Half-cell Reaction of Bienzyme-Based Glucose Bio cathode using Methylene Blue and Multiwall Carbon Nanotube on Modified Screen-printed Electrode

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Abstract—This paper describes the preparation of nanobiocomposite between methylene blue (MB) and bienzyme which was immobilized onto multi-walled carbon nanotubes (MWNTs) and then been applied on the screen-printed carbon electrode (SPCE) to form biocathode for biofuel cell. The half-cell reaction of biofuel cell was tested. The ratio of immobilized enzymes was varied to maximizing the output current density. It was found that the optimum ratio of bienzymes, HRP:GOx, on the electrode was 1:6. The developed cathodic electrode can obtain the current sensitivity of 0.88 µA/mM which greater than that used only the glucose oxidase (GOx) or horseradish peroxidase (HRP). Furthermore, the biemzyme-based electrode gives the best current density of 118 µA/cm² at lesser substrate concentration when it was compared with only one enzyme modified on SPCE at room temperature.

Keywords—Bienzyme, Biofuel Cell, Carbon nanotubes, Glucose oxidase, Horseradish Peroxidase, Methylene blue

I. INTRODUCTION

OVER the decades, the bioelectrochemical power generation via biological process has been used to enhance the process of generating electric power from various sources of energy. The production of electrical power uses a wide variety of fuels not only to biomass conversion, but also alternative energy source such as glucose in body for implantable in the form of medical devices. Enzymatic fuel cell basically uses glucose as fuel and oxidoreductase enzyme which directly converts chemicals into electrical energy through electrochemical reaction pathway. Some enzymes are able to directly interact with an electrode because of short electron tunneling distances so that the majority of these are small redox protein that metalloccenters (e.g. heme, copper or iron-sulfur clusters) and in their active site close to the protein surface [1-3]. However, the problem is that an enzyme such as glucose oxidase has the active site deeply embedded in the protein shell. Consequently, it is difficult for electron to transfer between the active center and electrode. In this case, the problem can be solved by the use of azine redox mediators to facilitate electron transfer with the electrodes at a low overpotential and with a high catalytic reaction rate such as methylene blue (MB) [4-6], which is the main mediator of the phenothiazine group having a good electrochemical reversibility and greater stability than electropolymerized form [7].

Recently, carbon nanotube (CNT) has been a considerable attention because of the physical and chemical properties which can adsorb biomolecules enzyme and methylene blue onto the side wall of the carbon nanotubes to form the π - stacking interaction. Hence, the excellent electroactivity and the high stability of nanocomposite layer can be obtained. Also, there has shown that CNT is very useful for electronic devices development such as biosensors and biofuel cell for practical application [8-10].

This study looks into a system of enzymatic fuel cell which consists of bienzyme immobilized onto an electrode. Biocathode with modified crosslinked with the redox mediator methylene blue (MB) were coated, firstly, with multiwall carbon nanotubes (MWNTs) by non-covalent adsorption used with bovine serum albumin (BSA) and glutaraldehyde on the surface of screen-printed carbon electrode. Thus, the first enzyme glucose oxidase (GOx) perform the glucose, which can oxidize glucose to H₂O₂ (the source of the substrate for the second enzyme) horseradish peroxidase (HRP) reaction can in turn be reduced to produce water. Therefore, bienzyme system can theoretically be applied in the enzymatic fuel cell. One interesting key is that the bienzyme has different kinetic characteristics, for instance, HRP has very fast reaction kinetics compared to GOx so that the substrates and products would be produced at different rate. The optimum ratio of enzymes on the electrode 1:1(unit of HRP to unit of GOx) was found [11]. Therefore, in this study, the optimized enzyme ratio to enhance current density and the practical optimal ratio of bienzyme will be examined.

This paper aims to investigate experimentally electrochemically the biocathode on a modified screen-printed carbon electrode with two enzymes: horseradish peroxidase (HRP) and glucose oxidase (GOx). To the best of our knowledge, our study also discloses that MWNTs and
combination of bienzyme on SPCE can help to compress more electrons from a glucose molecule. At the end, a discussion will be given on the application with fuel cell system. The present method offers a simple work and easy to follow, and can also improve efficiency of enzyme fuel cell.

II. MATERIALS AND METHODS

A. Materials

Peroxidase from horseradish (HRP, EC 1.11.1.7 188 U/mg solid) and glucose oxidase type X-S from Aspergillus niger (GOx, EC 1.13.4.190 U/mg solid) were purchased from Sigma-Aldrich. Multiwalled carbon nanotubes (MWNTs, purity > 95% diameter 20-40 mm, length 10-30 µm) was purchased from Cheap Tubes Inc Co. Ltd. (China). D (+) glucose, Methylene blue, bovine serum albumin (BSA) and glutaraldehyde were purchased from Sigma-Aldrich. Screen-printed carbon electrodes (surface area 3 mm²) were purchased from Quasense (Thailand). All other chemicals were analytical grade.

All electrochemical measurements were carried out in phosphate buffered saline (PBS, pH 7.0) which was prepared with 0.1 M Na₂HPO₄ and NaH₂PO₄ containing additionally 0.1 M KCl and stored at 4°C.

B. Equipments

Electrochemical measurements were performed using an Autolab PGSTAT 10 computer-controlled potentiostat (Eco Chemic, Netherlands) with the general purpose electrochemical GPES software operating system. Cyclic voltammetry (CV) and Differential pulse voltammetry (DPV) were employed to characterize the biocathode’s redox reactions. The screen-printed carbon electrodes used as working electrode were presented carbon working area and Ag/AgCl reference/counter area.

