ผลของวิธีการเชื่อมขวางด้วยกลูตารัลดีไฮด์ต่อการตรึงเอนไซม์ในเมตริกซ์อนุภาคนาโนของเงิน/ไคโตซาน

นางสาวติรกา นวลจันทร์

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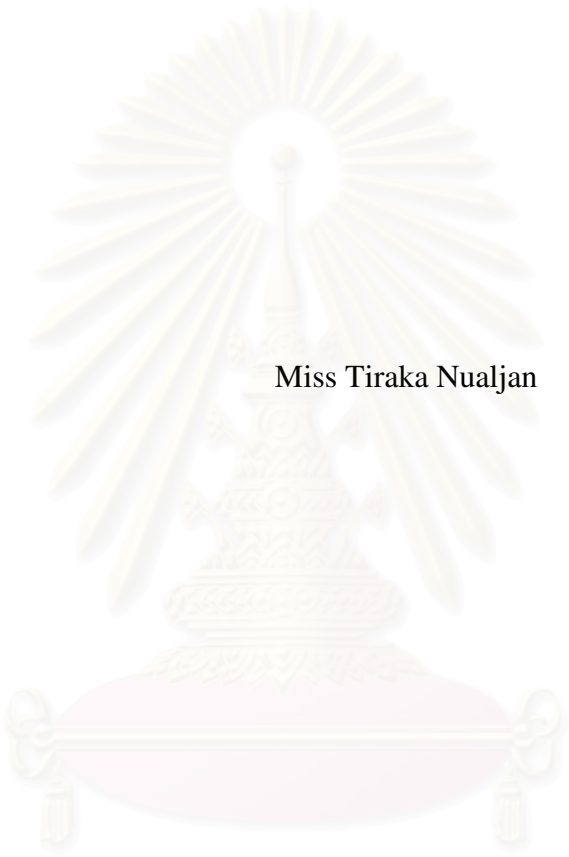
EFFECTS OF GLUTARALDEHYDE CROSS-LINKING METHODS ON ENZYME IMMOBILIZATION IN Ag NANOPARTICLE/CHITOSAN MATRICES

Miss Tiraka Nualjan



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ติรกา นวลจันทร์ : ผลของวิธีการเชื่อมขวางด้วยกลูตารัลดีไฮด์ต่อการตรึงเอนไซม์ในเมตริกซ์อนุภาคนาโนของเงิน/ไคโตซาน
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งานวิจัยนี้ศึกษาอิทธิพลของวิธีการเชื่อมขวางด้วยกลูตารัลดีไฮด์ต่อการตรึงเอนไซม์ในเมตริกซ์อนุภาคนาโนของเงิน/ไคโตซานด้วยวิธีแตกต่างกัน ดังนี้ ; A เอนไซม์ถูกกักในเมตริกซ์อนุภาคนาโนของเงิน/ไคโตซาน; B เอนไซม์ถูกกักในเมตริกซ์ กลูตารัลดีไฮด์/อนุภาคนาโนของเงิน/ไคโตซาน; C เอนไซม์ถูกกักในเมตริกซ์ อนุภาคนาโนของเงิน/ไคโตซาน จากนั้นถูกเชื่อมขวางด้วยสารละลายกลูตารัลดีไฮด์ ; D เอนไซม์ถูกกักในเมตริกซ์ อนุภาคนาโนของเงิน/ไคโตซาน จากนั้นถูกเชื่อมขวางด้วยไอกลูตารัลดีไฮด์ที่อิ่มตัว; E เอนไซม์ถูกดูดซับในเมตริกซ์ กลูตารัลดีไฮด์/อนุภาคนาโนของเงิน/ไคโตซาน งานวิจัยส่วนแรกศึกษาผลของอนุภาคนาโนของเงินต่อการตอบสนองทางไฟฟ้า พบว่า อนุภาคของเงินยังช่วยเพิ่มการตอบสนองสัญญาณทางไฟฟ้า และช่วยให้เอนไซม์กระจายตัวดีขึ้น อย่างไรก็ตามความเข้มข้นของอนุภาคนาโนของเงินที่มากเกินไปทำให้อัตราการถ่ายเทมวลสารของสารตั้งต้นและผลิตภัณฑ์ถูกจำกัด ส่วนที่สองศึกษาผลของการเชื่อมขวางด้วยกลูตารัลดีไฮด์ต่อการตอบสนองทางไฟฟ้าของไบโอเซนเซอร์ และการใช้ซ้ำ พบว่าการตอบสนองทางไฟฟ้าของอิเล็คโทรด A, B, C, D และ E มีค่า 7.9, 0.66, 3.3, 1.2 และ 4.3 ไมโครแอมแปร์ ตามลำดับ กลูตารัลดีไฮด์มีผลต่อการปริมาณเอนไซม์ที่ถูกตรึงและมีผลให้การทำงานของเอนไซม์น้อยลง แต่การเชื่อมขวางไบโอเซนเซอร์ด้วยไอกลูตารัลดีไฮด์ที่อิ่มตัว สามารถนำกลับมาใช้ซ้ำได้

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TIRAKA NUALJAN: EFFECTS OF GLUTARALDEHYDE CROSS-LINKING METHODS ON ENZYME IMMOBILIZATION IN Ag NANOPARTICIE/CHITOSAN MATRICES, ADVISOR : ASSOC. PROF. SEEROONG PRICHANONT, Ph.D., CO-ADVISOR : ASSIT. PROF. BUNJERT JONGSOMJIT, Ph.D. 71 pp.

Amperometric phenol biosensors were constructed by immobilization of Horseradish peroxidase (HRP, EC 1.11.1.7) in Ag-chitosan composite modified on glassy carbon electrodes. Five different methods of enzyme immobilization were investigated and compared: (A) HRP entrapment in Ag nanoparticle/chitosan composite; (B) HRP entrapment in glutaraldehyde solution/Ag nanoparticle/chitosan composite; (C) HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with glutaraldehyde solution; (D) HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with saturated glutaraldehyde vapor and (E) HRP adsorption in glutaraldehyde solution/Ag nanoparticle/chitosan composite. In this study, the experiments were divided into two parts. Firstly, the effects of Ag nanoparticle on electrochemical responses were studied. It was revealed that Ag nanoparticle enhanced the biosensor response and helped enzyme dispersion. However, too concentrated of Ag nanoparticles resulted in limited substrate or product mass transfer and led to lower responses. Secondly, effects of glutaraldehyde cross-linking methods on response currents and repeatabilities were determined and compared. It was found that the response currents of electrode A, B, C, D and E were 7.9×10^{-6}, 0.66×10^{-6}, 3.3×10^{-6}, 1.2×10^{-6} and 4.3×10^{-6} A respectively. The response current was found to decrease when using glutaraldehyde sine glutaraldehyde could cause enzyme inactivation. But the repeatability was found to be improved by cross-linking with saturated glutaraldehyde vapor.

Department : Chemical Engineering..... Student's Signature : .....
Field of Study : Chemical Engineering... Advisor's Signature : .....
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CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

1.1 Motivation

Phenolic compounds are byproducts of large-scale production such as coke ovens, petrochemical production and plastic industry. They often exist in wastewaters. Due to their toxicity, they constitute an acute environmental problem. Therefore, the control of these pollutants is important as shown by several European and EPA directives (Cosnier et al., 1999). Several techniques are used for determination of phenols, such as chromatography, spectrophotometry and amperometric biosensor (Li et al., 2006). Among these techniques, amperometric biosensor gives the highest sensitivity. Moreover, it gives fast responses at low costs.

The immobilization of enzyme in the fabrication of amperometric biosensors is a crucial step because it plays an important role for long term stability of sensors. Poor stability of biosensors is mainly caused by enzyme inactivation and leaching from immobile materials. Chitosan is widely used as matrices for enzyme immobilization due to its excellent properties such as biocompatibility and nontoxicity. However, there still exists enzyme leaching. There are several researches who involved improving the property of chitosan matrices to overcome this problem. Some researchers cross-linked chitosan and enzyme using glutaraldehyde (Hung et al., 2003) which established intermolecular cross-links with the amino groups of chitosan and enzyme. However, glutaraldehyde was found to cause enzyme inactivation. Therefore, combination of chitosan with other material or metal nanoparticles that have good interaction with the protein have been studied (Xu et al., 2004). Moreover, metal nanoparticles enhance the electron conductivity of chitosan.

In this work, we are interested in immobilization of horseradish peroxidase (HRP, EC 1.11.1.7) in Ag-chitosan composites and apply for phenol biosensors. In order to

prevent enzyme leaching while keeping high enzyme activity, five different methods of enzyme immobilization will be investigated and compared: (A) HRP entrapment in Ag nanoparticle/chitosan composite; (B) HRP entrapment in glutaraldehyde solution/Ag nanoparticle/chitosan composite; (C) HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with glutaraldehyde solution; (D) HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with saturated glutaraldehyde vapor and (E) HRP adsorption in glutaraldehyde solution/Ag nanoparticle/chitosan composite.

1.2 Objectives

To investigate effects of glutaraldehyde cross-linking methods on HRP immobilization in Ag nanoparticle/chitosan matrices for biosensor applications.

1.3 Working scopes

1.3.1 Determine optimum conditions for fabricating five different modified electrodes based on reduction current by using an amperometric method using platinum wire as a counter electrode and silver/silver chloride (Ag/AgCl) as a reference electrode. The operating conditions for biosensors are a recorded at scan rate of 50 mV/s, and the applied potential are ranged between -500 mV and 500 mV.

For five different working electrodes, chitosan and HRP concentrations will be fixed at 0.5 %w/v and 10 mg/mL respectively.

(A) HRP entrapment in Ag nanoparticle (20-1000 ppm)/chitosan composite.

(B) HRP entrapment in glutaraldehyde solution/Ag nanoparticle (optimum concentration from type A)/chitosan composite.

(C) HRP entrapment in Ag nanoparticle (optimum concentration from type A)/chitosan composite and cross-linked with glutaraldehyde solution (0.0025-0.1% (v/v)).

(D) HRP entrapment in Ag nanoparticle (optimum concentration from type A)/chitosan composite and cross-linked with saturated glutaraldehyde vapor.
(E) HRP adsorption in glutaraldehyde solution(0.0025-0.1% (v/v)/Ag nanoparticle (optimum concentration from type A)/chitosan composite.

1.3.2 Investigate electrode response characteristics by using an amperometric method.

- Repeatability
- Stability
- Sensitivity
- Linear range
- Response time

1.3.3 Characterize optimum immobilization matrix for each working electrode by SEM to study and FTIR for identifying the chemical structure of modified electrode.

1.4 Expected benefits

To define the best method for the immobilization of HRP in the Ag-chitosan composites that exhibits good reusability for amperometric phenol biosensor.

CHAPTER II

BACKGROUND & LITERATURE REVIEWS

This chapter describes the basic background of biosensor such as measurement method and other key operating parameters. The enzymatic reactions of HRP and method of enzyme immobilization including literature reviews about material for enzyme immobilization on biosensor are also included.

2.1 Biosensor

A biosensor is a device incorporating a biological sensing element connected to a transducer. The transducer converts an observed change (physical or chemical) into a measurable signal, an electronic signal whose magnitude is proportional to the concentration of specific chemical (Fig 2.1).

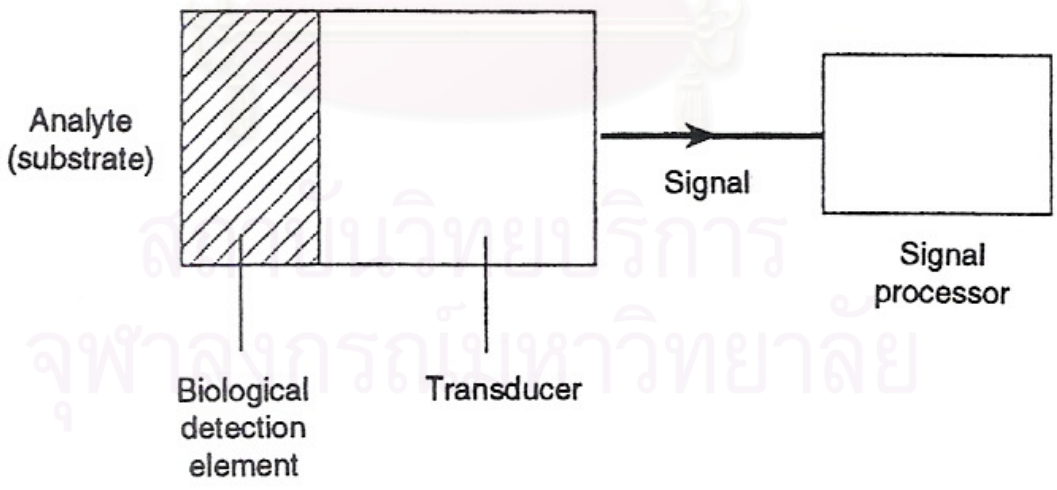


Figure 2.1 Schematic layout of a biosensor (Eggins, 1999).

Types of biosensors could be categorized depending on types of signal as indicated in table 2.1.

Table 2.1 Types of biosensor

Biological molecule	**Indicated Signal**	**Measuring system**	**Transducer**
Enzyme	Electron	Amperometric	Electrode
Microorganism or Cell organelles	Ion	Potentiometric	ISE, GSE
	Gas	Potentiometric	ISFET
Animal or plant tissue	Thermal	Calorimetric	Thermister
	Light	Optical	Optoelectronics
Antigen/Antibody	mass	Mass change	Piezoelectric crystal

ISE = ion selective electrode, GSE = gas sensing electrode,
ISFET = Ion selective field effect transistor

The complete biosensor should be cheap, small, portable and capable of being used by semi-skilled operators. There are clearly purposes developing a biosensor factors such as high sensitivity, long term stability.

2.1.1 The transducer

The transducer converts a chemical change into an electronic signal whose magnitude is proportional to the concentration of phenol. Some of electrochemical transducer are enzymatic based which involves redox reactions. The reaction is usually an oxidation or a reduction. There are many devices with different methods for determining the concentration of the sample. In this section, three devices are introduced as follows (Ngamukot, 2005)

(a) ***Potentiometry***

Potentiometry is a technique that measures the potentials of electrochemical cells under the condition of a little or no current flow. Potentiometric method requires a working electrode, a reference electrode and a device for measuring the potentials. The potential that develops in the electrochemical cells is a result of free energy change that would occur if the equilibrium condition of a chemical reaction has been satisfied. The potential varies with the concentration of the analyte. Potentiometry is widely used in analytical technique because of its simplicity, versatility and low cost.

(b) ***Voltammetry***

Voltammetry is one of the electroanalytical techniques that measures a current as a function of potential. The difference between potentiometry and voltammetry is that the latter comprises of three electrodes: working electrode (WE), reference electrode (RE) and counter electrode (CE) (Fig 2.2). The potential is applied to the working electrode as a function of time, and then the current is measure as potential varied. The signal in the form of current as a function of potential is called voltammogram.

Cyclic voltammetry

Cyclic voltammetry (CV) is an excellent technique for the study of electroactive species. The potential is applied to the electrode in a triangular waveform and the current at the working electrode is measured. The controlling potential is applied to the working electrode and after it reached the pre-determined value, the potential is scanned in reverse, causing a negative scan back to the original potential and thus the cycle is made. Single or multiple cycles can be used on the same surface. The forward scan (in the negative direction) produces the current peak for any analytes that can be reduced. The current will increase to the maximum value at the reduction potential and decrease as the concentration of the analyte near the electrode surface is depleted. Then the applied potential is reversed (in the positive direction), causing the maximum value in the reverse manner from the forward scan at the oxidation potential. A cyclic voltammogram is the

plot of the response current at the working electrode vs. applied excitation potential. As a result, the plot gives information about the redox potential and electrochemical reaction rate. The current-potential (i-E) plot or a cyclic voltammogram (Fig 2.2) shows four main parameters, the cathodic peak potential (E_{pc}), the anodic peak potential (E_{pa}), the cathodic peak current (i_{pc}) or reduction current, and anodic peak current (i_{pa}) or oxidation current. In this case, redox peak of HRP occurred which the anodic peak current or oxidation current occurred when the potential is scanned positively, which came from the oxidation of HRP by H_2O_2. The cathodic peak current or reduction current occurred when the potential is scanned negatively, which came from the reduction of phenol by electrode.

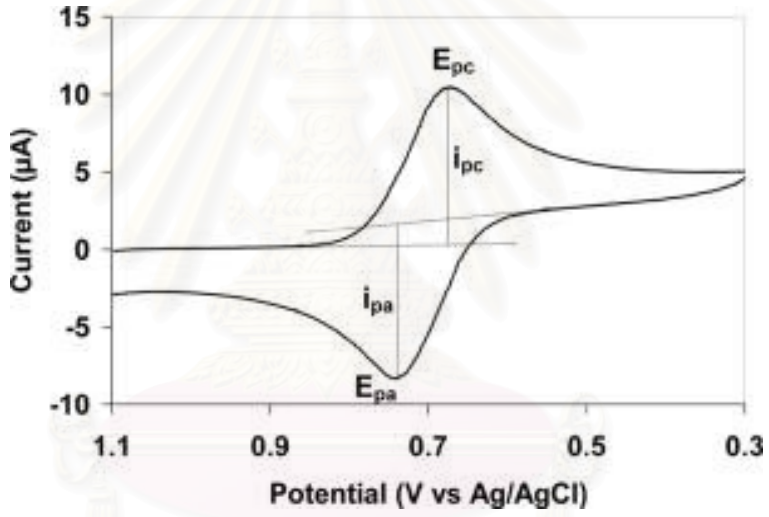


Figure 2.2 Cyclic voltammogram with four main parameters (http://upload.wikimedia.org/wikipedia/en/4/4c/Cyclovoltammogram.jpg)

(c) ***Amperometry***

In this technique, a constant potential is applied at the working electrode. At the potential applied, the analytes go through an oxidation or reduction at the electrode. The current responses are directly proportion to the concentration of the analytes. The disadvantage of amperometry is the lack of reproducibility due to the deposition of impurities on the electrode surface. To obtain reproducible results, the electrode surface

must be cleaned regularly either by polishing or performing electrochemical process. A simple current-time waveform for amperometry is shown in Fig 2.3.

