Enzyme Chemical Engineering and Its Application to Biosensors

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ABSTRACT

Enzyme chemical engineering is a fast growing area in biotechnology. It has been used to change the stability, solubility, activity and other properties of enzymes for more control over and wider application of enzymes.

In this thesis, this technology is applied to another new and fast growing area of research: biosensors. Over the last decade, biosensors are gaining increasing awareness as a highly attractive analytical tool. One of the current challenges in this area is to identify a universal and scaleable way to produce sensitive, stable, instantaneous, and easy to prepare biosensors for mass production

In this study, enzyme chemical engineering is adopted as a new approach and glucose oxidase is served as a model to build a biosensor system in attempting to address the above challenge.

In the study, glucose oxidase was used as the catalyst to chemically amplify the redox reaction of glucose. Haemin was employed as the bifunctional promoter to act as a "bridge" to connect glucose oxidase (GOD) and electrode. Haemin, similar to ferrocene also acts as a mediator to transfer electrons between the active center of the enzyme and the electrode.

In the construction of a haemin-glucose oxidase biosensor, haemin was covalently bound with glucose oxidase. The haemin-glucose oxidase conjugate was then chemisorbed on to the platinum electrode to modify the electrode surface and form an "enzymatic redox center-bridge-electrode" system. The modification of the glucose oxidase with haemin comprised of two steps: converting the haemin carboxyl group to the reactive enol ester and then covalently bonding to an amino group of glucose oxidase. For chemisorption, the electrode was soaked in a solution of the haemin-glucose oxidase conjugate in phosphate buffer solution (pH 7.0) at 4°C for 16 hours. The same experiment was carried out by using unmodified glucose oxidase as a blank.

The following facts proved that the covalently bound haemin-glucose oxidase system was formed successfully: 1) The large molecule fractions eluted from the Sephadex G-10 gel column had the enzyme activity and other characteristics of glucose oxidase. 2) The same fractions retained about 2/3 to 3/4 of the specific activity of original glucose oxidase. 3) The absorbance spectra of these fractions showed the peaks corresponding to both haemin and glucose oxidase.

The following evidence suggests that the haemin-glucose oxidase conjugate was successfully chemisorbed on to the electrode surface: 1) The cyclic voltammogram of the electrode chemisorbed with conjugate was completely different from that adsorbed with glucose oxidase alone. 2) The cyclic voltammogram of the conjugate chemisorbed electrode in the solution with glucose was quite distinct from that without glucose. Thus a different species from either glucose oxidase or haemin was chemiabsorbed on to the electrode.

Furthermore, the conjugate chemisorbed electrode showed linearity between current response and glucose concentration at a range from 0mM to 10mM. The ratio of the current response to glucose concentration was about 1.6μA/mM. However, the platinum electrode adsorbed by GOD alone had a poor response to glucose. The response time of the system of platinum electrode-haemin-glucose oxidase was very rapid at less than one minute, and the response fell initially but then remained stable over a period of 14 days.

Thus the experimental data proved that the system of platinum electrode-haemin-glucose oxidase met the requirements for a glucose sensor in the factors of the sensitivity, linear response range, lifetime, ease of preparation, convenience of operation, non-toxicity and low cost. In other words, it demonstrated the characteristics of a glucose biosensor.

Finally, using the preparation of this glucose biosensor as a model, the electrochemical mechanism of the biosensor system was proposed. The model was also used to suggest a systematic approach for constructing amperometric biosensors. The extension of this approach and the potential applications of

this type of biosensor are also discussed.

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LIST OF ABBREVIATIONS

DCC	Dicyclohexycarbondi-imide
DEC	1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride
EFGP	Electrode-ferrocene-GOD entrapped within polypyrrole film
EHG	Electrode-haemin-GOD
FAD	Flavin adenine dinucleotide
$FADH_2$	Reduced flavin adenine dinucleotide
GOD	Glucose oxidase
HGOD	Conjugated haemin-glucose oxidase
HOSu	Hydroxysuccinimide
Mox	The oxidised form of mediator
Mred	The reduced form of mediator
Na-HEPES	Sodium 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonate
PDR	Protein determination reagent
POD	Horseradish Type II peroxidase
SGE	"Sandwiched" glucose oxdase electrode
TTF	Tetrathiafulvalene
UV	Ultraviolet
Vis	Visible

CHAPTER ONE

INTRODUCTION

The twenty first century is already being considered the biotechnology century. More and more people are realizing the importance of biotechnology and the biological sciences. The two areas of endeavor are advancing at a speed never seen before. In the meantime, concern for health and environmental issues are urging many to develop analytical instrumentation that is sensitive, accurate, specific, portable, inexpensive and fast in response. This need has led to the birth of biosensors (Griffiths and Hall, 1993).

1.1 Biosensors

Biosensors or biotransducers are types of apparatus that convert a biological event into an electrical or optical signal. Before the emergence of the biosensor, there were four major types of transducers: electrochemical (electrodes), mass (piezoelectric crystals or surface acoustic wave devices), optical (optrodes) and thermal (thermistors or heat-sensitive sensors) (Copper and Hall, 1988; Coulet, 1991).

Biosensors as a new class of sensor, convert a biological recognition process into a quantifiable analytical signal (Turner, 1996). They were born out of combination of existing sensors, such as amperometric, potentiometric, thermal, piezoelectric, acoustic and optical sensors, with biological systems, such as enzymes, cells, and microorganisms (Kost and Hague, 1995).

The most common biosensor is the enzyme electrode, a hybrid device consisting of an enzyme immobilized in close proximity to the active surface of an electrochemical transducer (Clark, 1987; Vadgama, 1990; Phadke, 1992).

1.2 Amperometric biosensor

The most common biosensors are the electrochemical biosensors.

Electrochemical sensors based around transducers offer a convenient route for measuring a chemical concentration and converting it into an electrical signal. This signal can be readily interfaced to monitoring and control circuitry.

There are two types of electrochemical biosensors, potentiometric and amperometric. The latter is more common. Amperometric sensors are electrochemical devices in which an electrode is set at a specific potential and any subsequent charges generated by oxidation or reduction processes at the electrode are recorded (Albrey *et al.*, 1984).

The amperometric sensor functions as follows:
A constant potential, with respect to a stable reference electrode, is applied to the detection electrode, which is also called the working electrode. The applied potential between these two electrodes causes an electrochemical oxidation or reduction of the measured substance at the working electrode. The original method employed a two-electrode system. The flow of current between the working and reference electrodes can cause the reference electrode to become unstable. Therefore, modern amperometry uses a three-electrode system in which the applied potential between the working and the reference electrode causes a current to flow to a third electrode, the auxiliary electrode. No amperometric current is allowed to flow to the reference electrode. Thus the stability of the reference electrode can be assured (Marcus and Sutin, 1985).

In both two electrode and three electrode systems, if the reaction is under diffusion control, the measured amperometric currents are directly proportional to the concentration of the detected electroactive species. As the amperometric sensor is set at a potential within the plateau region of the current versus potential curve of the electrode, it has the advantage, at least in theory, that small variations in the potential of the reference electrode will have little effect on the probe's response (Jaffari and Turner, 1995).

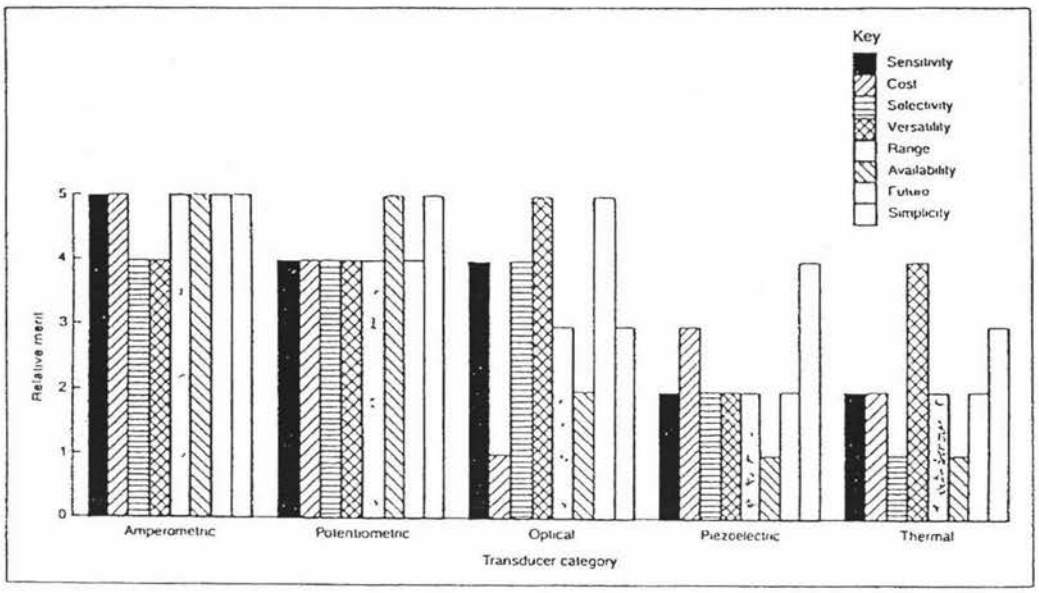


Figure 1.1 A comparison of the relative merits of the various transducer technologies used in the construction of biosensors (David Griffiths and Geoffrey Hall, 1993)

Griffiths and Hall (1993) made a comparison of the relative merits of the various transducer technologies used in the construction of biosensors. Figure 1.1 shows that comparing with others on the factors of sensitivity, cost selectivity, responsive range, availability and simplicity, the amperometric transducer has the higher profile than the others (potentiometric, optical, piezoelectric and thermal). This is the reason that the majority of commercial biosensor devices are amperometric enzyme electrodes. Thus it is thoughtful to choose the amperometric technique to construct biosensor in this thesis.

1.3 Glucose oxidase (GOD) sensors

Glucose oxidase sensors are the most popular biosensors (Wilson 1992) and have been used in number of areas such as:

a) Determination of glucose, one of the most frequently performed routine analyses in clinical chemistry, microbiology and food industries, makes amperometric glucose biosensors an attractive approach for glucose detection.

For example, fluctuating body glucose levels in a diabetic patient's body need to be monitored, preferably continuously, by an *in vivo* glucose sensor.

b) In combination with other enzymes, glucose oxidase sensors are applicable to the measurement of di- and polysaccharides arising from amylase and cellulase activity. Measurement of polysaccharides is required in many biotechnological processes.

This could be one of the explanations why numerous researchers worldwide are concerned with the continued development and optimization of glucose sensors (Sternberg *et al.*, 1989; Turner *et al.*, 1986, Taylor and Schultz, 1996).

The development stages of sensors used to measure glucose are as follows:

1.3.1 Direct detection method

Glucose oxidase (GOD) catalysis the oxidative reaction of glucose.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

Based on this reaction, the first generation of glucose sensors was designed in the direct detection mode and was comprised of either an oxygen sensor, or a pH electrode or a H_2O_2 detector.

The first glucose sensor appeared in 1962, when Clark and Lyons (Clark and Lyons, 1962) had the idea of determining glucose by immobilizing a layer of glucose oxidase on a pH or pO_2 electrode. The pH electrode detects the gluconic acid produced by the reaction, and the O_2 electrode measures the consumption of oxygen by the enzyme.

1.3.1.1 Oxygen sensor

As oxygen is consumed during the enzymatic reaction, the decrease in the oxygen concentration in the glucose oxidase membrane is a linear function of the glucose concentration. The oxygen concentration can be measured with a Clark cell (Clark and Lyons, 1962). This technique is used by several research groups and is described, for example, in the work of Gough's group (Gough *et al.*,1982). Because the concentration of dissolved oxygen will change during the measurement process, the oxygen concentration in the sample has to be measured to correct the result in this method. Thus a differential set-up is needed.

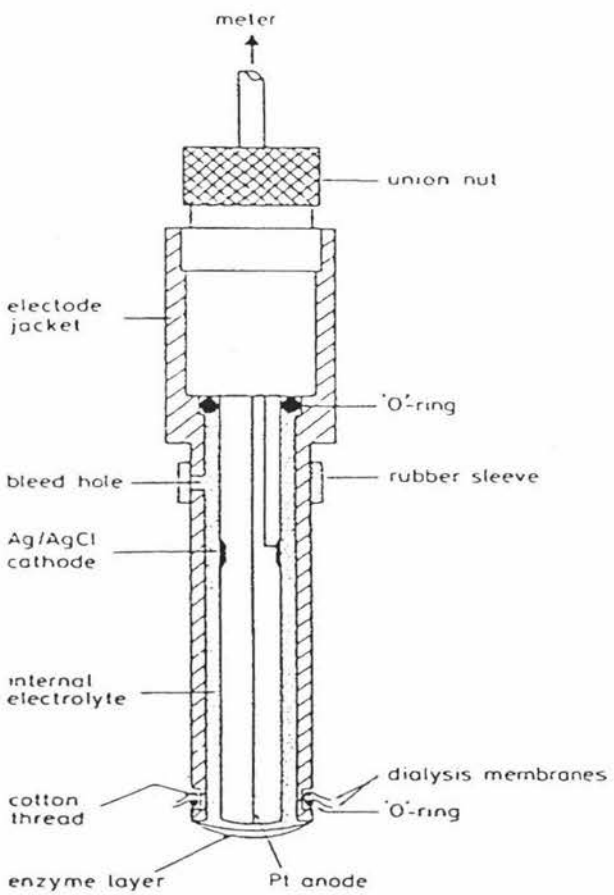


Figure 1.2 Amperometric enzyme electrode based on a Radiometer E5046 Clark oxygen sensor
(Tang and Vadgama,1990)

This results in a complicated device. Furthermore, because the rate of the reaction is a function of the oxygen concentration, the response of the biosensor to glucose (Tran-Minh and Broun, 1975) depends on the initial partial pressure of oxygen in the sample. This also explains why the detectable glucose concentration range increases with the partial pressure of oxygen. A stable oxygen concentration has to be maintained to ensure proper operation of the enzyme electrode. This can be achieved either by using samples with a large volume, or by using an external supply of oxygen. The presence of reducing compounds, such as ascorbic acid, must be avoided.

1.3.1.2 pH electrode

As gluconic acid is generated during the enzymatic reaction, the pH value will decrease as a function of the glucose concentration. The pH in the glucose oxidase membrane can be measured with a conventional pH electrode. Since the pH value of the solution does change during the measurement, a differential set-up is also necessary in this case. The major disadvantage of this method however is the dependence of the response of the glucose sensor to changes in the buffering capacity of solution (Coulet, 1991).

1.3.1.3 H_2O_2 detector

As H_2O_2 is generated during the enzymatic reaction, the H_2O_2 concentration will increase linearly as a function of the glucose concentration (Gough *et al.*, 1982). Normally, as H_2O_2 is not present in samples to be analyzed, a single H_2O_2 detector in the glucose oxidase membrane is sufficient for the determination of the glucose concentration in the solution. A Pt electrode biased at 0.7V versus a Ag/AgCl electrode can be used as an H_2O_2 detector. The major drawback to this approach is the fact that oxidation of hydrogen peroxide requires a large over potential, thus making these sensors susceptible to interference from electroactive species in the solution, such as ascorbic acid, uric acid and Fe (II) (Higgis, 1988). Also, as with the other two methods of detection, this method

does not eliminate the dependence of the biosensor on oxygen, which is still required for the enzymatic oxidation of glucose. During the reaction, only a small part of the oxygen consumed in reaction is regained, as not all the H_2O_2 formed in the reaction is oxidized at the electrode. If there is no continuous and stable supply of oxygen either via an air supply (Riopon *et al.*, 1990; Kasuno,1989) or electrolysis (Enfors, 1987) to compensate for the consumption in the enzymatic reaction, the signal emitted by the electrode does not give a correct reading of the glucose concentration. In other words, at high glucose concentration or low oxygen concentration, all oxygen in the GOD membrane is consumed, and then changes in the glucose concentration will not contribute to variations in the H_2O_2 concentration.

1.3.2 Indirect detection involving a mediator

The mediator is a low molecular weight redox group with fast electrode kinetics, and which can also undergo homogeneous electron transfer with a solution species (Hill et al., 1988).
The mediator reacts according to the following scheme:

$$GOD-FADH_2 + Mox \longrightarrow GOD-FAD + Mred + 2H^+$$
$$Mred \xrightarrow{anode} Mox$$

The oxidized form of mediator (Mox) is reduced to its reduced form (Mred) by the cofactor, and then oxidized to Mox when it comes into contact with the anode. It is therefore possible in principle to develop an electrode that responds to glucose concentration without interference from O_2 (Cass *et al.*, 1984).

The principal properties of a suitable mediator can be summarized (Frew and Hill, 1987; Tran-Minh, 1993):

1. Fast heterogeneous electron-transfer kinetics;
2. Rapid homogeneous electron-transfer with the redox protein;
3. Stable in the oxidized and reduced forms;
4. No reaction with other solution species (particularly O_2);

5. Regeneration potential must be low and pH-independent;
6. Must be non-toxic for in vivo applications;
7. Has the potential to capitalize on the use of synthetic organic chemistry to custom design the properties of the mediator.

A variety of electron acceptors have been used as mediators in solution; for example, quinones, organic dyes, and ferricyanide (Mahenc and Aussaresses, 1979). Their use is limited because they are toxic, pH-dependent, and often undergo autooxidation.

Transition metal cations and their complexes can be used as mediators. Many mediators reported in the literature were based on iron ions or its complexes. However free iron ions (Fe^{3+}) do not make good mediators as they are subject to hydrolysis and precipitation. Among the studied mediators, the most successful one was ferrocene (Cass *et al.*, 1984; Hall, E. A. H. 1990). Ferrocene (η 5-bis-cyclopentadienyl-iron) consists of an iron ion between two cyclopentadienyl anions as show in Figure 1.3 (Eggins, B. 1997).

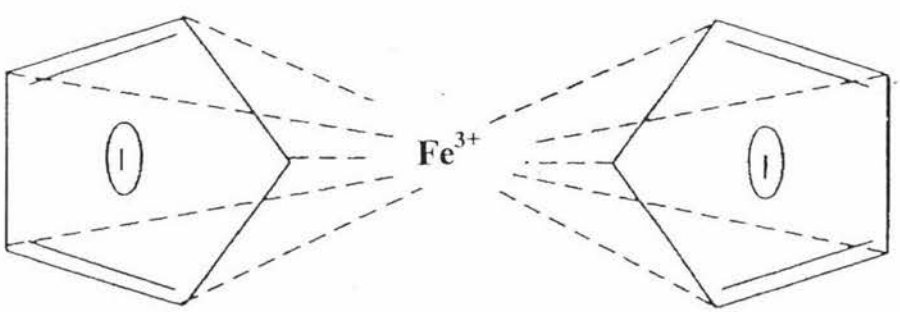


Figure 1.3 The structure of ferrocene

A biosensor using ferrocene as a mediator gives a current that is virtually independent of both pH and oxygen partial pressure. The range of the electrode response is quite large (Cass *et al.*, 1985; Dicks *et al.*, 1986).

Unfortunately, the oxidized form of ferrocene, the ferricinium ion, is quite soluble and will release into the sample. This leads to the disadvantages of instability and short lifetime of the biosensor. In order to ensure the electron transfer, the mediator must be present in both oxidized and reduced forms, which must remain immobilized in the vicinity of the electrode surface (Degani and Heller, 1987).

The construction of a biosensor requires the immobilization of an additional component, the mediator, at the electrode surface, together with the enzyme (Phadke, 1992). Thus modification of electrode is quite necessary in the development of biosensor techniques (Geckele and Muller, 1993).

