THE DEVELOPMENT OF AMPEROMETRIC BIOSENSORS FOR THE DETECTION OF GLUCOSE, LACTATE AND ETHANOL

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Abstract

Amperometric biosensors, also commonly known as enzyme sensors or enzyme electrodes, are a growing and very progressive area of research. Biosensors are analytical devices that contain a biological sensing element connected to a physical transducing element. The physical transducer "senses" the change in the biological element as it undergoes a chemical reaction. The physical transducer then converts chemical equivalents from the enzyme reaction in a dependent relationship to electrical equivalents that can be measured. Biosensors combine the power of electrochemistry with the specificity of enzymes to produce sensors that are specific to particular enzyme substrates. Some have wide specificities and others are quite narrow. Considering the wide range of enzymes available, the choice depends on the end use of these sensors.

The aim of the current study was to design biosensors for the detection of glucose, lactate and ethanol. The method for attaching enzymes to electrodes was based on the carbodiimide method. The carbodiimide method activates haeme which then is able to be covalently attached to enzymes. Enzyme-haeme conjugates were then allowed to absorb onto platinum electrodes by exploiting the knowledge that haeme can bind irreversibly to platinum by sharing *pi*-electrons with the *d*-orbitals of platinum. The enzymes involved were glucose oxidase, lactate dehydrogenase and alcohol dehydrogenase.

The use of flow injection analysis for evaluating biosensors was described and was found to be a fast, efficient method and the results were highly reproducible. In testing electrodes, the results of the present study showed it was possible to obtain current response that was dependent on the concentration of substrate when these enzyme electrodes were used. A particularly significant result in this study was the achievement of current responses that were dependent on substrate concentration in the absence of NAD^+ for lactate and alcohol dehydrogenases using the substrates lactate and ethanol respectively. There is however much work to be done to improve the success rate of making these enzyme electrodes. Several factors were found to cause variable results whilst making and using these enzyme electrodes, such as the absorption of unbound enzyme to the sensing surface of the electrode that may produce significant current response, the formation of aggregated haeme during the enzyme-haeme conjugation process and most importantly, and the ability to make successful enzyme-haeme conjugates to be absorbed onto the sensing surface of the electrodes.

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Contents

Abbreviations

ADH	Alcohol dehydrogenase
AO	Alcohol oxidase
BSA	Bovine serum albumin
CV	Cyclic voltammogram
DMF	Dimethyl formamide
DCC	Dicyclohexylcarbodiimide
GO	Glucose oxidase
HS	6-hydroxysuccinimide
LDH	Lactate dehydrogenase
LOD	Lactate oxidase
NAD^+	β-nicotinimide adenine dinucleotide
NADH	β-nicotinimide adenine dinucleotide (reduced form)
Pt	Platinum
R.O. water	Reverse osmosis water
TLC plates	Thin layer chromatography plates

Chapter 1

Part 1

1.1. An Introduction to Biosensors

The search for and development of new analytical procedures and equipment that provide fast, reliable and relatively low operating costs has been going for a few decades. Over the period, a large range of non-specific analytical equipment has been developed. In the last thirty years, a new era in the development of analytical sensors was heralded with the design of analytical systems that were capable of differentiating between different analytes through the incorporation of biological materials. The current concern with any analytical procedure is the cost of the reagents and the man hours utilised to analyse the sample.

The work of Clark and Lyons (1962) has been credited with the first bio-sensing chemical sensor. It involved entrapping an enzyme, glucose oxidase, within a semi-permeable membrane onto an oxygen electrode. The principle was quite simple. The decrease in oxygen, measured using the oxygen electrode, was proportional to the amount of glucose in solution. The term enzyme sensor was later coined by Updike and Hicks in 1967, when they immobilised glucose oxidase using a polyarcylamide gel, placing the enzyme in extremely close contact with an oxygen electrode. Since then, there have been numerous publications, describing the use of different enzymes to detect other analytes, different methods of immobilising enzymes, and different approaches to biosensor design as the electronics technology widens.

1.1.1. Principles of biosensors

The term biosensor is given to physical transducers whose sensing area contains a biological component and converts the concentration of a specific (bio-)chemical to an electrical or optical output signal (Hampp *et al*, 1994). For the sensor to be useful, the signal generated can be the result of an electroactive species formed from the binding of analyte and biological component. Alternatively, a signal may result from binding of the

analyte and the biological component giving a change in conformation in the biological component, which in turn causes changes in charge, density, temperature, colour, fluorescence (Scheller *et al*, 1989), or any other detectable changes using the present technology. There are two parts to chemical sensing, recognition and amplification (Janata, 1989). The incorporation of biological enzymes extends the range of analytes that could be measured by chemical sensors. The sensing layer can be made more selective with the use of enzymes, thereby conferring a degree of specificity for detection of analytes. The reactions between enzymes and substrates are specific. However, this does depend on the type of enzyme and the specificity of the enzyme. Some enzymes have wide specificities, such as carboxyesterase (hydrolyses various esters of carboxylic acids), while some have with very specific substrate requirements such as aspartate-ammonia lyase (specific only for L-aspartate) (Lehninger, p 218, 1975). Therefore depending on the requirements of the biosensor, different specificites of enzymes can be selected to suit.

1.1.2. Classification of Electrochemical Biosensors

Scheller *et al* (1989) classified biosensors into three different categories: first, second and third generation sensors. First generation sensors were defined as biosensors requiring membranes to trap the enzyme near the electrode sensing area. An example was the Clark and Lyon (1962) polymer membrane which entrapped the enzyme glucose oxidase onto an oxygen electrode. The second generation sensors consisted of covalently bound or directly absorbed enzymes, sometimes with the immobilisation of cofactors with the enzymes. With this method, the need for membranes becomes redundant. An example of this is the covalent immobilisation of glucose oxidase with FAD^+ on Teflon bonded to carbon electrodes (Sonawat *et al*, 1984). The third generation sensors use relatively new concepts involving the immobilisation of enzymes onto an electronic component, therefore incorporating biological information about changes to the enzyme with electronic information that could be electronically filtered to eliminate unwanted signals. One of the first few biosensors of this type was developed by Danielsson *et al* (1979), where a urease-based ENFET (enzyme-based field-effect transistor) was used for the detection of ammonia. These examples do not preclude the

importance of other types of biosensors. They included other physical detection methods as part of biosensor designs such as thermometric, piezoelectric, photometric and chemical bio-detection. Thermometric biosensors utilise either thermistors (mixtures of metallic oxides and polycrystalline semiconductors) or thermopiles (generates passive signals which are detectable) for measurement of variation in reaction enthalpies. Piezoelectric measurements devices are able to detect small variations in mass (enzyme-inhibitor associations or enzyme-antibody coupling). Photometric detection depends on the variation in light intensity due to absorption, fluorescence or luminescence. Chemical bio-detection biosensors include detection of the coupling between antibodies and antigen. This results in a change in electric charge, mass or optical properties which can be detected by potentiometric (Yamamoto *et al*, 1987), photometric (Tronberg *et al*, 1987), piezoelectric (Thompson *et al*, 1986) or thermometric transducers (Mattiasson, 1977).

1.1.3. Controlled-Potential Experiments using Potentiostats

Potentiostats essentially act to maintain the potential required depending on the needs of the study. They can be programmed to deliver potentials between different ranges or to maintain a fixed potential. Potentiostats are able to provide a constant potential at the working electrode with respect to the reference electrode, irrespective of changes in the current passing through the electrolytic cell and/or in the solution resistance (Souto, 1994).

Two commonly used methods in electrochemical biosensors are either potentiometric or amperometric. Potentiometric sensors makes use of the relationship between the emf of the electrochemical cell and the concentration of the chemical species in the sample. The first potentiometric sensor described by Guilbault and Montalvo (1969) for urea monitored the voltage produced without any applied potential. Potentiometric sensor measurements could be performed in both liquid and gaseous phase (Janata *et al*, 1994). The working electrode essentially probes the solution for oxidation and reduction reactions. Whilst the reference electrode has a constant and reproducible potential that is independent of environmental changes that are occurring. An example of a potentiometric sensor is the glass pH electrodes that monitor the concentration of

hydrogen ions or monovalent cations (Canh, 1993). Other studies involving enzymes immobilised to potentiometric electrodes, such glucose oxidase on a platinum electrode gave linear responses to increasing glucose concentrations (Wingard *et al*, 1983; Wingard and Castner, 1987), immobilising the enzyme urease for the detection of NH_4^+ ions as a result from the enzyme reaction (Joseph, 1984; Canh, 1993).

During an amperometric experiment a potential is applied between the working electrode and the reference electrode which gives rise to a current which is proportional to the concentration of the electroactive species in solution. This is the difference between a potentiometric and an amperometric sensor. Amperometric sensors detects the current flowing through the working electrode (Janata, 1994), while potentiometric sensors measures the potential difference between two electrodes. A major difference between potentiometric and amperometric sensors is the consumption of reaction products (Canh, 1993). Amperometric biosensors utilising only a working and a reference electrode are prone to changes in solution resistance, if the solution resistance increases, the applied potential will decrease. Two electrode amperometric measurements are adequate when currents are small relative to the size of the electrodes. The problem can be overcome by the addition of a third electrode known as the auxiliary electrode.

1.1.4. The Working Electrode (Physical Transducer)

In traditional electrochemistry, the working electrodes were commonly either mercury or amalgam, as both have reproducible homogenous surfaces, which were easy to clean (Greef *et al*, 1985). Mercury and amalgam electrodes have a large hydrogen over-voltage (meaning the electrolysis of water is not observed except at high negative and positive potentials), allowing these electrodes considerable working ranges (Greef *et al*, 1985). However, although the electrochemical properties of these two metals were excellent, they are not suitable as working electrodes in biosensors, as these metals are liquid in nature. The alternatives are solid electrodes. Solid electrodes are easier to handle in comparison to mercury and amalgam electrodes. Solid electrodes can constructed from different conducting materials, generally noble metals, such as platinum, gold, or less commonly palladium, and various forms of carbon (Cass, 1990).

However, the properties that make mercury and amalgam successful electrochemical electrodes also apply to solid electrodes. The surface must be easily maintained and a good range in working potential is needed. The ease with which metals form oxide layers at different potentials must also be taken into consideration. Therefore platinum, gold, palladium and various derivatives of carbon are the choice of many biosensor investigators. Platinum and other noble metals are used as electrodes because these metals are less prone to corrosion (Carr, 1993). This maybe relevant when the end use of these electrodes is directed towards biological samples.

1.1.5. Auxiliary Electrode

The primary function of an auxiliary electrode is to supply the current required by the working electrode, which completes the circuit (Cass, 1990), without taking part or influencing the measured response at the working electrode (Greef *et al*, 1985). The surface area of the auxiliary electrode must be large enough to avoid problems of driving the current through the auxiliary electrode (Cass, 1990) that results in large overpotentials (Greef *et al*, 1985). Similar materials to those used for making working electrodes can also be used in auxiliary electrodes.

1.1.6. Reference Electrode

The main consideration for a reference electrode is whether the electrode can hold a stable and reproducible redox potential (Cass, 1990). Its role in an electrochemical experiment is to provide a fixed potential that does not vary. Inevitably during the course of the experiment current will be drawn from the reference electrode (Greef *et al*, 1985), and the reference electrode must be able to hold its potential constant during that time. The saturated calomel electrode or Ag/AgCl electrode is commonly used as a reference electrode in amperometric experiments (Cass, 1990). The reference Ag/AgCl electrode can be made by depositing a thin layer of AgCl onto a spectroscopically pure silver wire. The preference for using Ag/AgCl stems from the fact that Ag^+ and Cl^- ions are able to freely move between the interfacial region of the silver metal and the electrolytic solution (KCl). The exchange of Ag^+ and Cl^- ions is important because

otherwise a potential difference would develop as positive ions associate with the negative electrolytic solution and negative ions associate with the positive metal surface (Carr, 1993). This can lead to a decrease in the sensitivity of the electrochemical measurement.

1.2. Methods for Immobilising Enzymes onto Electrodes

Barker (1987) described some of the important considerations associated with enzyme immobilisation. Reliability of the enzymes after immobilisation is one of the high priorities, and others are high degree of specificity of the enzyme, good stability to temperature, ionic strength, pH changes and redox potential changes. It is also ideal to be able to increase the stability of biological activity of the enzyme and limit the amounts of degradative agents present in solution. The method of immobilisation chosen for a particular system will greatly depend on the type of end use requirement. The ideal immobilisation technique would be applicable from enzyme electrode to multiple enzyme electrodes, antibodies, micro-organisms, or even whole cells (plant and animal systems). However, one important point remains the same, that the enzyme remains biologically active when immobilised on the electrodes. There are four types of immobilisation procedures (Barker, 1987).

1.2.1 Immobilisation of Enzymes by Absorption

This is one of the simplest methods of attaching enzymes to the sensing surface of an electrode. The advantage of this method is it relies on binding using Van der Waal's forces, hydrogen bonding and or salt bridges. There are limited needs for preparative reagents or clean-up steps. There are minimal changes to the 3-D conformation of the enzyme when bound to the electrode in this way. However the simplicity of this method for attaching enzymes to electrode surfaces has also its drawbacks. The binding of these enzymes depends on weak binding forces such as Van der Waals, which can be disrupted by changes in pH, temperature, ionic strength or even with the presence of the substrate. This makes the method unsuitable for making robust biosensors. This method is also prone to leaching of enzymes and other components co-absorbed onto the

electrode, though this can be minimised if the enzymes and components are cross-linked (Yacynych, 1992).

1.2.2. Immobilisation of Enzymes by Physical Entrapment

Enzymes can be also be immobilised within a gel. The first account using this method was in a study by Updike and Hicks (1967), trapping glucose oxidase in a polyacrylamide gel. Since then materials such as starch gels, gelatine, nylon and silastic gels had been used (Beh *et al*, 1989). This method can be applied to any enzyme as it involves trapping the enzyme within a 3-D lattice of the gel or membrane. An example of an enzyme trapped within a membrane can be seen in Guibault and Shu (1972), where urease was spread over a nylon net and covered with a dialysis membrane to a urea-sensitive electrode. The advantages of this method are that it is mild in conditions compared with covalent modification, and other cofactors or enzymes can easily be co-entrapped with the enzymes (Yacynych, 1992). The main faults of this method of entrapment are that a large diffusion barrier exists, the entrapment is not perfect, as enzyme can leach out from the gels (Barker, 1987), and deactivation of cofactors and or enzymes can occur with the formation of radicals. The preferred method for immobilising enzymes is through chemical immobilisation using covalent bonds. It ensures greater long term enzymic stability (Mascini and Guilbault, 1986).

1.2.3. Immobilisation of Enzymes by Cross-linking

Enzymes can also be immobilised using a combination of either enzyme absorption or enzyme entrapment in conjunction with a cross-linking agent. Cross-linking between enzymes and enzyme supports is induced using bi-functional agents such as glutaraldehyde, bisdiazobenzidine-2,2'-disulphonic acid, N-ethyl-5-phenylisoxazolium-3'-sulphonate, toluene-2-isocyanate-4-isothiocyanate, hexamethylene diisocyanate or 1,5-Difluoro-2,4-dinitrobenzene (Barker, 1987). This method of enzyme immobilisation has been favoured for many years as it confers the added advantage of increasing enzyme stability (Bajpai and Margaritis, 1985). It favours enzymes with small substrates, which tend to have better access than large enzyme substrates to the enzymes

buried within the entrapment matrix. A disadvantage of this method is that some of the enzyme may not be available to the substrate as either the conformation of the cross-linked enzyme has changed so the active site is inaccessible to the substrate, or the enzyme is buried too deep for the substrate. Therefore the enzyme activity may decreased as some of the enzymes are only acting as supporting structures.

1.2.4. Immobilisation of Enzymes by Covalent Binding

There are documented accounts of different reagents available for covalent modification of enzymes in preparation for immobilisation onto the sensing surface of the biosensor. The covalent bond between the enzyme and the physical support must occur in areas that are not essential to the catalytic activity of the enzyme. Coupling of enzyme and support usually occurs in low temperature, low ionic strength and usually within the physiological pH of the enzyme (Barker, 1987). Some of the common coupling methods are the cyanogen bromide method, the carbodiimide method, coupling by thiol groups, coupling via diazozium groups from aromatic amino groups, and using cyanuric chloride (Barker, 1987, Canh, 1993). The main advantage of using covalent binding is the enzyme is unlikely to be released during its use as a biosensor.

Part 2

1.3. Introduction to the Current Research

The main aim of this study was to evaluate and characterise the behaviour of biosensors designed to measure glucose, lactate and ethanol. Glucose is an important analyte both clinically and industrially. There is a large proportion of the population who suffer from diabetes melititus. The degree of severity differs for each sufferer for some it is life threatening. The rapid and exact determination of blood glucose can help determine the amount of insulin needed to alleviate the effects of diabetes. A number of portable glucose sensors are already commercially available, such as the ExacTech glucose analyser by Genetics International, U.K., 2300 Stat and 1500 G by Yellow Springs Instrument Co, USA (Alvarez-Icaza and Bilitewski, 1993). A glucose sensor is also

important in process control for the fermentation industry which includes dipolysaccharides, polysaccharides and amylase determinations (Suleiman and Guilbault, 1992). A lactate biosensor has a future role for control cardiac pacemakers, defibrillators and online monitor of critically ill patients for signs of acidosis (Scheller, Pfeiffer, 1987). Lactate is also an important metabolite in sports medicine (both in humans and animals), in the fermentation industries. Lactate biosensors can also measure meat quality. There would be applications for an alcohol biosensor in medico-legal matters, fermentation process control, as well as to food and beverage industries (Verduyn *et al*, 1983).

1.3.1. Use of Haeme for Enzyme Immobilisation

As previously mentioned, there are different ways of immobilising enzymes to an electrode's surface. There are advantages and disadvantages associated with each method of immobilisation. However, the work of Large (1993) had shown promising results in covalently linking to a molecule which is strongly absorbed to platinum(Pt) electrode surface. The original idea was conceived by Blackwell and Greenway (Personal communication, 1992), and the method was further developed by Large (1993). The idea was based on work by Lane and Hubbard (1973), where it was found conjugated double bonds can bind irreversibly to Pt by sharing pi-electrons from compounds with conjugated double bonds with the d-orbitals of the Pt surface. Haeme was suggested as it contained conjugated double bonds and therefore might bind irreversibly to the Pt electrode's surface. The added advantage of using haeme was it also contained two carboxyl groups that could be used to attach to an enzyme molecule containing amine groups forming amide bonds between the haeme molecule and the enzyme. The conjugation method used 6-hydroxysuccinimide and dicyclohexylcarbodiimide to activate the haeme molecules to bind to the enzymes. The conjugation method was initially derived from those used by Al-Bassam *et al* (1979), Rajkowski and Cittanova (1981), and Sauer *et al* (1986, 1989).

In Large's study (1993) haeme was suggested to not only act as a method for immobilising enzymes but as an electron mediator as well. The results from Large (1993) for conjugated haeme to glucose oxidase showed linear response to increasing

concentrations of glucose standards. The results presented for conjugated haeme to alcohol dehydrogenase showed a linear response to an increasing range of methanol standards especially at low methanol concentrations. Those results for conjugated haeme to alcohol dehydrogenase did not show any linear electrochemical response to ethanol standards in the same way. The results presented in the study of Large (1993) did not show conclusive evidence of haeme acting as an electron mediator.

1.3.2. What is Haeme?

Porphyrins are initially synthesised from glycine. Porphyrins have a role in haemoglobin, in cytochromes, and in chlorophyll. They can complex with iron, magnesium, zinc, nickel, cobalt or copper. The metal complex is held by four co-ordination bonds to nitrogen. In the case of iron, there are two more co-ordination bonds perpendicular to the square planar complex of the four nitrogen co-ordination sites (called fifth and the sixth co-ordination sites). In myoglobin or haemoglobin, the fifth co-ordination site binds to a histidine residue in the protein and the sixth site can be occupied by oxygen, carbon monoxide or cyanide. In cytochromes, which contain a central iron complex, the fifth and sixth co-ordination sites of iron are bound to the protein.

1.3.3. The Current Status of other Known Electron Mediators in Biosensors

Biosensors dependent on the detection of oxygen depletion, such as that designed by Clark and Lyons (1962), or by Updike and Hicks (1967), suffer from the requirement for presence of oxygen in the sample. Fluctuations in partial pressures of dissolved oxygen can lead to erroneous results (Cardosi and Turner, 1991). The system can be redesigned to increase the amount of oxygen diffusion to the sensing surface, as has been done by Gough *et al* (1985). The Clark type electrode have both glucose and oxygen diffusing axially into the enzyme layer. The improvement made by Gough *et al* (1985) to increase oxygen diffusion was to add a further hydrophobic membrane attached radially around the Clark type electrode where only oxygen was able to diffuse into the enzyme gel

radially as well as axially. This meant a greater amount of oxygen from the glucose oxidase reaction was able to be detected.

The use of mediator molecules to transport electrons or redox equivalents could reduce or completely negate the need for oxygen dependence (Cardosi and Turner, 1991). The main problem with transferring electrons between the biological component and the surface of the physical transducer is that the active site centres are usually buried within the polypeptide structure. Therefore, examples of direct electron transfers between the large biological components of biosensors and the physical transducers are quite rare (Schuhmann and Schmidt, 1992). One of the current ideas on designing reagentless biosensors uses the technique of having soluble mediators trapped within a membrane with the enzyme and the mediator in as close to the sensing surface to the electrode as possible. Other techniques involve the co-immobilisation of mediators with the enzymes to the sensing surface, or chemically modified electrodes that promote the orientation of enzymes on the electrode surface. The use of mediators confers several advantages, provided certain criteria's are fulfilled.

The putative mediator must fulfil some or all the criteria below to be a successful mediator:

1. It does not react with oxygen, which enables the biosensor to work in anaerobic conditions and the analysis is independent of oxygen limitations.
2. The new working potential is determined by the mediator couple, that is preferably lower than the original redox couple, to reduce interference from the other electroactive species which may be present.
3. The reoxidation of the mediator does not produce protons. The electrode is then relatively insensitive to pH.
4. The two states of the redox couple must be stable between being reduced at the active site of the enzyme and oxidised at the surface of the electrode.
5. The enhancing behaviour of the mediator can be further amplified if the mediator is closer to the active site during the reduction of the mediator. This means the surface charge of the mediator must be compatible to that of the active site.

There is a growing list of mediators and putative mediators. One of the more successful mediators for oxidases to date is ferrocene(bis(η^5-cyclopentadienyl)iron, $FeCp_2$) and its derivatives (Cass *et al*, 1984, 1985; Frew and Hill, 1987). The redox couple of ferrocene versus the standard calomel electrode is $E^o = 0.165$ V. Ferrocene replaces oxygen as a cofactor and acts as an electron acceptor. It is then reoxidised at the electrode surface. There are others which mediate electrons for oxidases, such as hexacyanoferrate-(III) (Schlapfer and Racine, 1974), tetrathiafulvalene (Turner *et al*, 1987), tetracyano-p-quinodimethane (Kulys and Cenas, 1983), and various quinones (Ikeda *et al*, 1985; Senda *et al*, 1986). The disadvantage in using ferrocene or its derivatives as mediators is the amount of mediator leakage that occurs during its use. The work by Schuhmann *et al* (1990), showed that 1,1'-dimethylferrocene (which is insoluble), after it had been reduced at the electrode surface, produces a soluble species (ferricinium) that in time leaches away from the electrode. The study by Schuhmann *et al* (1990) was able to provide evidence to that effect.

A method that had gained wide interest of recent years is "molecular wiring". The combination of redox mediators and conducting polymers provides an interesting alternative for the use of large molecular weight enzymes with buried active sites. Early examples of this type of work, such as Degani and Heller (1987; 1988) suggested by increasing the length of the ferrocene derivative, the reduced species may hop between mediator molecules without diffusing across the interfacial region.

The situation is much simpler with oxidases, as the cofactor (FAD/ $FADH_2$) is buried within the enzyme. The problem with pyridine nucleotide dependent enzymes or dehydrogenases is that the cofactor does not lie within the protein matrix. However, the potentials required to reoxidise the reduced form of cofactor generally involve large overpotentials, thus increasing the production of radical intermediates (Schuhman and Schmidt, 1992). For the reoxidation of NAD^+/ NADH couple for an unmodified bare platinum electrode the potential has to be more than 550 mV vs. SCE (Jaegfeldt, 1980) and more than 450 mV vs SCE for carbon electrodes (Laval *et al*, 1987). Schuhmann and Schmidt (1992) suggested the reason for high overpotentials needed was due to the irreversibility of the anodic oxidation of NADH. The goal of the work in this area had been to reduce the working potentials needed to make this reaction feasible for use in biosensors. There have been several promising compounds which had been used for this

purpose: quinones formed directly onto carbon surfaces (Cenas, Rozgaite and Kulys, 1984) or directly absorbed on carbon surfaces (Cenas, Kanapieniene and Kulys, 1984), meldola blue (Marko-Varga *et al*, 1986), organic conducting salt N-methyl-phenazinium tetracyanoquinodimethanide ($TTF^{+}TCNQ^{-}$) (Kulys, 1986), ferrocenium salts (Carlson and Miller, 1983), and ferricyanide ions (Powell *et al*, 1984). The other approach is to use a membrane to entrap the cofactor, though this leads to disadvantages in response times due to the diffusion barrier created by the membrane.

Sensors based on the use of redox proteins provide an interesting possibility for the development of biosensors. Betso *et al* (1972), described the reduction of horse-heart cytochrome c at mercury, platinum, and gold electrodes, though the electron transfer rates observed were quite slow. It wasn't until the study by Eddowes and Hill (1977) which described a method that combined 4,4'-bipyridyl (a molecule known to promote electron transfer between transition metals) with horse heart ferricytochrome c that faster electron transfer was observed. Hill and Sanghera (1990) suggested that the reason for the slow rate of electron transfer is due to the active sites not accessible for the transfer of electrons to occur and the absorption of the enzyme could result in the denaturation of the enzyme. Eddowes and Hill (1977) adsorbed 4,4'-bipyridyl on the surface of gold electrodes that interacted with cytochrome c (which is the mediator protein). Cytochrome c belong to a class of protein called redox proteins known to be involved in cell respiration and in photosynthetic systems. Hill and Sanghera (1990) suggested the dipole moment in cytochrome c created an environment that enabled the haeme to take part in electron transfer processes.

1.3.4. Flow Injection Analysis (FIA)

The fast and reproducible nature of the flow injection analyser (FIA) makes it a good choice as an instrument for testing and characterising putative biosensors. They confer many advantages such as controlled flow rate, and the timing of the sample introduction can be kept constant (Ruzicka and Hansen, 1981). Various cofactors that may be needed for enzymic activity may be added as part of FIA set-up. Changes can be made in sampling times and reaction volumes. It is possible to manipulate the dilution factors, develop multi-channel detectors that enable device miniaturisation and simplification of the FIA technique (Schmid and Kunnecke, 1990). Thus expensive reagents are not wasted inappropriately. The main advantage in this system is its flexibility. The system can be easily be incorporated into biosensor design, hence improving repeatability in between testing periods. The FIA technique in contrast to other techniques will still give reproducible results even under conditions of incomplete mixing of sample, a transient signal and the chemistry does not need to reach equilibrium (Ruzicka and Hansen, 1981). Hence its popularity in its use in testing characteristics of sensor applications.

1.3.5. Specific aims of this Research

The aims of the current study are:

1. To develop a system in which biosensors can be tested using Flow Injection Analysis.
2. To further investigate the behaviour of biosensors made from haeme conjugated to glucose oxidase, lactate dehydrogenase or alcohol dehydrogenase.
3. To investigate the possibility of haeme acting as an electron mediator in place of the cofactor NAD^+/ NADH in relation to the lactate dehydrogenase and alcohol dehydrogenase.

Chapter 2

2. Materials and Methods

2.1. Preparation of Buffers and Standard Solutions.

2.1.1. Preparation of Phosphate Buffer

A stock of 0.1 M phosphate buffer, pH 7.5 was made up from K_2HPO_4. A stock of 1 L was prepared for use as carrier stream in FIA, as a zero blank for electrode calibrations, and for preparation of standard solutions for electrode calibrations.

2.1.2. Preparation of Tris/HCl Buffer

A stock of 0.1 M Tris/HCl buffer, pH 7.5 was made up from Trizma® Base (Sigma; T-1503). The pH was adjusted using HCl. A stock of 1 L was prepared for use as carrier stream as flow injection analysis, as a zero blank for electrode calibrations, and for preparation of standard solutions for electrode calibrations.

2.1.3. Preparation of Standard Solutions

2.1.3.1. D-Glucose

A stock of 100 mM glucose was prepared using D-Glucose (BDH AnalaR®; 10117). The same buffer that was used to make use the carrier stream in the flow injection analyser was used in the making of these standards. The appropriate dilutions were made to make up different glucose concentrations.

2.1.3.2. Ethanol

A stock of 100 mM ethanol was prepared using Ethanol 99.7-100% v/v (BDH AnalaR®; 10107). The same buffer that was used to make use the carrier stream in the flow injection analyser was used in the making of these standards. The appropriate dilutions were made to make up different ethanol concentrations.

2.1.3.3. Methanol

A stock of 100 mM methanol was prepared using Methanol (BDH AnalaR®; 10158). The same buffer that was used to make use the carrier stream in the flow injection analyser was used in the making of these standards. The appropriate dilutions were made to make up different methanol concentrations.

2.1.3.4. 2-Propanol

A stock of 100 mM 2-propanol was prepared using 2-Propanol (BDH AnalaR®; 10224). The same buffer that was used to make use the carrier stream in the flow injection analyser was used in the making of these standards. The appropriate dilutions were made to make up different 2-propanol concentrations.

2.1.3.5. L-Lactate

A stock of 100 mM lactate was prepared using L(+) Lactic Acid (Sigma; L-2250). The same buffer that was used to make use the carrier stream in the flow injection analyser was used in the making of these standards. The appropriate dilutions were made to make up different lactate concentrations.

2.2. Preparation of Pt electrodes

Perspex or 6 mm diameter glass tubing was used to construct the outer casing of the electrodes. Experiments that referred to flow cell version 2 used electrodes that were made from a perspex outer casing. Experiments involving the recording of electrochemical measurements using batch collection and all flow injection analysis experiments involving flow cell version 3 used electrodes that were constructed from glass outer casings.

2.2.1. Preparation of Bare-Pt electrodes

A piece of electrical wire was soldered to one side of the platinum disk for each electrode. The piece of electrical wire was then connected to the potentiostat using a Crocodile clip with standard rubber shrouds or Push On Terminals with corresponding Blades (RS Components, 433-090 and 433-129) with transparent polythene covers (RS Components; 534-799). The platinum disk (diameter = 3.8 mm) present on each electrode were held in place using epoxy resin (Araldite®; Super Strength).

The terms "end on" and "embedded" electrodes referred to the construction style of the electrodes. "End on" electrodes were made of metal disks placed at the end of the ground glass tubes, and sealed using Araldite epoxy cement and allowed to set overnight. "Embedded" electrodes referred to electrodes with the metal disks place approximately 2 mm from the edge of the ground glass tubing. The metal disk was then set on place with Araldite.

The bare platinum electrodes were cleaned prior to use in electrochemical measurements. There was a two step procedure for cleaning unless the electrodes were of the "embedded" type. If the electrodes were constructed in the style of "embedded" electrodes then the electrodes were only cleaned using the procedure described for electrocleaning electrodes.

The bare platinum electrodes were initially cleaned with alumina. The polishing kit was purchased from BAS. The felt provided in the kit was initially wetted with R.O. water. A drop of alumina powder provided in the kit was spotted on the felt. The electrodes were polished on the piece of felt in a figure eight motion. The procedure was carried

out for approximately 2 minutes for each electrode. The electrodes were rinsed immediately under R.O. water and followed by the electrodes being polished using a diamond slurry provided in the polishing kit. The polishing procedure followed the same as that polishing using alumina. The electrodes were then sonicated to remove any alumina or diamond slurry particles. The next step was to electroclean the electrodes to removed any other absorbed particles not removed by polishing. An function generator was set up to deliver a square wave between the potentials of +1.5 V and -1.5 V. The electrodes were then rinsed in MilliQ water and is ready for use.

2.2.2. Preparation of Haeme-Pt electrodes

Haeme-Pt electrodes were prepared in the following manner. 6.82 mg of haeme was dissolved in 22.18 µL of 50 mg/ml dicyclohexycarbodiimide (DCC) in DMF, 12.38 µL of 50 mg/ml hydroxysuccinimide (HS) in DMF and 15.44 µL DMF. This mixture was left to activate for 1 hour. This solution of activated haeme was added to a solution containing buffer (0.1 M phosphate buffer, pH 7.5 or 0.1 M Tris/HCl buffer, pH 7.5). This solution was left stirring overnight at 4°C. Previously polished and electrocleaned Pt electrodes were dipped in the solution containing activated haeme for 20 hrs. Cyclic voltammograms and electrochemical data were then collected.

2.2.3. Preparation of GO-Pt electrodes

25 mg/ml of glucose oxidase in 0.1 M phosphate buffer, pH 7.5 was prepared. Previously polished and electrocleaned Pt electrodes were dipped in a this solution for 20 hrs. Cyclic voltammograms and electrochemical data were then collected.

2.2.4. Preparation of Toray Paper electrodes

Toray paper electrodes were made using the "embedded" electrode design. The cavity between the ground glass tubing edge and the Pt disk was filled with conducting paste (Fluka Biochemika Hydroxyethyl-cellulose medium viscosity 1 no.54290 ['Cellosize WP-40']) or with silver epoxy resin (Two Part Silver Loaded Epoxy Stock no. 567-604;

hardener (contains Polyamide complex) and resin (contain Epoxy Resin)). A piece of Toray paper was cut (6 mm diameter). The piece of Toray paper was then soaked into a solution of ethanol. The edges of the Toray paper and the glass edge were sealed with nail polish.

2.3. Preparation of FIA

The make of FIA used was Tecator (model 5020). The type of tubing the tubing used from the sample injection valve and from the carrier stream used the black/black tygon tubing provided by Tecator. This provided a final flow rate of 1.2 ml/min. The injection timing loops that were used most often for collecting electrochemical data of electrodes were, for Pump 1 ($T_1 = 4$ and $T_2 = 0$), Pump 2 ($T_1 = 0$ and $T_2 = 0$) and the length of injection time was 10 seconds (or $T_{inj} = 1$, and Mode =1). This meant that the FIA's carrier stream would continue pumping at all times. The sample injection loop $T_1 = 4$ meant that sample pump would only work for 40 seconds. This was enough time for the sample injection loop to be filled with sample. The sample injection time was 10 seconds ($T_{inj} = 1$), this meant that injection valve rotated and repositioned itself for 10 seconds in sync with the valve of the carrier stream before returning back to its previous position.

2.3.1. Instrumentation- Potentiostat

The potentiostat used was made by BAS. The model was CV-27. With some of the measurements, a preamplifier was used in addition to the CV-27. The model of the preamplifier was PA-1 also made by BAS. The potentiostat supplied either a triangular wave form (for cyclic voltammetry) or a constant potential (for amperometry).

2.3.2. Instrumentation- Chart Recorders

For amperometry, a chart recorder made by Rikadenki (model R-102) was used to record current measurements. For cyclic voltammetry, an X-Y chart recorder was used

to record the events. The X-Y chart recorder was made by Hewlett Packard (model 7004B).

2.4. Conjugating Haeme with Enzymes

2.4.1. Gelatine bound GO

5 % gelatine was dissolved in 0.2 M phosphate, pH 7.5 by incubating at 37°C. Then 20 mg of glucose oxidase was measured and dissolved in 5 % gelatine in an Eppendorf tube. The bare platinum electrodes were polished and electrocleaned as described in 2.2.1. 5 µL of the enzyme/gelatine mixture was applied to the sensing surface of the electrode. The enzyme/gelatine mixture was allowed to set at room temperature. The electrodes were then immersed in 1 % glutaraldehyde (stock 25 %) in 0.2 M phosphate buffer, pH 7.5 for a timed 60 seconds, followed by a wash for 1 minute under running R.O. water. 5 µL of enzyme/gelatine mixture was again applied to the electrodes and allowed to set at room temperature. The electrodes were then immersed in 1 % glutaraldehyde for a timed 60 seconds, followed by a wash for 1 minute under running R.O. water. Then the electrodes were immersed in 0.1 M lysine for at least 20 minutes.

2.4.2. Conjugating Haeme to GO

6.8 mg of haeme (Sigma, H-2250) was dissolved in 22.2 µL of 50 mg/ml dicyclohexylcarbodiimide (DCC) in DMF, 12.4 µL of 50 mg/ml hydroxysuccinimide (HS) in DMF and 15.44 µL DMF. It was important to add the HS before adding DCC. This was left for 1 hour for haeme to be activated. The activated haeme was then slowly added to a stirring solution of glucose oxidase (1.95 mls at 25 mg/ml) in 0.1 M phosphate buffer, pH 7.5 at 4°C. The solution was left stirring for 20 hrs. After 20 hrs, 200 µL of the glucose oxidase-haeme conjugate mixture was added to the top of a Sephadex G-25 column (column dimensions, 1 cm x 19 cm). The column was then washed with 0.1 M phosphate buffer, pH 7.5 and fractions of 1.5 mls were collected.

The fractions were examined spectrophotometrically between 250 nm and 500 nm. The fraction containing the highest protein absorbance (at 280 nm) coinciding with peak absorbance of haeme (at 380 nm) were set aside for enzymatic assay of the activity of glucose oxidase. Once the activity had been determined of the fractions, the highest enzymatic fraction that coincided with an absorbance at 380 nm (haeme), the prepared bare platinum electrodes were dipped into the fraction containing glucose oxidase-haeme conjugates for 20 hours.

2.4.3. Conjugating Haeme to LDH

The same procedure as that used for making glucose oxidase-haeme conjugates were used for making lactate dehydrogenase-haeme conjugates. The LDH was purchased from Sigma (L-2881), 50,000 units from porcine heart. 6.1 mg of haeme was dissolved in 19.9 µL DCC (50 mg/ml stock), 11.1 µL of HS (50 mg/ml stock) and 19.0 µL DMF. The rest of the procedure was the same as section 2.4.2. The enzyme assay used for LDH is described in section 2.6.2.

2.4.4. Conjugating Haeme to ADH

The same procedure as that used for making glucose oxidase-haeme conjugates were used for making alcohol dehydrogenase-haeme conjugates and as described by Large (1993). The ADH was purchased from Sigma (A-7011), 150,000 units from yeast. 8.6 mg of haeme was dissolved in 27.9 µL DCC (50 mg/ml stock), 15.6 µL of HS (50 mg/ml stock) and 6.5 µL DMF. The rest of the procedure was the same as section 2.4.2. The enzyme assay used for ADH is described in section 2.6.3.

2.5. Method for Cyclic Voltammogram (CV) analysis

The CV analysis of electrodes were recorded using the potentiostat CV-27 from BAS. The potential was cycled between the potentials required, that was dependent on the type of enzyme involved. Prior to the any data being recorded, the solution containing

either buffer or standards were degassed using N_2 (for 1 hr) until CV data was about to be collected. The results were collected using an X-Y chart recorder.