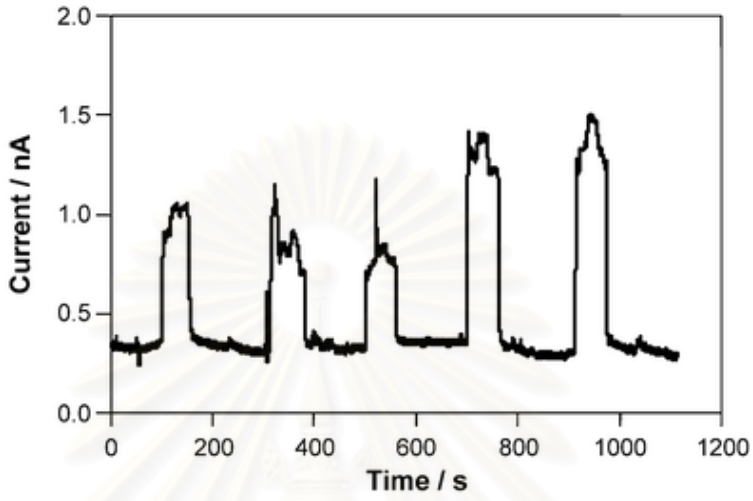


Figure 2.3 Current-time for constant potential amperometry (www.rsc.org/ej/AN/2007/b611920d/b611920d-f5.gif)

2.1.2 Performance Factors

Biosensor can be considered as a new technique in analytical field. Thus, methods for determining the performance of biosensor and factors involved are necessary. Five important factors are defined to use in measuring of biosensor's performance. (Cooper and Anthony, 2004)

(i) *Selectivity* – a range of chemical substances that responses to the sensor. Broad range of substances refers to low selectivity sensor and narrow range is vice versa.

(ii) *Range and Linear range* – the concentration range of substances that can be measured. The lowest measurable concentration is called a detection limit, which is normally more than 10^{-5} M (0.01 mM). The detection limit can be found by plotting the relationship between electrical potential and analyte's

concentration. Then, extending a liner portion of the graph to intersect the baseline. The intersection between these two lines is a detection limit as shown in Fig 2.4.

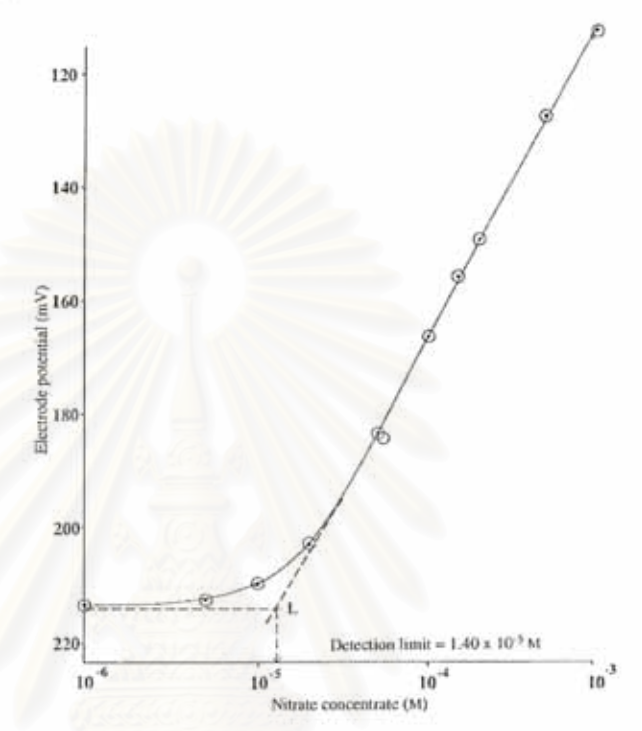


Figure 2.4 Method of determining the detection limit
(Cooper and Anthony, 2004)

(iii) *Reproducibility* – the ability of the biosensors to give a similar outcome for the repeating experiment. The result is meaningless if the error of the experiment cannot be defined. Repetition of numbers of experiment must be performed followed by standard deviation in order to compare the result from new experiment to the standard value. The expected reproducibility of the biosensor for the repeated experiment should be within ±5 to 10 %.

(iv) *Response time* – the amount of time required for the system to approach equilibrium. This response time can be varied for each biosensor; however, the typical value is less than 5-10 minutes.

(v) *Life time* – the duration that the biological component (or enzyme) on the biosensor can perform reaction and yield reasonable response before it is deteriorate or lost it activity. There are three aspects of lifetime related to biosensor; the lifetime of the biosensor in use, the lifetime of the biosensor in storage and the lifetime of the biological material stored separately.

2.2 Amperometric biosensors for phenol detection

An amperometric biosensor is an analytical device used in order to determine the concentration of analyst which converts an electron transfer as a biological response into an electrical signal (Fig. 2.5). The biological response of the biosensor is determined by the biocatalytic membrane which accomplishes the conversion of reactant to product. Immobilised enzymes possess a number of advantageous features which makes them particularly applicable for use in such systems. They may be re-used, which ensures that the same catalytic activity is present for a series of analyses. This is an important factor in securing reproducible results and avoids the pitfalls associated with the replicate pipetting of free enzyme otherwise necessary in analytical protocols. Many enzymes are intrinsically stabilized by the immobilization process, but even where this does not occur there is usually considerable apparent stabilization.

Figure 2.5 Schematic diagram of an amperometric biosensor

2.2.1 The biological substance for phenol detection

Over the past few decades, amperometric biosensors modified with tirosinase, horseradish peroxidase or laccase have been developed for phenol detection. Among all, it was found that horseradish peroxidase based biosensor are the most sensitive for a great number of phenol compounds due to its ability of electron donating for peroxides (Marko-Varga et al., 1995). Morever, it shows a high stability (Korbut et al., 2008).

Horseradish peroxidase (HRP)

HRP is one of many enzymes used in detection of phenol that can be used as electron donating compound in the reaction of horseradish peroxidase with peroxide (Ruzgas et al., 1995).

$$HRP(Fe^{3+}) + H_2O_2 \longrightarrow HRP(Fe^{5+}) + H_2O \quad 2.1$$

$$HRP(Fe^{5+}) + AH_2 \longrightarrow HRP(Fe^{4+}) + AH^{*} \quad 2.2$$

$$HRP(Fe^{4+}) + AH_2 \longrightarrow HRP(Fe^{3+}) + AH^{*} + H_2O \quad 2.3$$

According to the above reactions and Fig 2.6, it starts with oxidation of peroxidase by hydrogen peroxide and formation of an intermediate compound, HRP (Fe^{5+}) (Equation 2.1). Then the oxidized enzyme is reduced to its native form in the two following steps (Equation 2.3 and 2.4), involving two kinetically distinct enzyme intermediates, the HRP(Fe^{5+}) and HRP(Fe^{4+}) state. In each step, the electron donor species (AH_2) such as phenols is oxidized and converted to free radicals (AH^{*}) (Ruzgas et al., 1995; Ruzgas et al., 1996). These free radicals are electroactive and can be reduced on electrode surface (Rosatto et al., 1999). Therefore, the reduction current is proportional to the phenol concentration in the solution.

However, peroxidase can also do the direct electron transfer between enzyme itself and electrode (Fig 2.7). This leads to the unnecessary of the electron mediator (such as phenol) for electron transfer (Gorton et al., 1992). Thus, this phenomenon can limit the

sensitivity of the biosensor for monitoring phenol due to the background current of the direct electron transfer from peroxide.

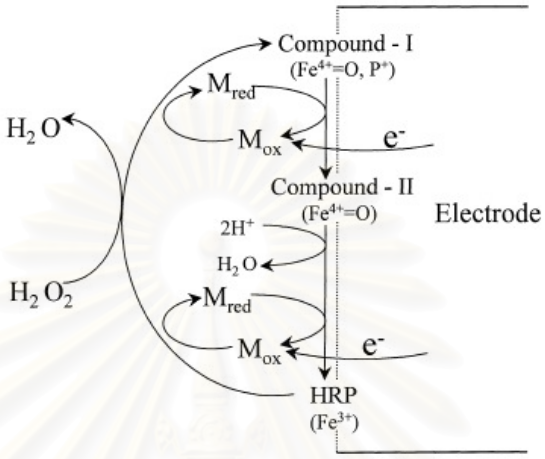


Figure 2.6 Mechanism of mediated electron transfer at HRP modified electrode. M_{ox} and M_{red} are the oxidised and reduced forms of the mediator respectively (Rosatto et al., 1999)

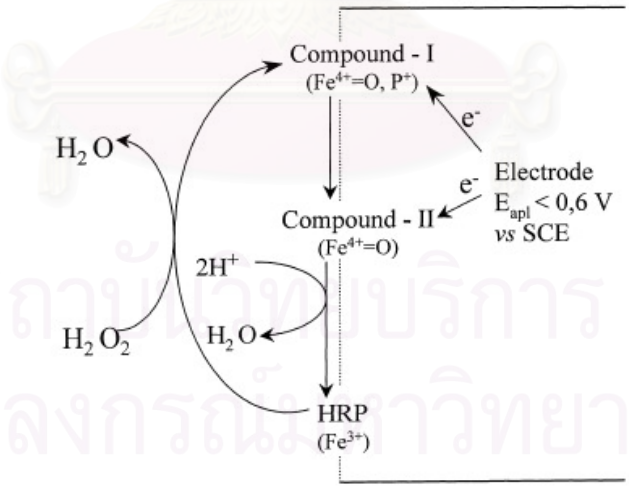


Figure 2.7 Mechanism of the direct electron transfer between HRP and base electrode (Rosatto et al., 1999)

Tyrosinase

Tyrosinase, also known as polyphenol oxidase (PPO), is a widely used enzyme for phenol detection biosensor. Its important function is the ability to catalyze the oxidation of phenolic compounds (i.e. catechol) to the corresponding quinones (*o*-quinone) in the present of oxygen (Stephanie, 1994; Li et al., 1998) (Equation 2.4-2.6) (Fig. 2.8). This liberated quinone species can be electrochemically reduced to phenolic substances and the reduction signal is measured, usually at low potential, without any mediator (Li et al., 1998). The enzymatic reactions are presented by the following scheme (Duran and Esposito, 2000; Liu et al., 2000):

$$\text{Phenol} + \text{tyrosinase } (O_2) \longrightarrow \text{catechol} \qquad 2.4$$

$$\text{Catechol} + \text{tyrosinase } (O_2) \longrightarrow o\text{-quinone} + H_2O \qquad 2.5$$

$$o\text{-quinone} + 2H^+ + 2e^- \longrightarrow \text{catechol (at electrode)} \qquad 2.6$$

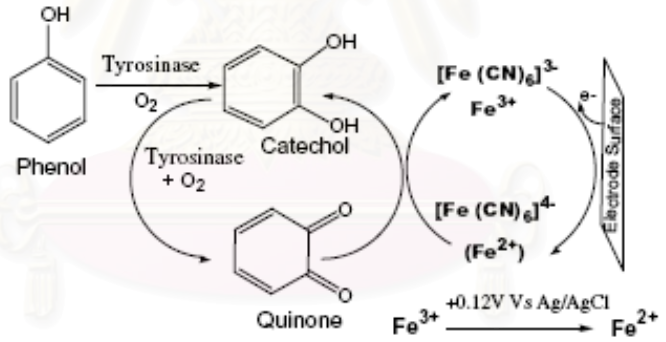


Figure 2.8 Mechanism of tyrosinase enzymatic reaction (Rajesh et al., 2005)

2.3 Enzyme immobilization

Enzymes are protein molecules, which serve to accelerate the chemical reactions. Without enzymes, most biochemical reactions would be too slow to even carry out life processes. Enzymes display great specificity and are not permanently modified by their participation in reactions. Since they are not changed during the reactions, it is cost-effective to use them more than once. However, if the enzymes are in solution with the reactants and/or products it is difficult to separate them. Therefore, if they can be attached to the reactor in some way, they can be used again after the products have been removed. The term "immobilized" means unable to move or stationary. And that is exactly what an immobilized enzyme is: an enzyme that is physically attached to a solid support over which a substrate is passed and converted to product.

The biological component has to be intimately attached to the transducer. There are five regular methods of doing this, as follows (Eggins, 1999).

(i) *Adsorption* – the enzyme can be adsorbed to some substances without any reagents required.

(ii) *Microencapsulation* – the biomaterial is trapped behind a membrane, giving close contact between the biomaterial and the transducer.

(iii) *Entrapment* – the biological component is blocked in a matrix of a gel or a paste or a polymer.

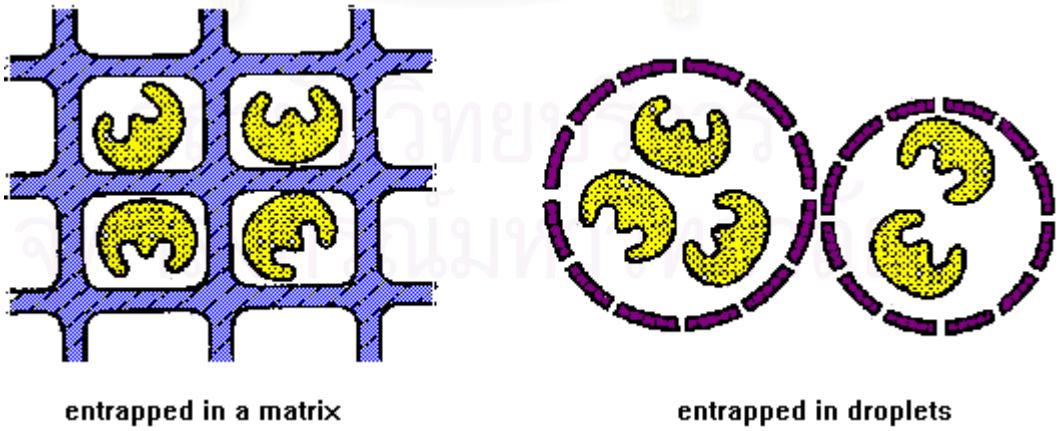


Figure 2.9 Entrapping Enzyme

http://www.rpi.edu/dept/chem-eng/Biotech-Environ/IMMOB/Immob.htm

(iv) *Cross-linking* – the biological material is chemically bonded to the solid support by using a bifunctional agent.

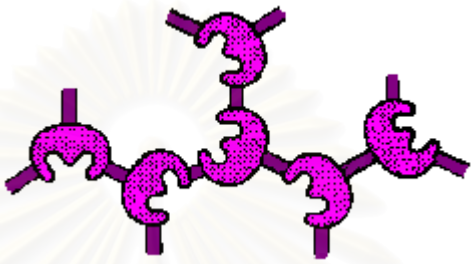


Figure 2.10 Cross-linking enzyme

http://www.rpi.edu/dept/chem-eng/Biotech-Environ/IMMOB/Immob.htm

(v) *Covalent binding* – a functional group of the biological component is covalently bonded to the support matrix. The functional groups that may take part in this binding are listed below:

Amino group	Carboxyl group	Sulfhydryl group,
Hydroxyl group	Imidazole group	Phenolic group
Thiol group	Threonine group	Indole group

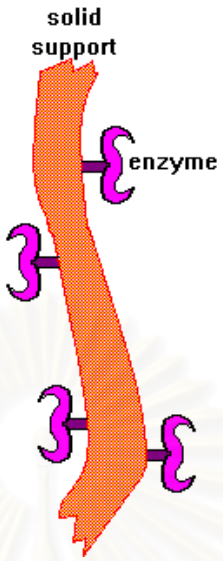


Figure 2.11 Covalent binding enzyme
http://www.rpi.edu/dept/chem-eng/Biotech-Environ/IMMOB/Immob.htm

2.4 Enzyme Kinetics

An enzyme is a large, complex macromolecule, consisting mostly of protein. Particularly for the enzyme used in biosensor, the mode of action involves oxidation or reduction which can be detected electrochemically.

The basic enzyme catalysis mechanism:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad 2.7$$

Where S = substrate(s)
E = enzyme
ES = enzyme-substrate complex
P = product(s)

Apply the steady-state approximation of kinetic theory to the system shown in equation 2.7; thus,

Rate of formation of complex = $k_1[S][E] - k_{-1}[ES]$

Rate of breakdown of complex = $k_2[ES]$

This approximation assumes that the concentration of the enzyme-substrate complex is usually steady or constant, so the rate of formation of the complex is balanced by the rate of its breakdown back to enzyme and forward to products. Therefore,

$$k_1[S][E] - k_{-1}[ES] - k_2[ES] = 0$$

The enzyme concentration is usually described in terms of the total quantity: $[E_0]$ rather than the unknown amount: [E], so that $[E_0] = [E] + [ES]$. Then,

$$k_1[S][E_0] - k_1[S][ES] - k_{-1}[ES] - k_2[ES] = 0$$

Hence,

$$[ES] = \frac{k_1[E_0][S]}{k_{-1} + k_2 + k_1[S]}$$

$$\therefore [ES] = \frac{[E_0][S]}{K_M + [S]}$$

Where $K_M = (k_{-1}+k_2)/k_1 =$ *Michaelis constant*

Thus, the overall rate of reaction (rate of formation of products) is given by the *Michaelis-Menton equation*:

$$v = \frac{d[P]}{dt} = -\frac{d[S]}{dt} = k_2[ES] = \frac{k_2[E_0][S]}{K_M + [S]} \qquad 2.8$$

When $[S] >> K_M$, a maximum rate constant: V_{max} is reached and $V_{max} = k_2[E_0]$. On the other hand, when $[S] << K_M$, $v = V_{max}/2$. Figure 2.12 shows this relation in a curve. However, it is experimentally more convenient to plot the data in straight-line form which can be done by inverting the Michaelis-Menton equation:

$$\frac{1}{v} = \frac{K_M + [S]}{k_2[E_0][S]} = \frac{K_M}{k_2[E_0][S]} + \frac{1}{V_{max}} \qquad 2.9$$

When $1/v$ is plotted against 1/[S], it will obtain a straight line with a slope of K_M/V_{max} and an intercept of $1/V_{max}$; hence, both K_M and V_{max} can be solved. This is called the *Lineweaver-Burk plot*.

