a) but these also caused the largest loss in catalytic signal by disrupting the electrostatic interactions immobilising protein (Figure 4.1b). Conversely the smaller surfactants octanol, butanol, ethylene glycol, and PVS caused smaller losses in catalytic signal, (Figure 4.1b) but also effected a smaller reduction in non-specific real-time protein adsorption (Figure 4.1a).



(a)



(b)

Fig. 5.1 (a) Catalytic signal due to real-time non-specific protein adsorption in presence of surfactants, and catalytic signal following surfactant adsorption (b) Catalytic signal loss caused by surfactant disruption of electrostatic immobilisation.

5.2.1.1 *Non-ionic surfactants*

Tween-20, Triton-X 405, and triton-X 100 are all non-ionic surfactants. As such they rely primarily on hydrophobic interactions, and hydrogen bonding to adsorb. Tween-20 is a common laboratory surfactant often employed as an ingredient of wash solutions for immunoassays, and has been used as a blocking agent in other immuno-diagnostic set-ups. Testing began at 0.1 % Tween-20 in buffer. Results were encouraging with real-time non-specific signals dropping significantly compared to control values. The tests were expanded to higher, and lower concentrations of Tween-20 (0 – 0.2 %). At concentrations above 0.125 % Tween-20, non-specific signal decreased. However, in tests probing non-specific catalytic activity in the presence of a layer of immobilised protein results indicated that Tween-20 was removing protein from the surface. The most likely explanation of these results was that Tween-20 was adsorbing to the PANI/PVS layer itself, and in the process disrupting the electrostatic bonds, which were used to immobilise protein. Once adsorbed, Tween-20 opposed any further interaction between protein and the PANI/PVS surface.

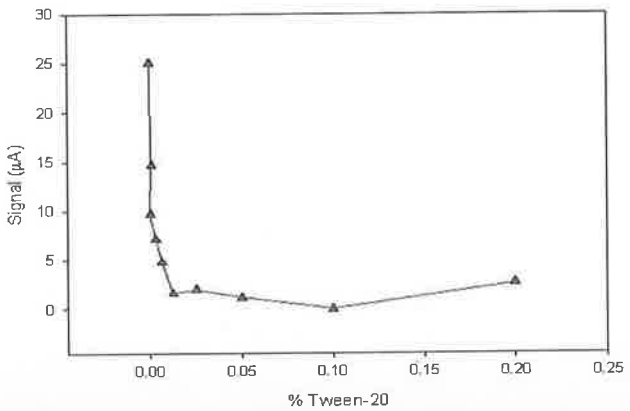
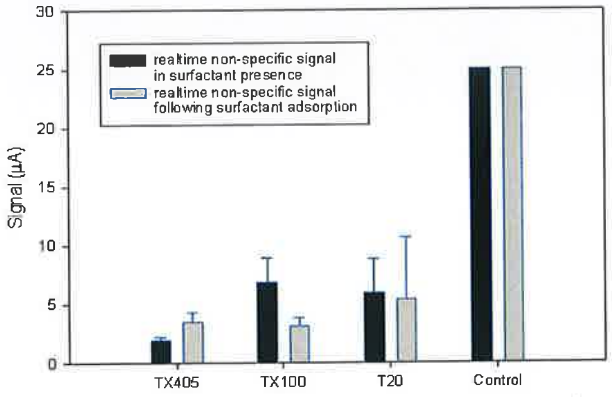


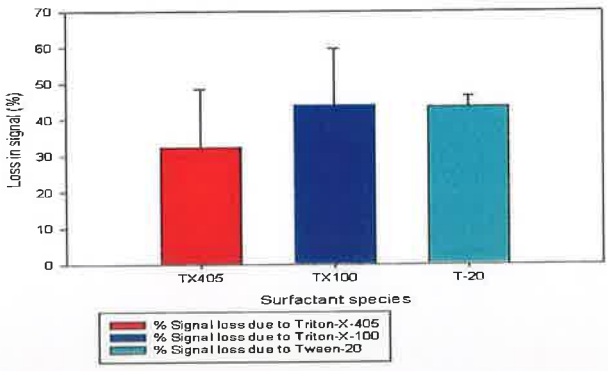
Fig. 5.2 Catalytic signal due to real-time non-specific protein adsorption in presence of Tween-20.

