Improvement of Glucose Biosensor Performances Using Poly(hydroxyethyl methacrylate) Outer Membrane

Norhana Jusoh, Azila Abdul-Aziz, Eko Supriyanto

***Abstract*—**A glucose biosensor was developed based on immobilization of glucose oxidase and poly(allylamine) ferrocene by layer-by-layer covalent attachment. Factors that can affect current response, response time, enzyme kinetics and membranes stability membrane were discussed. The apparent Michaelis-Menten constant, K_m^{app} obtained were quite low but was increased up to 28.68mM due to effect of nafion layer to the multilayer membrane. To further improve the glucose biosensor performances, a cross-linked poly(hydroxyethyl methacrylate) (pHEMA) membrane was attached to the multilayered-nafion membrane to extend the linearity of sensor response and also to protect the biosensor. Multilayered-nafion membrane with cross-linked pHEMA demonstrated acceptable K_m^{app}, which was around 40.58mM with high sensitivity and fast response time towards the glucose.

***Keywords*—**Biosensor, Enzyme Kinetics, Outer Membrane, Cross-linking, Poly(hydroxyethyl methacrylate).

I. INTRODUCTION

For decades, the search for an ideal glucose biosensor continues to be one of the main motivations in sensor research due to its biological importance [1]. Glucose is involved in human metabolic processes as the primary source of energy for the brain as well as all other cells in the body [2, 3]. The consequences of poor glucose regulation are, long term damage to organs from too much glucose (hyperglycemia) and coma or death caused by too little glucose reaching the brain (hypoglycemia) [3]. The insulin dose must be adjusted to minimize hyperglycemia while avoiding serious hypoglycemia [4].

Numerous attempts have been made to fabricate glucose biosensors because of the clinical significance of measuring blood glucose levels [5]. Half of biosensor research papers published is on glucose detection [6]. For example, a comparative study was proposed by previous researcher to evaluate the use of fuzzy logic controllers over other conventional controllers to maintain the blood glucose level within a normoglycemia (normal concentration of glucose in blood) average especially when diabetic patient is subjected to different condition [7].

A biosensor is a device incorporating a biological sensing element connected to a transducer [2]. The use of biological component for molecular recognition contributes to the high specificity of the biosensor. The biological component will transform the analyte to a quantifiable property that can be transformed into an electrical signal by the transducer. Researchers have shown a keen interest in the application of biosensors due to the characteristic of biosensors that are easy-to-use, cheap, fast and highly sensitive methods for recognition of biomolecules [8].

Biosensors have been divided into optical, calorimetric, piezoelectric, electrochemical, field effect transistor (FET) and surface acoustic waves based on the type of transducer used [2, 9]. The most commonly used biosensor is electrochemical biosensors [9]. The main advantage of biosensor over other conventional analytical methods is the simplicity of analysis and possibility of continuous detection of the analyte [10].

Electrochemical biosensors can be divided into conductometric, potentiometric and amperometric biosensors. Amperometric have been widely used because of its capability to directly transduce the rate of reaction into current. Amperometric devices detect the production of current when constant potential is applied between two electrodes. Currents are generated when electrons are exchanged between a biological systems and electrode. Potentiometric and amperometric are mostly adopted transducers since both methods are simple to use and electrodes based on these transducers can be miniaturized without great difficulty [10].

Generally, glucose biosensors are based on the fact that the enzyme glucose oxidase (GOD) catalyzes the oxidation of glucose to gluconic acid [2]. Even though expensive, GOD enzymes are stable over a long period of time and are also highly selective for glucose in the presence of other sugars in blood solution [6]. With a combination of advanced electrochemical technique and high substrate specificity,

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amperometric glucose biosensor represents the most successful commercial biosensor development with rapid and accurate diagnosis [11].

Electrochemical sensing of glucose has been classified according to three generations. In the first generation, GOD enzyme catalyzes the oxidation of glucose to gluconic acid with the production of hydrogen peroxide. However, by using an artificial electron acceptor or mediator to replace the natural acceptor oxygen, this second generation approach is preferable as it is capable of overcoming tissue oxygen dependence problem suffered by first generation glucose biosensors. In addition, the oxidation of the reduced mediator occurs at low potential thus reducing the sensitivity of the sensor to interfering substances [12, 13, 2, 9]. The third generation biosensors are based on directly coupled enzyme electrode using conducting salt electrodes [2].

There are three main steps in the reaction of the mediators with glucose oxidase [14]. The first step is the diffusion of the substrate from the bulk solution to the surface of the enzyme. Then, the second one is the transfer of the electron from the reaction centre of the enzyme to the mediator. Finally, electron is transported from the mediators to the electrode. Meanwhile, the distance between the electrode and the reaction centre of enzyme will influence the membrane response time. Other restraining factor in glucose biosensor is limited linearity of the biosensor response to higher glucose concentrations. Since glucose level in diabetic blood can reach up to 30mM [15, 16], limited linearity of the calibration curve will pose a problem as direct measurement without any dilution is impossible.