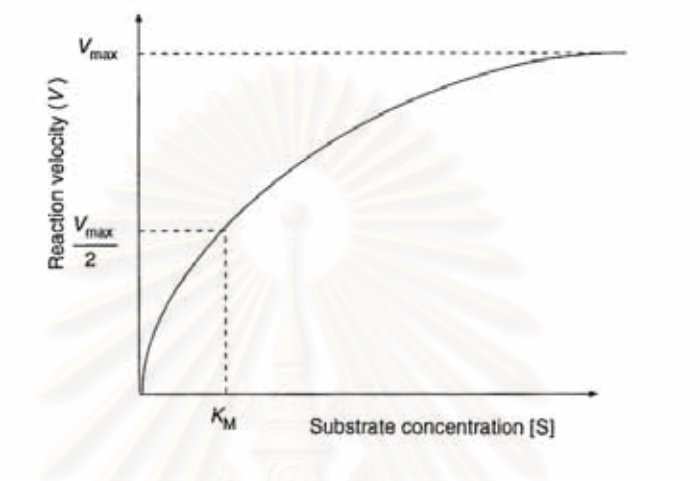


Figure 2.12 Dependence of reaction rate on substrate concentration for an enzyme-catalyzed reaction at constant enzyme concentration (Eggins, 1999)

2.5 Biosensor performances reviews of various material for enzyme immobilization

The immobilization of enzyme is a crucial step in the fabrication of phenol biosensor. Numerous immobilization methods have been developed to stabilize some enzymes in the electrodes. Recently, materials such as polyvinyl alcohol (PVA), sol gel, conducting polymer, nafion, chitosan and glutaraldehyde have been proven to be promising as the immobilization matrices. Advantage and disadvantage of these materials were summarized in Table 2.2, including their performances. There had been modification of each material with biocompatible materials which can help improve the stability and bioactivity of an enzyme-based biosensor. They could retain the activity of enzyme well because of their desirable microenvironment and enhance the direct electron transfer between the enzyme's active sites and the electrode.

By an electrochemical doping technique, there have been some researches on electropolymerized conducting polymers such as polyaniline and polypyrrole (Mu et al.,1991; Xue and Mu, 1995; Mu and Xue, 1996; Gerard et al.,1999; Cosnier, 1999; Chaubey et al., 2000); however, they showed a large reduction background current at working potential values < 100 mV (vs. SCE) and were not able to be applied directly to mostly negative potential amperometric biosensors (Xue and Shen, 2002). Later, Xue and Shen (2002) constructed a biosensor using polyaniline-polyacrylonitrile (Pan-PAN) composite matrix as an enzyme immobilization support to solve the above problems.

Recently, the sol-gel technique is widely studied. Silica sol-gel immobilized amperometric biosensor was developed by Li et al. (1998). In addition, Al_2O_3 sol-gel has been found to be a suitable matrix for improving the stability of the immobilization of enzyme (Liu et al., 2000a; Liu et al., 2000b). Still, there are some problems of the shrinkage and cracking of sol-gel matrix. In order to prevent these difficulties, some polymers such as poly(ethyleneoxide) (Nakanisi and Soga, 1997), poly(ethyleneglycol) (Zi, 1999), polyhydroxyl (Gill and Ballesteros, 1998), natural-polymer chitosan (Miao and Tan, 2001), and a grafting copolymer of poly(vinylpyridine) and poly(vinyl alcohol) (Wang et al., 1998; Wang et al., 2000; Wang and Dong, 2000) were mixed with sol-gel derived silicate matrix to form organic-inorganic hybrid materials. Moreover, to retard the shrinkage and overcome the brittleness of the pure sol-gel derived silicate matrix, the use of a sol-gel derived silicate/Nafion (a perfluorosulfonated ionomer) composite film was reported (Kim and Lee, 2003). The latest technique which can avoid the evaporation of sol-gel component; thus, can solve the problem of matrix shrinkage was proposed. It was called Sonogel-Carbon electrodes (Kaoutit et al., 2007).

Some of the methods mentioned above are complicated, since they are multi steps and time consuming process, while others are simple and sensitive. Nevertheless, there are still many problems affecting practical application of the biosensor. Therefore, the search for a simple and reliable method to immobilize enzyme for biosensor application is still of interests and development of good immobilization method and materials to improve the biosensor stability is very signicant.

Table 2.2 Biosensor performances reviews of various materials for enzyme immobilization

Modified electrode		Analytical performances				
		Detection Limit (mol L^{-1})	Linear range (mol L^{-1})	Sensitivity	Stability	Response time (s)
Poly(vinyl alcohol)(PVA) ***Advantages*[Kim et al.,2006]** 1. Biocompatibility 2. Non toxicity 3. Low price 4. Easy availability ***Disadvantage***[Kim et al.,2006] 1. Mass transfer limitation and less phenol reach the electrode surface. 2. PVA has high swelling index so has weak mechanical strength and is dissolved readily in water.	PVA- regenerated silk fibroin (RSF)-methylene blue/HRP/H_2O_2 [Liu et al.,1997]	5×10^{-6}	1×10^{-5} to 1.5×10^{-3}	-	Retained 89.7% after 60 days	<50 s
	PVA-Mesoporous Silica/Tyrosinase-HRP/Phenol [Dai et al.,2004]	4.1×10^{-9}	2×10^{-7} to 2.3×10^{-4}	14×10^{-12} (A M^{-1} cm^{-2})	Retained 93% after 60 days	<10 s
	PVA-Glutaraldehyde/Tyrosinase/Phenol [Kim et al.,2006]	-	0.5×10^{-6} to 1×10^{-4}	130.56×10^{-3} (A M^{-1})	7 days	-
Sol-gel ***Advantages*** 1. The porous sol-gel matrix have physical rigidity, chemical inertness. 2. Inexpensive	Silica sol gel/Tyr/Phenol [Wang et al.,2000]	0.1×10^{-6}	2.0×10^{-7} to 1.6×10^{-4}	23.1×10^{-3} (A M^{-1})	Retained 73% after 21 days	17 s
	Alumina sol gel/Tyr/Phenol [Liu et al.,2000]	0.2×10^{-9}	1.5×10^{-9} to 3.5×10^{-4}	127×10^{-3} (A M^{-1})	Retained 70% after	4 s

3. Excellent matrix for the immobilization. ***Disadvantages*** 1. Sol-gel matrix the shrinkage and cracking.					90 days	
	SiO_2 sol gel/HRP/Phenol [Rosatto et al.,2002]	0.5×10^{-6}	5×10^{-6} to 25×10^{-6}	32×10^{-3} ($A\ M^{-1}$)	240 days	-
	ZnO sol gel/Try/Catechol [Liu et al.,2005]	50×10^{-9}	1×10^{-7} to 5×10^{-5}	166 $\times 10^{-3}$($A\ M^{-1}$)	Retained 75% after 14 days	15 s
Conducting polymer ***Advantages*** 1. Easy deposition on electrode 2. Easy to control of thickness by deposition charge. 3. biocompatibility ***Disadvantages*** 1. Some is a carcinogenic monomer 2. The inherent thickness of film inhibited its potential.[Morrin et al.,2005]	Polyaniline-polyacrylonitrile/Try/Phenol [Xue et al.,2002]	-	1×10^{-7} to 7.5×10^{-5}	0.96($A\ M^{-1}\ cm^{-2}$)	320 days	-
	p-toluene sulfonate-doped polypyrrole/Tyr/Phenol [Rajesh et al.,2004]	0.8×10^{-6}	3.3×10^{-6} to 2.2×10^{-4}	24.3×10^{-3} ($A\ M^{-1}$)	90 days	40 s
Nafion ***Disadvantages*** 1. The rate of charge transfer was slow [Pan et al.,2007].	Nafion-Multiwalled carbon nanotube/Tyr/Phenol[Tsai et al.,2007]	0.13×10^{-6}	1×10^{-6} to 1.9×10^{-5}	303×10^{-3} ($A\ M^{-1}$)	-	< 8 s

Chitosan ***Advantages***[Lei et al.,2002, Wang et al.,2008] 1. Biocompatibility 2. Low toxicity 3. High permeability 4. Inexpensive 5. High mechanical strength 7. Excellent membrane-forming ability 8. Large microscopic surface area and porous morphology of chitosan matrix lead to high enzyme loading. ***Disadvantages*** 1.Cracking	Chitosan-Nano Au/HRP/H_2O_2 [Lei et al.,2002]	6.3×10^{-6}	1.22×10^{-5} to 2.43×10^{-3}	0.013 ($A\ M^{-1}\ cm^{-2}$)	Retained 60% after 30 days	-
	Chitosan-Nano ZnO/Tyr/Phenol [Li et al.,2005]	5×10^{-8}	15×10^{-8} to 6.5×10^{-5}	182×10^{-3} ($A\ M^{-1}$)	Retained 91% after 20 days	10 s
	Chitosan-Laponite clay/Tyr/Phenol [Fan et al.,2006]	1.1×10^{-8}	1.1×10^{-8} to 4.0×10^{-5}	284 ± 2(mA M^{-1} cm^{-2})	Retained 55% after 75 days	30 s
	Chitosan- nano Fe_3O_4/Tyr/Phenol [Wang et al.,2008]	2.5×10^{-8}	8.3×10^{-8} to 8.3×10^{-5}	0.225 ($A\ M^{-1}$)	Retained 53% after 60 days	-
Glutaraldehyde ***Disadvantages***[Kim et al.,2006] 1. GA is toxic for some enzyme.	Silica-titanium/HRP/Phenol [Rosatto et al.,1999]	-	10×10^{-6} to 50×10^{-6}	-	Retained 53% after 60 days	3 s
	Nano $CaCO_3$/Tyr/Phenol [Shan et al.,2007]	0.62×10^{-9}	1×10^{-8} to 1×10^{-5}	336×10^{-3} ($A\ M^{-1}$)	Retained 70% after 56 days	<12 s

2.6 Reviews of chitosan on biosensor

The enzyme peroxidase has been covalently bonded to a wide variety of water-insoluble supports. Chitosan a natural-polymer, is obtained by the alkaline deacetylation of chitin which composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) (Fig 2.13). Both biopolymers are chemically similar to cellulose, differing only in the functional group situated at carbon-2 of the monomeric unit. The presence of free amine groups in chitosan enhances the greater solubility and reactivity than that of chitin and cellulose. In addition, this biopolymer is susceptible to chemical modification due to the amine and hydroxyl groups that facilitate the covalent binding of enzyme.

Chitosan, its pKa is about 6.3. At lower pH solutions (<pKa), most of the amino groups are protonated, making chitosan a water-soluble polyelectrolyte. When the pH is higher than pKa, the amino groups are deprotonated, and chitosan becomes insoluble.

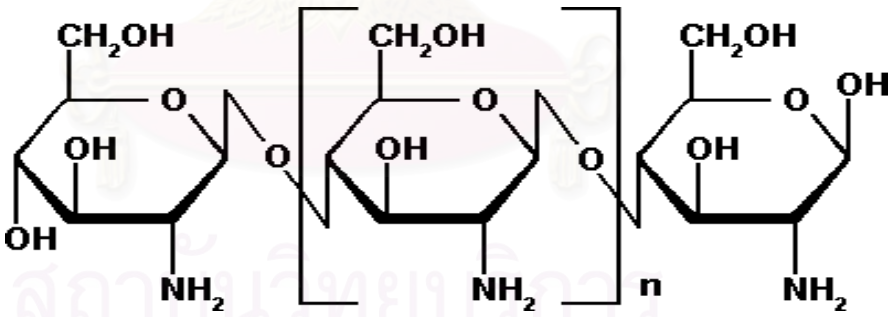


Figure 2.13 Structure of chitosan (http://en.wikipedia.org/wiki/Chitosan)

Recently chitosan has attracted considerable attention and has been used as a support for enzyme because of excellent film-forming ability, high permeability, nontoxicity, biocompatibility, low cost and easy availability. Enzymes immobilized in these polymers possess an increased stability and are more temperature resistant. Immobilizing enzyme is often achieved using the crosslinking agent such as glutaraldehyde, although the enzymes have also been entrapped in chitosan to provide

a high enzyme loading. During cross-linking, the aldehyde group of glutaraldehyde reacts with amino groups of chitosan and the enzyme molecule to form covalent linkages (Fig 2.14-2.15). However, certain amount of glutaraldehyde causes biocatalyst inactivation (Wilson et al., 2008). Wang et al., (2002) studied immobilization of tyrosinase in cross-linked chitosan with (3-aminooryloxypropyl) dimethoxymethylsilane. The large microscopic surface area and porous morphology of chitosan matrix led to high enzyme loading, and retaining its bioactivity. In the study of Fernandes et al., (2008), immobilized laccase on microspheres of chitosan crosslinked with tripolyphosphate (TPP) was investigated. The TPP can form crosslinking by ionic interaction between positively charged amino ($–NH_3^+$) groups of chitosan and negatively charged TPP molecules in acid medium. This biosensor showed excellent long-term stability. Hung et al., (2003) studied the immobilization of lipase in chitosan beads by activating its hydroxyl groups with carbodiimide followed by cross-linking more lipase to the amino groups with glutaraldehyde. Thermal and pH stabilities and reusability of the lipase increased considerably by the binary method of immobilization. Besides, Oliveira et al.,2006 have introduced a novel procedure to successfully immobilize peroxidase on the chitosan by the bifunctional glutaraldehyde reacts with the amine groups of chitosan to form Schiff bases, and with the addition of the epichlorohydrin, through the opening of the epoxide ring links are formed with carbon atoms and the chloride group is discharged. In addition, the chemical crosslinking of chitosan with glutaraldehyde/epichlorohydrin and peroxidase immobilization showed strong interactions, long-term stability, thermostability and high sensitivity compared with other recently constructed biosensors.

However, these methods require the use of solvent and cause enzyme inactivation. Therefore, the search for a simple and reliable method for enzyme immobilize enzyme is interests. In recent years, there have been an introduction of a biocompatible nanomaterials in chitosan with electrostatic interactions which can help improve the stability and bioactivity of an enzyme based biosensor. Moreover, they improved dispersion of enzyme in chitosan. They could retain high activity of enzyme because of their desirable microenvironment and enhance the direct electron transfer between the enzyme's active sites and the electrode (Gorton et al., 1999; Jia et al.,

2002). For these purposes, ZnO nanoparticle were used by Khan et al. (2006). Also, clay was employed for the same objectives (Zhao et al., 2006). In addition, the use of $Fe_3O_4$was reported (Wang et al.,2008).

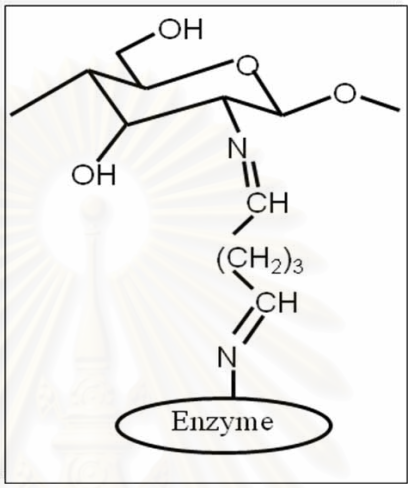


Figure 2.14 Structure of chitosan-enzyme cross-linking with glutaraldehyde

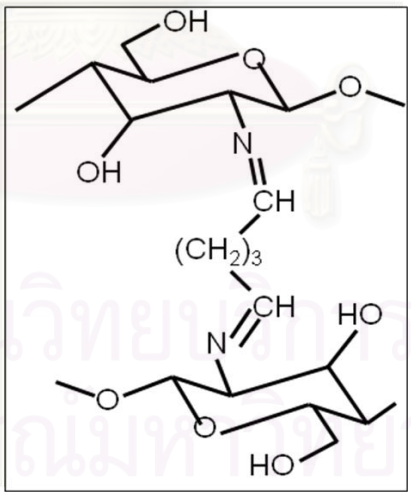


Figure 2.15 Structure of chitosan cross-linking with glutaraldehyde

2.7 Reviews of Metal Nanoparticle on biosensor

Various metals such as silver (Ag), gold (Au), platinum (Pt), and palladium (Pd) have been studied as additives for enzyme immobilization. These metal nanoparticles have good interaction with the protein. Among these metals, gold nanoparticles (AuNPs) have excellent conductivity and biocompatibility. They are suitable for acting as "electronic wires" to enhance the electron transfer between redox centers in enzymes and electrode surfaces, and as catalysts to increase electrochemical reactions (Zhao et al., 2006). However, AuNPs are inherently instable and expensive.

Silver nanoparticles have advantages to enhance the electron conductivity of biosensor and the other can adsorb the protein by the interaction between the enzyme. Several methods have currently been applied to synthesize silver nano-particles, such as physical processes of atomization or milling, chemical methods of thermal decomposition, chemical reduction, sol–gel, water-in-oil (W/O) microemulsions, or electrochemical processes. However, the method of chemical reduction from aqueous solutions is most preferable for obtaining nano-sized particles of silver. The essential feature of this chemical reduction method is to give a desirable particle shape and size at high yield and low preparation costs (Kim et al., 2004). By chemical reduction method

reducing agent such as $NaBH_4$ and etc. is added to $AgNO_3$ aqueous solution for oxidation and reduction. In the formation of silver nano-particles by chemical reduction method, several factors are important for preparation of nano-sized powder of silver. Properties of silver nano-particles obtained by chemical reduction method are affected by various parameters such as the molar concentration ratio of R ($[AgNO_3]$/[reducing agent]) value (Kim et al., 2004). To ensure the entire reduction, the concentration of $NaBH_4$ was 10 times that of metal salt (Huang et al., 2004).

$$4AgNO_3 + NaBH_4 \longrightarrow 4Ag + 4HNO_3 + NaB$$

2.8 Reviews in our previous work

Suksompong, 2006 immobilized horseradish peroxidase into the chitosan incorporated silver nanoparticles with entrapment method. The focus was given on the study of effects of horseradish peroxidase, chitosan, and silver nanoparticles concentrations on efficiency of immobilized enzyme based on reaction rate, maintenance and storage stability. In this study, the experiment was divided into two parts. First, the effect of pH of chitosan solution (4, 5 and 6), size of chitosan film (delicately cut , 0.3 x 0.3 and 0.5 x 0.5 cm2), and substrate concentrations (pyrogallol, 0.03 - 0.10 M and hydrogenperoxide, 0.10 - 0.60 M) were studied . The optimum conditions for enzyme activity were determined at pH 5 of chitosan solution, 0.5 x 0.5 cm2 of chitosan film size, 0.075 M pyrogallol , and 0.50 M hydrogenperoxide . Data from the first part were further applied to investigate with experimental design for optimum conditions of enzyme immobilization in chitosan incorporated silver nanoparticles. The silver nanopartilces , synthesized using reducing agents, had average size of 37 nm. The concentrations of enzyme solution (0.05, 0.10, and 0.15 mg/ml), chitosan solution (0.5, 1.0, and 1.5% w/v), and silver nanoparticles (0.4 x10-2, 0.8 x10-2, and 1.2 x10-2nM) were studied. The optimum conditions for enzyme reaction was found at 0.15 mg/ml of horseradish peroxidase, 0.5% w/v of chitosan, and 0.4 x10-2 nM of silver nanoparticles with the specific activity of 230 U/mg-enzyme. However, maintenance and storage stability of immobilized enzyme under this optimum condition was quite low. The residue activity of immobilized enzyme was 21.38 % after 3 cycles of operation. After storing the immobilized enzyme at 4 $^{\circ}$C and room temperature for 2 weeks, the residue activity were determined at 4.68 % and 6.44 %, respectively.