1.3.3 Modification of electrode

The electron transfer reactions of biological molecules are frequently very slow at ordinary electrodes, including the oxidative reaction of glucose. To overcome this problem, methods of immobilizing enzymes onto electrodes have attracted attention (Rossi, 1987).

Modified electrodes can be divided into monolayer or multilayer structures, depending on the amount of material present on the electrode surface. A multilayer structure has the advantage that many more catalytic (or binding) sites are present, and so the electrode activity can be more stable. On the other hand, the multilayer electrode has the concomitant disadvantage that electrons must move through the multilayer film if the sites are on the outer layer. Monolayer modified electrodes are subdivided into those prepared by the covalent attachment of species to the electrode surface and those in which the molecules are adsorbed to the surface (Bartlett, 1990).

The electrodes can also be modified by the deposition of polymeric electrolyte species on the electrode surface, and the application of conductive polymers or conductive organic salts as electrode materials (Dumont, 1996). Thus, the

redox species of opposite charge are entrapped and strongly held by electrostatic interaction with the fixed charged sites (Espenscheid *et al.,* 1986; Szentirmay and Martin, 1984; Anson *et al.,* 1983 a, b). Finally, an alternative approach, which looks very promising, is to modify the enzyme by the covalent attachment of redox centers to the protein (Degani and Heller, 1987; Bartlett, 1990). In this case, the covalently attached redox centers act as "stepping stones ", allowing the electron to get from the active site out to the electrode by a number of short steps. In short, the modification of electrode and the appropriate enzyme is one of the most popular approaches to the construction of biosensors (Hall, 1987; Zull *et al.*, 1994).

1.4 Aim and content of this thesis

Considering the current trends in the development of biosensors and the technological demands of their applications, the study of chemical modification of enzyme proteins and their application to biosensors became the aim of this thesis.

Based on knowledge of the structure and properties of haemin and glucose oxidase, the following system was chosen:

- Glucose oxidase as a chemical amplifier to catalyze the redox reaction of glucose;
- Haemin as the bifunctional promoter to be a "bridge" to bind glucose oxidase to the electrode and mediate the transfer of electrons between glucose oxidase and electrode.

In construction of a haemin-glucose oxidase biosensor, haemin was to be covalently bound to glucose oxidase for achieving a modified glucose oxidase. The haemin-glucose oxidase conjugate was then chemisorbed on to the platinum electrode to modify the electrode surface and form an "enzyme redox center-bridge-electrode" system.

In this thesis, different approaches and conditions were experimented first to

identify the optimum techniques for chemical modification of enzyme, chemisorb enzyme to electrode and analysis of resulted conjugated enzyme and biosensor. Then the enzyme conjugation and biosensor were prepared under optimized condition and characteristics of the modified enzyme proteins and electrode were studied.

At the last, preparation of the glucose biosensor was used as a model to illustrate the biochemical mechanism, approach and probably extending clues for constructing biosensors. Their current and future applications to biosensors were also discussed.

CHAPTER TWO

METHODS AND EQUIPMENT

The experiments that were carried out and optimized in this thesis include:

- Haemin assay by atomic absorption spectrometry;
- Haemin assay by UV/Vis spectrometer;
- Glucose oxidase concentration assay;
- Assay the activity of glucose oxidase;
- Conjugating haemin to glucose oxidase;
- Separation of haemin–GOD conjugate from free haemin;
- Preparation of platinum wire auxiliary electrode;
- Preparation of reference electrode;
- Preparation platinum disc electrode;
- Preparation of working electrode;
- Pretreatment of electrode surface;
- Chemisorption of haemin-glucose oxidase conjugate to platinum electrode;
- Analysises of the resulted biosensor

2.1 Haemin assay

2.1.1 Haemin assay by atomic absorption spectrometry

2.1.1.1 Instrument and reagent

Instrument: GBC Atomic Absorption Spectrometry.

Instrument Parameters:

System Type	Flame.
Lamp Current (mA)	7.0.
Wavelength (nm)	248.3.
Slit Width (nm)	0.2.
Slit Height	Normal.

Gas Control Parameters:

Flame Type	Air-Acetylene.
Acetylene Flow	5.0 ml/minute.
Air Flow	5.0 ml/minute.

Reagents:

Buffer solution	0.05M sodium phosphate pH 7.0.
Haemin	Servia 24410 cryst. Pure.

Ferric standard solution (Fe 5.0 μg/ml):

12.1mg of $FeCl_3 \cdot 6H_2O$ was dissolved in 100ml of Milli-Q water, into which 5.0ml of 1.0M HCl was added, then transferred into a volume flask, Milli-Q water was added to final volume of 500ml.

Haemin stock solution: 4.5mg of haemin was dissolved in 10.0ml of the buffer solution and kept at 4 °C.

2.1.1.2 Procedures

Calibration graph of ferric concentration in haemin: added 0.0ml, 1.0ml, 2.0ml, 5.0ml of the ferric standard solution into test tubes respectively, diluted with Mill-Q water to a final volume of 5.0 ml, then recorded on the atomic absorption spectrometer.

Determination of ferric content of haemin: took 1.0ml of haemin stock solution and diluted it with 0.05M phosphate buffer solution (pH 7.0) to 10.0ml, added 0.40ml, 0.80ml, 1.16ml, 1.60ml, 2.00ml, 2.40ml of the diluted haemin solution into test tubes respectively, diluted with the buffer solution to a final volume of 5.0 ml, then run on the atomic absorption spectrometer.

2.1.2 Haemin assay by UV/Vis spectrometer

Instrument: Shimadzu 160 Ultraviolet-Visible Spectrometer.

Reagents:

Buffer solution: 0.05M sodium phosphate pH 7.0.

Haemin standart solution: 15.7 mg of haemin was dissolved in 10.0ml of the buffer solution and kept at 4°C.

Procedure: Absorbance spectra of haemin.

The haemin standard solution was diluted by the phosphate buffer pH 7.0 to a final volume of 3.0ml, then 20mg of solid sodium dithionite was added and the sample shaken vigorously. The absorbance spectrum between 250nm and 650nm was recorded.

2.2 Glucose oxidase concentration assay

2.2.1 Instrument and Reagent

Instrument: Shimadzu 160 Ultraviolet-Visible Spectrometer.

Reagent:

Reagent A: consists of an aqueous solution of 1% bicinchoninic acid, sodium salt (B-9643, Sigma), 2% $Na_2CO_3 \cdot H_2O$, 0.16% Na_2-tartrate and 0.95% $NaHCO_3$ in 0.1N NaOH (pH 11.2);

Reagent B: consists of an aqueous solution of 4% copper (II) sulfate pentahydrate (Sigma);

Other Reagent: 4% cupric sulfate $5H_2O$ solution; 0.05% sodium azide solution; 0.05M phosphate buffer pH 7.0; glucose oxidase (GOD) (EC 1.1.3.4 Type VII from Aspergillus niger Sigma); GOD standard solution: 1.0mg/ml in the 0.05M phosphate buffer, pH 7.0. Milli-Q water was used for preparing all reagent solutions.

2.2.2 Procedures

The required amount of protein determination reagent (PDR) was prepared weekly by adding 1 part (volume) of Reagent B to 50 parts (volume) of Reagent A at room temperature.

Added 40μl, 80μl,100μl,120μl, 160μl and 200μl of the GOD standard solution into 6 test tubes which pre-contained 1.0ml of the phosphate buffer and 3.0ml of PDR solution respectively, adjusted the total volume to be 5.0ml by adding the phosphate buffer. Then the mixture was incubated at 37 °C for 30 minutes. After cooling at room temperature for 15 minutes, the absorption at 562nm was recorded.

2.3 Assaying the activity of glucose oxidase

2.3.1 Instrument and reagents

Instrument: Shimadzu 160 Ultraviolet-Visible Spectrometer.
Reagents: 0.1M phosphate buffer (pH 6.0); phenol (MW 94.11); 4-amino-phenazone (MW 203.25); glucose oxidase (GOD) (EC 1.1.3.4 Type VII) from Aspergillus niger, Sigma; peroxidase (POD) (Horseradish Type II), Sigma; glucose (MW 180); GOD standard solution: 1.0 mg/ml in 0.1M phosphate buffer solution pH 6.0; substrate solution: add 51.8mg phenol, 7.8mg 4-amino-phenazone, 3.6g glucose and 0.5mg POD in 50ml 0.1M phosphate buffer (pH 6).

2.3.2 Procedures

Substrate solution 3ml was pipetted into 6 test tubes respectively and pre-incubated in a water bath at 25 °C for 5 minutes. Buffer solution was added to adjust the final volume to be 5.0ml. Then the varied volume of GOD standard solutions were added to the test tubes and readings at 512nm were taken every 30 seconds for 6 minutes.

2.4 Conjugating haemin to glucose oxidase

The procedures for the conjugation of haemin to glucose oxidase can be

divided into two steps. The first step is to convert the carboxyl group of haemin to the reactive enol ester. The second step is to form the peptide bond between the reactive enol ester of haemin and the amino group of glucose oxidase.

There are several ways to convert carboxyl group to active enol ester. Pikuleva *et al.* (1992) used Woodward's Reagent (N-ethyl-5-phenylis- oxazolium-3-sulfonate) as the activator. Another common one is the DCC- HOSu system. For our experiments the disadvantage of the DCC-HOSu system was that DCC is not soluble in aqueous solutions. DCC reactions are usually carried out in dry organic solutions. However the second step of the conjugation, glucose oxidase reacting with activated haemin, should be carried out in aqueous solution. Thus the solution transfer made the procedures lengthy.

In Deagani and Heller's work (1987), the system of DEC [1-(3-dimethylamino-propyl)-3-ethyl carbodi-imide hydrochloride] and Na-HEPES [sodium 4-(2-hydr-oxyethyl)-1-piperazine ethane sulfonate] can activate the haemin in aqueous. Therefore the both steps can be carried out in aqueous solution.

It should noted here that the pH optimum for the conjugation of haemin with glucose oxidase equaled 8.2, but glucose oxidase will be deactivated when pH > 8.0, so pH 7.2 was used here.

2.4.1 Reagent

Glucose oxidase EC 1.1.3.4 from *Aspergillus niger* product No. G-2133 activity 168.2 units/mg (Sigma), haemin (Servia 24410 cryst. pure), Na-HEPES [Sodium 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonate], and DEC [1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride] (Aldrich). Milli-Q water was used for preparing all reagent solutions.

2.4.2 Procedure

Haemin (80mg) was dissolved in 4 ml of 0.1M HEPES (pH = 7.6), 0.1N HCl and 0.1N NaOH were used to adjust pH to 7.2. DEC (100mg) was then added to the solution and the pH adjusted back to pH 7.2 again, then it was left to react for 1 hour. Then the sample vial was placed in an ice bath and 50mg of glucose oxidase was added with shaking. If the pH moved out of the 7.2 – 7.3 range, it was adjusted back to pH 7.2 again. Then the glass vial was sealed with a parafilm and the reaction solution was kept in 4 °C for overnight (about 16 hours).

2.5 Separation of haemin –GOD conjugate from free haemin

2.5.1 Reagent

Buffer solution:	0.05M phosphate, pH 7.0.
Gel:	Sephadex G-10.
Sodium-azide:	0.020 %.
Milli-Q water.	

2.5.2 Procedure

Prior to the column packing, the Sephadex G-10 gel was swollen for three hours in the 0.05M phosphate buffer pH 7.0. A Sephadex G-10 gel column 2cm × 26cm was then packed.

The reaction solution was centrifuged and filtered through a 0.20μm-pore size filter, and added to the top of the Sephadex G-10 gel column. The buffer solution pH 7.0 was used as the eluent to separate the conjugate from the unreacted haemin. It was obvious that a brown band could be readily eluted out by the buffer.

The fractions with strong absorption peaks at 280nm were collected. Sodium azide (0.02%) solution was added to prevent growth of bacteria. A deep dark

brown band remained at the top of the column while the fractions with high absorption at 280nm were collected. There were three fractions in the collection and the volumes of the three fractions were 10.0ml, 8.5ml and 5.4ml respectively.

2.6 Electrode preparation

2.6.1 Preparation of platinum wire auxiliary electrode

A 10cm length of glass tube with a 2.5mm diameter was heated in a flame at the center of the tube and drawn out to form two electrode bodies. The tip that was drawn out is cut at where a 0.6mm diameter is achieved to prepare auxiliary electrode and reference electrode

A 1cm length of platinum wire (0.25mm diameter) was inserted in to the end of 0.6mm diameter glass tube, and the tube end was melted to seal around the platinum wire. Solder was introduced down inside the tube, which was melted in the flame after a connecting wire pushed into the solder (Ruth Large,1993).

2.6.2 Preparation of reference electrode

A 10cm length of 0.5mm diameter silver wire was coated with silver chloride by making the silver wire anode of an electrolytic cell with a platinum cathode in 0.1M HCl and applying 1.5V for 15 minutes. This wire was inserted into the end (0.6mm diameter) of the glass tube and the end of the tube was melted in flame to seal around the silver wire. A connecting wire was soldered to the top end of the silver wire. The glass tube was filled with 1M KCl solution.

2.6.3 Preparation platinum disc electrode

A 1cm platinum wire was spot-welded onto one side of a 4mm diameter disc (0.5mm thickness) and a connecting wire was soldered to the platinum wire.

The assembly was glued with epoxy cement into a glass tube (6mm diameter, 6cm length). The tube was filled with polyurethane resin. Then the glass tube with epoxy cement was ground back until the platinum disk was exposed (Belanger *et al.*, 1989).

2.6.4 Preparation of working electrode

2.6.4.1 Pretreatment of electrode surface

Electrode surface pretreatment was very important and was made particularly difficult by the fact that chemical materials are readily adsorbed onto the surface. It was necessary to clean the chemisorbed materials away completely for efficient formation of Pt-C bonds between haemin and the surface of platinum electrodes and gaining highly reproducible results.

Procedures: The platinum disk electrode was soaked in deaerated 1M perchloric acid, and a cyclic current-potential curve was recorded at 2mv sec^{-1} (0.4 to 1.3V, then back to -0.3V), which served to remove the last traces of adsorbed organic materials, by anodic and cathodic treatment (Gileadi *et al.*, 1975). If it is necessary, this cyclic voltammetry technique can be used several times to confirm the purity of the surface. The electrode was then washed with Milli-Q water.

2.6.4.2 Chemisorption of haemin-glucose oxidase conjugate to platinum electrode

The platinum disc electrode was placed in the haemin-glucose oxidase solution (Fraction 1 solution, Section 2.3.2.2 of this thesis) at 4 °C for 16 hours and then washed with the 0.05M phosphate buffer solution (pH 7.0) and stored at 4 °C in the buffer. It was applied as a working electrode for electrochemical detection and measurements.

In addition, another platinum disc electrode processed through the above

procedure was pretreated and soaked in the 0.1mg/ml of glucose oxidase solution at 4 °C for 16 hours and then washed with the phosphate buffer solution (pH 7.0) and stored at 4 °C in the buffer (pH 7.0). This electrode was used as a working electrode in the electrochemical detection and comparison measurements.

2.6.5 Cyclic voltammetry assay

The experimental conditions were as follows:

- Working electrode: the platinum disc electrode chemisorbed by glucose oxidase-haemin conjugate (GOD- H).
- Reference electrode: the silver-silver chloride electrode.
- Auxiliary electrode: the platinum wire electrode.

The experiments were carried out in a three-electrode configuration in an electrochemical cell at room temperature in 0.05M phosphate buffer solution (pH 7.0) with glucose or without glucose. The applied voltage was swept up from -200mV to 1000mV then back to -200mV at 20mV sec^{-1}. The voltammograms were measured over 4-6 times of potential cycles to confirm the stability of the voltammogram with time. For comparison, a clean platinum disc electrode and the platinum disc electrode adsorbed by GOD were taken place of the working electrode and the cyclic voltammetries in respect of were also recorded.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Structure and properties of glucose oxidase and haemin

3.1.1 Structure and properties of glucose oxidase

Glucose oxidase (β-D-glucose: Oxygen 1-oxidoreductase EC1.1.3.4, from Aspergillus niger) is a glyco-protein. By three methods, Bennett and Vincent (1965) estimated the average molecular weight of Aspergillus niger glucose oxidase was 186,000. The enzyme contains 2 molecules of bound flavin adenine dinucleotide (FAD), and the FAD content of glucose oxidase was found to be 10.9mμ moles per mg (dry weight of glucose oxidase). Native Aspergillus niger glucose oxidase has no titratable sulfhydryl groups and is not inhibited by mercurial at concentrations less than 10^{-2} M. However, following denaturation by heat or 8M urea, one sulfhydryl group and two disulfide groups could be detected (Bennett and Vincent 1965). In the native enzyme, the disulfide bonds help to maintain the three dimensional structure of the protein. The carbohydrate content of the enzyme was found to be approximately 16%. The large carbohydrate content of the Aspergillus niger glucose oxidase could confer on the enzyme some of its unusual properties, such as, nonprecipitation at 100 °C, high stability and high solubility in water. When a solution of glucose oxidase was heated in a sealed tube protected from light at 100°C for 2 to 15 minutes, no protein precipitate was formed. However, all the enzyme activity was destroyed. FAD is cleaved from the enzyme under these conditions (Bennett and Vinaent, 1965).

Hecht *et al.* (1993) studied the crystal structure of glucose oxidase and suggested that the refined model include 580 amino acid residues, the FAD cofactor, six *N*-acetylglucosamine residues, three mannose residues and 152 solvent molecules (Prior to measurement the crystals of glucose oxidase were transferred from the crystallization drop to a storage solution containing 2.5M ammonium sulfate in 50mM sodium acetate buffer pH 5.6). Figure 3.1 shows

the representation of the overall folding of glucose oxidase.

