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A Sol-gel Biosensor for the Detection of Maltose in Starch Hydrolysis†

A. K. M. Shafiqul Islam[1*], Tan Chia Shin[2] and Mohd Noor Ahmad[1]
[1]School of Chemical Sciences, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia
[2]School of Bioprocess Engineering, Universiti Malaysia Perlis (UniMAP), 02600 Arau, Perlis, Malaysia
*Corresponding author (e-mail: shafiqul@usm.my)

A hybrid sol-gel nano-biosensor has been developed to determine maltose produced by starch hydrolysis in the presence of α-amylase. Glucose oxidase (GOx) and glucosidase (GD) were covalently immobilized on the sol-gel sensor via carbodiimide reaction. The kinetic study confirmed a successful immobilization of the enzymes. Immobilization conditions such as the amount of enzyme, immobilization time; and pH in working buffer were optimized. The biosensor showed the maximum response at glucose oxidase concentration of 20 U/ml and immobilization time of 90 minutes. The optimum pH of the working buffer was pH 7 at 45°C. From the Lineweaver-Burk plot, the K_m value for the immobilized enzymes was 12.72 mM which was much lower than enzyme activity in solution (24.6 mM). The biosensor showed a linear response to maltose hydrolysis in the range of 0.5 - 5 mM. The sensitivity and correlation coefficient of the sensor were 29.15 μA/mM/cm^2 and 0.9975, respectively.

Key words: Sol-gel; covalent immobilization; α-glucosidase; glucose oxidase; maltose hydrolysis

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Salivary and pancreatic α-amylases were commonly used to catalyze starch hydrolysis which led to the significant release of maltose [1]. Salivary amylase was also reported to be associated with certain physical and mental stresses induced by sports activities and therefore it had been considered as a stress related biomarker. A disposable tri-enzymatic biosensor had been prepared [2] for the determination of α-amylase in human saliva wherein the detection was based on the amount of maltose generated by the hydrolysis of maltopentose in the presence of salivary α-amylase. The biosensor was constructed by immobilizing α-glucosidase which hydrolysed maltose to glucose. Glucose was determined by an oxygen electrode coated with glucose oxidase [3] membrane (Figure 1).

In the reductive half reaction, GOx catalyzed the oxidation of β-D-glucose to D-glucono-δ-lactone and FAD was reduced to $FADH_2$ while the reduced GOx was reoxidized by oxygen to produce H_2O_2 in the oxidative half reaction and H_2O_2 was cleaved by catalase (CAT) to yield water and oxygen (Figure 1). The quantification of glucose was achieved via electrochemical detection of the enzymatically liberated H_2O_2.

However, the sensitivity problem is very common in the detection of oxygen concentration and significant interference signals will be generated. This issue was prevented by using a mediator to replace oxygen as electron transfer [4] (Figure 2).

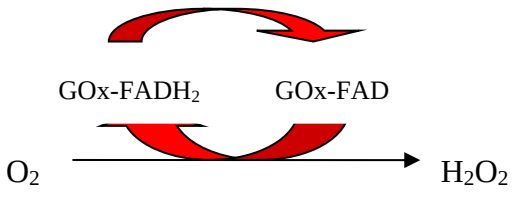

Figure 1. Reaction of GOx with glucose [3]

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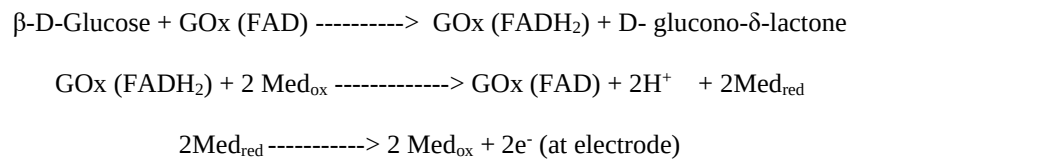


Figure 2. Schematic diagram of sensing mechanism

Enzymes are regarded as bio-catalysts with the advantages of high catalytic activity, selectivity, specificity, and biodegradability [5]. However, enzymes are not recoverable and reusable, making it not economical. This disadvantage could be overcome by using robust immobilization techniques [6] to enhance enzymatic properties such as resistance to extreme reaction conditions (e.g., pH and temperature far from their physiological range), increase enzyme activity, multiple reusability or continuous use, and improvement of substrate specificity and enantio specificity, or product selectivity [5, 7-9].