Recently, mesoporous silicas (MPSs) materials have been studied and found to be very interesting for immobilization matrices. They have pore diameters in the range of 2-50 nm which is suited for bioimmobilization. They have exhibited many good characteristics, such as order and uniformity, adjustable pore size, large surface area, chemical and mechanical stability, and resistance to microbial interferences. These materials are considered suitable for physical adsorption of molecules, especially through hydrogen bonding or covalent bonding. Moreover, they can be

used for reactive points for the attachment of functional groups (Chouyyok et al., 2007). Punwittayakool, 2008 choosed MCF which is mesoporous silica possesses a system of interconnected pores with diameters of 22-42 nm because of HRP immobilized in MCF was high activity and stable under storage (Chouyyok et al., 2008). The ultimate aim of this research was to investigate the immobilization of Horseradish peroxidase in mesoporous silica/silver nanoparticle/chitosan composite material using electrochemical method with glassy carbon electrode. In this study, the experiments were divided into three parts. Firstly, mesoporous silica type MCF was synthesized and silver nanoparticles were attached on MCF. The synthesized MCF has average pore size of 23.7 nm and total surface area of 629.97 m^2/g. Secondly, the effects of amount of mesoporous silica (0.1 – 1 % w/v), chitosan concentration (0.1 – 1 % w/v), and silver nanoparticle concentration (20 – 100 ppm) on electrochemical response were studied. It was revealed that additives such as MCF and Ag particles helped enzyme dispersion to a certain concentration, however, higher concentration of additives resulted in higher substrate/product mass transfer limitation. In addition, Ag particles were found to help enhancing electrical response. Optimal compositions of modified electrode were 20 ppm Ag solution, 0.5 %w/v chitosan, 0.7%w/v modified MCF and 10 mg/ml HRP. The optimal composition of enzyme immobilization was further applied to investigate the reusability of immobilized enzyme. The use of modified electrode was limited to only once. The main cause was probably enzyme leakage from chitosan matrix.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

1. Horseradish peroxidase (HRP, EC 1.11.1.7), available from Toyobo, Japan.
2. Chitosan (deacetylation degree 95 %, MW 1,450 kDa), available from Seafresh chitosan Co., Ltd., Thailand.
3. Acetic acid (CH_3 COOH), available from BDH laboratory supplies.
4. Silver nitrate ($AgNO_3$), available from Poch S.A.
5. Sodium Borohydride ($NaBH_4$), available from Ajax Fine Chem.
6. Sodium Hydroxide (NaOH), available from Ajax Fine Chem.
7. Potassium hydrogen phosphate (K_2 HPO_4), available from Ajax Fine Chem.
8. Potassium dihydrogen phosphate (KH_2 PO_4), available from Ajax Fine Chem.
9. Phenol (C_6 H_5 OH), available from Carlo Erba Regent Co.
10. Hydrogen peroxide (H_2 O_2) 30%, available from E.Merck, Darmstadt.
11. Glutaraldehyde (C_5 H_8 O_2), available from Sigma chemical Co.

* All chemicals were of analytical grade and used without further purification.

3.2 Methods

3.2.1 Synthesis of Ag nanoparticles (Punwittayakool, 2008)

The preparation of Ag solution was obtained by chemical reduction of metal salts to yield the corresponding zero valent metal nanoparticles with $NaBH_4$. 50 ml of $AgNO_3$ solution mixed with 50 ml of $NaBH_4$ which $NaBH_4$ concentration was 10 times of $AgNO_3$ concentration in flask 250 ml and was controlled at 70 °C under stirring for 90 mins. The mixtures changed to yellow. The obtained silver nanoparticle solution was then stored in dark brown bottle at 4°C before use.

3.2.2 Preparation of chitosan solution

In this study, chitosan 0.5 %w/v was prepared by dissolving 0.5 g of chitosan powder in 100 mL of acetic acid (1%, v/v). The viscous chitosan solution was stirred

by magnetic stirrer overnight at room temperature. Then, adjustment of chitosan solution to pH 5.0 with 0.1 M NaOH solution was then carried out. Chitosan solution was stored in bottle at 4˚C.

3.2.3 Fabrication of enzyme electrode

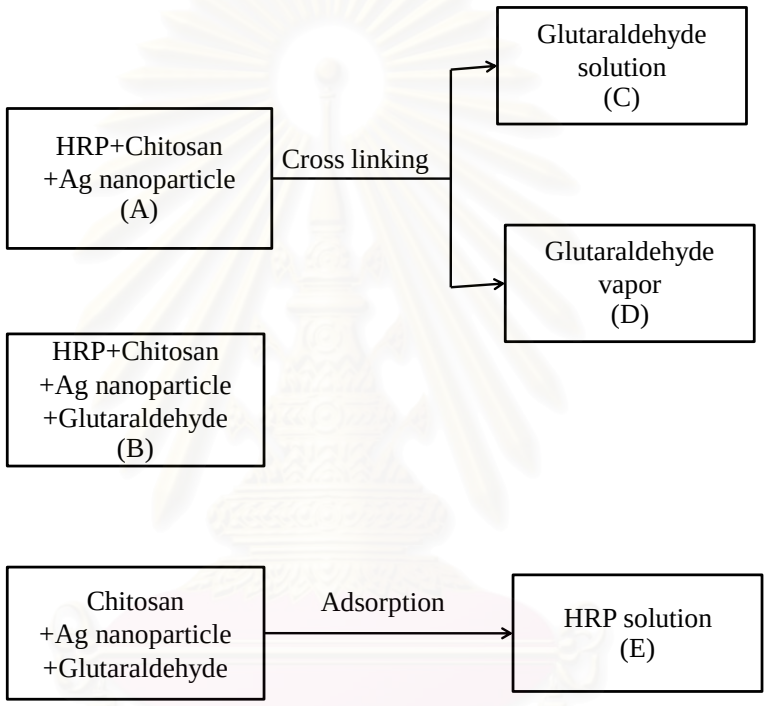


Figure 3.1 schematic diagrams of five different procedures of fabrication of modified electrodes

3.2.3.1 Procedure A (HRP entrapment in Ag nanoparticle/chitosan composite)

HRP in 0.1 M PBS solution was prepared to a final solution of 10 mg/mL. This HRP solution, Chitosan solution and Ag nanoparticles (20 -1000 ppm) with a volume ratio of 1.5:5:1 were mixed thoroughly to the final volume of 152 μL and stirred at 4 °C for 1 h. Then, 5 μL of this mixture was dropped on the surface of the cleaned GCE. This electrode was dried at room temperature for 45 mins. Before experiments, this electrode was immersed once in 0.1 mol/L PBS (pH 6) to wash out the nonimmobilized enzyme from electrode surface. The prepared electrode was next air dried for 15 mins be for being used.

3.2.3.2 Procedure B (HRP entrapment in glutaraldehyde solution/Ag nanoparticle/chitosan composite)

HRP in 0.1 M PBS solution was prepared to a final solution of 10 mg/mL. This HRP solution, Chitosan solution, Ag nanoparticles (Optimum concentration in type A) and glutaraldehyde with a volume ratio of 1.5:5:1:1 were mixed thoroughly to the final volume of 172 μL and stirred at 4 °C for 1 h. Then, 5 μL of this mixture was dropped on the surface of the cleaned GCE. This electrode was dried at room temperature for 45 mins. Before experiments, this electrode was immersed once in 0.1 mol/L PBS (pH 6) to wash out the nonimmobilized enzyme from electrode surface. The prepared electrode was next air dried for 15 mins be for being used.

3.2.3.3 Procedure C (HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with glutaraldehyde solution)

Optimum matrix mixture obtained in type A was used for fabricating electrode C. 5 μL of this mixture was dropped on the surface of the cleaned GCE. This electrode was dried at room temperature for 45 mins. Next, immersed this modified electrode in glutaraldehyde solution (0.0025-0.1%v/v) for 15 min and then this electrode was immersed in distilled water to wash out the nonimmobilized enzyme from electrode surface. The prepared electrode was next air dried for 15 mins be for being used.

3.2.3.4 Procedure D (HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with saturated glutaraldehyde vapor)

Optimum matrix mixture obtained in type A was used for fabricating electrode C. 5 μL of this mixture was dropped on the surface of the cleaned GCE. This electrode was dried at room temperature for 45 mins. Next, This modified electrode was placed in saturated glutaraldehyde vapor for 15 min and then this electrode was immersed in distilled water to wash out the nonimmobilized enzyme from electrode surface. The prepared electrode was next air dried for 15 mins be for being used.

3.2.3.5 Procedure E (HRP adsorption in glutaraldehyde solution/Ag nanoparticle/chitosan composite)

Chitosan, (0.0025-0.1% (v/v)) glutaraldehyde, and Ag nanoparticles with a volume ratio of 5:1:1 were mixed thoroughly to the final volume of 140 μL and stirred at 4 °C for 1 h. After that, 5 μl of this mixture was dropped on the surface of the cleaned GCE. This electrode was dried at room temperature. Next, this electrode was immersed in the HRP solution 10 mg/mL for 15 min. Before electrochemical experiments, this electrode was immersed in 0.1 mol/L PBS (pH 6) to wash out the nonimmobilized enzyme from electrode surface. The prepared electrode was next air dried for 15 mins be for being used.

3.2.4 Electrochemical measurements

Electrochemical measurements of cyclic voltammetry and amperometry were performed with a Glucosen potentiostat (Chulalongkorn University, Thailand). The electrochemical cell consists of a three-electrode system with a glassy carbon electrode (GCE) as a working electrode, a platinum wire as a counter electrode, and a silver/silver chloride (Ag/AgCl) electrode as a reference electrode (Fig 3.2-3.3). Before each experiment, working electrode was first polished with 0.3 μm alumina slurry and rinsed with distilled water. The electrode was then sonicated using ultrasonic cleaner (CREST, model D, Malaysia) at the frequency of 40 kHz in absolute ethanol for 5 minutes and distilled water for 5 minutes, respectively. After dried in ambient condition for 30 minutes, the electrode was ready to be used for each experiment.

Magnetic stirrer were performed by Barnstead Thermolyne, Canada that using to provide continuous convective transport during the amperometric measurement.

Figure 3.2 Glucosen potentiostat

(a) (b) (c)

Figure 3.3 (a) working electrode (WE), (b) counter electrode (CE) and (c) reference electrode (RE).

3.2.4.1 Cyclic voltammetric

The cyclic voltammogram response of modified electrode to phenol was measured by adding 800 µl hydrogen peroxide 0.1 mM (into 5 ml of 0.1 M PBS pH 7 which containing 0.1 mM phenol in beaker 10 ml as substrate solution. The solution was stirred at a constant rate using a magnetic stirrer bar. Cyclic voltammogram was recorded at scan rate 50 mV/s and the potential between -500 mV and 500 mV. A cyclic voltammogram is the plot of the response current at the working electrode vs. applied excitation potential. As a result, the plot gives information about the redox potential and electrochemical reaction rate.

3.2.4.2 Amperometric

The amperometric response of modified electrode to phenol was measured same as cyclic voltammetric but in this technique, a constant potential is applied at the working electrode. In this study, fixed potential at -0.05 V (Dai et al, 2004). Current was measured as a function of time. At the potential applied, the analyte go through an oxidation or reduction at the electrode. The current responses are directly proportion to the concentration of the analytes. The amount of response current for the system to approach equilibrium.

We chose the best composite film of each procedure to investigate reuse of modified electrode using amperometric method to determine response current for successive assay. Before reuse of each electrode, they were washed with water and dried in air.

We chose the best type of biosensor to investigate stability of biosensor using amperometric method. We stored this electrode at 4 °C in a refrigerator.

3.2.5 Characterization of modified electrode

Scanning Electron Micrographs (SEM) were obtained with model JSM-6400. Fourier transform infrared (FT-IR) spectrometer was obtained with model 1760x.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Matrix characterization

In this study, chitosan was used as the supporting matrix for the immobilization of HRP because of its excellent properties such as biocompatibility and nontoxicity. However, there still exists enzyme leaching and poor stability (Punwittayakool, 2008). We improved the property of chitosan matrices by cross-linked chitosan and enzyme using glutaraldehyde which established intermolecular cross-links with the amino groups of chitosan and enzyme (Fig 4.1-4.2). Moreover, we modified chitosan with Ag nanoparticles to helped enzyme dispersion because metal nanoparticles have good interaction with the protein (Xu et al., 2004) and enhance the electron conductivity of chitosan that resulting in high response current.

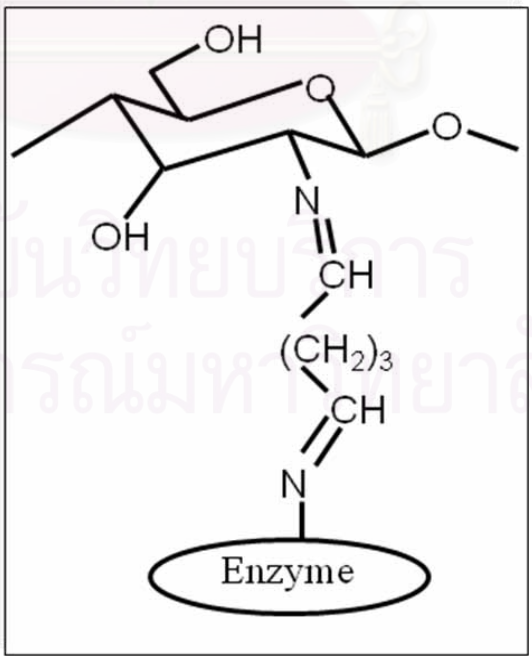


Figure 4.1 Structure of chitosan-enzyme cross-linking with glutaraldehyde

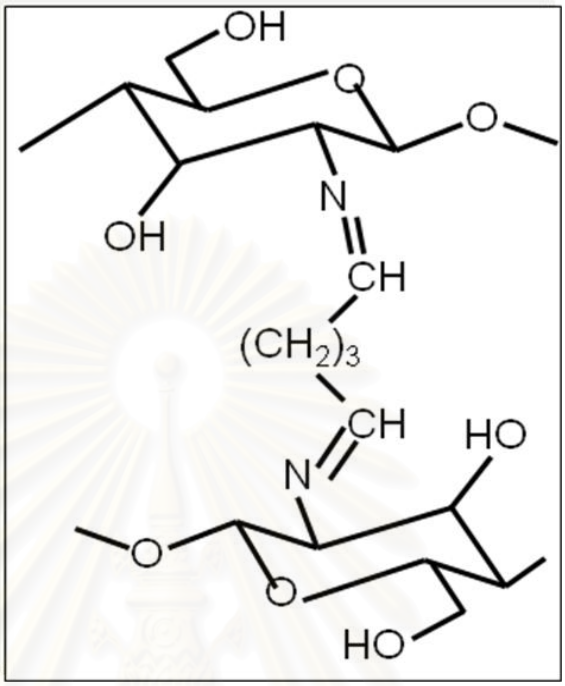


Figure 4.2 Structure of chitosan cross-linking with glutaraldehyde

Five different methods of enzyme immobilization were investigated and compared. Method A, HRP entrapment in Ag nanoparticle/chitosan composite. It had no cross-linking by glutaraldehyde. Chitosan has pKa about 6.3. At pH 5 of solution (<pKa) which optimum conditions for HRP activity (Suksompong et al., 2006), most of the amino groups are protonated (Fig 4.3, 4.4 (A)). For method B, HRP entrapment in glutaraldehyde solution/Ag nanoparticle/chitosan composite. Whereas, during cross-linking, the aldehyde group of glutaraldehyde may be reacted with the amino group of chitosan and HRP by covalent bonding (Fig 4.4 (B)). In case of method C, HRP was entrapped in Ag nanoparticle/chitosan composite and then cross-linked with glutaraldehyde solution. The aldehyde group of glutaraldehyde may be reacted with the amino group of chitosan and HRP by covalent bonding which similar to the film from method B (Fig 4.4 (C)). While method D, HRP was entrapped in Ag nanoparticle/chitosan composite and then cross-linked with saturated glutaraldehyde vapor. The aldehyde group of glutaraldehyde may be reacted with the amino group of chitosan and HRP by covalent bonding which similar to the film from method B and

C (Fig 4.4 (D)).Method E, HRP was adsorbed in glutaraldehyde solution/Ag nanoparticle/chitosan composite. First, chitosan cross-linked with glutaraldehyde by covalent bonding and then some of residual aldehyde group of glutaraldehyde may be reacted with the amino group of enzyme (Fig 4.4 (E)).

