Characterization of Enzyme Electrode from Nanochitosan Immobilized Glucose Oxidase on Carbon Paste Modified with Nanofiber Polyaniline for Biosensor Application

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Abstract
Quantitative glucose determination is very important in medical application, pharmaceutical and food industry. Biosensor based on glucose oxidase enzyme has been developed to detect glucose levels precisely, rapidly, and sensitive. On this research, we have fabricated enzyme electrode for amperometric biosensor to determine glucose. The enzyme was glucose oxidase (GOD) and was immobilized on chitosan nanoparticle, and then embedded into carbon paste modified with nanofiber polyaniline. The GOD electrode has been characterized by cyclic voltammetry and the result showed that GOD electrode performed on optimum pH 5.0 and optimum substrate (glucose) concentration was 40 mM. The Michaelis-Menten constant Km and Vm of enzyme was determined by amperometric method and calculated as 1.934 mM and 0.937 mA respectively. These results will be further used for development of glucose biosensor.

Keywords: glucose oxidase; chitosan; polyaniline; nanoparticle; enzyme electrode; biosensor

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Introduction
Biosensor is an analytical device that converts the concentration of the analyte into a signal (e.g. electrical) through biochemical reaction mediated by enzyme, amino acids, nucleic acids, immunochemicals, organelle, tissue, etc (Di Wei and Ari Ivaska, 2006). Glucose biosensor mainly used in pharmaceutical industry, food industry, and medical application to determine level of glucose precisely. It has been developed since 1962 by Leland C. Clark using glucose oxidase enzyme (GOD). Glucose biosensor is an enzyme sensor that uses GOD on electrodes as a sensing probe. GOD will catalyse the oxidation of D-glucose (as target analyte) to D-glucono-δ-lactone and will be further hydrolysed non enzymatically to gluconic acid and H₂O₂ (Sabir et al, 2007). The amount of released H₂O₂ is usually determined by amperometric/electrochemical methods by oxidation H₂O₂.at the working electrode. Electrons that were released from this reaction will be detected as an electrochemical signal.

Enzyme electrode consist a combination of electrochemical probe (amperometric, conductometric, or potentiometric) with immobilized enzyme. Enzyme immobilization can be produced through several mechanisms namely crosslinking, entrapment, adsorption, encapsulation, or carrier-binding (Gorecka dan Jastrzebska 2011). Glutaraldehyde usually used as matrix for glucose oxidase immobilization (Colak et al. 2012), but unfortunately glutaraldehyde has toxic effect. Due to this issue, on this research we used nanoparticle chitosan as matrix to immobilize GOD. Chitosan is a biopolymer that has ability to form good membrane, no toxic effect, low immunogenicity, high mechanical strength, consists amine group, and has high stability due to its hydrophilic effect. Because of its advantages, chitosan is a promising immobilization matrixes (Nakorn, 2008). Nanoparticle of chitosan even has more advantages to enhance the bio catalytic of immobilized glucose oxidase due to increase of surface area and number of functional group that bond to the enzyme.

Another way to improve biosensor capability is by enhance the movement of electron pair from enzymatic reaction on the electrode. On this research we used carbon paste modified by nanofiber polyaniline. Polyaniline is conductive polymer that can help movement of electron pair from enzymatic reaction on electrode (Keyhanpour et al., 2012), hopefully this will make glucose biosensor has better response and stability.

Researches of glucose biosensor have been widely carried out, but the development of glucose oxidase immobilized on chitosan nanoparticle for enzyme electrode modified with nano polyaniline has not been conducted before. Our research was aimed to characterize the enzyme electrode made from...
immobilized glucose oxidase with nanochitosan and embedded into nanopolyaniline modified carbon paste electrode. The information gained from this research hopefully can be used for further glucose biosensor development.