The immobilization methods are based on the distinctive characteristics of functional groups of the amino acid side chains of the enzymes, which interact or react with the support in several ways. The enzymes could be attached to the support surface by adsorption via reversible bonds, like van der Waals forces, hydrophobic or ionic linkages, or by irreversible chemical bonds such as covalent attachment [10]. The various characteristics of supports, reactive groups, and immobilization protocols allow the preparation of immobilized enzymes with different properties [9, 11]. Therefore, suitable supporting matrices and immobilization approaches are vital steps to overcome current obstacles of enzymes immobilization for biosensor applications.

Dicyclohexylcarbodiimide, diazotization, bromide cyanogen, and glutaraldehyde are maturely developed coupling methods [5]. Dicyclohexylcarbodiimide method is known as l-ethyl-3-(dimethyl-aminopropyl) carbodi-imide hydrochloride (EDC)/N-hydroxysuccin-imide (NHS) method. In a previous research, Mohiuddin *et al.* [12] used EDC and NHS as catalysts to facilitate the amine coupling reactions which initiated the covalent attachment of α-glucosidase onto amine-functionalized multi-walled carbon nanotubes (MWCNTs-NH_2). The amide bonds were formed from the reactions between carboxyl groups of polyamide layers and amino groups of α-glucosidase enzyme. Glutaraldehyde coupling method is commonly used to immobilize enzymes onto an amino-functionalized support matrix based on Schiff (condensation) reaction. In this process, one aldehyde group of glutaraldehyde molecule reacts with the amino group of the enzyme molecule, and the other aldehyde group reacts with the amino group of the support matrix [13-15]. The simple adsorption of lipase on carbon nanofibers showed a poor immobilization efficiency of 36%, while covalent coupling using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC)/ N-hydroxysuccinimide (NHS) showed better immobilization efficiency of 56% [16]. The supporting materials are also of crucial importance for enzyme immobilization. Inert polymers and inorganic materials are usually used as carrier matrices for enzymes immobilization [6].

In this research, the α-amylase activity was determined by analysing maltose from starch hydrolysis using glucose oxidase and α-glucosidase immobilized sol-gel/Fc/MWNTs composite electrode. The main advantage of this system lies relatively on the simple construction and enzyme immobilization technique.

EXPERIMENTAL

1. Instruments

Dropsens μStat 200, a hand-held USB powered laboratory research instrument obtained from Dropsens, S.L., Oveido, Spain, was used as electrochemical biosensor and voltammetric analysis.

A three-electrode system was used to perform the electrochemical measurements. The electrodes consisted of a platinum wire auxiliary electrode (0.5 mm diameter and 7.5 cm length with gold-plated connector, from Bioanalytical System, Inc., USA), a silver/silver chloride (Ag/AgCl) reference electrode (from Bioanalytical System, Inc., USA), and an in-house fabricated sol-gel carbon nanotube working electrode which consisted of a PVC tube and a copper wire to enable electrical contact.

2. Reagents

Glucose oxidase from *Aspergillus niger* (EC 1.1.3.4), 3-aminopropyltriethoxysilane (APTMOS), trimethoxy-methylsilane (MTMOS), N-(3-dimethyl-aminopropyl)-N'- ethylcarbodimide hydrochloride (EDC), and N-hydrosuccinimide (NHS) were obtained from Sigma, St. Louis, USA. α-glucosidase (EC 3.2.1.20) was purchased from Toyobo Co., Ltd., Osaka Japan. Ferrocene (Fc) (98%) was purchased from Acros Organics, New Jersey, USA.