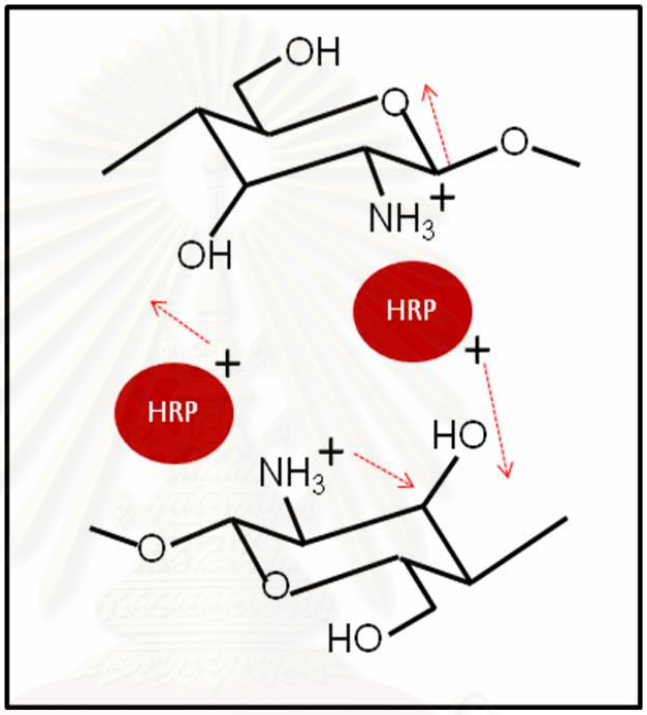


Figure 4.3 Structure of chitosan cross-linking with glutaraldehyde

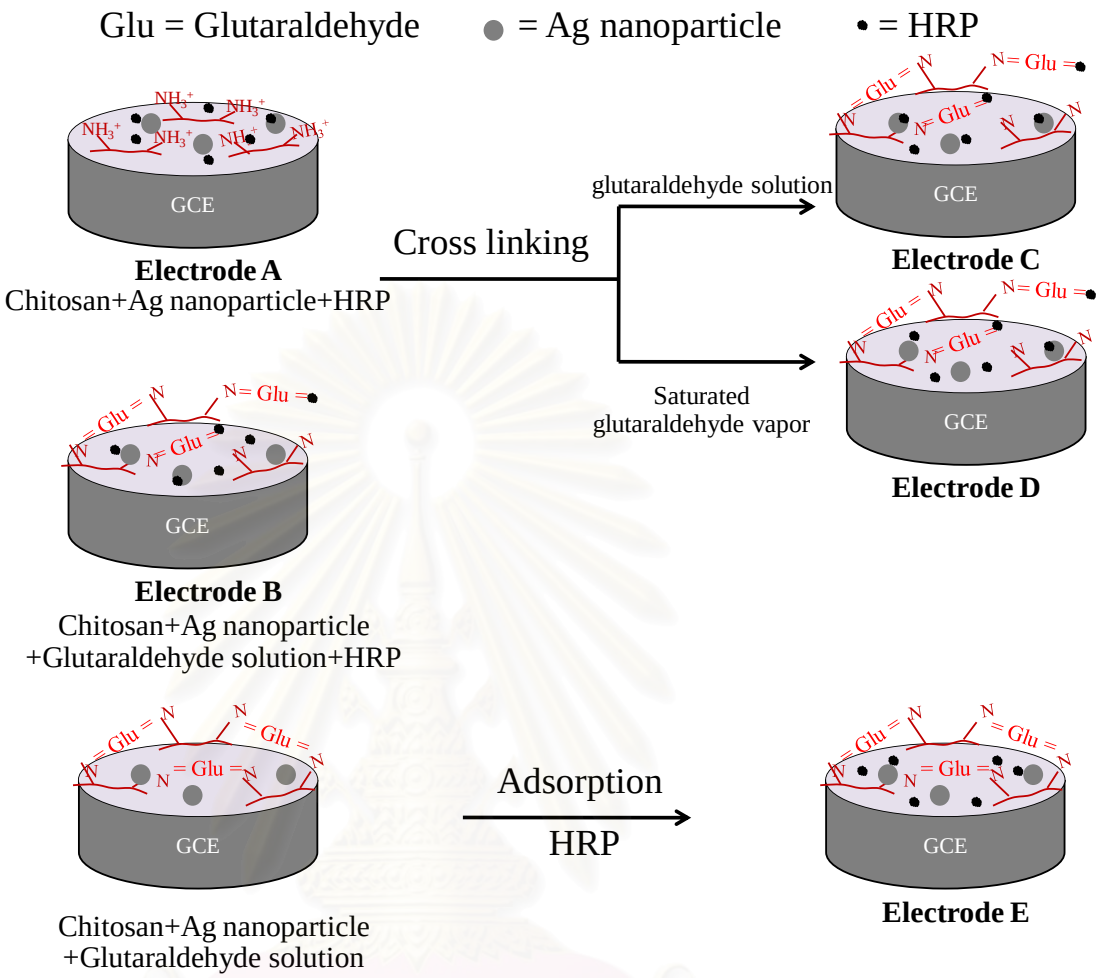


Figure 4.4 Schematic illustration of five different method of HRP immobilization

(A) HRP entrapment in Ag nanoparticle/chitosan composite

(B) HRP entrapment in glutaraldehyde solution/Ag nanoparticle/chitosan composite

(C) HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with glutaraldehyde solution

(D) HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with saturated glutaraldehyde vapor

(E) HRP adsorption in glutaraldehyde solution/Ag nanoparticle/chitosan composite

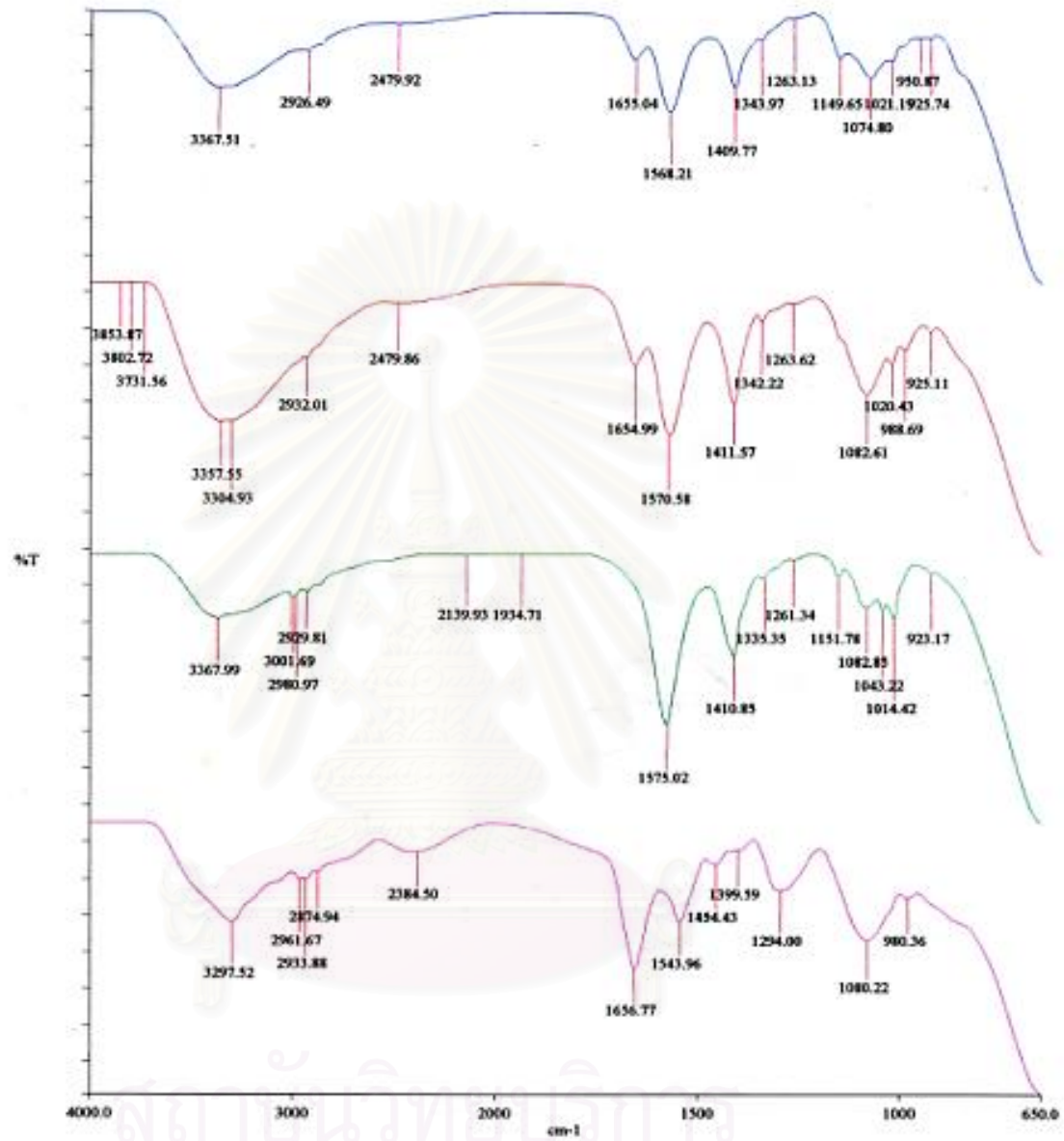


Figure 4.5 FTIR spectra of electrode A (blue line), electrode B (red line), chitosan (green line) and HRP (pink line)

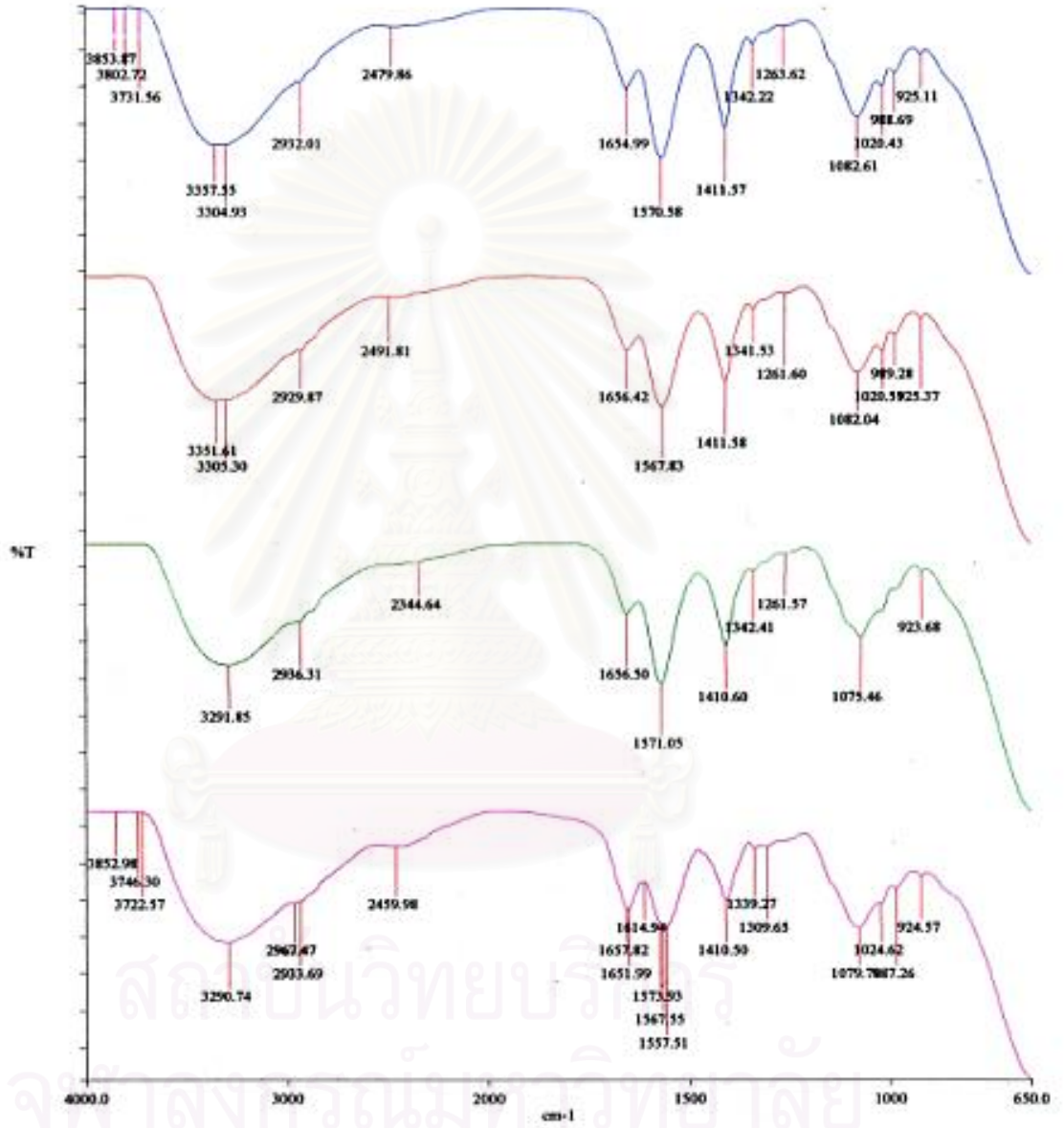


Figure 4.6 FTIR spectra of electrode B (blue line), electrode C (red line), electrode D (green line) and electrode E (pink line)

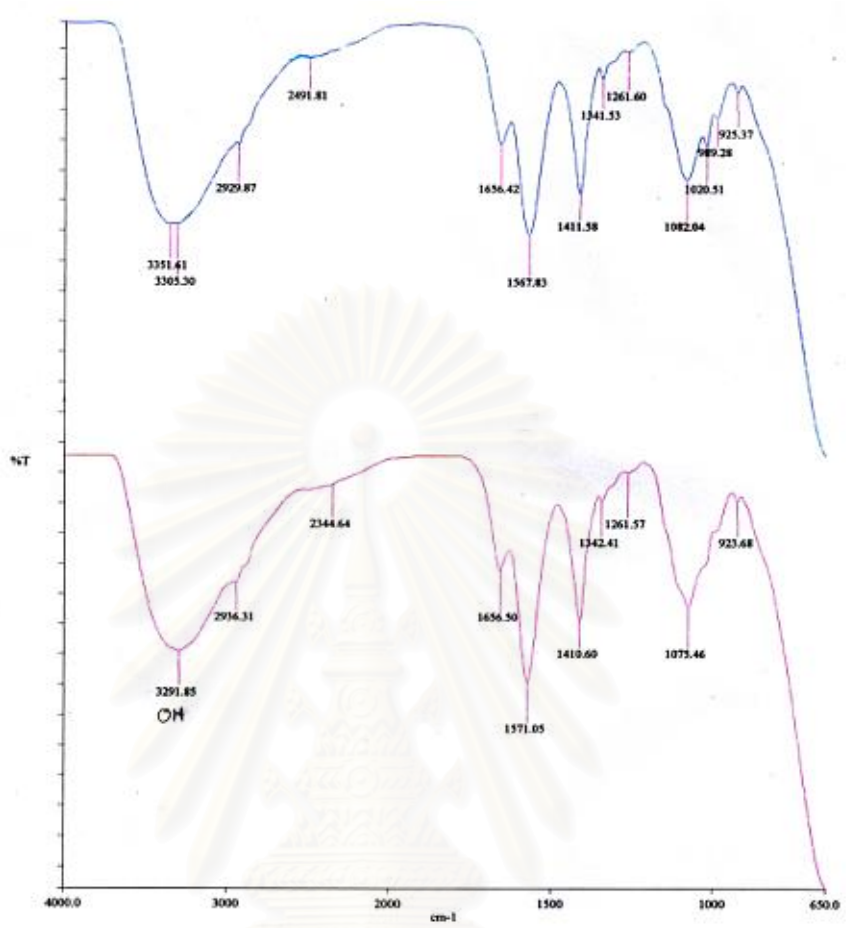
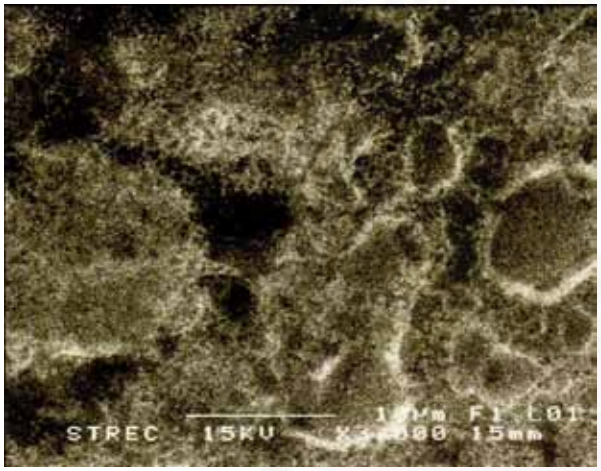


Figure 4.7 FTIR spectra of electrode C (blue line) and electrode D (pink line)

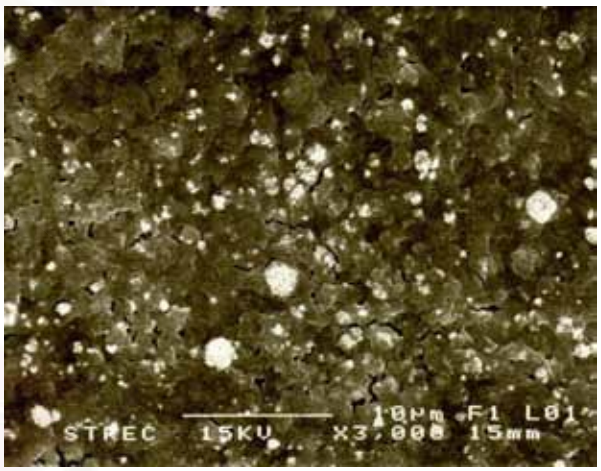
The aldehyde group of glutaraldehyde reacted with the amino group of chitosan and HRP by covalent bonding (C=N). The interactions of chemical structure of modified electrode were investigated by IR spectra. The FT-IR spectra of modified electrodes, chitosan and HRP are shown in Fig 4.5-4.7. In Fig 4.5 (green line), the adsorption band at 1575.02 cm^{-1} is the typical spectrum of chitosan. FT-IR bands of chitosan shift to a lower wave number in electrode A and B (1575.02 cm^{-1} shift to 1568.21 cm^{-1} and 1570.58 cm^{-1} respectively). Besides, in Fig 4.5 (pink line), the adsorption band at 1656.77 cm^{-1} is the typical spectrum of HRP. FT-IR bands of HRP shift to a lower wave number in electrode A and B (1656.77 cm^{-1} shift to 1655.04 cm^{-1} and 1654.99 cm^{-1} respectively). A 1630-1680 cm^{-1} is assigned to C=N bending. This

fits well with the increase in intermolecular which decreased the wave number of chitosan and HRP. This suggests that there are interactions between glutaraldehyde and some of enzyme and chitosan. Resulting in other electrodes (Fig 4.6-4.7) which similar to electrode A and B.

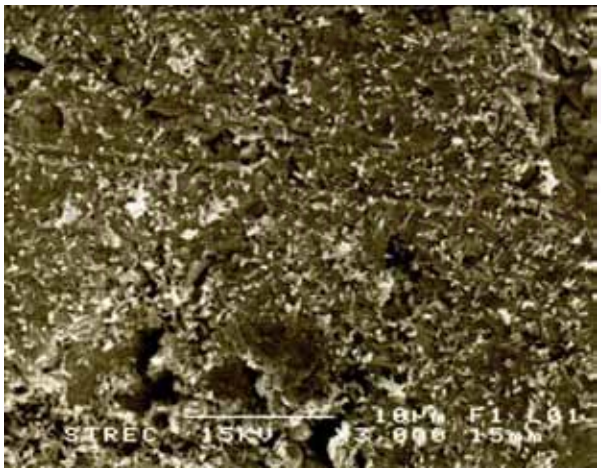
The surface morphology of five nanobiocomposite films which have different method enzyme immobilization was characterized by scanning electron micrograph in order to investigate the homogeneity of the film. The results were shown in Fig 4.8. It was found that, they had different uniformly. The film of D which its cross-linking with saturated glutaraldehyde vapor was rougher than others. The different structure of the film effected different substrate diffusion and mass transport, resulting in different sensitivity, linearity and stability of biosensors.