2.6. Enzyme assay systems

2.6.1. For the enzymatic detection of Glucose Oxidase

Reagents:

Glucose oxidase buffer (0.1 M phosphate, pH 6.0). Dipotassium hydrogen phosphate (17.4g) was dissolved in MilliQ water. The pH was adjusted to 6.0 with 1 M H_2SO_4 and made up to 1 L.

Substrate/chromogen reagent:

207 mg	phenol	(11 mM)
31.2 mg	4-aminoantipyrene	(0.77 mM)
14.4 g	glucose (anhydrous)	(0.4 M)
2.0 mg	peroxidase	(1.5 kU/ml)

These were dissolved in 200 ml of glucose oxidase buffer.

Standard glucose oxidase. A stock of 20 mg/ml glucose oxidase of known activity (Sigma, G-6766). A 1:100 dilution was made of the stock for assay. 50 µL of this dilution contained 10 mg enzyme (6×10^{-2} sigma units).

Procedure:

3.0 mls of substrate/chromogen was pipetted into test tubes and preincubated in a water bath at 35°C. 50 µL of diluted (1:100) glucose oxidase standard was pipetted into reagent tube at zero time, mixed well and left at 35°C for 5 minutes. The absorbance after 5 minutes was recorded at 512 nm. The electrodes were dipped into individual test tubes containing 3 mls substrate/chromogen at 35°C. The electrodes were swirled for 5 minutes and the absorbance at 512 nm was recorded. A blank of substrate/chromogen was recorded at 512 nm and subtracted from the readings. This method was taken from Large (1993).

2.6.2. For the detection of Lactate Dehydrogenase

Reagents:

Lactate dehydrogenase buffer (1 M Tris (hydroxymethyl aminomethane, pH 9.5). Standard NAD^+ (0.5 mM), NAD^+ (0.0010 g) was dissolved in 10 mls of LDH buffer. Standard Lactate (0.275 M), 1.32 g lactate dissolved in 50 mls LDH buffer.

Substrate:

1.5 mls	LDH buffer (73.2 mM)
200 µL	NAD^+ (0.12 mM)
1.0 mls	Standard lactate (40 mM)

Standard LDH, a 1:100 dilution of 50,000 units LDH (L-2881) with buffer was made.

Procedure:

The amounts as indicated above of substrate were pipetted and preincubated at 37°C. For standards, 100 µL of diluted LDH (Sigma L-2881; 50,000 units) was pipetted into a reagent tube at 37°C at zero time, mixed well and absorbances were continually recorded over 5 minutes. Electrodes were placed in test tubes containing substrate, swirled continuously, and absorbance was recorded at 30 seconds interval for 5 minutes. For both the standards and electrodes, the rate of absorbance per minute was calculated. One unit will reduce 1.0 µmole of pyruvate to L-lactate per min at pH 7.5 at 37°C.

2.6.3. For the detection of Alcohol Dehydrogenase

Reagents:

Alcohol dehydrogenase buffer (1 M Tris (hydroxymethyl aminomethane, pH 7.5). Standard NAD^+ (30 mM), NAD^+ (0.2 g) was dissolved in 10 mls of ADH buffer. Standard Ethanol (1 M), 61.61 mls 95 % ethanol in 1 L ADH buffer.

Substrate:

1.5 mls	ADH buffer
0.3 mls	NAD^+
0.3 mls	Standard ethanol
0.895 mls	Water

Standard ADH, a stock of 10 mg/ml ADH of known activity was made up. A 1:100 dilution with buffer was made. 145 Sigma units.

Procedure:

3.0 mls of substrate was pipetted and preincubated at 37°C. For standards, 5 µL of diluted ADH was pipetted into a reagent tube at 37°C at zero time, mixed well and absorbances were continually recorded over 5 minutes. Electrodes were dropped into a test tube containing substrate, swirled continuously, and absorbance was recorded at 30 seconds interval for 5 minutes. For both the standards and electrodes, the rate of absorbance per minute was calculated. One unit of ADH will convert 1 µmol of ethanol to NADH per min at pH 8.8 at 25°C. 1 unit will convert 0.73 µmol of ethanol to NADH per min at pH 7.5 at 37°C.

2.7. Software used

The data (TLC plates, FIA results, and CV's) that were shown in the current study was scanned using Mircotek Scanmaker (model II HR) and the image was then processed in Adobe Photoshop 3.0, Corel Photopaint (version 5.0) and Corel Draw (version 5.0). There were no modifications made to the original results.

Statistical analysis of results were done using Microsoft Excel 5.0 and in Vital (statistical analysis programme, written by R. Fletcher, Agresearch, Palmerston North, New Zealand).

Curve-fitting of data used IgorPro (Macintosh version, from WaveMetrics, Inc, P.O. Box 2088, Lake Oswego, OR 97035 or E-mail WaveMetrics@AppleLink.Apple.com).

Chapter 3

3.1. Equipment Design- test beds for Biosensors

There are two important methods used to evaluate amperometric biosensors. One method is known as the "batch method" and the other "flow injection analysis (FIA)". The method used depends on the type of end results required. The batch method requires less equipment compared to the FIA method.

3.1.1. Determination of sample using Batch method

For amperometric measurement using the batch method the equipment required is a three electrode system immersed in a sample of interest, connected to a potentiostat to hold the potential at a set value, and finally connected to a recorder to record the events. The monitored response from the amperometric measurements is a progression towards a steady state. The advantage of this method was that the volume of sample can be large. A disadvantage of the batch method is it cannot be automated easily. The length of time required for steady state to be reached can limit the number of samples analysed (Canh, 1993). The main use for this method is in initial evaluation of biosensor designs. It provides useful information on response time and calibration curves.

3.1.2. Flow Injection Analysis (FIA)

The reasons for the growth in popularity of Flow Injection Analysis(FIA) as a tool for analysis of solutions are the simplicity and versatility of the system. One of the strengths of FIA is the ability to inject a defined volume of sample into an unsegmented flow stream (carrier stream) with highly reproducible results (Ruzicka and Hansen, 1981). Therefore, injections of both standards and samples can be controlled to produce high degrees of repeatability. Another advantage in using FIA is the degree of control it provides for the amount of dilution required of the sample. Conditions can be organised so that highly concentrated samples can be diluted or similarly, the degree of dilution could be limited for already diluted samples.

Different flow rates can be achieved by varying the tubes size. The length of time which the samples stayed in the detector would be varied to produced the optimum response (Ruzicka and Hansen, 1981). In comparing FIA with batch measurements, the response curve recorded in a FIA never reaches a plateau (steady state) unlike batch measurements (Ruzicka and Hansen, 1981; Canh, 1993). FIA relies on both the same sample size and the same time at which the sample reaches the detector after each injection. Therefore, instead of the plateau region being important as in batch measurement, peak height recorded corresponds to sample concentration. FIA also has the advantage of being able to work with small volumes and since steady state conditions do not have to be reached, FIA was capable of high rates of sample measurement. FIA had been reported capable of analysing up to 200 samples/hr (Canh, 1993), although this is dependent on the response time of the biosensor. The FIA setup used for later experiments involving either H_2O_2 detection or biosensor evaluations is shown in Figure 3-1.

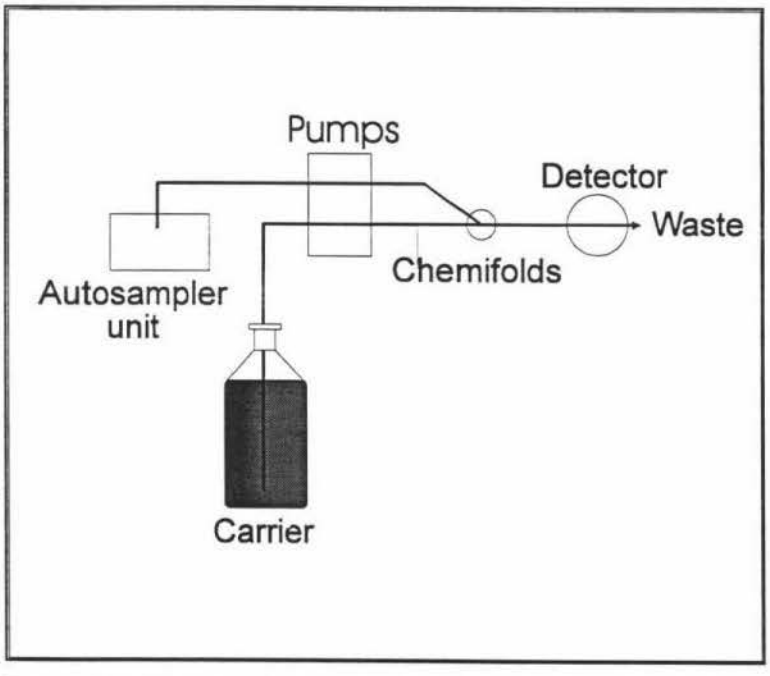

Figure 3–1. The setup of the Flow Injection Analyser in relation to the amperometric detector. The samples were set up in the autosampler unit. There were two independent pumps controlling the flow of the carrier stream and the sample stream. The chemifold was redundant during the testing for H_2O_2 but was important when other reagents (cofactors of the reaction) were required. The detector unit consisted of a three electrode system connected to a potentiostat and a recorder.

3.2. Reasons for the Development and Manufacture of Flow Cells

A flow cell was needed to house the reference, auxiliary, and working electrodes for FIA. As well as that, flow cells were designed to engineer the geometry of flow and sensing surfaces to give sensitive and well resolved responses between samples, and to minimise sample carry over. In contrast with liquid chromatography, where the dead volume space and the actual detectable area are synonymous as it relies on UV-Vis absorption for detection of analytes, in the case of electrochemical detection, the flowing stream of analytes immediately surrounding the working electrode (hydrodynamic boundary) is critical. Analyte that is distant from the hydrodynamic boundary of the sensing surface would not be detected (Johnson *et al*, 1986). The flow cell designs also needed to be compatible with the operations and fittings of the FIA.

Flow cells designs have been based on tubular flow (Matsuda, 1967), thin layer (Matsuda, 1967), wall jet (Yamada and Matsuda, 1973) and rotationally symmetric system (Matsuda, 1967) configurations. Currently, two types of cell designs predominate, thin layer and wall jet. In thin layer systems, a thin layer of solution passes over the surface of the working electrode. In comparison to thin layer, the wall jet system relies on a flowing stream of analyte flowing perpendicular to the working electrode's surface, thus spreading radially over the working electrode's surface.

The work of Elbicki *et al* (1984) showed the type of flow cell used was not an issue, but whether the flow cell allowed natural development of the hydrodynamic boundary was important in defining resolution and response of the electrodes. Elbicki *et al* (1984) maintained that wall-jet designs were theoretically and experimentally similar to thin layer designs when small spacers were used, thus not allowing the natural development of the hydrodynamic flow of a wall jet design.

Flow cell designs have to take into consideration certain design constraints inherent in flow cell construction. Therefore fundamental questions such as size of reaction chamber, where amperometric detection of sample occurs, the angle at which the inlet from the FIA comes in to pass over the working electrode, the distance between the auxiliary electrode and working electrode, were considered.

For a functional amperometric flow cell, the main constraints were:

1. Flow cell design had to incorporate three electrodes. It also had to have an inlet and outlet to allow sample through the flow cell.
2. The material used to manufacture flow cells had to be rigid, but yet allow modifications to the structure to be made to; i.e. holes to be drilled.

Perspex is such a material commonly used. There were three versions of the flow cell made. Version 1 flow cell was found to be inadequate for its designed purpose. Version 2 flow cell was found to be adequate for its designed purpose. The design of Version 3 flow cell was a gift from Cranfield University. The results and progress of the development of flow cells are discussed under headings "Version 1 Flow Cell", "Version 2 Flow Cell" and " Version 3 Flow Cell".

3.2.1. Version 1 Flow Cell

The original design of Version 1 flow cell was taken from was Staden (1986). There were modifications that were made to suit an amperometric design, the inclusion of an auxiliary and a reference electrode. The original study was concerned with the detection of chlorides using a coated tubular solid-state chloride ion electrode. Staden (1986) looked at several factors, such as length of line used between FIA pumps and detector, flow rate, sample volume, and factors inherent in detector cell design (design, contact area and dead space volume). The overall final design by Staden (1986) was a FIA linked to a flow through detector. The factors discussed by Staden (1986) were taken into consideration for the design of the present FIA linked amperometric detector.

Version 1 housed a three electrode system; the working, auxiliary and reference electrodes. A schematic diagram of flow cell design is shown in Figure 3-2. Version 1 did not work with the precision needed. Figure 3-3 showed it produced quite broad sample peaks in response to H_2O_2 at 600 mV, merging one sample peak with the next. This was unsatisfactory, and there was the possibility of the previous sample interfering with the detection of the current sample (carry over effect).

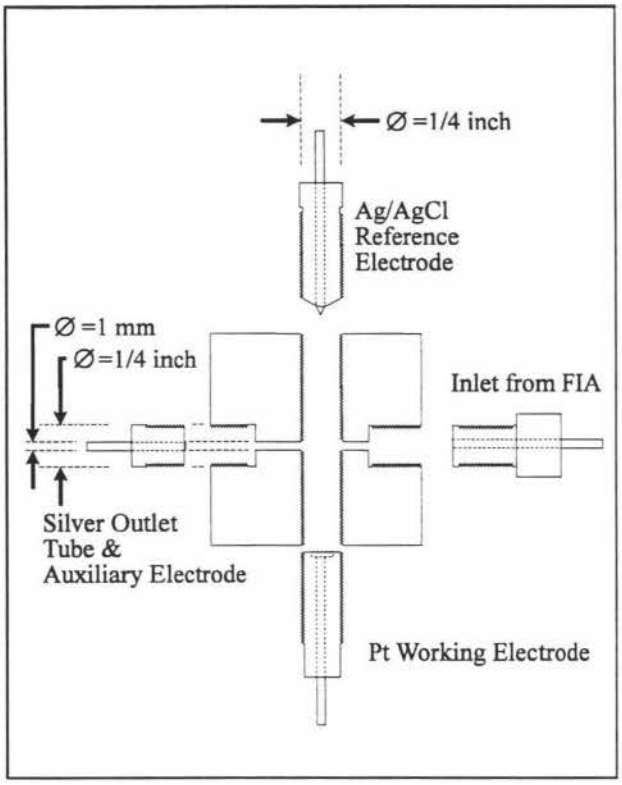

Figure 3–2. Version 1 Flow Cell. The size of the Pt disk electrode was 3 mm in diameter. The reference and the auxiliary electrodes were held with epoxy glue inside a perspex holder.

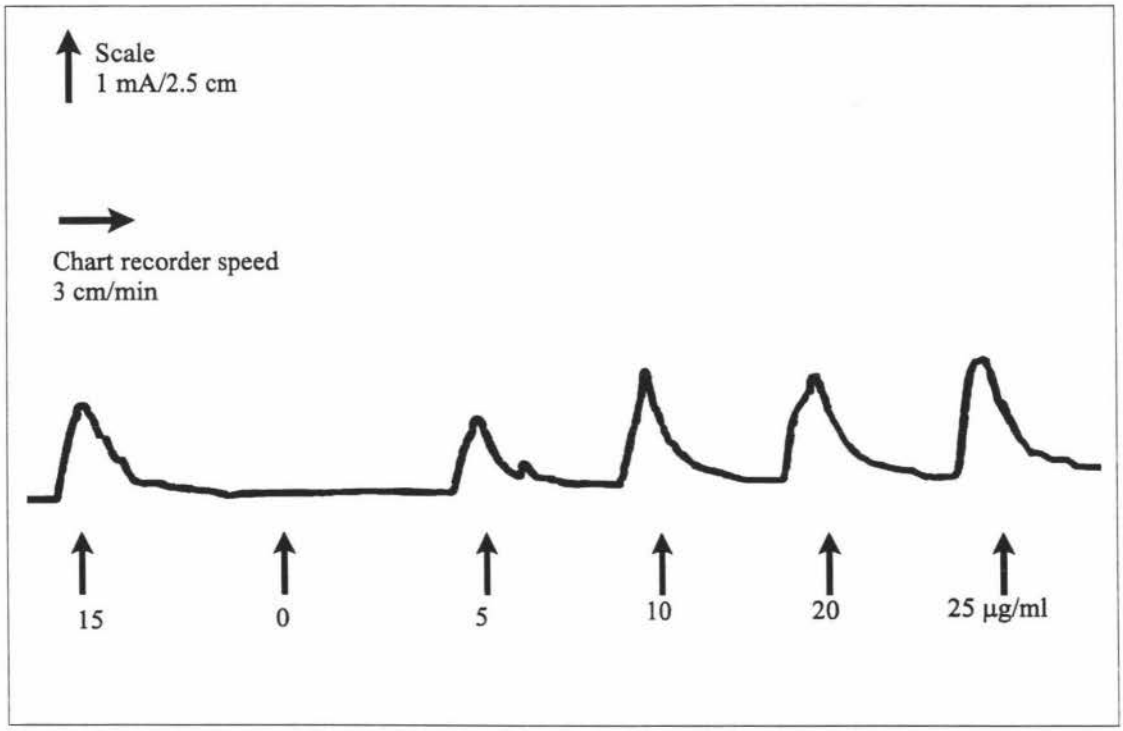

Figure 3–3. A representation of results obtained using Version 1 Flow Cell. Hydrogen peroxide was used for calibration of flow cell. The results were obtained in conjunction with FIA. The buffer used was 0.1 M phosphate buffer, pH 7.5. Potential was poised at 600 mV.

The graph in Figure 3-4 shows signal response linearity up to 10 μg/ml H_2O_2 for Version 1 flow cell. Improvements were needed to give linearity past 10 μg/ml H_2O_2. There were several factors which could have produced non-linearity and carry over. The larger diameter of tubing used may have increased the amount of radial dilution occurring between sample and carrier stream. The other problem could be a larger than necessary dead volume space between the working and reference electrode. This could result in a broader peaks, such as those seen in Figure 3-3. Another problem encountered was the inability to determine exactly the position of both the reference and working electrodes, as there were no stops to allow the electrodes to be positioned reproducibly between experiments. A second flow cell was developed.

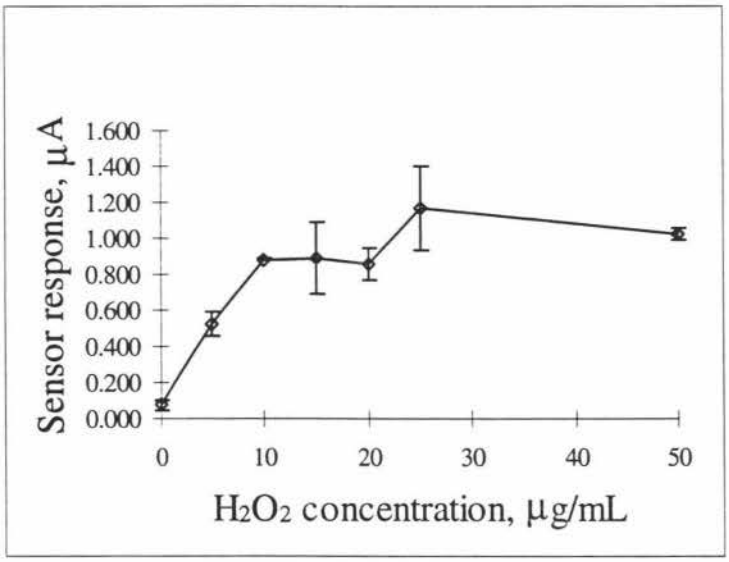

Figure 3–4. Results from testing Version 1's effectiveness for the detection of H_2O_2 using a Pt working electrode. Potential poised at 600 mV. Error bars represent the amount of variation from sampling three sets of H_2O_2 calibrated concentrations.

3.2.2. Version 2 Flow Cell

Version 2 design was based on the design of Frenzel and Bratter (1986). The cell from the study by Frenzel and Bratter (1986) was modified to fit into the FIA connector's configuration. Later, it was discovered that the position of the reference and auxiliary electrodes ought to be placed downstream in relation to the working electrode (Wang, 1994), so that any leakage or side reaction from the reference and the auxiliary would not interfere with the detection on the working electrode. The design of the second two

did place the reference electrode somewhat downstream in relation to the working electrode. As previously mentioned, the wall jet design was preferred by some researchers as it may provide more well defined hydrodynamic properties (Huang *et al*, 1993). Huang *et al* (1994) showed the distance between the inlet nozzle and the working electrode could be separated as much as 6 mm without interfering with the development of the hydrodynamic flow of the wall jet.

In Version 2 flow cell, efforts were made to get the reference electrode closer to the working electrode, to reduce the amount of dead volume space. This was achieved by angling the reference electrode with respect to the working electrode, thus decreasing the amount of overall volume within the amperometric detection area. A diagram of Version 2 flow cell is shown in Figure 3-5.

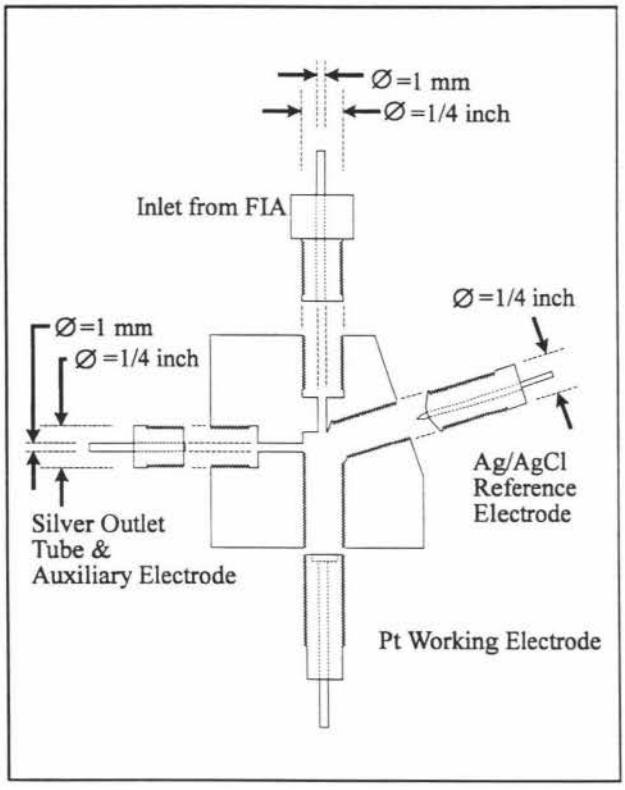

Figure 3–5. Version 2 Flow Cell. The size of the Pt disk electrode was 3 mm in diameter. The reference and the auxiliary electrodes were held with epoxy glue inside a perspex holder.

The changes made to the design of the second generation flow cell and the use of smaller diameter tygon tubes in FIA have improved the results considerably. The peaks were sharp with the addition of H_2O_2 (see Figure 3-6). There was also a higher response to H_2O_2, at 50 µg/ml the response was nearly up to 10 µA (see Figure 3-7) compared to

1 μA response in Version 1 flow cell. Higher responses make easier detection. Subsequent experiments involved Version 2 flow cell.

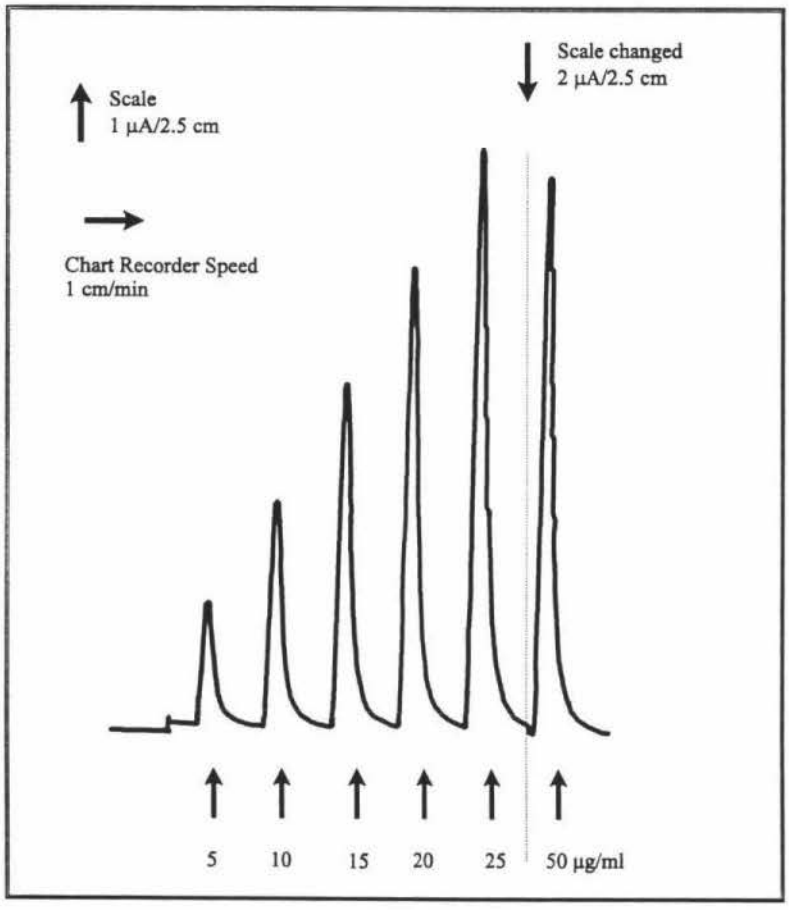

Figure 3–6. A scanned trace of injection peaks using Version 2 flow cell. The calibration used H_2O_2 in conjunction with FIA. Carrier stream 0.1 M phosphate, pH 7.5.

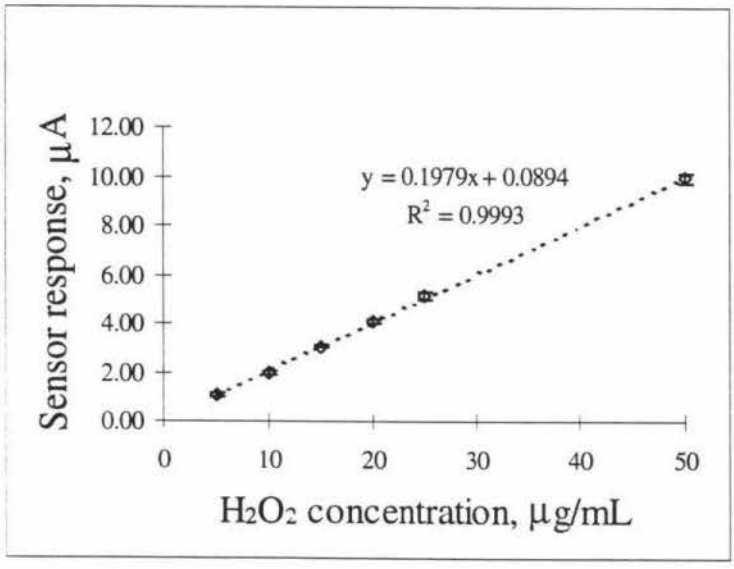

Figure 3–7. Calibration curve for Version 2 flow cell's detection of H_2O_2 using a Pt electrode. Potential poised at 600 mV. Buffer used was 0.1 M phosphate, pH 7.5.

3.2.3. Version 3 Flow Cell

A third flow cell was evaluated because it was a gift from Cranfield University. Its main advantage in the design was the ability to use glass encased Pt electrodes. A diagram of Version 3 flow cell is shown in Figure 3-8. The raw data results of the amperometric detection of H_2O_2 using Version 3 flow cell are shown in Figure 3-9. The cause of the flow injection peaks not being smooth as in Figure 3-7 was later discovered to be due to the condition of the Ag/AgCl reference electrode. Prior to this, the silver coated platinum wire used in the Ag/AgCl reference electrode was kept dry. It was later discovered that if the silver coated silver wire was kept in a solution of 3 M KCl, it was adequate to obtain FIA results that did not have any spikes.

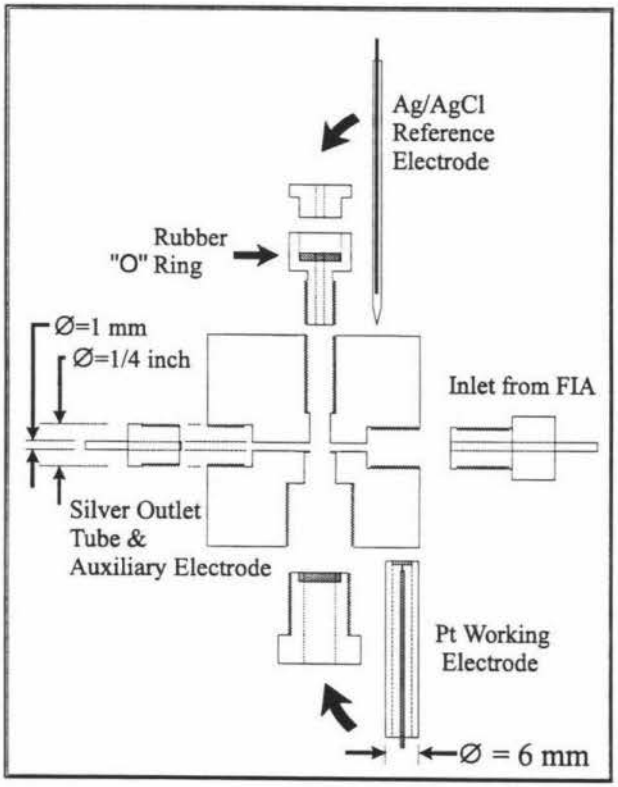

Figure 3–8. Schematic representation of Version 3 flow cell. Courtesy of Cransfield University.

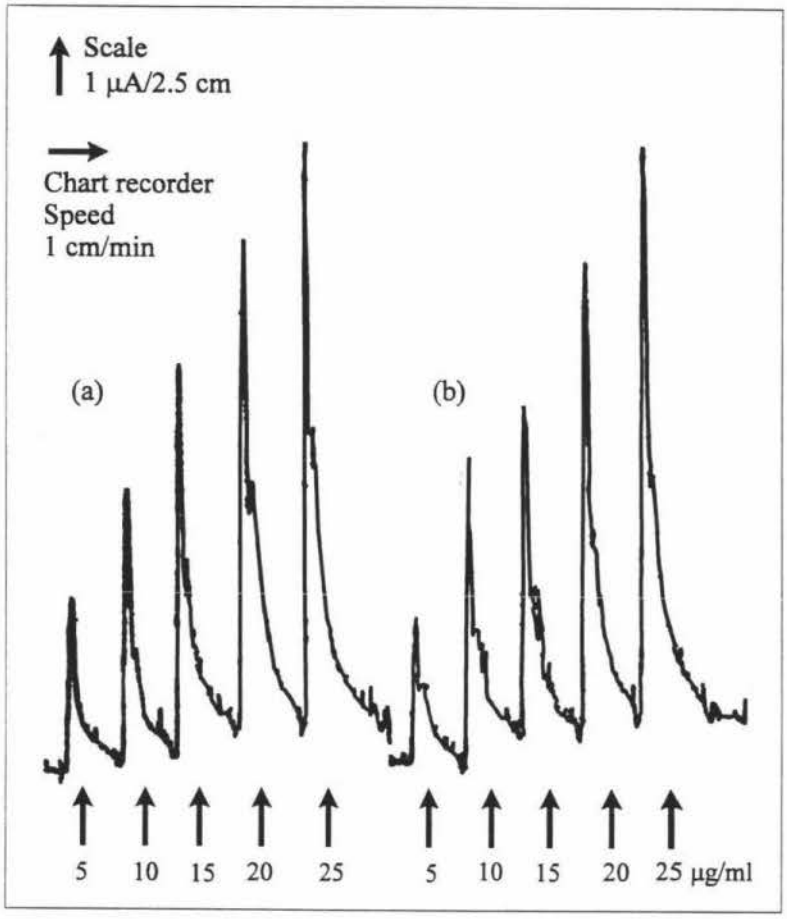

Figure 3–9. A comparison of raw FIA data results between Version 2 flow cell and Version 3 flow cell. (a) Denotes raw data of amperometric detection of H_2O_2 using Version 2 flow cell. (b) Denotes raw data of amperometric detection of H_2O_2 using Version 3 flow cell. The carrier stream was 0.1 M phosphate buffer, pH 7.5.

3.3. The Use of Toray Paper as an Alternative to Platinum Disk for Construction of Electrodes

3.3.1. Reasons for Replacing Platinum with Toray Paper

Electrodes manufactured with platinum disks are expensive. There are cheaper alternatives. One alternative was Toray carbon paper. Toray paper used was a carbon (trade name: XC-72) base with 10% platinum loading.

3.3.2. Design of Electrodes Incorporated with Toray Paper

There were two parts to consider in the design of these electrodes:

1. The design must be able to isolate the interior of electrodes from the outside environment, without compromising the connection between electrode and potentiostat.
2. The electrodes would ideally be designed to fit into a FIA flow cell.

The dimensions and design can be seen in Figure 3-10a. Electrode casing was made with glass, approximately 5 mm internal diameter. This provided sufficient room to insert a piece of electrical wire soldered onto a platinum disk (∅=5 mm). The platinum disk was inserted in the glass tube, leaving a gap of approximately 3 mm between the platinum disk and end of the glass tube. Although the aim was to use Toray paper in place of Pt, the presence of Pt was for the sake of convenience rather than as a sensing surface. The function of Pt was to provide an electrical contact between the Toray paper via hydroxyethyl-cellulose (conducting paste) to the potentiostat. In a subsequent design (Figure 3-10b), the Pt disk was replaced by a brass disk.

The gap between the Toray paper and the Pt disk was filled with conducting paste. Toray paper was cut into sizes of 6 mm in diameter using a hole punch. It was then soaked overnight in 95% ethanol before attachment to electrodes. Selleys Superglue was used to attach the Toray paper to the glass tube. Nail varnish was used to coat the sides of the Toray paper and glass tube to prevent sample solutions leaking into the hydroxyethyl-cellulose chamber. A plastic-forming spray (Plastic Kote) applied using a paint brush was also used. The varnish or plastic coat was allowed to dry overnight.

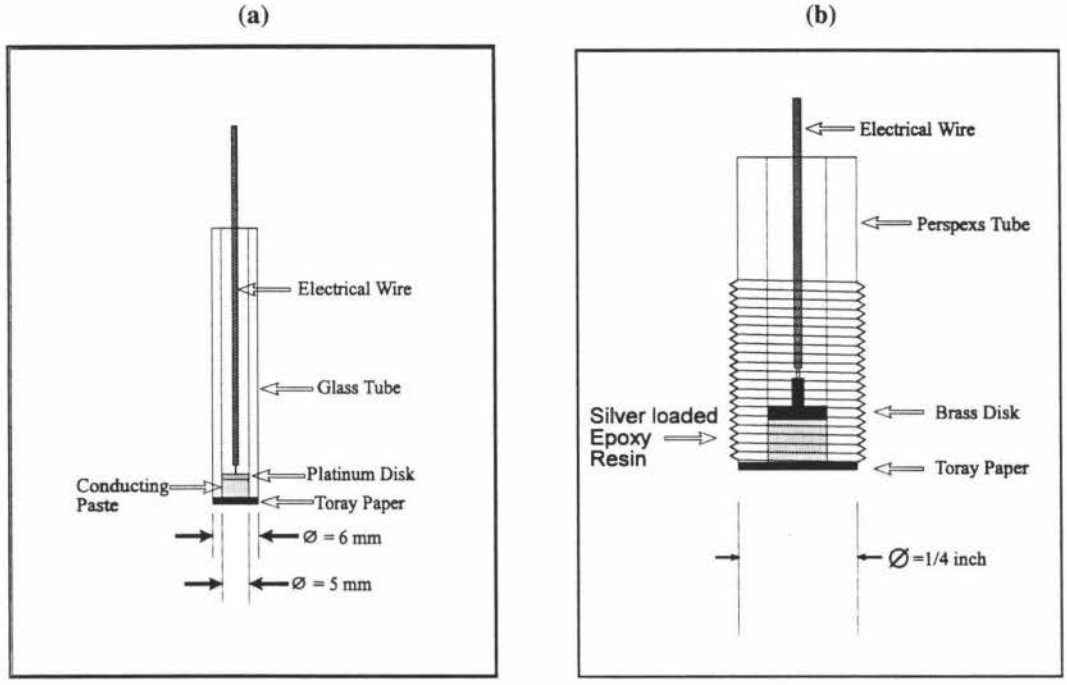

Figure 3–10. (a) Electrode design for incorporation of Toray paper. This type was designed for data collection using batch designed experiments involving potentiostat CV-27. (b) Electrode designed for use of Toray paper in FIA.

Data collection began with the use of potentiostat CV-27 under batch conditions. The work was done inside a Faraday cage which was earthed. The potential was poised at 600 mV. The reference electrode used was a commercially available Ag/AgCl electrode from BAS. A platinum wire was used as an auxiliary electrode. There were nine trials altogether. Each time the piece of Toray paper became disconnected from the rest of the electrode, the piece of Toray paper was replaced with a new piece. The used piece of Toray paper was discarded because it was too wet to be rejoined to the electrode holder. This was a common theme to all the problems encountered with making Toray paper electrodes. The piece of Toray paper would eventually come away from the glass tube thus severing the contact to potentiostat CV-27. This was probably the reason why only one set of readings gave results which were linear (see Figure 3-11). Silver loaded epoxy resin was used in placed of conducting paste, without any further success. There were three trials using this method (data not shown).

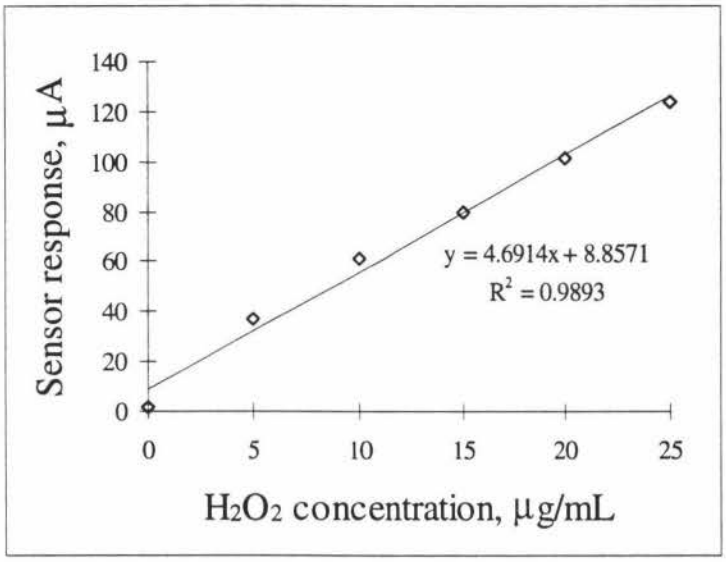

Figure 3–11. Calibration curve for Toray paper designed for batch experiments. This involve the use of potentiostat CV-27 without being attached to a FIA. After each addition of sample it was followed by waiting for a new baseline to be reached. After each reading a it was followed by a wash using R.O. water. This was the only linear sensor response reading.

3.3.3. Incorporation of Toray paper into FIA

This work began with modifying the electrodes used in batch experiments for incorporation into FIA. The outer casing was made out of perspex instead glass, this allowed screw threads to be made. The dimensions were designed to fit into 1/4 inch holes in the flow cell. A diagram is shown in Figure 3-10b. The electrodes were poised at 600 mV, referenced to Ag/AgCl with a silver tube acting as an auxiliary electrode (Setup was the same as those using Pt disk electrode in Figure 3-6).