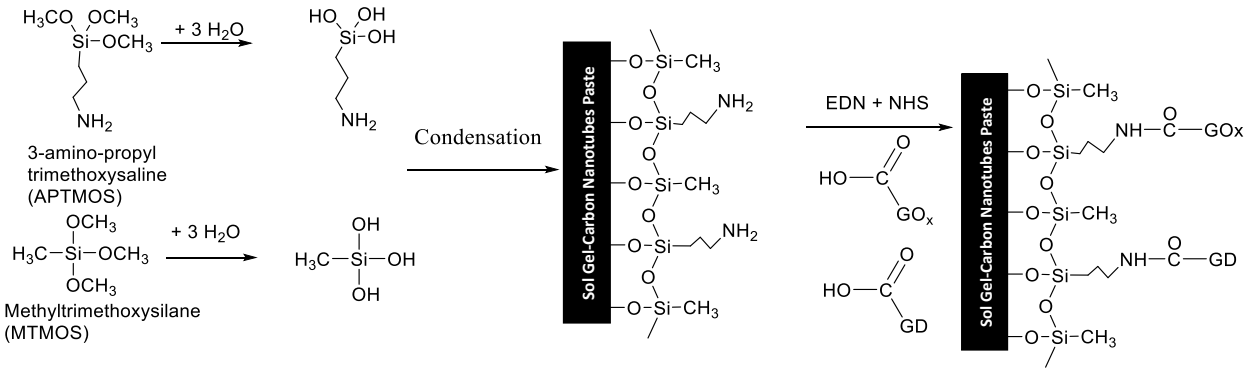


Figure 3. Schematic diagram of sol-gel nanocarbon composite biosensor fabrication

MWNTs (average diameter: 10-40 nm, length: 1-25 μm, purity by weight: 93%) were obtained from Fibermax Composites, Greece. All other reagents and chemicals used were of analytical grade.

3. Design of Sol-Gel Nanocarbon Biosensor

Hybrid sol-gel was prepared through a two-step hydrolysis of two precursors, APTMOS and MTMOS, in an acidic pH followed by condensation reaction between silanol moieties. The later resulted in the formation of siloxane (Si-O-Si) polymers [17] as shown in Figure 3.

Two different enzymes, α-glucosidase (GD) and glucose oxidase (GOx), were covalently immobilized onto the exposed surface of the sol-gel/Fc/MWNTs modified working electrode through carbodiimide coupling reagents EDC and NHS which have the ability to weaken the amine bond of the sol-gel and hence forming covalent bonds between the enzymes and sol-gel. The binding sites of the enzymes are dependent on the amount of EDC and NHS. In addition, free carboxylic groups on the enzymes were also converted into reactive intermediates by using EDC and NHS [17]. These intermediates would be easily attached to the amine groups of the sol-gel to form covalent bonds between the enzymes and sol-gel.

4. Preparation of Sol-Gel Nanocarbon Electrode

Homogeneous sol-gel solution was prepared by vigorously mixing 0.6 mL of MTMOS, 0.4 mL of APTMOS, 0.35 of mL distilled water, 1.6 mL of methanol, and 0.02 mL of hydrochloric acid (HCl) on a hot plate stirrer. The MWNTs and sol-gel solution were thoroughly hand-mixed in a mortar until a homogenized blackish smooth paste was obtained. The composite mixture was tightly packed into a 3 mm in diameter by 5 mm in length PVC tube through one end of the tube. A copper wire was inserted through the other end of the PVC tube to allow an electrical contact. The prepared electrodes were polished with 0.05 μm Alumina slurry on 1500# and 2000# metallographic sand papers, and rinsed with double-distilled water until a shiny surface was obtained.

5. Preparation of Ferrocene (Fc) Modification of Electrode

The ferrocene solution was prepared by dissolving it in methanol and added into the sol-gel, whereupon a yellowish solution was formed. 0.3 mL of Fc/sol-gel and 0.05 g of MWNTs were hand-mixed in a mortar until a homogenized mixture was obtained and tightly packed into a 3 mm in diameter by 5 mm in length PVC tube.