Material and Methods

Nanofiber polyaniline (particle size of110-120 nm based on SEM 7500x) was received from Akhiruddin Maddu (2014), Glucose oxidase (Sigma Aldrich: EC.1.1.3.22, purified from Aspergillus niger, 7200 Unit/mL), sodium tripolyphosphate (STPP), graphite powder, liquid paraffin, bovine serum albumin, acetic acid, sodium acetic, Na3PO4, NaH2PO4, KCl, potassium ferricyanide.

Homogenizer (Dispergierstation TB.10 IKA), ultrasonic processor (130 Watt 20 kHz, Cole-Parmer), VASCO particle size analyser equipped with nanoQ software, pH meter (HANNA pH 21), glass cylinder (outside Ø 1.2 cm, inside Ø 0.9 cm) connected with Cu-wire, eDAQ Potensiotstat-Galvanostat (Ecoder 410) equipped with Echem v2.1.0 software.

Chitosan Nanoparticle Preparation

Nanoparticle chitosan were prepared according to Nakorn 2008 with modification. 20 mg of Chitosan solubilized on 40 mL acetic acid 2%v/v, homogenized with 20 mL tripolyphosphate (TPP) 0.75 mg/mL by magnetic stirrer at 13500 rpm for 10 minutes at room temperature. Solution than sonicated using ultrasonicator at amplitudo 40% for 5 minutes. Nanoparticle chitosan was obtained after centrifugation at 12000 rpm for 30 minutes and then kept in aquabidest at 4 °C. Size of chitosan nanoparticle was determined by particle size analyser (PSA).

Carbon Paste Electrode and Modified Carbon Paste Electrode Preparation

Carbon paste electrode (CPE) was prepared by mixing 0.15 mg graphite powder and 100 µL liquid paraffin on mortar and filled into electrode tube (0.9 cm diameter, 4 cm length, and 0.7 cm height) which already connected with Cu wire. The modified carbon paste electrode (MCPE) was made from 2 mg polyaniline and 0.15 gram graphite powder that already mixed with 100 µL liquid paraffin. The electrode surface was smoothed with paper. (Colak et al. 2012)

Enzyme Electrode (GOD/MCPE)

Enzyme electrode of GOD/MCPE was prepared according to Colak et. al 2012. First, enzyme was immobilized with nanochitosan. Buffer that used for enzyme immobilization were acetic buffer 0.1 M pH 4.0-6.0 and phosphate buffer 0.1 M pH 6.5-8.0. GOD immobilization were made by mixed37 mL glucose oxidase (7200 Unit/mL), 1 mg bovine serum albumin (BSA), 63 µL buffer pH varied4-8, 30 µL nanochitosan 2.5% and then left for 15 minutes at 30 °C. The immobilized GOD than poured into MCPE, allowed it to dry at 4 °C and rinsed by the same pH buffer to remove the free GOD and nanochitosan. Enzyme electrode must be kept in buffer at 4 °C until use.

Amperometric Measurements

Electrochemical signal were measured by eDAQ potensiotstat-galvanostat equipped with Echem v2.1.0.software. Comparison electrode was Ag/AgCl, Pt was auxiliary electrode, and enzyme electrode was the working electrode. A mixing of buffer 0.1 M 1 mL, 1 mL potassium ferricyanide 100 mM as mediator (Fadhilah 2013), and 180 µL glucose 250 mM that has been lass for 24 hours to mutarotated, were added to the electrochemical cell. MCPE without enzyme was used as blank. (Colak et al. 2012)

Determination of Optimum pH

Acetic buffer and phosphate buffer 0.1 M with varied pH 4-8 were used to determine optimum pH of GOD/MCPE. MCPE without enzyme was used as blank. 1 mL buffer, 1 mL potassiam ferricyanide 100 mM, 180 µL glucose 250 mM were added to the electrochemical cell, and the current were measured. (Colak et al. 2012)

MaximumSubstrateConcentration (Colak et al. 2012), Sensitivity (Fadhilah 2013), and Enzyme Kinetics (Hoshino et al. 2012)

1 mL buffer, 1 mL potassiam ferricyanide 100 mM, 180 µL glucose with varied concentration 0.1 mM-60 mM were added to the electrochemical cell, and the current was measured.