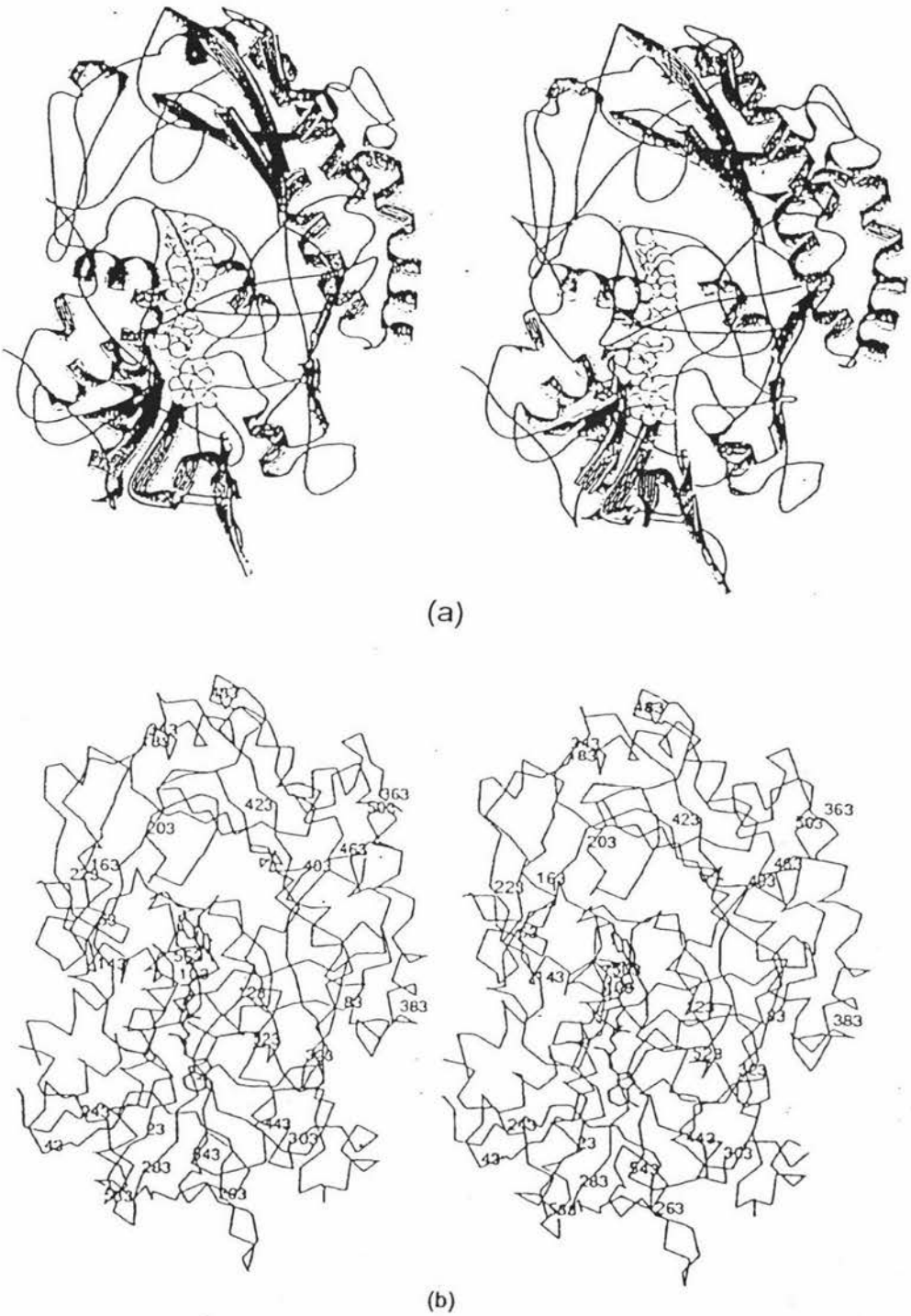


Figure 3.1 Representation of the overall folding of glucose oxidase (a) Stereo ribbon drawing of the glucose oxidase monomer; (b) C^{12} tracing of glucose oxidase monomer. Labels are included at every 20th residue. (Hechet *et al.*, 1993).

Glucose oxidase acts as a chemical amplifier to catalyze the oxidation of glucose. Because glucose oxidase only reacts with β-D-glucose and not with δ -D-glucose or other sugars, the catalytic reaction is highly selective. Glucose oxidase is a large protein that contains two active redox centers ($FAD/FADH_2$) which catalyze the oxidation of glucose.

In an electrode-mediated process, the enzymatic reaction catalyzed by glucose oxidase can be divided into two separate steps. Glucose is involved in the reduction of the FAD (oxidized flavin adenine dinucleotide) at the active site of glucose oxidase to $FADH_2$ (reduced flavin adenine dinucleotide) in the reductive half reaction. Then the $FADH_2$ bridged by mediators is re-oxidized at the surface of electrode in the oxidative half reaction (Weibel and Bright, 1971; Bright and Poter, 1975; Degani and Heller, 1987).

$$\beta - D - \text{glucose} + \text{glucose oxidase} - FAD \longrightarrow \delta - D - \text{gluconolac tone} + \text{glucose oxidase} - FADH_2$$

$$\text{Glucose oxidase} - FADH_2 \longrightarrow \text{Glucose oxidase} - FAD + 2H^+ + 2e^-$$

Because glucose is not an electroactive substance, under normal conditions it is difficult to oxidize or reduce glucose electrochemically. The $FAD/FADH_2$ redox active centers of glucose oxidase translate the oxidation of glucose into a reaction that can be measured amperometrically. If factors such as temperature, buffer systems, working potentials are constant, the current that flows is in response to the concentration of glucose. Thus, a sensitive and highly selective amperomertric biosensor might be designed for glucose.

However, glucose oxidase does not directly transfer electrons to conventional electrodes. This is because, even on closest approach, the distance between the redox centers of the enzyme and the electrode surface exceeds the distance across which electrons can be transferred at sufficient rates (Degani and Heller 1987). Thus, without assistance, the $FADH_2$ at the enzyme active center is difficult to oxidize electrochemically through the electrode reaction (Degani and Heller 1987).

As a result, a "bridge" is necessary to immobilize the glucose oxidase on the surface of electrodes and to mediate electron flow between electrode and the active site of glucose oxidase.

3.1.2 Structure and properties of haemin

Haemin as a derivative of porphyrin is among the most versatile compounds in nature. On the basis of 1 iron atom per molecule, the haemin molecular weight value of 1131 can be calculated out (Winslow and Landal, 1963). Haemin tends to aggregate in both aqueous and non-aqueous solutions (Falk, 1964). In all cases the aggregate is much less active than the monomeric form.

In association with specific proteins, haemin is able to carry out a tremendously wide range of functions. Combined with specific proteins, the haemin group can take part in several other types of reaction including hydroxylation (as in cytochrome P450), dioxygenation (as in tryptophan pyrrollase), oxidation by peroxide (as in catalase) and self-oxidation (as in haemin oxygenase). In all of these examples, the haemin group is at the active center of the protein (Brown *et al.*, 1980). The structure of haemin group is as follows:

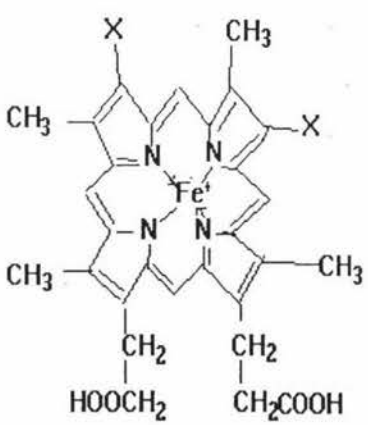
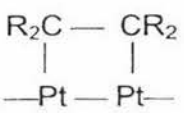


Figure 3.2 The structural formula of ferrihaems

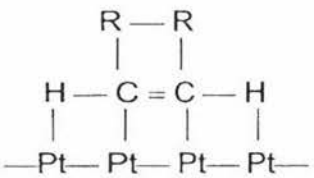
X = protoferrihaem, -CH = CH_2; deuteroferrihaemin, -H; mesoferrihaem, -CH_2CH_3; haematoferrihaem, -CH,OH,CH_3; coproferrihaem, - CH_2 CH_2CO_2H.

(Brown *et al.*, 1980)

Protoferrihaemin contains conjugated double bonds (olefinic) and carboxyl group. In this work, the haemin being mentioned is the abbreviated form of protoferrihaemin. The conjugated double bonds of substances such as haemin can react with the surface of a platinum electrode to form two Pt-C bonds. The two commonly accepted types of bonding at the surface of platinum are the associative and dissociative models.



(a) The associative model



(b) The dissociative modle

The associative model stresses the loss of double bond character with formation of two Pt-C bonds. The dissociative model emphasizes retention of double bond character with cleavage of C-H bonds and Pt-C and Pt-H bonds. Both models lead to essentially the same predictions concerning the location of the carbon chain within the double layer (Ross *et al.*, 1973).

Haemin can react with platinum surfaces to form irreversible chemisorbed species, which can not be removed from the surface by treatment such as rinsing or electrolysis apart from strongly oxidization (Lau and Hubbard, 1970, 1971). A chemical reaction between the π system of conjugated double bond and the surface platinum atoms occurs in which the metal acts as an acceptor of electrons (Somorjai and Szalkowski, 1971; Byfield and Abuknesha, 1994).

Furthermore, the carboxyl groups of haemin can react with the amino groups of proteins to form peptide bond. Haemin and glucose oxidase are thus covalently bound via peptide bonds (Pikuleva *et al.*, 1992).

The mechanism of bond formation is that if one or two carboxyl group of haemin are converted to reactive enol ester by a carbodi-imide, then the amino groups of glucose oxidase react with the enol ester and the haemin bound to glucose oxidase through the peptide bond. Haemin then acts as a bridge to connect

glucose oxidase with the platinum electrode.

3.2 Assay haemin

3.2.1 Solvent system

In order to achieve accurate and reproducible data for haemin, it is important to have a good solvent system to protect the haemin from precipitation, aggregation and re-oxidation. Brown and Lantzke (1969) indicated that haemin could self-aggregate. They added pyridine to prevent aggregation.

Phosphate buffer (pH = 7.0) was chosen for these experiments due to the following reasons:

1. Haemin has negligible solubility in pure water and aqueous acid.
2. The pH value is an influencing factor for the measurement of haemin.
3. The sensitivity of the measurement in the solution at pH = 7.0 is higher than that at pH > 7.0.

To prevent re-oxidation, N_2, He or sodium dithionite can be chosen as the antioxidant. The sodium dithionite was chosen in this research, due to its effectiveness, availability and convenience.

3.2.2 Assay haemin by determination of ferric content in haemin

According to the nature of haemin, it should be possible to determine the ferric content of haemin by atomic absorption spectrometry.

Figure 3.3 shows that the calibration of ferric concentration is reproducible and when the ferric concentration is less than 2μg/ml there is a linear relationship between ferric concentration and absorbance.

However, comparing the calculated ferric concentration value with the

experimental atomic adsorption value and comparing the calculated haemin value with added haemin content (Table 3.1), it is clear that the small error in the determination of iron led to a large error in the calculated value of haemin. Because the ratio of haemin molecular weight to the iron atomic weight is 1131/56, equal to 20.2. Thus, the experimental value of ferric with atomic adsorption is not suitable for the calculation of haemin content accurately.

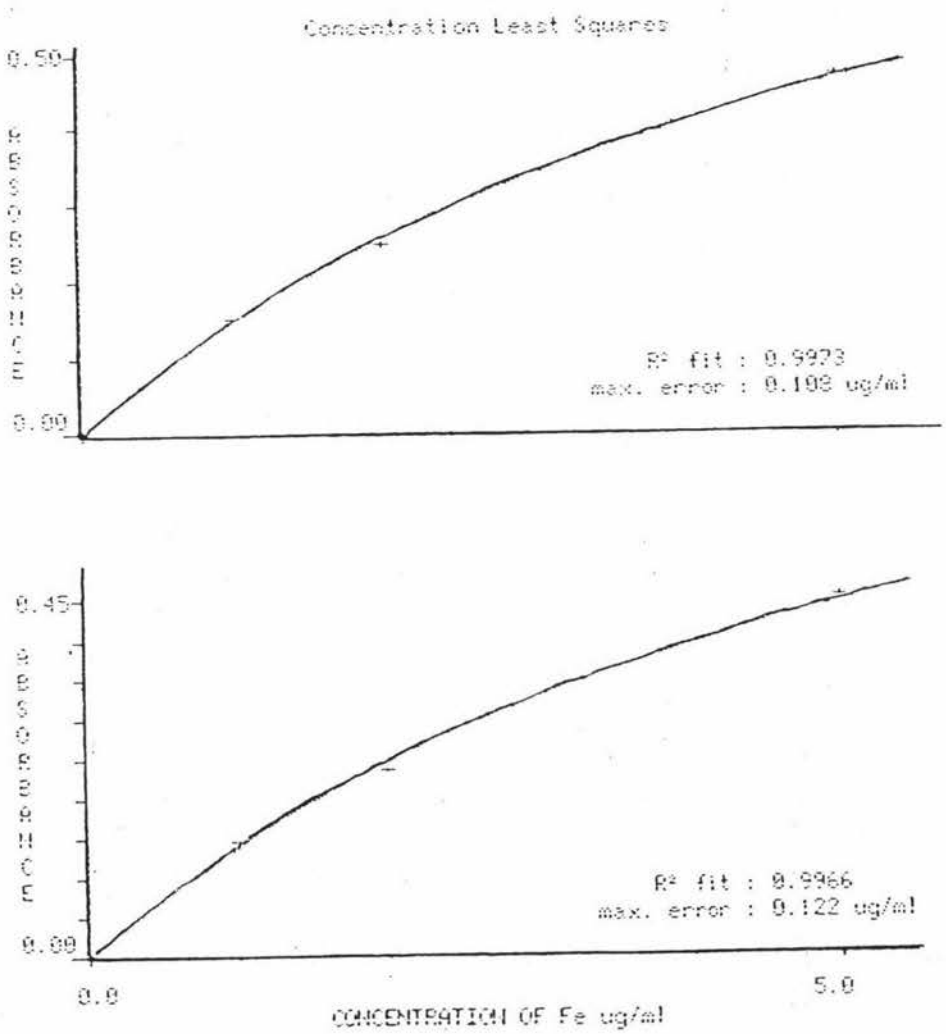


Figure 3.3 Calibration graph for ferric concentration in haemin comparison of two determinations

Table 3.1 Comparison of the calculated value with the determinated value of iron and the calculated value with added value of haemin

Haemin Added (μg/ml)	Fe Calc. (μg/ml)	Fe Meas. (μg/ml)	Haemin Calc. (μg/ml)
3.6	0.18	0.20	4.04
7.2	0.36	0.40	8.08
10.4	0.53	0.67	13.53
14.4	0.71	0.91	18.35
18.0	0.89	1.10	22.22
21.6	1.07	1.41	28.48

3.2.3 Assay haemin by UV/Vis spectrometer

3.2.3.1 Absorbance spectrum of haemin

Figure 3.4B shows that the haemin spectrum has well resolved peaks with maxima at 348nm, 420nm, 480nm, 528nm and 557nm.

However the glucose oxidase spectrum also has high absorbance at 348nm and 420nm. Therefore peaks at 348nm and 420nm of haemin are not suitable for analysis even if they are the two highest peaks in the absorbance spectrum. Among the rest of the peaks, peak at 557nm is higher than the others, so the spectral absorption at 557nm was used to quantitatively analyze the haemin content.

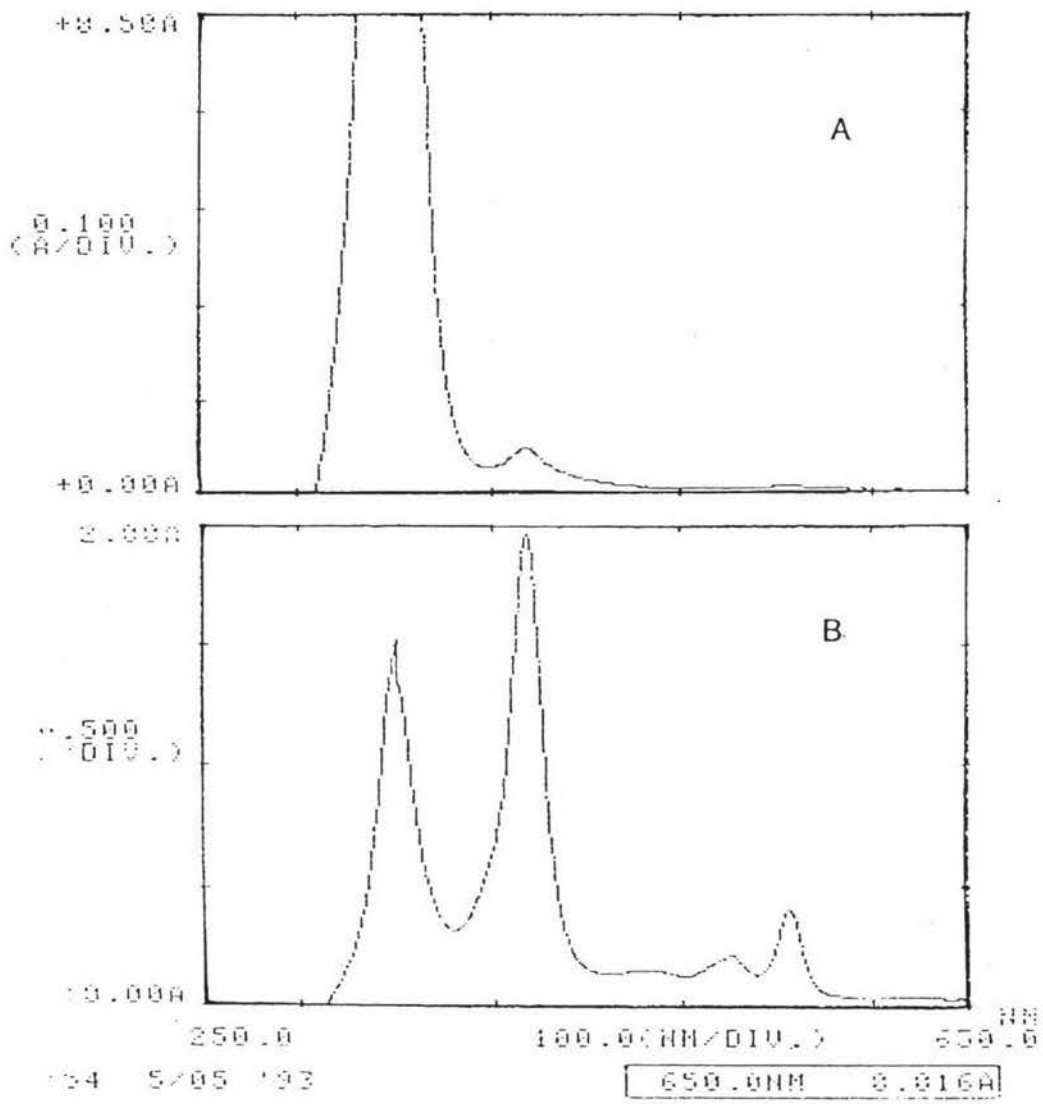


Figure 3.4 Absorbance spectra for haemin and glucose oxidase
(A) glucose oxidase; (B) haemin

3.2.3.2 Calibration for haemin

Procedure:

Variable volumes (from 7.0μl to 42.0μl) of the haemin standard solution were diluted with sodium phosphate buffer (pH 7.0) to a final volume of 3.0ml. To these were added 20mg solid sodium dithionite, shaken vigorously and then the excess of dithionite was allowed to settle. Absorbance spectra between 250nm

and 650nm were recorded. Quantitative assays for haemin were carried at 557nm.

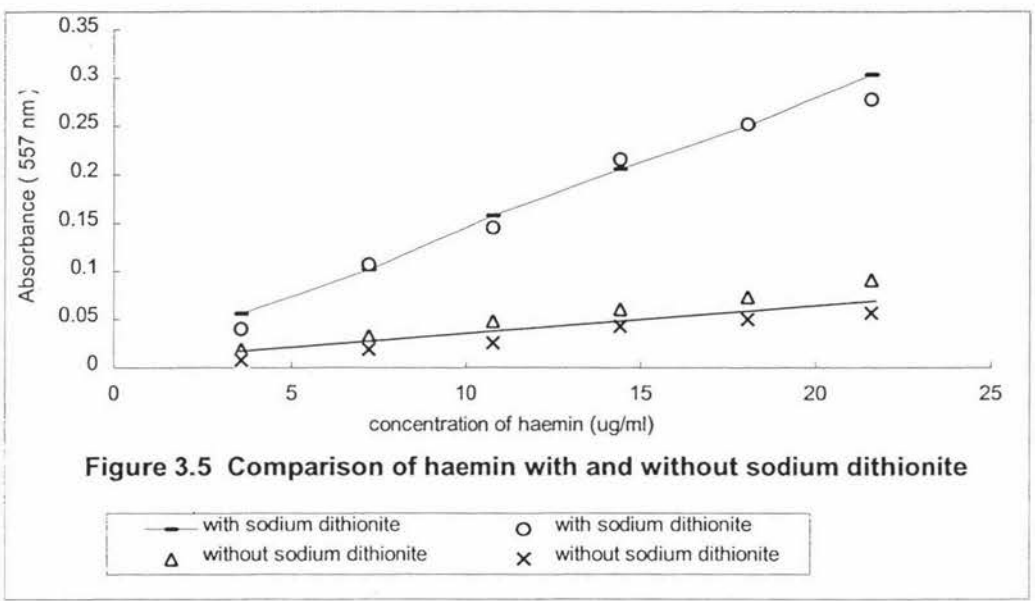


Figure 3.5 Comparison of haemin with and without sodium dithionite

Figure 3.5 shows that there is a good linear relationship between haemin content and absorbance (at 557nm). It also demonstrated that, comparing to analyzed value without dithionite, the one with dithionite is more sensitive and the results are more reproducible.

