PREPARATION OF GLUCOSE SENSOR BY IMMOBILIZATION OF GLUCOSE OXIDASE INTO POLYPYRROLE USING $K_3Fe(CN)_6$ AS AN ELECTRON MEDIATOR

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หัวข้อวิทยานิพนธ์	การเตรียมกลูโคสเซนเซอร์โดยวิธีการตรึงเอนไซม์กลูโคสออกซิเดสในพอลิไพโรล โดยใช้ $K_3Fe(CN)_6$เป็นตัวกลางส่งผ่านอิเล็กตรอน
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บทคัดย่อ

วิทยานิพนธ์นี้เป็นการเตรียมกลูโคสเซนเซอร์โดยการตรึงเอนไซม์กลูโคสออกซิเดสในฟิล์มพอลิไพโรลบนอิเล็กโทรดทองขนาดไมโคร และมีเฟอร์ริไซยาไนด์เป็นตัวกลางส่งผ่านอิเล็กตรอน เพื่อเลี่ยงปัญหาการจำกัดของปริมาณออกซิเจนในสารละลาย และการถูกรบกวนจากตัวแทรกสอดเนื่องจากการใช้ศักย์ไฟฟ้าสูง กลูโคสเซนเซอร์ดังกล่าวถูกเตรียมด้วยวิธีอิเล็กโตรเคมิคัลพอลิเมอร์ไรเซชันที่ศักย์ไฟฟ้า 0.70 โวลต์ เทียบกับขั้วอิเล็กโทรดอ้างอิง Ag/AgCl กระแสตอบสนองกลูโคสได้จากปฏิกิริยาออกซิเดชันที่ศักย์ไฟฟ้าคงที่ 0.45 โวลต์ ของกลูโคสกับเอนไซม์กลูโคสออกซิเดสเมื่อมีเฟอร์ริไซยาไนด์เป็นตัวกลางส่งผ่านอิเล็กตรอนในสารละลายปราศจากออกซิเจน จากการทดลองพบว่าภาวะที่เหมาะสมในการเตรียมคือ ไพโรลมอนอเมอร์เข้มข้น 40 มิลลิโมลาร์ เอนไซม์กลูโคสออกซิเดส 25 ยูนิต/มิลลิลิตร และ 25 มิลลิโมลาร์ ของเฟอร์ริไซยาไนด์ ที่ความหนาของฟิล์ม 0.09 ไมครอน (42 มิลลิคูลอมบ์/ตารางเซนติเมตร) กลูโคสเซนเซอร์นี้มีพิสัยเชิงเส้นในช่วงความเข้มข้น 1 ถึง 10 มิลลิโมลาร์ และสามารถเลี่ยงการรบกวนจากตัวแทรกสอดบางชนิดได้ที่ศักย์ไฟฟ้า 0.45 โวลต์ เช่น กรดแอสคอร์บิก และ 4-acetaminophenol หัววัดน้ำตาลกลูโคสที่เตรียมนี้มีความว่องไว 79 นาโนแอมแปร์/มิลลิโมลาร์/ตารางเซนติเมตร หัววัดน้ำตาลกลูโคสมีเสถียรภาพดีขึ้น เมื่อเก็บไว้ในสารละลาย 100 นาโนโมลาร์ ของเฟอร์ริไซยาไนด์ใน 0.1 โมลาร์ ฟอสเฟตบัฟเฟอร์ pH 7 ที่ 4 องศาเซลเซียส

Thesis Title	Preparation of Glucose Sensor by Immobilization of Glucose Oxidase into Polypyrrole Using $K_3Fe(CN)_6$ as an Electron Mediator
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ABSTRACT

The purpose of this research is to prepare a novel glucose sensor using electrochemical polymerization of pyrrole on a gold microelectrode in the presence of the enzyme glucose oxidase and ferricyanide solution at a potential of 0.70 V *versus* Ag/AgCl. The enzyme and ferricyanide were entrapped into the polypyrrole films during the electrochemical polymerization process. Glucose responses were evaluated by measurment of the amperometric oxidation of ferricyanide at 0.45 V in the absence of oxygen. The optimal condition for the film preparation was found to be a pyrrole concentration of 40 mM, in the presence of 25 units/ml of glucose oxidase and 25 mM ferricyanide aqueous solution. The optimal response was obtained from a film thickness of 0.09 μm (42 mC/cm^2). The linearity of the glucose sensor response ranged from 1 to 10 mM glucose. The PPy/GOD/Ferri enzyme electrode prevented the autooxidation of interferences such as ascorbic acid and 4-acetaminophenol at 0.45 V. The sensitivity of the glucose sensor was 79 nA/mM/cm^2. The PPy/GOD/Ferri enzyme electrode was found more stable by storing in 100 nM ferricyanide and 0.1 M phosphate buffer pH 7 at 4 $^{\circ}$C.

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LIST OF ABBREVIATIONS

A	ampere
Ag/AgCl	silver coated with silver chloride
nA	nano ampere
μA	micro ampere
C	coulomb
E_{pa}	anodic peak potential
E_{pc}	cathodic peak potential
Ferri	ferricyanide
GOD	glucose oxidase
H_2O_2	hydrogen peroxide
I	current
I_{pa}	anodic peak current
I_{pc}	cathodic peak current
M	molar
mL	milliliter
mM	milimolar
nM	nano molar
NHE	normal hydrogen electrode
ox	oxidized form
PPy	polypyrrole
red	reduced form
SHE	standard hydrogen electrode

CHAPTER I

INTRODUCTION

1.1 Research Motivation

An amperometric enzyme electrode is a device for measuring the current generated by an electrochemical reaction at a fixed potential. For an amperometric glucose sensor, the reaction of glucose and oxygen in the presence of enzyme glucose oxidase will generate hydrogen peroxide. It is an electroactive species which is oxidizable at an electrode surface. The response to hydrogen peroxide is proportional to the concentration of glucose. Amperometric glucose sensors have good sensitivities and linear ranges. However, they can sometimes lack of selectivity [1]. Potential at 0.7 V does not only oxidize the hydrogen peroxide but also any other oxidizable species at the working potential. Other oxidizable species produce a higher current response and error. Non-electroactive, high molecular weight species such as proteins are the sources of fouling by adsorption on the electrode surface. Fouling adsorption decrease sensor response over time [2].

There are some advantages of the amperometrical sensors that make them very useful in bioprocess control. Amperometric sensors are inexpensive, simple to make and operate, and adaptable to process control instrumentation. The sensors can often operate in a wide concentration range.

Sometimes the selectivity of electrochemical sensors needs to be improved. The use of membrane [3,4] is a common way to eliminate the adsorption of proteins on an electrode surface and reduce the interferences. However, this method is limited by mass transport through the membrane, thus the response time may be used for longer. It is difficult to control thickness, reproducibility and uniformity of the membrane. The application of conducting polymers can overcome this problem. Various conducting polymers have been

extensively considered as the materials for the entrapment. Polypyrrole has been a particular choice for entrapment of protein molecules because films can be grown from aqueous solutions compatible with most biological elements [5,6]. The thickness of films is controlled by the amount of charges passed during the electrochemical polymerization procedure [7].

More recently systems involving mediators have been described in the literature. Electron mediators can replace oxygen as electron donor-acceptors, resulting in a mediated enzyme sensor. The ideal mediator should react rapidly with the reduced enzyme. To avoid the problem of interferences in real samples the oxidation potential of the mediators should be low and independent of pH.

Ferricyanide (hexacyanoferrate(III)) is a water soluble electron mediator used with some soluble enzymes. The ferrocyanide (hexacyanoferrate (II)) product is oxidized at a fixed potential on the electrode.

In this study, preparation of a glucose sensor by immobilization of glucose oxidase and ferricyanide into polypyrrole by electrochemical polymerization was investigated. The glucose sensor is expected to determine glucose concentration at low potentials, in order to avoid interferences.

1.2 Objectives of the Research

1. Preparation of gold microelectrode used as the working electrode.

2. Preparation of glucose sensors by immobilization of glucose oxidase and ferricyanide into conductive polypyrrole films.

3. Determination of glucose concentration by the amperometric glucose sensor.

4. Study of the sensitivity, stability, reproducibility, and lifetime of the entrapped ferricyanide glucose sensor.

1.3 Scope of the Research

1. Investigation of the parameters for immobilization of enzyme glucose oxidase(GOD) and entrapment of ferricyanide by electrochemical polymerization; such as concentration of monomer, concentration of ferricyanide and charge passed during electrochemical polymerization.

2. Determination of conditions required for the best sensitivity and selectivity.

1.4 Expected Results

We should be able to prepare an amperometric glucose sensor on a gold microelectrode. Glucose concentrations would be determined at low potentials, where there is relatively little interferences.

CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 The Amperometric Enzyme Electrode

An amperometric enzyme electrode measures the current which is generated by the electrochemical reaction of an electroactive substance at a fixed potential. The application of potential between a reference and a working electrode allows one to measure a current when an electroactive analyte is oxidized or reduced, depending on the voltage, at the working electrode [8].

The enzymes used for sensors often require oxygen as a electron acceptor. Glucose oxidase (GOD) is used for the amperometric sensor which oxidizes glucose to gluconic acid: [9]

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{GOD} \text{gluconic acid} + H_2O_2 \qquad (2.1)$$

$$H_2O_2 \xrightarrow{0.70\ V} 2H^+ + O_2 + 2e^- \qquad (2.2)$$

This reaction is monitored amperometrically either by measuring the current generated by the increase of hydrogen peroxide [10] or by decrease of oxygen. The limited solubility of oxygen in solution creates a problem at high substrate concentrations often encountered in bioprocesses because it limits the linear range of the sensor. Oxygen can be generated from hydrogen peroxide at the electrode surface, when it is at a positive potential. However, this regenerated oxygen is not enough to meet the demands of the enzymatic reaction at high substrate concentrations due to losses from diffusion. In addition it may be interfered by another electroactive substances at the higher polarization voltages. Substances such as ascorbic acid, acetaminophenol and uric acid can interfere with the measurement which autooxidation at high potential. The result from this approach is a large current response and positive error.

Potentials at the lower range are often used to minimize this interference. Thus the problem of limited oxygen solubility can be solved by using redox mediators that allow electron to transfer from reduces species to working electrode. Moreover, the mediator is often used at a lower range potential to overcome the interference.

2.2 Introduction to Conducting Polymers

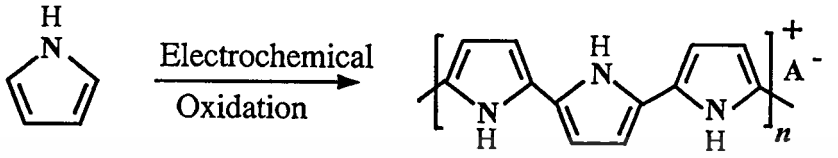
Conducting polymers are polymeric materials containing extended conjugated π-electron backbones. They display unusual electronic properties, such as low energy optical transition, low ionization potentials and high electron affinities [11]. Such polymers are easily oxidized or reduced by charge-transfer agents (*dopants*) that act as electron acceptors or electron donors, respectively. This results in a class of polymers that can be oxidized or reduced more reversibly than conventional polymers [12,13]. These materials may be prepared by electrochemically, typically by oxidation of their monomers in the presence of an electrolyte in aqueous or organic solutions [14]. Passage of current through a solution results in the loss of electrons, and compounds are oxidized at the anode. Electrons are gained and compounds reduced at the cathode. This process is referred to as electrochemical polymerization when polymer is formed [15].

Charge transfer agents affect this oxidation or reduction by entering or leaving the film as the oxidation state changes. By this process insulating polymers can be converted to conducting polymers with near metallic conductivity in many cases. During the polymer synthesis, the incorporation of extended π-electron conjugation is of foremost importance. The monomeric starting materials therefore typically contain aromatic or multiple carbon-carbon bonds, that must be preserved in the polymer backbone. Interesting conducting polymers include polypyrrole, polyaniline and polythiophene.

Polypyrrole (PPy) has been a particular choice for the entrapment of protein molecules because it has a low oxidation potential, and has a water-

soluble monomer, which enables films to be grown from aqueous solutions compatible with most biological elements.

It is generally accepted that the electrochemical polymerization of heterocyclic monomers proceeds equation (2.3): [16]



(2.3)

The mechanism for polymerization (Figure 2.1) involves oxidation of pyrrole at the α-position to form a radical-cation (I), which undergoes radical coupling to yield the dimer-dication (II). The latter loses two protons to yield the dimer (III). The dimer repeats the same reaction sequence-loss of an electron to form a dimer radical-cation, coupling with itself and I to form the tetramer-dication and trimer-dication, respectively, followed by two protons loss to yield tetramer and trimer. Propagation to polymer proceeds via repetition of the same sequence-one electron loss, coupling of different-sized radical-cations, deprotonation. Electrochemical polymerization, as usually carried out, does not yield the neutral form. One cycle back and forth between the conducting and nonconducting forms, colored black and light yellow, respectively, by reversing polarity. The doped polymer would have a structure such as IV, where A^- is the anion of the electrolyte. The doped polymer precipitates out and coats the surface of the anode during polymerization. The polymerization reaction and polymer properties (conductivity and mechanical strength) are dependent on such parameters as identity and concentration of electrolyte, reaction temperature, and current density. The dimerization rate increased as the square of the radical cation concentration increased, leading to a much greater consumption of this species as the rate of pyrrole oxidation and the active electrode area increased with pyrrole concentration [17].

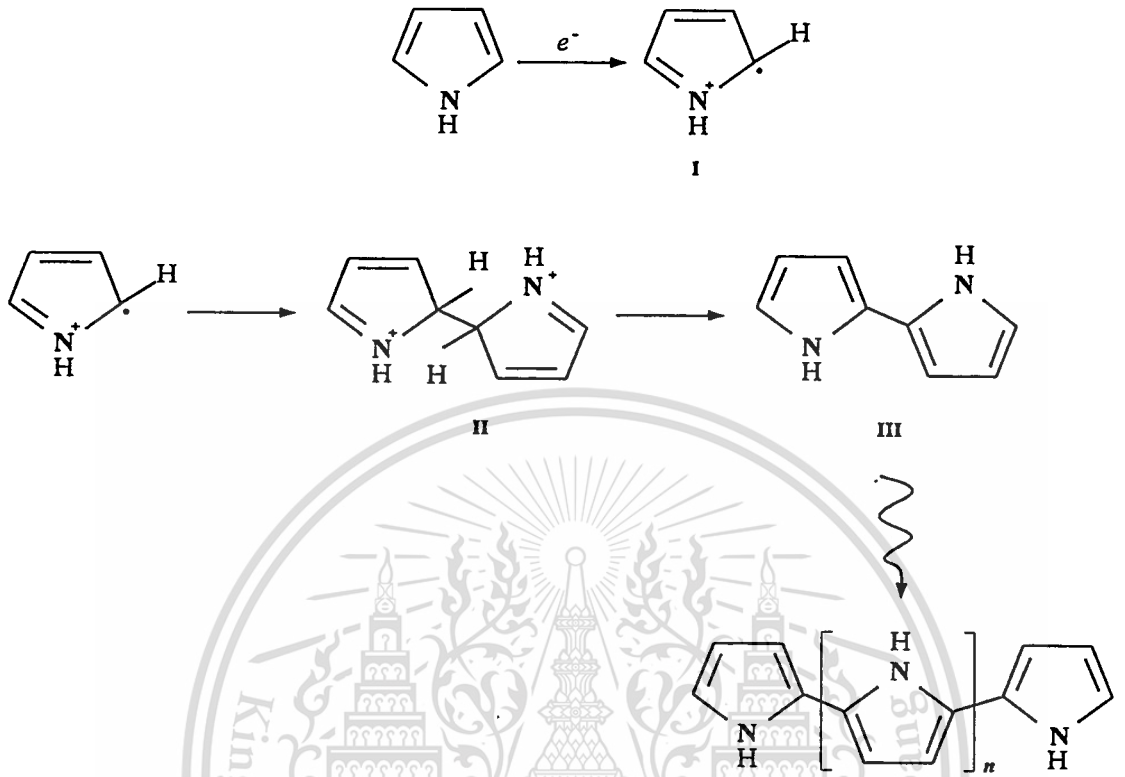


Figure 2.1 Mechanism of polypyrrole formations

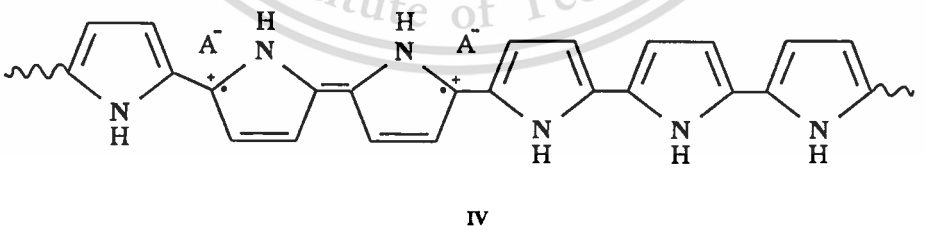


Figure 2.2 Structure of the doped polypyrrole

2.3 Permselective Film Conducting Polymers for Enzyme Electrode

Permselective films greatly enhance the selectivity and stability of amperometric probes by rejecting interference from the electrode surface [18]. The processing of polypyrrole highlights the utility and the limitations of most conducting polymers as far as biosensors are concerned. Polypyrrole can be electrochemically polymerized from aqueous media at neutral pH, which allows the incorporation of a wide range of counter ions. Other conducting polymers are more limited in this regard [9].