Kinetics of immobilized GOD were determined using Michaelis-Menten equation:

\[ I = \frac{I_{\text{max}}[D - \text{glucose}]}{K_m + [D - \text{glucose}]} \]

\[ I_{\text{max}} \] is maximum measured current response, \[ K_m \] isMichaelis-Menten constant, dan [D-glucose] is glucose concentration. Obtained Michaelis-Menten then was derivated to be a plot Lineweaver-Burk plot.

Results and Discussion

Performance of CPE and MCPE

CPE was prepared from graphite powder and liquid paraffin while MCPE was added polyaniline nanofiber. Graphite powder function were to strengthen the electrode, minimize friction, and enhance the current stability. Liquid paraffin on the other hand was added to bound the graphite powder to make it remain stable duringamperometric measurement (Svancara et al. 2002).
Performance of CPE and MCPE were compared to justify which electrode will be used to prepare the GOD electrode. Cyclic voltamogram from CPE and MCPE are shown in Figure 1. Current produced from CPE and MCPE were 0.276×10^{-4} A and 1.209×10^{-4} A respectively (Table 1). MCPE resulted in a higher current peak on anode than CPE and this result indicated that MCPE was better than CPE, and will be used to prepare GOD electrode.

![Cyclic Voltamogram CPE and MCPE at 100 mVs⁻¹, potential 1 V, Initial E 0 mV, final E 0 mV, upper E 1000 mV, and lower E -500 mV.](image)

**Figure 1.** Cyclic Voltamogram CPE and MCPE at 100 mVs⁻¹, potential 1 V, Initial E 0 mV, final E 0 mV, upper E 1000 mV, and lower E -500 mV.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Potential energy (V)</th>
<th>Maximum current (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPE</td>
<td>-0.304</td>
<td>0.276×10^{-4}</td>
</tr>
<tr>
<td>MCPE</td>
<td>-0.085</td>
<td>1.209×10^{-4}</td>
</tr>
</tbody>
</table>

Table 1. Peak anode current of CPE and MCPE

The increase current of MCPE was because of polyaniline addition. Polyaniline that added to the carbon paste filled the porous between graphite particle and hence resulted the electrical conductivity on electrode because the movement of electrons were unbreakable (Suriyant et al. 2013). Polyaniline was a conductive polymer which have capability to deliver electrons from biochemical reaction (Gaikward et al. 2006). The conductivity of polyaniline was resulted from HCl addition as protonic acid during polymerisation (Maddu et al. 2008).

**Optimum pH of GOD/MCPE**

pH influence to GOD/MCPE response has an important role in spreading charges on GOD active site which in the end will determine the enzyme activity. Enzymatic reaction of GOD and glucose was measured amperometrically resulted as current. Correlation between pH and current value on GOD/MCPE was described in Figure 2. The highest current value on peak anode (1.8655 mA) was shown on pH 5.0 while the lowest (0.2898 mA) was shown on pH 6.0. It means that maximum response of immobilized GOD on MPCE was reached at pH 5.0. Optimum pH of our immobilized GOD/MCPE was the same with Colak et al. (2012) which immobilized GOD with glutaraldehyde/polyaniline. On the other hand the optimum pH of immobilized GOD was different to free GOD which was 5.5 (Triana 2013).

![pH influence to current measurement of GOD/MCPE (Temperature 25°C, glucose concentration 0.25 M).](image)

**Figure 2.** pH influence to current measurement of GOD/MCPE (Temperature 25°C, glucose concentration 0.25 M).

Chitosan that was used to immobilized GOD was a polycationic polymer and also consist amine group on its structure, enable it to immobilize many enzymes (Tanget al 2006). Particle size of chitosan nanoparticle that was prepared to immobilize GOD was 152.9 nm. Nanoparticle of chitosan provided more surface area and probability of functional group to bind with enzyme produced increase in enzyme immobilization on the surface of nanochitosan (Nakorn et al. 2008). Immobilization of enzyme could lead to conformation changes of enzyme yet to ionisation of side chain of amino acids, and in the end also resulted in alteration of pH optimum. Ionisation at specific pH influenced the complex enzyme-substrate and resulted in increasing current. Polyaniline also played a role, it has higher conductivity at pH 5.2 dan pH 7.3 (Colak et al. 2012).