Various coating solutions have been employed as outer membranes to extend sensor linearity and to protect the membrane. Negatively charged polymer nafion has also been widely to control the substrate diffusion into the membrane. With negatively charged sulfonate groups, nafion can be used to reduce the diffusion of small neutral or negatively charged interfering species such as ascorbic acid and uric acid to the catalytic electrode surface. Nafion is biocompatible to the enzymes since it has both hydrophilic and hydrophobic properties, is chemically inert and is subjected to relatively little adsorption of species from the solution [17, 18]. Besides, nafion layer preparation is simple and the permeability of this layer in whole blood is reproducible [18].

Using nafion as external diffusion and additional interfering eliminating layer, Yang *et al*. [18] was able to extend the linear range of the sensor response to over 25mM glucose concentration. Nafion also gave short response time, which was only about 10s and a low background current, which was around 10–40nA as a consequences of the nafion effect that restricted the access of the larger molecules and anions to the electrode [18].

Liu and Deng also used nafion film as an outer membrane in order to protect the biosensor from fouling agents and interferents as well as an inner membrane to prevent the leaking of tetrathiafulvalene mediator [19]. As the results, the biosensor responds with linear range from 0.3mM to 10mM. In addition, when a nafion film was employed, the linear range was extended from 6 to 30mM glucose [20].

Pickup *et al.* has used cellulose dialysis membrane as an outer membrane and able to obtain a linearity up to 30mM [21]. Choi *et al.* also reported that the glucose biosensor response was linear up to 10mM by using cellulose acetate with poly(ethylene-glycol) (CA-PEG) as an outer membrane [22]. Yu *et al.* used epoxy resin-polyurethane outer membranes and the sensor gave a linear range from 1-25mM [23]. Ihab *et al.* reported that linear range up to 26mM, 31.1mM and 37.7mM were obtained by using cellulose acetate, polyurethane and polyvinylchloride coating solutions as the outer membrane, respectively [24].

Cross-linked poly(2-hydroxyethyl methacrylate) (pHEMA) has also been studied as a potential protection membranes since these hydrogels have good mechanical strength with controllable glucose solute permeability. Kermis *et al.* have investigated the transport properties of pHEMA at different cross-linking ratio as potentially retaining and protection membranes for optical glucose affinity sensors [25]. The cross-linked pHEMA hydrogels membranes exhibited good protein rejection with small glucose solute permeability. Besides, cross-linked pHEMA was quite promising in eliminating electrochemical interferences, such as ascorbic acid and acetaminophen, that commonly faced by a hydrogen peroxide based biosensor [26].

Hydrogels are hydrophilic polymers that can absorb water or other biological fluid [27, 28]. These pHEMA hydrogels have been widely used in biomedical and pharmaceutical applications since these biocompatible hydrogels have good mechanical strength, chemical resistance, high water content and are similar to natural tissues [25, 28]. For example, pHEMA hydrogels have been widely used as a soft contact lens material and in implantable applications such as artificial tendons, artificial liver, artificial skin, drug delivery systems and insulin delivery systems. Therefore, pHEMA hydrogels can be an appropriate outer membrane since these hydrogels have ability to limit glucose substrate substrate by controlling glucose solute permeability.

In this work, glucose oxidase and redox poly(allylamine) ferrocene was immobilized by layer-by-layer covalent attachment with the addition of nafion. The membranes was constructed by the formation of Schiff base bonds between aldehyde groups of periodate-oxidized GOD and amino groups of PAA-Fc as proposed by Zhang *et al.* [29]. To improve the glucose biosensor performances, a cross-linked poly(2-hydroxyethyl methacrylate) (pHEMA), external membranes was attached to the multilayered-nafion membranes to extend the linearity of sensor response and also to protect the biosensor. All electrochemical studies were done by attaching the membranes to platinum electrode. The performance of this glucose biosensor was evaluated amperometrically at 0.363V by using a potentiostat.