6. Covalent Immobilization of GOx and GD

The modified electrodes were immersed in 0.05 M phosphate buffer solution (PBS) containing 0.03 M EDC and 0.06 M NHS at pH 6.5 for 90 minutes. Then, sensors were immediately placed in different amounts of GOx and GD in the same buffer solution to immobilize the enzymes onto the working electrode surface. The biosensors were rinsed with phosphate buffer at pH 7 under constant stirring to remove the excess free enzymes. When the biosensors were not in use they were stored in a refrigerator under a dry condition at 4 °C.

7. Enzyme Kinetic Study of GOx/GD Biosensor

Five different concentrations (1, 5, 10, 15, and 20 mM) of maltose were prepared to study the Michaelis-Menten kinetic mechanism. Based on the current signal of the biosensor, an enzymatic kinetic study was performed to determine the enzyme activity on the electrode using the modified [18] Lineweaver-Burk equation:

$$\frac{1}{I_S} = \left(\frac{K_m}{I_{max}}\right) \cdot \left(\frac{1}{S}\right) + \left(\frac{1}{I_{max}}\right) \qquad [1]$$

A Lineweaver-Burk plot of the GOx/GD biosensor was plotted. K_m and I_{max} were obtained from the slope and intercept of the Lineweaver-Burk plot, respectively.

8. Starch Hydrolysis

2.0 % w/v of starch in 20 mL of 0.1 M PBS at pH 7 and 0.1 M KCl was used as working solution. The background current signal caused by starch in PBS and KCl with α-amylase was recorded. Various reaction times were studied using 0.1 mL of α-amylase (63.6 KNU).

RESULTS AND DISCUSSION

1. Sol-Gel and Fc Modified Electrode

The combined effects of Fc and MWNTs enabled efficient electron transfer. The concentration of Fc is a crucial factor for enhancing the electron transfer in electrodes. The cyclic voltammograms exhibited the performance of the modified electrode with various concentrations of Fc solution.

In Figure 4(b), the redox peaks presents reversible oxidation and reduction reaction of covalently bonded Fc derivatives. The 0.045 M Fc solution exhibited a pair of well-defined anodic and cathodic peak curves at 0.42 V and 0.22 V with the high oxidation peak current response of 109.86 µA in the cyclic voltammogram. The shape of the peaks in Figure 4 is approximately symmetrical and this proves a well electron redox reaction of Fc+/Fc couple was achieved by a mediated electron transfer process. The Fc modified electrode was electrochemically active [19].

Figure 4(a) exhibits the sol-gel-MWNTs lower peak current response for oxidation and reduction. After the electrode was modified with Fc, peak current increased (Figure 4(b)). Fc increased the active surface area of the modified electrode. At the same time, the peak-to-peak separation (ΔEp) decreased which showed that Fc had a good conductivity. The ΔEp became smaller than that of the sol-gel-MWNTs electrode, showing the rapid electron-transfer kinetics and Fc played a main role in promoting electron transfer, which effectively promoted electrode transfer between Fc/Fc+ and the electrode.

2. Study of Analytical Parameters of Biosensor

The effectiveness of the two co-immobilized enzymes, GOx/GD, was studied using glucose and maltose as separate substrates to characterize the behavior of each enzyme. Calibration curves were obtained by the same electrode for glucose and maltose. The GOx/GD biosensor was fabricated by covalent immobilization of 10 U/mL GOx and 10 U/mL GD on a modified electrode.

2.1. Electrochemical Response of GOx/GD Biosensor to Glucose and Maltose

Different concentrations of glucose were prepared to check the oxidation peak current response using the GOx/GD biosensor. The cyclic voltammograms of the GOx/GD biosensor in various concentrations of glucose and maltose are shown in Figure 5. The stability and repeatability of the GOx/GD biosensor were studied for 5 cycles at a scan rate of 0.02 V/s.