**Enzyme Kinetics**

Amperometric measurement of GOD/MCPE at variation substrate concentration was done at room temperature to facilitate practical biosensor application. Correlation between substrate concentration and current measured of GOD was presented in Figure 3 as Michaelis-menten curve. Current value on peak anode was equivalent to GOD activity. After substrate concentration reached 20 mM, GOD showed its maximum activity at 1.44765 mA. At this point, enzyme was saturated with substrate and more addition of substrate will not significantly change enzyme activity (Mikkelsen and Corton 2004). Linearity of this measurement was required to determine maximum activity of enzyme electrode (Figure 4) and showed that sensitivity of GOD/MCPE was 0.20 mAmM⁻¹. Linearity of substrate concentration lied at range 0.6-1.8 mM, obtained from the higher determination coefficient (0.9955) which closely to requirements of determination coefficient by ICH (0.99700 (Safrizal, 2011)).
Michaelis-Menten constant, $K_m$ was determined by Lineweaver-Burk method (Figure 5), and calculated as 1.934 mM. Enzyme reaction rate was expressed as maximum current ($I_{max}$), calculated as 0.973 mA. Km of GOD/MCPE was lower than Km of free GOD (33–110 mM, Sigma Aldrich 2014). This result indicated that immobilized GOD had higher affinity to substrate than free GOD. Tang et al. (2006) also reported that lipase immobilized on nanochitosan had lower $K_m$ (0.37 x $10^2$ g/L) than free lipase (1.01 x $10^2$ g/L). Interaction of enzyme-nanoparticle chitosan protected enzyme from inactivation, therefore the activity of enzyme increased. Nanofiber polyaniline on MCPE also function as matrix that immobilized GOD, because polyaniline has ammine group which can interact with GOD (Tang et al., 2006).

Compared to $K_m$ of GOD/MCPE from Colak et al 2012, ours is still higher. The difference was on immobilization matrix. Colak used glutaraldehyde as matrix to immobilized GOD. Enzyme that bond to ammine group of glutaraldehyde during immobilization produced high stability and not easy to solubilise so prevent inactivation of enzyme because of enzyme folding (Migneault et al., 2004).

After all, $K_m$ of our GOD/MCPE if converted to mg/dL was 34.8 mg/dL, this indicated that our enzyme electrode was potential to develop further into glucose biosensor even to determine hypoglycaemia (blood glucose level below 60 mg/dL) (Shan et al., 2010). Biosensor fabrication require optimum condition and kinetic parameter of enzyme to determine the performance of enzyme electrode. Another parameter that needed is sensitivity of electrode. Sensitivity describe the response of instrument to changes of analyte concentration. Sensitivity of our GOD/MCPE was 0.20 mA/mM: means every elevation of 1 mM of glucose will elevated the current of 0.20 mA. This value showed that the enzyme electrode quite responsive to slightly changes of analyte (Fadhillah 2013).

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Conclusion

GOD/MCPE worked optimum at pH 5.0 and substrate concentration 20 mM. Linearity for working range of GOD/MCPE was between 0.6–1.8 mM, and the sensitivity was 0.20 mA/mM. $K_m$ dan $I_{max}$ were calculated as 1.93 mM dan 0.97 mA respectively, indicated that the GOD/MCPE potential to be further developed to blood glucose biosensor.

Citation and References


Hoshino T, Sekiguchi S, Muguruma H. 2012. Amperometric biosensor based on multilayer containing carbon nanotube, plasma-polymerised film, electron transfer mediator phenothiazine, and glucose