Polymer modified electrodes have received extensive interest since they can be designed to provide important improvements in stability and selectivity. Selectivity of polymer modified electrodes can be attained by different mechanisms such as size exclusion [19], ion exchange, and hydrophobic interactions. Polymer modified electrodes obtained from the electrochemical polymerization of pyrrole have received great attention in view of several promising applications including the development of chemical and biochemical sensors.

The entrapment of enzymes within electrochemical polymerized films offers the possibility of attaching the biocatalysts to conducting surfaces. Electrochemical polymerization of polypyrrole proceeds via highly reactive π-radical cations which react with other pyrrole molecules to form chains with mainly α,α'-coupling. Since the polymer is positively charged, anions from the solution are accumulated in the film. The negative charge of enzyme glucose oxidase at pH 7.0 decisively influences the positively charged matrix and polymer-enzyme stabilization occurs from the electrostatic interaction between the positively charged polymer and the negatively charged enzyme [6]. In addition, the porosity of the films is generally controlled by the counter ions [20]

2.4 Mediators

A mediator is a low-molecular-weight redox couple, which transfers electrons from the redox center of the enzyme to the surface of the working electrode. During the catalytic cycle the mediator first reacts with the reduced enzyme and then diffuses to the electrode surface where it undergoes rapid charge transfer. This can be illustrated with reference to glucose oxidase. Electron transfer mediators, such as ferri/ferrocyanide, ferrocene and benzoquinone can take the place of oxygen that electron acceptor for oxidases. This can sometimes overcome the problem of limited availability of oxygen [1]. Also, the focus of recent research is the coupling of an electron mediator with a species that electrochemically polymerizes to form a film over the electrode. The film can mediate electron transfer, serve to screen out interferences and prevent electrode fouling as follows : [21]

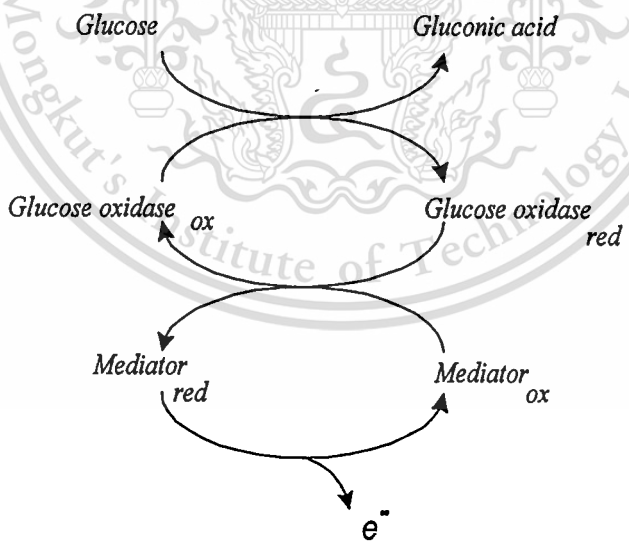


Figure 2.3 Schematic reaction pathway utilizing a redox enzyme transfered amperometric measurement via a redox mediator.

Although ferrocene is a good mediator for glucose oxidase it is not confined within the polymer. It would be more desirable if both the mediator and enzyme could be entrapped within the polymer film. It is known that ferro/ferricyanide can be incorporated into electrochemical polymerized films of polypyrrole and that it will remain entrapped within the films as long as the polymer is not reduced [20, 22]. Ferricyanide is also known to act as a mediator for the oxidation of glucose oxidase as follows : [23, 24]

$$GOD_{red} + [Fe(CN)_6]^{3-} \longrightarrow GOD_{ox} + [Fe(CN)_6]^{4-} \quad (2.4)$$

$$2[Fe(CN)_6]^{4-} \longrightarrow [Fe(CN)_6]^{3-} + 2e- \quad (2.5)$$

Mor and Guarnacia, for example, used ferricyanide as an electron acceptor dissolved in the sample solution but with glucose oxidase entrapped at the working electrode [22]. Morover, Bartlett, Ali and Eastwick-Field used ferrocyanide as a redox mediator in poly(N-methylpyrrole) for the oxidation of the enzyme at 0.45 V *versus* SCE. Glucose responses were measured at a rotating electrode (9Hz) at 0.45 V *versus* SCE, grown on a platinum disc electrode (0.39 cm^2) [24].

In this research work, the overall glucose sensor reaction will be controlled by diffusion of solutes as main elementary process. The homogeneous electron transfer from glucose to $[Fe(CN)_6]^{3-}$ via GOD, and oxidation of $[Fe(CN)_6]^{4-}$, which could be generated current by electron transfer from glucose to $[Fe(CN)_6]^{3-}$ at the electrode and solution interface [23, 24, 25, 26]

The homogeneous electron transfer between $[Fe(CN)_6]^{3-}$ and GOD plays an especially important role for the sensor characteristics, because it is a rate-limiting process for the mediator type enzyme sensor. Thus, kinetic data for this process are very significant for the development of a rapid responsive, Quantitative results regarding $[Fe(CN)_6]^{3-}$/GOD system immobilized in

poly(N-methylpyrrole) films were reported by Bartlett, Ali and Eastwick-Field [25].

2.5 The Electrochemical Cell

An electrochemical cell consists of at least three components, i.e., two electrodes and an electrolyte [27, 28]. The electrolyte is a phase through which charges are transferred by the movement of ions. An electrolyte may be a liquid solution or fused salts. The second phase is the electrode, in which charge is transferred by electronic movement. Electrodes can be metals or semiconductors, and can be solid or liquid. The electrochemistry processes depend on the transport of reactants in the electrolyte to the electrode surface. Therefore this transport involves a change from ionic conduction to electronic conduction. When two different electrodes are placed in an electrolyte, a potential difference appears and can be measured between the two phases in the current path. Hence the electrochemical cell must contain at least two interfaces. Each interface is called a half-cell. The electrode part of the interface under study is called the working (or indicator) electrode. Therefore the half-cell standardizes by using the other electrode made up of phases having constant composition, called the reference electrode. The internationally accepted primary reference is the standard hydrogen electrode (SHE), or normal hydrogen electrode (NHE), which is the interface at which protons are in equilibrium with hydrogen gas. Potentials are often measured and quoted with respect to reference electrodes other than the NHE, which is not very convenient. The common reference is Ag/AgCl which is

$$AgCl + e^- \longrightarrow Ag + Cl^- \qquad (2.6)$$

Its potential is 0.22 V *versus* NHE.

The electrode potential is linked to the concentration of oxidised (O) and reduced (R) species at the interface by the Nernst equation:

$$E = E^0 \pm RT/nF \ln[O]/[R], \qquad (2.7)$$

in which E is cell potential (V), E^0 is standard electrode potential (V), R is the gas constant (J /mol K), T is temperature (K) and F is the Faraday constant (C/mol) and n is the number of electrons transferred.

Electrochemical cell is classified as both galvanic and electrolytic cells. Galvanic cell reactions occur spontaneously at each electrode when a conductor connects them. Electrolytic cell reactions occur only by applying an external voltage greater than reversible potential of the cell. For example, let consider the cells shown in Figure 2.4. The reaction $Cu^{2+} + 2e^- \longrightarrow Cu$ is the same in both cells. The galvanic cell, by using a counter half-cell with a more negative potential than Cu/Cu^{2+} so called internal electrolysis occurs. In the electrolytic cell, any counter half-cell can be used, via connection to an external power supply. Analytical electrochemistry is concerned with electrolytic cells. The working electrode can either be held at equilibrium and changes in potential can be measured (potentiometry), or an overpotential can be applied and the resulting current measured (voltammetry).

However the current is also dependent on the transport of electroactive species to and from bulk solution. The mass transport may be controlled by one, or a combination, of the following components: i) migration is transport of electroactive species to away from the electrode surface under the influence of an electric field; ii) convection is the transport of a solute species caused by hydrodynamic motion of the solution; and iii) diffusion is transport brought about by a concentration gradient. The effect of migration can be suppressed by using a high concentration of supporting electrolyte. Convection can be limited by working in quiescent solutions and performing measurements over

relatively short timescales. By doing this, only mass transport by diffusion needs to by considered.

2.6 Voltammetry

Voltammetry is the measurement of the current, which flows at an electrode, as a function of time and the potential applied to the electrode. As a result of a voltammetric experiment, a current-time, or current-potential curve, is recorded. These curves can be used for qualitative and quantitative determinations and for thermodynamic and kinetic studies.

2.6.1 Cyclic voltammetry

Cyclic voltammetry is a technique for the measurement of current potential relationships. The potential is varied linearly with time at an electrode in quiescent solution. The current-potential curve is governed by the rate of transfer of electrons across the interface as a response to the variation in potential along a triangular waveform, as shown in Figure 2.5. Change in oxidation state, or charge transfer, is a feature common to many chemical and biochemical reactions.

As the potential of the electrode is moved positive, the reductant begins to be oxidized. The electron transfer is from solution to the working electrode. The anodic current is observed (i_a). For a reversible process, as the potential of the electrode is then moved in a negative direction. The reduction occurs, and the surface concentration of the oxidant progressively decreases as electrons flow from the electrode to the solution. So a reduction current is observed (i_c). A typical cyclic voltammogram for reversible process is shown in Figure 2.6.

The reversible process means that the ratio of O/R at the interface follows E according to the Nernst equation (2.7). A redox process can be quasi-reversible or irreversible because of either slow rates of electron

transfer or chemical instability. This produces broader, flatter peaks and causes the redox potentials to shift with scan rate.

2.6.2 Amperometry

Amperometry is a voltammetric technique where the potential is kept constant and current is recorded as a function of time. The concentration of oxidise form of electroactive species were increased at the surface of electrode, when they were overoxidized. The amperometric current reaches a steady-state if left for long time. This current is a linear function of the concentration of the electroactive species. The required measuring equipment is simpler than for cyclic voltammetry.

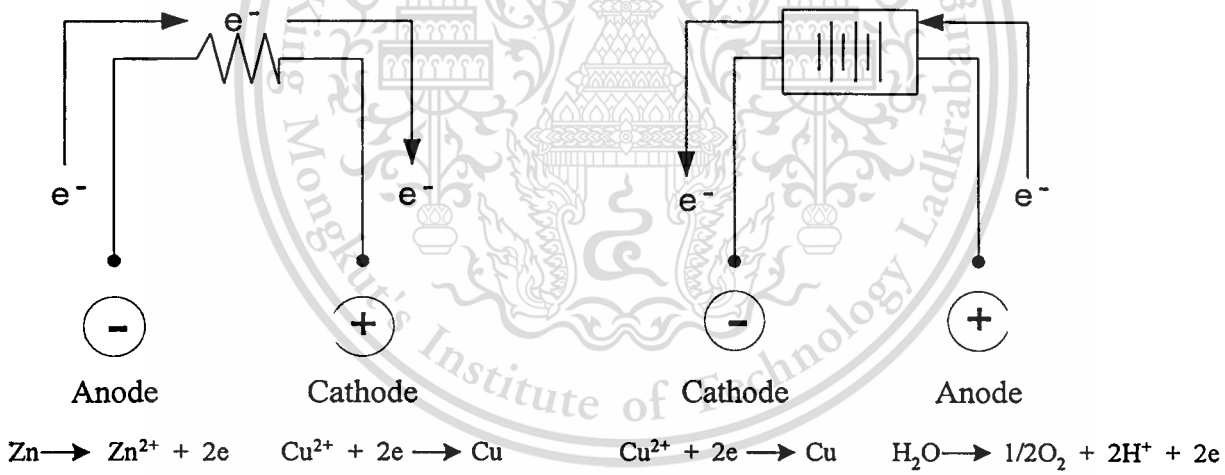


Figure 2.4 Schematic diagrams of (a) Galvanic and (b) Electrolytic cell

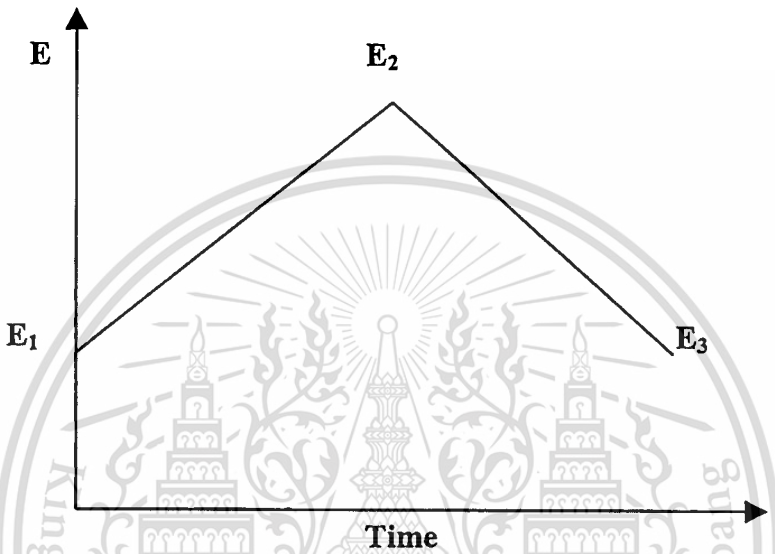


Figure 2.5 Triangular potential wave form for cyclic voltammetry

Where E_1 = initial potential

E_2 = switching potential

E_3 = final potential

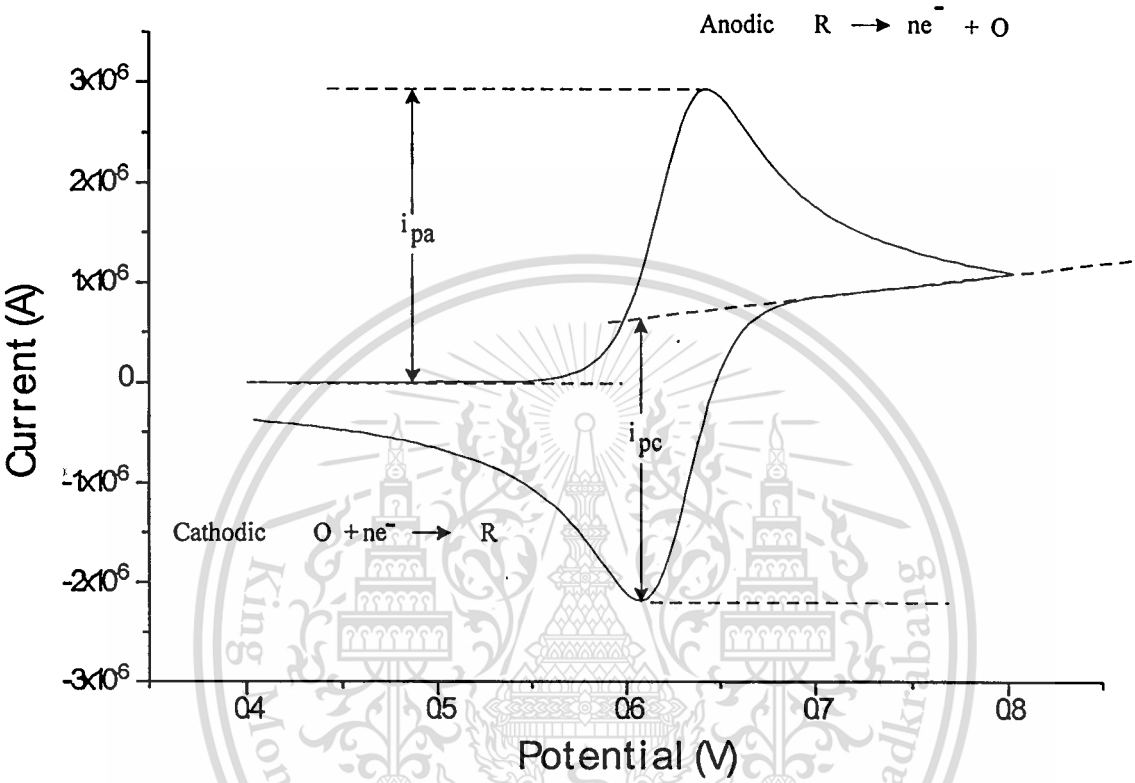


Figure 2.6 A typical cyclic voltammogram for a reversible process

2.7 Electrochemistry at Microelectrodes

Microelectrodes are electrodes of micrometer or smaller dimensions. A property from which a very small electrode benefits is convergent transport by diffusion and/or migration. As illustrated, this permits a higher flux density at the interface than is possible at larger electrodes. Because of its relative ease of fabrication, the most popular microelectrode shape is the inlaid disk [29].