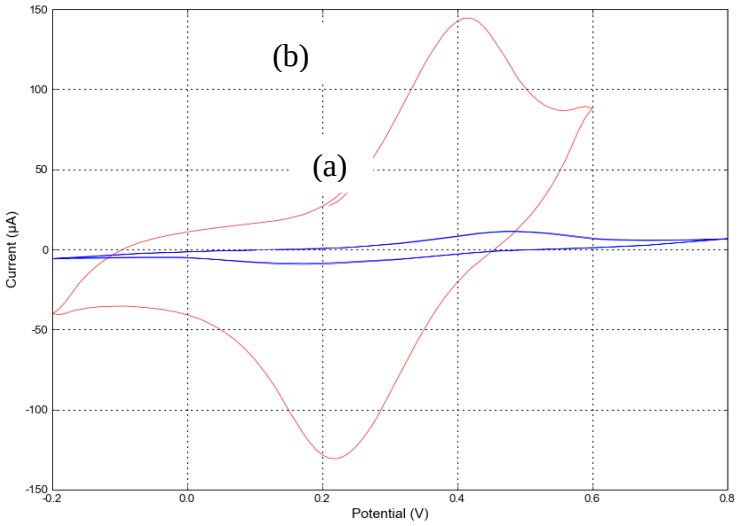
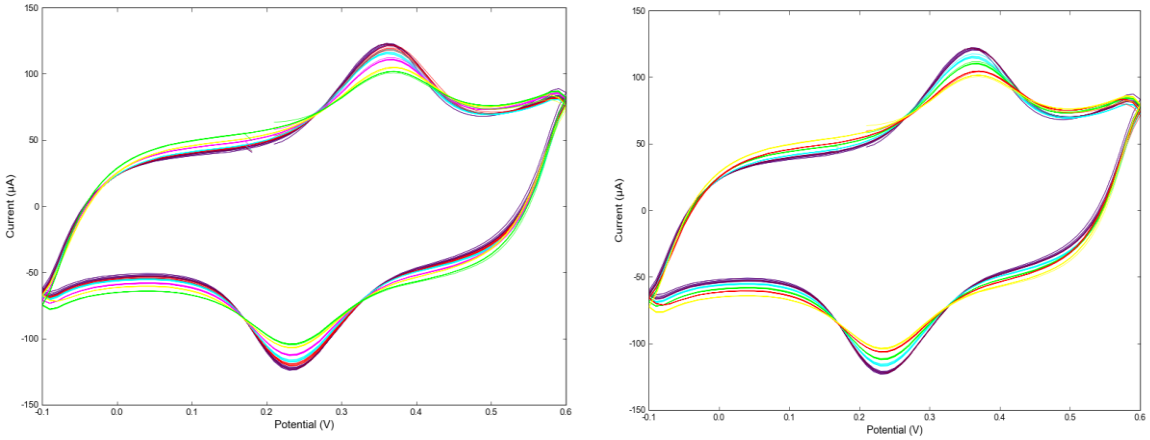


Figure 4. Cyclic voltammograms of 0.05 M KCl at a scan rate of 25 mV/s: (a) sol-gel/MWNTs, and (b) sol-gel Fc and MWNTs modified electrode



(a) (b)

Figure 5. Cyclic voltammograms of the GOx/GD biosensor in the absence and presence of various concentrations of (a) glucose and (b) maltose in 0.05 M PBS and 0.05 M KCl.

Without glucose, the GOx/GD biosensor showed a pair of redox peaks of Fc. Upon addition of glucose, the oxidation peak current increased, which exhibited electrocatalytic oxidation of glucose. Fc increased the current response and efficiency performance of the enzyme biosensor by promoting the electron transfer between active sites of the enzymes and the electrode [19]. Where Fc and Fc^{+} represented reduction and oxidation of ferrocene, GOx (FAD) and GOx ($FADH_2$) were the oxidized and reduced forms of GOx. Two electrons were transferred from glucose to GOx (FAD), and then electrons of GOx ($FADH_2$) were transferred to Fc on the electrode surface and produced current that was directly proportional to the glucose concentration in the solution. The GOx/GD biosensor showed a good stability and repeatability.

Figure 5(b) shows the current-potential oxidation peak response obtained by cyclic voltammeter for various concentrations of maltose. The biosensor exhibited increased pairs of redox peaks of Fc directly proportional to maltose concentration. The increased current was due to maltose being converted to glucose by glucosidase and then glucose hydrolyzed to D-gluconolactone by glucose oxidase. The oxidation peak was due to the reduction of ferricinium (Fc+) to Fc produced at the oxidizing potential.