Figure 3-12 shows the type of result obtained using Toray paper electrodes within Version 2 flow cell. There were a total of nine attempts to use these electrodes. Similar problems were encountered as with Toray paper electrodes designed for batch experiments. The piece of Toray paper would consistently be discovered unattached from the electrode holder. There was also the problem of baseline drift as seen in Figure 3-12.

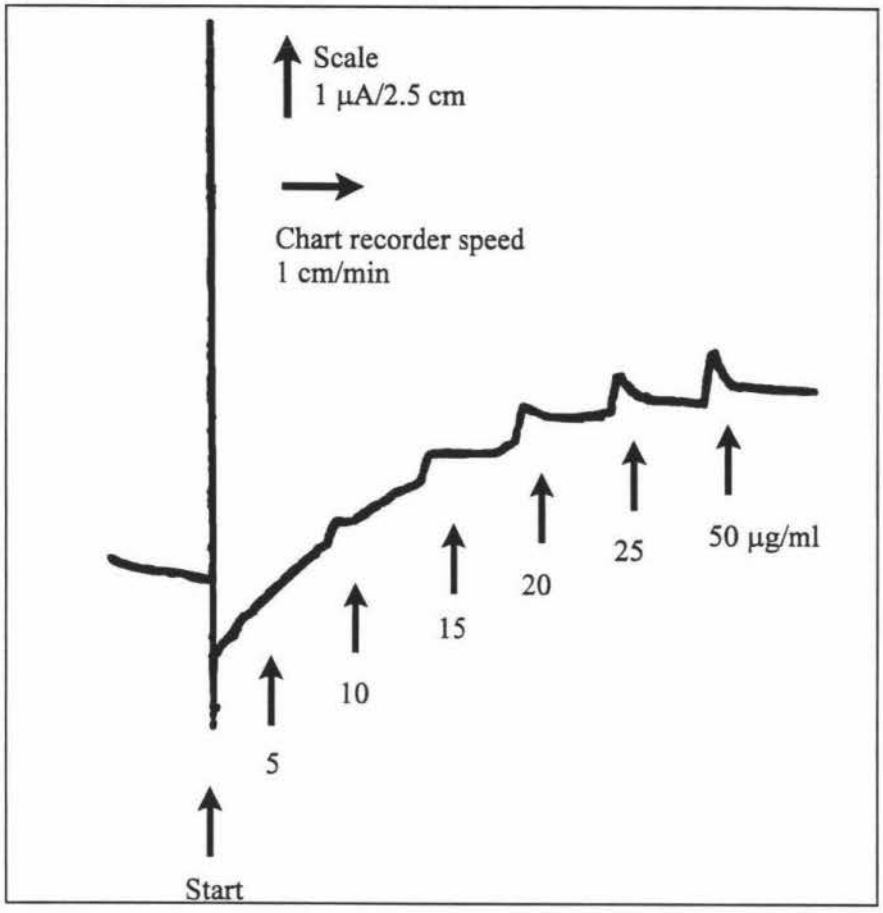

Figure 3–12. Raw data obtained using Toray paper electrodes designed for use with FIA. The potential was poised at 600 mV.

3.3.4. Improving Electrical contact between Toray Paper and Potentiostat

The screw electrode was the second type of electrode designed to be incorporated into a FIA. A schematic construction is shown in Figure 3-13. The screw (now acting as an electrode holder) was soldered to a piece of electrical wire to allow attachment to potentiostat CV-27. Silver loaded epoxy resin was used to attach Toray paper to the screw. The edges of the Toray paper and the screw were then coated with Plastic Kote using a brush to prevent interference between silver loaded epoxy resin and samples. Finally, it was incorporated into FIA's setup for sample analysis. The electrode was poised at +600 mV, with a Ag/AgCl reference electrode, and a silver tube for an auxiliary electrode.

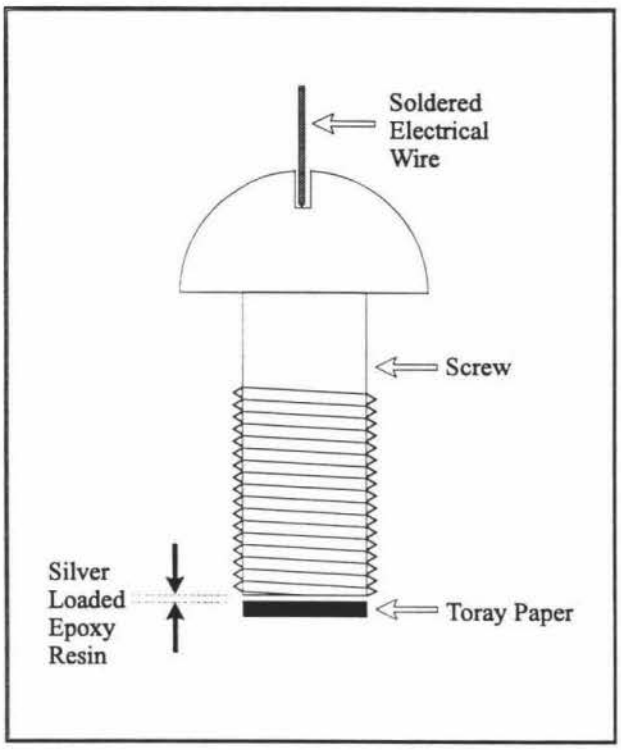

Figure 3–13. Construction of a "screw" electrode for use with Toray paper within a FIA flow cell.

In Figure 3-14, the calibration curve for a platinum disk electrode in a FIA is linear up to 25 μg/ml H_2O_2. The platinum disk electrode was used as a control for both Toray paper attached with hydroxyethyl-cellulose (conducting paste) and with silver loaded epoxy resin attached screw. The calibrations using H_2O_2 for Toray paper attached either by conducting paste or silver loaded epoxy resin (screw electrode) were not as good as with the platinum disk electrode. The size of the response obtained using Toray paper electrodes gave only 1.99 μA as opposed to 9.04 μA with platinum disk electrode. The linearity of the calibration curve for Toray paper electrodes (r^2 = 0.9668) was better compared to Pt disk electrode (r^2 = 0.8675), although the platinum disk electrode calibration curve was linear between 5-25 μg/ml H_2O_2 (r^2 = 0.999). The curve "Toray paper" represents data collected using conducting paste to link Toray paper to an embedded Pt disk inside a perspex electrode holder (see Figure 3-10b). The conducting paste was prevented from escaping by coating the sides with nail varnish. In this case, the Toray paper electrodes worked with good linearity in the calibration curves, but the problems encountered with making a successful linear calibration reading makes this method unfeasible. The common theme of the piece of Toray paper becoming disconnected from the rest of the electrode holder as seen in section 3.3.2. (batch

measurement) occurs with Toray paper electrodes made for FIA. In Figure 3-11, the figure showed a single calibration curve out of nine curves that gave a linear response in batch measurements of Toray paper electrodes. In Figure 3-14 however, three calibration runs of a single Toray paper electrode were successfully made. The alternative was using silver loaded epoxy resin to link the electrode holder (screw) to a piece of Toray paper, the advantage being that the epoxy resin was conductive as it was silver loaded upon curing. A screw was used to increase the conductive surface. However, only a single calibration curve was made due to the screw electrode suffering the same problems as some of the other Toray paper electrodes. Therefore compared to a Pt disk electrode, all versions of Toray paper electrodes did not perform as well nor was it as convenient as using Pt disk electrodes.

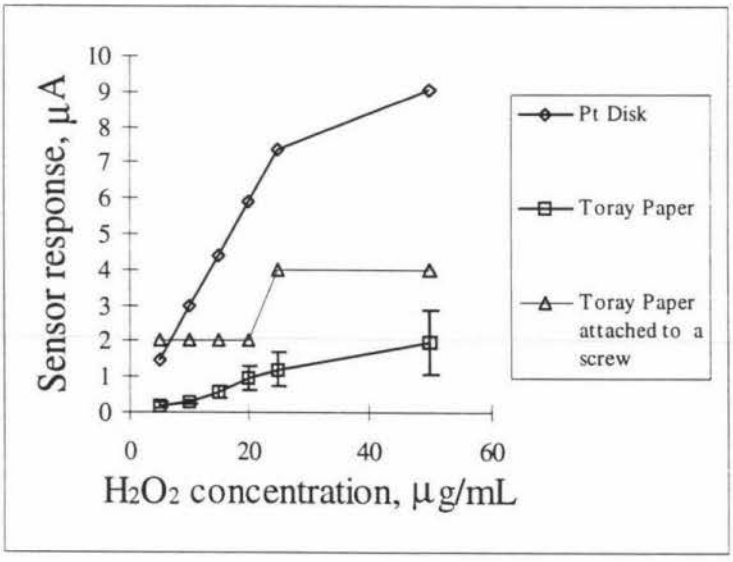

Figure 3–14. Comparison between different methods for detection of H_2O_2. Pt disk electrode (one calibration run) was used as an established method for H_2O_2 detection. In the legend "Toray Paper" (three calibration runs) indicates Toray paper electrodes designed for FIA used. "Toray Paper attached to a screw" (one calibration run) indicates electrodes made as in Figure 3-10, used in FIA. In all cases the potential was poised at +600 mV.

3.4. Discussion

There was a need to construct the equipment and select the best method for using the FIA in conjunction with an amperometric detector. The results for flow cell design between version 1 and version 2 clearly showed the advantage of version 2's design for H_2O_2 detection. There was a possibility that the version one's flow cell results could

have been improved. This would have involved a change in the internal diameter of the FIA tubing used to a smaller size. In some of the chart recordings obtained while detecting H_2O_2, there was a second but smaller peak present. It always occurred after the main H_2O_2 peak had been detected. This secondary peak can be attributed to an artefact caused by the electrical impulse on starting the sample pump so the next sample could be delivered to the injection valve (Lunte *et al*, 1985).

The basic design of version two flow cell was modelled after the wall jet design. The main advantage of the wall jet design is low dead volume and in addition, the jet impinging on the surface of the working electrode lessening the deposition of suspended solids and fouling of the biosensor, and gives well defined hydrodynamics. The disadvantage to this method is it may decrease the life of the biosensor, due to the mechanical stress it places on the immobilised enzyme as the jet of sample impinges on the working electrode (Canh, 1993), although this depends greatly on the mechanical strength of the immobilisation method.

The work done developing the FIA for use in sampling procedures had progressed to the point where it was considered to be satisfactory. This allowed a concentrated effort for developing electrodes to detect other electroactive species than H_2O_2. The development of flow cells was assessed and Version 2 Flow cell was considered to be satisfactory for use in further experimental studies. Version 3 flow cell was a later addition, which had an advantage over Version 2 flow cell as glass encased Pt disk electrodes could be used.

The work on Toray paper as a substitute for platinum disk as working electrodes had highlighted several problems. The main problem associated with electrodes fitted with Toray paper was maintaining physical and electrical contact between the piece of Toray paper and the rest of the electrode.

There were various attachment methods tried:

1. Glueing the edges of the Toray paper to the electrode which met with limited success.
2. Attempting to soldering a piece of wire to connect to the piece of Toray paper was unsuccessful.
3. Nail varnish and Plastic Kote were used around the edges of the electrode to hold the piece Toray paper in place. This was also to protect the silver loaded epoxy resin that was used in place of the solder to connect the wire to the Toray paper from contaminating the samples.

4. A screw was used as an electrode holder to increase connectivity to Toray paper.
5. Silver loaded epoxy resin was used to strengthen the connection between electrode and Toray paper. The other type of connector used was conducting paste, which was found to be unsuccessful.

However, none of these methods increased the useability of Toray paper. There were some results that may have suggested it was possible, but a lot more work would be required to address the problem of improving the connectivity between the electrode holder and Toray paper. Therefore it was decided to abandon Toray paper in preference to Pt disk electrodes.

Chapter 4

4.1. Glucose Analysis Using Glucose Oxidase Electrodes

The initial thrust in biosensor development was the development of glucose sensitive chemical sensors. The reason for the high level of research into a biosensor for glucose was the significance of glucose both in clinical and industrial sectors. There are a large number of diabetes patients in the world, who require information about their blood glucose levels. There is also the importance of on-line monitoring of critically ill patients. Glucose determination is not only important in the medical field, but glucose is an important analyte industrially as well. Glucose is important to the fermentation industry (Bartlett, 1990) as it determines the "feed times" for the yeast, or for analysis of different types of food produced industrially that have to meet certain levels of health and industrial limits (Suleiman and Guilbault, 1992).

The development of an enzyme based oxygen sensor made by Clark and Lyons (1962) made it possible to measure glucose in body fluids and tissues without the use of complicated analytical procedures. The next major advancement in biosensor design was the incorporation of mediator molecules. Cass *et al* (1984) showed ferrocene/ ferricinium ion couples capable of acting as electron mediators in place of oxygen. This made measurement of glucose independent of oxygen presence.

A common enzyme used for the development of glucose biosensors was glucose oxidase (GO). The reason being, glucose oxidase is readily available, highly active, and very stable. Glucose oxidase belongs to a class of enzymes called oxidoreductases. It contains two molecules of flavin adenine dinucleotide (FAD^+) per molecule of glucose oxidase (Nakamura *et al*, 1965).

It catalyses the reaction:

$$\text{Glucose} + O_2 \xrightarrow[\text{Oxidase}]{\text{Glucose}} \text{Gluconic acid} + H_2O_2 \qquad \text{Equation 1}$$

The mechanism of electron transfer suggested by Nakamura *et al* (1965) for GO was:

$$P\text{-}FADH_2 + O_2 \longrightarrow P\text{-}FAD + H_2O_2$$

$$P\text{-}FAD + S \longleftrightarrow S\text{-}P\text{-}FAD$$

$$S\text{-}P\text{-}FAD \longrightarrow P\text{-}FADH_2 + Lactone \qquad \text{Equation 2}$$

Where $P\text{-}FADH_2$ was the reduced form of the enzyme unit, P-FAD was the oxidised form, S-P-FAD the enzyme substrate complex and S was glucose. The results of the study by Nakamura (1965) showed convincing evidence of this reaction mechanism with data from observed and calculated values in good agreement.

The reaction at the platinum (Pt) electrode surface was:

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^- \qquad \text{Equation 3}$$

This reaction at the Pt surface can be monitored amperometrically with the potential poised between +500 mV and +800 mV (Luong *et al*, 1993).

The active site of glucose oxidase was found to be buried deep within the enzyme, as much as 50 Å (Bartlett, 1990). Therefore direct oxidation is not possible. The discovery of artificial electron mediators resulted in measurements that were independent of oxygen availability. The mediators also had the advantage of lowering the potentials required for electrochemical measurements, therefore lowering the interference effects from other electroactive species present. One such mediator described by Cass *et al* (1984) was ferrocene. It still remains as one of the successful mediators for glucose oxidase.

Turner (1987) categorised four methods of immobilising enzymes to electrodes. This section will be comparing glucose oxidase immobilised in a gelatine-glutaraldehyde cross link matrix to a covalent immobilisation technique for attaching glucose oxidase to haeme. One of the early methods for immobilising enzymes was with polyacrylamide gel (Updike and Hicks, 1967). Later immobilising techniques utilised gelatine or bovine albumin treated with glutaraldehyde to stabilised the enzymes (Mullen *et al*, 1986; Alva

et al, 1991). The use of glutaraldehyde prevented the lost of enzymes through leaching from the "pores" of the gelatine matrix (Barker, 1987), which was one of the concerns of this method of enzyme immobilisation. This method of immobilisation was also suitable for use of optimising the flow injection analyser because it is an established method. The method had also been optimised by Large (1993) to promote cross-linking and preserve enzyme activity of glucose oxidase.

The method of immobilisation currently under investigation is covalent binding of the enzyme and haeme to the sensing surface of the electrode. The method was originally conceived by Blackwell and Greenway (personal communication, 1992). Large (1993) had reported some preliminary experimental results of the behaviour of haeme bound GO using cyclic voltammetry and poised potential readings. The current study will further investigate the behaviour of haeme bound GO (Conjugate-GO), and seek to incorporate the conjugate within the framework of flow injection analysis (FIA), and finally to investigate the short and long term stability of the enzyme.

4.2. Use of Gelatine and Glucose Oxidase on Platinum Electrodes in a FIA System

Following the work done with the FIA setup (see section 3.2) and evaluation of a flow cell designed to carry out amperometric measurement of H_2O_2, it was deemed satisfactory to be able to concentrate on the development of a glucose sensor using GO on a Pt electrode. The arguments for incorporating biosensor testing within the framework of a FIA had previously been discussed in section 3.1. The evaluation of Gelatine-GO's behaviour within the framework of FIA was important to establish as a comparison for haeme linked GO. The concept of haeme bound GO (Conjugate-GO) is new.

The preparation of Gelatine-GO electrodes after immobilisation onto a Pt disk electrode for incorporation into FIA followed the same procedure as that of section 3.2.2. At the time, only flow cell versions 1 and 2 were available. Since the results showed that version 2 flow cell was more sensitive to hydrogen peroxide determination, the procedure for evaluating Gelatine-GO electrodes used the same procedure as that of version 2 flow cell. In Figure 4-1, the calibration curve for Gelatine-GO was linear up to

25 mM glucose for both Day 1 and Day 2 (shown by the correlation values of Day 1 and Day 2). After 24 hrs of storage at 4 °C in 0.1 M phosphate, pH 7.5, the percentage signal drop between Day 1 and Day 2 was 17.7% (at 25 mM glucose Day 1, gradient 0.1154; Day 2, gradient 0.095). However linearity of calibration curve to glucose was maintained as illustrated with high correlation values of 0.99 in Figures 1a and 1b.

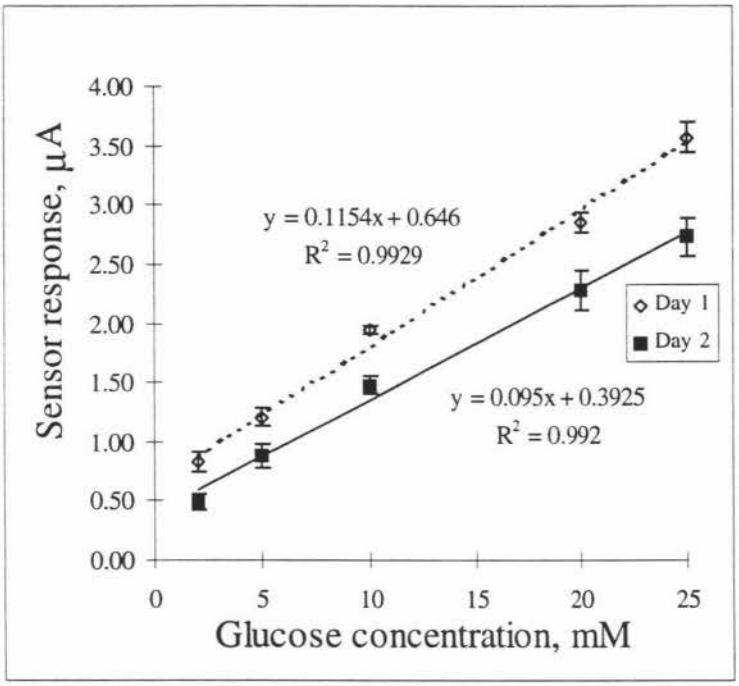

Figure 4–1. Calibration curve for glucose using glucose oxidase entrapped in gelatine on a Pt electrode. There were three calibration runs using FIA for each day. The same Pt electrode was used in all three runs. The electrode was poised at 600 mV. The error bars indicate standard error of the mean. The sensor response of the electrode was monitored over two days.

The calibration curves for Gelatine-GO were linear up to 200 mM glucose as shown in Figure 4-2. Standard errors of the means were higher at higher glucose concentrations.

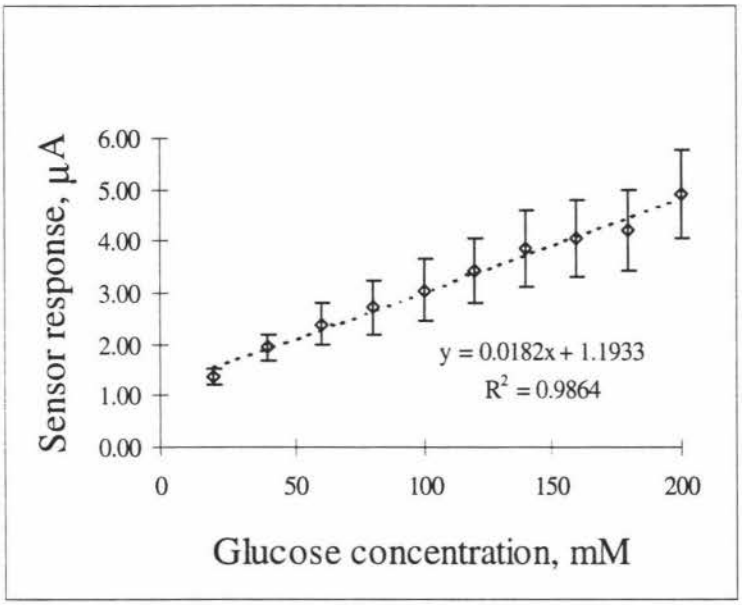

Figure 4–2. Calibration curve for glucose oxidase entrapped in gelatine on Pt electrode up to 200 mM glucose concentration. Potential poised at 600 mV. There were 5 calibration runs made on the same day, using the same electrode as in Figures 4-1. The error bars indicate standard error of the mean.

At this point, the method for amperometric detection of glucose using GO entrapped in gelatine was satisfactory; both setup of FIA and flow cell design in combination were able to produce good sensor response towards glucose in solution.

4.3. Glucose Oxidase-Haeme Conjugated Electrodes (Conjugate-GO)

Lane and Hubbard (1973) proposed that conjugated double bonds were able to share pi electrons with d-orbitals of a platinum surface. Haeme consisted of a rich source of conjugated double bonds (Large, 1993). The conjugated double bonds of haeme were exploited to link the enzyme glucose oxidase to a Pt surface, thus providing an alternative to using gelatine to immobilise the enzyme GO onto the surface of a Pt electrode.

There may be advantages to conjugating haeme to GO and allowing Conjugate-GO to absorbed directly to a Pt surface. A monolayer of Conjugate-GO was envisaged to absorb onto the Pt surface allowing faster amperometric responses to glucose, as the enzyme was closer to the Pt surface for H_2O_2 detection. The method for making Conjugate-GO can be found in section 2.4.2.

Figure 4-3 showed Conjugate-GO gave similar results to Gelatine-GO electrodes for glucose detection. The calibration curve was linear between 20 mM and 200 mM glucose. The sensor response of Conjugate-GO (Figure 4-3) had a gradient of 0.0239 compared to Gelatine-GO (Figure 4-2) that had a gradient of 0.0182. The calibration curve for Conjugate-GO ($R^2 = 0.9788$) was comparable in linearity to that of Gelatine-GO ($R^2 = 0.9864$) as illustrated in Figure 4-3. Both Conjugate-GO and Gelatine-GO electrodes did not intercept through zero if the regression line was projected back to zero concentration of glucose. This will be discussed in detail in section 4.8.

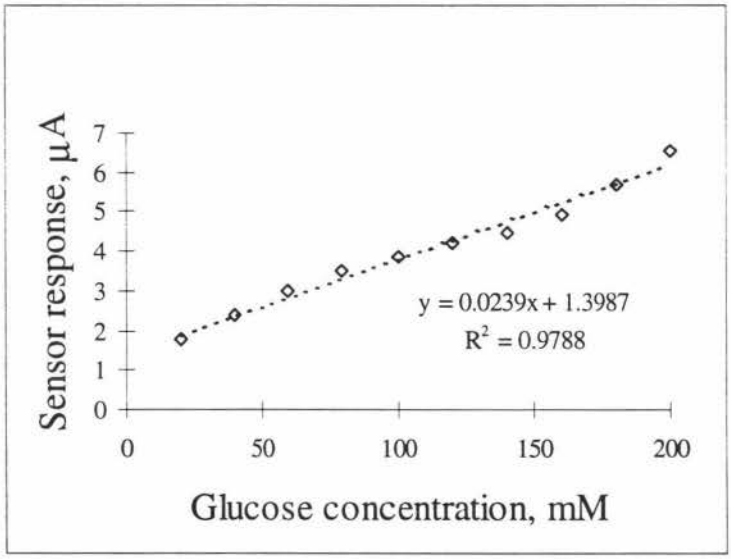

Figure 4–3. Calibration curve for Conjugate-GO showing signal response up to 200 mM glucose concentration for a single Pt electrode. Potential poised at 600 mV.

The results shown in Figure 4-4 illustrated Conjugate-GO electrodes showed a faster return to a nominated baseline than Gelatine-GO electrodes. This was possibly due to much faster interactions occurring between glucose and glucose oxidase and subsequent faster transfer of H_2O_2 to the platinum surface offered by the conjugate. Conjugate-GO was envisaged to form a monolayer on the surface of the Pt electrode. Glucose oxidase immobilised in gelatine had a matrix that could possibly slow down the diffusion of H_2O_2 to the Pt electrode surface. Therefore, Conjugate-GO did not seem to have the diffusion problem as seen in Gelatine-GO electrodes.

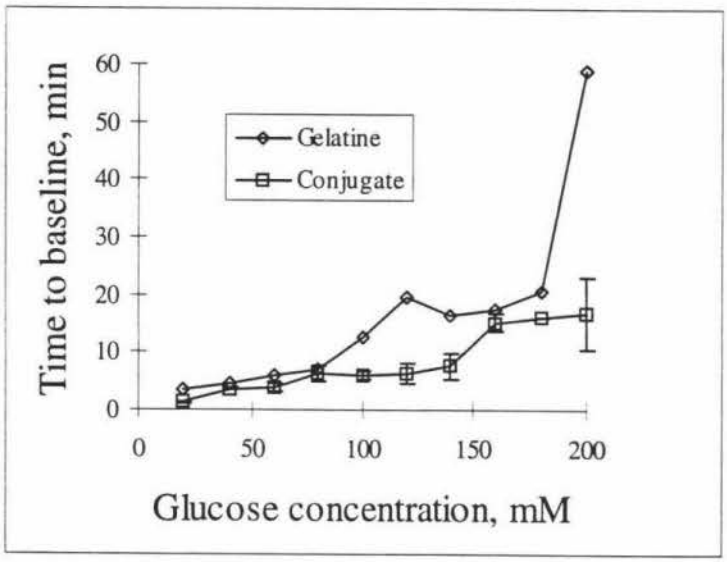

Figure 4–4. Comparison between gelatine and haeme immobilised glucose oxidase on Pt electrodes for time to return to baseline. Potential poised at 600 mV. Glucose oxidase immobilised in gelatine - 1 calibration run. Glucose oxidase immobilised with haeme - 2 calibration runs.

4.4. Evaluation of Viability of Conjugate-GO Electrodes Compared to Gelatine-GO Electrodes over a Five Day Period

There were two parts to this section. The first part looks at Conjugate-GO electrodes at higher glucose concentrations (between 20 mM and 200 mM glucose). The second part looks at Conjugate-GO at lower glucose concentration (between 0 and 25 mM glucose).

4.4.1. Performance of Conjugate-GO for amperometric glucose detection between the ranges of 20 mM and 200 mM glucose

The Conjugate-GO electrode was prepared as described in section 2.4.2. The electrode was stored in 0.1 M phosphate buffer, pH 7.5 at 4°C in between the days tested for amperometric response to glucose.

Figure 4-5 showed the effects of storage over time for the Conjugate-GO electrode. There was a large drop in sensor response within 5 days of preparation. There was a drop of 85% in signal response between Day 1 and Day 5 at 200 mM glucose concentration (at 200 mM glucose, Day 1 was 6.56 μA; Day 2 was 3.71 μA; and Day 5 was 1.01 μA). Therefore, storage under these conditions resulted in a large drop in sensor viability. Figure 4-5 also showed a "plateau" effect between 80 mM to 140 mM

followed by a sharp rise in the gradient after storage glucose concentrations in Days 1 and 2, however this effect did not seem evident in Day 5. This will be discussed further in section 4.8.

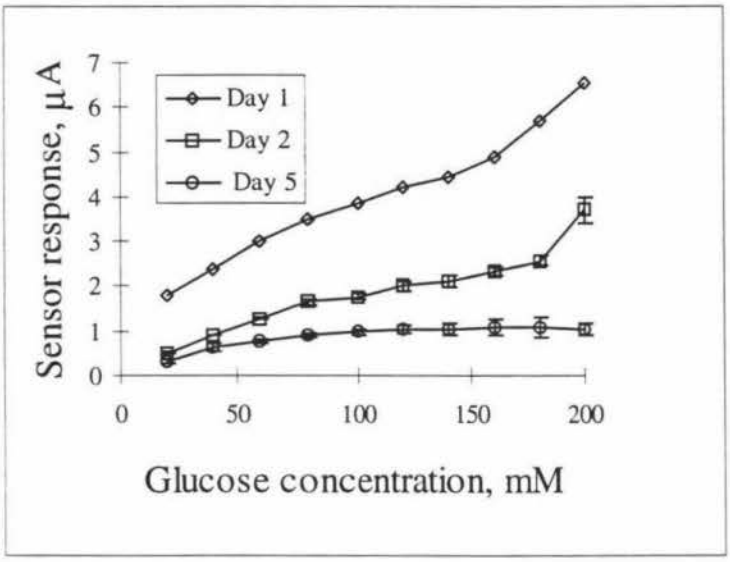

Figure 4–5. Calibration curve for glucose oxidase-haeme conjugates on Pt electrodes. Each line represents days after initial conjugate adsorbed onto Pt electrodes. The electrode was stored in 0.1 M phosphate buffer, pH 7.5 at 4°C. The error bars indicate standard error of the mean.

4.4.2. Performance of Conjugate-GO for amperometric glucose detection between the ranges of 0 mM and 25 mM glucose

This work was done over five successive days, at each day both electrochemical and enzyme activities were measured. There were only three Pt electrodes available for use that were similar in construction. One was used to construct an electrode with GO entrapped in gelatine, and two were used with haeme-GO conjugates.

The aim of this work was to compare:

1. Performance of gelatine and haeme entrapped GO stored at 4°C over 5 successive days.
2. Enzyme assays to measure enzyme activity to confirm amperometric response detected.
3. Linearity of calibration measurements.

The methods for the preparation of Gelatine-GO and Conjugate-GO electrodes were described in section 2.4.1. and 2.4.2. respectively. The electrodes were stored separately in 0.1 M phosphate, pH 7.5 at 4°C.

The electrochemical results for Gelatine-GO electrodes were shown in Figures 4-6a and 4-6b with corresponding data for Conjugate-GO electrodes shown in Figures 4-7a and 4-7b. Gelatine-GO had consistently higher sensor responses than Conjugate-GO (see Table 4-1 and Table 4-2 at the gradients from regression equations). However, there were similar trends shown between Gelatine-GO and Conjugate-GO. In both Gelatine-GO and Conjugate-GO electrodes over the five day period, both had lower sensor response at days 1 and 2. In the case of Gelatine-GO a large increase in sensor response was detected at day 3, followed with a drop in days 4 and 5, with day 5 showing the lowest response. In the case of Conjugate-GO at day 3, the gradient of the sensor response was twice that of day 2's gradient (Table 4-2). The main increase occurred in day 4 rather than day 3 as in Gelatine-GO. The decrease in sensor response at day 5 did not go as low as days 1 and 2 in Gelatine-GO in the case of Conjugate-GO.

The results for Gelatine-GO electrode acted as a control for the results of Conjugate-GO electrodes for performance evaluation over the same time period. The plateau region seemed to have suffered the greatest lost in response output; i.e. the region between 10 mM and 25 mM. At 15 mM glucose, for Gelatine-GO (shown in Figure 4-6a), Day 1 had 3.04 μA and Day 5 had 1.62 μA (53.3 % drop from Day 1 to Day 5). For Conjugate-GO (shown in Figure 4-7a) the opposite seemed to have occurred, the response increased over the same time period as Gelatine-GO, Day 1 had 0.37 μA and Day 5 had 0.86 μA (an increase of 232 % in sensor response between Day 1 and Day 5). The reason for this is not known as yet.

The sensor response increased and decreased as shown in Figure 4-6a and 4-7a with time for both Gelatine-GO and Conjugate-GO respectively, linearity of response (over 0-5 mM) were preserved in both cases (see Table 4-1 and 4-2). However, the linear region occurs between 0 and 5 mM, rather than between 20 mM to 200 mM as seen in Figure 4-4. This will be discussed in section 4.8.

The results shown in Figure 4-2 (Gelatine-GO), sensor response was comparable to those in Figure 4-7a. The gradient for Figure 4-2 is 0.0182 is quite smaller when compared to the gradient obtained in Table 4-2 for Day 1, which is 0.071 (a reduction of

74 %). The corresponding treatment for Gelatine-GO (Table 4-1) has a gradient of 0.360 which is a lot higher in comparison to both gradients of Gelatine GO (Table 4-2 and Figure 4-2).

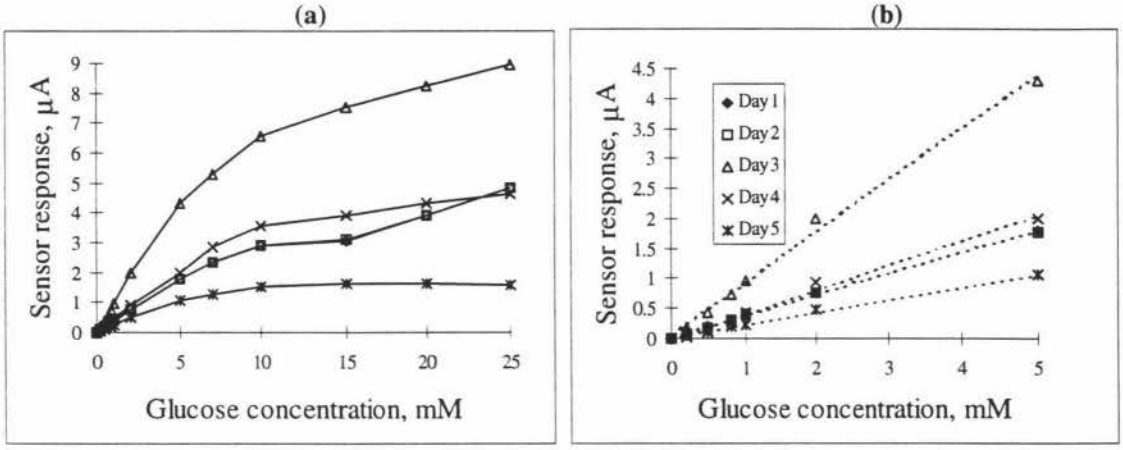

Figure 4–6. (a) calibration curve for Gelatine-GO obtained using FIA up to 25 mM glucose concentration. Potential poised at 600 mV. Each marker denotes different days. On each day, duplicate readings of each electrode were made before being stored in 0.1 M phosphate buffer, pH 7.5 at 4°C. (b) calibration curve showing Gelatine-GO linearity for glucose estimation up to 5 mM glucose concentration.

Table 4-1. Results of Regression Analysis for Gelatine-GO up to 5 mM Glucose concentration

Time (days)	Regression Equation	Regression Coefficient (r^2)
Day 1	y = 0.360x + 0.016	0.9996
Day 2	y = 0.354x + 0.023	0.9984
Day 3	y = 0.867x + 0.053	0.9957
Day 4	y = 0.412x + 0.016	0.9907
Day 5	y = 0.210x + 0.021	0.9948

Electrochemical events were monitored over a five day period. Gelatine-GO electrodes were stored in 0.1 M phosphate buffer, pH 7.5 at 4°C. The potential was poised at 600 mV.

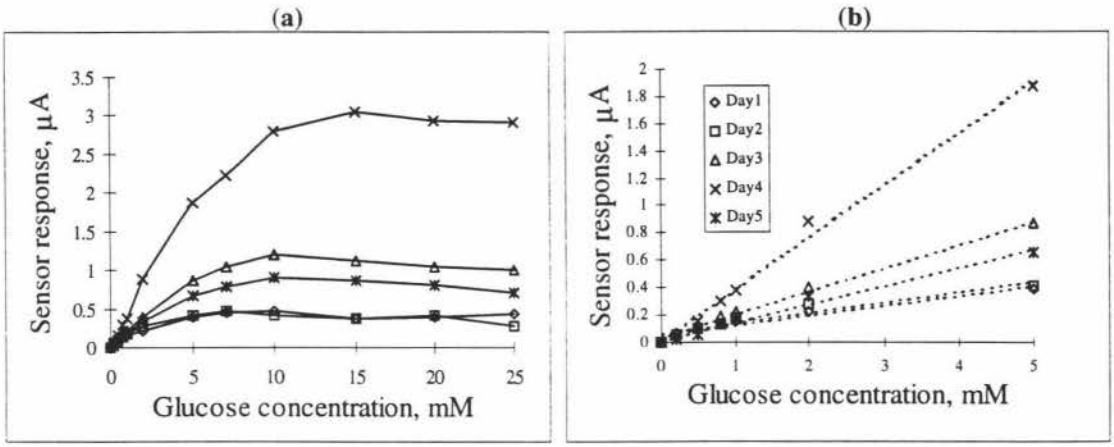

Figure 4–7a. (a) calibration curve for Conjugate-GO obtained using FIA up to 25 mM glucose concentration. Potential poised at 600 mV. The same conditions were used as in Figure 4-6a. Each electrode was read in duplicate over 5 successive days. (b) Calibration curve showing Conjugate-GO linearity for glucose estimation up to 5 mM glucose concentration.

Table 4-2. Results of Regression Analysis for Conjugate-GO up to 5 mM Glucose concentration

Time (Days)	Regression Equation	Regression Coefficient (r^2)
Day 1	y = 0.071x + 0.058	0.9424
Day 2	y = 0.077x + 0.062	0.902
Day 3	y = 0.169x + 0.038	0.9945
Day 4	y = 0.383x + 0.001	0.9938
Day 5	y = 0.134x + 0.014	0.9837

Electrochemical events were monitored over a five day period. Conjugate-GO electrodes were stored in 0.1 M phosphate buffer, pH 7.5 at 4°C. The potential was poised at 600 mV.

4.4.3. Ratio of sensor response to measured Enzyme activity

This section aims to compare enzyme activities of Gelatine-GO and Conjugate-GO electrodes with their respective sensor responses. Due to discrepancies between Gelatine-GO and Conjugate-GO enzyme assay results from these electrodes, an attempt was made to compare sensor responses of both types of electrode, by taking into account enzyme activity. Sensor responses for Gelatine-GO and Conjugate-GO electrodes were measured daily for five consecutive days, the results of which were presented in section 4.4.1. Immediately after sensor responses of these electrodes were measured, the activity of GO was measured using an enzyme assay for GO. The method used to detect GO activity on electrodes was described in section 2.6.1.

The comparison between Gelatine-GO and Conjugate-GO electrodes can be made more directly if enzyme activity was taken into account along with sensor response. This gives a result that compensates the low GO activity of Conjugate-GO electrodes

compared to Gelatine-GO electrodes. This method clearly shows that although the Gelatine-GO electrode had higher current responses that may well be due to a greater amount of GO immobilised. The evidence for this is shown in Figure 4-8a and Figure 4-8b. Using this type of analysis Conjugate-GO showed better performance compared to Gelatine-GO for the amount of enzyme present.