The reason is that haemin can exist in both oxidized and reduced forms. Figure 3.6 exhibits the spectra of oxidized and reduced haemin and only the reduced has absorption peak at 557nm. Thus it is essential to use an antioxidant to prevent the reduced haemin from re-oxidation.

In addition, no aggregation must appear in the concentration range of this assay. As haemin normally aggregates at high concentration, it was not deemed necessary to use pyridine in the haemin assay in this concentration range.

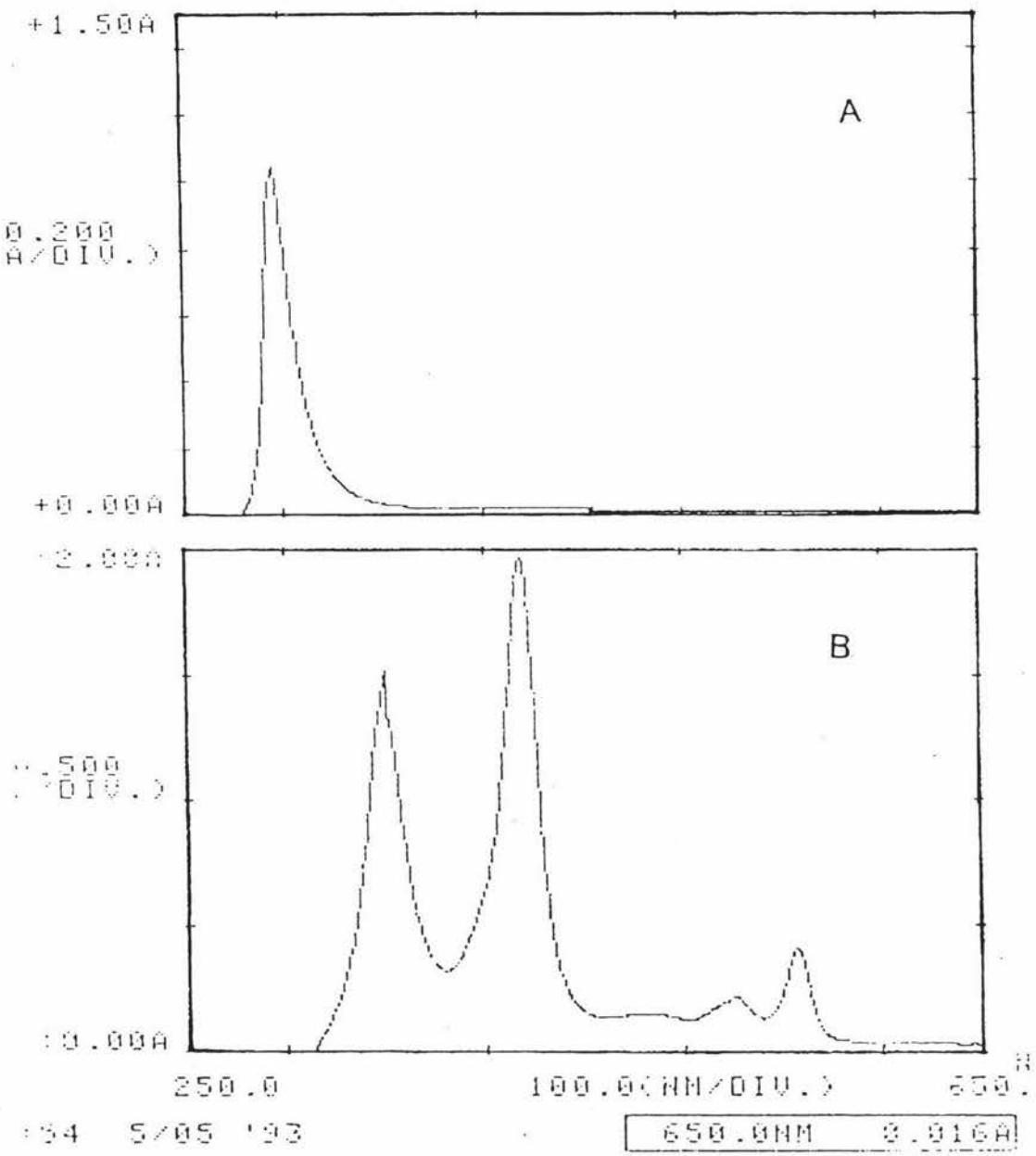


Figure 3.6 Absorbance spectra for oxidized (A) and reduced (B) haemin species in the buffer solution (pH 7.0)

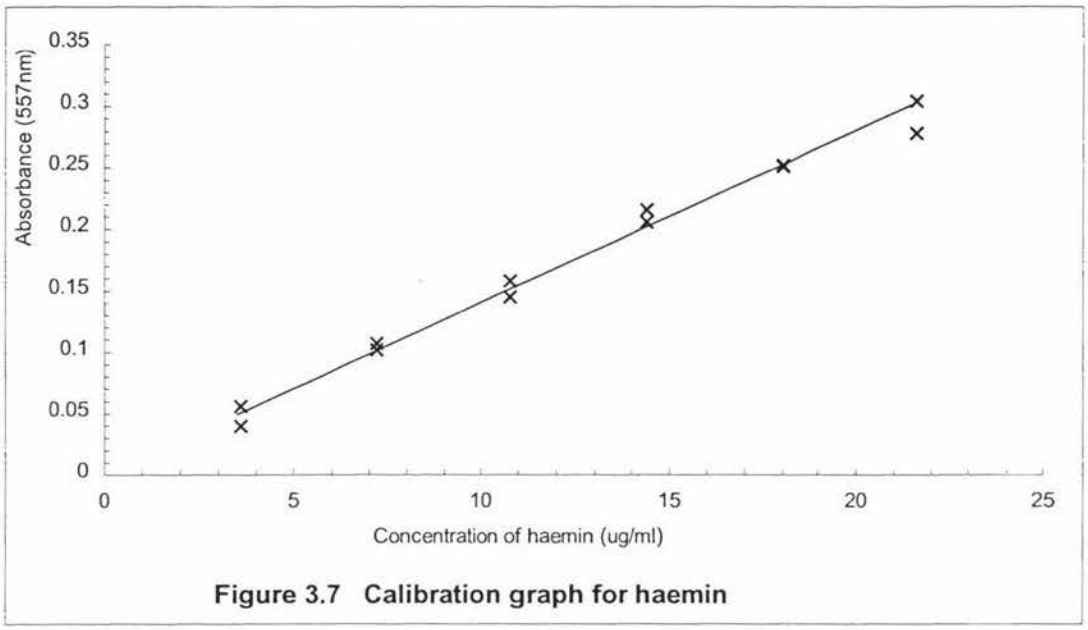


Figure 3.7 Calibration graph for haemin

3.3 Assay concentration of glucose oxidase

3.3.1 Principle of the assay concentration of glucose oxidase

Proteins reduce alkaline Cu (II) to Cu (I) in a concentration dependent manner. Bicinchoninic acid, sodium salt, is a stable water-soluble compound and a highly specific chromogenic reagent for Cu (I) forming an intense purple complex with an absobance maximum at 562nm in an alkaline environment. Because of this property, bicinchoninic acid can be substituted for the Folin-Ciocaltru reagent for the determination of protein, as the resultant absorbance at 562nm is directly proportional to protein concentration (Smith *et al.*, 1985; Sigma procedure No. TPRO-562). This procedure was used in this thesis for the determination of glucose oxidase.

3.3.2 Calibration graph for concentration of glucose oxidase

Figure 3.8 is the calibration graph for the concentration of glucose oxidase. It indicates that there is a linear relationship between GOD concentration and absorbance within GOD concentration range from 50μg/ml to 200μg/ml.

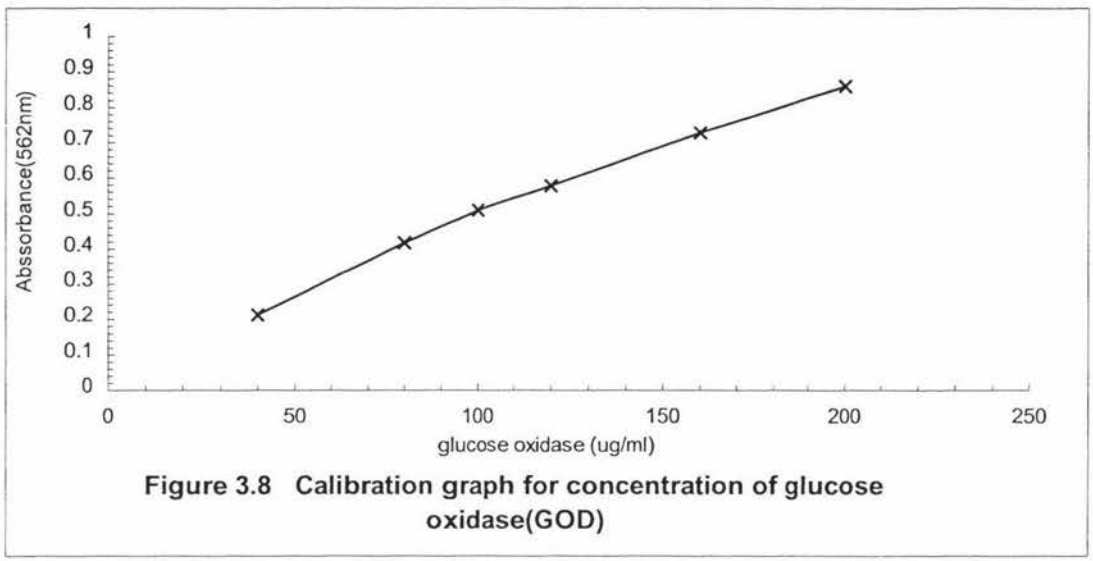


Figure 3.8 Calibration graph for concentration of glucose oxidase(GOD)

3.4 Activity calibration of glucose oxidase

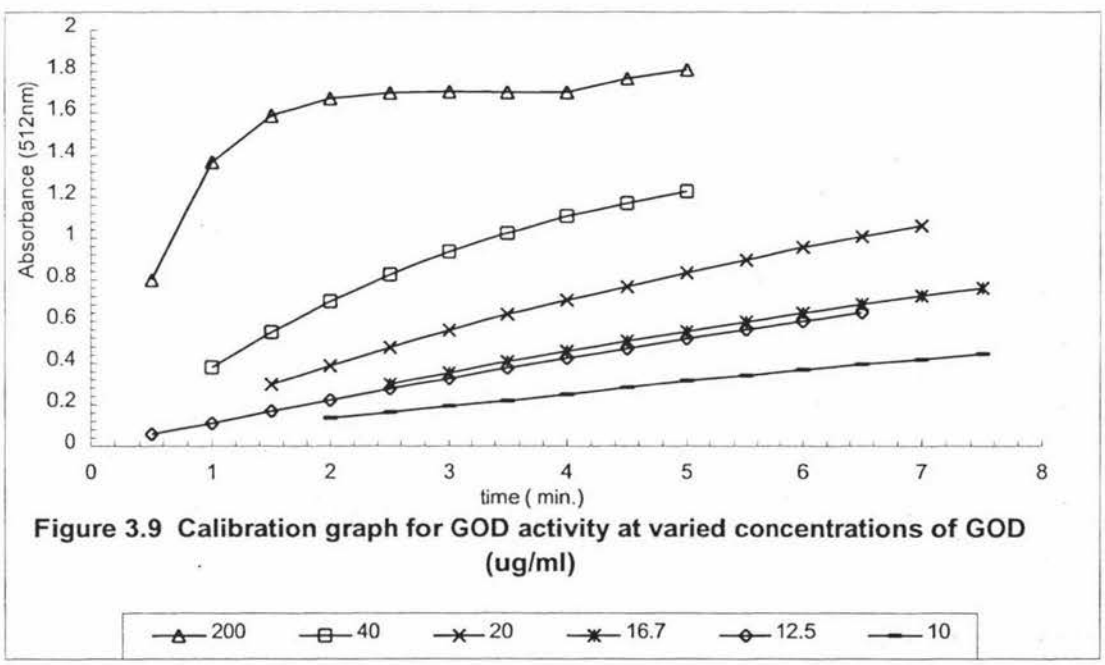


Figure 3.9 Calibration graph for GOD activity at varied concentrations of GOD (ug/ml)

Figure 3.9 is calibration graph for GOD activity (absorbance at 512nm versus determination time in minute). Every curve in Figure 3.9 represents the GOD activity curve at a fixed concentration of GOD. It can be seen that in this experiment, to work in the linear range of the assay, the concentration of glucose oxidase has to be less than 200 µg/ml

In this study, another kind of calibration graph for GOD activity was introduced (Figure 3.10). The absorbance at 512nm is plotted versus logarithm value of GOD concentration μg/ml in the graph. Every curve in Figure 3.10 represents GOD activity curve at a determination time (min.). It is clear that if the GOD concentration is fall out of the range between 10μg/ml to 200μg/ml, the absorbance is not dependent on the determinative time. As the conclusion, for the enzyme activity assay, the concentration of the enzyme should be kept within the range of 10μg/ml to 200μg/ml. Because the assay is linear and sensitive, the response is quick in this range.

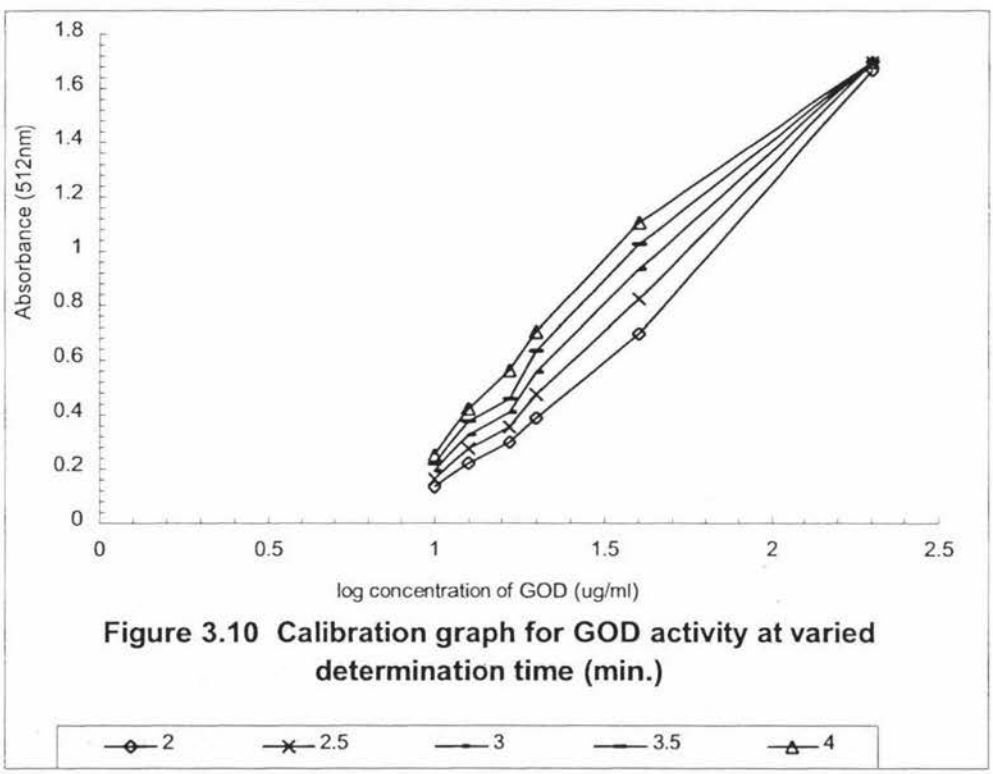


Figure 3.10 Calibration graph for GOD activity at varied determination time (min.)

3.5 Detection of haemin-GOD conjugate

3.5.1 Concentration of GOD in the conjugate

After the conjugation and separation, three fractions were collected. The GOD concentration assay of the haemin-GOD conjugate was carried out in the same

way as GOD concentration calibration.

Table 3.2 Concentration of GOD in the fractions

	Volume (ml)	GOD Concentration (mg/ml)	GOD Found (mg)	GOD Found/Added (%)
Fraction1	10.0	4.45	44.50	89.0
Fraction2	8.5	0.57	4.84	9.6
Fraction3	5.4	0.34	1.83	3.7

Note: The initial quantity of GOD (GOD added) was 50 mg.

Table 3.2 shows that the first part of elution (fraction 1) collected 44.50mg GOD (89.0 percent of glucose oxidase added) after conjugation.

3.5.2 GOD activity in haemin-GOD conjugate

Procedures: Dilute the fractions of haemin-GOD conjugate with buffer solution. Fraction 1 was diluted fifty times, fraction 2 ten times and fraction 3 ten times. The assay procedures for GOD activity in the fractions were the same as that of the GOD activity calibration

Figure 3.11 shows the GOD activity in the diluted fractions of GOD-haemin conjugate and shows that the haemin-GOD conjugate has GOD activity.

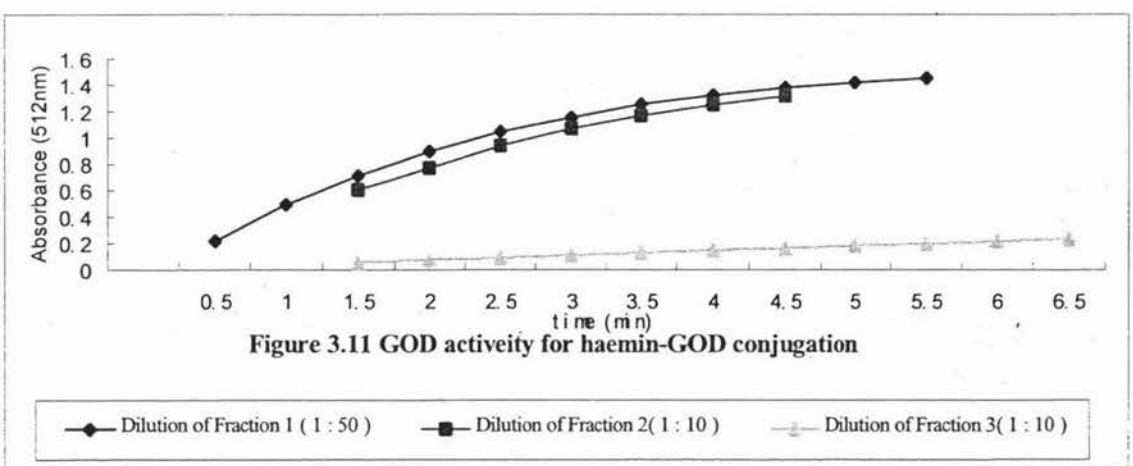


Figure 3.11 GOD activeity for haemin-GOD conjugation

3.5.3 Specific activity of haemin-GOD conjugated

For the calculation of the specific activity of the conjugated haemin-GOD, the relative data are shown as follows:

Original specific activity of GOD was168.2 Units/mg; GOD contents in Fraction 1 was 44.5mg; volume of Fraction 1 is 10.0ml; Diluted of Fraction 1 by 50 times (for determining the activity and concentration of GOD).