The hydrophobic character of surfactant molecules is related to the length of the alkyl or hydrocarbon chain, and hydrophobic interactions are essential to the adsorption of non-ionic surfactants (Somasundaran *et al.* (1991)). Thus, it was thought that a surfactant with a lower hydrophobic character would be less inclined to adsorb, and so would not interfere with protein immobilisation to the same extent, while it might still be able to

inhibit non-specific catalytic activity. Triton-X 100, and Triton-X 405 are non-ionic surfactants, but are smaller than Tween-20; thus, it was hoped that the reduced hydrophobicity of their shorter alkyl chains might reduce their adsorption at the PANI/PVS surface. However results for these smaller non-ionic surfactants were similar to those obtained for Tween-20, with reduced non-specific protein adsorption, but also disruption of the electrostatic interactions necessary to immobilise proteins to the PANI/PVS surface.



(a)

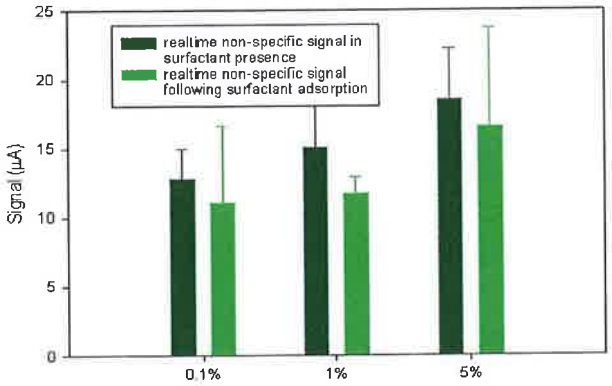


(b)

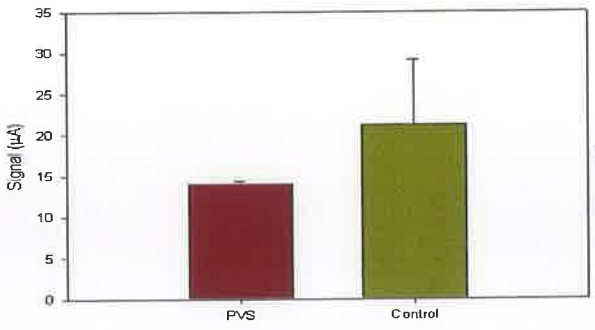
Fig. 5.3 (a) Non-specific signal in the presence of surfactant species and non-specific signal occurring due to persistence of surfactant species at electrode surface (b) signal loss due to surfactant disrupting immobilised protein at the PANI/PVS interface.

5.2.1.2 *Ionic surfactants*

PVS and SDS were the two ionic surfactants studied in this work. PVS is contained within the PANI/PVS matrix, thus an electrostatic repulsion existed between the negatively charged PVS within protein buffer, and the negatively charged PVS in the PANI/PVS layer. This should inhibit the electrostatic interactions, which are essential to the adsorption of ionic surfactants, predicting a low level of PVS adsorption. Indeed compared to the other surfactants PVS caused a smaller reduction in the level of non-specific catalytic activity, with real-time non-specific signal at 14 ± 0.3 μA compared to a control signal of 21 ± 7.8 μA. Similarly disruption of electrostatic bonds immobilising protein was also lower, with test signal close to control values at all percentages of PVS.



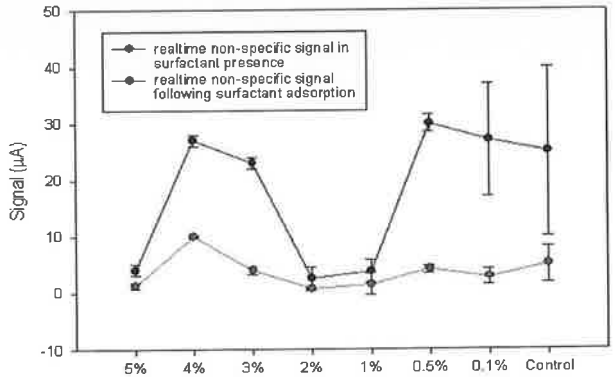
(a)