The results for Figure 4-8a and Figure 4-8b were obtained in the following manner. Using the data shown in Table 4-3 (which pertain to Figure 4-8b), sensor response for each day was divided by the corresponding enzyme activity for that day. The same calculations were performed on all the data and the results are presented in Figure 4-8b for each day at 10 mM of glucose. The results shown in Figure 4-8a were calculated using the readings at 5 mM glucose (data not presented). This was to show that the phenomena of Conjugate-GO electrodes performing better than Gelatine-GO electrodes did occur at different levels of glucose concentration. The difference between Gelatine-GO and Conjugate-GO electrodes was less in day 1 and day 2 compared to day 3, day 4 and day 5. The amount of difference expected between Gelatine-GO and Conjugate-GO was more like that seen on day 1 and day 2. It was not expected to see large differences (day 3, day 4 and day 5) between Gelatine-GO and Conjugate-GO. The reason for the large difference is that low current was detected from Conjugate-GO (day 1 and day 2) in the presence of high enzyme activities, and high currents were detected as the enzyme activity dropped. This unexpected trend was seen, but to a lesser extent, with the Gelatine-GO electrode.

Table 4-3. Comparison of Enzyme activity and Sensor response (10 mM glucose) for Gelatine-GO and Conjugate-GO for five consecutive days

	Gelatine-GO		Conjugate-GO 1		Conjugate-GO 2	
Time (days)	Enzyme activity (e.u.)	Sensor response (μA) 10 mM Glucose	Enzyme activity (e.u.)	Sensor response (μA) 10 mM Glucose	Enzyme activity (e.u.)	Sensor response (μA) 10 mM Glucose
Day 1	0.0660	2.915	0.0110	0.39	0.0090	0.535
Day 2	0.0850	2.915	0.0068	0.32	0.0057	0.520
Day 3	0.0730	6.565	0.0065	1.165	0.0060	0.900
Day 4	0.0530	3.560	0.0095	3.075	0.0043	1.380
Day 5	0.0460	1.510	0.0080	0.85	0.0035	0.970

Sensor response was measured as previously described. Potential was poised at 600 mV. Phosphate buffer 0.1 M, pH 7.5 was used. Enzyme assay for GO was used as an estimation of GO activity on the electrodes (section 2.6.1). Enzyme units denoted by e.u. - 1 unit of enzyme activity will oxidise 1.0 μmol of b-D-glucose to D-gluconic acid and H_2O_2 per min at pH 5.1 at 35°C.

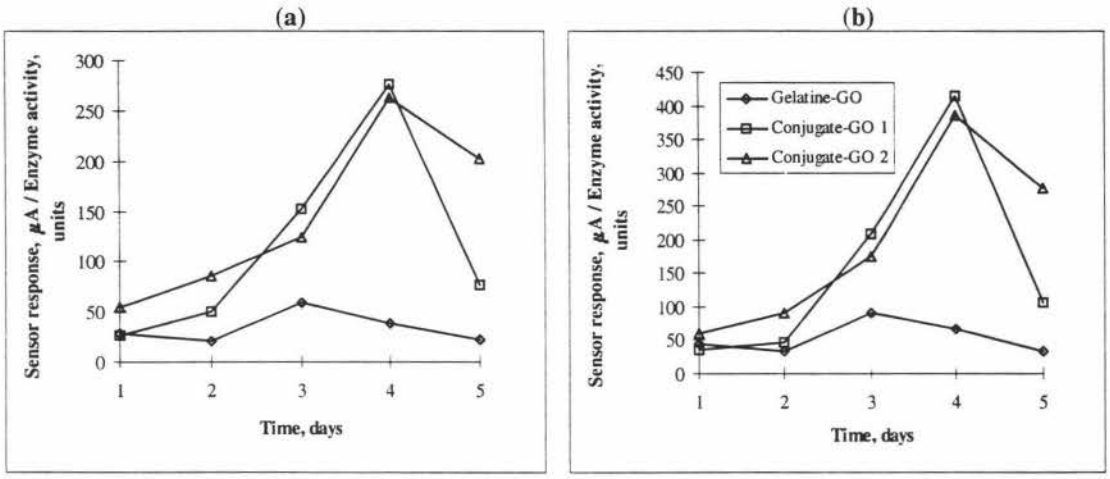

Figure 4–8. (a) Sensor response curves for Gelatine-GO and Conjugate-GO corrected for enzyme activity at 5 mM glucose. (b) Sensor response curves for Gelatine-GO and Conjugate-GO corrected for enzyme activity at 10 mM glucose. Enzyme activity was measured on the same day as when the electrochemical data was taken.

Although, the activity of GO detected on Conjugate-GO electrodes was low in comparison to Gelatine-GO, it is still possible to use Conjugate-GO electrodes in estimation of glucose concentration. This highlights the point about the efficiency of Conjugate-GO electrodes for responsiveness compared to a modified matrix (Gelatine). Gelatine entraps GO in a matrix, which was envisaged to slow down the process where electron transfer could be made between the enzyme and Pt disk; i.e. H_2O_2 is oxidised on the surface of Pt and an electron is generated. This gives Conjugate-GO electrodes an advantage compared to Gelatine-GO electrodes, as there is no matrix through which H_2O_2 has to pass. This result reflects those presented in Figure 4-4, where the return to a baseline was slower for Gelatine-GO than it was for Conjugate-GO electrodes.

4.5. The Effect of Long Term Storage on Viability of Conjugate-GO

The Figures 4-7a and 4-7b indicated decreases in signal response of Conjugate-GO over five days. Further studies were carried out to investigate an alternative storage method to preserve enzyme activity of Conjugate-GO once it had adsorbed onto the surface of the Pt electrode.

This experiment used five Pt electrodes onto which freshly made Conjugate-GO was absorbed. The method for preparation of Conjugate-GO electrodes was described in section 2.4.2. Preceding each amperometric analysis of Conjugate-GO electrodes following the schedule set out in Table 4-4, each electrode was rinsed under R.O. water

for two minutes. The electrodes were then placed in 0.1 M phosphate buffer, pH 7.5 at 4°C when the electrodes were not in use. The electrodes were then rinsed under R.O. water and placed into individually marked test tubes filled with 3 mls of 0.1 M phosphate buffer, pH 7.5 and were then placed in the freezer until the next testing period. During the subsequent testing of these electrodes, the electrodes were taken out of the freezer and thawed using a water bath set at 30°C. Once thawed the electrodes were again stored at 4°C while each individual electrode was tested for response to glucose.

Table 4-4. Protocol for Long Term Stability Study

Time	Electrode 1	Electrode 2	Electrode 3	Electrode 4	Electrode 5
Day 1	Tested	Tested	Tested	Tested	Tested
Day 4	Tested	Tested	Tested	-	-
Day 7	Tested	Tested	Tested	-	-
Month 16	Tested	Tested	Tested	Tested	Tested
Month 20	-	-	-	-	Tested

The electrodes were frozen in 0.1 M phosphate, pH 7.5. The dashes denote no testing of electrodes.

The work presented in Figure 4-9 is an accumulation of the data obtained by the use of five Conjugate-GO electrodes tested periodically over time (Table 4-4). Figure 4-9 showed high electrochemical activity on day 1 followed by a decline on subsequent testing. The decline in sensor response was expected after day 1, as GO enzyme activity was expected to decreased over time. The drop of sensor response was 70 % from day 1 to day 4.

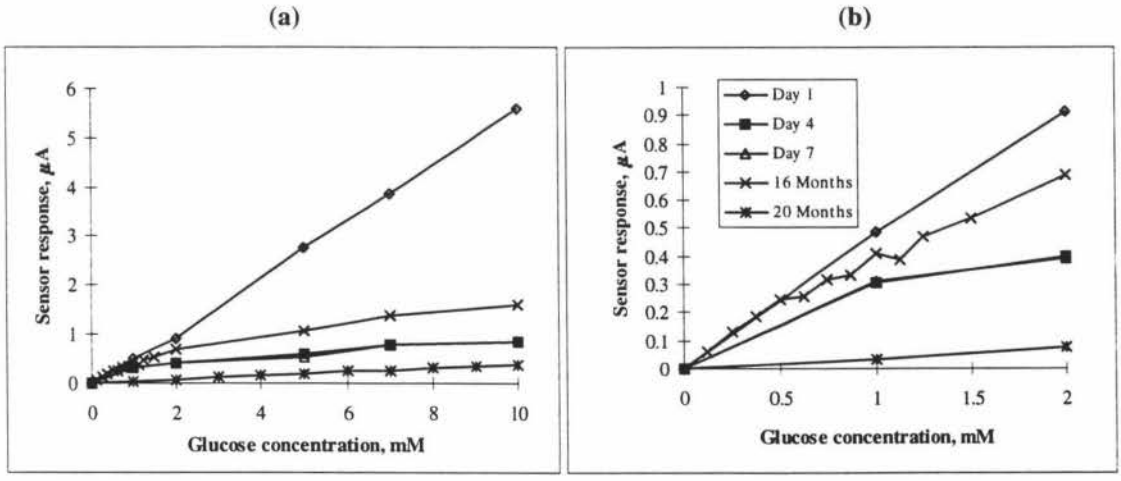

Figure 4–9. Response of Conjugate-GO following storage in freezer. Electrodes stored in 0.1 M phosphate buffer, pH 7.5. Refer to Table 4-4 for the number of electrodes tested per each day shown. (a) showed Sensor response between 0-10 mM. (b) showed sensor response between 0-2 mM.

Table 4-5. Regression Analysis for Viability of Conjugate-GO Electrodes After Long Term Storage

Time	Regression Equation	Regression Coefficient (r^2)
Day 1	y = 0.567x - 0.090	0.9989
Day 4	y = 0.078x + 0.163	0.8930
Day 7	y = 0.077x + 0.160	0.8955
Month 16	y = 0.160x + 0.182	0.9467
Month 20	y = 0.038x + 0.003	0.9988

Response of Conjugate-GO electrodes between 0-10 mM glucose stored 0.1 M phosphate, pH 7.5 in a freezer.

The results of Table 4-6 show that even Gelatine-GO electrodes were also prone to the similar characteristics of an increase in sensor response on the 3rd set of readings followed by a decreased on the 4th and 5th readings. The timing of the increase was not quite the same between Gelatine-GO and Conjugate-GO (stored at 4°C and frozen), but the characteristic phenomenon was still present. The results presented in Table 4-6 clearly shows which method of storage gave the least amount of sensor response drop. Conjugate-GO electrodes which were stored frozen in 0.1 M phosphate buffer, pH 7.5 showed the most drop compared to Gelatine-GO and Conjugate-GO electrodes stored at 4°C (at 10 mM glucose; Gelatine-GO and Conjugate-GO dropped between 0 % and 9 % relative to the 1st reading compared to a drop of 85 % for Conjugate-GO stored in a freezer). Note however that the frozen electrode was stored for months rather than days.

Table 4-6. Comparison of Electrodes between Two different Storage Conditions (4°C and frozen) showing percentage sensor response relative to Day 1 for 5 mM and 10 mM glucose concentration

	Glucose Concentration (mM)	1st reading	2nd reading	3rd reading	4th reading	5th reading
		Day 1	Day 2	Day 3	Day 4	Day 5
Gelatine-GO (stored at 4°C)	5 mM	100 %	98 %	239 %	111 %	58 %
	10 mM	100 %	100 %	225 %	122 %	51 %
Conjugate-GO (stored at 4°C)	5 mM	100 %	105 %	221 %	478 %	168 %
	10 mM	100 %	91 %	261 %	605 %	197 %
		Day 1	Day 4	Day 7	Month 16	Month 20
Conjugate-GO (stored frozen)	5 mM	100 %	21 %	20 %	39 %	7 %
	10 mM	100 %	15 %	15 %	29 %	7 %

Comparison between electrodes stored at 4°C and Frozen conditions. The values from 2nd to 5th readings were all relative to the sensor response of the 1st reading.

4.6. Confounding Problems associated with Conjugate-GO Electrodes

The results for Conjugate-GO presented to this stage showed some promise. However, there were obvious problems associated with variability in the results and degree of linearity of response. One problem was the change in linearity of response from up to 200 mM glucose shown in Figure 4-3 to only 5-10 mM glucose shown in Figure 4-7a. This could be due to different enzyme batches or perhaps the presence of a high K_m and a low K_m enzyme of GO (see section 4.8). One further problem recognised as work progressed was the lack of reproducibility in making successful conjugates. This section covers the work carried out in an attempt to resolve problems associated with making reliable Conjugate-GO electrodes.

4.6.1. Is the Signal Response due to Enzyme Absorption directly onto Pt or Is it due to the Response from Conjugate-GO linked absorbed onto the Pt surface?

In the experiments presented so far, it was assumed that the response from Conjugate-GO electrodes was from GO conjugated to Haeme. There was however another possibility. Some or all of the signal response from electrochemical experiments could be due to GO that was directly absorbed to the Pt surface. It was possible that GO could be absorbed in crevices on the surface of the Pt electrode. The electrochemical or enzymic assays carried out to this stage would not distinguish between amperometric signal responses from GO directly absorbed onto the Pt surface and Conjugate-GO binding to the surface. In order to test this possibility, the following study was carried out.

Nine Pt electrodes were polished and electrocleaned (see section 2.2.1. for preparation of Bare-Pt electrodes) prior to being dipped in a solution of GO, except for one electrode which was kept in reserve to serve as a blank for the duration of the experiment. The other eight electrodes were separated in two groups.

One group of four electrodes was dipped into 2 mls of 25 mg/ml of GO in 0.1 M phosphate buffer, pH 7.5 for 20 hrs (2 mls of 25 mg/ml GO was used as it was the same

concentration as when Conjugate-GO was prepared, see section 2.4.2.), followed by a two minute wash under running R.O. water. The electrodes were then stored in 0.1 M phosphate buffer, pH 7.5 at 4°C until used, with daily changes of buffer. This was to simulate the procedure of dipping Conjugate-GO electrodes into 2 mls of Conjugate-GO solution. The other group of four electrodes were dipped in 2 mls of 25 mg/ml of GO in 0.1 M phosphate buffer, pH 7.5, followed by a two minute wash under running R.O. water. The electrodes were then stored dry in a beaker at 4°C until used. The reason for this was to look at GO enzyme stability in a non-aqueous environment as a possibility for Conjugate-GO electrodes being stored in this way.

The other conditions such as buffer, standards and equipment were the same as those for Conjugate-GO and Gelatine-GO (results described in Table 4-3), the only difference being Conjugate-GO and Gelatine-GO electrodes were monitored enzymically and for sensor response daily for five days.

The results are shown in Table 4-7. Bare-Pt and GO-Pt both showed small amperometric response when compared with Conjugate-GO and Gelatine-GO. The results suggest on average sensor responses of Bare-Pt and GO-Pt result in negative currents. Sensor responses of these electrodes (GO-Pt), can be attributed to current drift as seen in the sensor responses for Bare-Pt, on average. The results for both enzymic and sensor responses of Bare-Pt were expected to be null results. Sensor response of Conjugate-GO and Gelatine-GO both exhibit positive currents. There was some residual activity detected on the electrodes of GO-Pt. The amount is very much lower when compared to Conjugate-GO, and lower still when compared to Gelatine-GO. It seems that non-conjugated GO was absorbed to a small extent relative to Conjugate-GO and Gelatine-GO.

Table 4-7. Comparison of Activity and Sensor response of Absorbed Glucose Oxidase and Bound Glucose oxidase after Five days at 4°C. Values given are mean and range.

Electrode Condition	Number of electrodes used	Storage Conditions (4°C)			
		In 0.1 M phosphate buffer, pH 7.5		In Air	
		Enzyme activity (e.u. x 10^{-3}) range (min-max)	**Sensor response (nA) at 20 mM glucose** range (min-max)	**Enzyme activity (e.u. x 10^{-3})** range (min-max)	**Sensor response (nA) at 20 mM glucose** range (min-max
Bare-Pt	1	**-0.54** (-0.7 to -0.43)	**-11.5** (-39.5 to 16.5)	-	-
GO-Pt	4	**0.158** (0 to 0.354)	**-11.5** (-105 to 70)	**0.084** (-0.62 to -1.09)	**-5.75** (-8 to -3.5)
Conjugate-GO	2	**5.75** (3.5 to 8.0)	**815** (790 to 840)	-	-
Gelatine-GO	1	**46** -	**1640** -	-	-

Buffer used was 0.1 M phosphate, pH 7.5, with daily changes of buffer stored in 4°C.

4.6.2. Analysis of the Results from Spectrophotometric Analysis of Conjugate

This section deals with the comparison of the results obtained through UV-Vis analysis of glucose oxidase, haeme only and Conjugate-GO. The concern was of making a false positive identification of enzyme-haeme conjugates, especially in the case of Conjugate-GO. Unconjugated glucose oxidase in comparison with alcohol dehydrogenase and lactate dehydrogenase may still give an amperometric response. The cofactor FAD^+ is buried within the framework of glucose oxidase. This is not the case for the dehydrogenses. The dehydrogenases require the cofactor NAD^+, but NAD^+ is a soluble cofactor and not trapped within the framework of the dehydrogenases. Therefore, the presence of FAD^+ and oxygen (within the carrier stream and the samples) make it possible for unconjugated glucose oxidase to be reduced and reoxidised as shown in Equation 1 and Equation 2 in section 4.1 but not so in the case for the dehydrogenases as the cofactor NAD^+ is not present (see Chapter 5 and Chapter 6). This reaction can be detected amperometrically. Therefore, there is more risk in associating amperometric response of Conjugate-GO to the actual presence of Conjugate-GO as opposed to the presence of absorbed glucose oxidase on the platinum electrodes compared to the dehydrogenases.

A spectrophotometer was used to evaluate spectrophotometrically (250 nm to 700 nm) the fractions collected after purification through G-25 Sephadex column. The

spectrophotometric method was used to determine the fractions containing Conjugate-GO. It was possible on the UV-Vis spectrophotometer used to automatically pick the presence of peaks and obtain absorbance values of those peaks. The presence of a peak at 280 nm indicates the presence of protein. The presence of a peak at 380 nm was used as a convenient indicator for the presence of haeme (Figure 6-10b). The Sigma catalogue (1993) lists the impurities present in glucose oxidase obtained from Sigma. It listed the presence of cytochromes as one of the impurities. Cytochromes contain haeme. Therefore a UV-Vis scan of glucose oxidase only would show the presence of haeme through the presence of a peak at 380 nm. A comparison of absorbance per mole ratio of glucose oxidase only, haeme only and Conjugate-GO, when dilution factors were taken into account, can indicate the presence of Conjugate-GO.

The results from UV-Vis scan of glucose oxidase only, haeme only and Conjugate-GO are shown in Figure 6-10a, Figure 6-10b and Figure 6-10c, respectively. The results of a UV-Vis spectrum of haeme only shown in Figure 6-10b indicate a maximal peak at 380 nm. The peak of 380 nm was used as an indicator as to the presence of haeme.

The absorbance of glucose oxidase only at 380 nm was 3 absorbance units (undiluted). The concentration of glucose oxidase used was 20 mg/ml. The molecular weight of glucose oxidase is 186 kD. Therefore, the absorbance per mole ratio for glucose oxidase is 27,900 absorbance/mole.

The absorbance of Conjugate-GO at 20 mg/ml concentration was 11.6 units (undiluted) at 380 nm. The molecular weight of Conjugate-GO is 186,452 D.

Therefore, the absorbance per mole ratio for Conjugate-GO for this preparation (Figure 6-10c) is 108,142 absorbance/mole. This was a successful conjugation as this preparation was carried through to electrochemical measurement of Conjugate-GO at the electrode stage. The results can be seen in Figure 4-7a and Figure 4-7b. Another example of a successful Conjugate-GO electrode preparation had UV-Vis absorbance of 7.5 units (undiluted) at 380 nm. Therefore, the absorbance per mole ratio is 69,602 absorbance/mole. When this preparation was measured electrochemically at the electrode stage, it gave a reading of between 0-472 nA for a glucose range of 0-10 mM ($y = 46.99x - 1.14$, $r^2 = 0.9987$).

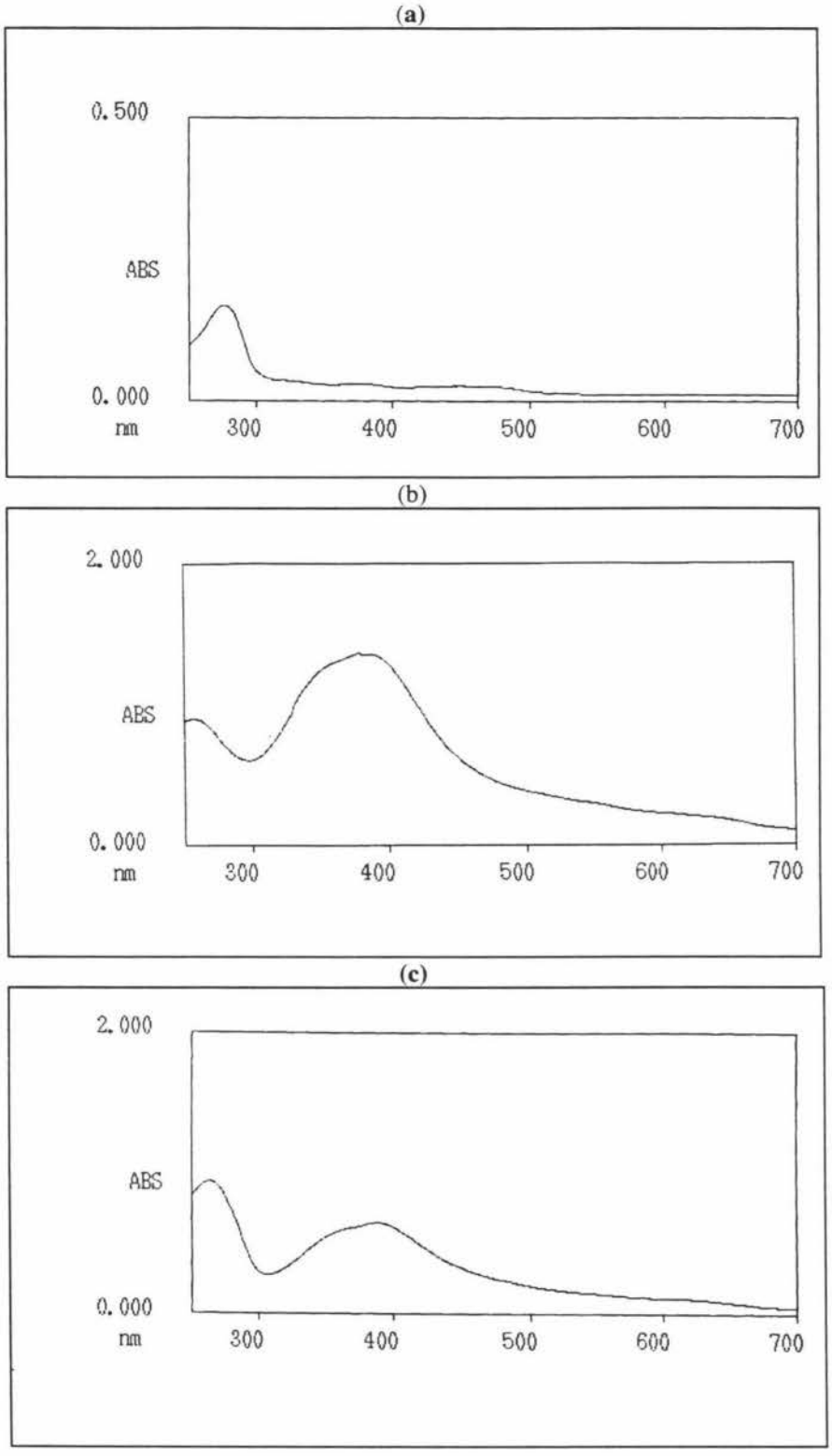

Figure 4–10. The comparison between the UV-Vis spectras of glucose oxidase, haeme only, and Conjugate-GO. (a) glucose oxidase only, (b) haeme only, (c) one of the fractions collected after purification through Sephadex G-25 column suspected of containing Conjugate-GO.

In an example of an unsuccessful conjugation, the UV-Vis absorbance of the preparation after G-25 Sephadex column was 1.48 units (undiluted) at 380 nm. Therefore, the

absorbance per mole ratio was 13,866 absorbance/mole. This value was actually lower than the absorbance per mole value for glucose oxidase only, 27,900 absorbance/mole. This demonstrates that some preparations that were supposed to be Conjugate-GO did not contain amounts of haeme greater than that of haeme present in glucose oxidase only. This indicates a problem with the conjugation processes.

4.6.3. Making Haeme Active Esters

This part of the work aims to look at the formation of haeme active ester prior to being combined with glucose oxidase, as there had been a number of problems that arose during the course of making electrochemical measurements using Conjugate-GO electrodes. One of the main problems was low sensor response during electrochemical studies associated with non-linearity in the calibration curve using glucose standards. This was taken to mean a successful conjugation between haeme and glucose oxidase did not occur. There were 13 out 21 occasions that were concluded to contain successful conjugates. YingQu (personal communication, 1994) suggested that the products after the activation process can be monitored using Thin Layer Chromatography (TLC) plates. TLC plates have been used to identify various compounds, such as various steroids, lipids or carbohydrates (Touchstone and Murawec, 1973). The method for running activated haeme on TLC plates was kindly provided by YingQu (personal communication, 1994). Figure 4-11 shows the results of the comparison between unreacted haeme and active esters of haeme. The TLC plates were ran in 1:1 mixture of chloroform and methanol.

Figure 4-11 clearly shows the difference between unreacted haeme and activated esters of haeme. There was a drift shown in the TLC plate with the unreacted haeme (Figure 4-11a), but this is most likely to be due to the presence of DMF (YingQu, personal communication, 1994). Figure 4-11b shows the presence of a mono-ester and di-ester spot (YingQu, personal communication, 1994). This confirms that haeme active esters were formed.

The rest of the haeme active esters were added into a solution of GO as described previously (section 2.4.2.), for use in the experiment described in section 4.6.3.

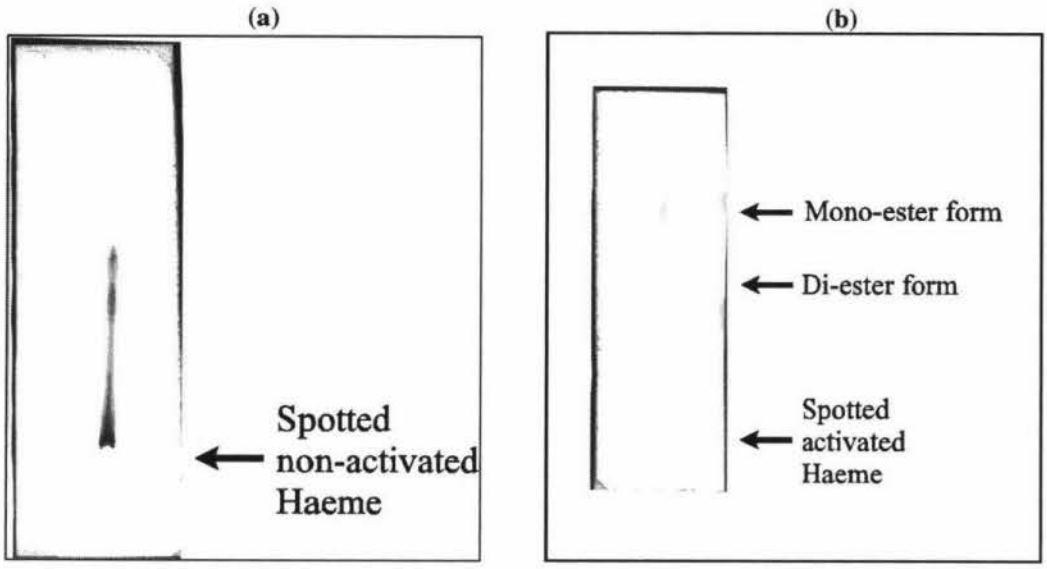

Figure 4–11. (a) Diagram showing non-activated haeme. (b) The presence of mono-ester and diester forms of haeme indicate haeme had been activated without residual non-activated haeme left at the position where it was spotted on the TLC plate. The amounts spotted on the TLC plates were not calibrated. A capillary tube was used to spot the samples onto theTLC plates.

4.6.4. Monitoring the Progress of Conjugate-GO after Purification through Sephadex G-25.

The pH of 0.1 M phosphate buffer, pH 7.5 (initial) in the presence of 50 mg of GO in solution can change with the addition of haeme active ester. This may affect the amount of Conjugate-GO formed as a consequence, especially if the pH drops more than a unit. The pH of the solution could be monitored during and after the additions of 10 μL quantities of active esters had occurred using a pH meter.

The presence of haeme active esters were confirmed using TLC plates as described in section 4.6.2. The rest of the procedure followed the method as described in section 2.4.2.

The Conjugate-GO mixture was left stirring at 4°C. Samples were taken at 3 hrs, 6 hrs and 20 hrs after initial addition of haeme active esters. The possibility of haeme forming haeme aggregates large enough to pass through a G-25 Sephadex column in the void volume with unbound enzyme or conjugated enzyme was considered, as suggested by Luo (personal communication, 1994). Samples of 200 μL were taken at appropriate times and processed immediately through a G-25 Sephadex column. The resulting 2 mls

fractions were collected and assayed for activity and UV-Vis spectrophotometric absorption.

Table 4-8. Monitoring pH of GO solution during additions of Haeme Active Ester

Time (min)	pH
0	7.471
10	7.425
20	7.391
30	7.363
60	7.282
120	7.286
180	7.271
240	7.260
300	7.254
360	7.262

The buffer used was 0.1 M phosphate, pH 7.5. Administration of 50 μL active ester were made in 10 μL aliquots every five minutes. This process was monitored at 4°C using a pH meter calibrated for 4°C. Samples of 200 μL were taken at 3 hrs, 6 hrs and 20 hrs.

Table 4-9 shows the results from a UV-Vis scan of the fractions containing the highest enzyme activity, detected using enzyme assays (section 2.6.1.). The fractions were scanned on the criteria of containing a main peak of 275-280 nm for enzyme and 380-390 nm for haeme. The UV-Vis spectra showed an increased amount of absorption detected in the region where haeme was expected to absorbed. This was an indication that either the conjugation process had gone as planned or aggregated haeme had formed and was passing through the column with Conjugate-GO and GO.

A suggestion was made to further purify the sample by using a Superose-12 column (Luo, personal communication, 1994). Superose-12 is a highly cross-linked agarose structure. It is generally used in conjunction with FPLC systems for purification, separation and molecular weight determination of proteins and peptides. It was hoped that Superose-12 had the ability to separate various sizes of aggregated haeme, enough so to produce a relatively pure sample of Conjugate-GO and GO.

Table 4-10, shows UV-Vis analysis of the fraction containing the highest enzyme activity after purification through a Superose-12 column. The ratio of other peaks (ie 275 nm, 450 nm and 583 nm) to peak 380 nm shows fairly similar ratios in all three cases of 3 hrs, 6 hrs and 20 hrs conjugation times (Table 4-9). This is possibly due to the Superose-12 column being able to separate aggregated haeme from Conjugate-GO, or

that no haeme aggregates formed in the first place. The final step after purification was to dip the recently cleaned Pt electrodes for GO sensor response evaluation.

The results from Table 4-9 were graphed and shown in Figure 4-12a, the corresponding graph after purification through Superose-12 column was shown in Figure 4-12b. The implications of these results will be discussed in section 4.8.

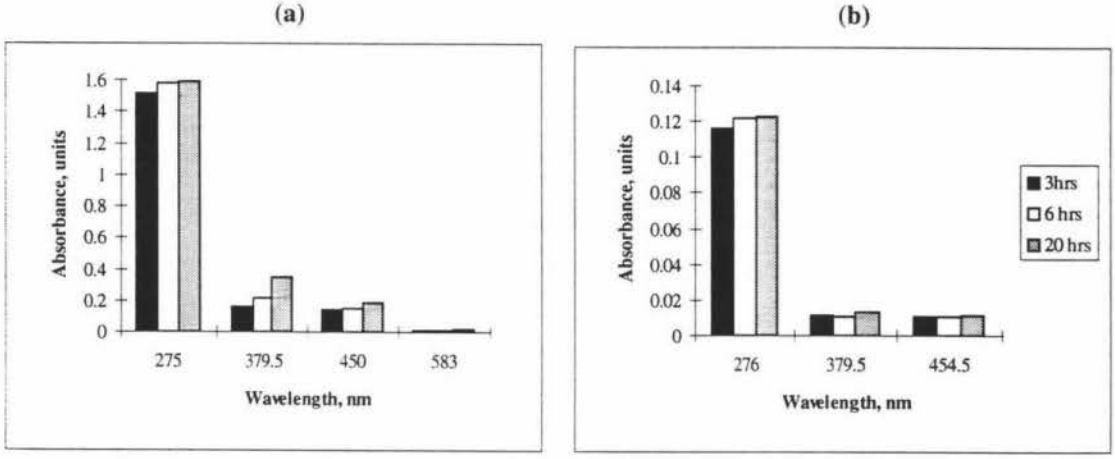

Figure 4–12. Major peaks detected from spectrophotometric scanned of 3 hrs, 6 hrs, and 20 hrs fractions containing highest enzyme activity. (a) shows UV-Vis scan fractions after purification using G-25 Sephadex column. (b) shows UV-Vis scan fractions after purification using Superose-12 column

The ratio absorbance between peak 280 nm (0.168 absorbance units) to 380 nm (0.030 absorbance units) for free glucose oxidase is 5.6. The ratio absorbance at 380 nm after 20 hrs conjugation time is 4.54 (Table 4-9). This is indicative that after 20 hrs more enzyme-haeme conjugates were formed compared to 3 hrs conjugation time (ratio 9.45, Table 4-9), as the absorbance ratio decreases between enzyme and haeme peaks more enzyme-conjugates were formed. The ratio to peak 380 nm goes up to around 9.5 for all the three time categories (3 hrs, 6 hrs and 20 hrs). It's now higher than the ratio given for free glucose oxidase (5.6 units) so conjugation had been obtained. The absorbance ratio for Table 4-9 for 3 hrs conjugation is also around the 9.5 units. Therefore, at three hours the amount of enzyme-haeme conjugates formed was more compared to after 20 hrs conjugation time.

Table 4-9. UV-Vis analysis of fraction containing the highest GO enzyme activity after 3 hrs, 6 hrs and 20 hrs of conjugation times after purification through Sephadex G-25 column

Time	Wavelength (nm)	Absorbance (units)	Ratio to peak 380 nm
3 hrs	583	0.006	0.038
	450	0.140	0.875
	379.5	0.160	1.0
	275	1.512	9.45
6 hrs	583.5	0.005	0.023
	452	0.150	0.701
	379.5	0.214	1.0
	276	1.580	7.38
20 hrs	583	0.021	0.060
	449.5	0.187	0.533
	379.5	0.351	1
	275	1.594	4.54

The results shown are the peaks detected after UV-Vis analysis of the fraction. Fractions of 2 mls were collected after purification through G-25 Sephadex column.

Table 4-10. UV-Vis analysis of fraction containing the highest GO enzyme activity after 3 hrs, 6 hrs and 20 hrs conjugation times after purification through Superose-12 column

Time	Wavelength (nm)	Absorbance (units)	Ratio to peak 380 nm
3 hrs	456.5	0.011	0.917
	379.5	0.012	1.0
	276.5	0.116	9.67
6 hrs	454.5	0.011	1.05
	379.5	0.011	1.0
	276.5	0.122	11.6
20 hrs	452	0.012	0.923
	379.5	0.013	1.0
	276.5	0.123	9.46

The results shown are the peaks detected after UV-Vis analysis of the fraction. Fractions of 2 mls were collected after purification through Superose-12 column.

The sensor responses of these electrodes were evaluated with the same procedure as those previously described. Figure 4-13a shows the results of the responses of these electrodes. It compares the different conjugation times, 3 hrs, 6 hrs and 20 hrs. The results appear to be quite promising. The data in Figure 4-13a are quite comparable to Figure 4-4 and Figure 4-7a. In Figure 4-4, Day 1 data were higher compared to data in Figure 4-12a. This difference could be explained due to the loss of activity of the enzyme over time. To produce data for Figure 4-13a, there were two extra days used for purification through Superose-12 column after purification using the G-25 Sephadex column compared to data for Figure 4-5, where only the G-25 Sephadex column was used. Day 2 and Day 5 data for Figure 4-5 may in fact be closer to the data obtained for

Figure 4-13a, at 20 mM (which was the lowest concentration used in Figure 4-5) for Day 5 was less than 0.5 μA in Figure 4-5. There was a similar occurrence in Figure 4-7a, at Day 3 sensor response was around 1.1 μA for 10 mM. The variability of sensor response could be due to the difference in preparation, the method may not vary but the enzyme used was from a different batch. Conjugate formation was maximal at 3 hours. Later increases in 380 nm absorbance were due to extra haeme aggregates forming not through the formation of glucose oxidase-haeme conjugates. The ratio to peak 380 nm for Table 4-9 after 3 hrs conjugation time reflects the results seen in Figure 4-13a, the 3 hrs conjugation time had the highest electrochemical sensor response compared to 6 hrs and 20 hrs.

Figure 4-13b shows the results of buffer injections in case, the results of what had been presented so far were due to additions of solutions rather than to a specific addition of enzyme substrate. Although one of the results shows a positive response to additions of buffer, the current detected were not significant compared to the sensor response obtained from Conjugate-GO electrodes with glucose.

The Conjugate-GO electrodes were used in a study into the behaviour of Conjugate-GO electrodes using cyclic voltammograms (CV) as the method of analysis.

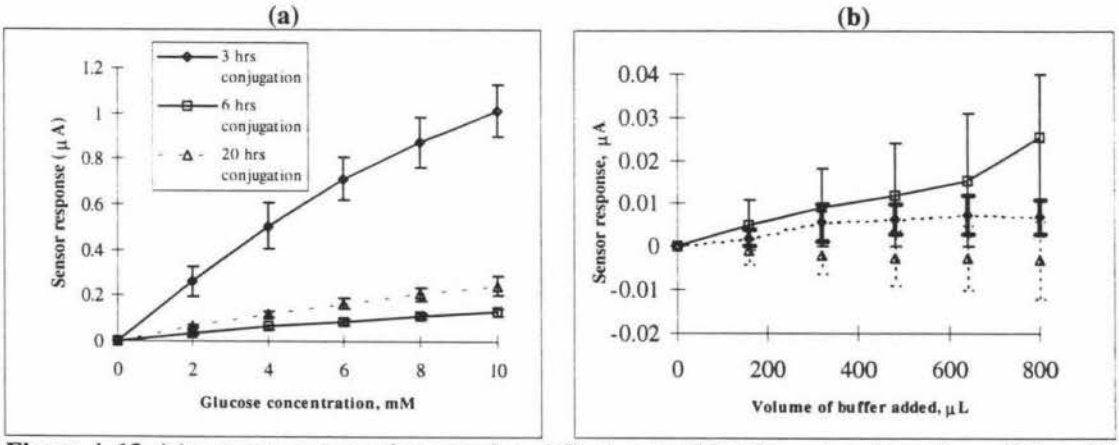

Figure 4–13. (a) sensor response for samples of Conjugate-GO taken at various times (3 hrs, 6 hrs and 20 hrs). (b) Standard addition of 160 μL buffer to act as blanks for Conjugate-GO electrodes. There was only one electrode per treatment, but two replicates were present in each condition.