From Figure 3.9, Figure 3.10 and Figure 3.11, it can be measured that the GOD activity in the diluted Fraction 1 corresponds to the activity of the original GOD concentrations between 50μg/ml and 60μg/ml. If it corresponds to the activity at 50μg/ml of GOD concentration, the specific activity of GOD in the fraction 1 can be calculated:

$$\text{Specific activity} = \frac{50 \times 10^{-3} \times 50 \times 10 \times 168.2}{44.5} = 94.5 \text{ units/mg}$$

If it corresponds to that at 60μg/ml, the calculation formula:

$$\text{Specific activity} = \frac{50 \times 10^{-3} \times 60 \times 10 \times 168.2}{44.5} = 113.4 \text{ units/mg}$$

Therefore, the specific activity had changed to 104.0 ±10 units/mg after the conjugation. Comparing with the original activity of the GOD enzyme (168.2 units/mg), thus 2/3 to 3/4 of the original activity was retained after the conjugation. These results show that after haemin conjugation with GOD, the specific activity of GOD decreased.

A similar result is presented in the paper of Degani and Heller (1987). They modified glucose oxidase with ferrocene carboxylic acid successfully and assayed the enzyme activity before and after the chemical modification. One of the results showed that after covalent attachment of an average of 12 ferrocene centers per enzyme molecule, the specific activity of the enzyme dropped from

120 units/mg) changed to 70 units/mg. Thus 1/2 to 2/3 of the original activity was retained after the chemical modification.

3.5.4 Comparison of the activity of haemin-GOD conjugate with that of GOD found

The question is whether the decrease of GOD specific activity in the above experiment resulted from the separation and other treatments or from the conjugation. To answer this question and to confirm the conjugation, a comparative experiment was carried out.

The comparative experiment was carried out simultaneously. In the comparative experiment, glucose oxidase "was reacted" with HEPES buffer (0.1M, pH7.6) alone instead of activated haemin ester in the buffer. Then the resulting solution was treated exactly the same way as the haemin-glucose conjugate. The test results of this "reference GOD" are compared with those of conjugate. The results are as follows:

3.5.4.1 Comparison of the activities

Comparing the GOD specific activity curves between haemin-GOD conjugate and the reference GOD (Figure 3.12), it is clear that the GOD specific activity curve of haemin-GOD conjugate was similar to that of the GOD. However, the GOD specific activity of the conjugate was 25%-35% less than that of the reference GOD.

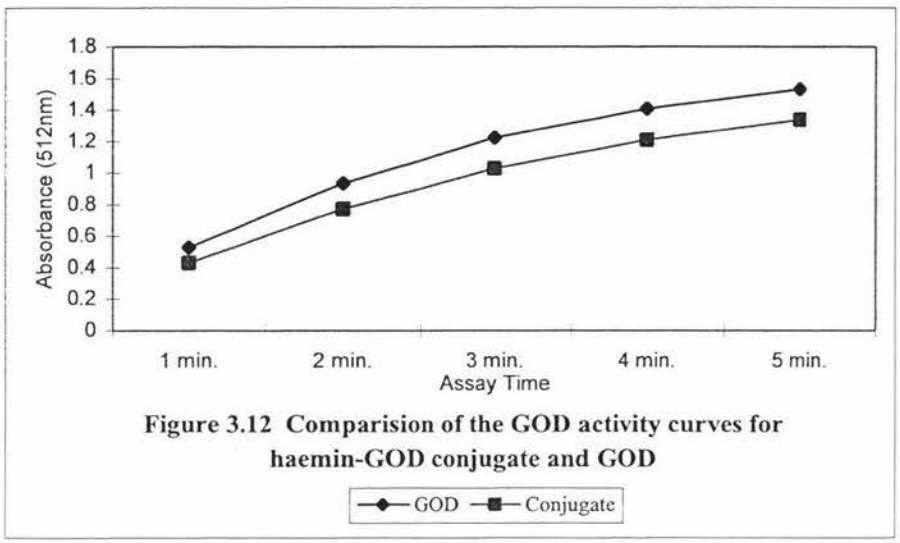


Figure 3.12 Comparision of the GOD activity curves for haemin-GOD conjugate and GOD

3.5.4.2 Comparison of the effect of storage

The effect of storage was studied by recording GOD activity curves at intervals over a period of 14 days (Figure 3.13 and Figure 3.14). The result indicates that the storage conditions were suitable for the conjugate and the reference GOD, and the effects of storage for both the reference GOD and the conjugate were similar.

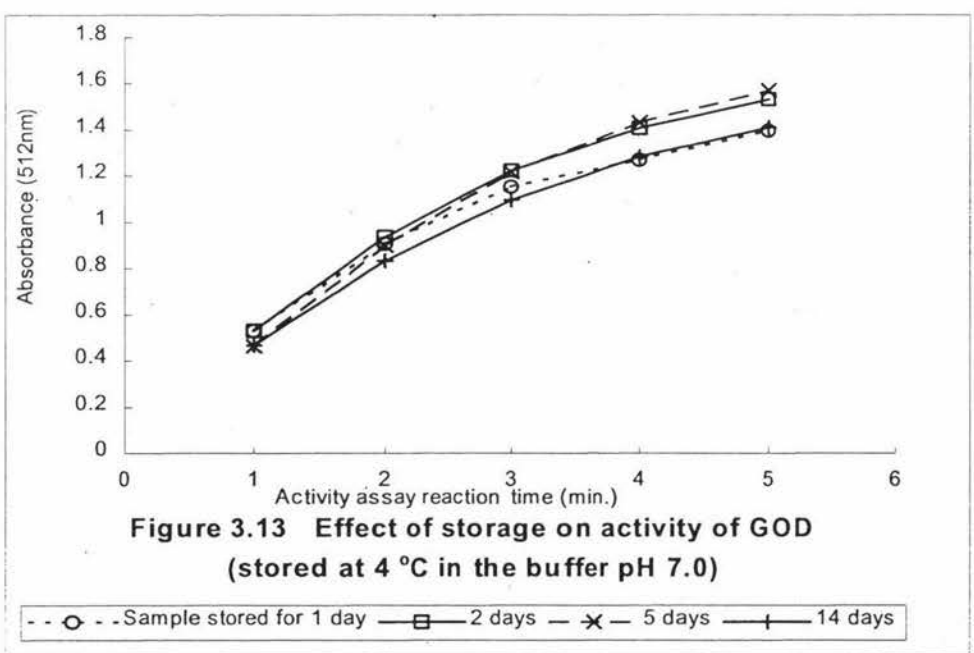


Figure 3.13 Effect of storage on activity of GOD (stored at 4 °C in the buffer pH 7.0)

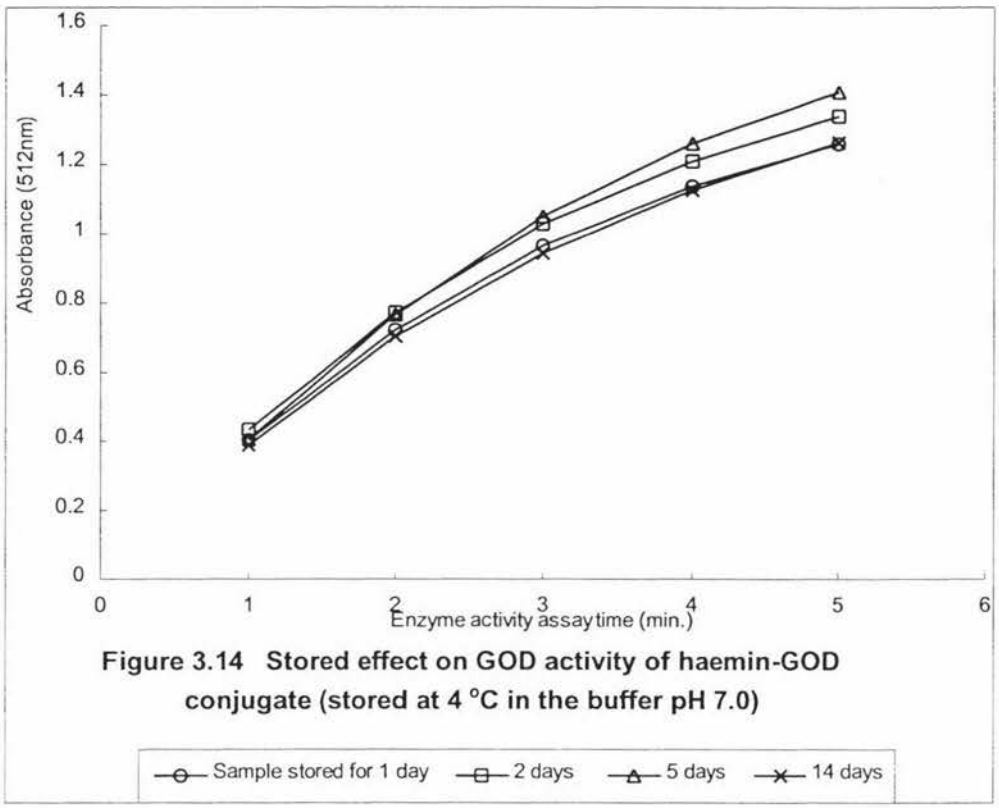


Figure 3.14 Stored effect on GOD activity of haemin-GOD conjugate (stored at 4 °C in the buffer pH 7.0)

3.5.4.3 Comparison of the stability of GOD activity

Figure 3.15 and Figure 3.16 are the stability graphs for GOD activity of the reference GOD and haemin-GOD conjugate respectively. Every curve in the figures represents the GOD activity curve at a fixed determination time. It is obvious that the GOD activity of haemin-GOD conjugate was similar to that of the reference GOD over a period of 14 days. In other words the behavior of the conjugate is similar to that of unconjugated GOD.

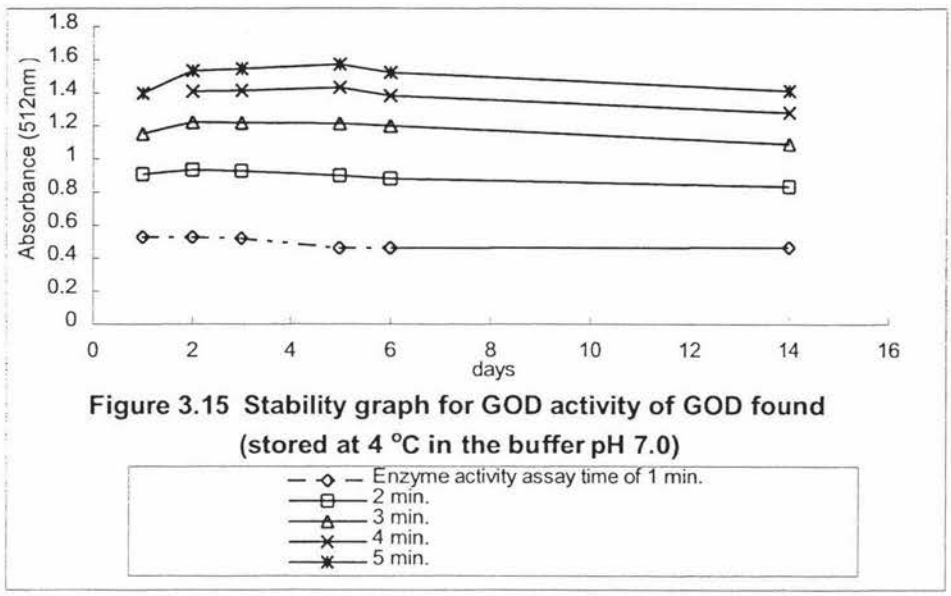


Figure 3.15 Stability graph for GOD activity of GOD found (stored at 4 °C in the buffer pH 7.0)

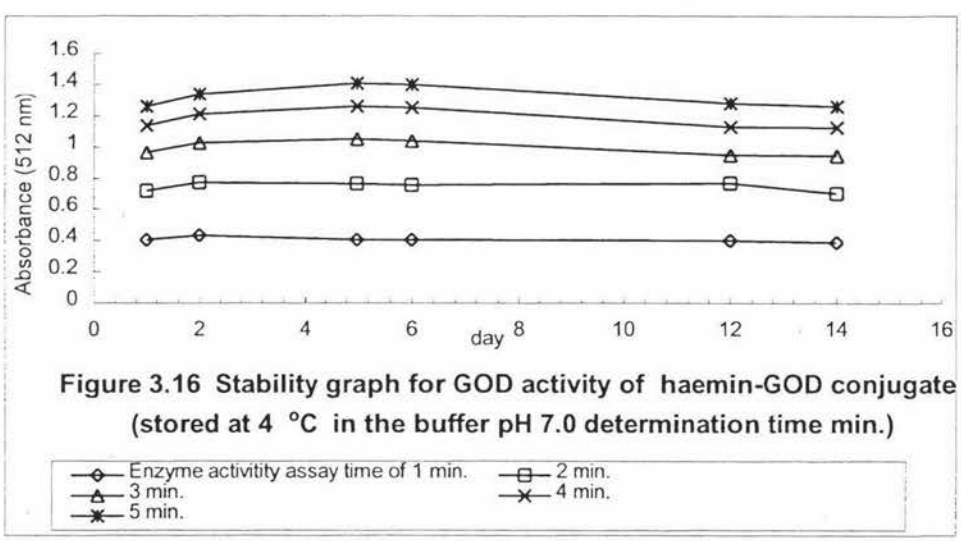


Figure 3.16 Stability graph for GOD activity of haemin-GOD conjugate (stored at 4 °C in the buffer pH 7.0 determination time min.)

3.5.4.4 Comparison the specific activity

From Figure 3.17, it is known that the specific activity of the conjugate and the reference GOD slightly changed initially then remained steady over the period of 14 days. However, the specific activity of the conjugate was less than that of the reference GOD.

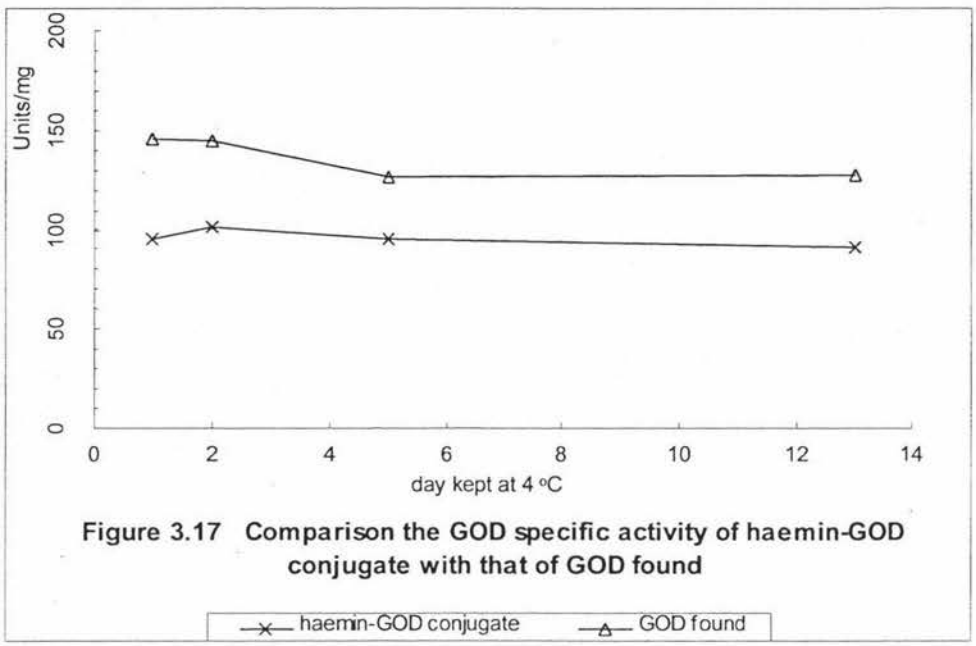


Figure 3.17 Comparison the GOD specific activity of haemin-GOD conjugate with that of GOD found

In summary, these comparative studies indicated that the haemin-GOD conjugate retained the activity characteristics of GOD. The specific activity of the conjugated GOD was found to be less than that of the reference GOD.

To study the reason for loss of enzyme activity, free enzyme was used as reference. It was treated exactly the same way as the GOD that was used to conjugate to haemin, apart from that instead of using activated haemin in HEPES buffer (0.1 M, pH7.6), 4.0 ml of HEPES buffer alone was used to react with the GOD. Then, as for the haemin-GOD conjugate, this reference GOD solution was passed through Sephadex G-10 column before further analysis was carried out.

The results confirmed that the decrease of GOD specific activity in the haemin-GOD conjugate was caused by the conjugation of haemin molecule to GOD and not the chemistry involved.

3.5.5 Absorbance spectra of the purified haemin-GOD conjugate

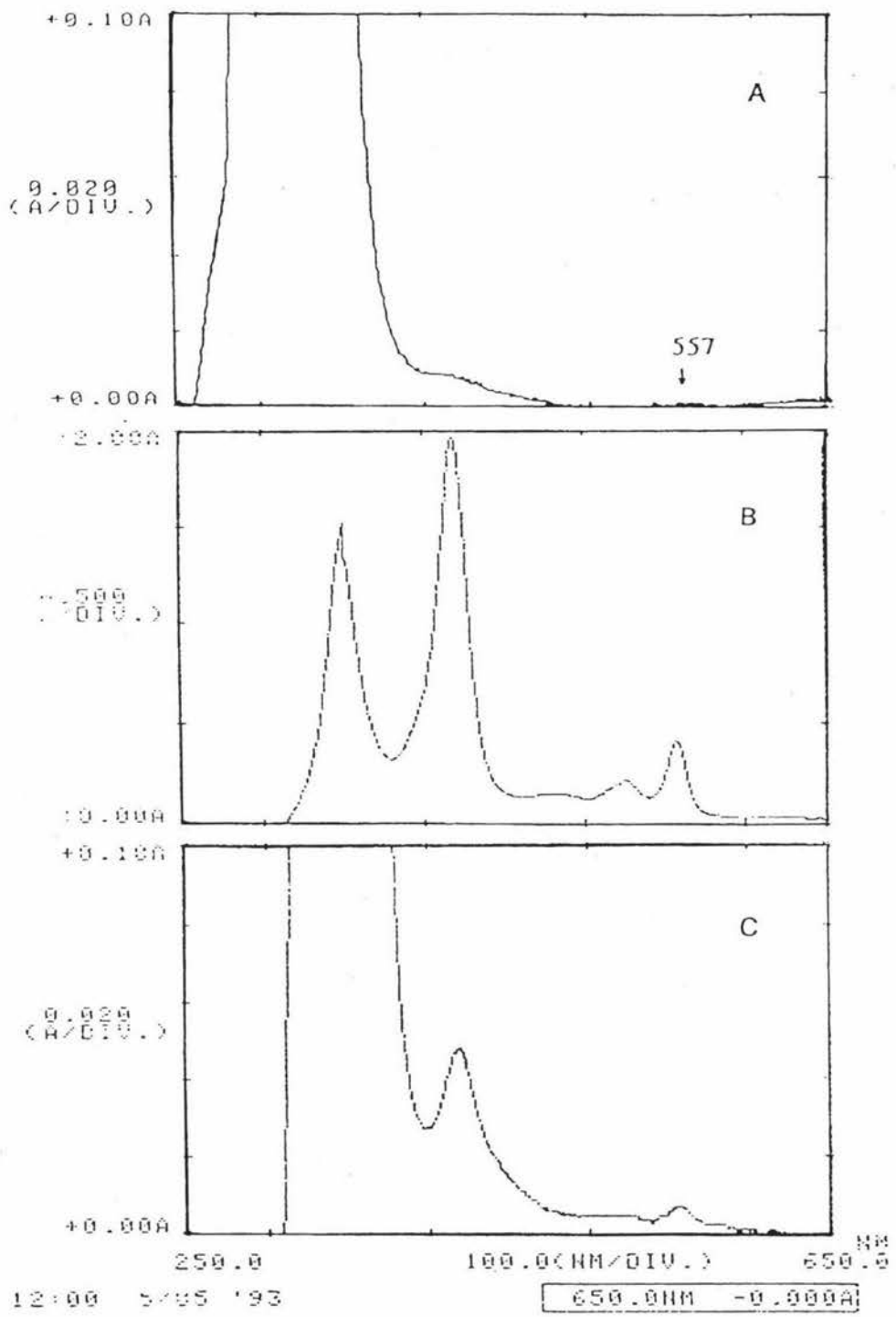


Figure 3.18 Absorbance spectra for GOD, haemin and conjugate
(A) GOD; (B) haemin; (C) haemin-GOD conjugate

The absorbance spectrum of purified haemin-GOD conjugate was recorded between 250nm and 650nm. The absorbance spectrum of the conjugate (Figure 3.18-C) exhibits characteristics of both haemin (Figure 3.18-B) and GOD (Figure 3.18-A). This indicates that haemin conjugated to the GOD molecule. Because haemin molecules are small molecules, they are retained longer on the gel filtration columns (Degani and Heller, 1987). However when the small haemin molecule is covalently attached to the large GOD molecule, it becomes too big for the pores of the gel column and therefore, moves rapidly and elutes off the column in the larger protein peak.