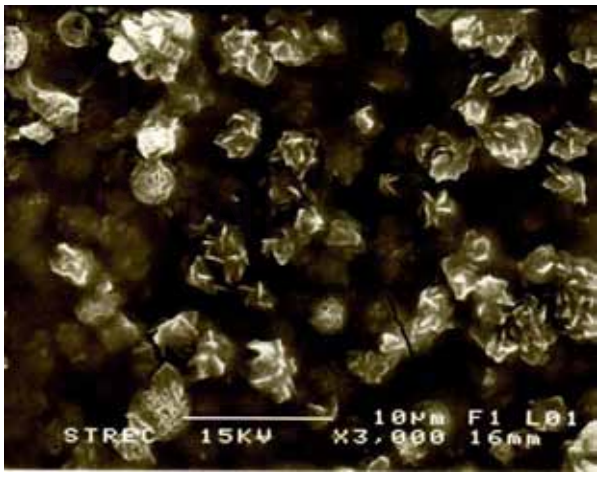
(A)



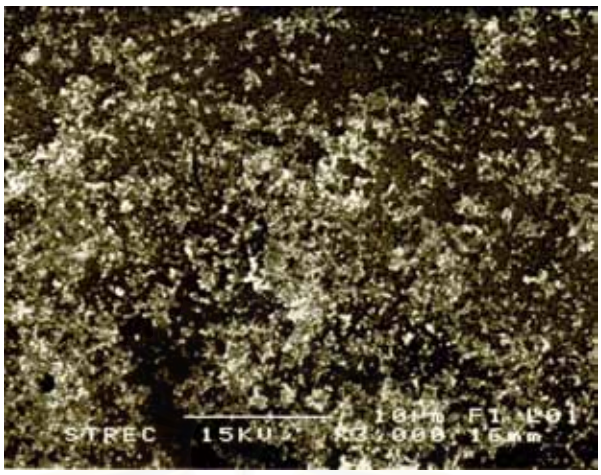
(B)



(C)



(D)



(E)

Figure 4.8 SEM of (A) HRP entrapment in Ag nanoparticle/chitosan composite; (B) HRP entrapment in glutaraldehyde solution/Ag nanoparticle/chitosan composite; (C) HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with glutaraldehyde solution; (D) HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with saturated glutaraldehyde vapor and (E) HRP adsorption in glutaraldehyde solution/Ag nanoparticle/chitosan composite.

However, we did not characterize matrix by EDX to study dispersion of enzyme and Ag nanoparticles and AA to determine amount of enzyme in matrix because this matrix got a little of these components. Also, we did not study dispersion of matrix by TEM because the matrix eroded the TEM's equipment.

4.2 Electrochemical characterization of Ag nanoparticle/ chitosan /HRP /GCE based on phenol detection

The enzymatic mechanism of the amperometric biosensor based on HRP for detection of phenol is shown as follows (Ruzgas et al., 1995):

$$HRP(Fe^{3+}) + H_2O_2 \longrightarrow HRP(Fe^{5+}) + H_2O$$
$$HRP(Fe^{5+}) + AH_2 \longrightarrow HRP(Fe^{4+}) + AH^*$$
$$HRP(Fe^{4+}) + AH_2 \longrightarrow HRP(Fe^{3+}) + AH^* + H_2O$$

In each step, phenol (AH_2) is converted to free radicals (AH^*), which are electroactive and can be reduced on the electrode surface. The reduction current is therefore proportional to phenol concentration. However, HRP (Fe^{5+}) can also be directly reduced on the electrode surface. Thus, the sensitivity of a biosensor for monitoring phenol is limited due to the background current of the direct electron transfer (Rosatto et al., 1999). Fig.4.9 shows the cyclic voltammogram of Ag nanoparticle /Chitosan/HRP/GCE in the absence and presence of phenol. It is clearly indicated that this biosensor performed high phenol sensitivity due to the low background current in absence of phenol.

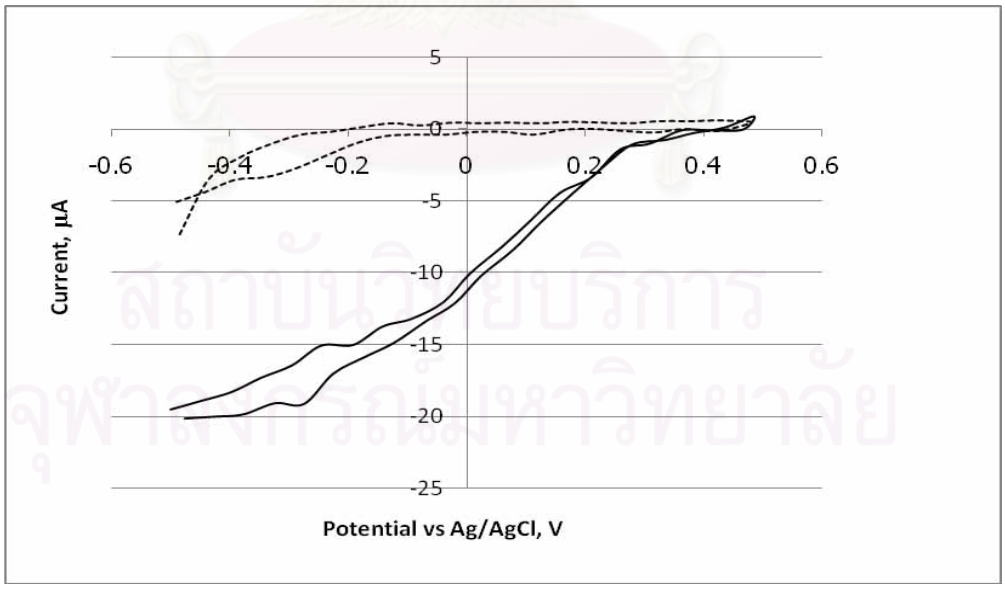


Figure 4.9 Cyclic voltammograms of Ag nanoparticle/Chitosan /HRP/GCE in the absence (a) and presence (b) of phenol at a scan rate of 50 mV/s and the potential between -500 mV and 500 mV in 0.1 M PBS(pH 7) at room temperature.

4.3 Optimization of experimental variables

The performance of biosensor is mainly affected by the electrocatytic activity of modified electrode material and composites. In addition to this, the response current of the modified electrodes depends on activity of enzyme and substrate or product mass transfers. The factor which is important contributing component to the performance of an immobilized enzyme system are chemical and physical properties of support. They have effect on activity and stability of enzyme. The support functional group which is chemical properties has effect on activity of enzyme whereas physical properties such as pore size, specific area and tortuosity have effect on amount of immobilized enzyme and dispersion of enzyme.

Moreover, physical properties have effect on Effective Diffusivity (D) as shown in equation 4.1 which concern in mass transfer limitation.

$$D_{eff} = D_s \frac{\varepsilon}{\tau} H \qquad (4.1)$$

Where D_{eff} is a function of bulk diffusivity (D_s), particle porosity (ε), tortuosity (τ) and hindrance factor (H)

High response current exhibits that high enzyme activity, high enzyme loading or high diffusivity.

4.3.1 Effect of silver nanoparticles

HRP was immobilized in chitosan which modified with Ag nanoparticles that have good interaction with the protein and enhance the electron conductivity of chitosan. Fig. 4.10, demonstrates a significant effect of silver nanoparticles on electrochemical responses. In this section, we tested the response of modified electrode in solutions which consist of PBS pH 7 as buffer solution for dissolve the substance solutions, Hydrogen peroxide and Phenol which acted as substance solutions to determine the interfere of each solution in electrochemical reaction. Different concentrations of Ag nanoparticle solution (20, 100, 500 and 1,000 ppm) within HRP /chitosan composite modified glassy carbon electrode were studied. It was found that response currents increased rapidly from 2.5×10^{-6} A to 8×10^{-6} A when concentration of Ag nanoparticle solution was increased from 0 up to 100 ppm.

It showed that Ag nanoparticles did help facilitating electron transfer. Moreover, they may be improved dispersion of enzyme (Fig 4.11 (a) and (b)), resulting in good enzyme activity led to high response current. However, the response currents were found to decrease when Ag nanoparticle concentration was increased from 100 to 500, and 1000 ppm. This was probably due to an increase hindrance of silver nanoparticles to substrate or product mass transfers between bulk solution and enzyme active sites (Fig 4.11 (c)). Therefore, 100 ppm Ag nanoparticle was the concentration chosen for all subsequent biosensor fabrication.

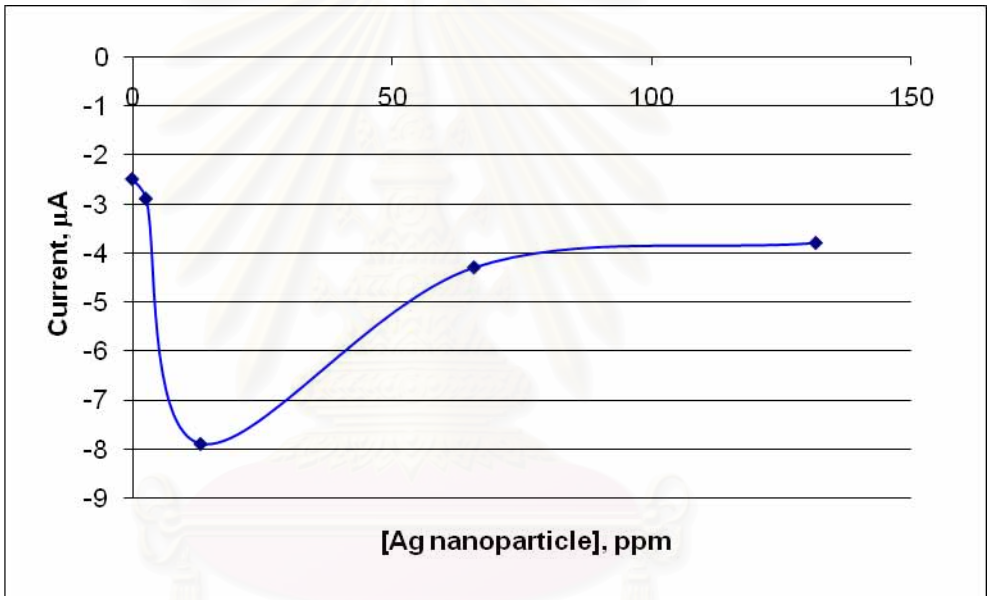
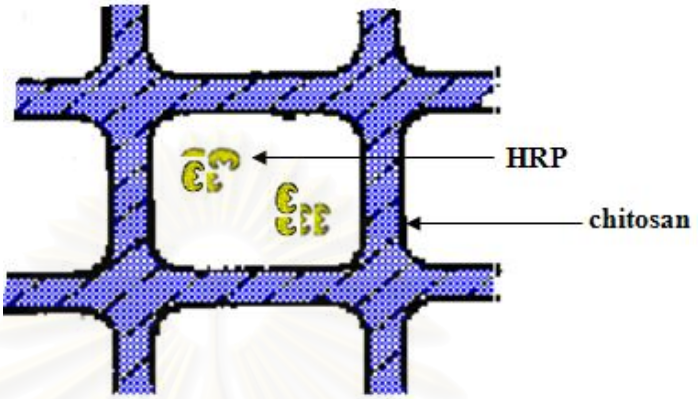
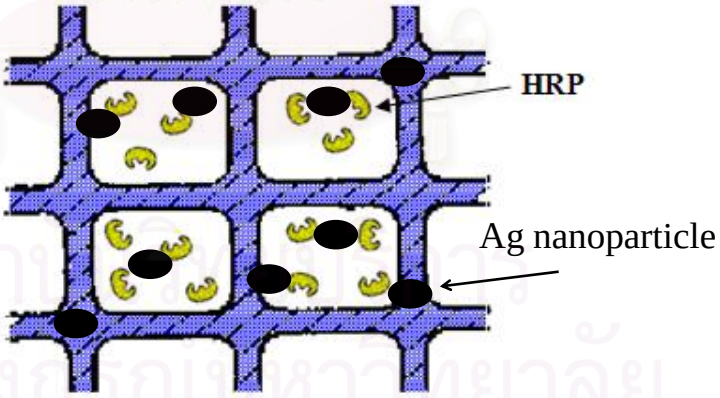


Figure 4.10 Effect of Ag nanoparticle concentration in modified electrode on response current
Experimental condition : 0.1 mM phenol in 0.1 M PBS(pH 7)
at an applied potential of -0.05 V versus Ag/AgCl



(a)



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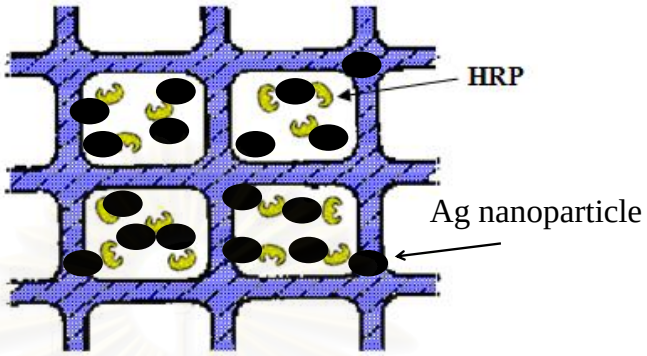

(c)

Figure 4.11 Proposed schematic diagrams for HRP were entrapped in chitosan (a), HRP were entrapped in Ag nanoparticles/chitosan (b,c)

4.3.2 Effect of cross-linking time

The effect of the cross-linking time on the response current for biosensors was studied from 0 to 360 mins in fabrication electrode B (Fig 4.12). It was found that cross-linking time did not effect to response current of biosensors which HRP entrapment in glutaraldehyde solution/Ag nanoparticle/chitosan composite. Due to during cross-linking, the aldehyde group of glutaraldehyde may be reacted with the amino group of chitosan and HRP by covalent bonding. Therefore amount of cross-linking depended on amount of aldehyde group and amino group of matrix and HRP.

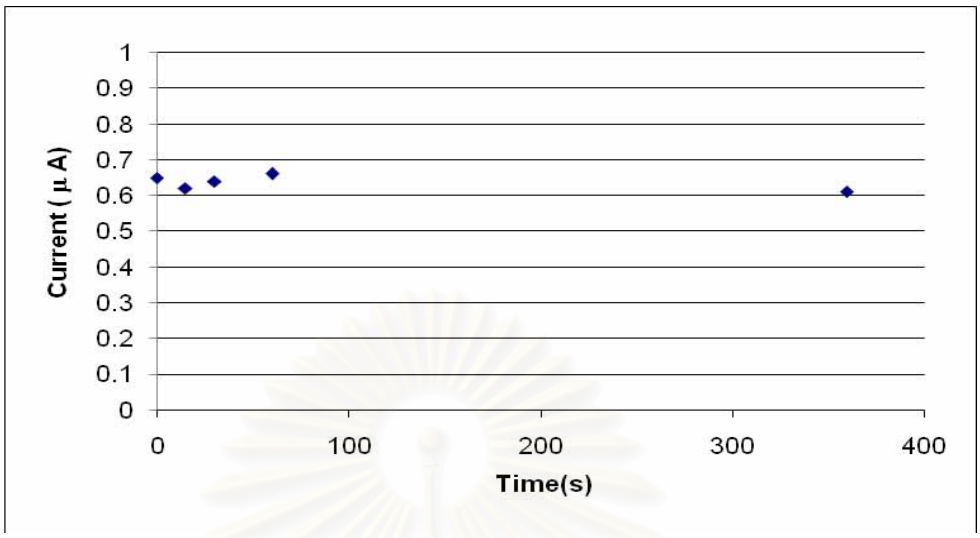

Figure 4.12 Effect of cross-linking time on response current
Experimental condition : 0.1 mM phenol in 0.1 M PBS(pH 7)
at an applied potential of -0.05 V versus Ag/AgCl

4.3.3 Effect of glutaraldehyde concentration

Poor stability of biosensors is mainly caused by enzyme inactivation and leaching from immobile materials. To overcome this problem, we cross-linked chitosan and enzyme using glutaraldehyde which established intermolecular cross-links with the amino groups of chitosan and enzyme. However, glutaraldehyde was found to cause enzyme inactivation. Therefore, glutaraldehyde concentration was important to use.

The response current of modified electrode with glutaraldehyde concentration range of 0-0.1 %v/v was studied. Fig. 4.13 shows effect of glutaraldehyde concentration on response current for electrode C that HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with glutaraldehyde solution by covalent bonding. It was found that, the current response decreased largely as the glutaraldehyde concentration changed from 0 to 0.025%v/v, following with consistent response current in the glutaraldehyde concentration range of 0.025–0.1%v/v. Glutaraldehyde was found to inactivation enzymes, resulting in bad response currents of biosensor. Moreover, the mount of cross-linking agent may produce biocatalyst inactivation.