It is known that either the time scale of the measurement or the radius of the electrode affects the characteristics of a voltammogram that one obtains in a quiescent solution [12, 29]. On a fast time scale or at an electrode of large radius, a peak-shaped voltammogram is obtained. Conversely, with a small radius or longer time scale of measurement, one obtains a sigmoidal-shaped voltammogram. The influence of electrode radius on the two types of voltammogram can be explained.

For macroscopic electrodes, the current is proportional to the flux of molecules at the electrode surface. The flux at the electrode surface arises from planar diffusion of species to the electrode (Figure 2.7). The current increase is proportional to the electrode area and then decreases with time. A peak-shaped voltammogram is obtained, because the electrolysis rate greatly exceeds the rate at which the species can diffuse to the electrode surface.

When the thickness of the diffusion layer is approximately equal to the radius of the electrode i.e. in the case of a microelectrode, the diffusion layer boundary will grow in a hemispherical fashion. Thus, non-planar diffusion predominates (Figure 2.7b). As the distance increases, more and more volume elements of solution are available to supply the electrode because of the spherical geometry of the diffusion field. This counteracts the increase in diffusion time (i.e. diffusion from further into the solution) so that the current does not fall and a steady-state wave is obtained. A sigmoidal voltammogram is revealed which is proportional to the edge or radius of the electrode.

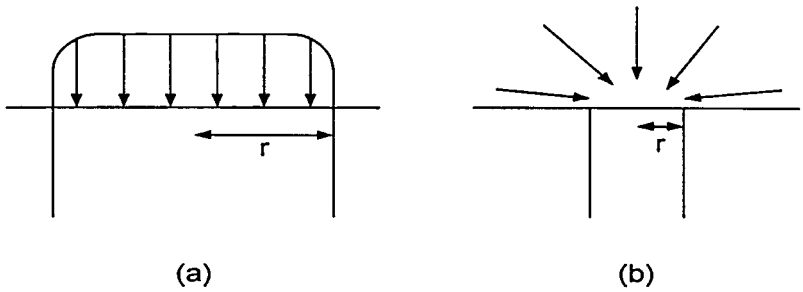


Figure 2.7 Representations of the (a) linear diffusion field and (b) spherical diffusion field at a microdisk electrode at short and long times respectively.

Since the microdisk is not uniformly accessible, the flux of species reacting at the surface is unequal. Electrolysis occurs mainly at the edges of the disk whereas spherical electrodes are unique in that all points on their surfaces are accessible. The rate of diffusion is not a function of position on the surface. Thus, diffusion of electroactive species to any spherical electrode can be described by Fick's second law in spherical co-ordinates.

Immobilization of enzymes by the anodic oxidation of monomers in the presence of the enzyme, is advantageous for the preparation of very small electrodes. Because of the surface of electrode was coveraged absolutely by electrochemical polymerization.

CHAPTER III

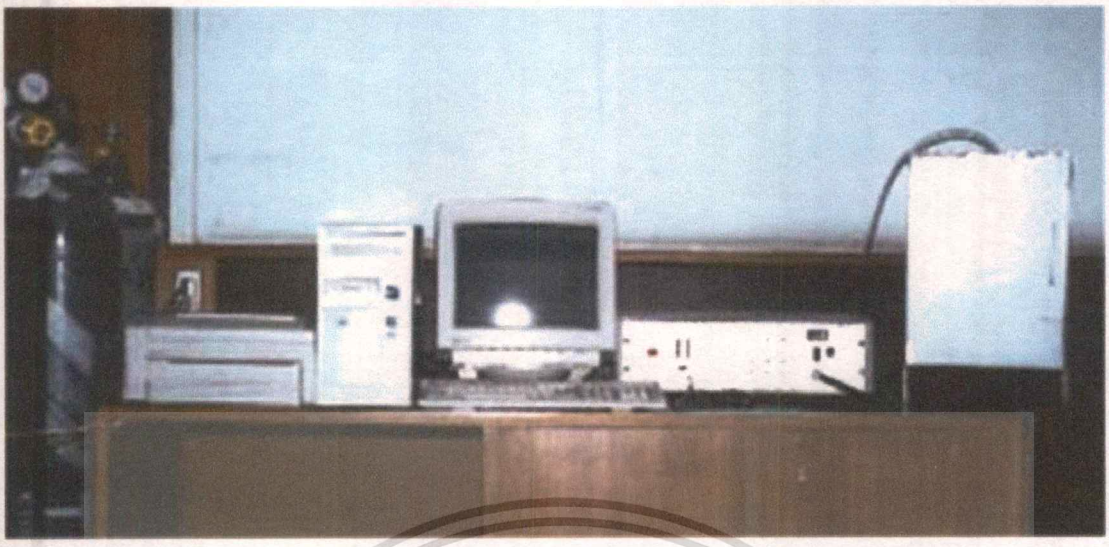
EXPERIMENTAL DETAILS

3.1 Chemicals

96% Pyrrole was received from Fluka Co. Ltd., and freshly distilled before used. Glucose oxidase type II derived from *Aspergillus niger* 10,000 unit from Sigma Chemical Company. β-D-Glucose purchased from BDH Ltd. was used to prepare the standard solutions of glucose. The solution was prepared 24 hour before used to ensure complete mutarotation between the α and β forms. Potassium ferricyanide was received from Merck. L(+)ascorbic acid was received from Merck. Solution of 10 mM phosphate buffer (pH 7) was used for preparing the glucose solutions and determination of glucose concentration. The pH of the buffer was adjusted by using either 0.1 M NaOH or 0.1 M HCl. Highly pure aluminium oxide (particle size 0.3 μm) with used for polishing was purchased from BDH Ltd. The electrodes were polished with polishing set obtained from Metrohm. All solutions were prepared by deoxygenated and deionized water. Other reagents were analytical grade.

3.2 Equipment

A Potentiostat/galvanostat (Autolab, model PGSTAT10) was used for the electrochemical polymerization, and current measurement. Output from the scanning potentiostat/galvanostat was connected to a personal computer and a printer, supported by GPES 4.4 operating software (Figure 3.1). A conventional three electrodes system consisted of an electrochemical polymerization of polypyrrole films onto gold microelectrode of 203 μm diameter, silver/silver chloride reference electrode, and platinum wire counter electrode.



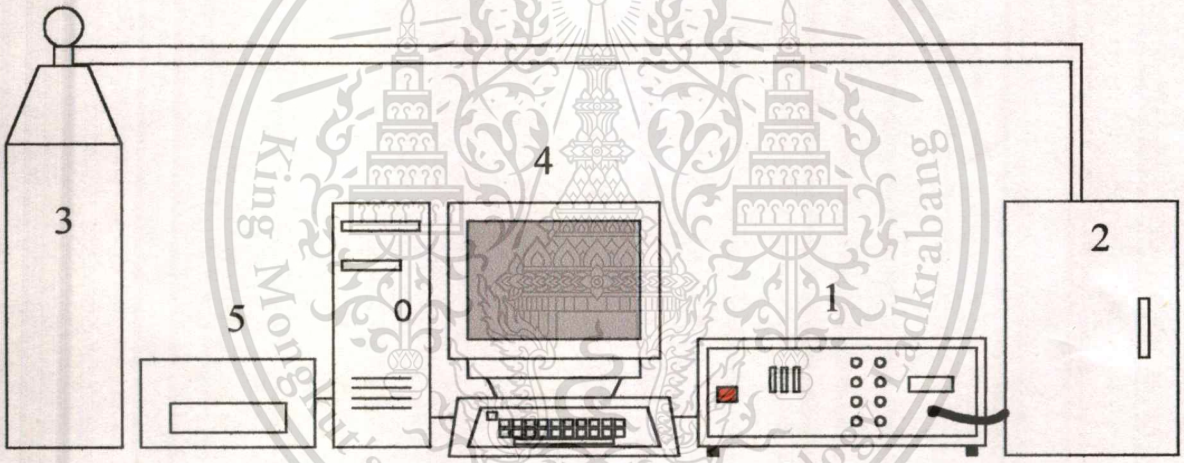


Figure 3.1 The apparatus for electrochemical experiments

1 = Potentiostat model Autolab PGSTAT 10

2 = Faraday cage

3 = Nitrogen tank

4 = Personal computer

5 = Printer

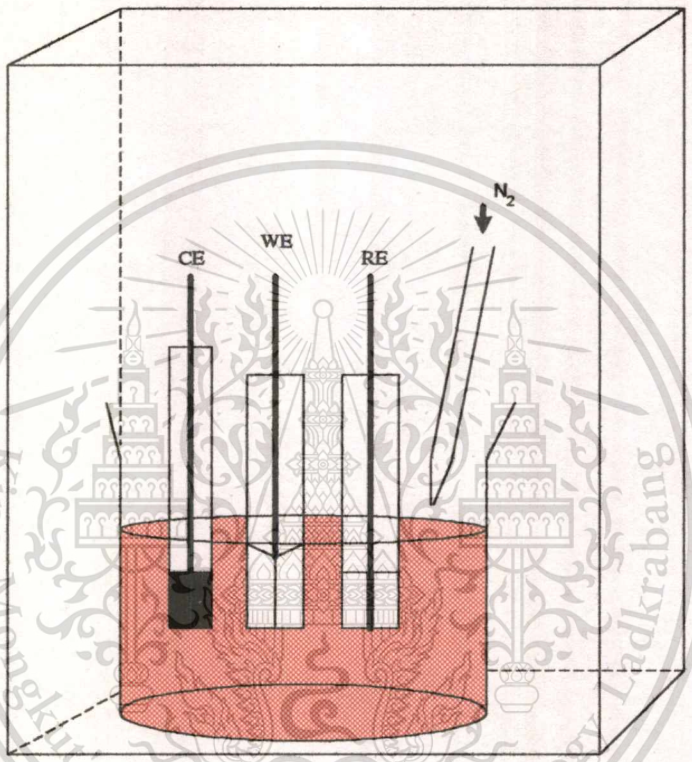


Figure 3.2 The configuration of measuring cell in Faraday cage.

WE = Working electrode

CE = Counter electrode

RE = Reference electrode

Electrochemical cell measured current in Faradaic cage under N_2 gas (Figure 3.2). Micropipets 20 and 500 μL from Grilson were used for measuring a very small volume of solution.

3.3 Procedures

3.3.1 Preparation of Gold Microelectrodes

The gold microelectrodes were prepared by sealing fine gold wire into capillary glass rod with a diameter of 0.6 cm (Figure 3.3). The gold wire (diameter of 203 μm) was cleaned in the solution of H_2O_2 and H_2SO_4 with the ratio 3 : 1. Then the electrode was polished with emery paper and then with aluminium oxide (0.3 μm) powder. The electrode was cleaned in ultrasonic bath for 5 minutes [26]. The electrodes were, when not use, stored in desiccator.

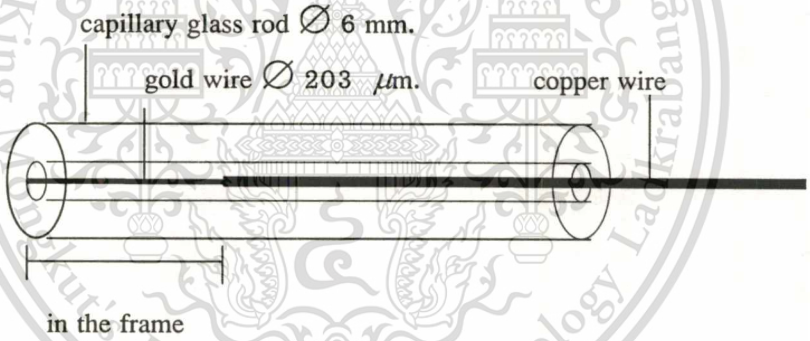


Figure 3.3 Preparation of gold microelectrode

All measurements were carried out using three electrodes, the gold microelectrode as the working electrode, the platinum wire as the counter electrode, and silver/silver chloride (Ag/AgCl) as the reference electrode. The picture of three electrodes was shown in Figure 3.4. Electrochemical measurements were performed by a potentiostat Autolab PGSTAT 10 at room temperature in a Faraday cage.

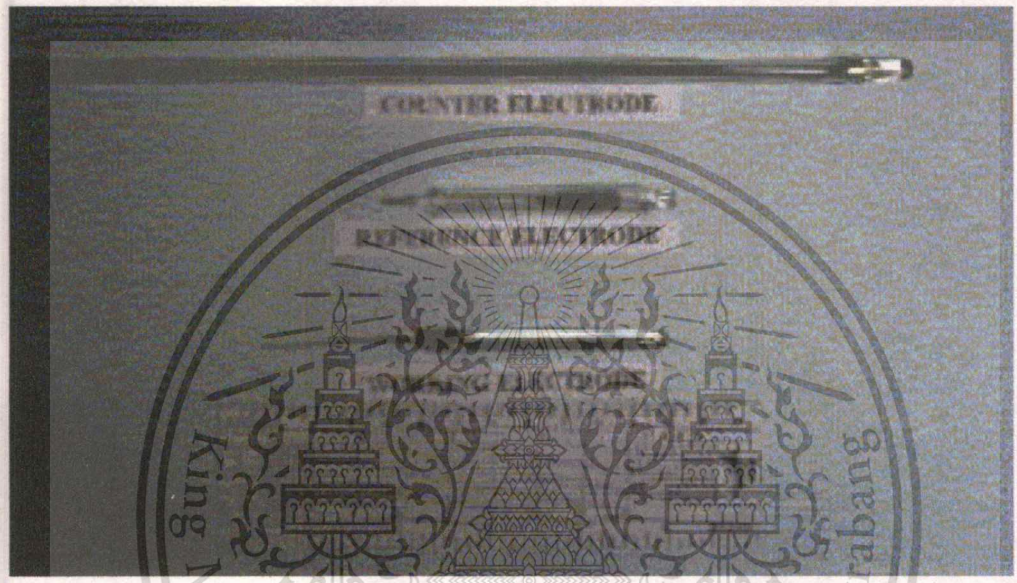


Figure 3.4 Picture of three electrodes

3.3.2 Pretreatment of Gold Microelectrodes

Gold microelectrodes were polished with the polishing set consisted of 0.3 μm aluminium oxide suspended on a wetted polishing cloth. Then the microelectrodes were cleaned in ultrasonic bath for 5 minute and pretreated by electrochemical cleaning in solution of 0.1 M H_2SO_4 by cyclic voltammetry from 0.2 to −1.6 V *versus* silver/silver chloride, at scan rate of 0.1 V/sec until the cyclic voltammograms provided the same shape. This mean that the impurities on the electrode surface were removed.

3.3.3 Determination of Electrode Area

The electrode surface area was calculated by equation 3.1 [28]. Using redox couple molecules and cyclic voltammetry technique, we obtained parameter for the equation. Thus the electrode surface area was investigated by measuring the anodic or cathodic peak current and concentration of ferricyanide. Solution of 0.1 to 0.5 M of ferricyanide were prepared and 0.1 V/sec was used as the scan rate. In general the electrode area can be calculated by the cross section area of gold wire but this area may not be correspond to the electrode reaction.

$$i_p = (2.69 \times 10^{-5})n^{3/2} AD_0^{1/2} v^{1/2} C_0^* \qquad (3.1)$$

i_p = peak current (A)

n = number of electron transfer

A = electrode area [cm^2]

D_0 = diffusion coefficient of ferricyanide in 0.1 M KCl (0.673×10^{-5} [cm^2/sec])

v = scan rate [V/sec]

C_0^* = concentration of ferricyanide [mol/cm^3]

3.3.4 Electrochemical Polymerization of Pyrrole and Immobilization Glucose Oxidase and Ferricyanide

Electrochemical polymerization of pyrrole onto a gold microelectrode in the presence of the enzyme glucose oxidase and ferricyanide solution was performed at a potential of 0.70 V *versus* silver/silver chloride. Then the film was characterized by cyclic voltammetry from -0.7 to 0.7 V at scan rate of 0.1 V/sec. The reversible peak of ferricyanide should be observed.