C. Preparation of MB-MWNTs

The fabrication of MB-MWNTs was done exactly as described previously [6-7]. In brief, MWNTs-COOH was added to MB solution in distilled water with sonication at room temperature. Then, the mixture solution was filtered by filtration (cellulose acetate filter, pore size 0.45 µm) and washed repeatedly until non-absorbed of MB can be observed. Finally the MB-MWNTs was dispersed in double distilled water.

D. Construction of the biocathode

The biocathodes were constructed by drop coating of an aqueous crosslinking precursor composed of HRP, GOx, BSA, MB-MWNTs and glutaraldehyde. Mixtures of HRP and GOx at different mass ratios were prepared in PBS prior to use. Then, BSA, MB-MWNTs and glutaraldehyde were added to enzyme solution and well mixed. For coating process, 5 µl of the precursor was applied on only a working electrode surface with an active area 3 mm². After drop coating, the electrodes were placed in a laminar hood at room temperature for 2 h to performing the crosslinking reaction. To remove the non-crosslinked, the electrodes were then washed with PBS pH 7.0. The constructed biocathodes were stored in PBS, pH7.0 at 4°C until use.

III. EXPERIMENTAL RESULTS

A. Electrocatalyst reduction of biocathode (GOx/HRP/MB-MWNTs on SPCE)

Cyclic voltammetry technique was used to examine the electrocatalyst activities of the GOx/HRP/MB-MWNTs on SPCE with and without glucose. Fig. 1 shows the cyclic voltammogram obtained from the modified electrode in the absence and in the presence of 2 mM glucose in 0.1 M of PBS (pH 7.0). In the absence of glucose (Fig. 2), the GOx/HRP/MB-MWNT shows the clear redox peaks. The oxidation and reduction peak potentials can also be observed at -0.25 and -0.35 V, respectively (Fig. 1, curve a). When 2 mM of glucose was added, the cathodic peak current was increased and caused to decrease in anodic peak current (Fig.1, curve b).

Fig. 1 Cyclic voltamograms of the GOx/HRP/MB-MWNTs on SPCE in 0.1 M PBS pH 7.0 (a) absent glucose and (b) present glucose of 2 mM at a scan rate of 40 mV/s

Fig. 2 Cyclic voltamograms of the GOx, HRP and GOx/MB-MWNTs on SPCE in 0.1 M PBS pH 7.0
In Fig. 1 and Fig. 2, the electrocatalyzed reduction of H₂O₂ by the HRP electrode and the effective electrobiochemical oxidation of glucose by the GOx in reaction mechanism allow the functionalized MB on MWNTs with GOx, HRP and biocatalysts. MWNTs obviously can serve as not only the connecting site to immobilize the enzyme adjacent to each other but also the bridge to effectively shuttle electrons to promote the electrical communication between the enzyme and electrode surface. The mechanism can be summarized as Eq. (1)-(3) and depicted as in Fig. 3.

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\begin{align*}
\text{Glucose} + \text{GOx} + \text{O}_2 & \rightarrow \text{gluconic acid} + \text{H}_2\text{O} \\
\text{HRP}_{(\text{red})} + \text{H}_2\text{O}_2 & \rightarrow \text{HRP}_{(\text{ox})} + \text{H}_2\text{O} \\
\text{HRP} + \text{MB}_{(\text{red})} & \rightarrow \text{HRP} + \text{MB}_{(\text{ox})} + \text{H}^+ + 2\text{e}^- 
\end{align*}
\]

GOx. It is clearly seen that the electrode prepared with HRP:GOx at a unit ratio of 1:6 has the highest current sensitivity. The biocatalyst response to glucose was, then, investigated and resulted in Fig. 5. The current response of the GOx/HRP/MB-MWNTs increases with glucose concentration which is proportional to the electric current. It can be seen that the current density of biocatalyst is higher than only the glucose oxidase (GOx) or horseradish peroxidase (HRP). When the glucose concentration was higher than 2 mM the current density decreases. However, at some point it will be fixed as called “saturation”. This shows that the ability of current generation is limited to the saturation point as the amount of substrate will not make the power up. Therefore, the concentration of glucose of 2 mM is suitable for use in our biofuel cell.

**B. Optimization of conditions for glucose sensitivity**

To improve the analytical characteristics of biocatalyst half cell reaction for building and developing a biocatalyst based fuel cell for glucose analysis, HRP and GOx were immobilized by crosslinking with MB-MWNTs, BSA and glutaraldehyde together. The different solutions containing the two enzymes were prepared at the unit ratio of enzyme from [11] to the unit enzyme this research the GOx adopted in the experiment has an activity 190 U/mg protein, and HRP 188 U/mg protein and expressing the two activities in unit of HRP to unit of GOx so vary enzyme 1:1 up to three times 1:15 as shown in Fig. 4.

![Fig. 3 Illustration of the reactions occurring at the SPCE electrode modified with GOx/HRP/MB-MWNTs](image)

**IV. CONCLUSION**

In summary, we have demonstrated the biocatalyst comprising of a carboxylated MB-MWNTs-modified on SPCE and a crosslinked matrix of biocatalyst, BSA and glutaraldehyde. It is found that the MB-MWNTs modification on SPCE is indispensable to achieve an active biocatalyst with high current density. The characteristic and efficiency for glucose concentration of nanobiocomposite showed good electrochemical activity than only enzyme. Additionally, it is suggested that the optimal ratio HRP:GOx of 1:6 provides a high sensitivity response to glucose. Finally, the application of the biocatalyst to biosensors and enzyme fuel cell are also demonstrated.

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**REFERENCES**