The haemin-GOD conjugate had the same absorption spectrum and functional properties as the free species, and the covalent binding of the haemin carboxyl group to glucose oxidase did not lead to the change of the properties of modified glucose oxidase.

3.6 Electrochemical detection of platinum electrode chemisorbed with the haemin-GOD conjugate

3.6.1 Cyclic voltammetry

Cyclic voltammetry experiment was applied to the platinum disc electrode that had been treated with the haemin-glucose oxidase conjugate solution. During the experiments, in relative to the reference electrode, the potential of the working electrode was cycling within a selected range. The resulted current flow brtween the working electrode and the auxiliary electrode was measured (Danilich *et al.*, 1993). A plot of current versus applied potential is a cyclic voltammogram and shows a characteristic increase in current at the potential where oxidation or reduction of an electroactive species occurs (Kemp, 1985; Higgins, 1988).

Cyclic voltammetry is a very sensitive technique and this is particularly true for the detection of the electroactive species bound on the electrode surface.

Thus, once the electrode surface has been modified by the redox groups, it is very easy to detect the presence of these species and the response is quick and sensitives (Bartlett, 1990).

3.6.2 Cyclic voltammetry assay

In this experiment, a three-electrode configuration was used, which consists of a silver-silver chloride electrode as reference electrode, a platinum wire as auxiliary electrode and a working electrode treated with haemin-glucose oxidase conjugate. In order to detect the redox species on the electrode, an appropriate potential of working electrode was selected. The potential was high enough to reduce the electroactive species bound on the surface of the working electrode. For comparison, a clean platinum disc electrode and a GOD adsorbed electrode were served as working electrode and the respective cyclic voltammograms were recorded.

3.6.3 Cyclic voltammetric characteristics before and after chemisorption at electrode

The cyclic votammograms for the clean platinum electrode and for the platinum electrode treated with GOD are exhibited in Figure 3.19. The cyclic votammogram in Figure 3.19-B is different from that in Figure 3.19-A, because GOD is adsorbed on the surface of the electrode in this case.

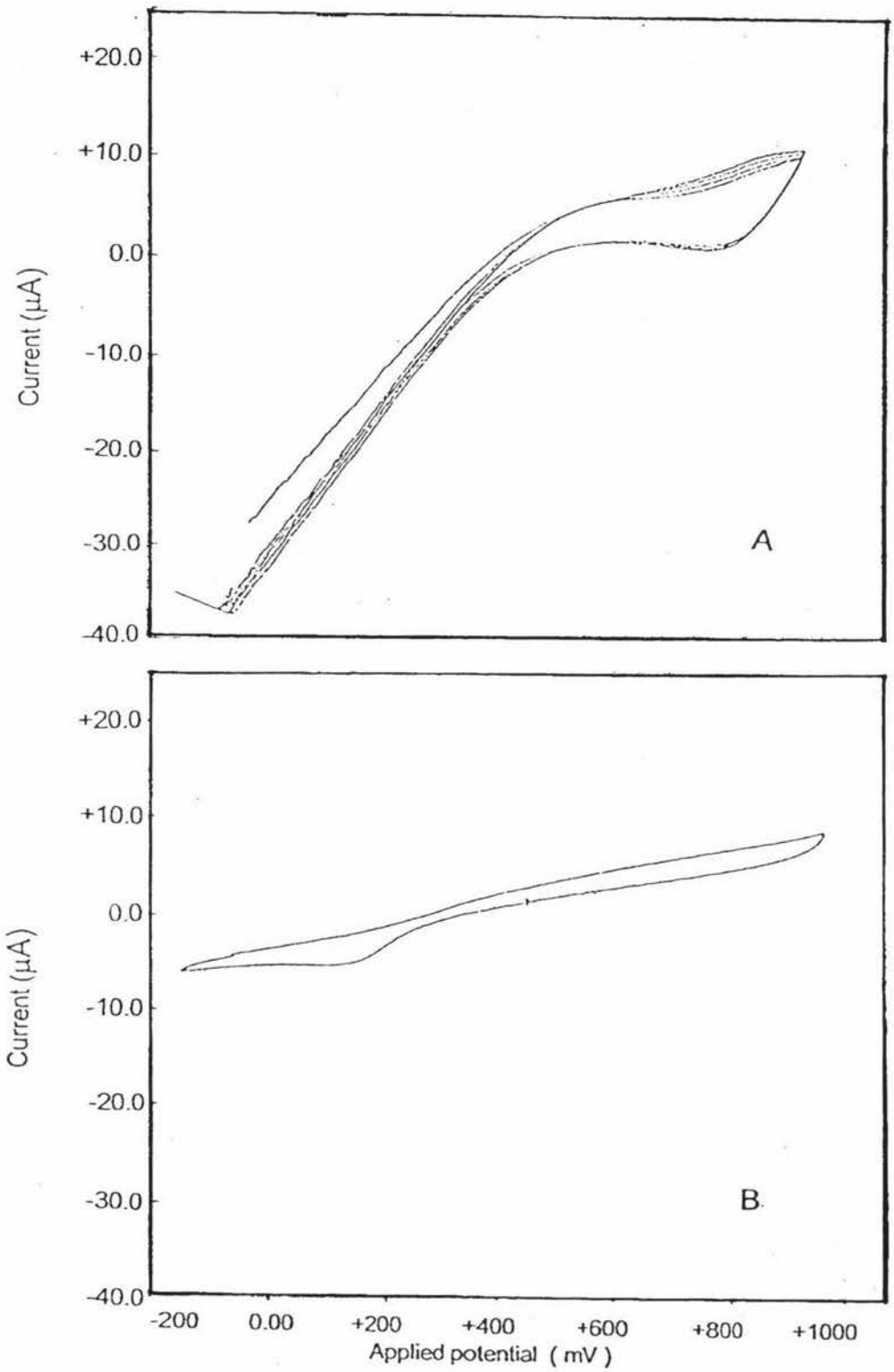


Figure 3.19 Cyclic voltammograms

A. Clean platinum electrode in the buffer (pH 7.0);

B. Platinum electrode with adsorbed GOD in the buffer (pH 7.0)

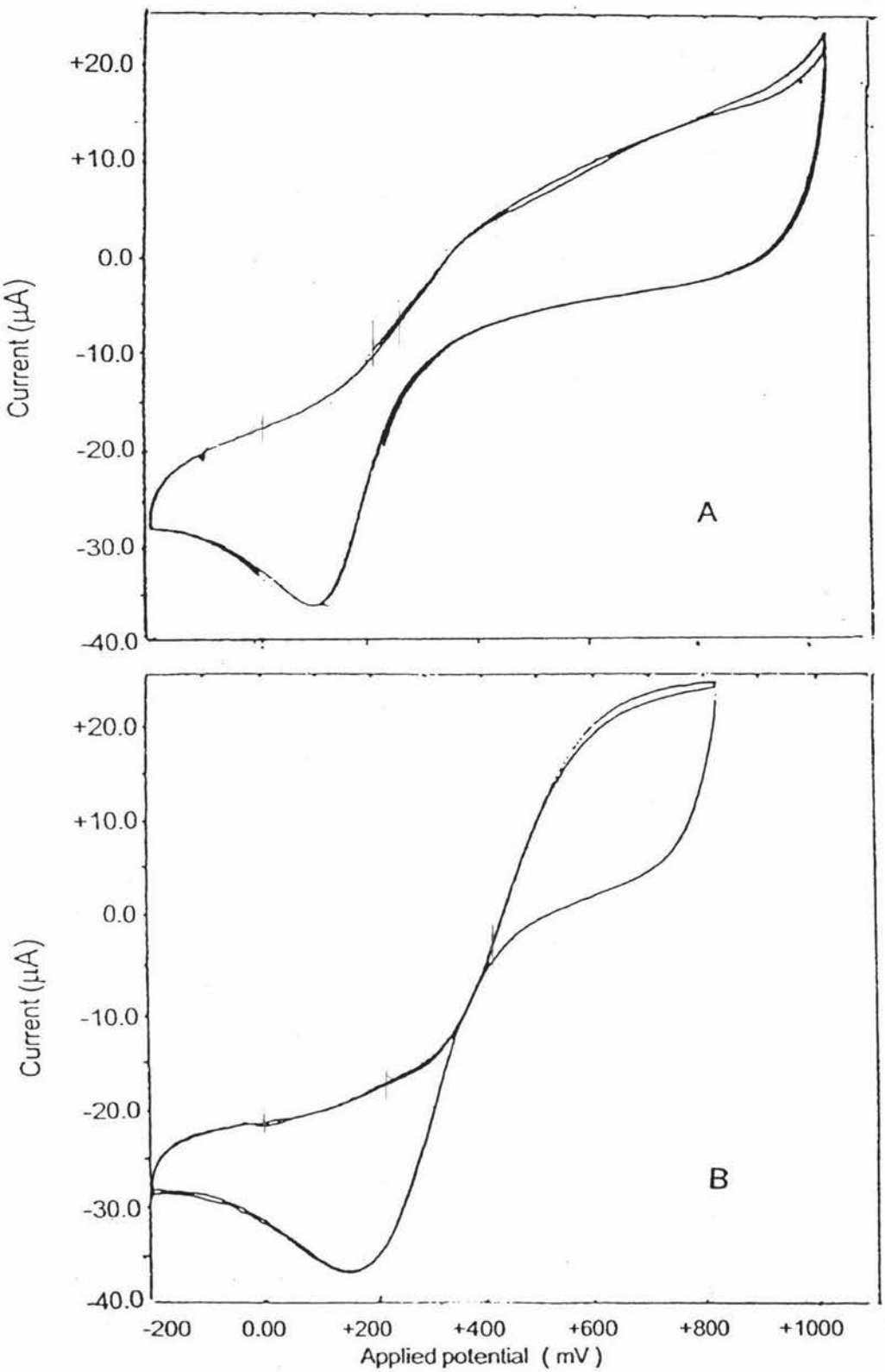


Figure 3.20 Cyclic voltammograms for the platinum electrode chemisorbed with the haemin-GOD conjugate.

A) In the buffer (pH 7.0) without glucose; (B) in the buffer (pH 7.0) with 10mM glucose

Figure 3.20 shows the cyclic voltammograms of platinum electrode treated by haemin-GOD conjugate. The cyclic voltammogram in Figure 3.20-B was recorded in the phosphate buffer with 10mM of glucose (pH 7.0) and the cyclic voltammogram in Figure 3.20-A was recorded in the buffer without glucose (pH 7.0).

The cyclic voltammograms in Figure 3.20 are quite different from the cyclic voltammograms in Figure 3.19-B. It is evident that the surface of the electrode was modified by the chemisorbed haemin-GOD conjugate, which is a substance distinctive from glucose oxidase. Comparing the cyclic voltammogram in Figure 3.20-B with the cyclic voltammogram in Figure 3.20-A, it is indicated that the electrode chemisorbed by haemin-GOD conjugate has a characteristic response to glucose.

The cyclic voltammograms of platinum/haemin-GOD conjugated electrode consisted of an anodic wave on the forward sweep at -200mV to 1000mV, and cathodic waves on the reverse sweep at 1000 mV to -200 mV, and at a sweep rate of 20 mV/S. The anodic wave is attributed to the oxidation of glucose, and the cathodic wave is attributed to the reduction of gluconic acid.

$$\underset{\text{glucose}}{C_6H_{12}O_6 + H_2O} \xleftarrow[\text{cathodic}]{\overset{\text{anodic}}{GOD}} \underset{\text{gluconic acid}}{C_6H_{12}O_7 + 2H^+ + 2e^-}$$

Bartlett and Bradford (1990) treated glucose oxidase with tetrathiafulvalene (TTF), an organic salt in aqueous buffered solution. This led to the modification of glucose oxidase by incorporation of TTF molecules. Figure 3.21 shows the cyclic voltammogrom for TTF modified glucose oxidase at a platinum-disc electrode recorded at 5mV/s in phosphate butter pH 7.0.

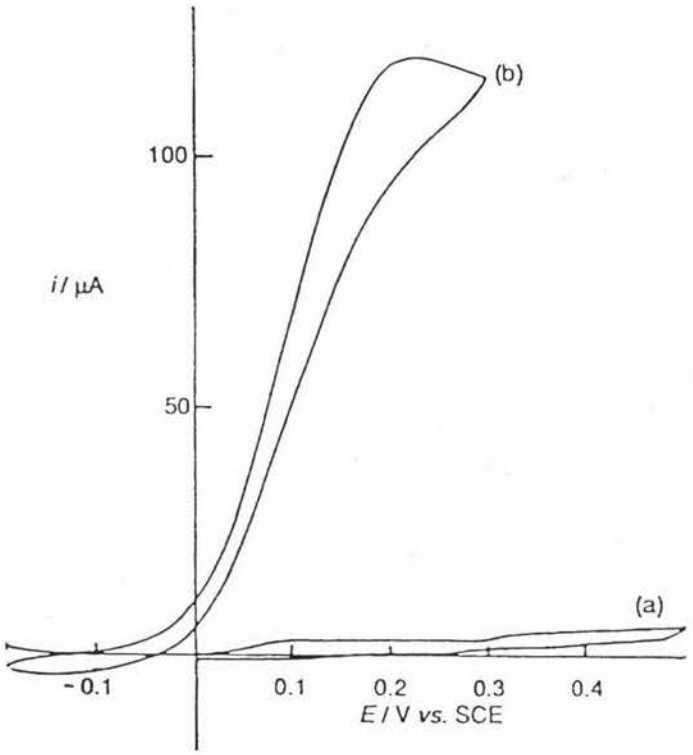


Figure 3.21 Cyclic voltammogram for TTF modified glucose oxidase at a platinum-disc electrode.
(a) no glucose; (b) 90 mmol dm^{-3} glucose. (Bartlett and Bradford, 1990)

3.7 Electrochemical measurement

Instrument:

The electrochemical cell included the Ag/AgCl reference electrode, the platinum wire auxiliary electrode and the platinum electrode chemisorbed by haemin-GOD conjugate as the working electrode. Using + 600mV of working potential, the experiments were carried out at room temperature in a 0.05M phosphate buffer (pH 7.0) with varied glucose concentration. The calibration graph for measurement of glucose is exhibited as the curve 1 in Figure 3.22.

For comparison, the platinum disc electrode adsorbed with glucose oxidase as a working electrode performed the electrochemical measurement in 0.05M

phosphate buffer (pH 7.0) with varied glucose concentration. Both working electrodes were always stored at 4°C in the buffer solution when not in use.

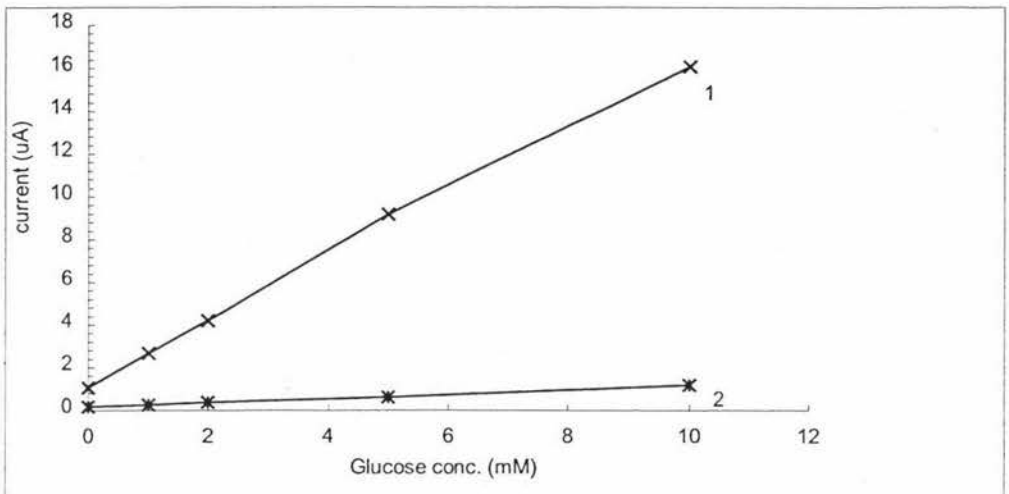


Figure 3.22 Current responses to glucose for:

(1) platinum electrode chemisorbed by haemin-GOD conjugate;
(2) platinum electrode adsorbed by GOD

Comparison of curve 1 with curves 2 in Figure 3.22, shows that the platinum electrode adsorbed by GOD had a poor response to glucose and its response was decreased to the lower limits of detection. The reason is the non-bonding interactions between the electrode surface and GOD molecules. Because the interactions with the surface are weaker, the modified surface would be less stable. The working electrode made in this manner would not give as sensitive and consistent response results.

On the contrary, it was obvious (curve 1 in Figure 3.22) that the platinum electrode chemisorbed by haemin-GOD conjugate had the enzyme activity of GOD and exhibited a linear response to the concentration of glucose at a range from 0mM to 10mM.

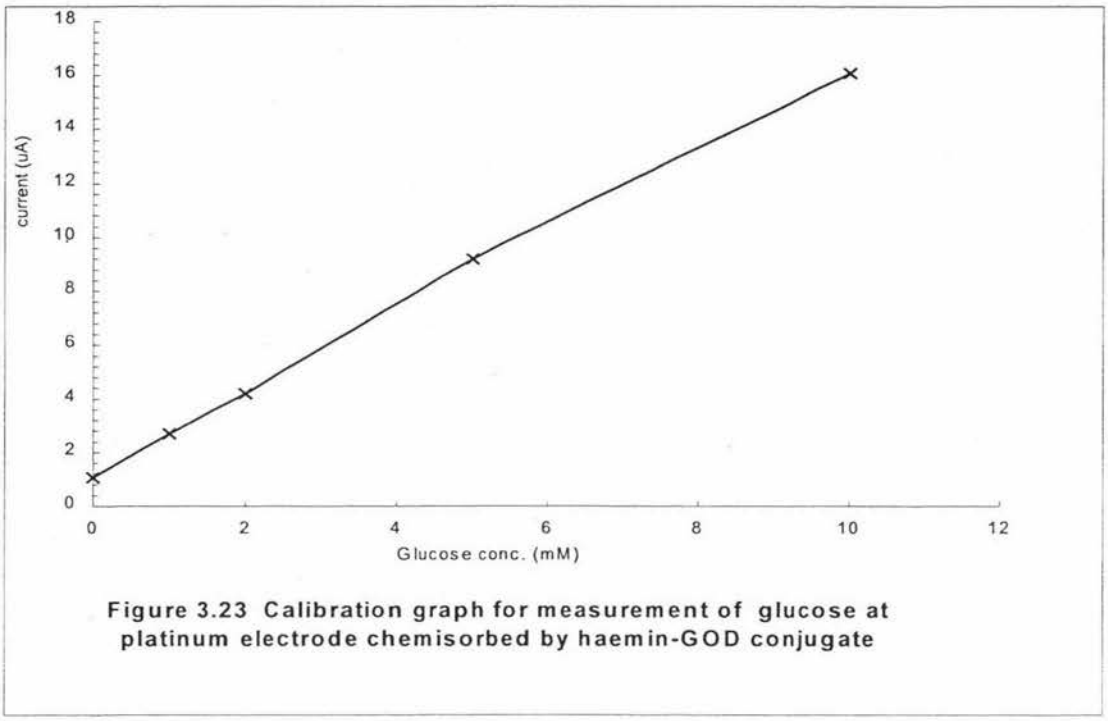


Figure 3.23 Calibration graph for measurement of glucose at platinum electrode chemisorbed by haemin-GOD conjugate

Furthermore, the ratio of the current response to glucose concentration is about 1.6 μA/mM. This indicates that the sensitivity of the platinum electrode chemisorbed by haemin-GOD conjugate is high and it is totally different from the electrode treated with GOD alone.