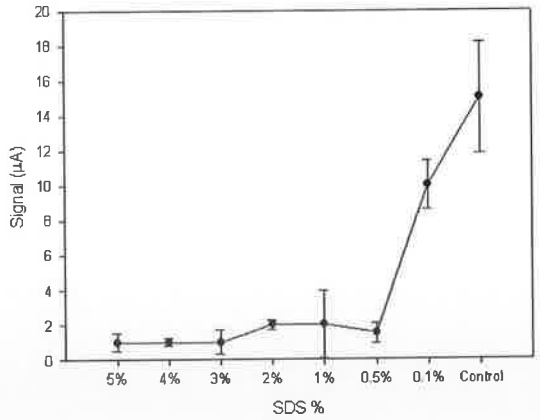
(b)

Fig. 5.4 (a) Non-specific activity in the presence of PVS (b) Signal following exposure of electrode surface to surfactant, and protein removal by surfactant.

SDS is a noted protein denaturant (Watanabe *et al.*, 2005), which possesses a negative charge that would inhibit the electrostatic interactions necessary for adsorption, again predicting a reduced contribution to eliminating non-specific catalytic activity. Based on a negligible reduction in non-specific activity, SDS appeared not to become adsorbed; however, it did appear to disrupt the electrostatic bonds, which held protein immobilised at the PANI/PVS surface. SDS impact on real-time non-specific catalytic activity was negligible at all percentages compared to control values.



(a)

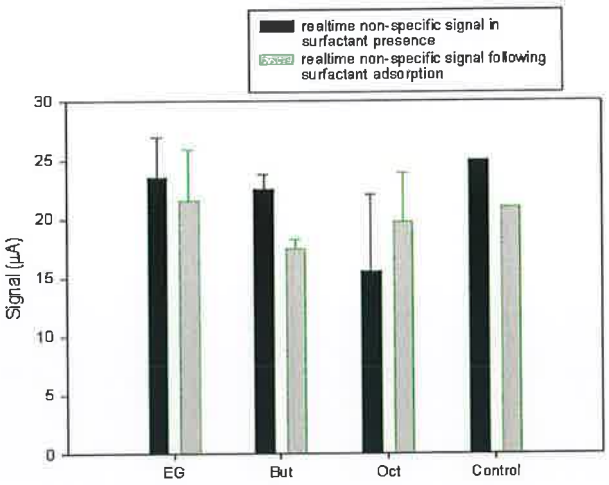


(b)

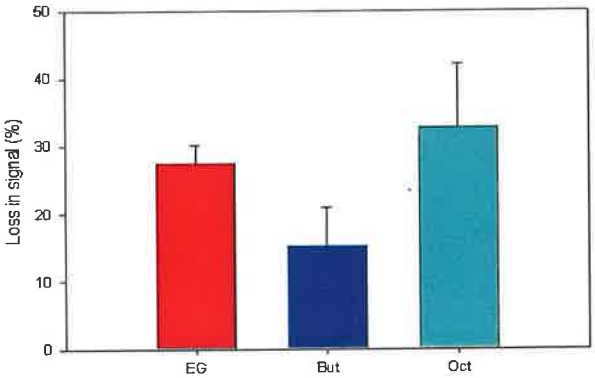
Fig. 5.5 (a) Real-time non-specific activity in the presence of SDS and Signal following exposure of electrode surface to SDS (b) Signal obtained following removal of protein from electrode surface by SDS.

5.2.1.3 Microsurfactants

The polar hydroxy (-OH) group makes the microsurfactants examined here water-soluble. Ethylene glycol with two hydroxy groups is more water-soluble than either of the alcohols (butanol and octanol) tested here which have one hydroxy group each. Results for tests with these three microsurfactants did not indicate a large amount of adsorption taking place, as evidenced by negligible change in non-specific activity, and no reduction in the immobilisation of protein to the PANI/PVS layer (Figure 4.6a). However, all three surfactants disrupted the electrostatic immobilisation of protein (Figure 4.6b). From this data it appears that the microsurfactants tested did not adsorb to the same extent as the larger ionic, and non-ionic surfactants tested. However, sufficient immobilisation did take place to disrupt the electrostatic interactions which immobilised proteins, but without reducing non-specific adsorption.