3.3.5 Determination of Glucose

The amperometric response could be detected as an electrical signal from electroactive species which were oxidised at the working electrode at a fixed potential. The current response of glucose was evaluated by placing the three electrodes in 5 ml of 0.1 M phosphate buffer pH 7.0. Determination of glucose was done in a Faraday cage for noise prevention. After setting potential, the current background was left to be constant and then glucose solution was injected into the measuring cell. The current-time response was recorded as the difference between the steady state current of sample (i) and the background current (i_0). [8] The value of $i - i_0$ was the current response related to concentration of sample in measuring cell.

3.3.6 Determination of Sensitivity

The sensitivity is usually defined as the final steady state change in the magnitude of the sensor output signal with respect to the change in concentration of a specific chemical species ($\Delta S/\Delta C$). The lowest concentration of substrate that can be detected by sensor is a limit of detection.

3.3.7 Determination of Selectivity (Interferences)

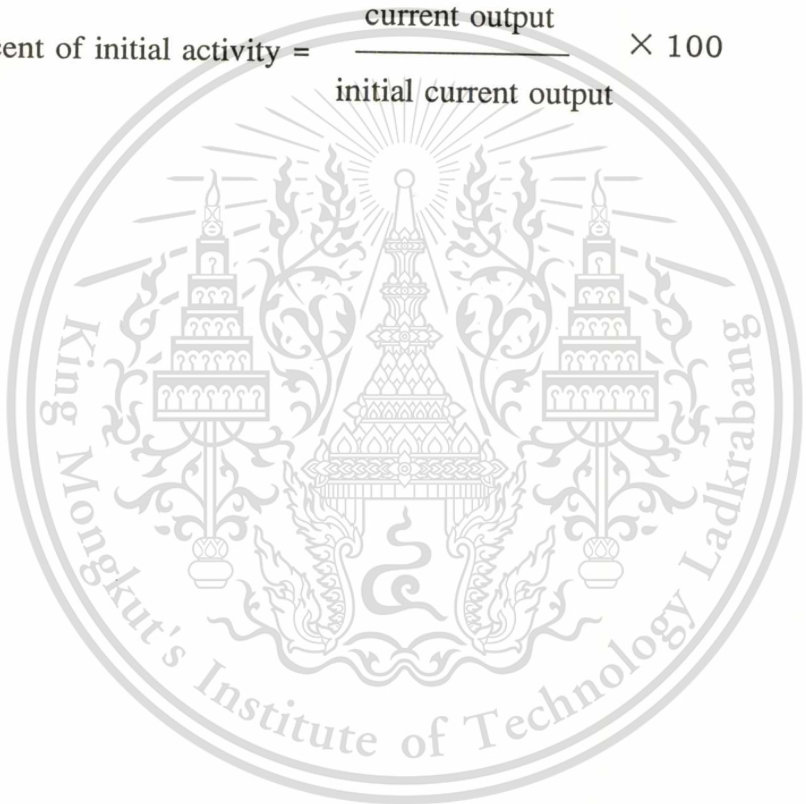
The ideal biosensor would only respond to changes in concentration of the target analyte, and would not be influenced by the presence of other chemical species. Thus the interferences such as ascorbic

acid and 4-acetaminophenol were used for determination of selectivity of glucose sensor.

3.3.8 Determination of Stability

The stability of glucose sensor was shown in terms of enzyme electrode lifetime in which enzyme electrode activity decreased by half of its initial activity. The results were shown as a plot of percentage of initial activity *versus* time.

$$\text{percent of initial activity} = \frac{\text{current output}}{\text{initial current output}} \times 100$$



CHAPTER IV

RESULTS AND DISCUSSION

4.1 Characterization of the Prepared Electrode

In this research work, simple microelectrodes were prepared for using as glucose sensors. The calculation of the electrode area and identification of the microelectrode were investigated. Because of the relationship between magnitude of current response and substrate concentration would specify the sensitivity of the glucose sensor. The electrode prepared for glucose sensor was identified as a microelectrode by using cyclic voltammetric measurement in a solution of 0.1 M $K_3Fe(CN)_6$ and 0.1 M KCl with scan rate of 0.001 V/sec. For the small radius electrode a sigmoidal-shaped voltammogram should be observed in order to slow scan rate in the quiescent solution [29]. The sigmoidal-shaped voltammogram is shown in Figure 4.1.

The electrode surface area can calculated from various methods [28]. In this research the electrodes surface area were calculated by equation 3.1., where i_p and C_0^* were obtained from the relationship between anodic peak current of redox couple molecules and their concentrations. The cyclic voltammograms of various ferricyanide concentrations are shown in Figure 4.2. The result can be ploted as a linear graph shown in Figure 4.3. Its slope could be used to calculate the area of electrode. In this research, the electrodes surface area were 1.88×10^{-4} to $3.40 \times 10^{-4} cm^2$.

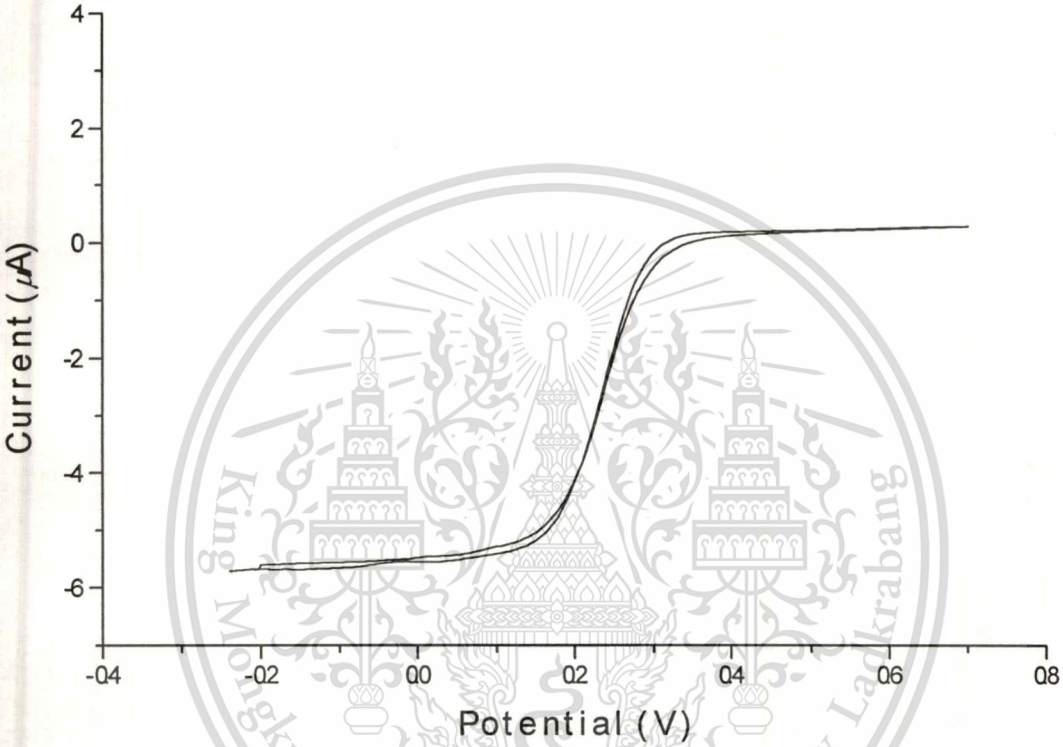


Figure 4.1 Typical sigmoidal shape cyclic voltammogram of 0.1 M $K_3Fe(CN)_6$ in 0.1 M KCl using microelectrode from −0.1 to 0.7 V *versus* Ag/AgCl, scan rate 0.001 V/sec. Counter electrode was platinum.

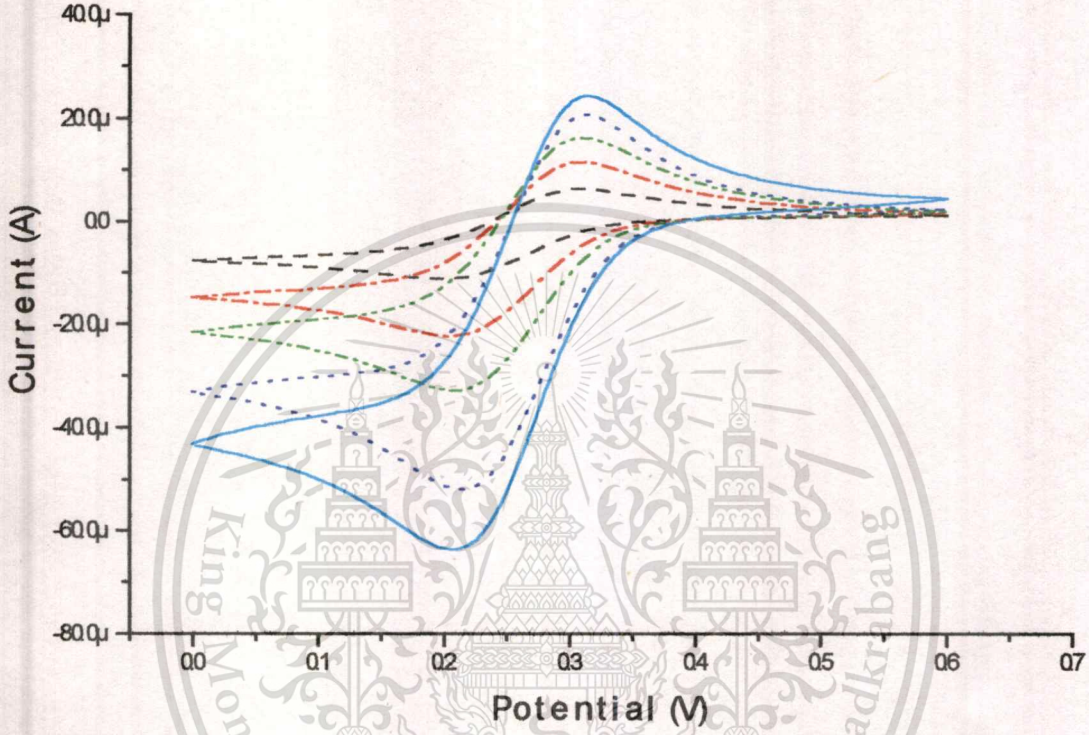


Figure 4.2 A typical cyclic voltammogram for a gold microelectrode obtained in various ferricyanide concentration in 0.1 M KCl, scan rate 0.1 V/sec. (_ _) 0.1 M, (_ . _) 0.2 M, (_ . .) 0.3 M, (.....) 0.4 M, and (____)0.5 M

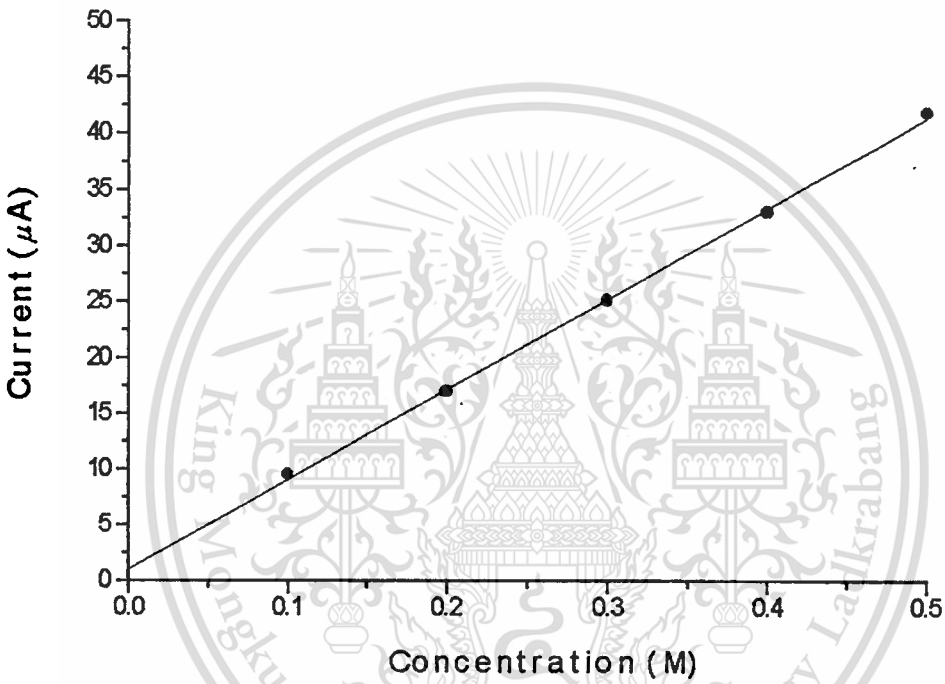


Figure 4.3 The relationship between anodic current and concentration of ferricyanide. The calculated slope was 0.08.

4.2 The Effect of Ferricyanide on the Polypyrrole Films

Ferricyanide entrapped in the polypyrrole films were prepared by electrochemical polymerization. Then the oxidation-reduction of ferricyanide in the polypyrrole films were observed by cyclic voltammetric technique. The cyclic voltammogram of the PPy/Ferri films in 0.1 M phosphate buffer pH 7 suggested that the ferricyanide could be incorporated into polypyrrole and entrapped within the films. The anodic current of ferricyanide refers to amount of them incorporated in the films. Figure 4.4 shows cyclic voltammogram for oxidation-reduction of ferricyanide in the films compared with that of the background. The amount of ferricyanide incorporated in the films increased linearly with increasing polymerization charge and ferricyanide concentration in the growth solution as shown in Figure 4.5. The result can be plotted in a linear graph as shown in Figures 4.6 and 4.7. Figure 4.8 shows the relationship between anodic current of ferricyanide in films and pyrrole concentration. Anodic current of ferricyanide increased because of the increasing of pyrrole concentration up to 40 mM. However when pyrrole concentration increased more than 40 mM, the anodic current did not show any significant. It can be calculated that at 40 mM pyrrole is the optimum concentration for entrapment of the ferricyanide. It can be explained that ferricyanide entrapment is limited by pyrrole concentration. The typical doped polypyrrole structure in Figure 2.2 shows that polypyrrole need certain amount of anions, ferricyanide. The PPy/Ferri electrode can be used as glucose sensor to determine glucose at 0.45 V *versus* Ag/AgCl. In short, ferricyanide has a feasibility to be used as electron mediator.

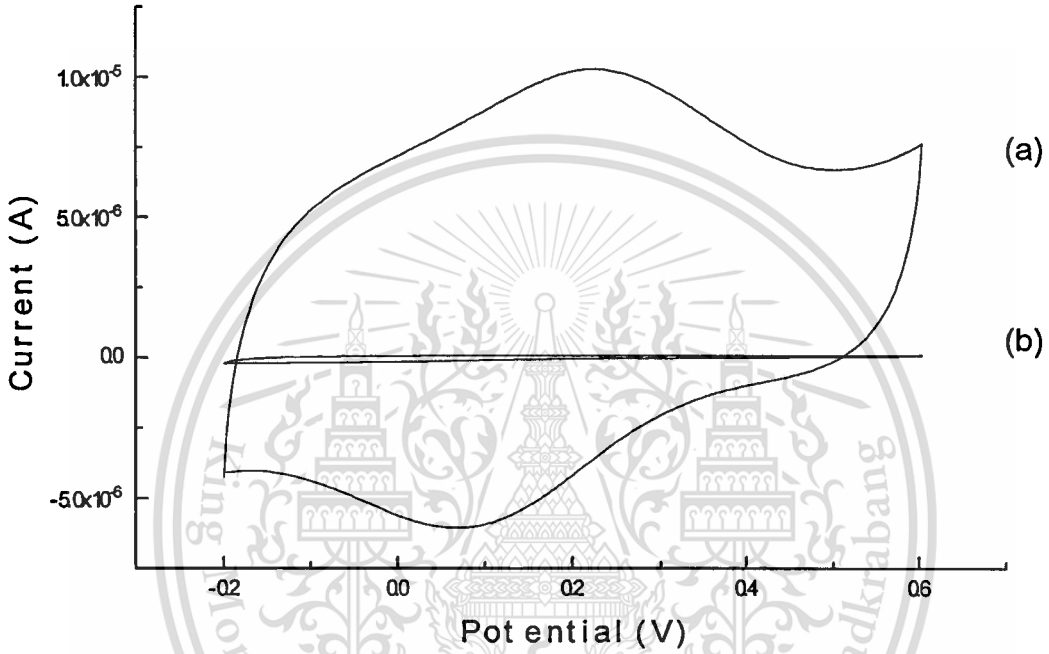


Figure 4.4 A cyclic voltammogram for gold microelectrode in 0.1 M phosphate buffer pH 7 from −0.2 to 0.6 V *versus* Ag/AgCl, scan rate 0.1 V/sec. Counter electrode was platinum.
(a) oxidation-reduction of ferricyanide in films and
(b) background