II. MATERIAL AND METHODS

A. Materials

Glucose oxidase (E.C. 1.1.3.4) from *Aspergillus niger* were purchased from Sigma (England). Ferrocene carboxaldehyde (98%), hydroxyehtyl methacrylate (HEMA), ethylene glycol dimethacrylate (EGDMA) and 2, 2-dimethoxy-2-phenylacetophenone (DMPP) were purchased from Aldrich (Germany). Cystamine dihydrochloride (98%) was purchased from Aldrich (China). Peroxidase horseradish (E.C. 1.11.1.7, type VI from Horseradish), glucose (corn sugar, 99.5%), sodium borohydride (98%), etylene glycol, poly(allylamine) hydrochloride (Average MW 70 000), triethlamine, sodium m-periodate) were purchased from Sigma (USA). Nafion solution (5% in a mixture of lower aliphatic alcohols and water) was bought from Fluka (USA). Kalium di-hydrogen phosphate, di-kalium hydrogen phosphate, kalium chloride, and hydrochloric acid were purchased from Merck (Germany). All chemicals were used as received.

B. Instrumentation

Electrochemical measurement was carried out using computer controlled potentiostat, Autolab with General Purpose Electrochemical System (GPES) software version 4.9 (Metrohm, Netherlands). A three-electrode electrochemical cell was used for the measurement. The working electrode (WE) used was a platinum electrode. A platinum auxiliary electrode was used as the counter electrode (CE). An Ag/AgCl/KCl was employed as the reference electrode (RE). All amperometric experiments were performed at a temperature of 25±1°C and under deoxygenated conditions.

Casting of pHEMA membrane coatings was using a G3P-8 Spin coater with a D6004 dispenser (Cookson Electronics Speciality Coating Systems Inc, Indianapolis).

C. Methodology

1. Synthesis of Periodate-oxidixed Glucose Oxidase

Carbohydrate groups on the surface of the glucose oxidase molecule were oxidized with sodium periodate to obtain carbaldehyde groups [19]. 0.05g of GOD in 15mL of 0.1M phosphate buffer solution (pH 6.8) was stirred slowly with 200mg of sodium metaperiodate for 1 hour at 4°C in the dark. The reaction was stopped with the addition of 7μL ethylene glycol for 30 minutes at 25°C. Then, the product was enclosed in dialysis tubing. The outer buffer phosphate solution was stirred and exchanged many times with fresh buffer. This dialysis procedure was carried out for 3 days.

2. Synthesis of Poly(allylamine) Ferrocene

Poly(allylamine) ferrocene (PAA-Fc) was synthesized according to the method by Zhang *et. al.* [29]. 70mg ferrocene carboxaldehyde was dissolved in 10mL methanol. Then, ferrocene was added drop wise within an hour to 60mL of methanol solution containing of poly(allyamine) hydrochloride (160mg) and triethylamine (1mL). The mixture was stirred for another hour at room temperature. Then, 40mg of sodium borohydride was carefully added at 0°C, and the stirring was continued for 90 minutes. Finally the mixture was freeze-dried and the residue was extracted with distilled water. The aqueous solution was further purified by enclosing in dialysis tubes against water. This dialysis procedure was carried out for 3 days.

3. Production of Covalently Linked Enzyme and Poly(allylamine) Ferrocene

Amino groups were introduced on the glass slide by pipetting 75μL of an aqueous solution of cystamine dihydrochloride (10mM). Then, 75μL of periodate-oxidized GOD solution in 0.1M phosphate buffer (pH 6.8) was added to the amino group's layer at room temperature. The GOD/PAA-Fc bilayer was formed by pipetting 75μL PAA-Fc solution in 0.1M phosphate buffer on the resulting enzyme monolayer. A covalently attached enzyme multilayer film was fabricated by repeating the coating of periodate-oxidized GOD solution and PAA-Fc solution in a cyclic fashion [19]. For multilayered-nafion membranes, 75μL of 0.5% nafion was added to the membrane as an initial and final layer. 0.5% nafion solution was prepared by diluting the commercial 5% (w/v) nafion solution with distilled water [19]. Every layer was dried under ambient condition after each casting before storage in a refrigerator at 4°C overnight. The enzymatic membranes were kept at 4°C in the refrigerator when not in use.

4. Detecting of Enzyme and Mediator Leakage

Enzyme leakage was measured colorimetrically. 150μL of 18% aqueous glucose solution and 50μL of 200μg/mL peroxidase solution were added to 1.25mL of chromogen solution at 25˚C. The chromogen solution was prepared by diluting 0.1mL of 1% o-Dianisidine in 12mL of 0.1M phosphate buffer, pH6.7. Then, 50μL of the washing solution was added to the mixture for 5 minutes at 25°C before 100μL of 4M HCl was added to stop the reaction. The absorbance value was read at 450nm. Leakage of ferrocene derivatives mediator was measured electrochemically by subjecting the washing solution to cyclic potentials [30] from 100-600mV with a scan rate of $10mVs^{-1}$.