4.7. Analysis of Cyclic Voltammograms for distinguishing between Conjugate-GO and GO-Pt.

As previously discussed, there was a problem in determining whether the sensor responses of Conjugate-GO electrodes were due to Conjugate-GO or to absorbed GO to the platinum surface. This section looks at this issue using cyclic voltammograms.

4.7.1. What is Cyclic Voltammetry?

Cyclic voltammetry (CV) is a potential-controlled "reversal" electrochemical experiment. A cyclic potential sweep of voltage is imposed on an electrode and the current response is observed. Analysis of the current response can give information about the thermodynamics and kinetics of electron transfer at the electrode-solution interface, as well kinetics and mechanisms of solution chemical reactions initiated by the heterogeneous electron transfer (Gosser, 1993, p. 27).

A potentiostat was used to control the type of potential cycle sweep. The type of cycle used depends on the information required. In this case it was important to gather information on the events that occur on the surface of the Pt working electrode. The signal from the working electrode can be amplified either with an analog or a digital instrument. The resulting response was plotted as current versus applied potential. Figure 4-14 shows the setup of a typical CV experiment.

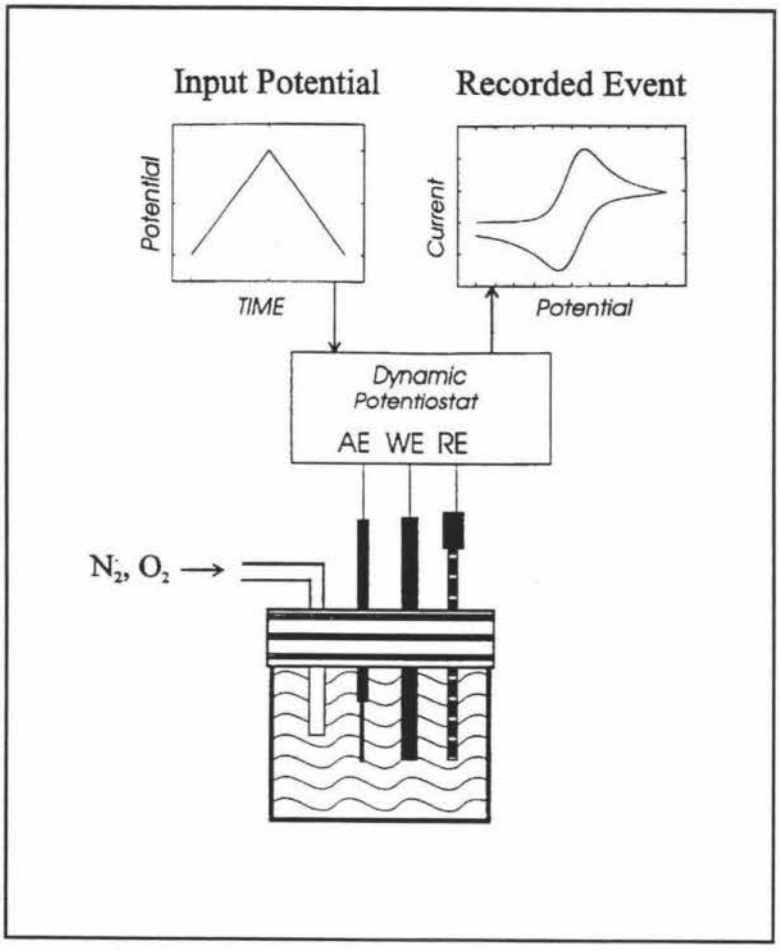

Figure 4–14. Overall view of CV experiment. AE denotes auxiliary electrode, WE denotes working electrode, and RE denotes reference electrode (diagram adapted from Gosser, 1993). Oxygen and nitrogen, both had been used to collect CV results.

4.7.2. Why were Cyclic Voltammograms used?

The study by Zhang et al. (1992) examined the cyclic voltammograms of haeme and artemisinin. Currently, artemisinin is used for the treatment of malaria, the combination of artemisinin and haeme (from malarial parasites) generates free radicals that interfere with the functions of malarial parasites. Artemisinin was found to have a reduction peak (forward sweep) between -873 mV and -1054 mV. An oxidation peak was not found with artemisinin (reverse sweep). Zhang et al. (1992) found haeme having reduction and oxidation peaks at approximately the same potential (reduction peak potential between -380 mV and -390 mV). The combination of artemisinin and haeme resulted in the disappearance of peak -1054 mV and appearance of a new reduction peak between -300 mV and -400 mV. The results suggest with the incorporation of haeme an otherwise

irreversible reaction (artemisinin) could be made reversible (artemisinin and haeme). The study by Large (1993) looked at CV differences between Bare-Pt, Haeme-Pt and Conjugate-Pt. The results showed differences in the shape of CV's between Bare-Pt and Haeme-Pt in buffer. Large (1993) also showed CV differences between Conjugate-GO in buffer and in 100 mM glucose. However, Large (1993) did not show a CV of GO-Pt in 100 mM glucose. Previously, in the study of Large (1993) the method used for studying the behaviour of Conjugate-GO did not differentiate between the response of Conjugate-GO and absorbed GO. This section deals with the comparison between cyclic voltammograms of Bare-Pt, Haeme-Pt, GO-Pt and Conjugate-GO (refers to conjugates on Pt electrode).

4.7.3. How Cyclic Votammogram data are Collected and Assessed

Currently, the potential used for oxidising H_2O_2 on the Pt surface for Conjugate-GO was 600 mV. There had been a range of potentials used for this purpose ranging from 300 mV to 600 mV. The lower the potential used, the lower the amount of interference from other substances that could be oxidised on the electrode surface; i.e. uric acid, ascorbic acid and some other substance that could interfere with the desired metabolite (H_2O_2). The diagram Figure 4-15 shows which points were collected for assessment.

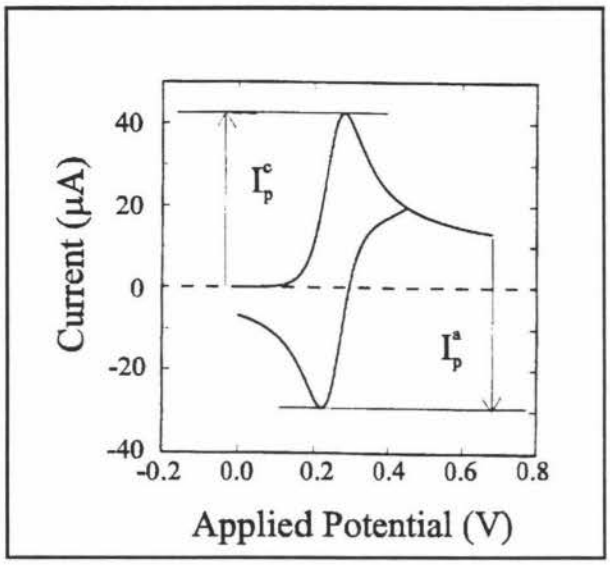

Figure 4–15. An idealised Cyclic voltammogram showing the position on the curve where Ipa and Ipc values are taken for further analysis.

The points on the CV's that were deemed to be important for assessing the different electrode conditions of Bare-Pt, GO-Pt, Haeme-Pt and Conjugate-Pt were indicated as Ipc and Ipa values (Figure 4-15). The preparation of Bare-Pt, GO-Pt, Haeme-Pt and Conjugate-Pt electrodes were described in section 2.2.1., 2.2.2., 2.2.3., and 2.4.2.

4.7.4. Comparing Results of Cyclic Voltammograms between different Conditions of Electrode Absorption

Currently, the use of Conjugate-GO for amperometric detection of glucose, despite its success (as shown previously) is subject to arguments that the response detected amperometrically maybe due to GO directly absorbed on the Pt electrode surface. The purification through Sephadex G-25 or through Superose-12 columns does not separate Conjugate-GO and free GO, as both methods rely on separation by size. The molecular weight of haeme was 650 compared to molecular weight of GO which was 186,000. Therefore conjugation of haeme to GO does not increase the molecular weight of the conjugate to where it could be separated from free GO using the Sephadex G-25 purification procedure.

The sequence of events was initiated by CV readings of Bare-Pt electrodes, followed by CV's of GO-Pt, Haeme-Pt and finally Conjugate-GO-Pt electrodes. There were six

electrodes used in each condition. The electrodes were initially polished with an alumina polish followed by an ultrasonic treatment, and finally electrocleaned (potential cycled between -1.5 V and +1.5 V) before CV's were taken. For a description of the method of CV experimental setup refer to section 2.5.

The points that were important in this case were Ipa and Ipc as described in Figure 4-15. They were collated and compared against each individual electrode in each of the four situations described. The overall results showed significant statistical differences between buffer and glucose for Bare-Pt, GO-Pt and Conjugate-Pt but not for Haeme-Pt. The results were tabulated in Table 4-11.

Table 4-11. Results from ANOVA analysis of Ipa values for four Pt electrode treatment

Condition of Pt electrode	Treatment condition	Overall mean sensor response for 6 electrodes	Mean difference between treatment condition	F-statistic
Bare-Pt	Buffer	-0.4145	5.107	6.3652 (a)
	Glucose	4.6925		
Haeme-Pt	Buffer	6.229	-1.0309	n.s.
	Glucose	5.1981		
GO-Pt	Buffer	6.8786	3.1685	20.0289 (c)
	Glucose	10.0471		
Conjugate-GO	Buffer	11.7167	2.8466	12.0052 (a)
	Glucose	14.5633		

a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$, n.s. = not significant

There was a problem in getting reproducible results for each treatment condition of Bare-Pt, Haeme-Pt, GO-Pt and Conjugate-Pt. There were significant differences found between each electrode for each treatment condition ($F = 23.1159$; $p < 0.001$). The reason for this was and still is unclear but it did compromise the results. The results of the ANOVA analysis showed it was not possible to distinguish between Bare-Pt, Haeme-Pt, GO-Pt and Conjugate-GO electrodes using this type of CV analysis for this set of electrode treatments. The results of a single electrode were shown in Figure 4-16 showing the variability of sensor response between each electrode condition. At best, the expected result for this section was a difference between buffer and glucose for Conjugate-GO electrodes for both Ipa and Ipc profiles. The expected Ipc profile for Haeme-Pt was no difference between the conditions when glucose was present or absent. In case of GO-Pt it was expected to see similar results as in Conjugate-GO,

except when Ipc values were taken into consideration. The plot of Ipc values of GO-Pt and Conjugate-Pt was expected to be different as the Ipc region is where haeme is said to be in the reduced form. Although, the work by Zhang *et al* (1992) showed haeme had a reduction peak between -380 mV and -390 mV. The work from conjugated lactate dehydrogenase to haeme (section 5.3.2.) suggests redox behaviour can be seen at the potential of -200 mV. GO-Pt did not contain haeme. The overall results of all the electrodes as calculated using ANOVA analysis show that for this sample preparation it was not possible to distinguish between Bare-Pt, Haeme-Pt, Go-Pt and Conjugate-Pt.

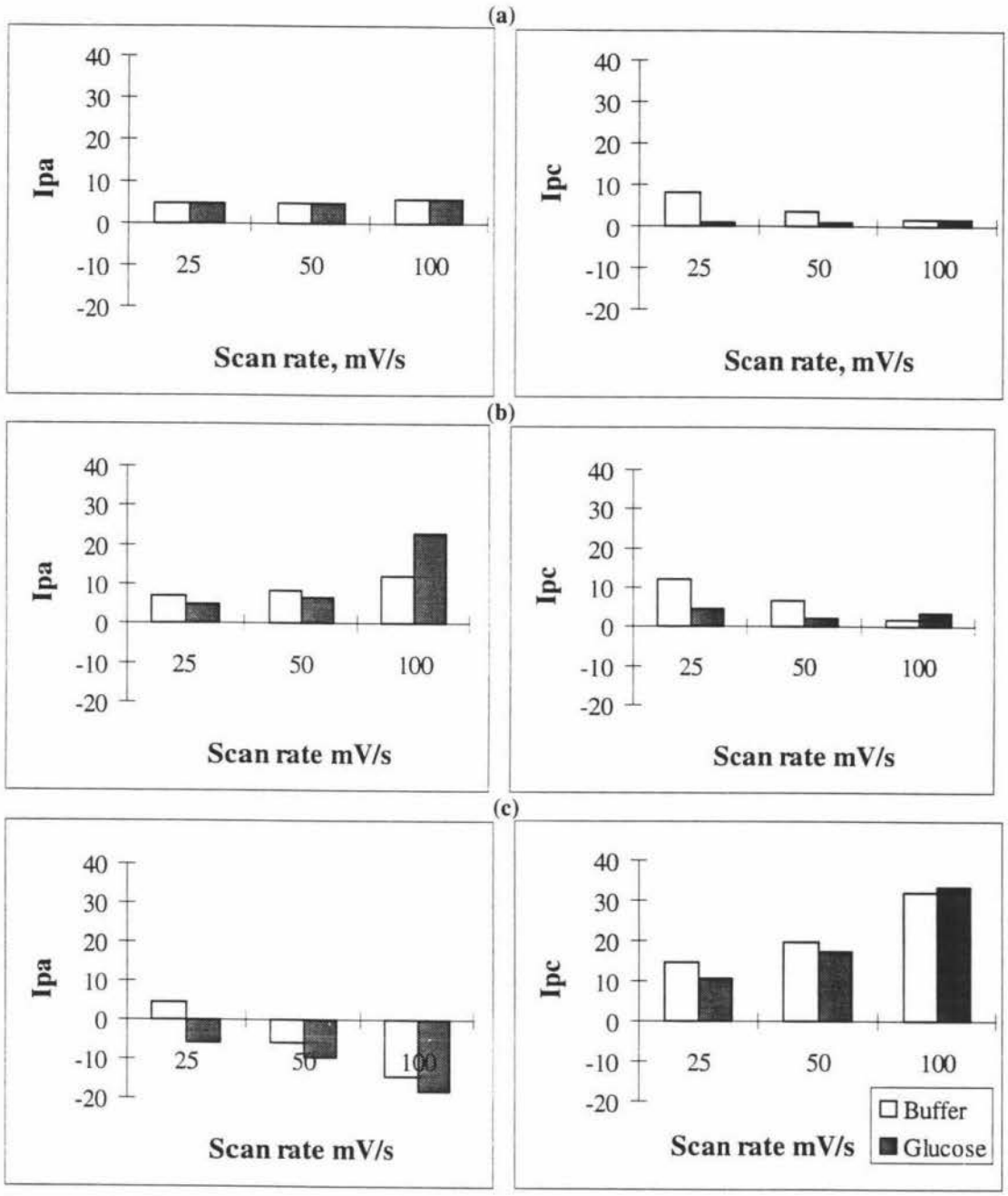

Figure 4–16. Comparison of Ipa and Ipc values of buffer and 10 mM glucose for Haeme-Pt, GO-Pt and Conjugate-GO relative to Bare-Pt Ipa and Ipc values for a single electrode. (a) denotes Haeme-Pt; (b) denotes GO-Pt; and (c) denotes Conjugate-Pt. The CV was scanned between potentials of -200 mV and +800 mV. The graphs on the right contained the plots for Ipa values and the graphs on the left contain plots for Ipc values.

4.8. Discussion

The aim of the current study was to investigate the behaviour of Conjugate-GO electrodes with respect to the determination of glucose. Gelatine-GO electrodes were prepared as a comparison method by which Conjugate-GO electrodes could be

evaluated. The initial work on Gelatine-GO electrodes showed although the response time for the detection of glucose was slower in comparison to Conjugate-GO electrodes, Gelatine-GO electrodes were found to be more robust in terms of stability over time and current responses were consistently higher compared to Conjugate-GO electrodes. It has been noted that the production of Conjugate-GO electrodes was not consistent. That may be due to several factors that are not known as yet. The results of the thin layer chromatography plates indicate it is likely that the root of the problem lies within the process of binding haeme active esters to the enzyme glucose oxidase. The thin layer chromatography plates indicate the active ester of haeme was formed. During the addition of haeme active ester, the pH dropped slightly from pH of 7.41 to 7.262. This was not a large drop and may not account for haeme active esters not binding to the enzyme. A comparison between the absorbance values obtained in the study by Large (1993) and the present study showed a large difference between the amount of spectrophotometric haeme absorption seen in Large (1993) and the present study. The spectrophotometric results of Conjugate-GO fractions collected and the final electrochemical evidence showed that if the peak at 380 nm was high, then it was likely that Conjugate-GO had been formed and the evidence from electrochemical activity can support this. Once the pH drops below 6 then it is less likely that a conjugate would form between haeme and glucose oxidase (Blackwell, personal communication, 1994). The pH where conjugation between glucose oxidase and haeme took place at pH 7.0 in the study by Large (1993). The addition of haeme active esters to glucose oxidase causes the pH to drop. It is conceivable that during the addition of haeme active esters to glucose oxidase the pH unit dropped low enough for the conjugation to proceed as per usual and the pH was low enough that it causes the enzyme structure to unfold slightly, thus allowing haeme bind and interact with the reactions occurring at the active site. The addition of 6 M urea can also cause the same effect. This might explain why some enzyme-haeme conjugates made successful electrodes and why some failed.

Luo (personal communication, 1994) suggested that aggregated haeme could be formed during the conjugation process between enzyme and haeme. In Figure 4-12a, conjugate was purified through G-25 Sephadex and the most active fraction collected was examined spectrophotometrically. The peak absorbances from the Conjugate-GO fraction showed an increase in absorbance at 380 nm from 3 hrs conjugation time to 20

hrs. When the fraction was again purified through a Superose-12 column, the peak at 380 nm showed similar absorbance values at 380 nm from 3 hrs conjugation time to 20 hrs. When compared to the previous result after the fraction was purified through only the G-25 Sephadex column, the suggestion of haeme aggregates made by Luo (personal communication, 1994) may be relevant. These aggregates should have been removed by the Superose-12 column. Haeme aggregates may form and interfere with the absorption of Conjugate-GO to the platinum electrodes. The haeme aggregates can absorb to the platinum surface by the same mechanism as with Conjugate-GO.

During the early studies involving Conjugate-GO electrodes in the present study, it was noted (refer to Figure 4-5) that the calibration curve was not quite linear. The curve for Day 1 (Figure 4-5) between the ranges of 20-140 mM seems to exhibit Michealis-Menten behaviour. It seems about to plateau around the region of 180 mM glucose. However, this did not occur as the curve started to increase linearly between 160 mM to 200 mM. A curve fitting programme (called IgorPro) was used to estimate the K_m of the results from Figure 4-5. The value obtained for K_m was 159.67 mM ± 54.3. The publish data for K_m was 110 mM (Gibson *et al*, 1964, Frederick *et al*, 1990). The curve fitting results for Gelatine-GO (Figure 4-2) was a K_m of 108.39 mM ± 21.9. It could be concluded from these results that the enzyme glucose oxidase was not modified a great deal by gelatine or conjugated with haeme as the K_m value remained the within similar to the published data. However in Figures 4-6a and 4-7a, it appears that saturation being reached at a glucose concentration that would indicate a much lower K_m.

The study of stability of Conjugate-GO electrodes showed whether stored frozen or in a fridge (4°C), the Conjugate-GO electrodes retained linear response in a glucose calibration curve albeit within a limited glucose calibration range. It was interesting to note that Conjugate-GO electrodes that were stored frozen in phosphate buffer were still usable for the determination of glucose, they showed a larger drop in current output compared to Gelatine-GO and Conjugate-GO electrodes stored at 4°. The stability may possibly be improved through the use of stabilising compounds such as poly-4-vinylpyridine. Poly-4-vinylpyridine was cross-linked to 2,2`-bipyridyl on gold electrodes to stabilise electrodes that contained absorbed or covalently attached monolayers (Doherty *et al*, 1994).

One of the significant differences between the response times of Gelatine-GO compared to that of Conjugate-GO electrodes is that Conjugate-GO electrodes take less time to be ready for another sample injection compared to Gelatine-GO, especially at high glucose concentrations (more than 140 mM glucose). This is consistent with other studies. Recently, Schereth *et al* (1994) used toluidine blue covalently immobilised on gold electrodes. The significance of this study was cystamine or mercaptopropionic acid was absorbed onto gold electrode surfaces. Toluidine blue was covalently attached to the monolayer of mercaptopropionic on the gold electrodes. The toluidine blue electrodes oxidised NADH at a potential between 0-100 mV(versus standard calomel electrode). The response times for lactate, malate, alcohol and glucose dehydrogenases were evaluated with stirring and found to be relatively quick especially for lactate and malate dehydrogenases. Quick response times were consistent with enzymes absorbed as monolayers on electrodes as is the case for Conjugate-GO electrodes.

There was a total of 21 conjugation trials made using glucose oxidase, and only 13 trials were deemed to be successful conjugations to haeme. During purification of these conjugates using Sephadex G-25 and after scanning in the UV-Vis absorbance region of the fractions collected, it was noticed that the spectrophotometric absorbance for haeme was generally quite low in comparison to the UV-Vis spectra seen in the study by Large (1993) for Conjugate-GO. This could indicate that very little haeme was conjugated to glucose oxidase. This could also be a factor to the low current responses obtained for Conjugate-GO electrodes in parts of this study. In a study by Gunaratna and Wilson (1990), they had found that when their enzyme choline oxidase was coupled directly to a HW-65 gel, 99% of the enzyme activity was lost. In the same study, Gunaratna and Wilson assessed the addition of spacer molecules and concluded spacer molecules allowed freer movement of the enzyme so the active site is less likely to be hindered.

The results from thin layer chromatography (TLC) plates of the formation of active esters of haeme clearly demonstrated that active esters of haeme were formed. This step was repeated a total of three times and in all three cases, haeme active esters were found to be formed. The step after making haeme active esters is to couple the haeme esters to the enzyme glucose oxidase. Since there were no other steps after conjugating haeme to glucose oxidase and through the evidence of spectrophotometric assays, the reason for

not producing consistent Conjugate-GO preparations could be the lack of binding occurring between haeme and enzyme.

The attempt to use cyclic voltammograms to differentiate between Bare-Pt, Haeme-Pt, Go-Pt and Conjugate-GO did not meet with much success. The problem could lie with the use of the wrong potential range to examine the electrodes. The study by Zhang *et al* (1992) described the reduction potential of haeme as being between -380 mV to -390 mV. The inconsistent ability to make Conjugate-GO electrodes could have also affected the final results.

The development of biosensors has to include developing methods that reduce the effect of interfering substances. A study by Johnson *et al* (1994) looked at the optimisation of platinum black glucose sensors for use in *in vivo* measurement of blood glucose. The conclusion from the study by Johnson *et al* (1994) suggests by decreasing the poised potential from 600 mV to 350 mV, the effect of interfering substances could be minimised to a satisfactory level without any additional membrane layers, enzymes, electrodes, or mediators. The construction of the platinum black electrode used by Johnson *et al* (1994) consisted of an enzyme layer of glucose oxidase and bovine serum albumin cross-linked with glutaraldehyde with a commercially bought outer membrane. Downard and Roddick (1994) suggested pretreating samples with glassy carbon surfaces to prevent the absorption of high molecular weight non-electroactive compounds on the surface of working electrodes. The results showed slightly higher current response obtained for samples pretreated with glassy carbon surfaces compared to samples not pretreated with glassy carbon electrodes. Other workers in this area had tended to look at the effect of interfering substances through firstly identifying possible interfering substances and secondly, analysing the affect of these substances through experimental trials (Lowry *et al*, 1994).

Beh *et al* (1989) reported the use of spacer molecules, such as lysine, asparagine, arginine, ornithine, glutamine, *m*-phenylenediamine and *p*-phenylenediamine, and concluded that using lysine coupled with *p*-Benzoquinone (cross-linking agent) gave the best linearity and current response for the detection of glucose. Gunaratna and Wilson (1990) used either avidin-biotin complex or antibodies to immobilise choline oxidase. The results showed the two spacer molecules improved detection limits, though it was found that the avidin-biotin complex to be more successful of the two spacer molecules.

The inclusion of spacer molecules between the enzyme and the electrode surface could extend the life of Conjugate-GO electrodes and improve detection limits.

In conclusion, the work on the making and characterising Conjugate-GO electrodes met with limited success in some areas. The initial results showed that Conjugate-GO can easily be incorporated into a flow injection analyser for fast and efficient analysis of glucose. The advantages of Conjugate-GO had been shown to be the fast response and recovery times that ultimately allows high sample outputs and the ease preparing Conjugate-GO electrodes as opposed to other methods of immobilisation. Conjugate-GO electrodes also have a respectable linear range for glucose analysis and Conjugate-GO electrodes have good stability so they can be stored for long term use. The work also highlighted the need for more research to be done on making the Conjugate-GO. The inconsistency between preparations of Conjugate-GO could be due to the conditions not being quite right for optimal binding between haeme and glucose oxidase. The other problem that could interfere with making "good" Conjugate-GO electrodes could be due to the competition occurring between aggregated haeme and Conjugate-GO for absorption on the platinum surface. Despite some of the setbacks that had occurred, this method of conjugation holds much promise for the development of a glucose sensor and for other enzyme based sensors.

Chapter 5

5.1. Lactate Analysis Using Lactate Dehydrogenase Electrodes

L-Lactate is of major importance both clinically and industrially. Lactate measurement is important clinically as an increase in blood lactate concentration can indicate a the condition of acidosis (Scheller, Pfeiffer *et al*, 1987). The condition of acidosis can develop from respiratory, hemodynamic or metabolic abnormalities. An accurate measurement of blood lactate level is also important in sports medicine for exercise control in both man and animals, particularly in horses. Lactate is an important industrial product as it aids in process control in milk and other food products (Canh, 1993).

There are four types of enzymes suited for the measurement of lactate, lactate dehydrogenase (LDH), lactate oxidase (LOD), lactate monooxygenase (LMO), and cytochrome b_2 (Cyt b_2). These enzymes catalyse different reactions and have different cofactor requirements.

The four different enzyme pathways for the metabolism of lactate are presented:

$$NAD^+ + \text{lactate} \xrightarrow{LDH} \text{pyruvate} + H^+ + NADH$$

$$2(Fe(CN)_6)^{3-} + \text{lactate} \xrightarrow{\text{Cyt b2}} \text{pyruvate} + 2H^+ + 2(Fe(CN)_6)^{4-}$$

$$O_2 + \text{lactate} \xrightarrow{LOD} \text{pyruvate} + H_2O_2$$

$$O_2 + \text{lactate} \xrightarrow{LMO} \text{acetate} + CO_2 + H_2O$$

Mascini *et al* (1984) immobilised LMO on a nylon net to a Clark-type oxygen electrode and measured lactate concentrations in reconstituted human sera and obtained a linear response up to 0.25 mM with a correlation coefficient of r = 0.995. However, as mentioned previously in Chapter 1, oxygen electrodes are prone to changes in oxygen content of buffer and sample.

There are a number of studies involving the use of LOD as the biospecific transducer in the construction of a biosensor (Mascini *et al*, 1983;1987; Scheller *et al*, 1989). The commercially available OMRON HER 100 and YSI Model 23L both use LOD. The enzyme LOD is a good choice for many due to its stability both during preparation and in common buffers such as chloride, phosphate, and Tris (Mascini and Moscone, 1991). The reason for its preferred choice over other enzymes for biosensors for measuring lactate is the lack of a requirement for a cofactor. The commercial preparation is available lyophilized and has relatively high activity. Biosensors made from lactate oxidases, like other oxidases, are prone to interference due to the presence of other electroactive species as the reduction of H_2O_2 occurs at relatively high potentials (see Chapter 1). This however does not mean that LOD based biosensors are not accurate, for example the YSI Model 23L sensor correlates with r = 0.997 with respect to the Boehringer photometric determination of lactate (Scheller *et al*, 1987). Similar results were obtained using the OMRON HER 100 sensor (Tsuchida *et al*, 1985).

Biosensors based on dehydrogenases have attracted considerable attention due to the number of dehydrogenase enzymes available. For example there are over 250 NAD^+ dependent dehydrogenases. If a general method for incorporating dehydrogenases into biosensor designs can be found, it should have wide applications both clinically and industrially. The difference between the oxidases and the dehydrogenases is the need for a soluble cofactor that becomes reduced in the presence of the substrate, unlike with the oxidases where the enzyme is reduced (Cardosi and Turner, 1991).

Burnett and Underwood (1965) showed it was possible to reoxidise NAD^+ from NADH following electrochemical oxidation at platinum electrodes, although, the reoxidation of NAD^+ on a metal surface such as platinum (Pt) poses a few problems. It requires potentials of over 1 V that results in the formation of radical intermediates (Braun *et* al, 1975; Albery & Bartlett, 1984; Blankespoor and Miller, 1984; Gorton, 1986; Yon Hin & Lowe, 1987; Matsue et al., 1987).

There are two general methods that others have used to overcome the problem of high potentials required to regenerate NAD^+. These include the introduction of another electron transfer mediator into the solution being tested. The electron transfer mediator reacts with NADH produced in the enzymic reaction and regenerates NAD^+, and the mediator is then re-oxidised electrochemically at a potential much lower than that

required for oxidation of NADH. Some of these electron transfer mediators are ortho- and para quinones, quinodiimines, phenlyenediimines, indophenols, phenazines and phenoxazines (Huck *et al*, 1984). The first method involves entrapping the mediator molecules using one of the immobilising methods discussed in Chapter 1. The second method involves chemical modification of the electrode surface to directly bind the mediator molecule to the surface of the electrode. One of the more successful methods used for dehydrogenase-based electrode are conducting salts, particularly those derived from N-methylphenazine (NMP) and tetracyanoquinodimethane (TCNQ) (Albery and Bartlett, 1984; Bryce and Murphy, 1984). Crystal structures of NMP.TCNQ complexes exhibit donor and acceptor complexes that form different configurations to allow electron transfer. NMP.TCNQ complexes form stacks with considerable pi-electron overlap and delocalization and this is thought to allow efficient electron transfer to occur between NMP.TCNQ complexes (Bryce and Murphy, 1984; Bartlett, 1990).

In the early 1980's, organic conducting salts such as N-methyl-phenazinium tetracyanoquinodimethanide ($TTF^{+}TCNQ^{-}$) gained prominence for the oxidation of NADH at the potential of -200 mV (versus Ag/AgCl electrode) (Kulys, 1981). Karyakin *et al* (1994) described another compound, poly(methylene blue), capable of oxidising NADH. The dehydrogenase was entrapped in a Nafion film on poly(methylene blue) modified electrodes and NAD^{+} was coimmobilised as N^{6}-aminoethyl-NAD^{+}. The disadvantage of this method was it was likely that the electrode could only be used for a short time. Skoog and Johansson (1991) described a modification to the biosensor electrode design to incorporate cofactor NAD^{+}. The cofactor would diffuse from an internal hollow filled with 10 mM NAD^{+} that was pressurised. This helped decrease the amount of the cofactor used. Wang and Chen (1994) described a method where LDH and NAD^{+} was incorporated into a graphite-epoxy matrix. This meant by polishing the electrode surface, new LDH and NAD^{+} are renewed for use.

The aim of the current study is to investigate and characterise the electrochemical behaviour of haeme-conjugated lactate dehydrogenase. It had been previously suggested by Large (1993) that haeme can not only act to bind the enzyme conjugate to a platinum electrode surface, but it may also act as an electron mediator. Haeme had not been previously bound to the enzyme LDH.

5.2. Control Experiments for Lactate Dehydrogenase Electrodes

As previously discussed in section 4.3, Conjugate-GO electrodes had been shown to be capable of estimating glucose concentrations with linear calibration curves ($r = 0.9788$; Figure 4-3). The same technique used for making Conjugate-GO electrodes was applied to making Conjugate-LDH electrodes. It was necessary to investigate whether a response to lactate required the presence of the enzyme LDH and not just haeme on its own.

5.2.1. Sensor Response and Cyclic Voltammogram (CV) of Haeme-Pt electrodes in the Presence of L-Lactate

Haeme-Pt electrodes were prepared as previously described with the same method as with conjugate-enzyme, except the enzyme (LDH) was omitted from the procedure (section 2.2.2). Haeme-Pt electrodes were then treated with the same procedure as for conjugate-enzyme electrodes, i.e. the electrodes were washed in R.O. water and stored in buffer.

The procedure was as follows. Haeme-Pt electrodes were initially treated with the potential poised at -200 mV in 0.1 M Tris/HCl, pH 7.5 buffer for 3 hrs and bubbled with nitrogen to condition the electrode before any electrochemical data was recorded. Then Haeme-Pt electrodes were poised at -200 mV in 0.1 M Tris/HCl, pH 7.5 buffer for the recording of electrochemical response in the absence and presence of LDH (used 100 enzyme units per 8 mls of buffer) in solution. The potential was poised at -200 mV, the same as in the work with Conjugate-LDH electrodes (section 5.2.1).

Figure 5-1 shows that a Haeme-Pt electrode did not interact with the substrate lactate, but in the presence of lactate dehydrogenase (cofactor NAD^+ was absent) there was a dependent relationship between sensor response and lactate concentration. The correlation coefficient for Haeme-Pt only was $r^2 = 0.357$ compared to when LDH was present in the same configuration; $r^2 = 0.9253$. The gradient of the line for Haeme-Pt in the presence of LDH is $y = 10.571x + 4.043$ compared to the absence of LDH, the gradient is $y = 0.76x - 1.19$. The results shown in Figure 5-1 are significant because they show a difference with the presence or absence of LDH. In both cases, the cofactor

NAD^+ was not present. A current was generated only when LDH was present in solution. This was repeated three times with the two other results not reproducing the initial response shown in Figure 5-1. The response of the Haeme-Pt electrode for the other two occasions showed similar results with lactate as that obtained in Figure 5-1 for Haeme-Pt electrode even in the presence of LDH. Therefore, the results found should be regarded with caution. The small response seen (nanoamps range, Figure 5-1) indicates that this effect is negligible when compared to responses observed with LDH-haeme conjugate electrode that is working well (see Figure 5-6).

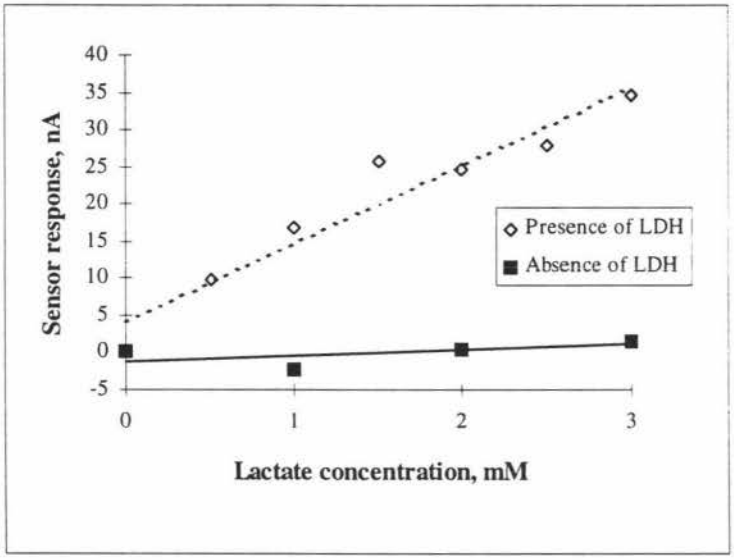

Figure 5–1. Response of Haeme-Pt electrode to increasing amounts of lactate in solution. The potential was poised at -200 mV, and the buffer was 0.1 M Tris/HCl, pH 7.5. NAD^+ was absent in all solutions. The legend "absence of LDH" refers to a Haeme-Pt electrode sensor response to lactate, in the absence of LDH and NAD^+ in solution. The legend "presence of LDH" refers to a Haeme-Pt electrode sensor response to lactate, in the presence of LDH (100 enzyme units per 8 mls of solution used) and absence of NAD^+.

After the electrochemical data had been obtained for a Haeme-Pt electrode in the presence and absence of LDH and lactate, cyclic voltammograms (CV) of these electrodes were recorded. Information on CV's can be found in section 4-7. The CVs shown in Figure 5-2a show a Haeme-Pt electrode in 0.1 M Tris/HCl, pH 7.5 without any LDH present in solution. The result of the first CV scan with buffer was overlayed onto another CV of a Haeme-Pt electrode with 4 mM lactate in buffer. There were no significant shifts in CV shape or area of CV in the presence of 4 mM lactate when compared to CV in buffer only. The CV results supports the results found in Figure 5-1, as no significant response to lactate by the Haeme-Pt electrode was detected. Following the results shown in Figure 5-2a, CV of Haeme-Pt in the presence of LDH was recorded.

Figure 5-2b shows CV's of Haeme-Pt electrode in 0.1 M Tris/HCl, pH 7.5 buffer, 4 mM lactate and 12.5 enzyme units /ml of LDH (100 enzyme units per 8 mls of buffer). There was a shift with the CV shape detected between buffer (grey line) and 4 mM lactate (red line). There was a shift of 55 mV in the position of the CV containing 4 mM lactate relative to buffer. The differences seen here are small in comparison to Figure 5-4 (Conjugate-LDH).

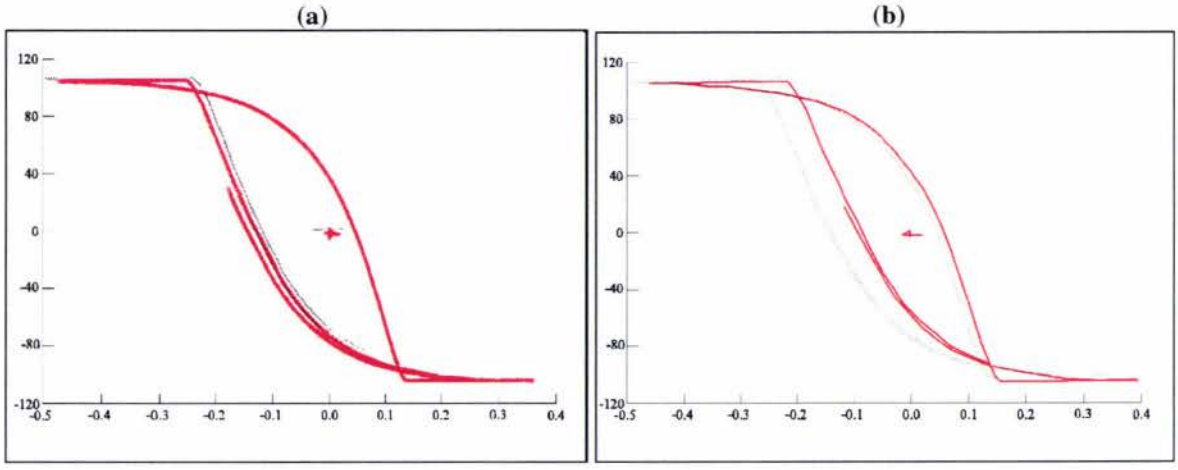

Figure 5–2. Cyclic Voltammograms comparing a Haeme-Pt electrode in buffer and in 4 mM lactate to a Haeme-Pt electrode in buffer and in 4 mM lactate with the addition of 100 enzyme units of LDH per 8 mls of buffer. (a) Shows Haeme-Pt in the absence of LDH. (b) Shows Haeme-Pt in the presence of LDH. NAD^+ was not present in either CV scans. The electrode was scan between -500 mV and +500 mV. Temperature 25°C. The grey line denotes electrode in buffer only. The red line denotes electrode in 4 mM lactate and buffer.