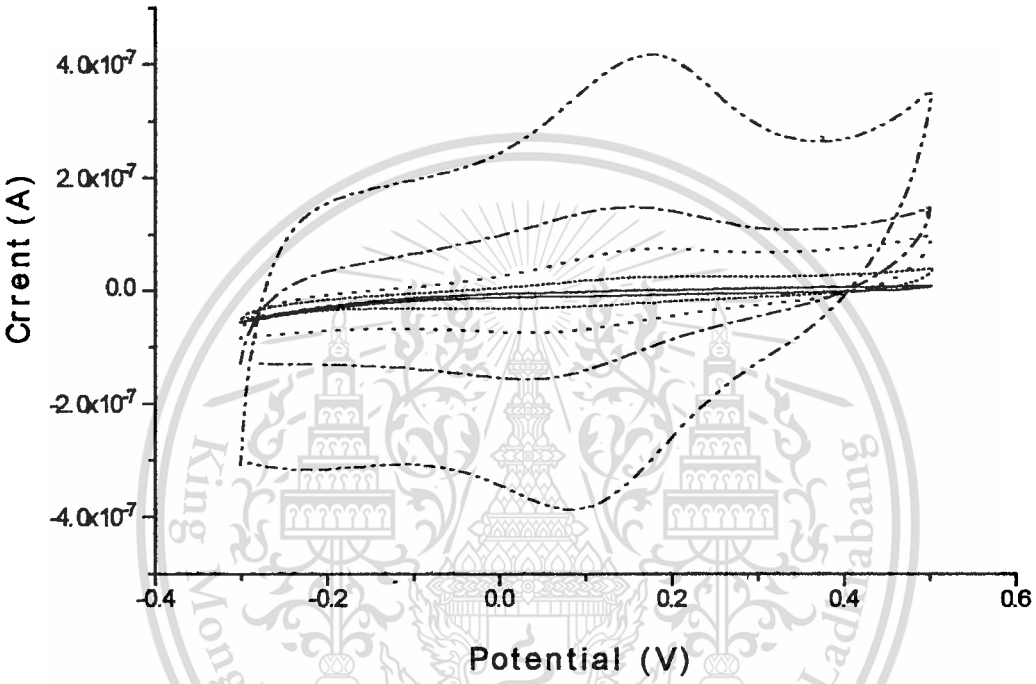


Figure 4.5 Typical cyclic voltammogram of 50 mM ferricyanide at various electrochemical polymerization charges in 0.1 M phosphate buffer pH 7 from −0.2 to 0.6 V *versus* Ag/AgCl, scan rate 0.1 V/sec. Counter electrode was platinum. ; (——) 2 μC, (·····) 4 μC, (·····) 8 μC, (·-·-·) 16 μC and (—·—) 32 μC

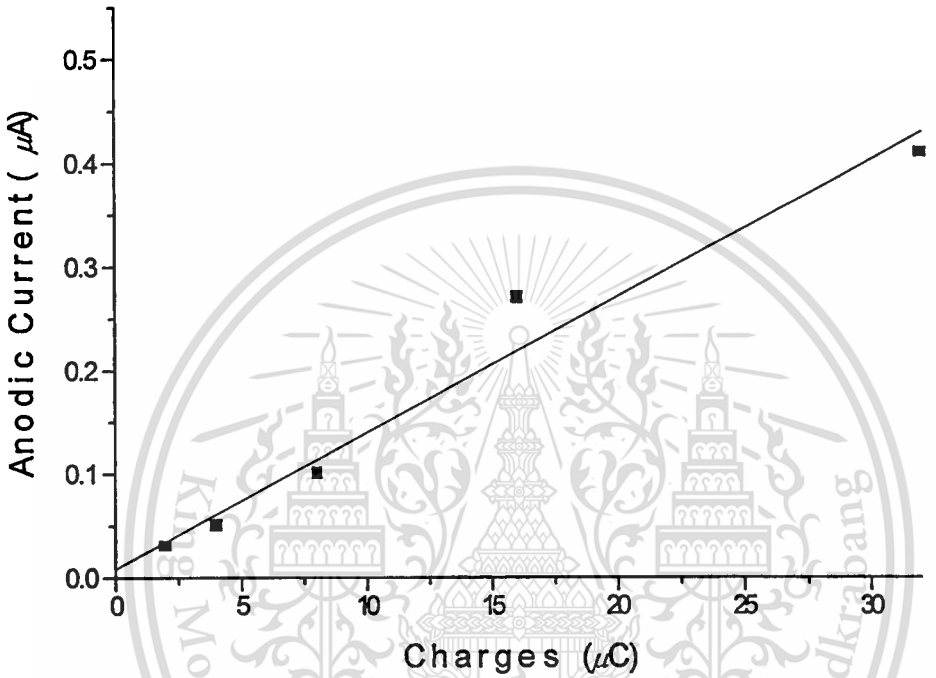


Figure 4.6 The relationship between anodic current of ferricyanide in film and electrochemical polymerization charges. The anodic current was determined by cyclic voltammetry from -0.2 to 0.6 V *versus* Ag/AgCl, scan rate 0.1 V/sec. Counter electrode was platinum.

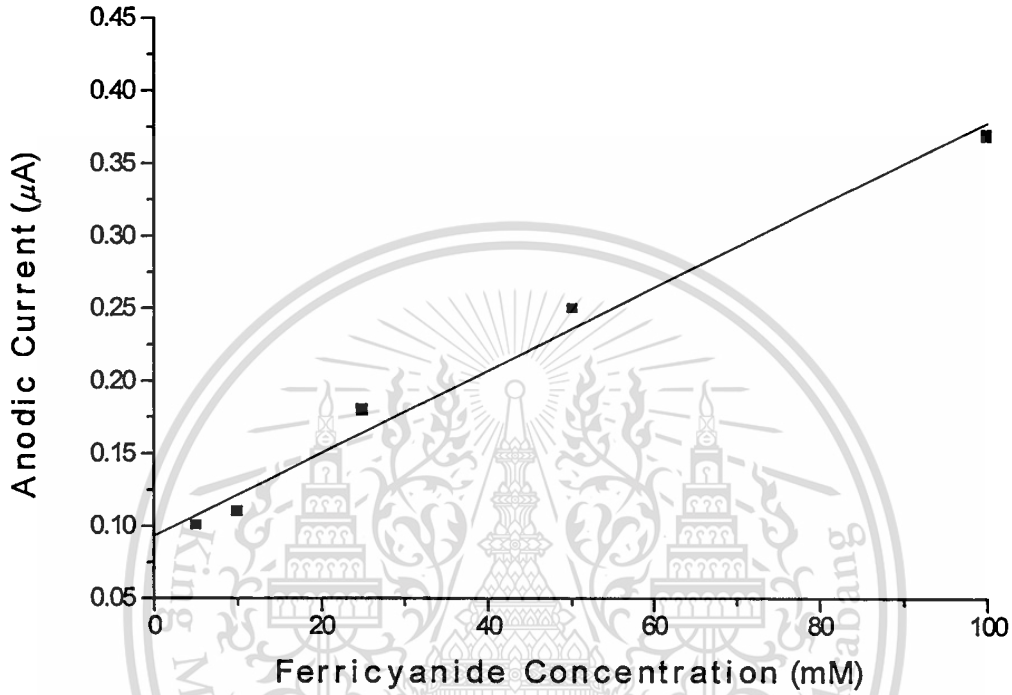


Figure 4.7 The relationship between anodic current of ferricyanide in film and ferricyanide concentrations. The anodic current was determined by cyclic voltammetry from -0.2 to 0.6 V *versus* Ag/AgCl, scan rate 0.1 V/sec. Counter electrode was platinum.

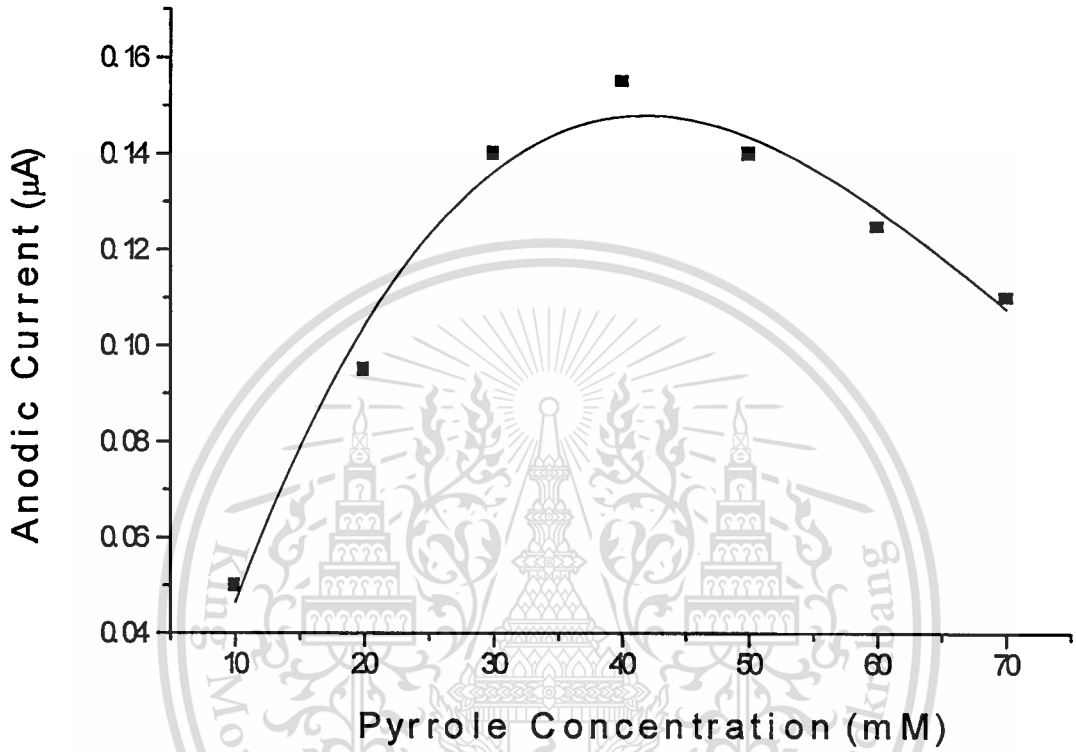


Figure 4.8 The relationship between anodic current and pyrrole concentration. The anodic current was determined by cyclic voltammetry from -0.2 to 0.6 V *versus* Ag/AgCl, scan rate 0.1 V/sec. Counter electrode was platinum.

4.3 The Optimization of Electrochemical Polymerization Charge for PPy/GOD/Ferri Electrode

The amperometric response of an enzyme electrode depends on several factors including the enzyme concentration in the polymer films, mass transport of the substrates and products through the polymer layers, and the enzyme reaction [8]. The electrochemical polymerization charge was identified as the thickness of films. The film thickness was estimated by assuming that 45 mC/cm^2 of charge yielded a 0.1 μm [7]. For these experiments, the concentration of glucose oxidase was kept constant at 25 units/ml and 50 mM ferricyanide in the growth solutions. The surface area of the working electrode was 1.88×10^{-4} cm^2. The PPy/GOD/Ferri enzyme electrodes were prepared by electrochemical polymerization at various charges, i.e., 2, 4, 8, 16 and 32 μC. The enzyme electrodes were used to determine current response of the standard glucose solution. The current response decreased as the charges were higher than 8 μC, and it did not show any significant current response at 32 μC. It was expected that the current response depended on the thickness of the films. Figure 4.9 demonstrates that the charge at 8 μC provided the highest current response for determination of glucose using PPy/GOD/Ferri enzyme electrode. The averaged of 42 mC/cm^2 equaled to the film thickness of 0.09 μm.

Sometime the current response decreased lower than steady state current whereas the films were thicker. It might be that the films were too thick for reduced species to diffuse into the film, so they could not oxidized back to reduced form. Therefore, amount of reduced species providing the current response would be reduced.

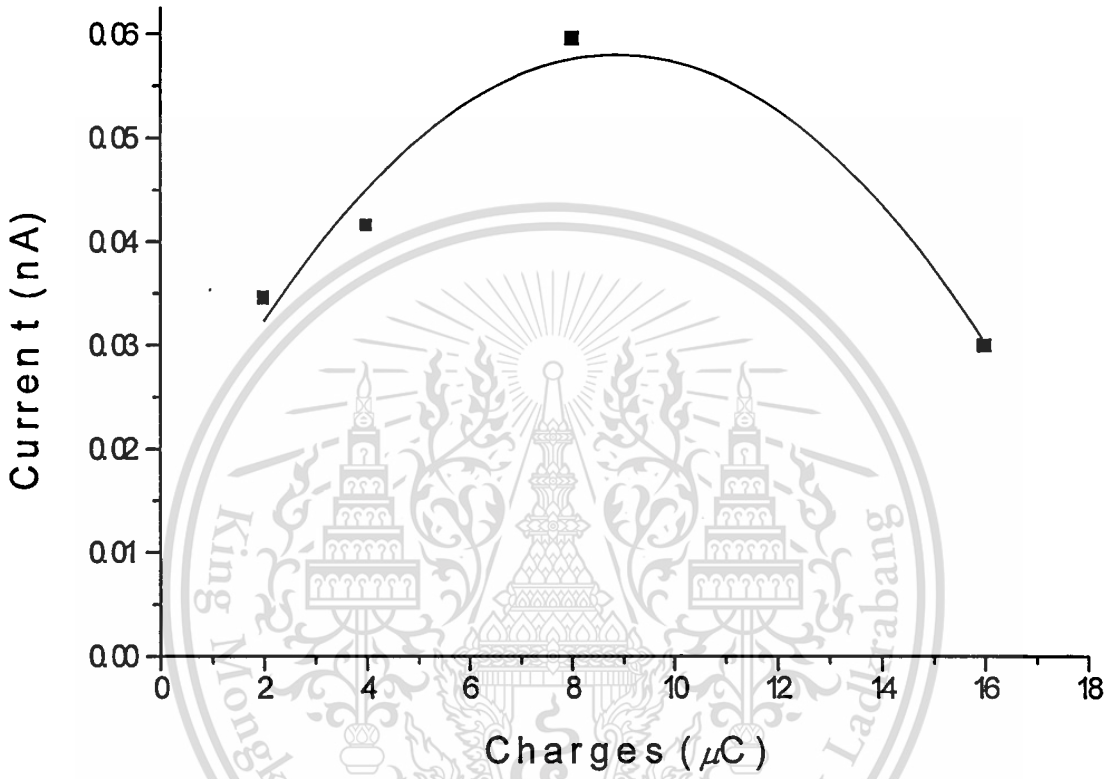


Figure 4.9 The relationship between current response of 20 mM glucose and electrochemical charges. The current responses were determined at 0.45 V *versus* Ag/AgCl in phosphate buffer pH 7 under nitrogen. Counter electrode was platinum.

4.4 The Optimization of Pyrrole Concentration for PPy/GOD/Ferri Electrode

The effect of pyrrole concentration on the current response of the glucose sensor was investigated. Polypyrrole can be polymerized by electrochemical reaction in aqueous media, which allows the incorporation of a wide range of counter ions [12]. For these experiments, the glucose oxidase and ferricyanide were used as counter ions. The glucose sensors were prepared by using various concentrations of pyrrole from 10 to 70 mM in the presence of 25 units/ml glucose oxidase and 50 mM ferricyanide solution. The applied charge was 42 mC/cm^2. Figure 4.10 demonstrates comparative currents for three electrodes with different area used for determination of glucose. It was found that the current response of 20 mM glucose was the highest at the 40 mM pyrrole for all electrodes. In addition, after applied potential, the growth solution changed to a dark yellow-green. It was expected that pyrrole monomers reacted with ferricyanide. However, we found that when freshly distilled pyrrole monomers were used, ferricyanide and glucose oxidase would immobilize into polypyrrole film very well and provided the good results.