5. Casting of pHEMA Outer Membrane

HEMA monomer with 40% (v/v) of deionized water [25] was mixed with ethylene glycol methacrylate (EGDMA) at cross-linking ratio of 0.0025. The photoinitiator, DMPP (2, 2-dimethoxy-2-phenylacetophenone) was added to the mixture solution in a vial to achieve a final concentration of 1.6wt% [31]. An aliquot of the mixture was transferred onto a multilayered-nafion layer on a glass slide. Then, the glass slide was placed under an UV light and irradiated for 5 minutes under continuous purging with nitrogen gas in a spin

coater. The membrane layer was then soaked in 0.1M phosphate buffer (pH6.7) for 48 hours to hydrate the pHEMA layer. Cross-linked structure of HEMA-EDGMA is illustrated in figure 1.

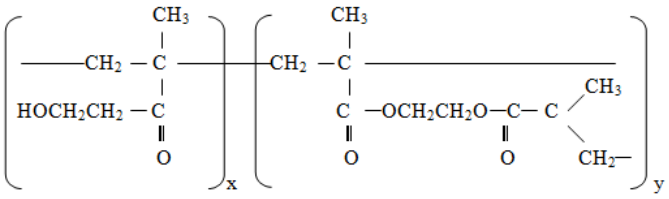

Fig. 1 Cross-linked structure of HEMA-EDGMA

6. Membrane Thickness Measurement

The thickness of the membrane was determined using a digimatic micrometer (Mitutoyo, Japan). Firstly, the thickness of two glass slides was measured. Then, membrane was sandwiched between two glass slides and the thickness of glass slides with membrane was measured. Finally, the thickness of membrane was obtained from the differences between the two measurements.

III. RESULT AND ANALYSIS

A. Retention of Enzyme and Mediator in Membranes

The washing solutions for the multilayer membranes were assayed for any sign of enzyme activity and mediator leakage in order to investigate the ability of the membranes in retainning GOD and ferrocene mediator. Figure 2 and 3 show the leaking profiles of GOD and ferrocene for the multilayered membranes.

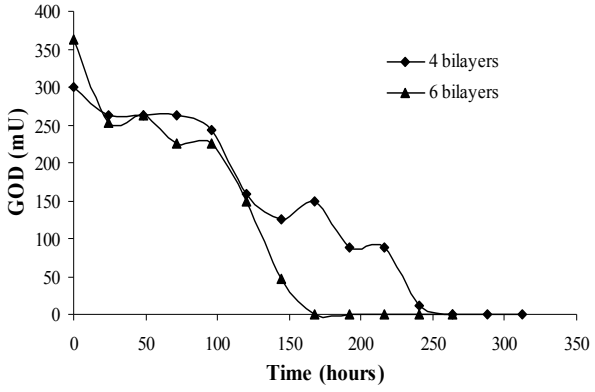

Fig. 2 Leaking profile of enzyme for multilayered membranes

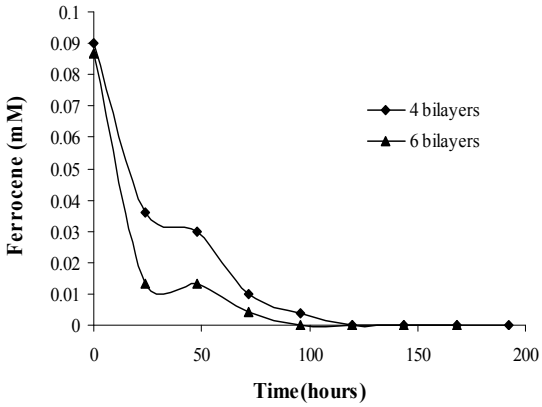

Fig. 3 Leaking profile of ferrocene mediator for multilayered membranes.

Figure 2 and 3 show that the leaking of enzyme as well as mediator decreased with time for the two types of membranes. No sign of enzyme activity was observed in the washing solutions after 11 and 7 days for membranes with 4 and 6 bilayers, respectively. Meanwhile, leakage of ferrocene from membranes with 6 bilayers stopped after 4 days, which was 1 day earlier than the membranes with 4 bilayers. For both membranes, the leakage of ferrocene stopped earlier compared to the enzyme.

Thus, these membranes cannot be applied for implantable glucose biosensor due to poor retention of the mediator and enzyme. Nevertheless, the leaking was not a problem for disposable glucose sensor. The leaking might be due to the possibility that the enzyme or mediator concentration might have exceeded the immobilization capacity of the membranes. The excess enzymes or mediator were not immobilized within the solid support and leached out easily from the membrane. The amount of leached enzyme was reduced by reducing the enzyme loading, but the problem with long leaking period is remained [32].