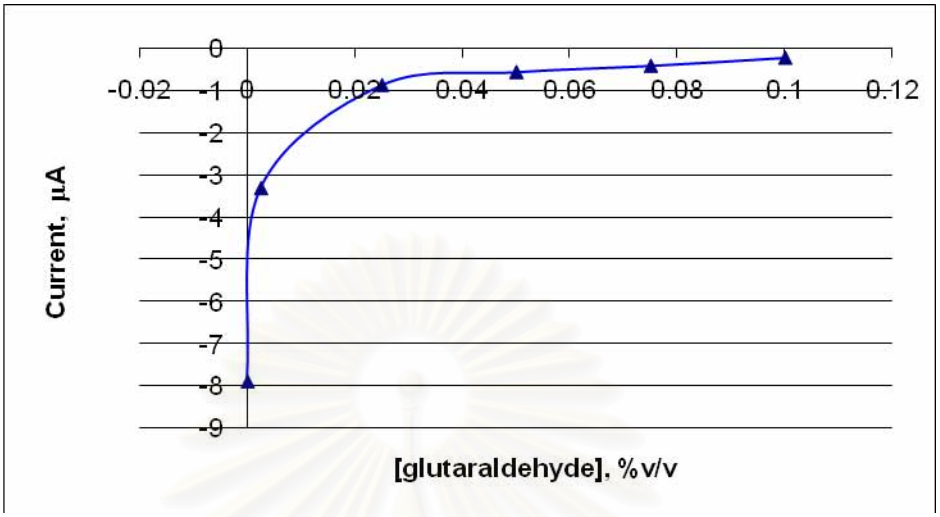

Figure 4.13 Effect of glutaraldehyde concentration for electrode C
Experimental condition : 0.1 mM phenol in 0.1 M PBS(pH 7)
at an applied potential of -0.05 V versus Ag/AgCl

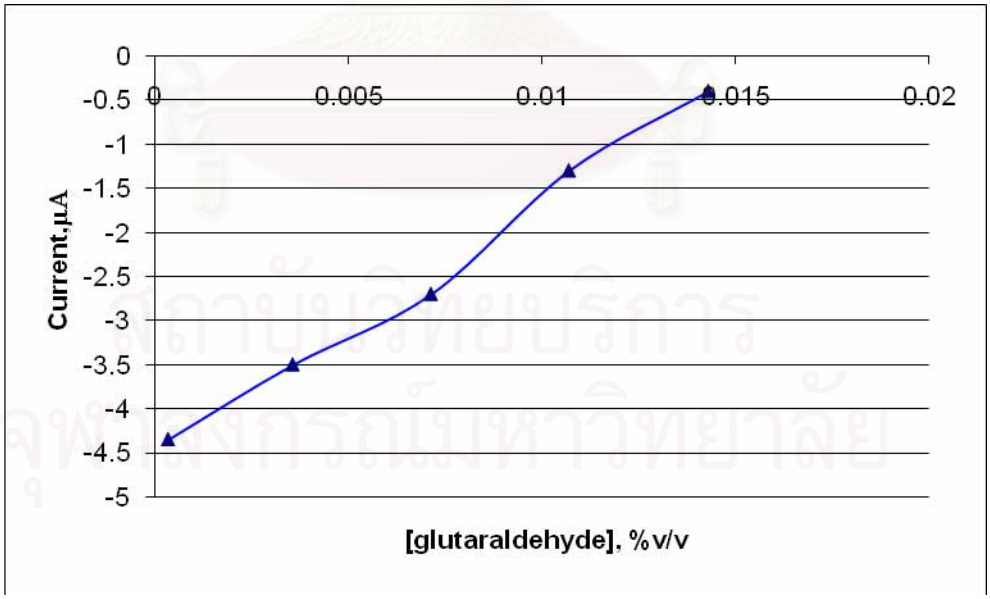

Figure 4.14 Effect of glutaraldehyde concentration for electrode E
Experimental condition : 0.1 mM phenol in 0.1 M PBS(pH 7)
at an applied potential of -0.05 V versus Ag/AgCl

Fig. 4.14 displays the response current of the modified electrode E which HRP was adsorbed on glutaraldehyde/Ag nanoparticle/chitosan composite. The aldehyde group of glutaraldehyde reacted with the amino group of chitosan by covalent bonding and then HRP was adsorbed in this film. Some of residual aldehyde group of glutaraldehyde may be reacted with the amino group of HRP by covalent bonding. It was found that the current response decreased when increased the glutaraldehyde concentration. Due to chitosan was more cross-linked when used more glutaraldehyde, resulting in more complex film led to enzyme was less adsorbed. This result is shown in Fig 4.15. We tested chemical reaction of residual enzyme from adsorption. It was found that, chemical reaction increased when increased the glutaraldehyde concentration. Resulting in decreasing of enzyme absorbed.

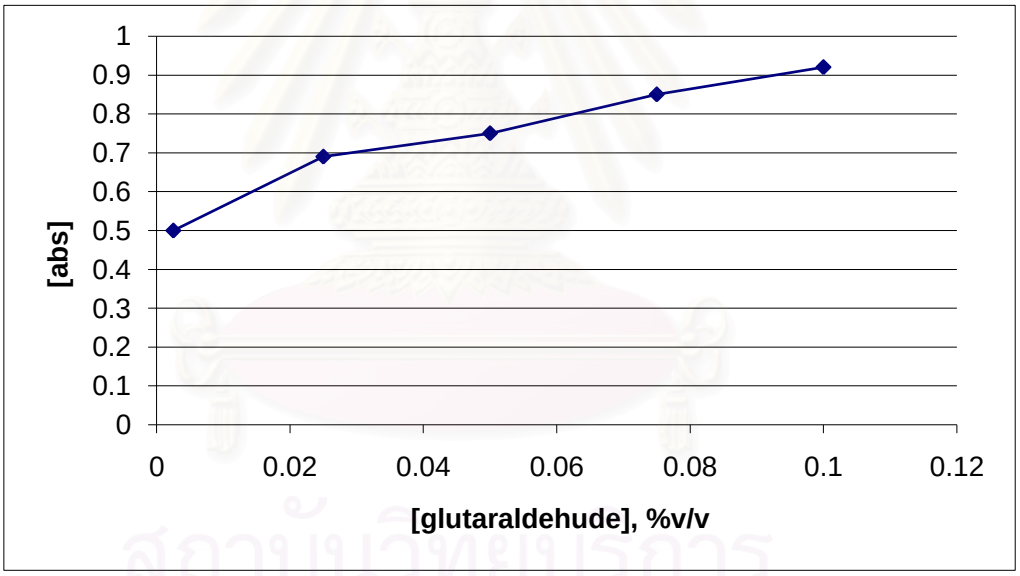

Figure 4.15 Chemical reaction of residual HRP immobilization of electrode E

4.4 Comparison of response current of modified electrodes

The thickness of film affected to the response time of biosensor. The response time of biosensor increased when the thickness of the film increased. This may be due to the increased of electron transfer distance from the electrode surface to redox center of the immobilized HRP (Xu et al., 2007). The response time of five modified electrodes were summarized in Table 4.1. It was found that the response time of the immobilized HRP from method C and E was nearly equal. Moreover, the result shown in SEM (Fig 4.8) revealed that the film from method C was similar to the film form method E. The highest response time was observed in the case of method D. On the other hands, the lowest response time was observed in the case of method B.

Table 4.1 Performance of the biosensors various immobilization method

Electrodes	Methods	Response current(A)	Response time(s)
A	HRP entrapment in Ag nanoparticle/chitosan composite	$7.9x10^{-6}$	85
B	HRP entrapment in glutaraldehyde solution /Ag nanoparticle/chitosan composite	$0.66x10^{-6}$	10
C	HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with glutaraldehyde solution	$3.3x10^{-6}$	95
D	HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with saturated glutaraldehyde vapor	$1.2x10^{-6}$	250
E	HRP adsorption in glutaraldehyde solution/Ag nanoparticle/chitosan composite	$4.3x10^{-6}$	90

The high surface area of the film supplied more sites for HRP immobilization, therefore, it increased the catalytic activity of sensor. The ordered porous character of the film increased the rate of mass transport, which resulted in current response (Xu et al., 2007). The optimum response current of five modified electrodes based on various glutaraldehyde cross-linking method were compared. It was found that the response current of electrode A, B, C, D and E were $7.9x10^{-6}$, $0.66x10^{-6}$, $3.3x10^{-6}$, $1.2x10^{-6}$ and $4.3x10^{-6}$ A respectively (Table 4.1). Therefore, each enzyme immobilization methods affect to substrate or product mass transfers between bulk solution and enzyme active sites, resulting in different response current. The biosensor with the best response current obtained from method A which had no cross-linking by glutaraldehyde. The response current decreased when using glutaraldehyde. Besides, we tested chemical reaction of modified electrodes that enzymes were immobilized. Fig 4.16 compared % relative response between chemical and electrochemical of various modified electrodes. It was found that, there is little different residual activity of enzyme of each electrodes when compared with different current response of each electrode. Electrode A had amount of initial HRP and amount of Ag nanoparticle equl to electrode C and D. But It had the response current and response chemical more than electrode C and D. Therefore, glutaraldehyde might be deactivate of some enzyme during cross-linking, resulting in less response current. Electrode C had amount of initial HRP and amount of Ag nanoparticle similar to electrode D. Moreover, Electrode C had response chemical similar to electrode D. So comparison of electrode C (using glutaraldehyde solution) to electrode D (using glutaraldehyde vapor), a lower signal was obtained ($1.2x10^{-6}$ A) in electrode C due to the mass transport was limited by the ordered porous character of the film.

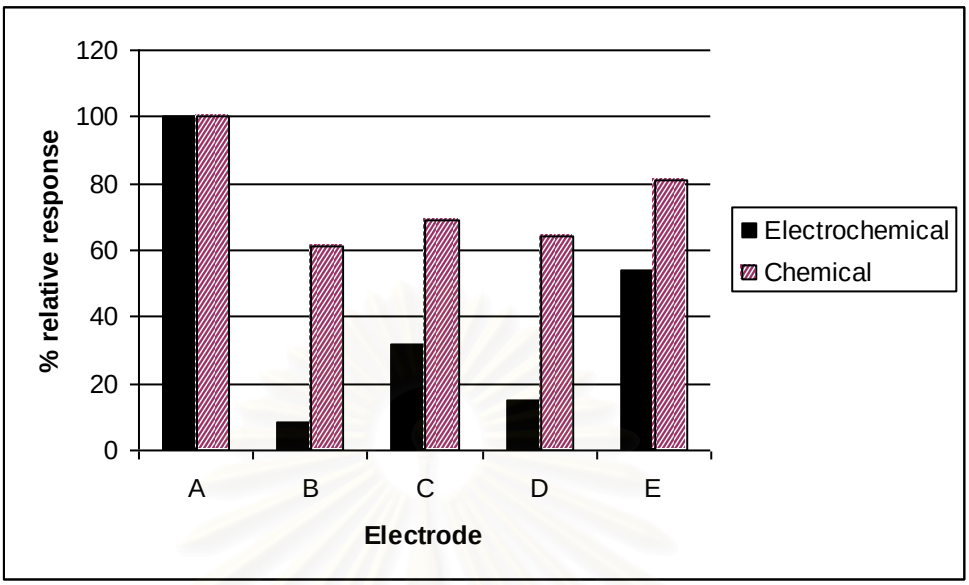

Figure 4.16 Chemical and electrochemical of various modified electrodes

4.5 Comparison of reusability of the biosensors

Under optimal condition in the determination of phenol (0.1 mM), the reusability of response current of all electrodes was also examined. The biosensor with the best reusability obtained by the procedure D which HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with saturated glutaraldehyde vapor. From fig 4.17, it was found that the response current decreased approximately to 15% of the original response in 9 successive assays, while electrode A which had no cross-linking by glutaraldehyde, could not repeatability. The results demonstrate that using glutaraldehyde improved leaching of enzyme from metric. On the other hands, the lower reusability was observed in the case of method D. It indicated that this modified electrode had still leaching of enzyme due to enzyme was absorbed, which absorption has strongly interaction less than cross-linking with glutaraldehyde. Resulting to modification electrode by cross-linked with glutaraldehyde vapor had the best procedure for HRP in chitosan/Ag nanoparticle matric.

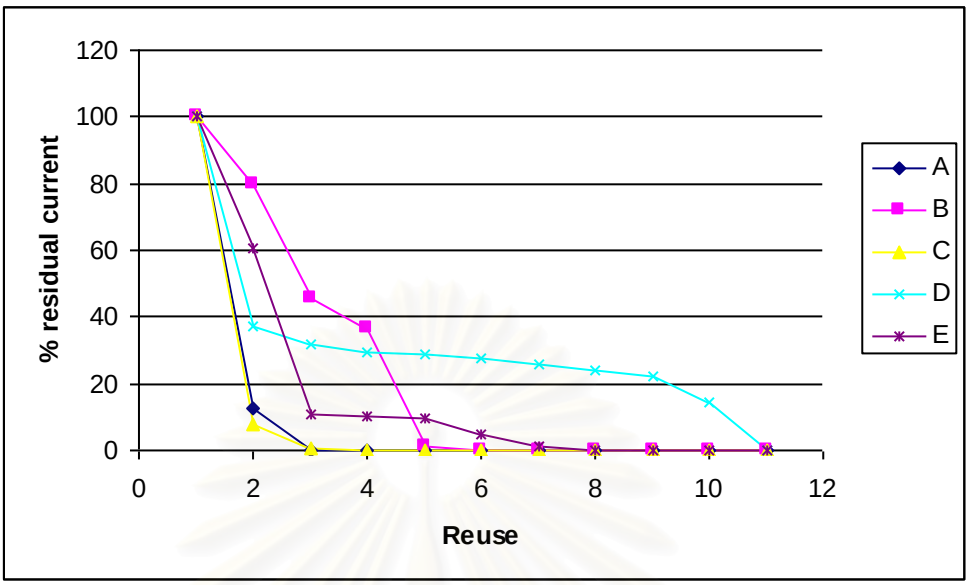

Figure 4.17 Reusability of the modified electrodes
Experimental condition : 0.1 mM phenol in 0.1 M PBS(pH 7)
at an applied potential of -0.05 V versus Ag/AgCl

4.6 Amperometric response characteristics of electrode D

4.6.1 Linear range

Fig. 4.18 shows the response current of the HRP electrode that was cross-linked with glutaraldehyde vapor is linear in the range between 0.05 – 0.87 mM. A lose in linearity at higher concentration of phenolic compounds is attributed to slow surface fouling by the reaction product.

4.6.2 Sensitivity

The sensitivity was calculated from slope of the calibration graph (Fig. 4.18).It was found to be 11.65 □A/mM.

4.6.3 Response time

The amount of time required for the system to approach equilibrium. This response time can be varied for each biosensor. However, the typical value is less than 5-10 minutes.

A slow response time within 250 s for phenol determination was observed. This indicated a slow diffusion of substrate and reaction products through the composite film.

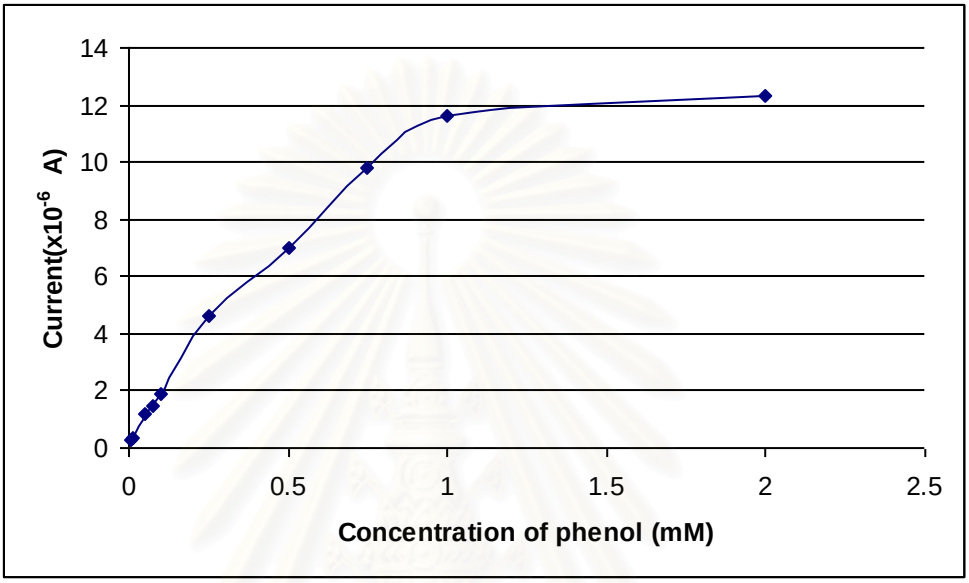

Figure 4.18 Calibration curves for modified glassy carbon electrode D to determine 0.1 mM phenol in 0.1 M PBS (pH 7) at an applied potential of -0.05 V versus Ag/AgCl

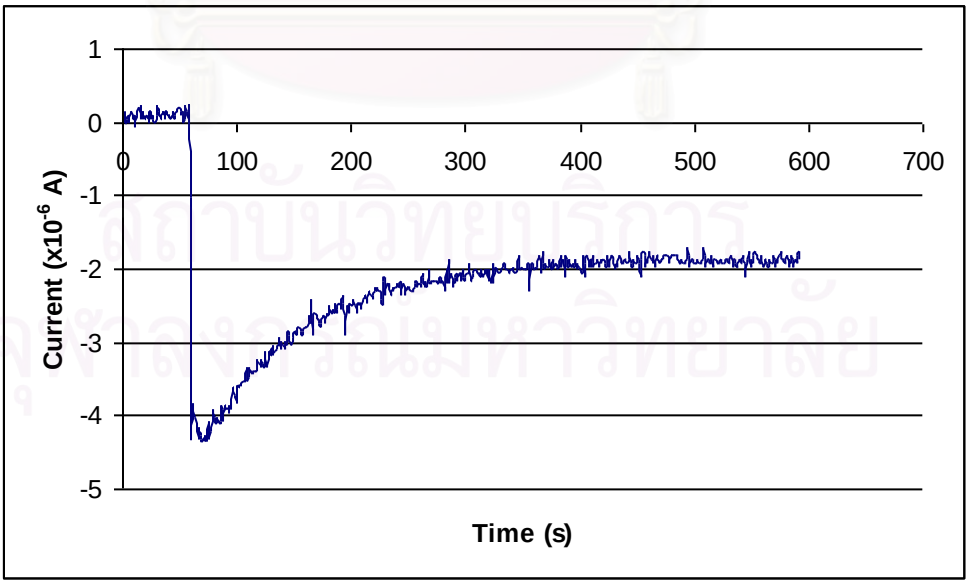

Figure 4.19 Amperometry of electrode D
Experimental condition : 0.1 mM phenol in 0.1 M PBS(pH 7)
at an applied potential of -0.05 V versus Ag/AgCl

4.6.4 Storage stability

HRP electrode was strode at 4 C when not use. The storage stability of the biosensor was investigated by measuring the biosensor response with 0.1 mM of phenol every five day. The response current of this biosensor decreased to 60% after storing 5 days. After 10 and 20 days of storage, the response current had about 40% and 10% respectively of the original response. It had stability more than previous our work.This could be due to the good biocompatibility of chitosan and Ag nanoparticle and strongly interaction between HRP and chitosan which was cross-linked with glutaraldehyde vapor.

CHAPTER V

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In this work, we are interested in immobilization of horseradish peroxidase in Ag-chitosan composites and apply for phenol biosensors. In order to prevent enzyme leaching while keeping high enzyme activity, five different methods of enzyme immobilization will be investigated and compared: (A) HRP entrapment in Ag nanoparticle/chitosan composite; (B) HRP entrapment in glutaraldehyde solution/Ag nanoparticle/chitosan composite; (C) HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with glutaraldehyde solution; (D) HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with saturated glutaraldehyde vapor and (E) HRP adsorption in glutaraldehyde solution/Ag nanoparticle/chitosan composite. Major findings from this work can be summarized as follows:

5.1.1 Matrix characterization

There are interactions between glutaraldehyde and some of enzyme and chitosan in modified electrodes. The different structure of the film effected different substrate diffusion and mass transport, resulting in different sensitivity, linearity and stability of biosensors.