5.3. Conjugating Haeme to Lactate Dehydrogenase (Conjugate-LDH)

As mentioned previously, the method for conjugating haeme to LDH was derived from the method of conjugating haeme to GO. A similar amount of LDH (3887 enzyme units) was used in the conjugation as had been used in conjugating GO (3750 enzyme units) to haeme (see section 2.4.2).

This section of work evaluates the work on Conjugate-LDH and its propensity for lactate determination.

5.3.1. UV-Vis determination of Conjugate-LDH

LDH-haeme conjugates were initially made according to the method described in section 2.4.3. The LDH-haeme conjugates were purified using a Sephadex G-25 column. Fractions of 1.5 mls were collected and characterised spectrophotometrically between 200 nm and 500 nm. The same criteria that was used for assessing which fractions contained Conjugate-GO spectrophotometrically was used for selecting the fractions that contained Conjugate-LDH, this criteria being the fraction showing the highest haeme absorbance peak (380 nm) coinciding with an enzyme peak (280 nm) when examined spectrophotometrically between 250 nm and 500 nm.

Figure 5-3 shows the results of a UV-Vis scan of the fraction containing the highest haeme and enzyme peaks of the fractions collected. In Figure 5-3, there was an enzyme peak detected around 270 nm and the characteristic broad peak of haeme between 300 nm and 500 nm. The fraction was also assayed for LDH activity to confirm it had the highest enzyme activity.

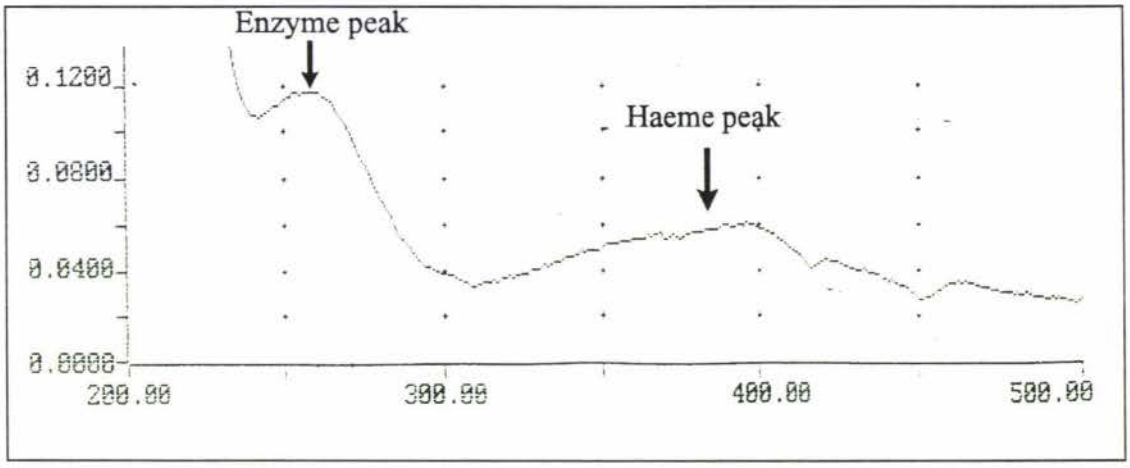

Figure 5–3. UV-Vis spectrophotometric scan of fraction containing Conjugate-LDH.

Four Pt electrodes were initially cleaned (section 2.2.1.). Immediately following the cleaning procedure, the electrodes were rinsed under running R.O. water and dipped in a solution of Conjugate-LDH. The electrodes were left in solution overnight at 4°C.

The following day, the electrodes were taken out of Conjugate-LDH solution and rinsed under running R.O. water. The electrodes were then stored in 0.1 M Tris/HCl, pH 9.5 during the rest of the period when the electrodes were examined amperometrically. The

decision to switch from 0.1 M phosphate, pH 7.5 to 0.1 M Tris/HCl, pH 9.5 was made for two reasons. The first was phosphate buffers tended to denature enzymes upon freezing (Blackwell, personal communication, 1993), so with respect to long term storage, Tris buffer was selected as an alternative. The pH was changed to 9.5 from 7.0 because Tris buffered better at 9.5 than 7.0. The conjugation process may also benefit from the higher pH as a low pH value affects successful conjugation between haeme and enzyme (Blackwell, personal communication, 1993).

The electrodes were initially treated by having all four electrodes in 0.1 M Tris/HCl, pH 9.5 buffer and the potential poised at -200 mV for 3 hrs before cyclic voltammograms were recorded.

5.3.2. Cyclic Voltammograms of Conjugate-LDH

As with Conjugate-GO, data from the CV's of Conjugate-LDH provided information on the region where there was the greatest difference in CV position relative to the control (buffer only CV) at a given potential. The data presented in Figure 5-4 was the result of one of the four Conjugate-LDH on a Pt electrode. The CV shown in Figure 5-4a was more uniform with respect to their response to lactate compared to the CV shown in Figure 5-4b, uniform meaning less signal noise around the region of -200 mV in CV for Conjugate-LDH compared to Conjugate-LDH (with NAD^+ present). The difference between Figure 5-4 and Figure 5-2b is, in Figure 5-4 LDH was conjugated to haeme and bound to the Pt electrode, rather than LDH being added into the solution bathing the Haeme-Pt electrode. In Figure 5-4, there was a large difference in the shape of the CV when lactate was present (red line) compared to buffer (grey line). There was also a discernible difference between having NAD^+ absent or present. The presence of NAD^+ (Figure 5-4b) increases the electrical noise.

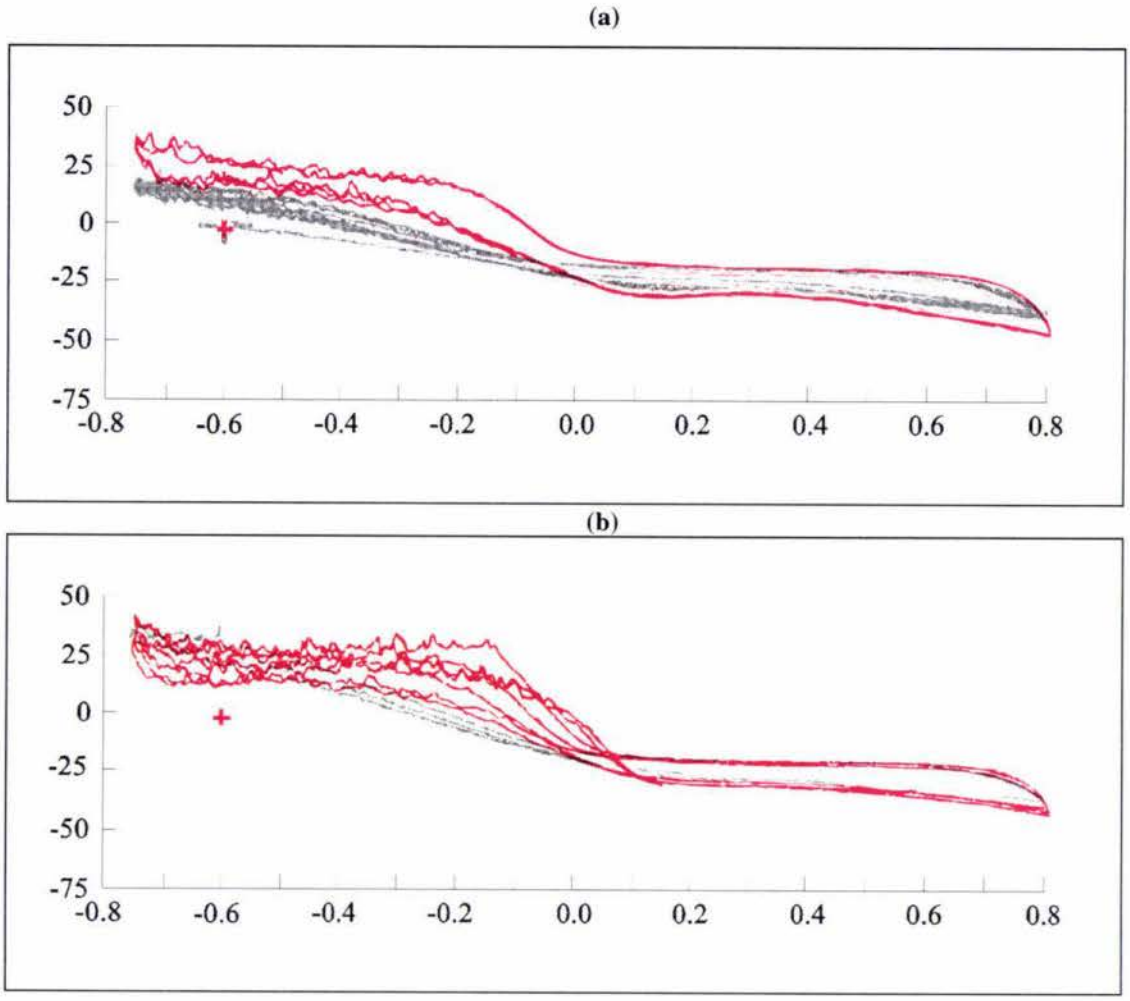

Figure 5–4. A Cyclic voltammogram of Conjugate-LDH absorbed onto a Pt electrode. (a) The comparison made between buffer and 10 mM lactate in the absence of any NAD^+. (b) The comparison between buffer and 10 mM lactate in the presence of 0.5 mM NAD^+. The electrodes were scanned between -800 mV and +800 mV. Buffer used was 0.1 M Tris/HCl, pH 9.5. Temperature 25°C. The grey line denotes buffer only. The red line denotes the presence of 10 mM lactate. The y-axis units are in nanoamps. The x-axis units are in volts.

The results from the other three Conjugate-LDH electrodes showed similar results to the one shown in Figure 5-4. The results shown in Figure 5-4 also showed that -200 mV is the potential where the difference between buffer and lactate is greatest.

5.3.3. Conjugate-LDH in a FIA for Lactate determination

One of the Conjugate-LDH electrodes that was used for CV acquisition, was used in a Version 3 flow cell (section 3.2.3.) for flow injection analysis (FIA). The carrier stream was 0.1 M Tris/HCl, pH 9.5. Lactate concentrations of 1, 2, 5 and 10 mM were made up in 0.1 M Tris/HCl, pH 9.5 buffer and degassed prior to use. The sensor response of

Conjugate-LDH to lactate is shown in Figure 5-5. The results from the trace shown in Figure 5-5 are also plotted in Figure 5-6. These results are quite significant as it shows Conjugate-LDH responding to lactate in the absence of cofactor NAD^+. This is a significant step towards achieving reagentless dehydrogenase based biosensors.

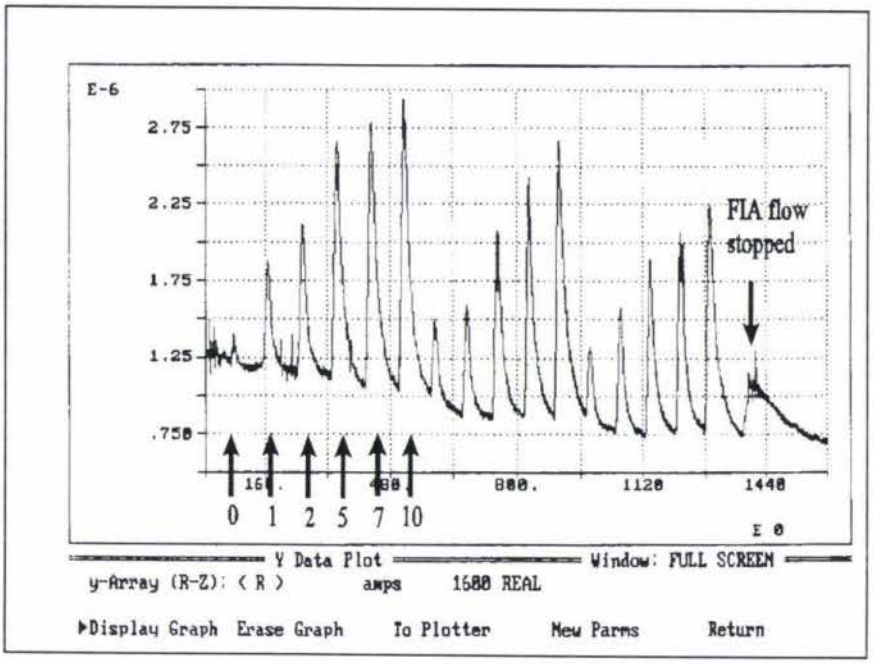

Figure 5–5. Sensor response to lactate of one of the electrodes was recorded using a FIA. Potential poised at -200 mV. Temperature 25°C. Buffer used 0.1 M Tris/HCl, pH 9.5. There were injections of 1, 2, 5, 7, and 10 mM lactate. There were three replicates of the series of standards made. This was done in the absence of NAD^+.

The results shown in Figure 5-6, show that the response to lactate is curvilinear between 0 mM and 10 mM lactate. The results are quite reproducible as seen in the small error bars present. The error is an average of 4.3% for each reading.

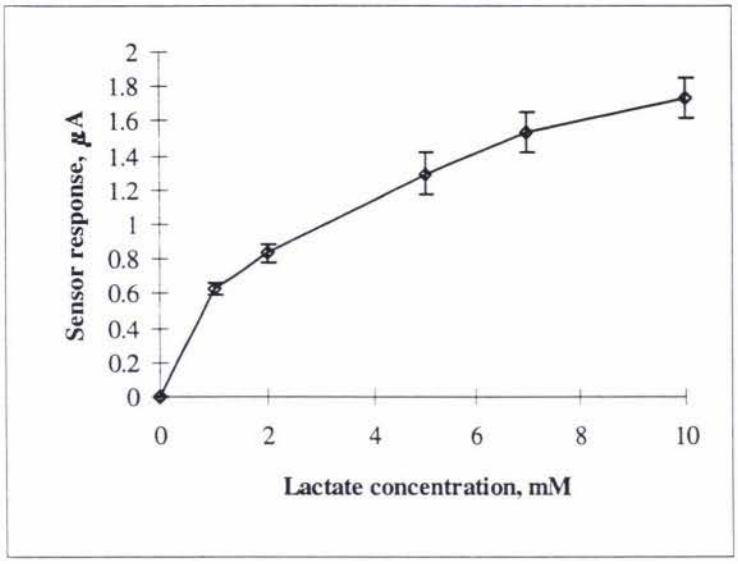

Figure 5–6. Conjugate-LDH sensor response to lactate using FIA. Potential poised at -200 mV. Carrier stream 0.1 M Tris/HCl, pH 9.5 buffer. Three replications were made from one Conjugate-LDH electrode. Error bars indicate standard error of the mean. NAD^+ absent.

From the CV results of Figure 5-4, it suggests that NAD^+ may not be needed for Conjugate-LDH to respond to lactate. The FIA was again used to examine this. A single Conjugate-LDH electrode that had been prepared using the method described previously was analysed using a FIA. The first series of lactate standards contained lactate and buffer. The second series of lactate standard that followed contained lactate, buffer and 0.5 mM NAD^+ (final concentration). The potential was poised at -200 mV. The results were plotted in Figure 5-7.

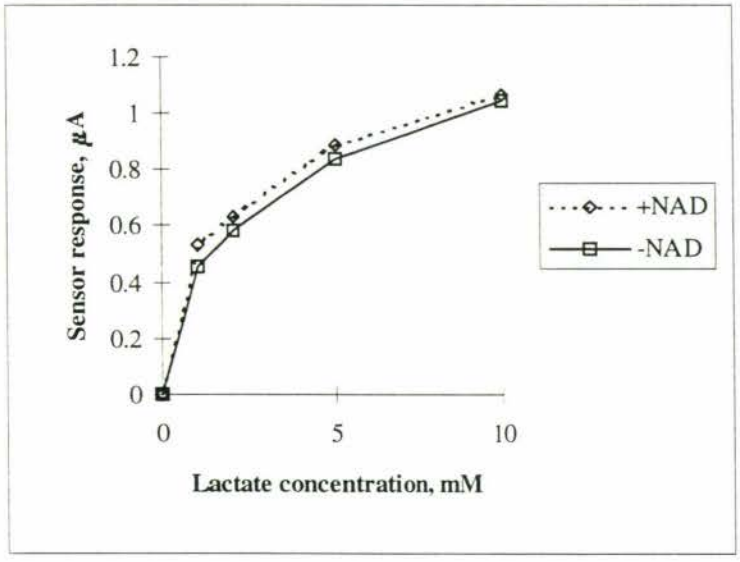

Figure 5–7. Comparison between the sensor response of Conjugate-LDH to NAD^+ in the presence and absence of lactate. Potential poised at -200 mV. Results obtained using FIA.

The results from Figure 5-7 show that NAD^+ may not be necessary for Conjugate-LDH to metabolise lactate. There was almost the same current response when NAD^+ was present and when it was not present. Figure 5-8, shows another trace from the FIA of a single Conjugate-LDH electrode where NAD^+ was being pumped alternately with the same sample containing no NAD^+. The trace shows the Conjugate-LDH electrode respond to lactate whether NAD^+ was present or not. This made NAD^+ redundant in the detection of lactate using Conjugate-LDH electrodes.

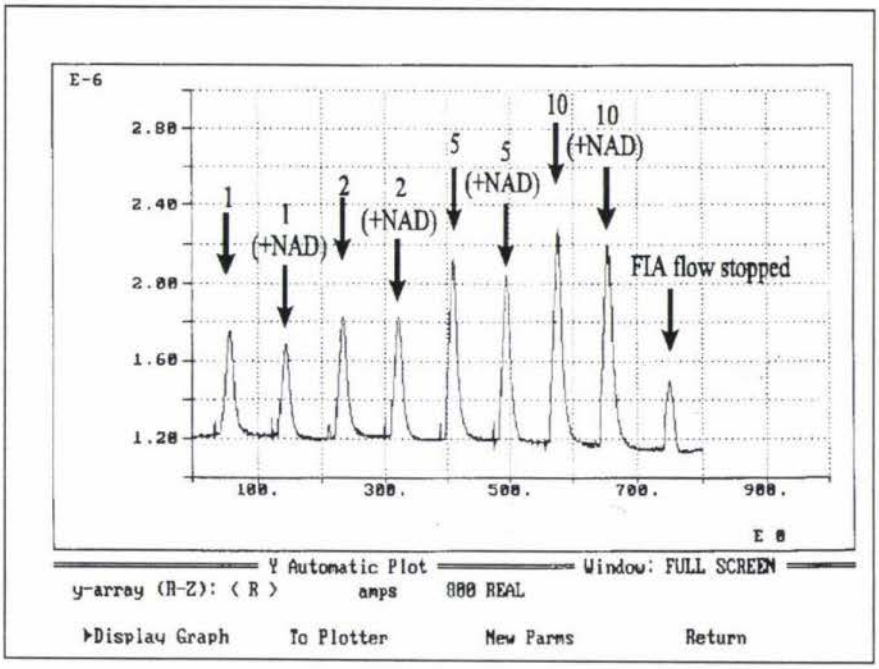

Figure 5–8. Trace from FIA showing sensor response of a Conjugate-LDH electrode to alternate additions of NAD^+. Buffer 0.1 M Tris/HCl, pH 9.5. Potential poised at -200 mV. Temperature 25°C.

5.4. Unsuccessful Conjugate-LDH Electrodes

The results presented so far show that Conjugate-LDH can be successfully made. However, out of thirteen conjugation trials of making Conjugate-LDH electrodes, only on three occasions was the conjugation process successful. Figure 5-9a shows one of the unsuccessful Conjugate-LDH preparations. Figure 5-9 clearly show that the electrodes prepared with Conjugate-LDH did not respond to lactate as compared to the results shown in Figure 5-8. On the other two successful occasions, the current was lower and required a preamplifier to record the signals. The effect of varying the conjugation conditions was investigated by varying the buffer, 0.1 M Tris/HCl at pH 7.5 and 0.1 M

phosphate at pH 7.5 was used. Neither produced conjugates that gave successful electrodes. The buffer was changed to phosphate at pH 7.5 because Tris does not buffer well at pH 7.5 as it is outside its buffering capabilities. Previously, it was mentioned that phosphate buffer can denature proteins, but this is only of concern when the enzyme-conjugates are stored in phosphate in the freezer. Since this section of work had not got to the stage of storing electrodes for long term use, the use of phosphate buffers would not affect the viability of enzyme-conjugates stored at 4°C. The factors involved in the lack of reproducibility of the initial success of the first conjugation between haeme and LDH are not known. However, during the thesis write up, it was realised that the activity of LDH is optimal around pH 9.5-10.0. Out of thirteen conjugation attempts between LDH and haeme, seven of those attempts were measured electrochemically at pH 7.5. In hindsight, this may be part of the reason as to why some of the electrochemical readings had been classified as unsuccessful. The electrochemical readings were not made in the pH range for optimal enzyme activity. This is not the complete reason however as there are still the remaining LDH-haeme conjugates that did not make successful conjugates.

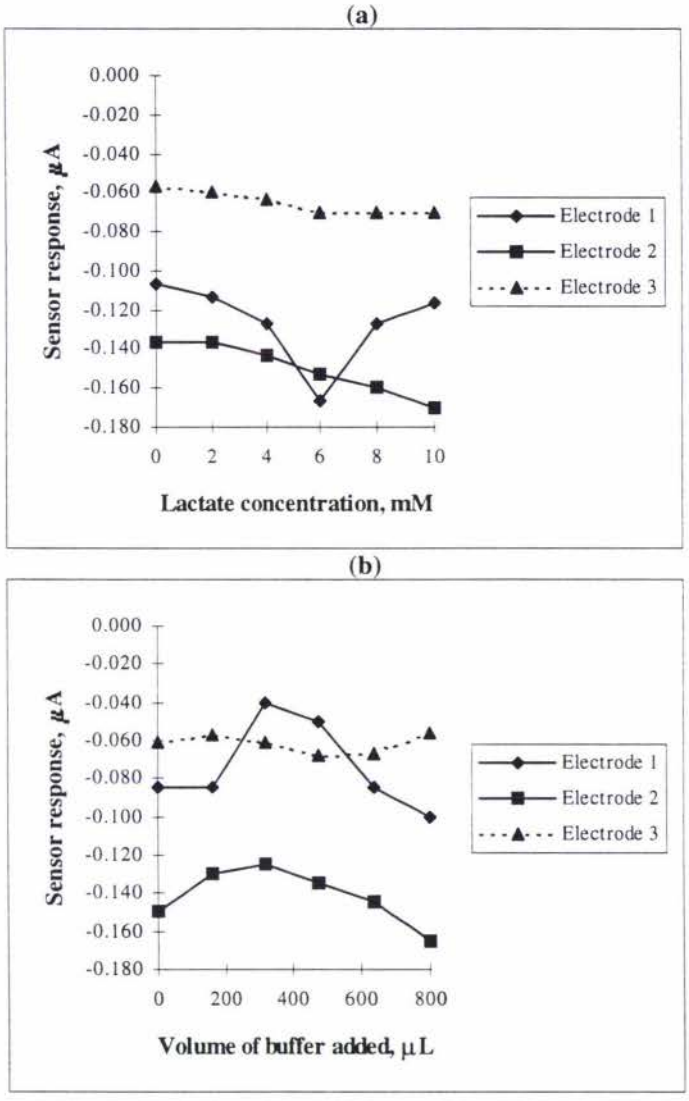

Figure 5–9. This shows the results of one of the Conjugate-LDH electrodes that was unsuccessful. Measurement was made using batch method. Buffer was 0.1 M phosphate, pH 7.5. Temperature controlled at 25°C. (a) Shows three Conjugate-LDH electrodes measuring lactate concentrations. (b) Shows the same three electrodes measuring injections of buffer only.

5.5. Discussion

The present study demonstrates a lactate sensor based on haeme conjugated to lactate dehydrogenase. The work has also shown there are still conditions of conjugation that could be improved to obtain more successful Conjugate-LDH electrodes. There may be other as yet unknown factors that determine the success of Conjugate-LDH electrodes. The success rate of Conjugate-LDH sensors was quite low, with only three out thirteen trials succeeding. During the three successful conjugation attempts, the data presented seemed quite conclusive for the presence of Conjugate-LDH on electrodes. Cyclic

voltammograms of haeme-only electrodes showed conclusive evidence that Haeme-Pt electrodes do not show significant changes with the addition of lactate. Unless LDH was present in the solution, the Haeme-Pt electrode showed no response to lactate in either the cyclic voltammograms or in batch measurement of the electrode. The Conjugate-LDH electrode showed convincing evidence of these electrodes acting without NAD^+ in CV's as well as in flow injection analysis measurements.

In the biosensor literature, there has not been any reported evidence of LDH acting without the cofactor NAD^+ present. With Conjugate-GO electrodes, the cofactor FAD^+ is buried within the GO structure and therefore could react with glucose to give detectable H_2O_2. In the case of Conjugate-LDH electrodes the soluble cofactor NAD^+ must be present, as NAD^+ is not trapped within the structure of the enzyme. The need for haeme in Conjugate-GO electrodes is as a linker to the Pt surface. In the case of Conjugate-LDH electrodes, the presence of haeme may serve two functions. Some haeme molecules may act to link LDH to the Pt surface. Some haeme molecules may somehow replace the function of NAD^+ in the dehydrogenase reaction.

A possible sequence of reaction events for Conjugate-LDH is that, during the making of the conjugate, the haeme molecules were able to enter the active site of LDH and perhaps occupy the same histidine residue as NAD^+ would have (histidine 195). Alternatively, the haeme may have been bound to another histidine residue near the active site. The reason for suggesting binding to a histidine residue is that in haeme, the four ligand groups of the porphyrin form a square-planar complex with iron; the remaining fifth and sixth coordination positions of iron are perpendicular to the plane of the porphyrin ring. In haeme proteins myoglobin and haemoglobin the fifth position is occupied by an imidazole group of a histidine residue and the sixth position is either unoccupied (deoxyheamoglobin and deoxymyoglobin) or occupied by oxygen (oxyheamoglobin or oxymyoglobin) or other ligands, such as carbon monoxide (Lehninger, 1975). The other alternative is, the haeme is bound close to the active site by virtue of the same carbodiimide reaction mechanism that links the haeme to enzyme. DiNello *et al* (1975) had suggested the importance of the role of histidine in interacting with halides giving two electrons to the haemeprotein of chloroperoxidase. They showed the possibility of an imidazole (histidine) group attacking a substrate, so that two electrons are available to return chloroperoxidase back to a more stable state. This

may not necessarily be the mode of action for Conjugate-LDH. The important part is that once a change had been initiated to the porphyrin or the iron complex, evidence from redox potentials suggests that the iron complex of the porphyrin can act as an electron sink. The redox potential for lactate to pyruvate is E_o = 0.185 V and the NAD^+ to NADH has a E_o value of -0.32. Therefore, NAD^+ is able to receive the hydride transfer from lactate. In the situation for haeme conjugated to LDH, the E_o value for Fe^{3+} + e- to Fe^{2+} is 0.770 V. It may be conceivable that the iron or even perhaps the porphyrin moiety (Gouterman and Stevenson, 1962; Mauzerall, 1965) is involved in the transfer of electrons to the Pt electrode. Although, the NAD^+ to NADH reaction has a negative E_o value and therefore suggest its a donor rather than an electron acceptor. In an excerpt from Lehninger (1975, p.485), it mentions that regardless of the E_o value of NAD^+/NADH, thermodynamic calculations are only concerned with initial and final states, not with the E_o values of the pathway. So, this suggests that because Fe^{3+}/Fe^{2+} reaction is more positive compared to lactate/pyruvate, then electrons will still flow towards Fe^{3+}/Fe^{2+}.

Other workers in this area have developed procedures for either providing the cofactor NAD^+ in the carrier stream (in FIA) or incorporating NAD^+ onto the lactate biosensor itself. Schelter-Graf *et al* (1984) described a method for analysing lactate and other substrates using dehydrogenases instead of oxidases. They had used epoxyacryl resin beads to bind the dehydrogenases. The cofactor NAD^+ was provided in solution. Wang and Chen (1994) incorporated NAD^+ and LDH into a graphite-epoxy matrix, by polishing the surface new NAD^+ and LDH are exposed.

The use of porphyrin-containing redox proteins has gained wider acceptance as more studies on their behaviour as electron mediators in biosensors have been carried out. The early work by Eddowes and Hill (1977) showed with the addition of 4,4'-bipyridyl, it was possible to improve the rate of electron transfer for cytochrome *c* on gold electrodes. Schmidt and Schuhmann (1996) suggested the importance of the alignment in orientation of redox centres for increasing the rate constant of the electron transfer reaction. Schmidt and Schuhmann (1996) also described the use of artificial promoters absorbed as a monolayer on the electrode surface to align and orient the protein on the electrode surface using complementary charge arrangements. Schmidt and Schuhmann (1996) covalently bound microperoxidase to a cystamin-monolayer on a modified gold

electrode using carbodiimide condensation. Microperoxidase consists of a haeme moiety bound to 11 amino acids. This method gives the porphyrin ring direct access to the electrode surface for electrochemical communication.

In conclusion, there is quite a lot of evidence that suggests our proposal that haeme acts both to bind LDH to the Pt surface and as an electron acceptor may be correct. However, there is still much work to be done. The inconsistency of making successful conjugates still may be related to what was found in Conjugate-GO. There it was likely that either active esters of haeme had failed to bind to the enzyme because the conditions were not optimal or aggregated haeme that formed competed for space with enzyme-conjugates on the Pt electrode surface.

Chapter 6

6.1. Ethanol Analysis Using Alcohol Dehydrogenase Electrodes

Ethanol is a major industrial product. Ethanol is produced in food, beverages, and fermentation processes, or as by products of industries, e.g. from pulping industries. Biosensors can be used to efficiently monitor the ethanol both as a product or a by-product of industries.

There are two types of enzymes suited for this task; alcohol oxidase and alcohol dehydrogenase (Canh, 1993). The advantage of alcohol oxidase is its similarity to glucose oxidase where oxygen will regenerate the reduced enzyme without any further need for other cofactors. Oxidases are dependent on adequate availability of oxygen in the sample. When the oxygen level is low the linear range of alcohol oxidase becomes limited to low concentrations of ethanol (Clark, 1972). Alcohol oxidase is the preferred choice for biosensor design as amperometric measurements using alcohol dehydrogenase have not been very successful in "real" sample determinations (Blaedel and Enstrom, 1980). Biosensors using alcohol dehydrogenase require the cofactor nicotiamide adenine dinucleotide (NAD^+) to be present. The high cost of NAD^+ and the increased potential for electrode fouling limits the use of alcohol dehydrogenase. The reoxidation of NADH to NAD^+ requires potentials of over 1 V which results in the formation of radical intermediates leading to fouling of the electrodes (Braun *et* al, 1975; Albery & Bartlett, 1984; Blankespoor and Miller, 1984; Gorton, 1986; Yon Hin & Lowe, 1987; Matsue et al., 1987).

6.2. Determination of Ethanol using Conjugate-Alcohol Oxidase (Conjugate-AO) Electrodes

Alcohol oxidase (AO) can be used for the detection of primary alcohols.

The enzyme catalyses the following reaction:

$$R\text{-}CH_2OH + O_2 \longrightarrow R\text{-}CHO + H_2O_2$$

The production of hydrogen peroxide can be determined amperometrically using a Pt electrode poised between +550 mV and +800 mV.

Guilbault *et al.* (1983) incorporated AO into an oxygen electrode. It produced good results in the measurement of ethanol in blood samples when compared to the results obtained using gas chromatography. Verduyn *et al* (1984) developed a sensor for direct and continuous ethanol measurement in fermentation processes using AO. The commercially available industrial alcohol sensor from Yellow Springs Instruments, YSI Model 27, can reliably measure ethanol concentrations up to 94 mM ethanol with an error of less than 2 % (Mason, 1983).

Large (1993) described preliminary experiments using AO conjugated to haeme. The response of a Conjugate-AO electrode to increasing concentrations of methanol was linear. Large (1993) reported that there was only a small response to ethanol. This section of work aims to further investigate the possibility of improving the results obtained by Large (1993) on the use of Conjugate-AO for ethanol measurement.

6.2.1. Electrochemical measurements of Conjugate-AO for the determination of Ethanol concentration

Figure 6-1 shows one of amperometric recordings made using Conjugate-AO. There was no discernible current response obtained from Conjugate-AO electrodes that was dependent on the concentration of ethanol.

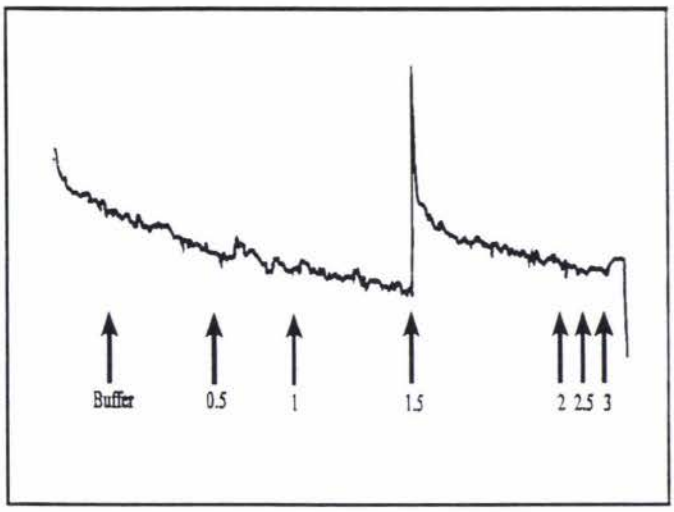

Figure 6–1. A scanned trace of amperometric analysis of Conjugate-AO with increasing amounts of added ethanol. The injection sites were indicated by an arrow. Each injection increased the concentration of ethanol in solution by 0.5 mM. The data was collected in batch measurement. The potential was poised at +600 mV with a temperature controlled at 25°C.

When the response to ethanol had not been satisfactory, Large (1993) substituted methanol in place of ethanol. This was repeated in the present study. The results obtained from methanol measurements were similar to those seen in Figure 6-1, i.e. no discernible linear current response to methanol was obtained. Two further preparations of Conjugate-AO electrodes were made and evaluated electrochemically (potential poised at +600 mV). The results were found to be similar to those obtained in Figure 6-1, which gave a total of three trials of Conjugate-AO electrodes that did not respond amperometrically to either ethanol or methanol. The results suggested that there maybe problems associated with making successful Conjugate-AO. This line of investigation was not pursued, instead attempts were made to develop alcohol electrodes using alcohol dehydrogenase conjugated to haeme. The initial aim of this study was to conjugate haeme to alcohol dehydrogenase (ADH) and not to AO, but an attempt to replicate the work of Large (1993) was thought to be useful at the time.

6.3. Determination of Ethanol using Conjugate-Alcohol Dehydrogenase (Conjugate-ADH) Electrodes

Alcohol dehydrogenase (ADH) as with lactate dehydrogenase (LDH) requires the cofactor NAD^+ to be present. As in the case with LDH, NADH cannot be reoxidised by

oxygen. The regeneration of NAD^+ from NADH can only occur through the use of high potentials or through electron mediators. The advantage of using ADH in preference to AO is that it eliminates the dependence on oxygen, but ADH's use is limited due to the high financial cost of the cofactor NAD^+ (Canh, 1993). Previous research by others on the use of ADH has concentrated on two main areas, immobilisation of cofactor and enzyme through entrapment or co-immobilisation of cofactor directly to the electrode. The cofactor NAD^+ can be either entrapped within a membrane such as a dialysis membrane (Kitagawa *et al*, 1989), entrapped in a layer of bovine serum albumin (Miyamoto *et al*, 1991) or be immobilised to an insoluble support such as sepharose with the aid of spacer groups so that the cofactor is flexible enough to take part in the enzymatic reaction (Ukeda *et al*, 1989).

ADH catalyses the following reaction:

$$CH_3CH_2OH + NAD^+ \longrightarrow CH_3CHO + NADH + H^+$$

After the early success of conjugating LDH to haeme (see section 5.3.2.), the same method of enzyme immobilisation was applied to ADH. The reason was to investigate the electrochemical behaviour of haeme as an electron mediator and as a possible substitute for cofactor NAD^+. Large (1993) made some preliminary evaluations using Conjugate-ADH for the purpose of measuring ethanol. One of the cyclic voltammograms showing haeme conjugated to ADH in the study by Large (1993) showed a difference between buffer alone and when the substrate ethanol was added. However, upon evaluation of the haeme-ADH biosensor amperometrically, it was found to response linearly to various concentrations of methanol but not to ethanol.

6.3.1. Evaluation of Conjugate-Alcohol Dehydrogenase Electrodes

The suitability of Conjugate-ADH was initially evaluated using cyclic voltammograms (CV) as had previously been done (Conjugate-GO and Conjugate-LDH, see section 4.7.1. and section 5.3.2., respectively). The procedure was as follows. An "end on" Pt electrode with platinum diameter of 3.7 mm encased in a glass tube (40 mm in length) was initially cleaned using an alumina polish followed by an electrochemical cleaning treatment (section 2.2). For a description of "end on electrodes" refer to section 2.2.

The CVs shown in Figures 6-2a, 6-2b and 6-2c were collected using a single platinum electrode. The electrode was polished with alumina and cleaned electrochemically in between collecting CVs of Bare-Pt, Haeme-Pt and Conjugate-ADH.

There was a major difference in the shape and position in the CV of Bare-Pt to the CV's of Haeme-Pt and Conjugate-ADH when only buffer was present. In Figure 6-2a, there was not a significant shift that occurred with the position of the CV containing 25 mM ethanol relative to buffer using a Bare-Pt electrode. In Figure 6-2b, there was a slight shift (approximately 0.1 μA) that occurred between the potential region of +100 mV and +800 mV in the presence of 25 mM ethanol when the CVs for Haeme-Pt were examined compared to buffer. In Figure 6-2c, there was a shift of 160 nA at potential region of -200 mV between the CV when 25 mM ethanol was present compared to buffer for Conjugate-ADH.