B. Kinetics Properties of the Membranes

The catalytic reaction that converts the substrate into product involves the formation of a transition state [33]. For glucose biosensor, the enzyme glucose oxidase catalyzes the oxidation of glucose (substrate) to gluconic acid (product). The enzyme substrate complex is the complex, when substrate is combined with the enzyme. Therefore, enzyme interfaced biosensors involves enzyme-substrate interaction that requires two significant applications which are monitoring of human glucose and also monitoring biochemical reaction at a single cell level [33].

Kinetics of simple enzyme-catalyzed reactions is often referred to as Michaelis-Menten kinetics or saturation kinetics that was developed by L. Michaelis and M. L. Menten in 1913 [34]. The parameter for Michaelis-Menten kinetics can be

determined from steady state currents and the electrochemical version of the Lineweaver-Burk equation [35, 36]. The apparent Michaelis-Menten constants, K_m^{app} and maximum current, I_{max} were determined from steady state currents and the electrochemical version of the Hanes-Woolf equation. I_{max} is the current at very high and saturated concentrations of substrate. Under these conditions, every enzyme molecule will have substrate attached to it and will be interacting with it to convert it to product as fast as it can. K_m^{app} corresponds to the substrate concentration at half–maximal current. K_m^{app} and I_{max} were calculated from below equations:

$$\frac{[S]}{I} = \frac{1}{I_{max}}[S] + \frac{K_m^{app}}{I_{max}} \quad (1)$$

$$I_{max} = \frac{1}{\text{slope}} \quad (2)$$

$$K_m^{app} = y - \text{intercept} \times I_{max} \quad (3)$$

where, [S] is substrate concentration, mM,

I is steady state current, A.

In this work, two types of enzymatic membranes were prepared, either with 4 bilayers or 6 bilayers.

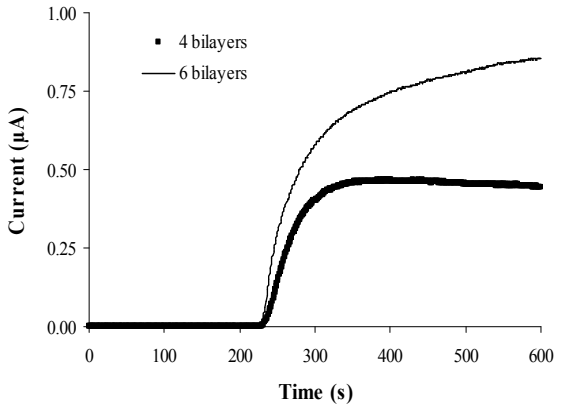

Fig. 4 Typical current responses of multilayered membranes

As shown in figure 4, by increasing the number of bilayers from 4 to 6, the current response was increased from 0.54μA to 0.83μA, respectively. By increasing the number of bilayers, the GOD concentration also increased and thus causes an increased in current response. Although ferrocene concentration also increased, extra ferrocene did not affect activity of the sensor since the effect of ferrocene was not large compared to that of enzyme [37].

Enzyme loading is one of the effects that complicate the application of enzyme sensors that can strongly influence the sensor signal. As catalysts, enzymes are not consumed by the reaction and thus, enzyme concentration is not crucial for the operation of biosensor. However, enzyme concentration is one of the limiting factors to a biosensor [2]. Considerable material is needed to provide sufficient units of enzyme activity if there is too much enzyme or the quality of the enzyme preparation is poor. Thus, the excess material can affect the rate of mass transport to the transducer.

Buerk has reported that biosensors are usually designed with high enzyme loading in an appropriate environment to make sure sufficient biocatalyst activity is available and to sustain its activity [4]. The biosensor response is usually dependent on the amount of active enzyme immobilized as reported by Chaubey and Malhotra [9]. Therefore, sensor response was more strongly influenced by enzyme. The addition of more glucose oxidase rejuvenated the sensor sensitivity to glucose. Thus, the sensitivity of multilayered membrane can be tunable by controlling the number of attached enzyme layers [29].

The response time to arrive of 95% at the steady state current for multilayered membrane with 4 bilayers and 6 bilayers were approximately 125s and 260s, respectively. The thicknesses of 4 bilayers and 6 bilayers membranes were 66.5 and 82.5 microns, respectively. As the number of bilayers increased, the membranes were thicker and thus the response time increased.

Guilbault *et. al.* reported that there are three steps in the mechanism of the response for enzyme electrodes [38]. Firstly, the substrate must diffuse through the solution to the membrane surface. Secondly, the substrate must diffuse through the membrane and react with the biocatalyst at the active surface. Finally, the products must diffuse to the electrode in order to be quantified. Thus, the membrane must be thin in order to obtain fast response times.

Furthermore, one of the factors that can affect response time was enzyme concentration [38]. As the amount of enzyme was increased, a shorter response time was observed until an optimum level was reached. After that level, the response time will increase due to a thickening of the membrane layer by the use of more weight of biocatalyst.