(a)



(b)

Fig. 5.6 (a) signal in the presence of microsurfactant and signal following PANI/PVS exposure to microsurfactants (b) signal loss by microsurfactant removing protein.

5.2.2 *Surfactant influence following covalent immobilisation*

Anti-p60, and p60-HRP conjugate proteins were used to investigate the Nickel-NTA system of immobilisation. Electrodes were prepared with immobilised antibody, and exposed to 500 µl of p60-HRP conjugate with H_2O_2, (1 mM); signal was validated with 500 µl of H_2O_2, (1 mM), and finally 500 µl of H_2O_2, with 0.1 % Tween-20. Deterioration of signal was observed following exposure of antibody-coated electrodes to Tween-20. This indicated that Tween-20 was adsorbing in the same manner as with the PANI/PVS layer. The results of these experiments are presented in Figure 4.7. The Nickel-NTA complex was distributed within the PANI/PVS matrix, which meant that the forces facilitating adsorption of Tween-20 were still present. The nickel-NTA strategy may exploit a stronger interaction with the histidine label of antibody proteins, but this was insufficient to prevent disruption by adsorbed Tween-20

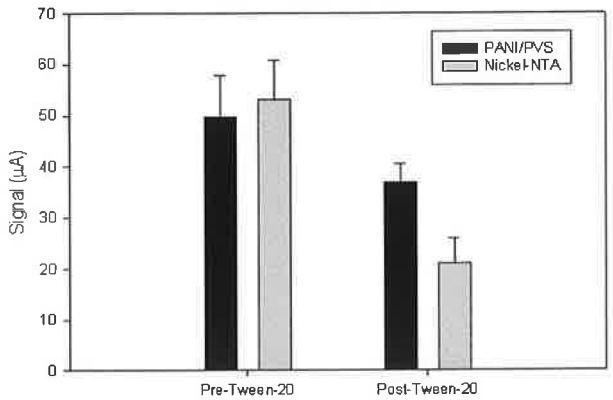


Fig. 5.7 Catalytic signal before and after passage of Tween-20 over a Nickel-NTA modified PANI/PVS electrode.

5.3 CONCLUSIONS

In this work, several surfactant species were investigated for their ability to prevent the non-specific adsorption of protein to the PANI/PVS layer. Work by other groups has demonstrated the ability of surfactant molecules to inhibit non-specific protein adsorption (and subsequent non-specific catalytic activity) by using them to exploiting available electrostatic, hydrophobic, and hydrogen bonds, making them unavailable to protein. In this way there is no capacity for protein to adsorb non-specifically since any regions facilitating adsorption are occupied by functionally immobilised protein, or surfactant molecules.

In this work the non-ionic surfactants Tween-20, Triton-X 100, and Triton-X 405 were examined, as well as the ionic surfactants SDS, and PVS, and the microsurfactants octanol, butanol, and ethylene glycol. For the non-ionic surfactants, adsorption by hydrophobic interaction took place, which did inhibit the non-specific adsorption of proteins, but also disrupted the immobilisation of protein by electrostatic means. The net result was reduced non-specific catalytic signal, but also reduced catalytic activity. This is thought to have been the result of surfactant adsorption at the PANI/PVS layer preventing the immobilisation of protein by electrostatic means. The microsurfactants caused a lower reduction in non-specific protein adsorption, and did not appear to adsorb in any appreciable quantities. The negatively charged ionic surfactants PVS, and SDS did not immobilise to the PANI/PVS layer, which was expected since their negative charges would result in repulsion from the negatively charged PANI/PVS layer. Both species demonstrated negligible reduction of non-specific protein adsorption.