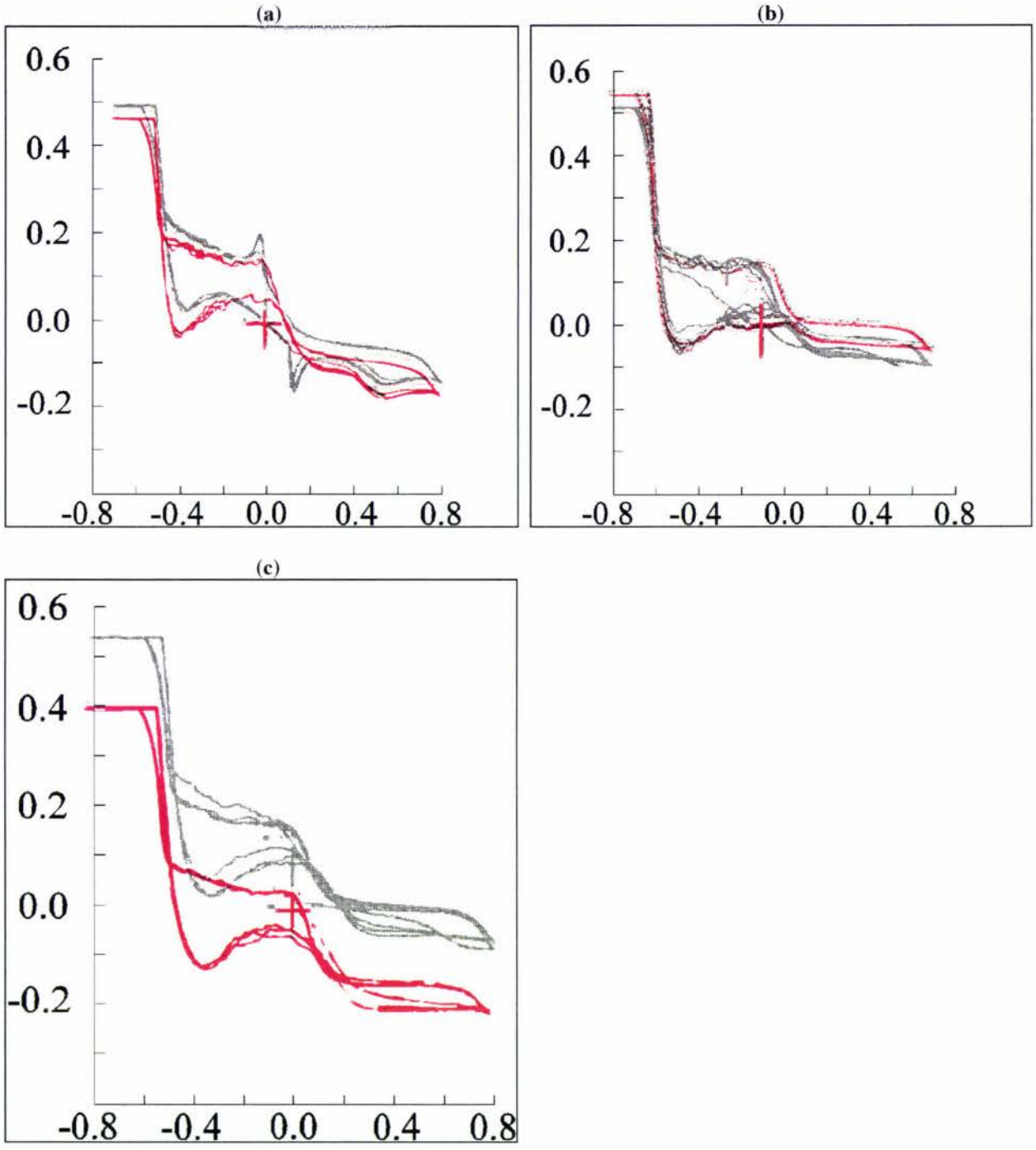

Figure 6–2. Cyclic voltammograms showing the comparisons between Bare-Pt, Haeme-Pt and Conjugate-ADH electrode's response, scanned between the potentials of +800 mV to -800 mV. (a) Bare-Pt. (b) Haeme-Pt. (c) Conjugate-ADH. The same Pt electrode was used in all three conditions. The buffer used was 0.1 M Tris/HCl, pH 7.5 at 25°C. The grey line denotes buffer only. The red line denotes the presence of 25 mM ethanol and buffer. NAD^+ was not present in any of the cases. The y-axis units are in microamps and the x-axis units are in volts.

In Figure 6-3, the CV's of Haeme-Pt and Conjugate-ADH were compared to each other in the presence of NAD^+. The comparison between Conjugate-ADH and Haeme-Pt in Figure 6-3 revealed smaller differences with the positions of the CV's when compared

to the CV of 25 mM ethanol to buffer at -200 mV. A difference of about 30 nA was seen at -200 mV between buffer and 25 mM ethanol for Conjugate-ADH in the presence of NAD^+ in solution (Figure 6-3b).

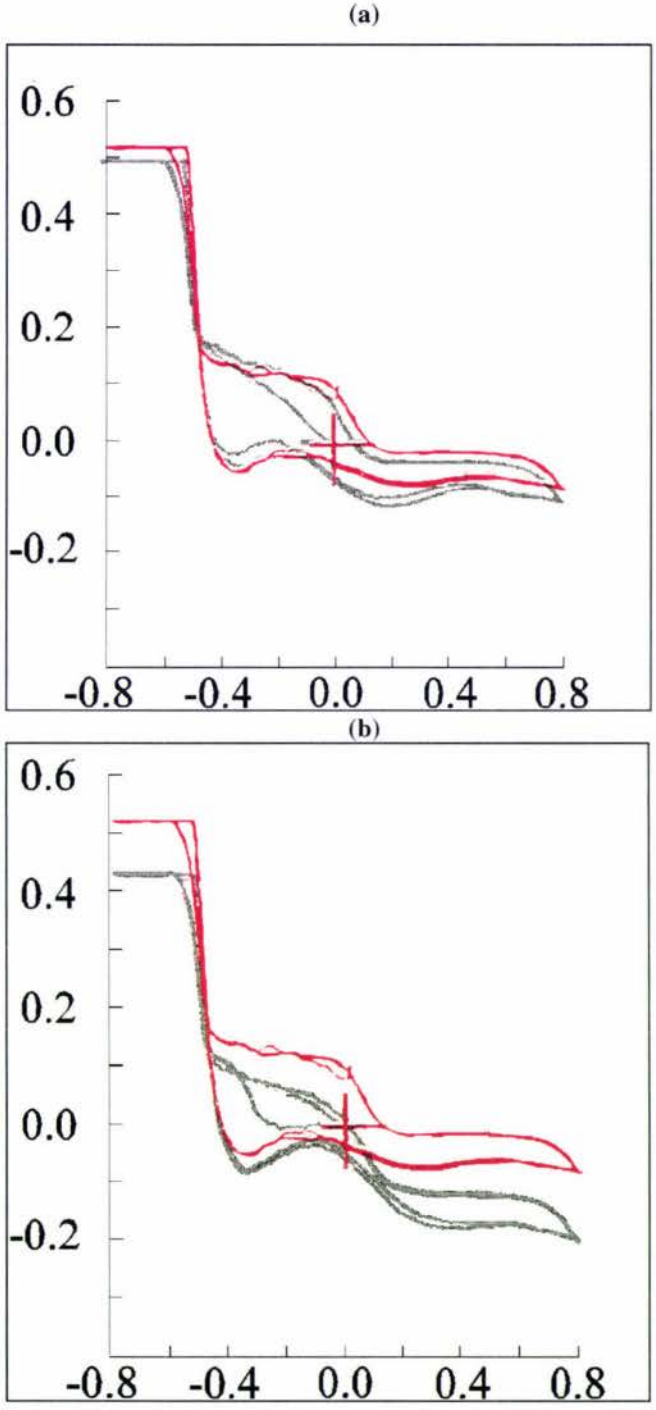

Figure 6–3. Cyclic voltammograms comparing Haeme-Pt and Conjugate-ADH electrodes in the presence of NAD^+. The electrodes were scanned between +800 mV to -800 mV. The buffer used was 0.1 M Tris/HCl, pH 7.5 at 25°C. The grey line denotes buffer and 10 mM NAD^+. The red line denotes 25 mM ethanol and 10 mM NAD^+ in buffer. The y-axis units are in microamps and x-axis units are in volts.

Next, the same Conjugate-ADH electrode as used in Figure 6-2 and Figure 6-3 was evaluated using the batch measurement method. The FIA was used initially to collect data for Conjugate-ADH, but the data collected was significantly affected by electrical noise. The electrical noise can be minimised more easily by collecting the data in a Faraday cage using batch measurement, where all the equipment used was grounded electrically. The results are shown in Figure 6-4. They show a definite increase in the amount of current detected at the electrode's surface when the enzyme ADH was present as compared to the readings for Bare-Pt or Haeme-Pt electrode. The response curve appears to show saturation kinetics.

It is also interesting to compare the results collected for CV analysis of Bare-Pt, Haeme-Pt, and Conjugate-ADH to the sensor response of each of these three conditions. As previously mentioned, the CV's showed differences, when ethanol was present for Conjugate-ADH but not for Haeme-Pt and Bare-Pt. In the results in Figure 6-4, Conjugate-ADH gave the highest response to ethanol compared to Bare-Pt and Haeme-Pt. However, the electrochemical response of the electrode in Figure 6-4 was in the picoamps range as opposed to nanoamps range (Figure 6-2) for the CV data. There isn't any obvious explanation for this discrepancy.

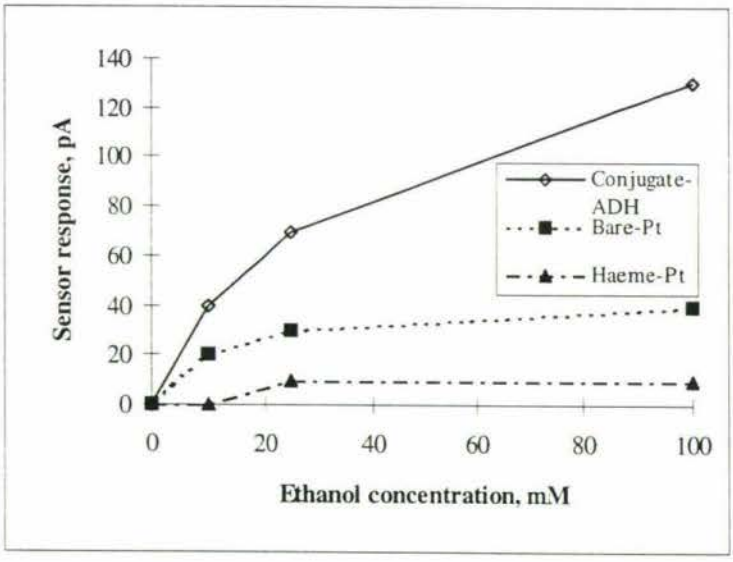

Figure 6–4. Comparison of sensor response between three conditions of a single Pt electrode. The potential was poised at -200 mV. The temperature controlled at 25°C.

The results for hydrodynamic potentiometry shown in Figures 6-5a and 6-5b both exhibited an atypical response. A typical response for hydrodynamic potentiometry is there should be an applied potential region where a plateau is reached. This obviously

did not occur with these Conjugate-ADH electrodes. This could be because the selected potential range was too narrow to see the plateau region. The sensor response for Conjugate-ADH was dependent on the concentration of ethanol (Figure 6-5a). This result concurs with that seen in Figure 6-2c. The electrochemical response was then evaluated. The results are not shown, as the electrochemical response was too electrically noisy to show any discernible response.

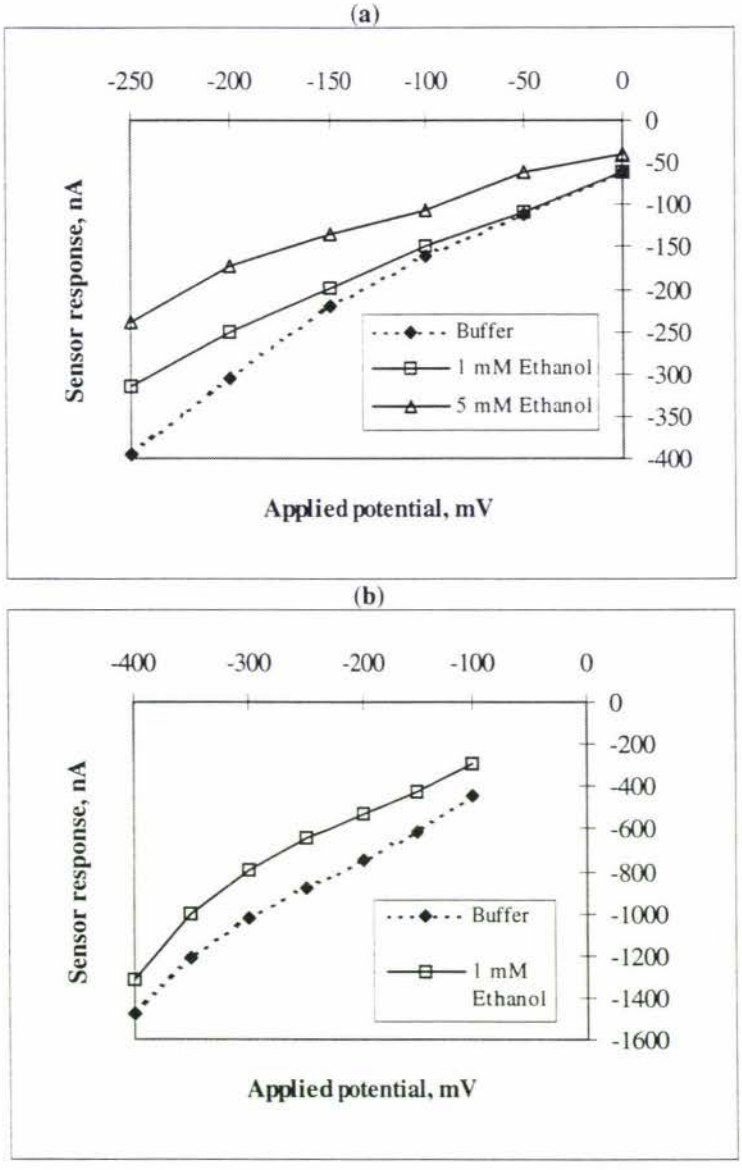

Figure 6–5. Results from Conjugate-ADH hydrodynamic potentiometric study. (a) Shows the range between 0 mV and -250 mV with three levels of ethanol concentrations. (b) Shows the range between 0 mV and -400 mV with two levels of ethanol concentrations. The buffer used was 0.1 M phosphate buffer, pH 7.5 at 25°C.

Based on the results obtained in Figure 6-6, a decision was made to change the poised potential from -200 mV to -100 mV. The change was made for two reasons; chiefly,

because higher sensor responses were not obtained, despite the μA differences obtained in the CV's and hydrodynamic voltammetry results seen in Figure 6-2 and Figure 6-5. The second reason, was due to the results shown in Figure 6-6. The second reason may later have proved to be misleading as the differences seen are not too large. Too much weight was placed on the small differences seen in Figure 6-6 but this is all in hindsight. However, six of the nine trials using Conjugate-ADH had the potential poised at -100 mV. The poised potential readings that followed after the CV responses were recorded as shown in Figure 6-6 did not show any linear response to increasing ethanol concentrations.

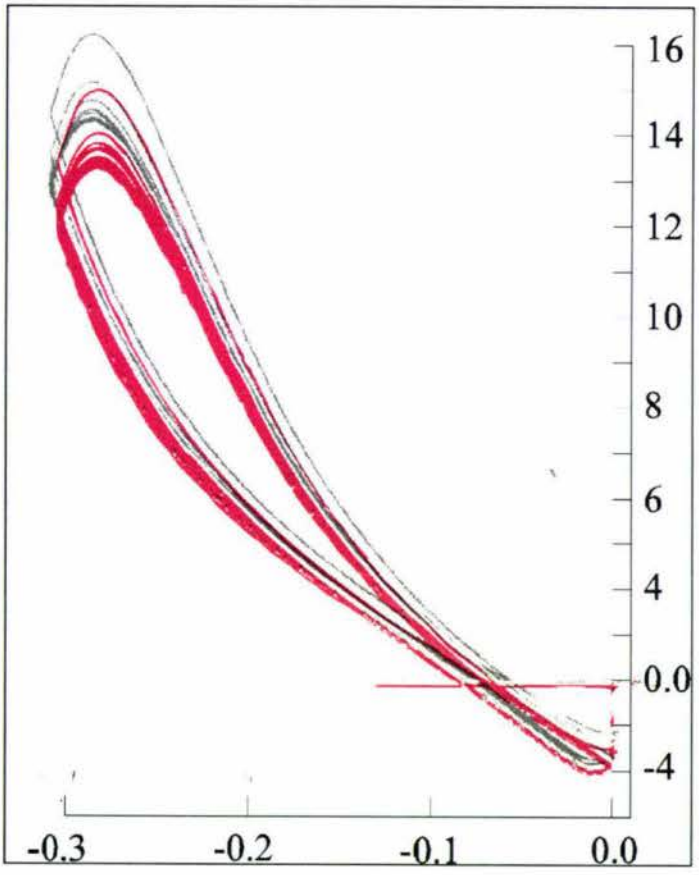

Figure 6–6. Cyclic voltammogram showing the reason for the decision to switch to a poised potential of -100 mV from -200 mV. The grey line denotes buffer and the red line denotes 50 mM ethanol. The y-axis units are in microamps and x-axis units are in volts.

The conjugation of Conjugate-ADH was repeated again, and the sensor responses to ethanol were again recorded. The results shown in Figure 6-7 showed again another Conjugate-ADH electrode that did not require NAD^+ to obtain a signal from ethanol. Only one reading is reported because repeated readings were electrically very noisy.

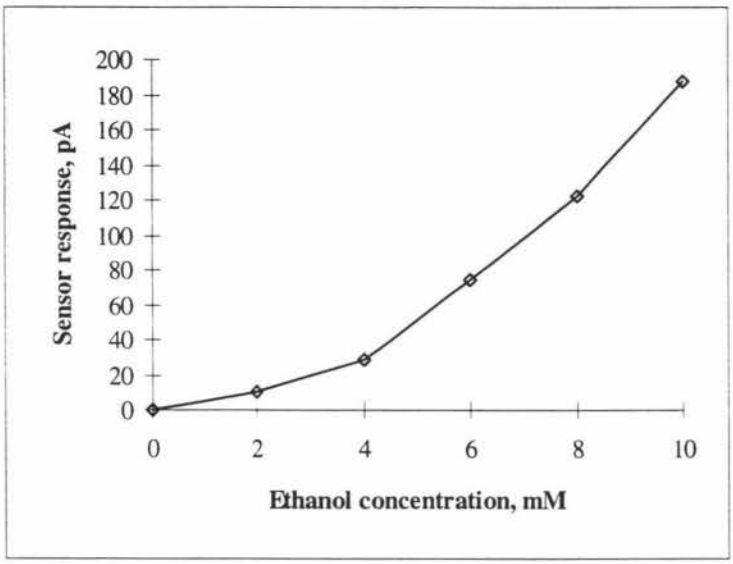

Figure 6–7. Sensor response of Conjugate-ADH to increasing concentrations of ethanol. Potential was poised at -100 mV. Buffer used was 0.1M Tris/HCl, pH 7.5 at 25°C. NAD^+ was absent.

As with Conjugate-LDH (section 5.3.3.) comparisons were made between the presence of NAD^+ and the absence of NAD^+. In the case of Conjugate-ADH, a preamplifier was required as the current responses obtained were very low. Circumstances, required the use of a commercial Pt microelectrode (10 microns in diameter). The microelectrode was initially cleaned using an electrochemical cleaning method (see section 2.2). The electrode was then dipped in a prepared solution of Conjugate-ADH and left overnight. The following day fresh buffers and standards were prepared. One set of standards contained NAD^+ and the appropriate ethanol concentration and the other set contained only the appropriate ethanol concentration. Batch measurements were made in a Faraday cage to minimise electrical noise and the temperature controlled at 25°C. The results are shown in Figure 6-8. The sensor response for the condition of NAD^+ absent was linear between 0 and 3 mM ($y = 0.35x - 0.36$, $r^2 = 0.957$), however the linearity for when NAD^+ was present was not as good ($y = 0.16x + 0.16$, $r^2 = 0.769$). The picoamps range of response seen in Figure 6-8 was not unexpected for a microelectrode.

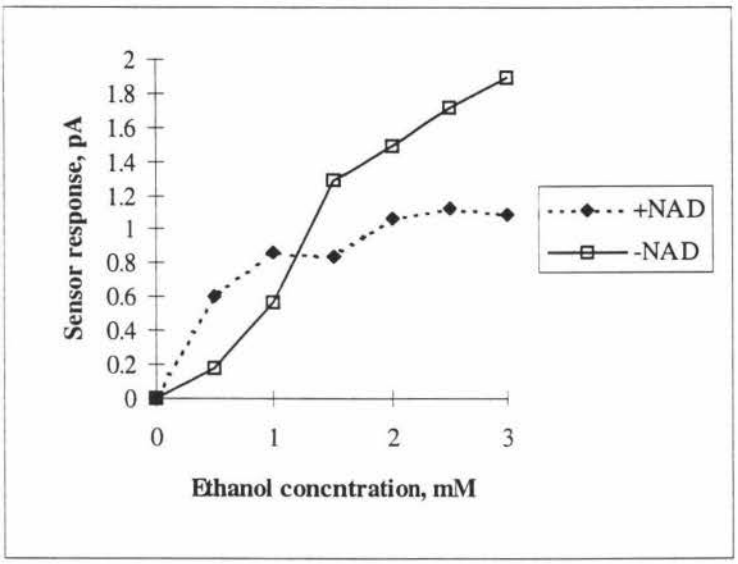

Figure 6–8. The comparison between when NAD^+ was present and when NAD^+ was absent using a single Pt microelectrode (10 microns in diameter). Potential was poised at -100 mV. Buffer used was 0.1 M Tris/HCl, pH 7.5 at 25°C.

6.4. Experiments Performed to show Interaction or Non-interaction of other Substances

The aim of this section was to look at the sensor response of Conjugate-ADH in the presence of other alcohols. This section also looks at the behaviour of Haeme-Pt and Bare-Pt electrodes in the presence of the same types of alcohols.

6.4.1. Interaction of Ethanol, Methanol and 2-Propanol on Conjugate-ADH electrodes

The responses of Conjugate-ADH electrodes were characterised in the presence of ethanol, methanol and 2-propanol (Figure 6-9.) and in the absence of NAD^+. The response to ethanol was fairly linear ($r^2 = 0.8660$) and the current responses were higher when compared to methanol. The response to methanol was also fairly linear ($r^2 = 0.8350$). In this experiment, there wasn't a significant response to 2-propanol, and it did not show linearity ($r^2 = 0.2724$). The source of ADH was from bakers yeast, so it can metabolise ethanol and methanol but not tertiary alcohols. The results from regression analysis shown in Table 6-1 indicate although the r^2 value for methanol showed the data fits a straight line, the regression analysis which compares the slope to zero slope finds

methanol and 2-propanol not significant. The regression analysis for ethanol was significant at the 95 % confidence interval. The results demonstrate that Conjugate-ADH significantly responds to ethanol, but not to methanol and 2-propanol.

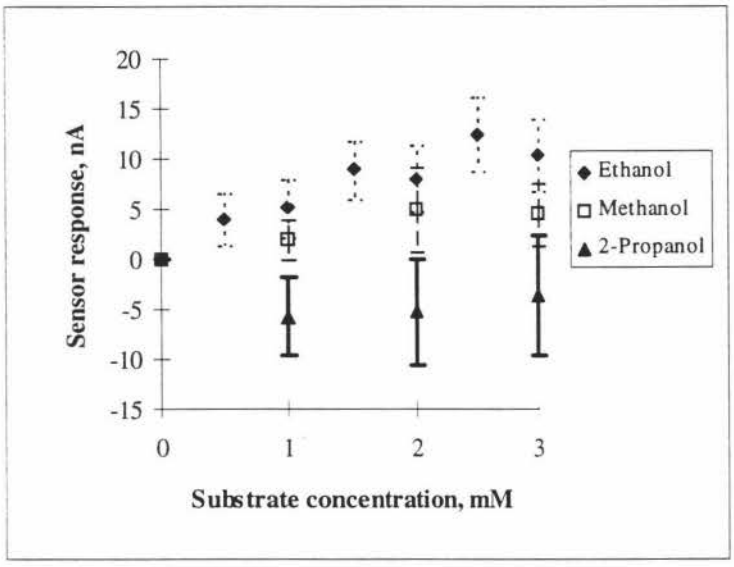

Figure 6–9 Response of Conjugate-ADH electrodes following treatment of various substrates. There were four electrodes involved for each condition (ethanol, methanol and 2-propanol). There were also two replicates for ethanol, one for methanol and one for 2-propanol for each of the four electrodes. Potential poised at -100 mV. Tris/HCl 0.1 M, pH 7.5 used as buffer. Error bars represent standard error for each condition.

Table 6-1. Results from Regression Analysis of Conjugate electrodes in Ethanol, Methanol and 2-Propanol

Treatment	Regression equation	r^2	Calculated F significance
Ethanol	y = 3.65x + 1.51	0.866	0.0023
Methanol	y = 1.66x + 0.42	0.835	0.0860
2-Propanol	y = -1.05x - 2.09	0.272	0.4781

There were four replicates for ethanol, methanol and 2-propanol.

6.4.2. Interaction of Ethanol, Methanol and 2-Propanol on Haeme-Pt electrodes

The integrity of an electrode can be compromised if it is able to detect or be influenced by the presence of another substance not specific to the enzyme conjugate. To check whether Haeme-Pt electrodes can produce a current response in the absence of Conjugate-ADH, Haeme-Pt electrodes were also tested in the presence of ethanol, methanol and 2-propanol. The preparation of Haeme-Pt electrodes is described in section 2.2.2. The results are shown in Figure 6-10.

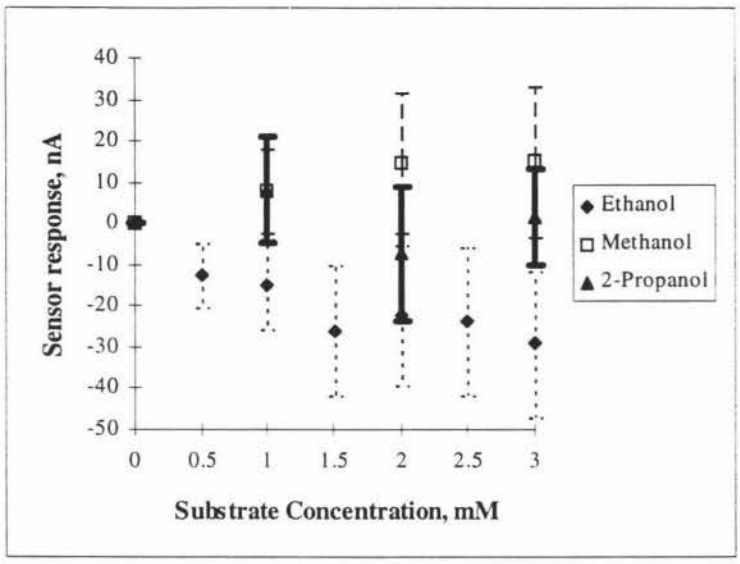

Figure 6–10. Response of Haeme-Pt electrodes following treatment of various substrates. There were four electrodes involved for each condition (ethanol, methanol and 2-propanol). There were also three replicates for ethanol, one for methanol and one for 2-propanol for each of the four electrodes. Potential poised at -100 mV. Tris/HCl 0.1 M, pH 7.5 used as buffer. Error bars are present indicating standard error for each condition.

Regression analysis were performed on all three data sets (ethanol, methanol and 2-propanol). In the results shown in Table 6-2, regression analysis of the data shows no significant responses for Haeme-Pt electrodes in ethanol, methanol or in 2-propanol. This would suggest that haeme Pt alone does not give a response to ethanol.

Table 6-2. Results from Regression Analysis of Haeme-Pt electrodes in Ethanol, Methanol and 2-Propanol

Treatment	Regression equation	r^2	Calculated F significance
Ethanol	y = -8.37x - 5.78	0.814	0.5737
Methanol	y = 5.22x + 1.58	0.895	0.5021
2-Propanol	y = -1.14 + 2.22	0.053	0.4755

There were four replicates for ethanol, methanol and 2-propanol.

6.4.3. Interaction of Ethanol, Methanol and 2-Propanol on Bare-Pt electrodes

The definition for a Bare-Pt electrode is a Pt electrode which has been cleaned and does not contain any other material on the surface of the Pt disk. Preparation of Bare-Pt electrodes are described in section 2.2.1. This set of readings looked at Bare-Pt electrodes responding to ethanol, methanol and 2-propanol. The ideal condition for Bare-Pt electrodes as with Haeme-Pt electrodes is no response obtained during their use.

In Figure 6-11 the results are as expected except when 2-propanol is used. It seems if 2-propanol is present with the test sample it is likely to give a false positive result.

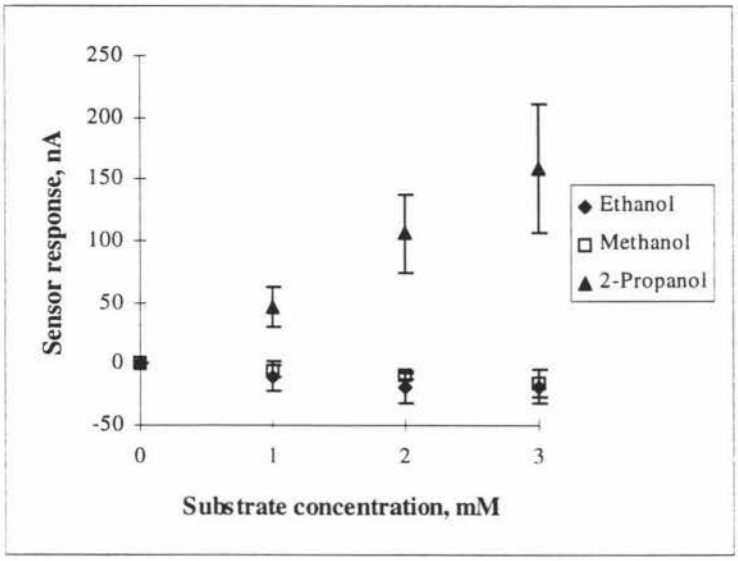

Figure 6–11. Response of Bare-Pt electrodes following treatment of various substrates. There were four electrodes involved for each condition (ethanol, methanol and 2-propanol). There were also three replicates for ethanol, one for methanol and one for 2-propanol for each of the four electrodes. Potential poised at -100 mV. Tris/HCl 0.1 M, pH 7.5 used as buffer. Error bars are present indicating standard error for each condition.

Regression Analysis was also performed on the data. The results are described in Table 6-3. The results in Table 6-3 show at 95% confidence interval, the responses of ethanol and methanol were not significant. This was as expected. The result from 2-propanol is significant at 95% confidence interval. This was not expected. Figure 6-12 shows the calibration data for Bare-Pt electrodes in 2-propanol. The data was significant at 95% confidence interval. There isn't any obvious explanation for this phenomenon. It was possible that a systematic artefact had developed during the course of the experiment.

Table 6-3. Results of Regression Analysis for Bare-Pt electrodes in Ethanol, Methanol and 2-Propanol

Treatment	Regression equation	r^2	Calculated F significance
Ethanol	y = -9.65x - 4.54	0.847	0.6456
Methanol	y = -3.23x - 2.44	0.717	0.6025
2-Propanol	y = 53.51x - 2.52	0.998	0.0177

There were four replicates for ethanol and 2-propanol, and one of methanol.

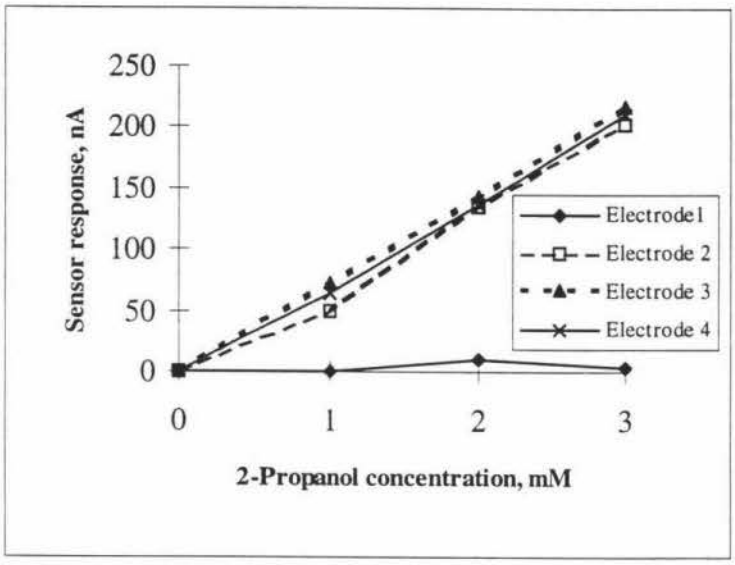

Figure 6–12. Bare-Pt electrode in 0-3 mM 2-Propanol. There were four Bare-Pt electrodes used. One calibration set per electrode. The buffer used 0.1 M Tris/HCl, pH 7.5.

6.5. Discussion

The results for Conjugate-ADH electrodes as a reagentless biosensor for ethanol show some promise. The current response obtained for Conjugate-ADH was quite low in comparison to Conjugate-LDH. The range of Conjugate-ADH was mainly in the picoamps range. There was a total of 9 trials of making Conjugate-ADH electrodes, 6 of those trials were successful (though the signals were in picoamps range but they did give a positive linear response to ethanol). So with Conjugate-ADH electrodes, a pre-amplifier was required to pick up the signals. The definition of a successful Conjugate-ADH response was defined as a dependent relationship between sensor response and ethanol concentration. The results must also be taken with care as out of the six trials that were successful, only one trial gave sensor responses in the nanoamps range. The picoamp responses detected, although correlated with ethanol concentration, may be the result of baseline drift in the positive range. During the six trials, appropriate care was taken to minimise false positive responses, meaning injections of buffer only were included before responses to ethanol were recorded. Conjugate-ADH electrodes were more successful than Conjugate-LDH electrodes, as even with the use of a pre-amplifier some of the preparations of Conjugate-LDH did not show any linear current responses as lactate concentrations were raised. However, the single batch of Conjugate-LDH

electrodes that gave sensor responses in the microamp range gave convincing evidence of these electrodes.

The comparison of CVs of Bare-Pt, Haeme-Pt and Conjugate-ADH showed that there may be some marginal differences between all three conditions. There was some concern about the CV obtained for Bare-Pt at 25 mM ethanol, as it was not expected to see a difference in the shape, although the relative position of the CV after addition of ethanol did not change except at high positive potentials. When the CVs of Haeme-Pt and Conjugate-ADH were compared, there was some concern raised as to the potential selected for poised potential studies of Conjugate-ADH. The selected potential of -200 mV (versus Ag/AgCl) or -100 mV was used as that was the same potential used for Conjugate-LDH. The initial Conjugate-LDH electrodes responded well at -200 mV.

With Conjugate-ADH electrodes, when NAD^+ was present, the current response seemed to be lower and less linear in comparison to when NAD^+ was absent. This result can be compared to the same situation as in Figure 5-7 for Conjugate-LDH. When NAD^+ was present the current response was slightly lower than when NAD^+ was absent. The recurring problem encountered with Conjugate-ADH electrodes was the number of times the electrodes were able to be used. It was common that only one or two readings could be made using one electrode. There isn't any obvious explanation for this as yet.

The results from the study of interfering effects from other alcohols such as methanol and 2-propanol showed that with Conjugate-ADH, the electrode was only specific for ethanol, and with Haeme-Pt no significant response was obtained for any of the three substrates tested. The results for Bare-Pt showed a significant response to 2-propanol. The reason for this not known.

During the study of stability of ethanol stored in human blood obtained from motorists who failed breath tests, Smalldon (1973) found human erythrocytes could oxidise ethanol to acetaldehyde. The mechanism was suggested to be linked to the oxyhaemoglobin-methaemoglobin redox system. If haemoglobin in blood is capable of oxidising ethanol to acetaldehyde, it is possible that a similar reaction could occur with a haeme moiety bound to alcohol dehydrogenase. This would explain the observation that ADH conjugated to haeme responds to ethanol in the absence of the cofactor NAD^+. These results suggest that with further research it may be possible to develop an ADH-haeme biosensor for ethanol measurement where the inclusion of NAD^+ was not

required. Such a biosensor would avoid the need to use an additional trapping system for NAD^+. For example, Miyamoto *et al* (1991) designed an alcohol sensor using alcohol dehydrogenase with the cofactor NAD^+ entrapped in a layer of bovine serum albumin (BSA). The method was successful in making a sensor for alcohol which required no further additions of NAD^+.

It is also clear that a large amount of research is still required to improve the sensitivity of the sensor responses to ethanol as most of the responses found in this study were quite low. This may be related to the problems faced with glucose oxidase conjugated to haeme electrodes, where responses were also low in some of the Conjugate-GO preparations. In writing up, it was also recognised that during the electrochemical readings, the pH of the solution can be raised to allow optimal activity of the enzyme. In this case for Conjugate-ADH electrodes, a pH of around 8.5 would have been more suitable for optimal activity of alcohol dehydrogenase to metabolise ethanol.

Chapter 7

7.1. Discussion and Conclusions

The current study extends the work of Large (1993) in using haeme to immobilise enzymes on platinum electrodes. The results from the work of Large (1993) showed that haeme could be conjugated to glucose oxidase (GO), alcohol oxidase and alcohol dehydrogenase. Large (1993) had also suggested that haeme was able to act as an electron mediator. The aim of the current study was to further evaluate the possibility of using haeme conjugated to various enzymes, such as alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH). The most significant result from the study of Large (1993) was that a response to methanol was seen with a ADH-haeme electrode in the absence of NAD^+.

The biosensors developed during the present study using haeme conjugated to enzymes were able to detect specific analytes in solution, though the reliability of the fabrication depended heavily on having successful haeme-enzyme conjugates formed in the first place. Work on the investigation as to why haeme enzyme conjugates were not formed was done in stages. The first stage looked at the process where haeme active esters were made. The formation of haeme active esters can be monitored using thin layer chromatography (TLC) plates. The results showed that haeme active esters were formed. The second stage in the haeme-enzyme conjugation process was the binding of activated haeme esters to the enzymes. It was not possible to check whether haeme enzyme conjugates were formed until after purification through a Sephadex G-25 column. Spectrophotometric analysis revealed that although there was some evidence of haeme peaks during UV-Vis analysis, the haeme peaks were not as large as the ones found in the study of Large (1993), relative to the protein peak (280 nm).

When electrochemical activity was not detected from these haeme-enzyme conjugate electrodes, a comparison was made between UV-Vis analysis of haeme only, glucose oxidase only and Conjugate-GO. Spectrophotometric analysis of fractions containing enzyme-conjugates, haeme only and enzyme only were always recorded after purification through a G-25 Sephadex column. The comparison of some Conjugate-GO and glucose oxidase fractions only revealed no difference between the amount of haeme

peak present in glucose oxidase only when compared to Conjugate-GO (where it was expected to be higher due to GO conjugated to haeme). Comparison between UV-Vis scans of the enzyme-haeme conjugates that failed to produce successful enzyme electrodes and the ones that did produce successful enzyme electrodes, revealed haeme peaks were found to have higher absorbance at 380 nm in comparison to that of glucose oxidase only for enzyme-conjugates that produced successful electrodes. This suggests that part of the problem of not being able to produce enzyme electrodes using this method is because of the lack of binding between haeme active esters and enzymes. Another possibility that must be considered for enzyme electrodes not working as expected, is that the conjugation process between activated haeme and enzymes did occur, but the enzyme-haeme conjugates failed to absorbed on the Pt electrode's surface for one reason or another. The use of cyclic voltammograms may help answer this question, as the CV recorded for haeme only absorbed to Pt electrodes and enzyme-haeme conjugates absorbed to Pt electrodes were more similar in shape compared to the CV recorded for Bare-Pt electrodes (see the work from Conjugate-ADH). However, the electrochemical work carried out for Conjugate-ADH resulted in either very little current detected or no response at all to ethanol concentrations. This was in spite of the CV results showing absorption of Conjugate-ADH onto the Pt electrodes. The work with Conjugate-GO suggested that haeme aggregates were formed during the conjugation process as suggested by Luo (1994). Aggregated haeme could also bind to the Pt electrode's surface. This could result in the change in CV shape as seen with Conjugate-ADH compared to Bare-Pt, which would explain the very low electrochemical response obtained. The enzyme-haeme conjugates would have to compete for space on the Pt electrode's surface with aggregated haeme.