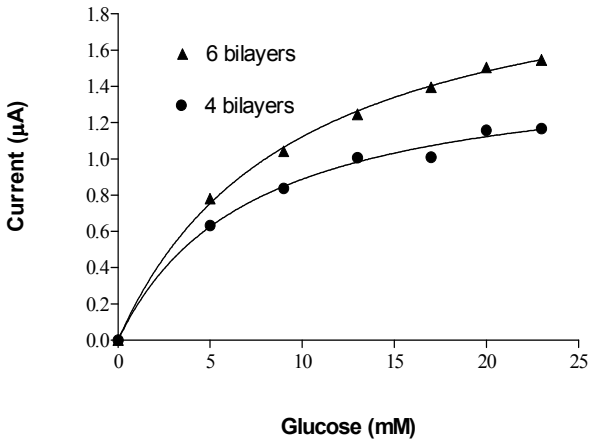

Fig. 5 Current responses to different glucose concentrations for multilayered membranes

The current responses increased stepwise with addition of glucose. For 4 bilayers membranes, the corresponding I_{max} and K_m^{app} for the membranes were 1.00μA and 5.24mM, respectively. Meanwhile, higher I_{max} and K_m^{app} were obtained for 6 bilayers membranes which were about 2.03μA and 8.69mM, respectively. The K_m^{app} obtained for both membranes were quite low. With such high sensitivity and small K_m^{app}, it shows that the immobilized glucose oxidase in multilayered membrane possessed a high enzymatic activity.

The kinetics constant, K_m^{app} and I_{max} of enzymes may be altered by the process of immobilization due to internal structural changes and restricted access to the active site. For an immobilized enzyme to catalyse a reaction, the substrates must be able to diffuse through the solution to catalytically active sites and the product diffuse away to into the bulk solution [39].

Generally, the K_m^{app} of an immobilized enzyme will be larger than that of the free enzyme in solution due to the effect of the diffusion of substrate to the active sites [35]. The K_m value depends on the strength of the bonds between the enzyme and substrate. The K_m value not only includes the affinity of substrate for enzyme, but also the rate at which the substrate is converted to product [40]. If these bonds are strong, the K_m^{app} will be low, indicating that the immobilized enzyme retains its bioactivity and possesses high biological affinity to glucose. Consequently, the active sites of the enzymes could be more readily available for enzymatic interactions. Thus, with high affinity of substrate for enzyme, low concentration of substrate is needed to achieve a specified conversion rate of substrate to product. K_m^{app} is independent of enzyme concentration.

Various K_m values for immobilized glucose oxidase also have been reported. Kunzelmann and Bottcher reported that K_m for immobilized enzyme in sol gel membranes varied from 0.04 to 0.11mM for various sol compositions [41]. With such lower K_m, analyte dilution or addition of diffusion barriers need to be considered since the relevant concentration values of glucose in blood and in urine are around 2-25mM and 0.2-0.7mM respectively. For bienzymatic GOD and horseradish peroxidase glucose biosensor with ferrocene carboxylic acid as the mediator, K_m for the immobilized enzyme was 0.25mM with linear range from 0.08 to 1.3mM [42].

C. Stability of Multilayered Membranes

The stabilities of SGS-CLPVA/Nafion membranes were investigated to determine the shelf life of the sensors. The current outputs of the membranes when subjected to 5mM glucose at certain periods were measure.

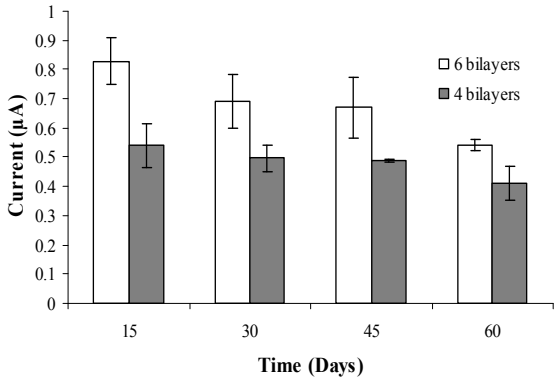

Fig. 6 Stability of multilayered membranes with different number of bilayers

As shown in figure 6, after 1 month, the membranes with 4 bilayers and 6 bilayers retained approximately 92% and 83% of the initial activities, respectively. After 2 months, the membranes with 4 bilayers were still quite stable with 76% of the initial activity remained. However, only 65% of the activities of the membranes with 6 bilayers remained.