The stability of this electrode was also studied by recording its response in 5mM glucose at intervals over a period of 14 days. Stability of response for the electrode chemisorbed by the conjugate in 5.0mM glucose of the buffer solution (pH 7.0) shows in Figure 3.24. It appears from Figure 3.24 that the response falls initially but then remains stable on storage of the electrode over 14 days.

The trend in the curve of Figure 3.24 reflects the enzyme activity of the electrode chemisorbed by the GOD-haemin conjugate during a period of 14 days. The first-order decay rate of the electrode (within 24 hours) was fast, then the decay rate became slow. In addition, the response time of the electrode treated by the conjugate was very rapid at less then one minute.

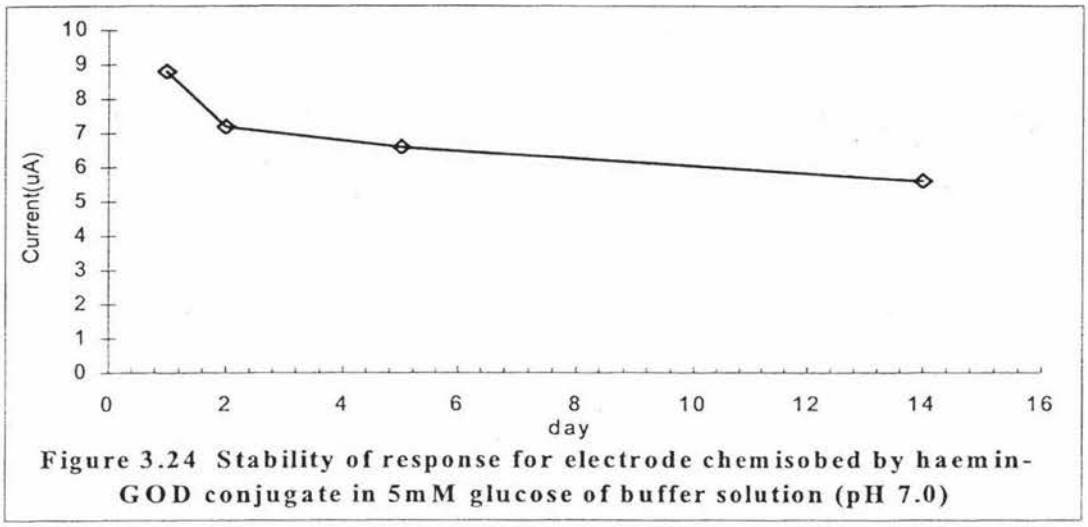


Figure 3.24 Stability of response for electrode chemisobed by haemin-GOD conjugate in 5mM glucose of buffer solution (pH 7.0)

Bartlett and Caruana (1992) constructed a microelectrode for the detection of glucose based on glucose oxidase immobilized in a poly(phenol) film. The microelectrode was stored at 4 °C in disodium hydrogen orthophosphate buffer (pH 7). The result indicates that the response falls initially but then remains stable on storage of the electrode, similar to the results of this thesis.

Figuer 3.25 shows the calibration graph of a glucose biosensor constructed by Schuhmann (1991). The biosensor was built of ferrocene modified glucose oxidase entrapped within a polypyrrole film.

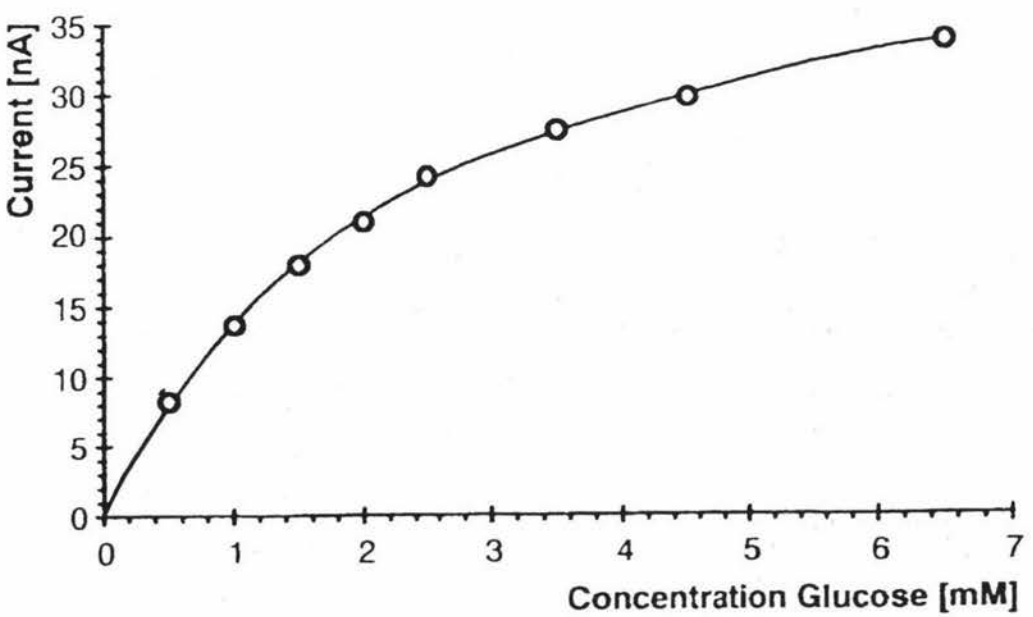


Figure 3.25 Calibration graph for glucose obtained with a ferrocene-modified glucose entrapped within a polypyrrole film (Schuhmann 1991)

Comparing Figure 3.25 with Figure 3.23, it is clear that the system of electrode-haemin-GOD (EHG) was more advantageous than the system of electrode-ferrocene-GOD entrapped within polypyrrole film (EFGP). The details are as follows:

(1). The ratio of the current response to glucose concentration for the EHG system was about 1.6μA/mM, however the ratio of that for the EFGP system was about 15nA/mM, thus the sensitivity of EHG system was about 100 times more than that of the EFGP system.
(2). The range of linearity for the EHG system was from 0mM to 10mM of glucose and that for the EFGP system was from 0mM to 2mM of glucose
(3). The construction procedure of the EHG system was very simple. The immobilization of GOD and conjugation of GOD with haemin were completed in one modification procedure. The immobilization of GOD onto the platinum electrode was also very simple. The electrode was only soaked in the conjugate solution at 4°C for 16 hours. However, in constructing of the EFGP system, the GOD was firstly modified with ferrocene, then the ferrocene-modified GOD was entrapped within a conducting-polymer film.
(4). No toxic materials are involved in the EHG system, because the haemin is a nontoxic substance in nature.
(5). The cost of constructing the EHG system was low.

Hall, E. A. H.(1990) listed seven requirements for an implantable glucose sensor as follows:

- Linear in 0-20mM range with 1 mM resolution
- Specific for glucose; not affected by changes in metabolite concentrations and ambient conditions
- Biocompatible
- Small-causes minimal tissue damage during insertion and there is better patient acceptability for a small device
- External calibration and <10% drift in 24h
- Response time <10min

- Prolonged lifetime-at least several days, preferably weeks in use

From this list, it is known that the EHG system can meet the requirements for an implantable glucose biosensor in terms of the sensitivity, linear response range, lifetime, response time, ease of preparation, convenience of operation, non-toxicity and low cost.

CHAPTER FOUR

CONCLUSION

The combination of enzyme redox systems with amperometric devices is currently an attractive approach to biosensor researchers. In most cases, glucose oxidase has been immobilized on to an electrode, and in an indirect detection system, a mediator is also added into the system. This thesis proposed and testified a simple and easy way to prepare an indirect glucose biosensor. The proposed biosensor consists of a platinum electrode chemisorbed by haemin-glucose oxidase conjugate. Therefore, in this system the mediator (haemin) is between the electrode surface and the enzyme active center, the most efficient position to transfer electron, and have the glucose oxidase immobilized (chemisorbed) onto the electrode surface through haemin.

The overall aim of the thesis was the construction of a model glucose biosensor.

The goals that were set included the following:

1. To find optimum conditions for the conjugation and the chemisorption,
2. To study the effects of modifying the enzyme, to find out why glucose oxidase modified by haemin is a good candidate for biosensor.
3. To evolve an approach for the construction of amperometric biosensors,
4. To identify obvious areas for further studies needed to further the knowledge of and extend clues for constructing the biosensor.
5. To discuss the current and future applications of this type of biosensor.

4.1 Finding optimum conditions for the conjugation and the chemisorption

According to the structure and properties of glucose oxidase and haemin, as well as the reference information, it is clear that glucose oxidase should be a good candidate for specific chemical amplification of the redox reaction of

glucose. This proposal would require immobilizing the enzyme on to the electrode and a bifunctional promoter to act as a bridge to connect the electrode surface with the glucose oxidase and as a mediator to transfer electron between electrode surface and active center of the enzyme.

In this thesis, the construction of glucose biosensor was divided into two steps. At first, glucose oxidase was conjugated with haemin, and then the haemin-glucose oxidase conjugate was chemisorbed onto the surface of a platinum electrode. In preparing haemin-glucose oxidase conjugate, firstly, the carboxyl group of haemin is converted to a reactive enol ester, then, the resulting enol ester reacted with the amino group of glucose oxidase to form a peptide bond.

The detailed experimental procedures of preparing glucose biosensor were as follows:

- The system of DEC [1-(3-dimethylaminopropyl)-3-ethyl carbodi-imide hydrochloride] and Na-HEPES [sodium 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonate] at pH 7.2 was chosen to convert the haemin carboxyl group to the reactive enol ester. The reason is that with the system of DEC and Na-HEPES the reaction can be carried out in aqueous solution. This is especially advantageous to the subsequent conjugation, which has to be carried out in the aqueous solution. This also avoided the possibility for the electrode surface being polluted by organic materials.

- The conjugation between the reactive enol ester of haemin and amino group of glucose oxidase was carried out at pH 7.2, 4 °C in a refrigerator for 16 hours.

- After the conjugation, the reaction solution was passed through the Sephadex G-10 gel column to separate haemin-glucose oxidase conjugate from unreacted haemin. The spectral absorption at 557nm was used to detect haemin content in the conjugate, as it is the biggest absorption peak of haemin that is not overlapping with the absorbance of glucose oxidase. However only reduced haemin has absorption peak at 557nm. As the

result, dithionite was use as an antioxidant to prevent the reduced haemin from re-oxidation in the procedures.

- The pretreated platinum disc electrode was soaked in the conjugate in phosphate buffer (pH 7.0) at 4°C for 16 hours.

- In this study, apart from using the traditional enzyme activity assay diagram, a new calibration graph for GOD activity is introduced. In the diagram, absorbance (512nm) is plotted versus logarithm value of GOD concentration (μg/ml), as showed in Figure 3.10. By using the diagram, the suitable range of GOD concentrations for GOD activity assay are easy to identify.

- The comparative experiments were carried out simultaneously. In the experiment, glucose oxidase "was reacted" with HEPES buffer (0.1M, pH7.2) alone instead of activated haemin ester in the buffer. Then the resulted solution was treated exactly the same way as the haemin-glucose conjugate. The tests results of this "reference GOD" are compared with those of conjugate.

As shown below, the experimental results indicated that both the conjugation and the chemisorption were successful.

1. The GOD activity curve for the haemin-GOD conjugate was similar to that of the GOD. Moreover, the GOD activity curve for haemin-GOD conjugate had similar stability characteristics to that of GOD over a period of 14 days. The conjugate therefore appears to have the characteristics of GOD. This means that the function of glucose oxidase was not significantly affected by conjugation.

2. The specific activity of the haemin-GOD conjugate was less than that of the unconjugated GOD. The conjugate retained about 2/3 to 3/4 of specific activity of the original GOD. The decrease of GOD specific activity in the conjugate was caused by the conjugation, as the unconjugated GOD had

been through all the procedures as the conjugate.

3. The UV/Vis absorbance spectra of the conjugate showed peaks corresponding to both haemin and GOD. The small molecule of haemin would travel slowly in the gel column, unless it has bonded to a much large molecule such as glucose oxidase. During the experiment, smaller unbound haemin molecules were retained on the Sephadex G-10 gel column, while the fraction of conjugate is the peak with glucose oxidase activity and moved through the gel column rapidly. Therefore, haemin that existed in this peak should have been bound to the GOD molecules.

4. The cyclic voltammogram of the electrode chemisorbed by haemin-GOD conjugate in the phosphate buffer (pH 7.0) with and without glucose was quite different from that of the electrode adsorbed by glucose oxidase alone. It is evident that the haemin-GOD conjugate is distinctive from glucose oxidase alone.

5. The cyclic voltammogram of the electrode chemisorbed by haemin-GOD in the solution with glucose is different from that without glucose. It is indicated that the surface of the electrode treated with the conjugate had a characteristic of responding to the glucose.

6. The platinum electrode treated with haemin-GOD conjugate had the characteristics of GOD activity to catalyze the redox of glucose specifically and exhibited a linear response to the concentration of glucose at a range from 0mM to 10mM. Contrary, the electrode treated with GOD alone had a poor response to glucose.

7. The sensitivity of the electrode chemisorbed by the conjugate was high. The ratio of current response to glucose concentration was about 1.6 μA/mM. Its features of a sensitive and linear response to glucose indicated that there was a rapid electron transfer between the active site of glucose oxidase and the electrode.

8. As for the stability of the electrode chemisorbed by the conjugate, the response fell initially but then remained stable over 14 days. In addition, its response time was very rapid at less than one minute.

Therefore, the facts above confirmed that both the conjugation and the chemisorptions were successful and the experimental data proved that the platinum electrode chemisorbed with the conjugate had the fundamental characteristics of a glucose biosensor. Its advantages include high sensitivity, wide linear response range, long lifetime, easy to prepare, convenient to operate, no toxic and low cost.

4.2 Studying the effects of the modifying the enzyme and the electrode

Some studies (Mclendon, 1988; Marcus and Sutin, 1985) showed that the probability of electron transfer of biological molecular falls as the distance increases. Bartlett (1990) suggested that:

$$K_{el} = K_{el,o} \exp (- \beta [r - r_o])$$

Where: K_{el} is the electronic transmission coefficient,
$K_{el,o}$ is the value when the separation is r_o
r is the distance between the centers, and
β is a constant.

Values of β estimated from experimental measurements were $12nm^{-1}$. This implies that the probability of electron transfer decreases by an order of magnitude for every 0.2nm increase in distance between the two centers. This indicates that decreasing the distance between the electrode surface and redox active sites of the enzyme is very important for the sensitivity and response speed of the resulting electrode.

Due to the fact that the molecular weight of glucose oxidase is quite big and the

redox active site of the enzyme is deeply buried within the protein shell, direct electron transfer between the enzyme and electrode surface is rarely encountered (Schuhmann, 1991; Degani and Heller, 1987; Hecht, *et al.*, 1993).

Therefore, researchers attempt to find the effective way to shorten the distance between the active center of the enzyme and the electrode. In the literature, ferrocene was conjugated to glucose oxidase to mediate the electron transfer (Degani and Heller 1987). Researchers also tried to entrap the ferrocene modified glucose oxidase within a conducting-polymer film to close the gap between ferrocene and the electrode (Schuhmann etc. 1991).

In this thesis, using haemin as the mediator shortened or eliminated the gap between the mediator and electrode. The bifunctional promoter, haemin, acted as a bridge to bond the electrode surface and the glucose oxidase. It also acts as a stepping-stone to mediate the electron transfer between the enzyme and electrode. The increasing sensitivity of the electrode chemisorbed by the conjugate was due to the shortening of the electron transfer distance. Thus this "sandwich" system shortened the electron transfer distance even further between the electrode surface and the glucose oxidase.

Haemin can be present in both oxidized and reduced forms and similar to ferrocene, can react as a mediator. Its reaction with the redox active center of GOD is shown in the following scheme:

$$GOD - FADH_2 + Haemin(ox) \longrightarrow GOD - FAD + Haemin(red) + H^+$$

$$Haemin(red) \xrightarrow{anode} Haemin(ox)$$

When the haemin covalently bond to the glucose oxidase and chemisorbed onto the electrode surface, the electrons transferred from the redox centers of enzyme to the haemin and then transfer to the electrode surface. Because the haemin was conjugated with the enzyme and the electrode surface, the electron transfer between the electrode and the active center of the enzyme was mediated by haemin. In the other words, electron transferred across a shorter

distance. As the result, the electron transference from the active site of the enzyme to the electrode surface was fast.

Figure 4.1 shows a sketch map for the entrapment of mediator (ferrocene) - modified enzymes within a conducting-polymer (Schuhmann, 1991). Figure 4.2 shows a sketch map for the haemin conjugate with the enzyme and chemisorbed onto the electrode surface.

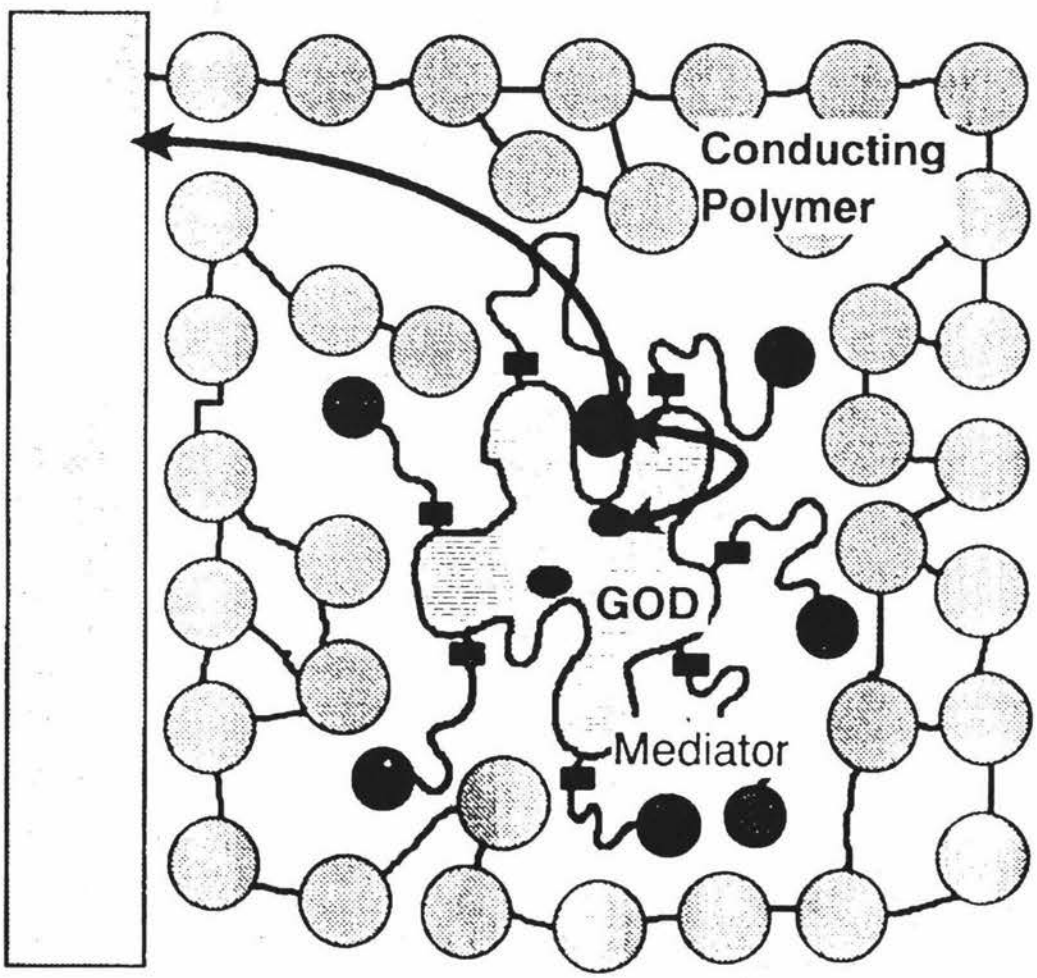


Figure 4.1 Entrapment of mediator-modified enzymes with a conducting film (Schuhmann 1991). Where mediator is ferrocene.