In the present study, conjugations were initially carried out at pH 7.0 in phosphate buffer, with some success, but for other reasons the pH and the type of buffer used was changed because it was suggested that during freezing, Tris was preferable to phosphate (Blackwell, personal communication, 1993). During the work by Large (1993), the conjugation process took place at pH 7.0 in 0.1 M phosphate buffer. It is possible that during the conjugation process for Large (1993), the pH may have dropped to as low as pH 6.2. It is possible that the acidic conditions relaxed the enzymes so that haeme was able to bind close to the active site. Urea could also serve the same purpose as slightly

acidic conditions. In retrospect, it seems possible that the conjugation reaction may have been inhibited by the higher pH. It is possible that at pH 7.0, or perhaps a lower pH for the duration of the conjugation process could promote relaxation of the conformation of enzyme molecules that allows haeme molecules to bind at or close to the active site of enzymes.

In the present study, when successful conjugation did occur, and Conjugate-ADH or Conjugate-LDH electrodes were successfully prepared, it was found that these electrodes responded to substrates (ethanol and lactate) in the absence of NAD^+, as Large's (1993) preliminary results had suggested. If successful conjugation requires a partial unfolding of the enzymes, with lower pH, it is possible that conjugation sites close to the active site will be exposed. If haeme is bound close to the active site, it might well act as an electron acceptor in place of NAD^+ in the ADH and LDH reactions. In this case, haeme is performing a dual role in the formation of these electrodes. It is providing a means to anchor the enzyme to the Pt electrode's surface, and acting in the transfer of electrons to the Pt electrode's surface. Bonding to the Pt surface could be achieved by haeme molecules conjugated to any point on the enzyme surface, while electron transfer is more likely to require haeme to be conjugated close to or at the active site. There is evidence in the literature to support both of these possible roles of haeme.

Porphyrins are generally found bound to proteins, and a lot of effort had been devoted to finding out how they interact. Many studies have focused on the 3-D structure and changes in 3-D structure. Such studies have supplied important facts, but also fundamental in these interactions are the energy relationships (Caughey *et al*, 1969). In the case for haeme binding, for example, consider the changes in protein structure that occur when the haeme iron of cytochrome c is oxidised and reduced. Similarly, when oxygen is bound to the haeme iron of haemoglobin, protein structure changes are generated at the exact expense of oxygen-haeme binding energy, and most interestingly, some of the binding energy is transmitted, in the form of energetic structure changes, to distant binding sites where it reappears as binding energy. The geometry of the structure will determine the pathways of energy flow and action, but large-appearing changes may have little free energy while sub-angstrom distortions may carry large energies. This suggests that changes to the enzyme conformation, as suggested earlier due to pH

changes, could result in increase binding energy for haeme and the work by Caughey *et al* (1969) stresses the importance of optimal geometry for energy flow.

There have been a large number of reports concerning the role of haeme in electron transfer citing the importance of pi-bonding between iron (Fe) of haemes and haemeproteins (haemoglobins and myoglobins) and ligands such as oxygen, carbon monoxide, nitric oxide, and alkylisocyanides. The iron (II) atom could serve as a donor (pi-donor) as well as an acceptor (sigma acceptor). This could be achieved by altering electron availability at the iron (II) atom, thereby changing the relative importance of the donor and acceptor roles of iron. Differences in the porphyrin structure or in the *trans* ligand could either decrease or increase the strength of O_2 (or CO) bonding. Reactions of iron (II) porphyrins with oxygen and carbon monoxide are known to be sensitive to both the electron withdrawing or donating power of substituent groups at the periphery of the porphyrin ring. The pi→pi transitions in porphyrin electronic spectra are markedly influenced by the type, oxidation state, and ligands of the central metal ion (Caughey *et al*, 1966). Changing substituents at the periphery of the porphyrin ring can effect ligand bonding to the central metal ion (Bearden *et al*, 1965). Participation of the porphyrin ring system in donor-acceptor type interactions is well known (Gouterman and Stevenson, 1962; Mauzerall, 1965).

In the work with enzyme-haeme conjugates especially Conjugate-LDH, it was found Conjugate-LDH was capable of interacting with the substrate lactate in the absence of the cofactor NAD^+, generally needed in a normal dehydrogenase enzyme functions. The work in the oxidation and reduction of iron may hold the key to the understanding of how Conjugate LDH and Conjugate-ADH are able to work in the absence of NAD^+.

The initial electrophilic attack takes place at the ferrous rather than the ferric porphyrin stage. This suggestion is supported by the observation that ferric porphyrins are not very susceptible to electrophilic attack (Paine and Dolphin, 1973). The suggestion that the oxidation of Fe(II) porphyrin might take place to give Fe (II) π-cation radical may also be extended to the function of the cytochromes (Dolphin *et al*, 1973). The electron transfer that accounts for the overall Fe(II)↔Fe(III) couple is unlikely to involve direct electron transfer at the iron, and Dolphin *et al* (1970) believed that electron transfer occurs initially at the porphyrin periphery followed by an internal electron transfer.

$$Fe\ (II) \underset{+e^-}{\overset{-e^-}{\rightleftharpoons}} [Fe(II)haeme\ ]^{+\cdot} \rightleftharpoons Fe(III)haeme$$

A comparison of the results of the redox behaviour in cytochromes found in previous studies provide an insight to the redox behaviour of haeme-enzyme conjugates in the present study. Cytochromes are of the redox chain whose activity serves to transport electrons so that the energy liberated during oxidation would be released in small units, coupled to synthetic reactions (Dickerson, 1974). One of the main components in cytochromes (as in haeme-enzyme conjugates) is an Fe atom. The Fe atom's behaviour is defined by the nature of the porphyrin binding and the protein structure, in this case cytochromes. During oxidation and reduction processes, cytochromes do not undergo major structural changes (Dickerson, 1974). The major structural change that occurs in cytochromes is an opening of the haeme pocket when the molecule is oxidised. In the oxidised state in cytochromes, only one edge of the haeme is exposed to the outside environment, the rest of the porphyrin ring remains enclosed in the hydrophobic pocket created by the internal cytochrome environment (Sanger and Hill, 1990). Phenylalanine 82 is highly conserved in cytochromes. When cytochromes are oxidised, phenylalanine 82 swings out, thus exposing the hydrophobic haeme pocket. It is thought that this hydrophobic pocket is solvent accessible, therefore the opening of the pocket makes this region less hydrophobic during oxidation, while an electron is transferred from cytochrome reductase to cytochrome *c* via haeme. At this stage there are two possible routes for the electron transfer to occur, either via the porphyrin periphery or with the iron complex. The next step looks at what could happen if the iron complex in haeme-enzyme conjugates had changed oxidation states as a result of electron transfer.

The study by Kassner (1973) provides an important clue as to how the reduction of Fe(III) can be achieved. Experimental results and calculations from model systems and cytochrome haeme peptides by Kassner (1973) suggest that the reduction potential of iron increases with the increase in hydrophobicity of the surrounding medium.

The reduction process suggested:

$$H^+ + e^- + Fe(III)porphyrin^+ + A^- \rightleftharpoons \underset{\text{(neutral state)}}{Fe(II)porphyrin^\circ} + HA$$

A^- symbolises a negative counterion. Takano *et al* (1973) found a tyrosine (Tyr 67) involved as the counterion. The porphyrin ring donates two electron pairs to the iron complex, filling two of the iron *d* orbitals, forming the neutral complex Fe(II) (Dickerson, 1974).

Massey and Ghisla (1974) suggested the charge-transfer interactions are expected to be dependent on the orientation of the donor-acceptor partners in the complex. The orientation of the donor-acceptor complex can be influenced by the protein.

Konicker and Vallee (1969) in their summary suggested that the redox behaviour of metallocinium cations (ferricinium, bis-benzene-chronium cation, cobalticinium) have been correlated with their ability to form charge-transfer complexes with π-electron donor groups present in the primary structure of nucleic acids and proteins. Guanine and adenine, tryptophan and histidine residues seems to play an important role as electron donors in nucleic acids and proteins and in the potential interaction between metallocinium cations as acceptors. The essential feature in regard to compounds important in charge transfers (such as FAD, FMN, quinones) is electronic delocalisation (Konicker and Vallee, 1969).

It may be interesting to note the actions of chloroperoxidase (a ferrihemoprotein) which reacts with H_2O_2 in a similar way to catalase and horseradish peroxidase. The significance of chloroperoxidase is the presence of iron and changes to oxidation state of iron as it undergoes electron transfer. The enzyme chloroperoxidase (Fe(III) is oxidised by H_2O_2 to yield chloroperoxidase (Fe(IV)) (DiNello *et al*, 1975). The restoration of the enzyme back to its resting state (Fe(III)) can be achieved by a halide (Br^-), donating two electrons to chloroperoxidase (Fe(IV)) through the interaction with iron. The study by Hager (1972, cited in DiNello *et al*, 1975) presented strong evidence that the interaction is likely to be through the iron with the halide bound to an oxygen atom coordinated to iron. DiNello *et al* (1975) suggests a base such as imidazole (e.g. from histidine) from a peptide chain attacks the halide which transfers two electrons from the halide to the porphyrin the bring the enzyme back to its resting stage (Fe(III)).

Another possibility that may be related to this action of iron or the porphyrin ring in reducing other compounds can be seen in the action of qinghaosu or artemisinin. Meshnick *et al* (1991) described a situation whereby red cell membranes become substantially oxidised when in the presence of haeme and artemisinin.

The role of haeme to haeme covalently bound, forming a chain (meaning haeme aggregates that are said to form during the conjugation process) could enhance electron transfer from the active site of the enzyme to the platinum electrode surface. Recently, a number of articles have discussed electron transfer between porphyrins and quinones (Macpherson *et al*, 1995), cyclic amino acids, cyclic alkanes, alklbipyridinium and aminochloronaphthquinone (Brun *et al*, 1992) showed by increasing the chain length and as long as the chain is flexible, electron transfer can occur more efficiently.

So far, the literature suggests that the results obtained from Conjugate-LDH and Conjugate-ADH is related to either iron in haeme or the porphyrin ring system. But whatever the case may be, there is evidence in the literature that haeme can transfer electrons, and if the oxidation state of iron is changed as part of the electron transfer process there is evidence in the literature as to how the oxidation state is returned to ground state.

The second aim of the current study was to look at the use of flow injection analysis (FIA) as a method for testing biosensors. One of the advantages of using FIA for testing biosensors is its versatility, since other reagents such as cofactors that maybe required as part of the biosensor enzymatic reaction can be added into the design of the FIA. The FIA is also capable of delivering reproducible sample sizes. This is important as putative sensors or sensor designs can be evaluated without the hindrance of different sample sizes delivered to the detection areas of the putative sensors. Recently, Rui *et al* (1993) described a method for the simultaneous detection of creatinine, glucose and urea with the use of a single channel potentiostat. This was achieved through the use of four enzyme reactors containing enzymes specific for creatine, glucose, urea and enzymes to further convert the products from the enzymatic reactions to O_2 and H_2O_2. Rui *et al* (1993) then varied the delivery times so that it was possible to analyse all three samples using a single oxygen electrode connected to a single potentiostat, as opposed to a multi-detecting potentiostat. There may be scope for the incorporation of columns to eliminate large molecular weight protein particles that could foul the biosensor electrode, for example the use of an acetate anion exchanger used to eliminate uric acid, ascorbic acid or acetaminophen (a common electroactive species in urine and blood plasma) (Male and Luong, 1993). There is also the possibility of using glassy carbon electrodes as a method of pretreating samples (Downard and Roddick, 1994; Ghawji and Fogg, 1986)

before reaching the biosensor, to remove smaller proteins not removed through the use of columns. The work of the current study in evaluating biosensors using FIA, found the FIA method to have high sample output rates, and low variation between samples which makes it less complicated when trying to evaluate new biosensor designs.

In conclusion, the method of using haeme to immobilise enzymes to Pt electrodes holds much promise. Although there are still many issues that need to be resolved, the principal aim of the thesis had been achieved. It was possible to incorporate enzyme-haeme biosensors into flow injection analysis, thereby increasing the reproducibility of the results. The work with Conjugate-GO had produced electrodes that were sensitive to changes in glucose concentrations. It showed that Conjugate-GO electrodes could be stored over long periods of time without any loss to the linearity, although the range of the linear region decreases over time. Conjugate-GO electrodes in comparison to Gelatine-GO electrodes were more rapid in their response to changes in glucose especially at high glucose concentrations. Conjugate-LDH electrodes showed with convincing evidence that it was possible to detect lactate in the absence of NAD^+. Conjugate-ADH also showed that it was possible to measure ethanol concentrations in the absence of NAD^+. The current work into "molecular wiring" of enzymes can provide insights into how electrons are passed from the enzyme active site to the electrode surface. Molecular wiring of enzymes may also in the future provide a method that allow electrons to "hop" between a chain of electron carriers and allow access to the active site of the enzymes. The use of columns and other devices as pretreatment procedures to eliminate unwanted electroactive species can prolong the life of the biosensor as well as lessening the risk of false results reported. Therefore, more work is required for improving the conjugation process so that the method is more reliable for making successful enzyme electrodes. From the literature, iron seems to feature prominently with a role in redox reactions, therefore, future work should consider how iron is able to perform such reactions in place of NAD^+ in ADH-haeme and LDH-haeme electrodes.

8. References

Al-Bassam M.N., O'Sullivan M.J., Bridges J.W. and Marks V. (1979). Improved double-antibody enzyme immunoassay for methotrexate. *Clin. Chem.*, **25**, 1448.

Albery, W.J. & Bartlett, P.N. (1984). An organic conductor electrode for the oxidation of NADH. *J. Chem. Soc. Chem. Commun.*, 234.

Alva S., Gupta S.S., Phadke R.S. and Gpvil G. (1991). Glucose oxidase immobilised electrode for potentiometric estimation of glucose. *Biosensor Bioelectronics*, **6**, 663.

Bajpai P. and Margaritis A. (1985). Improvement of the inulinase stability of calcuim alginate, immobilized K. marxianus cells. *Enz. Microb. Technol*, **7**, 34.

Barker S.A. (1987). *Immobilisation of biological component of biosensors - in biosensors fundamentals and applications*, edited by Turner A.P.F., Karube I. and Wilson G.S., Oxford University Press, New York.

Bartlett P.N. (1990). *Biosensor Technology- Applications of enzymes in amperometric sensors: problems and possibilities*. Edited by Buck R.P., Hatfield W.E. and Umana M. and Bowden E.F., Marcel Dekker, Inc, New York, 95-115.

Bartlett P.N. (1990). Conducting organic salt electrodes. *Biosensors: A practical approach*. Edited by Cass A.E.G., Oxford University Press, N.Y., 47-90.

Bearden A.J., Moss T.H., Caughey W.S. and Beaudreau C.A. (1965). *Proc. Nat. Acad. Sci.*, **53**, 1246.

Beh S.K., Moody G.J. and Thomas J.D.R. (1989). Studies on spacer molecules and coupling agents for immobilising glucose oxidase on nylon mesh for glucose oxidase electrodes. *Analyst*, **114**, 1421.

Betso S.R., Klapper M.H., and Anderson L.B. (1972). Electrochemical studies of heme proteins. Coulometric, polargraphic, and combined spectroelectrochemical methods for reduction of the heme prosthetic group in cytochrome *c*. *J. Am. Chem. Soc.*, **94**, 8197.

Blaedel W.J. and Engstrom R.C. (1980). Reagentless enzyme electrodes for ethanol, lactate and malate. *Anal. Chem.*, **52**, 1691.

Blankespoor R.L. and Miller L.L. (1984). Electrochemical oxidation of NADH. Kinetic control of product inhibition and surface coating. *J. Electroanal. Chem.*, **171**, 231.

Bonnett R. and Dimsdale M.J. (1968). The meso-hydroxylation and meso-benzoxylation of pyridine octaethylhaemochrome. *Tetrahedron Letters*, 731.

Braun R.D., Santhanam K.S.V. and Elving P.J. (1975). Electrochemical oxidation in aqueous and nonaqueous media of dihydropyridine nucleotides NMNH, NADH and NADPH. *J. Am. Chem. Soc.*, **97**, 2591.

Brun A.M., Hubig S.M., Rodgers M.A.J. and Wade W.H. (1992). Electron transfer in linked violoden-quinone molecules: rate constant enhancement with increased chain lenght. *J. Phys. Chem.*, **96**, 710.

Bryce M.R. and Murphy L.C. (1984). Organic metals. *Nature*, **309**, 119.

Burnett J.N. and Underwood A.L. (1965). Electrochemical oxidation of NADH. *Biochemistry*, **4**, 2060.

Canh T.M. (1993).*Biosensors*, Chapman & Hall, London.

Cardosi M.F. and Turner A.P.F. (1991). Mediated electrochemistry. A practical approach to biosensing. *Advances in Biosensors*, **1**, 125.

Carlson B.W. and Miller L.L. (1983). Oxidation of NADH by ferrocenium salts, rate-limiting one-electron transfer. *J. Am. Chem. Soc.*, **105**, 7453.

Carr J.J. (1993). *Sensors and Circuits*, PTR Prentice Hall, New Jersey.

Cass A.E.G. (1990). *Biosensors - a practical approach*, Oxford University Press, New York.

Cass A.E.G., Davies G., Francis G.D., Hill H.A.O., Aston W.J., Higgins I.J., Plotkin E.V., Scott D.L. and Turner A.P.F. (1984). Ferrocene-mediated enzyme electrode for amperometric determination of glucose. *Anal.Chem.*, **56**, 667.

Cass A.E.G., Davies G., Green M.J. and Hill H.A.O. (1985). Ferricinium ion as an electron acceptor for oxido-reductases. *J. Electroanal. Chem.*, **190**, 117.

Caughey W.S., Ebersaecher H., Fuchsman W.H., McCoy S. and Alben J.O. (1969). Pi-interactions in metalloporphyrins and hemeproteins. *Annals N.Y. Acad. Sci.*, **153**, 722.

Caughey W.S., Fujimoto W.Y. and Johnson B.P. (1966). Substituted deuteroporphyrins. II. Substituent effects on electronic spectra, nitrogen basicities, and ligand affinities. *Biochemistry*, **5**, 3830.

Cenas N.K., Rozgaite J. and Kulys J.J. (1984). Lactate, pyruvate, ethanol and glucose-6-phosphate determination of enzyme electrode. *Biotechnol. Bioeng.*, **26**, 551.

Cenas N.K., Kanapieniene J.J. and Kulys J.J. (1984). NADH oxidation by quinone electron acceptors. *Biochim. Biophys. Acta*, **76**, 108.

Clark L.C. Jr. (1972). A family of polargraphic enzyme electrodes and the measurement of alcohol. *Biotech. Bioeng. Symp.*, **3**, 377.

Clark L.C.Jr. and Lyons C. (1962). Electrode system for continuous monitoring in cardiovascular surgery. *Ann N.Y. Acad Sci*, **102**, 29.

Degani Y. and Heller A. (1987). Direct electrical communication between chemically modified enzymes and metal electrodes. 1. Electron transfers from glucose oxidase to metal electrodes via electron relays, bound covalently to the enzyme. *J. Phys. Chem.*, **91**, 1285.

Degani Y. and Heller A. (1988). Direct eletrical communication between chemically modified enzymes and metal electrodes. 2. Metals for bonding electron-transfer relays to glucose oxidase and D-amino acid oxidase. *J. Am. Chem. Soc.*, **110**, 2615.

Danielsson, B., Lundstrom I., Winquist F. and Mosbach K. (1979). On a new enzyme transducer combination: the enzyme transistor. *Analytical Letters B*, **12**, 1189.

Dickerson R.E. (1974). Redox state and chain folding in cytochrome c. *Annals of the New York Academy of Sciences*, **227**, 599.

DiNello R.K., Rousseau K. and Dolphin D. (1975). A model for the halogenating agent of chloroperoxidase. *Annals of the New York Academy of Sciences*, **244**, 94.

Doherty A.P., Buckley T., Kelly D.M. and Vos J.G. (1994). Stabilization of the redox polymer $[Os(bipy)_2(PVP)_{10}Cl]Cl$ in situ chemical cross-linking. *Electroanalysis*, **6**, 553.

Dolphin D., Muljiani Z. And Rousseau K. (1973). The chemistry of porphyrin π-cations. *Annals of the New York Academy of Sciences*, **206**, 177.

Downard A.J. and Roddick A.D. (1994). Effects of electrochemical pretreatment on protein adsorption at glassy carbon electrodes. *Electroanalysis*, **6**, 409.

Eddowes M.J. and Hill H.A.O. (1977). Novel method for investigation of the electrochemistry of metalloproteins: cytochrome c. *J. Chem. Soc. Commun.*, 771.

Elbicki J.M., Morgan D.M. and Weber S.G. (1984). Theoretical and practical limitations on the optimization of amperometric detectors. *Anal. Chem.*, **56**, 978.

Englander S.W. (1975). Measurement of structural and free energy changes in hemoglobin by hydrogen exchange method. *Annal. N. Y. Acad. Sci.*, **244**, 10.

Frederick K.R., Tung J., Emerick R.S., Masiarz F.R., Chamberlain S.H., Vasavada A. and Rosenberg S. (1990). Glucose oxidase from *Aspergillus niger*. *J. Biol. Chem.*, **265**, 3793.

Frenzel W. and Bratter P. (1986). Flow-injection potentiometric stripping analysis- a new concept for fast trace determinations. *Anal. Chim. Acta*, **179**, 389.

Frew J.E. and Hill H.A.O. (1987). Electrochemical biosensors. *Anal. Chem.*, **59**, 933.

Gauterman M. and Stevenson P.E. (1962). Porphyrin charge-transfer complexes with sym-trinitro benzene. *J. Chem. Phys.*, **37**, 2266.

Ghawji A.B. and Fogg A.G. (1986). Reduction in the size by electrochemical pre-treatment at high negative potentials of the background currents obtained at negative potentials at glassy carbo electrodes and its application in the reductive flow injection amperometric determination of nitrofurantoin. *Analyst*, **111**, 157.

Gibson Q.H., Swoboda B.E.P. and Massey V. (1964). Kinetics and mechanism of action of glucose oxidase. *J. Biol. Chem.*, **239**, 3927.

Gough D.A., Lucisano J.Y. and Tse P.H.S. (1985). Two-dimensional enzyme electrode sensor for glucose. *Anal. Chem.*, **57**, 2351.

Gorton, L. (1986). Chemically modified electrodes for the electrocatalytic oxidation of nicotinamide coenzymes. *J. Chem. Soc. Faraday Trans.* I, **82**, 1245.

* Refer to page 137

Greef R., Peat R., Peter L.M., Plether D. and Robinson J. (1985). *Instrumental methods in electrochemistry*, Ellis Horwood Ltd, Chichester.

Guilbault G.G., Danielsson B., Mandenius C.F. and Mosbach K. (1983). Enzyme electrode and thermistor probes for the determination of alcohols with alcohol oxidase. *Anal. Chem.*, **55**, 1582.

Guilbault G. and Montalvo J. (1969). A urea-specific enzyme electrode. *J. Am. Chem. Soc.*, **91**, 2164.

Gunaratna C. and Wilson G.S. (1990). Optimization of multienzyme flow reactors for the determination of acetylcholine. *Anal. Chem.*, **62**, 402.

Hampp N. Eppelsheim C. and Silber A. (1994). *Thick-film Sensors*, edited by Prudenziati M., Elsevier Science B.V., Netherlands.

Harris J. and Ritchie K. (1969). Cuproproteins: A model and system derived from tricyanoaminopropene (TRIAP) and copper. *Annal. N. Y. Acad. Sci.*, **153**, 706.

Hill H.A.O. and Sanghera G.S. (1990). Mediated amperometric enzyme electrodes. In *Biosensors: A Practical Approach*, edited by Cass A.E.G., Oxford University Press, N.Y., 19-46.

Huang Y.L., Khoo S.B. and Yap M.G.S. (1993). Flow-injection analysis-wall jet electrode system for monitoring glucose and lactate in fermentation broths. *Anal. Chim. Acta*, **283**, 763.

Huang Y.L., Khoo S.B. and Yap M.G.S. (1994). Characterization of a wall-jet electrode/ flow-injection analysis system. *Electroanalysis*, **6**, 1077.

Huck H., Schelter-Graf A., Danzer J., Kirch P. and Schmidt H. (1984). Bioelectrochemical detection systems for substrates of dehydrogenases. *Analyst*, **109**, 147.

Ikeda T., Hamada H., Miki K. and Senda M. (1985). Glucose oxidase-immobilised benzoquinone-carbon paste electrode as glucose sensor. *Agric. Biol. Chem.*, **49**, 541.

Jaegfeldt H. (1980). Absorption and electrochemical oxidation behaviour of NADH at a clean platinum electrode. *Electroanal. Chem.*, **110**, 295.

Janata J., Josowicz M. and DeVaney D.M. (1994). Chemical Sensors. *Anal. Chem.*, **66**, 207R.

Johnson K.W., Bryan-Poole N. and Mastrototaro J.J. (1994). Reduction of electrooxidizable interferent effects: optimization of the applied potential for amperometric glucose sensors. *Electroanalysis*, **6**, 321.

Joseph J.P. (1984). A miniature enzyme electrode sensitive to urea. *Mikrochim. Acta*, **2**, 473.

Kassner R.J. (1973). A theoretical model for the effects of local non polar heme environments on the redox potentials in cytochromes. *J. Am. Chem.Soc.*, **95**, 2674.

Kitagawa Y., Kitabatake K., Kubo I., Tamiya E. and Karube I. (1989). Alcohol sensor based on membrane-bound alcohol dehydrogenase. *Anal. Chim. Acta.*, **218**, 61.

Konicker W.A. and Vallee B.L. (1969). Metallocinium cations, nucleic acids and proteins. *Annal. N. Y. Acad. Sci.*, **153**, 689.

Kulys J.J. (1986). Enzyme electrodes based on organic metals. *Biosensors*, **2**, 3.

Kulys J.J. and Cenas N.K. (1983). Oxidation of glucose oxidase from *Penicillium Vitale* by one- and two-electron acceptors. *Biochim. Biophys. Acta*, **744**, 57.

Kulys J.J. (1981). Development of new analytical systems based on biocatalysers. *Enzyme Microb. Tech.*, **3**, 342.

* Refer to page 137

Laval J.M., Bourdillon C. and Moiroux J. (1987). The electrochemical regeneration of NAD^+ revisited. *Biotech. Bioeng.*, **30**, 157.

Lehninger A.L. (1975). *Biochemistry, 2nd edition*, Worth Publishers, New York.

Lowry J.P., McAteer K., El Atrash S.S., Duff A. and O'Neill R.D. (1994). Characterization of glucose-modified poly(phenylenediamine)-coated electrodes in vitro and in vivo: homogenous interference by ascorbic acid in hydrogen peroxide detection. *Anal. Chem.*, **66**, 1754.

Lunte C.E., Kissinger P.T. and Shoup R.E. (1985). Difference mode detection with thin layer dual-electrode liquid chromatography/electrochemistry, *Anal. Chem.*, **57**, 1541.

Luong J.H.T., Nguyen A.L. and Guilbault G.G. (1993). The principle and technology of hydrogen peroxide biosensors. *Adv. Biochem. Eng./ Biotechnol.*, **50**, 85.

Macpherson A.N., Liddell P.A., Lin S., Noss L., Seely G.R., DeGraziano J.M., Moore A.L., Moore T.A. and Gust D. (1995). Ultrfast photoinduced electron transfer in rigid porphyrin-quinone dyads. *J. Am. Chem. Soc.*, **117**, 7202.

Male K.B.and Luong J.H.T. (1993). Improvement of the selectivity of an FIA amperometric biosensor system for glucose. *Biosensors Bioelectronics*, **8**, 239.

Mascini M. and Guibault G.G. (1986). Clinical uses of enzyme electrode probes. *Biosensors*, **2**, 147.

Mason M. (1983). Determination of glucose, sucrose, lactose and ethanol in foods and beverages, using immobilised enzyme electrodes. *J. Assoc. Off. Anal. Chem.*, **66**, 981.

Massey V. and Ghisla S. (1974). Role of charge transfer interactions in flavoprotein catalysis. *Annals of the New York Academy of Sciences*, **227**, 446.

Marko-Varga G., Applqvist R. and Gorton L. (1986). A glucose sensor based on the glucose dehydrogenase absorbed on a modified carbon electrode. *Anal. Chim. Acta*, **179**, 371.

Mascini M., Iannello M. And Palleschi G. (1983). Enzyme electrodes with improved mechanical and analytical characteristics obtained by binding enzymes to nylon nets. *Anal. Chem. Acta*, **146**, 135.

Mascini M., Moscone D. and Palleschi G. (1984). A lactate electrode with lactate oxidase immobilised on nylon net for blood serum samples in flow systems. *Anal. Chim. Acta*, **157**, 45.

Mascini M., Moscone D. and Pilloton R. (1987). Pyruvate and lactate electrochemical sensor realised with immobilised enzymes for the control in artifial pancreas. *Ann. Chim.*, **77**, 813.

Matsue, T., Suda, M., Uchida, I., Kato, T., Akida, U. & Osa, T. (1987). Electrocatalytic oxidation of NADH by ferrocene derivatives and the influence of cyclodextrin complexation. *J. Electroanal. Chem.*, **234**, 163.

Mattiasson B. (1977). A general enzyme thermistor based on specific reversible immobilization using the antigen-antibody interaction. *FEBS Letters*, **77**, 107.

Mauzerall D. (1965). Spectra of molecular complexes of porphyrins in aqueous solution. *Biochemistry*, **4**, 1801.

Meshnick S.R., Thomas A., Ranz A., Xu C.-M. and Pan H.-Z. (1991). Artemisinin (qinghaosu): the role of intracellular hemin in its mechanism of antimalarial action. *Molecular and Biochemical Parasitology*, **49**, 181.

Miyamoto S., Murakami T., Saito A. and Kimura J. (1991). Development of an amperometric alcohol sensor based on immobilised alcohol dehydrogenase and entrapped NAD^+. *Biosensors & Bioelectronics*, **6**, 563.

Mullen W.H., Churchouse S.J., Keedy F.H. and Vadgama P.M. (1986). Blood glucose determination using an enzyme electrode based on the quinoprotein, glucose dehydrogenase. *Anal. Proc.*, **23**, 145.

Nakamura T., Yoshimura J., Nakamura S. and Yasuyuki O. (1965). *Oxidases and related redox systems - The action mechanism of flavin enzymes*, King T., Mason H.S. and Morrison M. (eds), pp 311-334, John Wiley & Sons, Inc., New York.

Paine J.B. and Dolphin D. (1971). Electrophilic attack at the porphyrin periphery. *J. Am. Chem. Soc.*, **93**, 4080.

Powell M.F., Wu J.C. and Bruice T.C. (1984). Ferricyanide oxidation of dihydropyridines and analogues. *J. Am. Chem. Soc.*, **106**, 3850.

Rajkowski K.M. and Cittanova N. (1981). The efficiency of different coupling procedures for the linkage of oestriol-16-α-glucuronide, oestrone-3-glucuronide and pregnmanediol-3-α-glucuronide to four different enzymes. *J. Steroid Biochem.*, **14**, 861.

Rui C.S., Ogawa H.I., Sonomoto K. and Kato Y. (1993). Multifunctional flow-injection biosensor for the simultaneous measurement of creatinine, glucose and urea. *Biosci. Biotech. Biochem.*, **57**, 191.

Ruzicka J. and Hansen E.H. (1981). *Flow Injection Analysis*, John Wiley & Sons, Inc, USA.

Sauer M.J., Foulkes J.A., Worsfold A. and Morris B.A. (1986). Use of progesterone 11-glucuronide-alkaline phosphatase conjugate in a sensitive microtitre-plate enzyme immunoassay of progesterone in milk and its application to pregnancy testing in dairy cattle. *J. Reprod. Fert.*, **76**, 375.

Sauer M.J., Foulkes J.A. and O'Neill P.M. (1989). A comparison of alkaline phosphatase, β-galactosidase, penicillinase and peroxidase used as labels for progesterone determination in milk by heterologous microtitre plate enzyme immunoassay. *J. Steroid Biochem.*, **33**, 423.

Scheller F., Schubert F., Pfeiffer D., Hintsche R., Dransfeld I., Renneberg R., Wollenberger U., Riedel K., Pavlova M., Kuhn M.and Muller H. (1989). Research and development of biosensors - a review. *Analyst*, **114**, 653.

Scheller F.W., Pfeiffer D., Schubert F., Renneberg R. and Kirstein D. (1987). Application of enzyme-based amperometric biosensors to the analysis of 'real' samples. *Biosensors- Fundamentals and Applications*, edited by Turner A.P.F., Karube S. And Wilson G., Oxford University Press, New York, 315.

Schelter-Graf A., Schmidt H.-L. and Huck H. (1984). Determination of the substrates of dehydrogenases in biological material in flow-injection systems with electrocatalytic NADH oxidation. *Anal. Chim. Acta*, **163**, 299.

Schlapfer P., Mundt W. and Racine P (1974). Electrochemical measurement of glucose using various electron acceptors. *Clin. Chim Acta*, **57**, 283.

Schlereth D.D., Katz E. and Schmidt H.-L. (1994). Toluidine blue covalently immobilised onto gold electrode surfaces: an electrocatalytic system for NADH oxidation. *Electroanalysis*, **6**, 725.

Schmid R.D. and Kunnecke W. (1990). Flow injection analysis (FIA) based on enzymes or antibodies- applications in the life sciences. *J. of Biotechnol*, **14**, 3.

Schmidt H.-L. and Schuhmann W. (1996). Reagentless oxidoreductase sensors. *Biosensors & Bioelectronics*, **11**, 127.

Schuhmann W. and Schmidt H.L. (1992). Amperometric biosensors for substrates of oxidases and dehydrogenases. *Advances in Biosensors*, **2**, 79.

Schuhmann W., Wohlschlager H., Lammert R., Schmidt., Loffler H.L., Wienmhofer H.D. and Gopel W. (1990). Leaching of dimethylferrocene, a redox mediator in amperometric enzyme electrodes. *Sensors and Actuators B*, **1**, 571.

Senda M., Ikeda T., Hiasa H. and Miki K. (1986). Amperometric biosensors based on a biocatalyst electrode with entrapped mediator. *Anal. Sci.*, **2**, 501.

Skoog M. and Johansson G. (1991). Internal supply of coenzyme to an amperometric glucose biosensor based on chemically modified electrode. *Biosensors & Bioelectronics*, **6**, 407.

Smalldon K.W. (1973). Ethanol oxidation by human erythrocytes. *Nature*, **245**, 266.

Sonawot H.M., Phadke R.S. and Govil G. (1984). Covalent immobilization of FAD and glucose oxidase on carbon electrodes studies using cyclic voltammetry. *Biotechnology/ Bioengineering*, **26**, 1066.

Souto R.C. (1994). Electronic configurations in potentiostats for the correction of ohmic losses. *Electroanalysis*, **6**, 531.

Staden J.F.V. (1986). A coated tubular solid-state chloride-selective electrode in flow-injection analysis. *Anal. Chim. Acta*, **179**, 407.

Suleiman A.A. and Guibault G.G. (1992). Biosensors for food analysis. *Biosensors Design and Application*. Mathewson P.R. and Finley J.W. (ed), American Chemical Society, Washington.

Takano T., Kallai O.B., Swanson R. and Dickerson R.F. (1973). The structure of ferrocytochrome c at 2.45 A resolution. *J. Biol. Chem.*, **248**, 5234.

Thompson M., Arthur C.L. and Dhaliwal G.K. (1986). Liquid-phase piezoelectric and acoustic transmission studies of interfacial immunochemistry. *Anal. Chem.*, **58**, 1206.

Touchstone J.C. and Murawec T. (1973). Methods for determination of steriods by thin layer chromatography. In *Quantitative Thin Layer Chromatography*, edited by Touchstone J.C. Wiley Interscience, New York, 131-154.

Tronberg B.J., Sepaniak M.J., Vo Dinh T. and Griffin G.D. (1987). Fiber-optic chemical sensors for competitive finding fluoroimmunoassay. *Anal. Chem.*, **59**, 1226.

Tsuchida T., Takasugi H., Yoda K., Takizawa K and Kobayashi S. (1985). Application of 1-(+)-lactate electrode for clinical analysis and monitoring of tissue culture medium. *Biotechnologyl and Bioengineering*, **27**, 837.

Ukeda H., Kamikado H., Matsumoto K. and Osajima Y. (1989). A new approach of the coimmobilization of alcohol dehydrogenase and NAD on glutaraldehyde activated sepharose and its application to the enzymatic analysis of ethanol. *Agric. Biol. Chem.*, **53**, 25.

Updike S.J. and Hicks G.P. (1967). The enzyme electrode. *Nature*, **214**, 986.

Verduyn C., Van Dijken J.P. and Scheffers W.A. (1983). A simple, sensitive, and accurate alcohol electrode. *Biotechnology & Bioengineering*, **25**, 1049.

Verduyn C., Zomerdijk T.P.L., van Dijken J.P. and Scheffers W.A. (1984). Continuous measurement of ethanol production by aerobic yeast suspensions with enzyme electrode. *Appl. Microbiol. Biotechnol.*, **19**, 181.

Wang J. (1994). *Analytical Electrochemistry*, VCH, New York.

Wang J. and Chen Q. (1994). Lactate biosensor based on a lactate dehydrogenase/nicotiamide adenine dinucleotide biocomposite. *Electroanalysis*, **6**, 850.

Wingard Jr.L.B., Cantin L.A. and Caster J.F. (1983). Effect of enzyme-matrix composition on potentiometric, response to glucose using glucose oxidase immobilized on platinum. *Biochim. Biophys. Acta*, **748**, 21.

Wingard Jr.L.B. and Castner J. (1987). Potentiometric biosensors based on redox electrodes. *Biosensors- Fundamentals and Applications*, edited by Turner A.P.F., Karube S. And Wilson G., Oxford University Press, New York, 153-162.

Yacynych A.M. (1992). Chemically constructed amperometric biosensors. *Advances in Biosensors*, **2**, 1.

Yamamoto N., Nagasawa Y., Shuto S., Sawai M., Sudo T. and Tsubomura H. (1978). The electrical method of investigation of the antigen-antibody and enzyme-enzyme inhibitor reactions using chemically modified electrodes. *Chem. Letters*, 245.

Yon-Hin B.F.Y. and Lowe C.R. (1987). Catalytic oxidation of reduced nicotinamide adenine dinucleotide at hexacyanoferrate-modified nickel electrodes. *Anal Chem.*, **59**, 2111.

Zhang, F; Gosser, D.K.Jr and Meshnick, S.R. (1992). Hemin-catalyzed decomposition of artemisinin (Qinghaosu), *Biochemical Pharmacology*, **43**, 1805.

* Gosser Jr.D.K. (1993). *Cyclic Voltammetry: simulation and analysis of reaction mechanisms*, VCH, New York.

* Lane R.F. and Hubbard A.T. (1973). Electrochemistry of chemisorbed molecules. I. Reactants connected to electrodes through olefinic substituents. *J. Phys. Chem.*, 77, 1401.

* Large R. (1993). *Development of an amperometric biosensor for the detection of alcohol*, Msc dissertation, Massey University, Palmerston North, NZ.