The degradation of enzyme might be due to contamination by bacteria that can destroy enzyme activity since the membrane was not covered with any protective membrane [38]. Besides, poor stability could be due to problems with the mediator. However, Brooks *et al.* reported the addition of extra ferrocene to spent electrodes did not affect activity but the loss of activity of ferrocene glucose sensors was more strongly influenced by the loss of enzyme by denaturation or detachment [37]. The addition of more glucose oxidase rejuvenated the sensitivity to glucose. Guilbault *et al.* also reported that the high voltages used in amperometric sensors can also inactivate the enzyme [38].

D. Effect of Additional Nafion Layers to the Multilayered Membranes

Nafion was added to the multilayered membrane as a barrier to the electroactive anionic interferents and also as an

additional layer in order to increase the linearity of current response to glucose. Multilayered membrane with 4 bilayers was added with additional nafion layers since this type of membrane were more stable and had shorter response time compared to 6 bilayers multilayered membrane. Figure 6 shows a typical current response of the multilayered-nafion membrane to 5mM glucose.

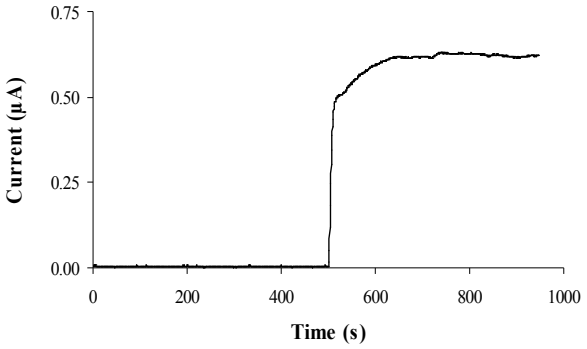

Fig. 7 Typical current response of multilayered-nafion membrane

As shown in figure 7, the response time to arrive at 95% of the steady state current for a multilayered-nafion membrane with a thickness of approximately 68 microns was approximately 156s. The current response to 5mM glucose was around 0.55µA. The thickness and current response value for the multilayered-nafion membrane was comparable to the multilayered membrane. Addition of nafion to the multilayer membrane only affect the response time with around 25% increase of the original value. Figure 8 shows typical calibration curve for multilayered-nafion membrane for kinetics study.

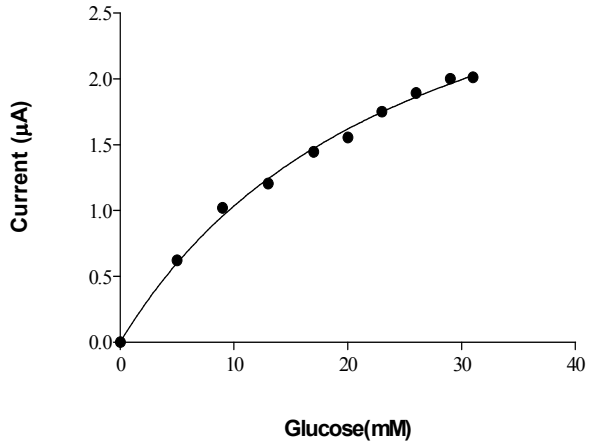

Fig. 8 Typical calibration curve for multilayered-nafion membrane

Meanwhile, higher K_m^{app} and I_{max} were obtained for multilayered-nafion membranes which were about 28.68mM and 4.16µA respectively. Thus, it shows that with the addition of nafion, linear range of the enzymatic membrane response was extended.

E. Effect of pHEMA Outer Membrane to the Multilayered Membranes

Outer membrane was added in order to extend the linear range of response to higher concentrations and to protect the enzymatic membrane [43]. The outer membrane limits the flux of the glucose substrate by controlling the glucose diffusion to the enzyme layer [44]. Other roles of the outer membrane are to preserve the enzyme activity, prevent interferences and improve lifetime of continuous monitoring of biosensor. Thus, in this research, the effect of cross-linked pHEMA protective outer layer to the kinetics properties of the multilayered-nafion membranes was studied. Cross-linked pHEMA hydrogels membranes can be an appropriate outer membrane since these hydrogels has the ability to limit glucose transport by controlling its mesh size. In addition, pHEMA hydrogels are biocompatible, have good mechanical strength and have been widely used in other biomedical and pharmaceutical applications.

Kermis *et al.* has investigated the transport properties of pHEMA at cross-linking ratios of 0.005, 0.01, 0.02, 0.03 and 0.04 as potential outer membranes for optical glucose affinity sensors [25]. The results showed that the cross-linked pHEMA hydrogels membranes exhibited good protein rejection but with small glucose solute permeability as permeability decreased with increased cross-linking ratio. Thus, in this study, cross-linked pHEMA with cross-linking ratio of 0.0025 was used as the outer membrane because high permeability was favored to ensure adequate diffusion of glucose large molecule (MW 150-186 kDa) through the membrane. Figure 9 shows the current response of multilayered-nafion membrane with cross-linked pHEMA.