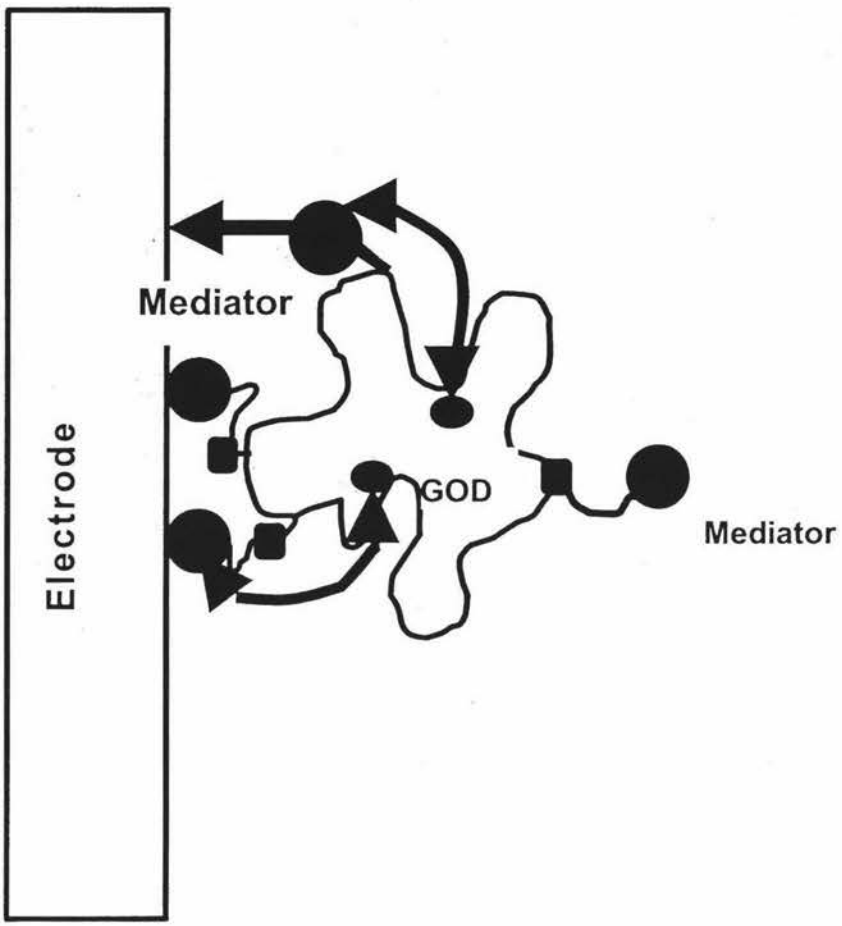


Figure 4.2 Haemin conjugate the glucose oxidase and the electrode surface

Figure 4.2 shows that the bifunctional effects of haemin led to the haemin-glucose oxidase conjugate strongly chemisorbed onto the electrode surface and act as mediator transfer the electrons between the redox active sites of glucose oxidase and electrode surface. Comparing with Figure 4.1 the distance between the electrode surface and the redox active sites of GOD in the system of electrode-haemin-GOD is shorter than both that in the system of ferrocene-modified GOD and the system of ferrocene-modified GOD entrapped within a conducting-polymer film. There was a fast electron transfer between the electrode surface and the redox active sites of GOD in the glucose oxidase-haemin-electrode system. It is the reason why the ratio of current response to glucose concentration was 1.6μA/mM in this system, comparing with 15nA/mM

in the system of ferrocene-modified glucose oxidase entrapped in the conducting -polymer film. It is also the reason why the linear response range was 0mM to 10mM of glucose in the glucose oxidase-haemin-electrode system comparing with 0mM to 2mM in the system of ferrocene-glucose oxidase entrapped within the conducting-polymer film. (See Figure 3.23 and Figure 3.25).

The lifetime of enzyme electrode depends on several factors including the life time of the modified enzyme, the storage and using condition, the interaction of the enzyme and the electrode, the strength of the bond between the enzyme and the electrode and so on. Owing to the fact that the GOD activity of haemin-GOD conjugate was stable and the conjugated double bonds of haemin interact strongly with the platinum surface to form irreversibly covalently chemisorbed species, the glucose oxidase-haemin-electrode system was stable at least over a period of 14 days. However, it should be mentioned that the lifetime of the electrode might be different under continuous operating conditions.

4.3 Evolving an approach for the construction an amperometric biosensor

A systematic approach for the construction of an easy to prepare, stable and more sensitive biosensor is suggested here using glucose oxidase as a model. At first, according to the demand from the application (for example, medicine, industry, agriculture, or environment), a stable enzyme is chosen to be a specific chemical amplifier for the specific substrate. Then a bifunctional promoter is chosen. It contains one functional group to modify the enzyme and a second functional group to immobilize the enzyme onto the electrode surface. Moreover, the bifunctional promoter should be present in both oxidized and reduced forms. It is a redox mediator to mediate the electrons transfer between the redox center of the enzyme and the electrode. Thirdly, the experiments should be done in an optimum physical, chemical, and biological conditions for conjugating, separating, chemisorpting and so on.

4.4 Identifying areas for further study

In this thesis, glucose oxidase-haemin conjugate chemisorbed electrode was studied as a model. It is proved from the experiments that the biosensor proposed and constructed by a straightforward technique is sensitive, stable and with a wider linear response range.

Glucose oxidase is a flavoprotein; some of the other flavoproteins and their applications are listed in Table 4.1.

Table 4.1 Some flavoproteins and their applications (Bartlett, 1990)

Enzyme	Analyte	Applications
Glucose oxidase	Glucose	Clinical
		Fermentation
		Meat industry
		Food industry
Xanthine Oxidase	Purines	Food Industry
D-Amino Acid Oxidase	D-Amino Acids	Clinical
Cholesterol Oxidase	Cholesterol	Clinical
Mono Amine Oxidase	Mono Amines	Clinical
		Food Industry
Galactose Oxidase	Galactose	Food Industry

As glucose oxidase, these flavoproteins contain flavin prosthetic groups as the redox activity center and amino groups that can be conjugated with the carboxyl group of haemin to form the peptide bonds.

Therefore, theoretically, the biosensors for determining purines, D-amino acids, cholesterol, monoamines, and galactose can be constructed in the way suggested in this thesis. The variety in activities and stabilities of flavoproteins will lead to the different responses for the corresponding electrodes. For

example, the currents for the D-amino acid system are about 100 times smaller than those for the glucose electrode. Partially, this is because D-amino acid oxidase is a much less active enzyme than glucose oxidase (Bartlett, 1992).

Another area worthy of further study is bifunctional molecules such as haemin. Some bifunctional promoters are listed in Figure 4.1 (Bartlett, 1996). These promoters contain one functional group to attach covalently to the electrode surface and another functional group to bind to enzymes. They served as "glue" to immobilize the enzyme onto electrode.

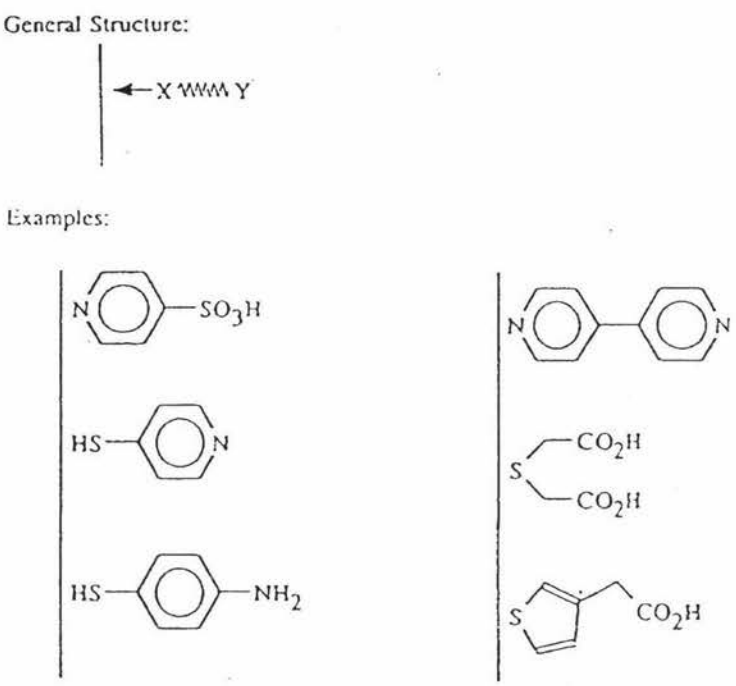


Figure 4.3 The general features and some examples of the biofunctional molecules.

X is a functional group that is able to interact with the electrode surface and Y is a functional group, which interacts with protein. (Bartlett, 1996).

The other area worthy of exploring is the design of the electrode. Electrode can be changed in size, shape and material. For example, a 25μm diameter poly (phenol)-glucose oxidase modified microelectrode was described by Bartlett

and Caruana (1992). Churchouse *et al.* (1986) and Garcia *et al.* (1983) presented a needle-type glucose biosensors. The carbon electrode can also be modified by chemisorptions. It is advantageous to use molecules with aromatic π electrode systems to interact with the π electrons on the graphitic carbon. For example, four redox dyes (4-[2-(1-pyrenyl)vinyl] catechol, meldola blue, 3-β naphthoyl-toluidine blue, and 4-[2-(2-naphthoyl)vinyl] catechol) are used to modify carbon surfaces (Jaegfeldt *et al.*, 1981, 1983; Bremle *et al, 1991*). Each dye molecule has an aromatic substituent attached in order to promote adsorption onto the basal plane regions of graphite through π - π interaction (Bartlett, 1996).

Other metal electrodes such as gold electrodes can take place of the platinum electrode for chemisorption and construction an electrochemical biosensor (Griffiths and Hall, 1993; Roe, 1992).

If combining enzymes, promoters and electrodes in variety ways to construct biosensors, it can be expected that a great number kind of biosensors will be constructed.

4.5 Discussion of the current and future applications of this type of biosensor

It has been reported that about half the research papers published on biosensors are concerned with glucose oxidase (Eggins, 1997). There are a number of reasons for this; notably the enzyme is readily available, highly active, and very stable. It makes a good standard compound on which to try out possible new biosensor techniques. Most importantly there is enormous interest in the development of diagnostic devices to monitor glucose levels for diabetics. Originally, most of glucose determination was done by taking samples of blood or urine. Usually it took several days to obtain, check and report full blood test results back to the doctors for assessment. Improvements on this technology for rapid glucose testing include the pinprick finger test, which involves a "color

stick" dye reaction (Turner and Pickup, 1985). Diabetic patients in intensive care, whose condition can change from minute to minute and especially diabetic patients on kidney dialyses need continuous on-line glucose monitoring. Therefore, recently, there has been a great deal of interest in the development of devices for glucose determination, which should be easy to operate, portable, inexpensive, accurate and reliable (Kost and Hague, 1995).

According to the statistics data (Taylor, 1996), the total biosensor market for 1994 was approximately $400 million. Over 50% of all biosensor sold were in the medical area, primarily due to sales of glucose biosensor. For example, MediSense (a kind of glucose biosensor) had sales of over $125 million in 1994 for its biosensor-based blood glucose monitors, capturing approximately 7% of the $2 billion blood glucose testing market. The success of blood glucose biosensors will stimulate the commercialization of other biosensors for rapid blood diagnostics, resulting in sales of nearly $1 billion by the year 2004 (Taylor, 1996). Since there are approximately 135million diabetic patients in the world and there may be 300million by the year 2025, the biosensor to measure blood glucose has been strongly desired (Kiyoshi, 2000).

" Artificial pancreas " is perhaps the most glamorous and sophisticated application of glucose biosensors. Scientists are attempting to design a device to meet the requirements of on-line measurement of blood glucose, converting the glucose level into an insulin required level, and injecting insulin continuously at a rate determined by the glucose level (Eggins, 1997; Sternberg *et al.*, 1989; Turner *et al.* 1986). The demanded for diabetic patients is to then maintain a balance between carbohydrate intake and insulin supplement conveniently and timely.

Biosensors may be used in medical prostheses and artificial organs. A neural-receptor-based biosensor is in effect an artificial nerve: it responds to specific, natural neurotransmitters to produce an electronic signal. If microsensors utilizing this technology could be integrated with body neurons, they could relay neural impulse signals across portions of damaged nerve tissue and restore functionality (Eggins, 1997; Wijesuriya and Rechnitz, 1993).

The current status and growth of biosensor products can be compared to the growth of immunoassays in the 1970s and 1980s. Today, immunoassay-based clinical diagnostic products capture approximately 35% of the 15 billion in vitro clinical diagnostic market. Traditional analysis methods, as well as immunoassay, are being challenged by new biosensor. Technology advances and revitalization of investment indicates that as biosensor technology matures and consistent manufacturing processes are developed, biosensors will become major players in diagnostic and measurement markets (Taylor, 1996; Kiyoshi, 2000).

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Appendix

This research was started in January 1993. Most experiments described in this thesis were completed in 1993, for example the conjugation of haemin to glucose oxidase plus the purification, qualitative and quantitative evaluations of the resultant product. Some of these results were reported in two posters (1995 and 1996) at biosensor conferences in New Zealand [1,2]. Due to a number of events in the author's private life, this thesis was not submitted until 2001.

Before 1993, Large [3] had carried out her work on a platinum electrode modified with a haemin-glucose oxidase conjugate. However there were theoretical and experimental problems that needed solving, which were essential to the success of building such kind of electrodes. These problems include: reliable, reproducible conjugation of haemin to glucose oxidase, purification and identification of the product, quantification tests of the starting materials and the product, adsorption of the haemin-glucose oxidase to platinum electrode and testing of the final electrode.

Because the enzyme is immobilized onto the electrode by haemin, and the electron transfer between the active site of the enzyme and the electrode is also mediated by haemin, the successful conjugation of haemin to glucose oxidase and the purification of the resulting product from the starting materials are fundamental to the success of the biosensor.

This thesis concentrates on the conjugating haemin to glucose oxidase, purifying the conjugate from starting materials, qualitatively and quantitatively studying the conjugate and exploring the mechanics of the adsorption of the conjugate to platinum electrode.

A summary of the key points in this thesis are listed as follows:

1. **The conjugation of haemin to glucose oxidase.** Based on preliminary experiments, a system of DEC-HEPES was chosen to activate haemin instead of using DCC. Because of the solubilities of DCC and NHS, haemin has to react with DCC in an organic solvent, while the subsequent reaction between activated haemin and enzyme is carried out in aqueous buffer. Experiments proved that the DCC-haemin active ester would precipitate out after addition to the aqueous buffer and the organic solvent could inhibit the enzyme. Using DEC-HEPES system however, the haemin activation and the subsequent reaction between haemin and glucose oxidase are both carried out in aqueous buffer. The reaction reached optimum yield after 12 hours. Haemin was used in excess to ensure a high yield of haemin-glucose oxidase conjugate. The whole reaction is controlled in such a way that the pH of the HEPES buffer is kept between 7.2-7.3 to minimize the hydrolysis of the active ester.

2. **The purification of the haemin-glucose oxidase conjugate.** During this research it was found that, under the chosen conditions, haemin will aggregate. This finding was communicated to Mr. Goh and was noted in his thesis (pages 65, 64, 76, 77 and 117) [4]. The coexistence of unreacted haemin with haemin modified glucose oxidase could cause several problems. Firstly, unreacted haemin will compete with the conjugate for adsorbance to the platinum electrode. This will decrease the enzyme/electrode binding rate and lead to lower electrode sensitivity. Secondly, the unreacted haemin could cause error in quantitative tests of the conjugate. Thirdly, the coexistence of unreacted haemin with the conjugate will effect the reproducibility of electrode manufacturing. To prevent any possible interference from aggregated haemin, careful size exclusion chromatography was carried out. During the chromatography, 280 nm was chosen to monitor the separation because both haemin and glucose oxidase have strong absorbance at this wavelength. Two separate peaks corresponding to glucose oxidase haemin conjugate and aggregated haemin were collected.

3. **The qualitative and quantitative study of conjugate.** Haemin absorbs at 295nm, 384nm, 420nm, 520nm and 557nm. Glucose oxidase absorbs at 280nm, 350nm, and 420nm. Absorbance peaks at 295nm, 384nm and 420 nm were therefore not suitable for identification and quantification of haemin in the haemin-glucose oxidase conjugate. The peak at 557nm was chosen for haemin identification and quantification in the conjugate. However, it was discovered through experiments that the peak at 557nm only appears when haemin is at reduced form. Since both reduced and oxidized forms of haemin existed in the conjugate, a reducing reagent, sodium dithionite was added to reduce haemin in the conjugate for reliable and reproducible haemin conjugate identification and quantification.

4. **Analysis.** During the research, TLC, HPLC, LC, electrophoresis, UV/VIS, CE, and AA were used to analyze haemin, glucose oxidase and the conjugate of the two. Apart from these techniques, electrode response and cyclic voltammograms were used to analysis the performance of the ultimate product, the haemin-glucose oxidase biosensor. The electrode tests were done at AgResearch laboratory at Palmerston North.

According to the theoretical and practical research in this thesis, a system was set up to produce a haemin glucose oxidase conjugate, to analysis the conjugate, and to adsorb the conjugate to the electrode. Thus a base was provided to build a biosensor, which served as a model to illustrate the biochemical mechanism, approach and extend existing research (at that time, i.e. 1993) toward the construction of this type of biosensors. The glucose oxidase biosensor built in this research showed sensitivity, stability and a good linear relationship with glucose concentration in the range of 0 -10 MM. The biosensor showed potential for future practical usage.

After having submitted this thesis in 2001, Mr. Goh's thesis was sent to me. This thesis had been embargo until the late 1990s. Both Mr. Goh's thesis and this thesis extend the work of Large (1993). In Mr. Goh's thesis, the content that relates to using haemin and glucose oxidase to build biosensors was concentrated on the electrode study, while in this thesis, the study concentrated on the chemical conditions and mechanics of conjugate haemin and glucose oxidase, the purification of the conjugate and character analysis of the conjugate. These two independent studies were designed separately, but they served as good references to each other. They illustrated the topic from different angle and plotted a bigger picture. It is regrettable that Goh's thesis and hence its contribution to this area was not discussed in the introduction of this thesis.

References:

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