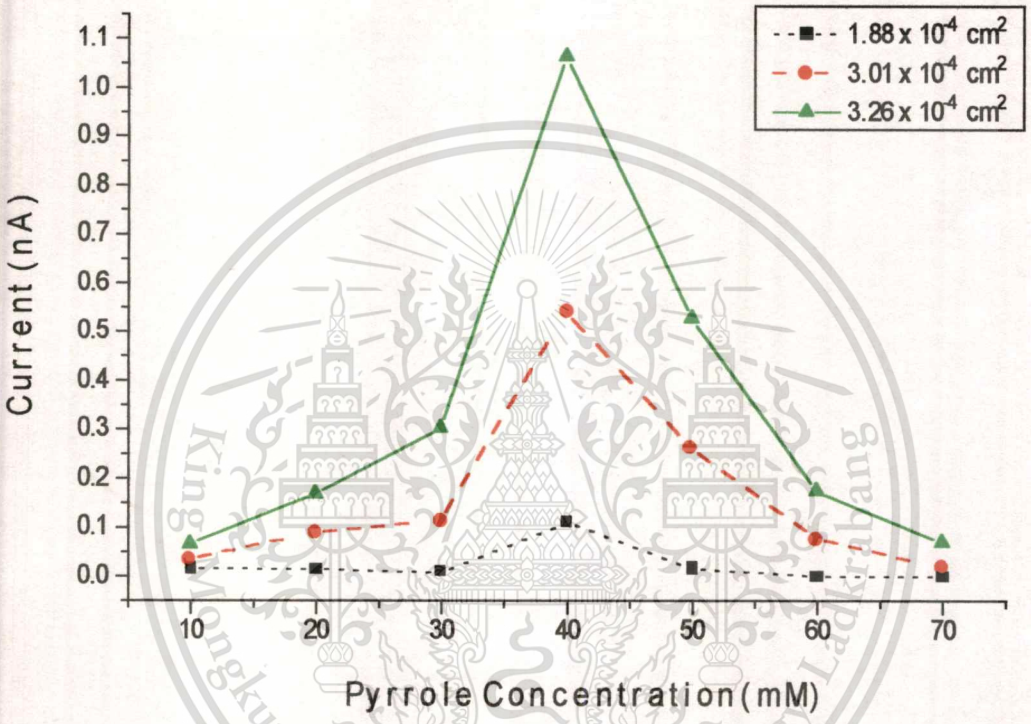


Figure 4.10 The relationship between current response of 20 mM glucose and pyrrole concentrations for three electrodes with different areas. The current responses were determined by amperometry at 0.45 V *versus* Ag/AgCl electrode. Counter electrode was platinum.

4.5 The Optimization of Ferricyanide Concentration for PPy/GOD/Ferri Electrode

The effect of ferricyanide concentration on the enzyme electrodes was investigated. The glucose sensors were prepared by electrochemical polymerization using various concentrations of ferricyanide, i.e., 5, 10, 25, 50, 75 and 100 mM in the presence of 25 units/ml glucose oxidase and 40 mM pyrrole aqueous solution. The applied charge was 42 mC/cm^2. Figure 4.11 demonstrates comparative currents for three working electrodes with different areas used for determination of glucose. The current response of 20 mM glucose was the highest at the 25 mM ferricyanide for all electrodes. As expected, the more the areas of the electrodes were, the higher the current response were obtained. Beyond the optimum point, the ferricyanide concentration increased, the current response decreased. It can be explained that the glucose oxidase competed with ferricyanide immobilize into polypyrrole films during electrochemical polymerization since they both have negative charge. The concentration of ferricyanide at 25 mM was found to be the optimum condition of PPy/GOD/Ferri electrode. In addition, we found that the polymerized time of pyrrole in ferricyanide solution was less than the time for polymerization of pyrrole in phosphate buffer pH 7. It could be possible that ferricyanide might be a catalyst of the polymerization. The ferricyanide will be immobilized into polypyrrole films by electrochemical polymerization only when polymerization occurs in the solution without phosphate buffer. It could be explained that phosphate is smaller than ferricyanide so that phosphate ion can diffuse into polypyrrole films faster than ferricyanide ions.

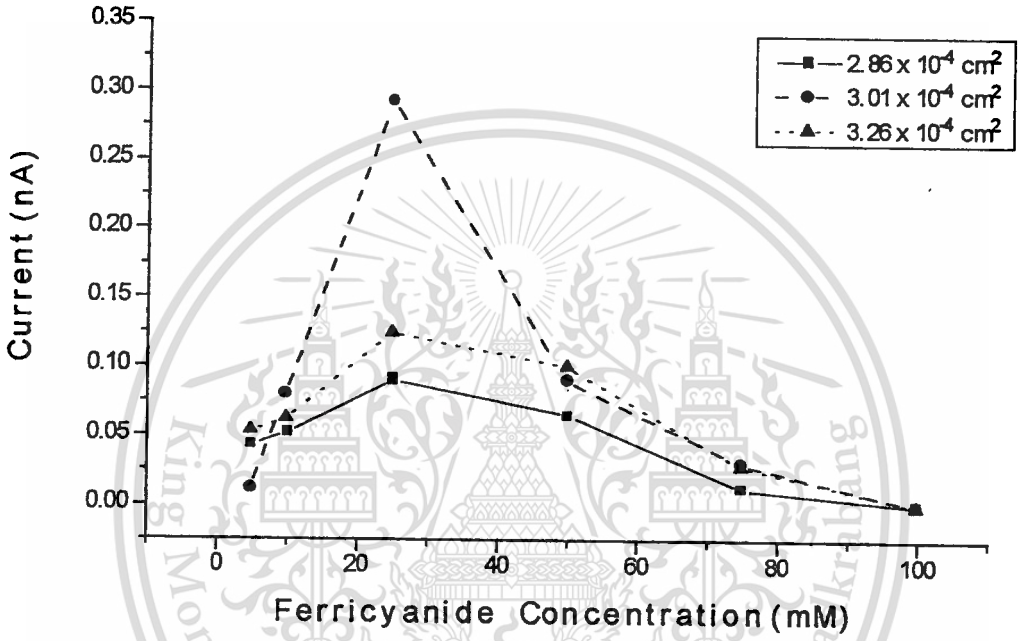


Figure 4.11 The relationship between current response of 20 mM glucose and ferricyanide concentrations for three electrodes with different areas. The current responses were determined by amperometry at 0.45 V *versus* Ag/AgCl. Counter electrode was platinum.

4.6 Sensitivity and Calibration Curve

The optimized PPy/GOD/Ferri electrode was prepared by electrochemical polymerization of 40 mM pyrrole at 0.70 V in the presence of 25 units/ml glucose oxidase and 25 mM ferricyanide solution. The optimized thickness of films was 0.09 μm (42 mC/cm^2). The glucose sensor prepared as described previously was investigated for sensitivity, linearity, selectivity and response time. The sensitivity of the glucose sensors was studied by determination of the current response for each electrode corresponding to 79 nA/mM/cm^2. Before testing for glucose, the PPy/GOD/Ferri electrode was conditioned at a constant potential of 0.45 V until the background current decreased and finally leveled off at a small current (less than 1 nA). The background current corresponded to the oxidation of the polymer. A typical calibration curve is shown in Figure 4.12. The slope was linear up to 10 mM. The response times were 25 to 500 sec. It also was found that the glucose sensor was able to determine glucose at 0.30 V.

4.6.1 Study of the Interferences

The determination for interferences such as ascorbic acid and 4-acetaminophenol are refered to selectivity of glucose sensor. The interferences from ascorbic acid and 4-acetaminophenol were always observed with an amperometric sensor due to an autooxidation at a high working potential. The Table 4.1 shows the current from ascorbic acid using amperometry technique. The PPy/GOD/Ferri glucose sensor can prevent the oxidation of both compounds as shown in Tables 4.2 and 4.3. The solution of 1 μM ascorbic acid and 1 μM 4-acetaminophenol have no effect on the PPy/GOD/Ferri electrode at 0.45 V. Ascorbic acid can be prevented 95% and 48% by using PPy/GOD/Ferri electrode at 0.70 and 0.45 V respectively. The PPy/GOD/Ferri electrode was used to determine glucose in carrot juice as shown in Figure 4.13.

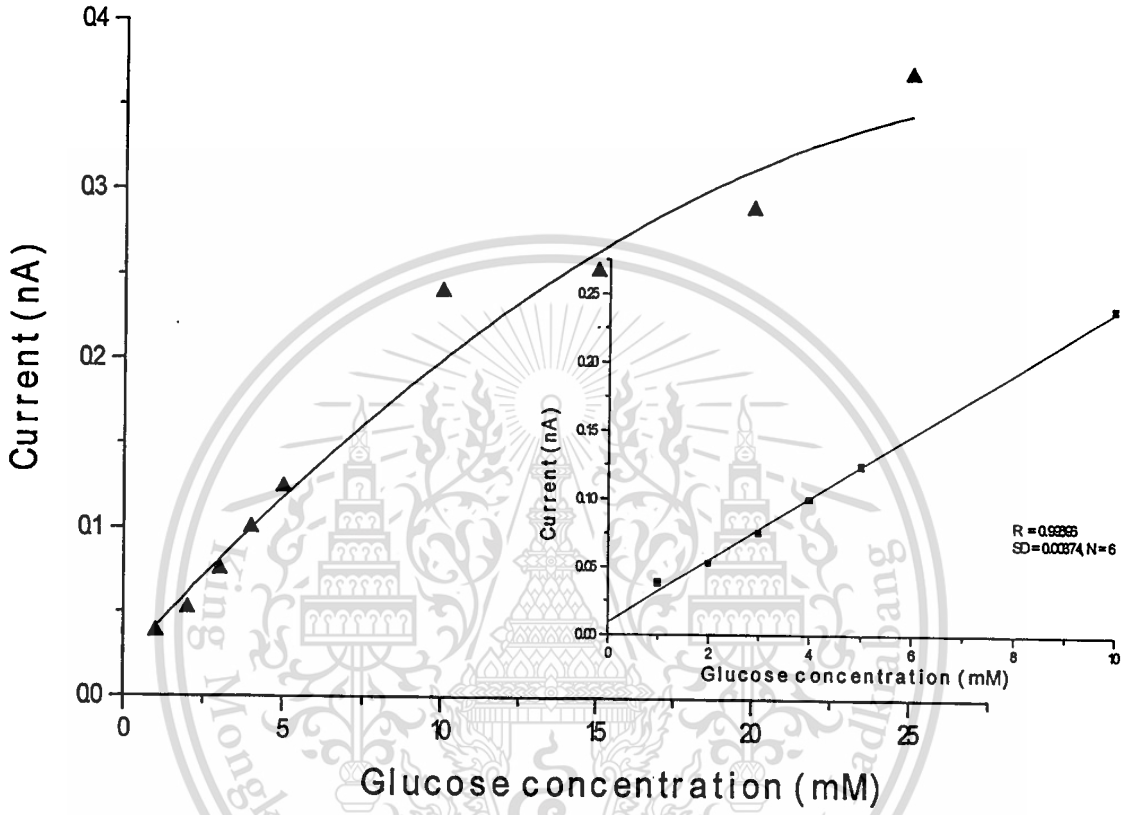


Figure 4.12 The calibration curve of glucose for PPy/GOD/Ferri electrode using amperometry at 0.45 V *versus* Ag/AgCl. Counter electrode was platinum.

Table 4.1 Current measurement of 40 μM ascorbic acid using the bare electrode

E (V)	I (nA)
0.70	200
0.45	3.0
0.30	0.27
0.10	0.10
0	none

Table 4.2 Current measurement of ascorbic acid using the PPy/GOD/Ferri electrode at 0.45 and 0.70 V

Ascorbic acid (μM)	I (nA) 0.45 V	I (nA) 0.70 V
40	1.57	10.19
20	1.45	4.70
10	1.19	1.64
5	0.12	0.20
1	none	none

Table 4.3 Current measurement of 4-acetaminophenol using the PPy/GOD/Ferri electrode at 0.45 and 0.70 V

4-Acetaminophenol (μM)	I (nA) 0.45 V	I (nA) 0.70 V
10	0.16	0.25
5	none	0.14
1	none	none

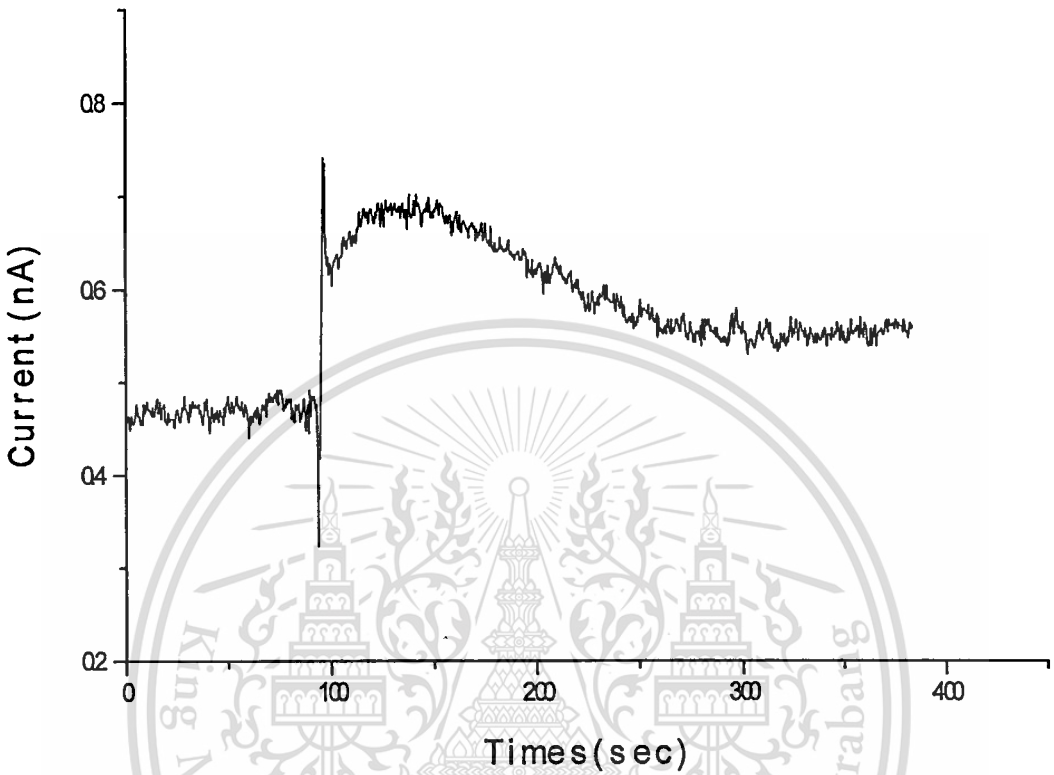


Figure 4.13 Current response of glucose sensor obtained from injection of 3 μL carrot juice into 0.1 M phosphate buffer 5 mL, pH 7 with PPy/GOD/Ferri electrode using amperometry at 0.45 V *versus* Ag/AgCl. Counter electrode was platinum.

4.7 Study of Stability

4.7.1 Operational Stability

For these experiments, the determinations of 5 mM glucose solution continuously were referred to the stability of glucose sensor. The Figure 4.14 demonstrates the relative activity of glucose sensor for operational stability. It was found that the half lifetime of glucose sensor was short when the % relative activity decreased rapidly. The reproducible results were obtained if freshly prepared, deoxygenated buffer solutions were used in determination of the glucose. Therefore, the glucose sensor can be regarded as an inappropriate sensor on operational stability. The cyclic voltammogram of PPy/GOD/Ferri electrode after determination of the glucose at various times of measurements is shown in Figure 4.15. The results can be plotted as an exponential decay in Figure 4.16. This can be explained that the decrease of stability due to the leaching of the ferricyanide out of the films.

4.7.2 Storage Stability

The results from current response can be regarded as an inappropriate on operational stability since the ferricyanide leached out of the films. Therefore the glucose sensors should be stored in a few amount of ferricyanide in phosphate buffer pH 7. It could be explained by schematic drawing in Figure 4.17. For these experiments, determination of ferricyanide entrapment was carried out to improve storage stability as shown in Figure 4.18. The storing solution were consisted of a 0.1 M phosphate buffer pH 7, and 0.1 M phosphate buffer pH 7 with various ferricyanide concentration, i.e., 50, 100, 500 nM 1.0 and 2.5 mM. It was found that ferricyanide entrapped in the films still maintained in the films when stored in 100 nM ferricyanide and phosphate buffer pH 7. In addition, the glucose sensor can not provided current response from glucose when stored in ferricyanide concentration higher than 100 nM in 0.1 M phosphate buffer pH 7. It might be explained that the glucose oxidase in the films was destroyed by ferricyanide in the solution. The

measurement of glucose response using PPy/GOD/Ferri electrode which stored in 100 nM ferricyanide and 0.1 M phosphate buffer pH 7 as shown in Figure 4.19. The glucose sensors were kept under 4°C after determination.