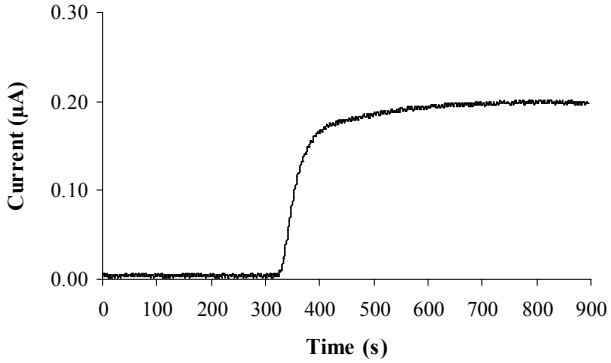

Fig 9 Typical current response of multilayered-nafion membrane with cross-linked pHEMA outer layer

For multilayered-nafion membrane with cross-linked pHEMA outer layer, the current response was around 0.21μA, which was smaller than the multilayered-nafion membrane without other outer layer. Meanwhile, membrane thickness was around 87 microns. The response time to arrive at 95% at the steady state current for multilayered-nafion membrane with cross-linked pHEMA was around 402s. The addition of cross-linked pHEMA to the multilayered-nafion membrane negatively affects the current response and also response time. Figure 10 shows typical calibration curve for multilayered-nafion membrane with cross-linked pHEMA outer layer.

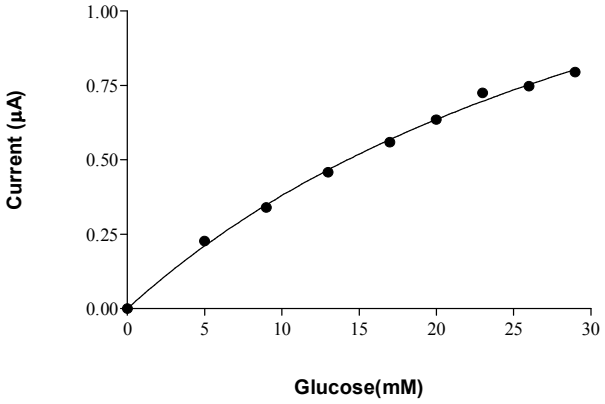

Fig 10 Typical calibration curve for multilayered-nafion membrane with cross-linked pHEMA outer layer

Higher K_m^{app} and I_{max} were obtained for multilayered-nafion with cross-linked pHEMA membranes which were about 40.58mM and 1.60μA respectively. The results show that the cross-linked pHEMA layer was able to extend the linear range for glucose sensor by limiting the glucose flux to the enzyme layer due to the small pore size of the cross-linked pHEMA layers. Brazel and Peppas reported that at 0.01 cross linking ratio, pHEMA had a mesh size of about 17.17Å, which was smaller than the mesh size of PVA hydrogels at the same cross-linking ratio [45].

Nevertheless, the mesh size of the matrix can be modified by varying the amount of cross-linking agent during the hydrogel preparation. Kermis *et al.* demonstrated that by increasing the EGDMA, the mesh size of cross-linked pHEMA can be reduced [25]. EGDMA was used in the polymerization reaction mixture in order to connect the macromolecular chains by shorter chemical cross-links to get a tighter mesh. In contrast, in the absence of cross-linking agent, pHEMA membranes prepared will contain macromolecular chains with a mesh that arises from physical entanglements only [25].

A plot of the concentration profiles of glucose diffusion through outer, enzyme and inner layer of a glucose biosensor showed that as the substrate diffused through the external layer, the amount of substrate was reduced in accordance with the permeability of the layer [35]. Thus, the amount of substrate that reached the enzyme layer was less compared to the original amount of glucose that was injected in bulk solution. Thus, as expected, the enzymatic membrane with an outer membrane will give lower current response than the membrane without an outer layer.

With an additional barrier to the multilayered-nafion membrane, the response time increased as expected since the outer layer reduced the diffusion rate of glucose from the bulk solution to the enzyme layer. The response time will be more adversely affected if the permeability of the outer layer was further reduced [35].

IV. Conclusion

In this method, glucose oxidase and redox poly(allylamine) ferrocene was immobilized by layer-by-layer covalent attachment. The result of this study indicated that enzyme loading was one of the main factors that can affect the performance of multilayer biosensor in terms of current response, response time, enzyme kinetics, membranes stability, and enzyme leakage. For enzyme kinetics, additional cross-linked poly(hydroxyethyl methacrylate) outer membrane improve the glucose linearity response with K_m^{app}, was around 40.58mM with high sensitivity and fast response time towards the glucose.

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