If a stronger means of immobilising protein was used it is possible that the adsorption of surfactant molecules would not have such a deleterious impact on electrostatic immobilisation. With this in mind the non-ionic surfactant Tween-20 was tested with the Nickel-NTA method of protein immobilisation for reduction in non-specific activity. It was thought that the stronger method of immobilisation employed by the Nickel-NTA system might not be as susceptible to disruption by surfactant molecules. However, significant loss in specific signal due to disruption of interactions between

protein, and the PANI/Nickel-NTA matrix still persisted.

While these results demonstrate the potential of surfactants to inhibit non-specific activity in a real-time scenario, this accompanied by a reduction in catalytic signal. Coupled to a stronger means of immobilisation real-time, application of surfactants may be more effective.

5.4 REFERENCES

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Chapter 6

Future work

6 FUTURE WORK

Based on study of the work published to date, and the results presented in the body of this thesis, I believe the most significant improvement in the sensing capabilities of this biosensor can be achieved by adopting a different means of protein immobilisation.

I believe that the immobilisation process as it is applied by Smyth's group amounts to a physical adsorption process, taking place at the PANI/PVS interface. It seems unlikely that any significant mass of antibody which may be entrapped within the PANI/PVS matrix contributes to catalytic signaling, if such entrapped species were generating the bulk of the catalytic response the adsorption of surfactant molecules would not evoke such a severe disruption to catalytic activity. However, the interactions that are involved in physical adsorption, are the same interactions which are cited as contributing to non-specific protein immobilisation, and these are also the same interactions which are necessary for the adsorption of surfactant molecules. This indicates that the protein, which has previously been demonstrated to immobilise at the surface of the PANI/PVS matrix, is there by a process of physical adsorption.

Physical adsorption is a process permitting very little control over the immobilisation of protein to a solid substrate, the bonds involved are extremely weak, with proteins maintained immobilised by the sheer number of such interactions. These interactions can be easily disrupted, and protein removed from a surface, which makes uniform reproducible immobilisation almost impossible to achieve. Furthermore there is no means by which a functional orientation of antibody molecules can be assured using physical adsorption, which means there is also no way to determine whether activity is due to antibody binding or non-specific protein association with the surface. As has been demonstrated by the results presented in this thesis there is a considerable amount of non-specific activity taking place within the biosensor in spite of the previously reported monolayer of protein being immobilised at the PANI/PVS interface. If an effective monolayer of protein is in fact being immobilised at the PANI/PVS interface, it should insulate the sensing surface against non-specific bulk electrochemical activity. As the concentration of antibody used during the immobilisation phase is increased up to 0.7

mg/ml there appears to be insulation of the PANI/PVS surface, however catalytic activity is still taking place. What is the source of this activity? Is it possible that protein is still associating with the PANI/PVS interface at some regions of the electrode? Is oxidation or reduction of species taking place within the PANI matrix itself? The latter seems unlikely given the lower potential of 0.1 V used during steady-state amperometry, however there are still issues to be addressed using the immobilisation protocols outlined in this thesis.

There are methods of antibody immobilisation which permit a greater level of control over the final orientation of antibody molecules, and are also more stable in terms of the avidity of the bonds maintaining antibody immobilised to the solid phase. Protein A has an affinity for the Fc portion of antibody molecules, and can be employed in the immobilisation of antibodies producing a layer of antibody anchored by its Fc fragment, leaving the Fab fragment free to interact with, and bind analyte present in samples. This immobilisation protocol also employs stronger bonds than physical adsorption to immobilise antibody, resulting in a layer of antibody with increased stability. Such a layer would be more robust to the disrupting influence of surfactant molecules, which would facilitate their use as a blocking species.

In summary, the electrostatic method of immobilisation devised by Mu *et al.* is ideally suited to the immobilisation of enzyme molecules within a PANI matrix, and the use of such structures for biosensing purposes. However, when antibodies are employed the immobilisation that results does not appear to be as effective in terms of orientation, or the avidity of immobilisation. That protein is immobilised to the surface of the PANI/PVS matrix has been demonstrated, however the exact mechanism behind this immobilisation has not been elucidated. At the very least a more comprehensive study of the protein layer than has previously been conducted is necessary, and most likely some other means of immobilising protein will be needed to facilitate the development of viable immunosensing platforms in the future.