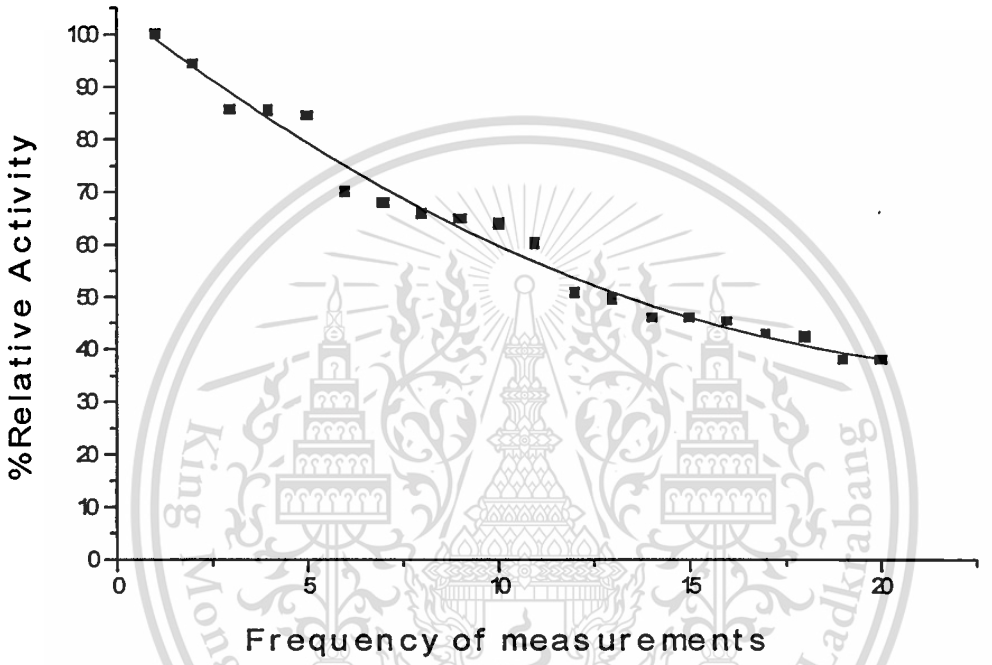


Figure 4.14 Operational stability of glucose sensor to 5 mM glucose % Relative currents were determined by amperometry at 0.45 V *versus* Ag/AgCl using PPy/GOD/Ferri electrode. Counter electrode was platinum

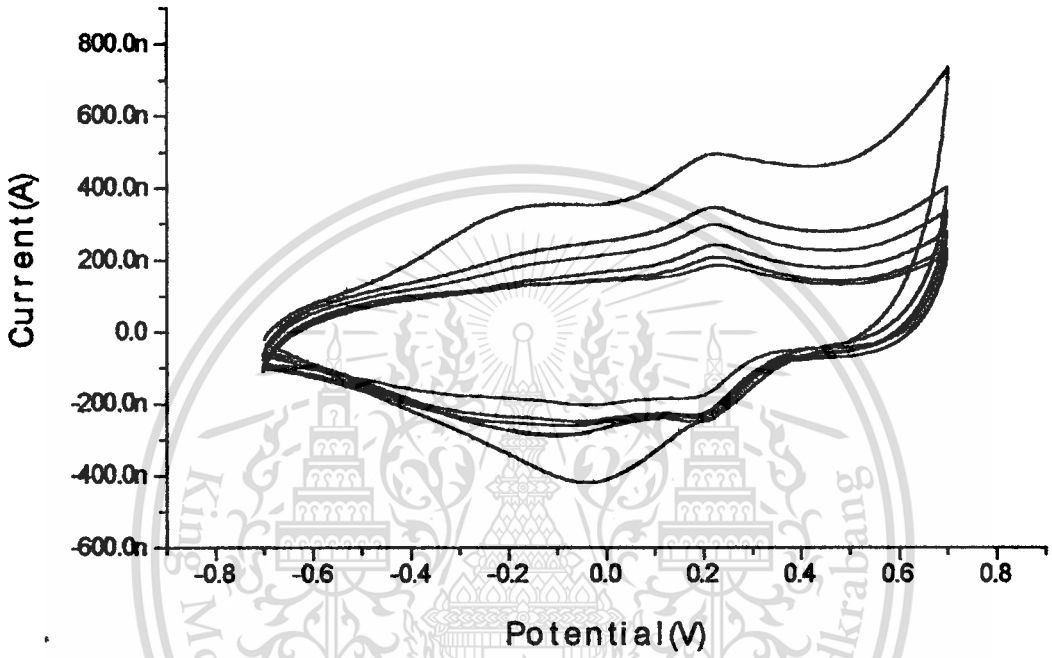


Figure 4.15 Typical cyclic voltammograms of the glucose sensor at different frequency for determination of glucose in 0.1 M phosphate buffer pH 7 from −0.7 to 0.7 V *versus* Ag/AgCl, scan rate 0.1 V/sec. The frequency for determination of glucose; (——) 1 time, (——) 2 times, (——) 5 times, (——) 10 times, (——) 15 times (——) and 21 times respectively.

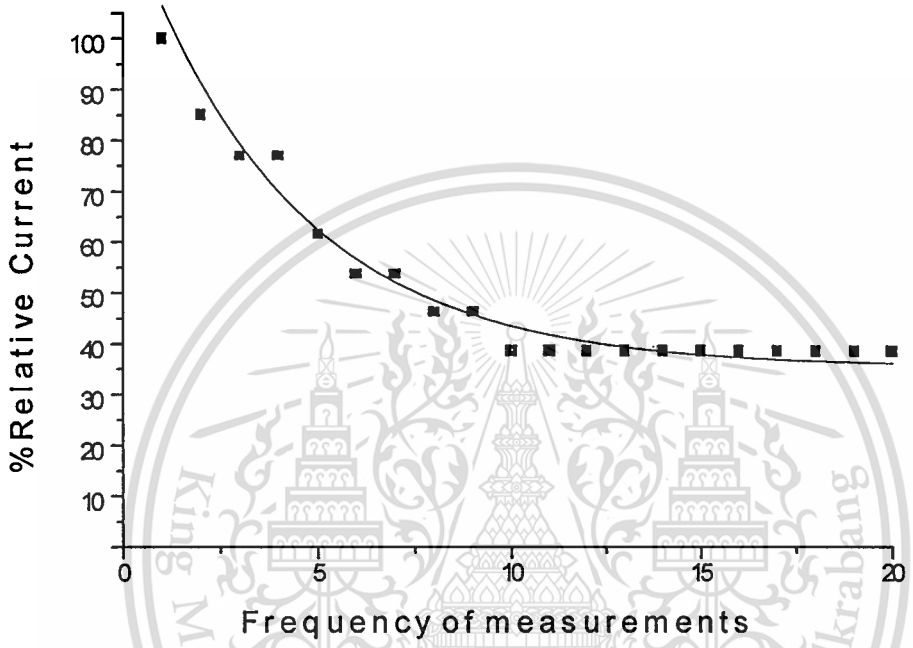


Figure 4.16 Graph shows operational stability of ferricyanide in films % Relative currents were determinated by amperometry at 0.45 V *versus* Ag/AgCl using PPy/GOD/Ferri electrode. Counter electrode was platinum.

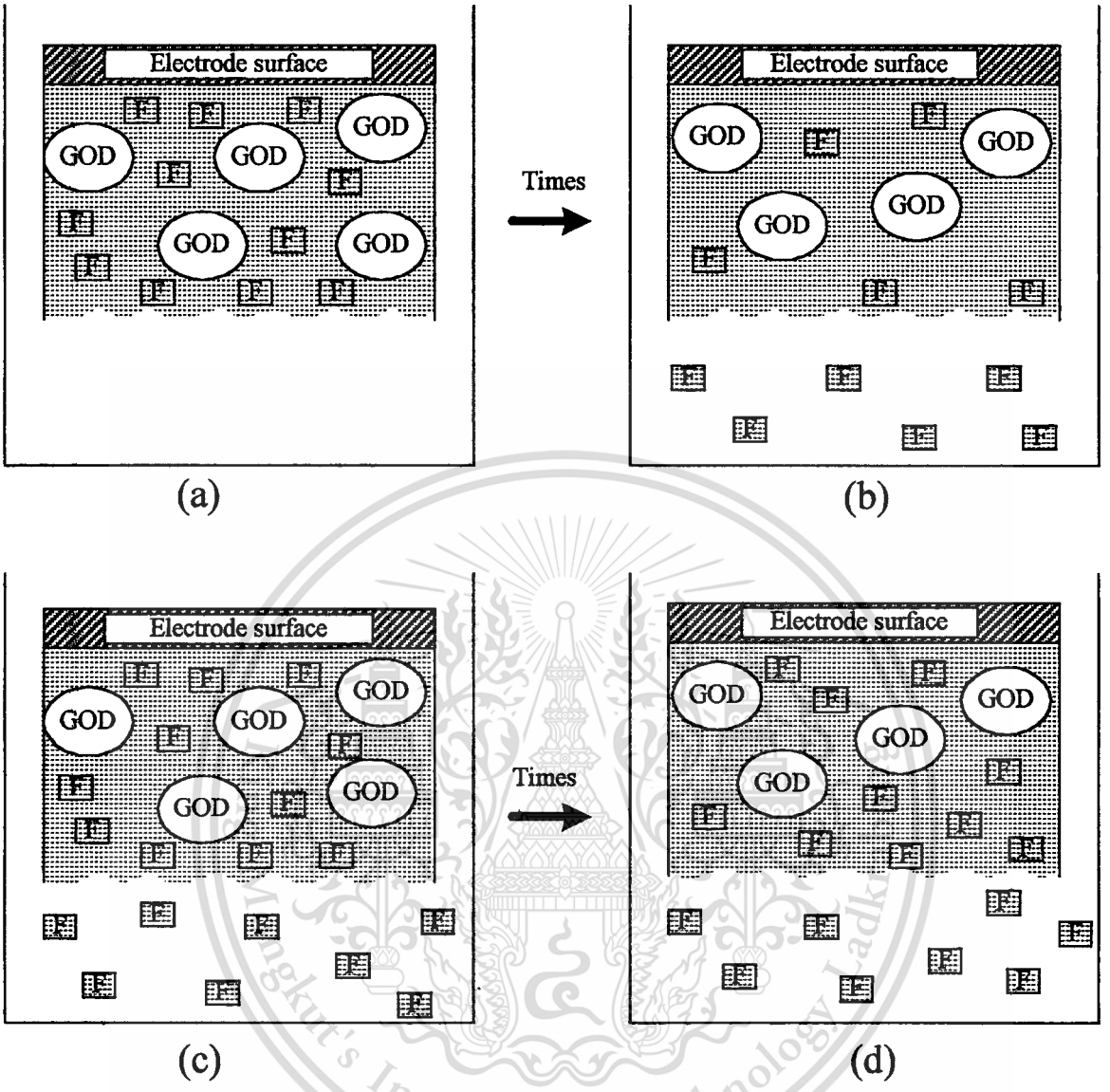


Figure 4.17 The schematic drawings of the electrodes (a) and (b) electrodes are before and after storing in phosphate buffer solution (c) and (d) electrodes are before and after storing in the solution of phosphate buffer and ferricyanide

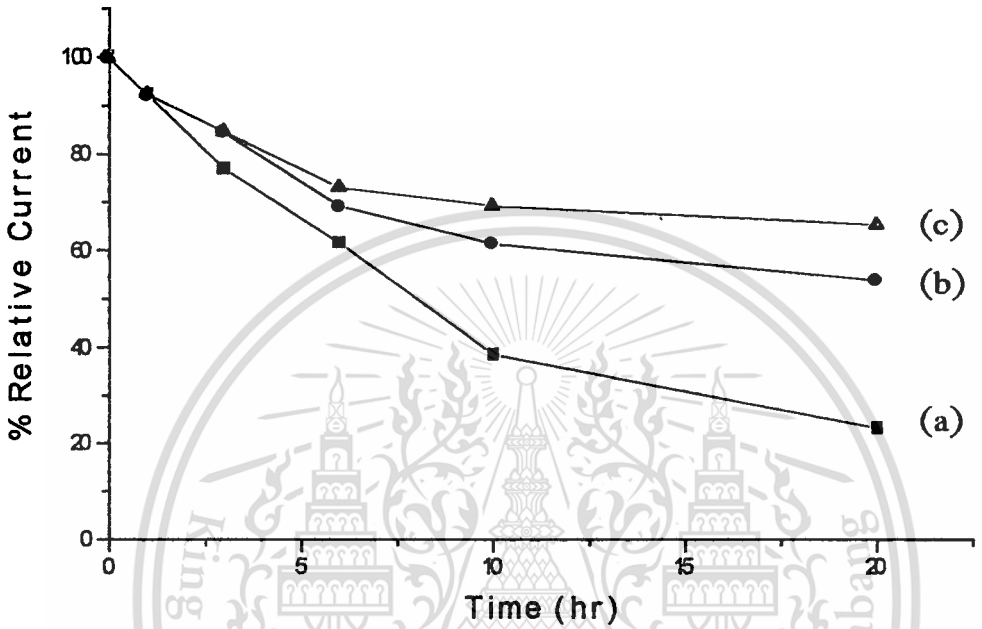


Figure 4.18 Storage stability of ferricyanide in films after storing at 4 °C in (a) 0.1 M phosphate buffer pH 7, (b) 0.1 M phosphate buffer pH 7 and 50 nM ferricyanide, (c) 0.1 M phosphate buffer pH 7 and 100 nM ferricyanide

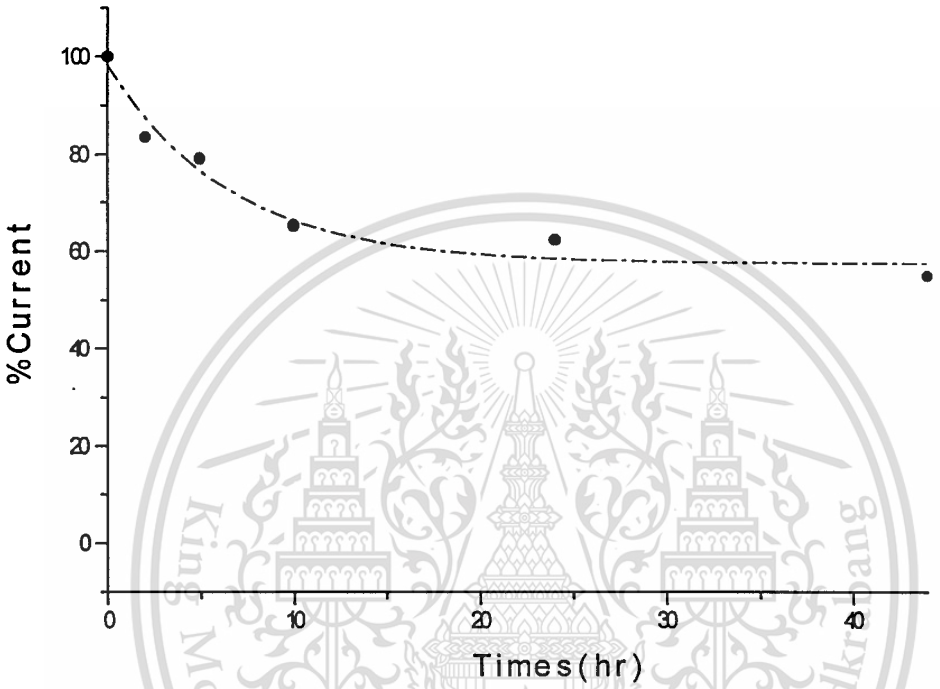


Figure 4.19 The storage stability of glucose sensor in 100 nM ferricyanide and phosphate buffer pH 7 at 4 °C.

CHAPTER V

CONCLUSION

An amperometric glucose sensor was prepared by the electrochemical polymerization of pyrrole onto a gold microelectrode in the presence of the enzyme glucose oxidase and ferricyanide solution at a potential of 0.70 V *versus* Ag/AgCl. The enzyme and ferricyanide were entrapped into the polypyrrole films during the electrochemical polymerization process. The electrodes with surface areas of 1.88×10^{-4} to 3.40×10^{-4} cm^2 were used as the bases for glucose sensors. Glucose responses were determinated by measurement of the amperometric oxidation of ferricyanide at +0.45 V *versus* Ag/AgCl in the absence of oxygen. Electrochemical experiments were carried out to determine the optimal conditions for the pyrrole monomer, glucose oxidase and ferricyanide films preparation. The concentration of 40 mM pyrrole in the presence of 25 units/ml of glucose oxidase and 25 mM ferricyanide were used for the preparation of the glucose sensor. The optimal response was obtained for a film thickness of 0.09 μm (42 mC/cm^2). The linearity of the glucose sensor response ranged from 1 to 10 mM glucose. The operational stability of the optimized PPy/GOD/Ferri enzyme electrode was for only a few assays, because the ferricyanide leached out during glucose measurement. The presence of oxidisable compounds such as ascorbic acid and 4-acetaminophenol did not interfere the PPy/GOD/Ferri enzyme electrode during measurement of glucose at 0.45 V. The sensitivity of the glucose sensor was 79 $nA/mM/cm^2$. The glucose could be stored in 100 nM ferricyanide and 0.1 M phosphate buffer pH 7 at 4 ^{o}C.