5.1.2 Electrochemical characterization of HRP based on phenol detection

Biosensor performed high phenol sensitivity.

5.1.3 Effect of silver nanoparticles

Ag nanoparticles did help facilitating electron transfer, caused hindrance to substrate or product mass transfers if too concentrated Ag nanoparticles were applied.

5.1.4 Effect of Effect of cross-linking time

Cross-linking time did not effect to response current of biosensors.

5.1.5 Effect of glutaraldehyde concentration

The response current decreased when using glutaraldehyde. Glutaraldehyde was found to cause enzyme inactivation. But the repeatability improved by the cross-linking with saturated glutaraldehyde vapor.

5.1.6 Comparison of response current of modified electrodes

It was found that the response current of electrode A, B, C, D and E were $7.9x10^{-6}$, $0.66x10^{-6}$, $3.3x10^{-6}$, $1.2x10^{-6}$ and $4.3x10^{-6}$ A respectively. The response current decreased when using glutaraldehyde.

5.1.7 Amperometric response characteristics of electrode D

Linear range

The response current of the HRP electrode that was cross-linked with glutaraldehyde vapor is linear in the range between 0.05 – 0.87 mM.

Sensitivity

It was found to be 11.65 □A/mM.

Response time

A slow response time within 250 s for phenol determination was observed.

Storage stability

The response current of this biosensor decreased to 60% after storing 5 days. After 10 and 20 days of storage, the response current had about 40% and 10% respectively of the original response. It had stability more than previous our work.

5.2 Recommendations for the future studies

5.2.1 The effect of potential should be studied. This parameter is important for amperometry, resulting in response current.

5.2.2 The thickness of the film is important for mass transfer. The thinner and more homogenous film do a good response time and response current. We should be use spay-roller instead of dropping solution on electrode.

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APPENDICES

Appendix A
Conference

International conference

Tiraka Nualjan, Bunjerd Jongsomjit and Seeroong Prichanont, " Effects of glutaraldehyde cross-linking methods on enzyme immobilization in Ag nanoparticle/chitosan matrices" , Extended Abstract for The 2nd Thammasat University International Conference on Chemical, Environmental and Energy Engineering, Thailand, 3-4 March 2009, Paper ID ChE-034.

The 2nd Thammasat University International Conference
on Chemical, Environmental and Energy Engineering
TU ChEEE 2009

Effects of glutaraldehyde cross-linking methods on enzyme immobilization in Ag nanoparticle/chitosan matrices

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Abstract

An amperometric phenol biosensors was constructed by immobilization of Horseradish peroxidase (HRP, EC 1.11.1.7) in Ag-chitosan composite modified on a glassy carbon electrode. This research has four method for immobilization: (A) HRP entrapment in Ag nanoparticle/chitosan composite; (B) HRP entrapment in Ag nanoparticle/chitosan composite and cross-linked with glutaraldehyde solution; (C) HRP entrapment in Ag nanoparticle/chitosan composite and cross-linked with saturated glutaraldehyde vapor and (D) HRP entrapment in Ag nanoparticle/ glutaraldehyde cross-linked chitosan. Effects of glutaraldehyde cross-linking methods on response currents and repeatabilitys were determined and compared. The response current depends on the method used for enzyme immobilization. It found that the response current of electrode A, B, C and D were 7.9×10^{-6}, 3.3×10^{-6}, 1.2×10^{-6} and 4.3×10^{-6} A respectively. The repeatability improved by the cross-linking with saturated glutaraldehyde vapor.

Keywords: biosensor, horseradish peroxidase, phenol, chitosan, glutaraldehyde

1. Introduction

Phenolic compounds are byproducts of large-scale production such as coke ovens, petrochemical production and plastic industry. They often exist in wastewaters. Due to their toxicity, they constitute an acute environmental problem. Therefore, the control of these pollutants is important as shown by several European and EPA directives [1]. Several techniques are used for determination of phenols, such as chromatography, spectrophotometry and amperometric biosensor [2]. Among these techniques, amperometric biosensor gives the highest sensitivity. Moreover, it gives fast responses at low costs.

The immobilization of enzyme in the fabrication of amperometric biosensors is a crucial step because it plays an important role for long term stability of sensors. Poor stability of biosensors is mainly caused by enzyme inactivation and leaching from immobile materials. Chitosan is widely used as matrices for enzyme immobilization due to its excellent properties such as biocompatibility and nontoxicity. However, there still exists enzyme leaching. There are several researchers whose

works involve improving the property of chitosan matrices to overcome this problem. Some researchers cross-linked chitosan and enzyme using glutaraldehyde [3] which established intermolecular cross-links with the amino groups of chitosan and enzyme. However, glutaraldehyde was found to cause enzyme inactivation. Therefore, combination of chitosan with other material or metal nanoparticles that have good interaction with the protein have been studied [4]. Moreover, metal nanoparticles was found to enhance the electron conductivity of chitosan.

In this work, we were interested in immobilization of horseradish peroxidase (HRP, EC 1.11.1.7) in Ag-chitosan composites and apply for phenol biosensors. In order to prevent enzyme leaching while keeping high enzyme activity, four different methods of enzyme immobilization were investigated and compared: (A) HRP entrapment in Ag nanoparticle/chitosan composite; (B) HRP entrapment in Ag nanoparticle/chitosan composite and cross-linked with glutaraldehyde solution; (C) HRP entrapment in Ag nanoparticle/chitosan composite and cross-linked with saturated glutaraldehyde vapor and (D) HRP entrapment in Ag nanoparticle/ glutaraldehyde cross-linked chitosan. The characteristics of response current of phenol biosensors depended on the glutaraldehyde cross-linking method used for enzyme immobilization.

2. Experimental

2.1 Reagent

Horseradish peroxidase (HRP, EC 1.11.1.7) was purchased from Toyobo, Japan and used as received. Chitosan (deacetylation degree of 95 %, MW 1,450 kDa), was purchased from Seafresh chitosan Co., Ltd., Thailand. Phenol was from Carlo Erba Regent Co. All other chemicals were of analytical grade and used without further purification. 0.1 M phosphate buffer solution (PBS) was prepared by mixing stock standard solutions of potassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) which were obtained from Ajax Fine Chem.

2.2 Apparatus

Electrochemical measurements of cyclic voltammetry and amperometry was performed with a Glucosen potentiostat (Chulalongkorn University, Thailand). The electrochemical cell consisted of a three-electrode system with a glassy carbon electrode (GCE) as the working electrode, a platinum wire as the counter electrode, and a silver/silver chloride (Ag/AgCl) electrode as the reference electrode. Before each experiment, the glassy carbon electrode (GCE) was first polished with 0.3 μm alumina slurry and rinsed with distilled water. Then, the electrode was sonicated respectively for 5 mins in absolute ethanol, and 5 mins in distilled water using an ultrasonic cleaner (CREST, model D, Malaysia). After the pretreatment, the GCE was dried in air before being dropped by specified chitosan matrices.

2.3 Synthesis of Ag nanoparicles

The preparation of Ag nanoparticle(20 - 1000 ppm) were obtained by chemical reduction of metal salts to yield the corresponding zero valent metal nanoparticles with $NaBH_4$. To ensure the entire reduction, the concentration of $NaBH_4$ was 10 times of metal salt. The reduction with this solution was carried out at 70 °C for 90 minutes and mixtures will change to yellow. Silver nanoparticle solution was stored in dark brown bottle at 4°C.

2.4 Fabrication of enzyme electrode

Schematic illustrations of the four different methods are shown in Fig. 1.

2.4.1 Method A

Chitosan solution (0.5 %w/v) was prepared by dissolving 0.5 g of chitosan powder in 100 mL of acetic acid (1%, v/v) HRP solution with a concentration of 10 mg/mL was prepared in 0.1 mol/L PBS (pH 6). Next, Chitosan, HRP, and Ag nanoparticles with a volume ratio of 5:1.5:1 were mixed thoroughly and stirred at 4 °C for 1 h. 5 μl of this mixture was dropped on the surface of the cleaned GCE. Then, this electrode was dried at room temperature. Before use, this electrode was rinsed with 0.1 mol/L PBS (pH 6) to wash out the nonimmobilized enzyme from electrode surface.

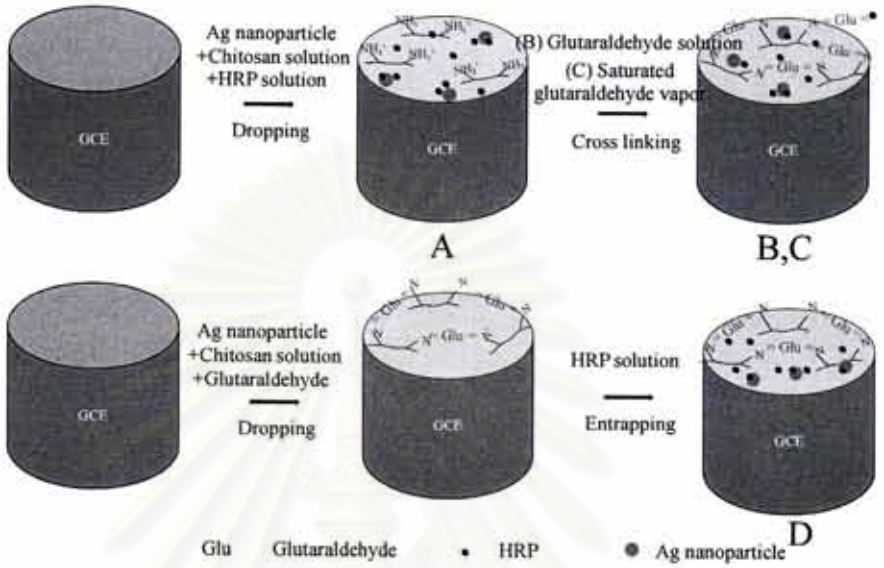

Fig. 1. Schematic illustration of four different methods for HRP immobilization.

2.4.2 Method B

Optimum matrix composition obtained from type A was used to fabricate type B electrode. And then, the modified GCE was the nonimmobilized enzyme from electrode surface.

2.4.3 Method C

Optimum matrix composition obtained from type A was also used to fabricate type C electrode. And then, the modified GCE was placed in saturated glutaraldehyde vapor for 15 min and then this electrode was rinsed with 0.1 mol/L PBS (pH 6) to wash out the nonimmobilized enzyme from electrode surface.

2.4.4 Method D

First, Chitosan, (0.0025-0.1% (v/v)) glutaraldehyde, and Ag nanoparticles with a volume ratio of 5:1:1 were mixed thoroughly and stirred at 4 °C for 1 h. 5 µl of this mixture was dropped on the surface of the cleaned GCE. Then, this electrode was dried at room temperature. Next the modified GCE was immersed in HRP solution for 15 min. Before use, this electrode was rinsed with 0.1 mol/L PBS (pH 6) to wash out the nonimmobilized enzyme from electrode surface.

immersed in glutaraldehyde solution (0.0025-0.1%v/v) for 15 min and then this electrode was rinsed with 0.1 mol/L PBS (pH 6) to wash out

3. Results and Discussion

3.1. Electrochemical characterization of Ag nanoparticle/chitosan/HRP/GCE based on phenol detection

The enzymatic mechanism of the amperometric biosensor based on HRP for detection of phenol is shown as follows [5]:

$$HRP(Fe^{3+}) + H_2O_2 \longrightarrow HRP(Fe^{5+}) + H_2O \quad (1)$$

$$HRP(Fe^{5+}) + AH_2 \longrightarrow HRP(Fe^{4+}) + AH^{\bullet} \quad (2)$$

$$HRP(Fe^{4+}) + AH_2 \longrightarrow HRP(Fe^{3+}) + AH^{\bullet} + H_2O \quad (3)$$

In each step, phenol (AH_2) is converted to free radicals ($AH^{\bullet}$), which are electroactive and can be reduced on the electrode surface. The reduction current is therefore proportional to phenol concentration. However, HRP(Fe^{5+}) can also be directly reduced on the electrode surface. Thus, the sensitivity of a biosensor for monitoring phenol is limited due to the background current of the direct electron transfer [5]. Fig. 2. shows the cyclic voltammogram of Ag nanoparticle /Chitosan/HRP/GCE in the absence and presence of phenol. It is clearly indicated that

this biosensor performed high phenol sensitivity due to the low background current in absence of phenol.

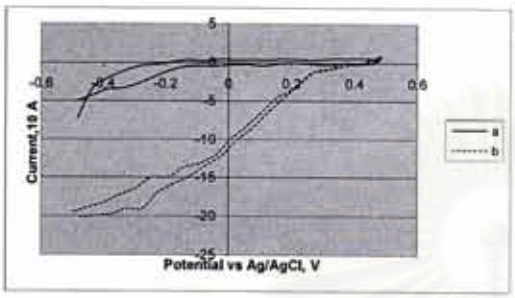

Fig.2. Cyclic voltammograms of Ag nanoparticle/Chitosan /HRP/GCE in the absence (a) and presence (b) of phenol at a scan rate of 50 mV/s and the potential between -500 mV and 500 mV in 0.1 M PBS(pH 7).

3.2 Optimization of experimental variables

3.2.1 Effect of silver nanoparticles

Fig. 3., demonstrates a significant effect of silver nanoparticles on electrochemical responses. HRP entrapment in Ag nanoparticle /chitosan composite without cross-linking by glutaraldehyde. The increase in response currents from 3 x 10^{-6} A to 8 x 10^{-6} A using respectively 20 and 100 ppm of Ag nanoparticles showed that Ag nanoparticles did help facilitating electron transfer. And then decreased when increased amount of Ag nanoparticle, due to increase hindrance to substrate or product mass transfers. Therefore, 100 ppm Ag nanoparticle was chosen for all subsequent biosensors.

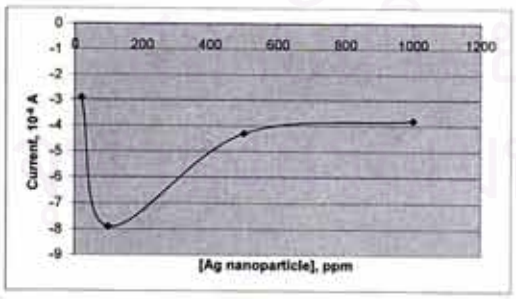

Fig. 3. Effect of amount of Ag nanoparticle modified electrode to 0.1 mM phenol in 0.1 M PBS(pH 7) at an applied potential of -0.05 V versus Ag/AgCl

3.2.2 Effect of glutaraldehyde concentration

The response current of modified electrode with the glutaraldehyde concentration range of 0.0025-0.1 %v/v was studied. Fig. 4., HRP entrapment in Ag nanoparticle/chitosan composite and then cross-linked with glutaraldehyde solution. The aldehyde group of glutaraldehyde reacted with the amino group of chitosan and HRP by covalent bonding. Glutaraldehyde effect to biocatalyst inactivation of enzyme, resulting in a bad responses current of biosensor. Moreover, the mount of cross-linking agent may produce biocatalyst inactivation [6]. The maximum response current was obtained at 0.0025 %v/v ($3.3x10^{-6}$A). Using saturated glutaraldehyde vapor, a lower signal was obtained ($1.2x10^{-6}$ A). Due to mass transfer was limited by thickness of saturated glutaraldehyde vapor film. Fig. 5., displays the response current of the modified electrode D in glutaraldehyde concentration range 0.0025-0.1 %v/v. The aldehyde group of glutaraldehyde reacted with the amino group of chitosan by covalent bonding and then entrapped HRP. It found that the current response decreased when increased the glutaraldehyde concentration, due to the enzyme entrapment was decreasing.

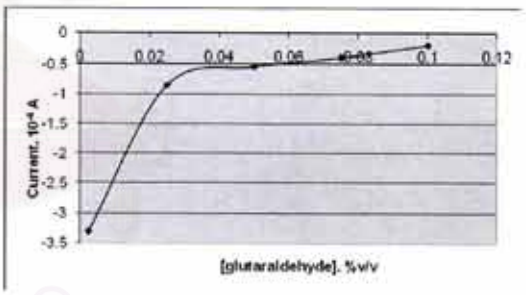

Fig. 4. Effect of glutaraldehyde concentration for electrode B to 0.1 mM phenol in 0.1 M PBS(pH 7) at an applied potential of -0.05 V versus Ag/AgCl

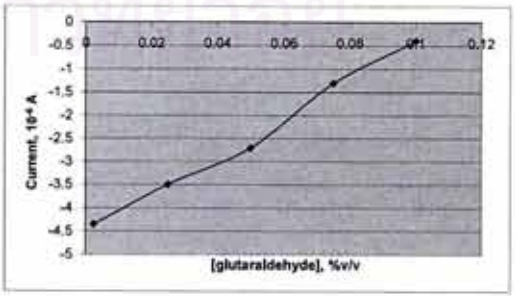

Fig. 5. Effect of glutaraldehyde concentration for electrode D to 0.1 mM phenol in 0.1 M PBS(pH 7) at an applied potential of -0.05 V versus Ag/AgCl

3.3 Comparison response current of modified electrodes

The optimum response current of four different electrode based on various glutaraldehyde cross-linking method were compare. It was found that the response current of electrode A, B, C and D were $7.9x10^{-6}$, $3.3x10^{-6}$, $1.2x10^{-6}$ and $4.3x10^{-6}$ A respectively. The biosensor with the best response current, obtained by the method A that without cross-linking by glutaraldehyde. The response current decreased when using glutaraldehyde due to enzyme may be inactivation. Electrode D had the response current more than electrode B and C due to enzyme entrapped in cross-linked chitosan which enzyme did not contact and react with glutaraldehyde.

3.4 Repeatability

Under optimal condition in the determination of phenol (0.1 mM), the repeatability of response current of all electrodes was also examined. The biosensor with the best repeatability obtained by the procedure C. It was found that the response current decreased approximately 50% in 5 successive assays, while electrode A could not repeatability. The results demonstrate that using glutaraldehyde improved leaching of enzyme from metric.

4. Conclusions

In this work, we are interested in investigating effects of glutaraldehyde cross-linking methods on enzyme immobilization in Ag nanoparticle /chitosan matrices for fabrication biosensor for the detection of phenol. The characteristics of response current of phenol biosensors depend on the method used for enzyme immobilization. The biosensor with the best response current, obtained by the method A. The response current decreased when using glutaraldehyde. But the repeatability improved by the cross-linking with saturated glutaraldehyde vapor.

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