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APPENDIX

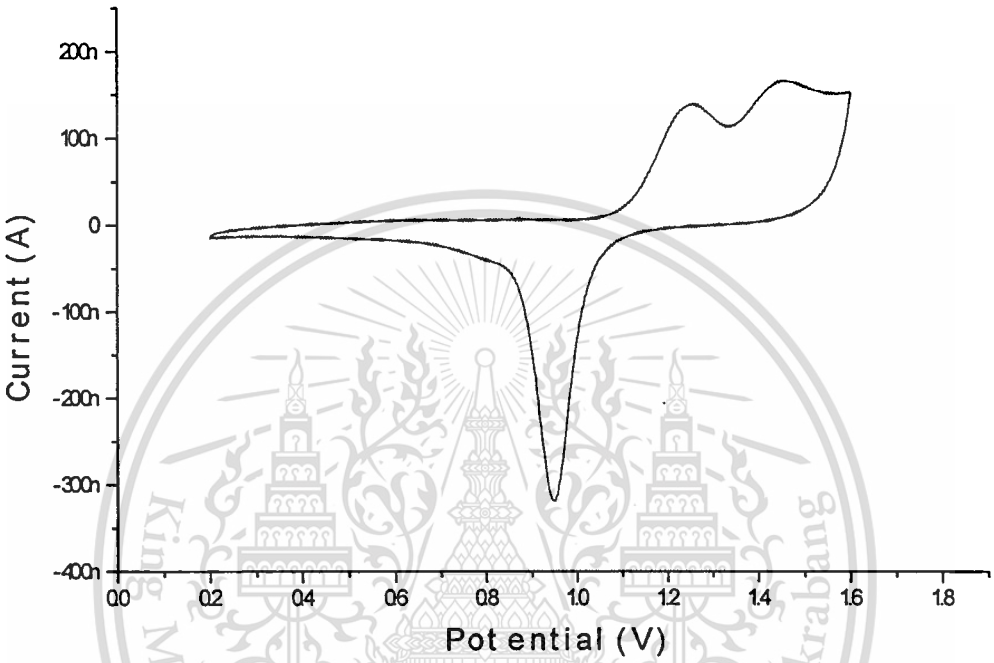

Figure 1 Cyclic voltammogram at bare gold in 0.1 M H_2SO_4 from 0.2 to −1.6 V *versus* Ag/AgCl, scan rate 0.1 V/sec. Counter electrode was platinum.

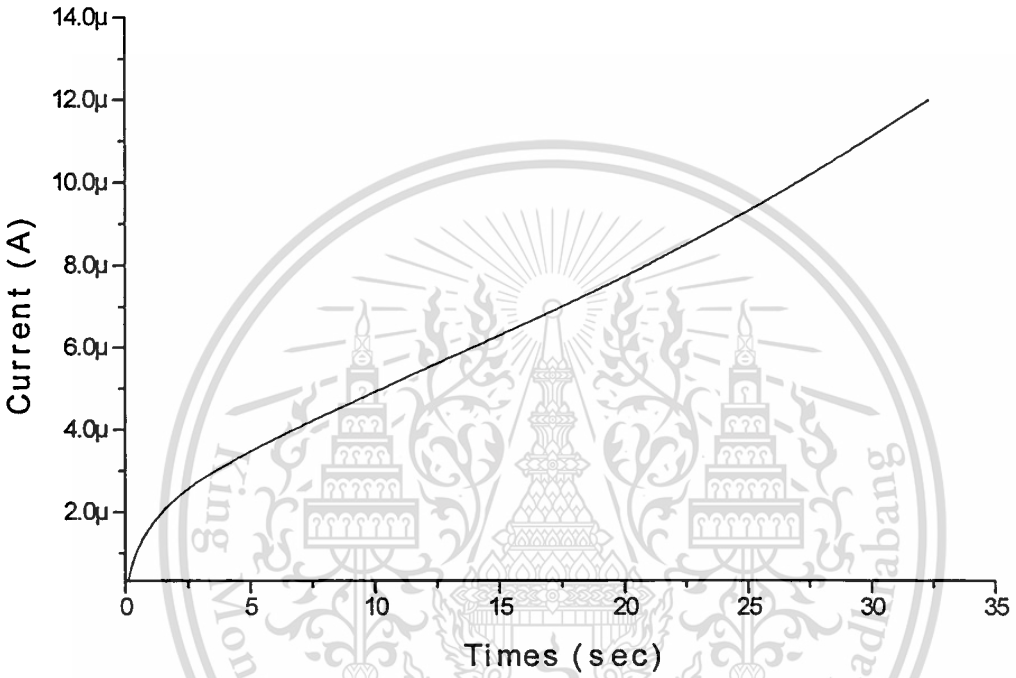

Figure 2 Electrochemical polymerization curve for preparation of PPy/GOD/Ferri films on microelectrode at 0.70 V *versus* Ag/AgCl. Counter electrode was platinum.

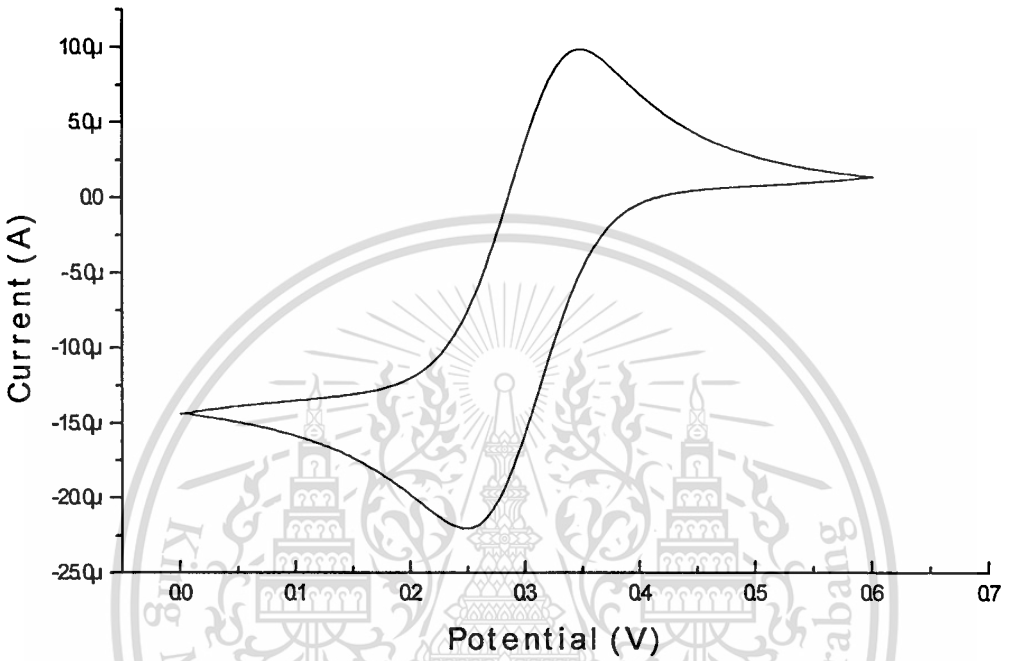

Figure 3 Cyclic voltammogram at bare gold microelectrode in 0.1 M $K_3Fe(CN)_6$ from −0.7 to 0.7 V *versus* Ag/AgCl, scan rate 0.1 V/sec. Counter electrode was platinum. The anodic and cathodic peaks potential are shown at 0.34 and 0.26 V respectively.

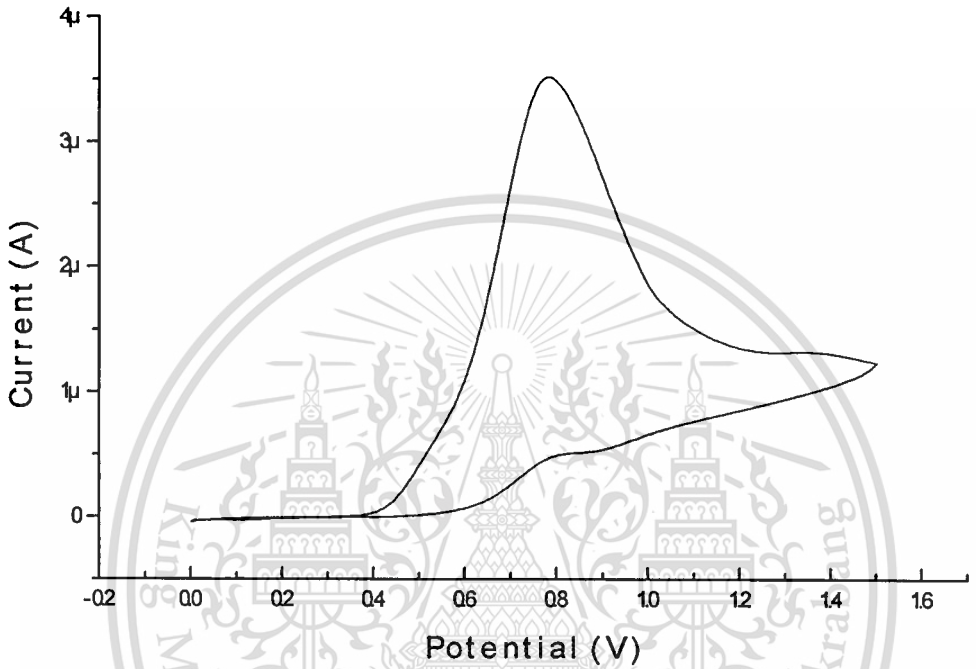

Figure 4 Cyclic voltammogram of 0.5 mM 4-acetaminophenol in 0.1 M phosphate buffer pH 7 from 0 to 1.5 V *versus* Ag/AgCl, scan rate 0.1 V/sec. Counter electrode was platinum.

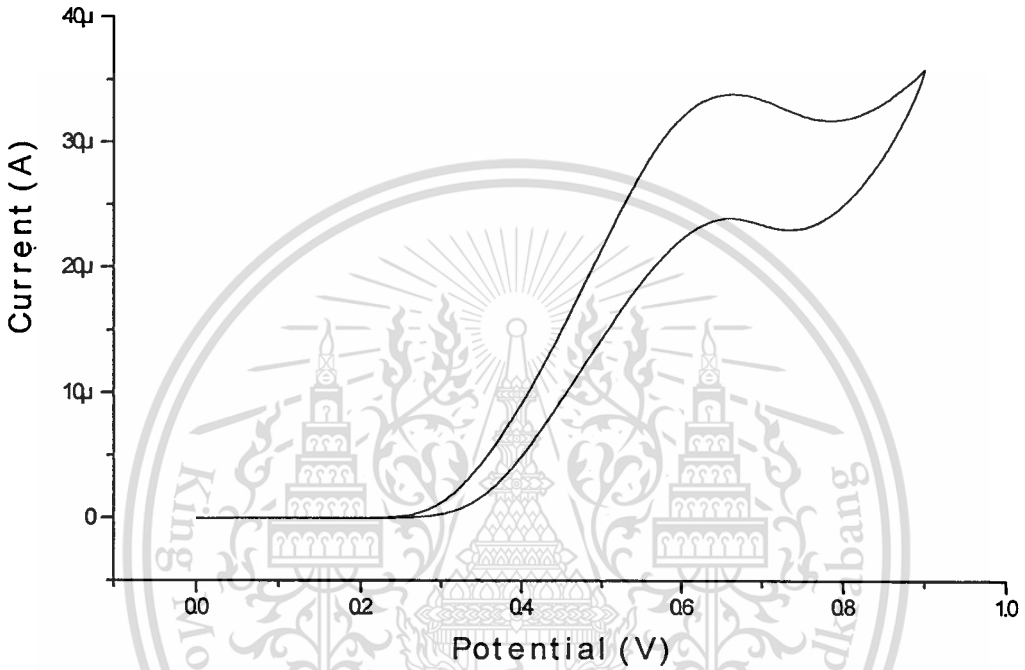

Figure 5 Cyclic voltammogram of 0.5 mM ascorbic acid in 0.1 M phosphate buffer pH 7 from 0 to 1.5 V *versus* Ag/AgCl, scan rate 0.1 V/sec. Counter electrode was platinum. The anodic peak potential of ascorbic acid is shown at 0.76 V.

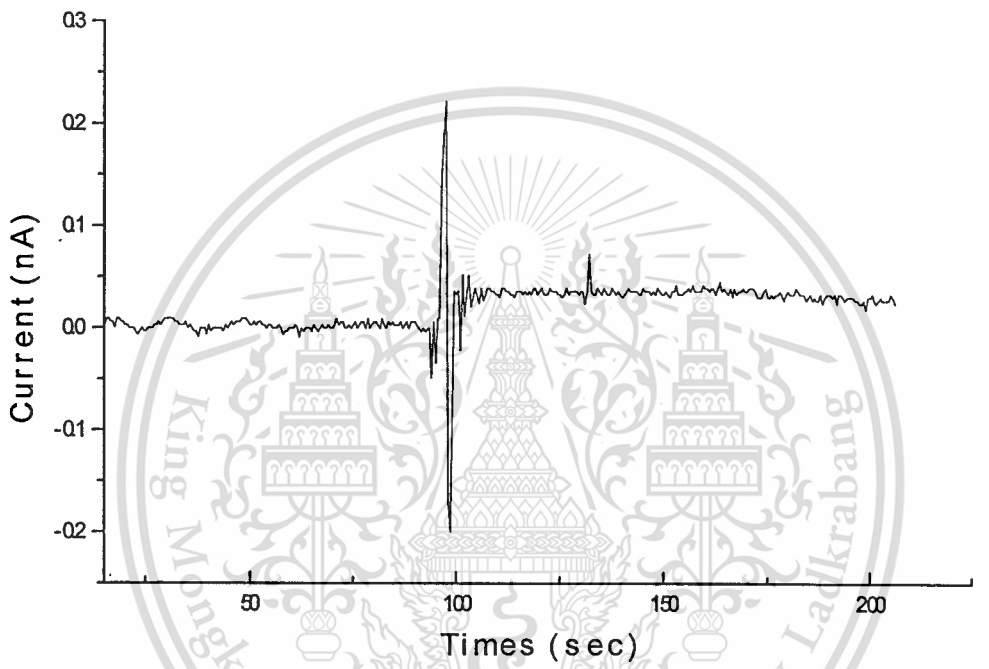

Figure 6 Current response obtained from injection of 25 μL 0.2 mM glucose into 0.1 M phosphate buffer 5 mL, pH 7 using PPy/GOD/Ferri electrode at 0.45 V *versus* Ag/AgCl. Counter electrode was platinum.

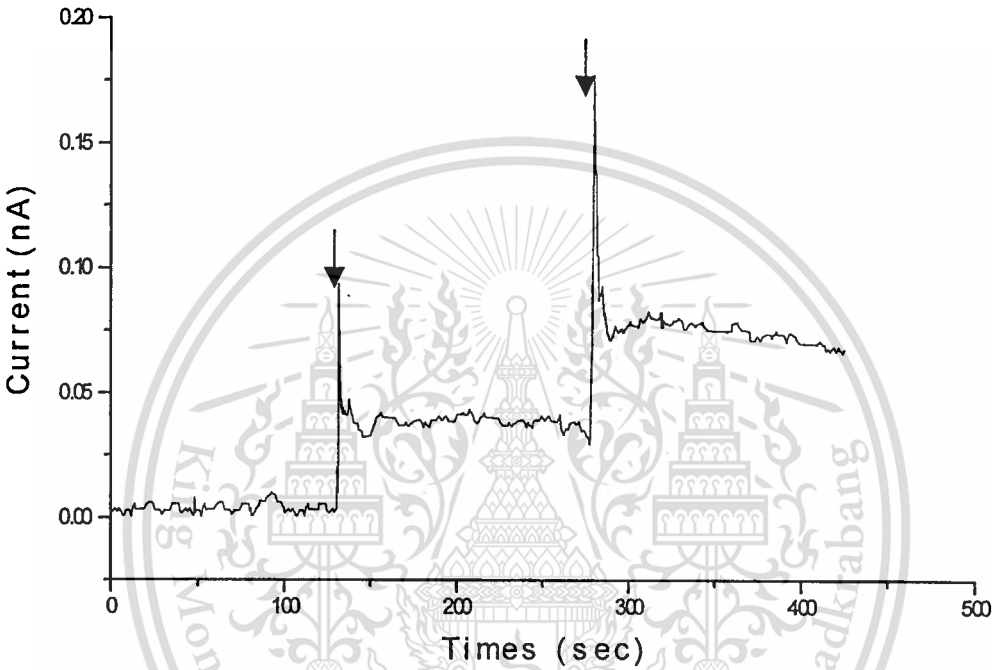

Figure 7 Current response of glucose sensor to a stepwise addition of glucose. An arrow shows injection of 25 μL of 0.2 M glucose into 0.1 M phosphate buffer 5 mL, pH 7 using PPy/GOD/Ferri electrode at 0.30 V *versus* Ag/AgCl. Counter electrode was platinum.

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