Calibration curve showed the oxidation peak responses of the GOx/GD biosensor increased considerably upon the addition of glucose and maltose (Figure 6). It exhibited an obvious electrocatalytic oxidation of glucose with increasing

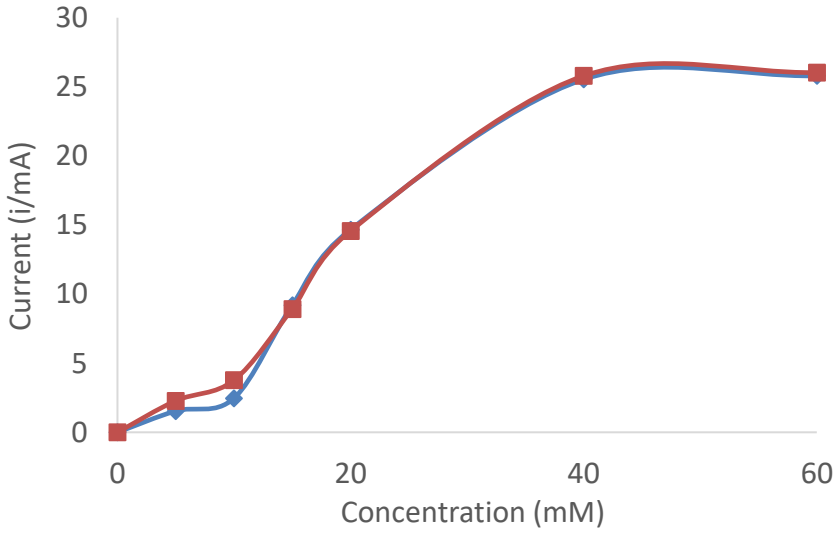


Figure 6. Calibration curve of oxidation peak response upon addition of (a) ■ glucose and (b) ♦ maltose

glucose concentration from 5 to 40 mM and reached an equilibrium at 40 to 60 mM of glucose concentration. The amount of GOx ($FADH_2$) generated was dependent on glucose concentration within the limit of GOx (FAD) concentration available in the solution. For relatively high concentration of glucose of more than 40 mM, the availability of GOx (FAD) became the limiting factor for the formation of GOx ($FADH_2$). In such case, the ferricinium cations and the corresponding oxidation current response became constant. A similar result was obtained by Creange and Murr (2010) [20]. In addition, higher concentration of glucose up to 40 mM did not show any significant increase which indicated that glucose had almost completely covered the surface of GOx as reported by Im *et al.* [18]. From Figure 6, it shows that the limiting effect of GOx (FAD) has significantly affected the current response.

In the first system, maltose was hydrolyzed to glucose by GD. Glucose was hydrolyzed by GOx (FAD) and then generated D-gluconolactone and GOx ($FADH_2$). The ferricinium cations produced by oxidation of Fc were reduced at the electrode into Fc when appropriate potential was applied on the working electrode with GOx ($FADH_2$) in the presence of GOx (FAD). In addition, the current response increased linearly with the increase of maltose concentration and reached a saturation value at 40 mM, which showed that 40 mM of maltose had converted to glucose and the active sites of GOx were saturated at the glucose level, similar to the results reported by He *et al.* [21]. The flattening of the calibration curve obtained at 40 mM of maltose concentration was almost the same with the calibration curve for glucose indicating that 40 mM of maltose was hydrolyzed by 10 U/mL of GD and generated almost 40 mM of glucose.

2.2 Amperometric Response of the GOx/GD Biosensor

Figure 7 shows the amperometric responses in different times for various maltose concentrations in 0.05 M PBS pH 7 and 0.05 M KCl at 0.4 V. The current response reached a saturation value in 2 min 20 sec when maltose was hydrolyzed to glucose by GD and glucose was detected by GOx. The active sites of GOx were saturated with glucose level by 2 min 20 sec. With the increase of maltose concentration, the GOx/GD biosensor responded rapidly as shown in Figure 7.

The calibration plot from the current response of the GOx/GD biosensor to different concentrations of maltose is illustrated in Figure 7. The calibration for current response increased linearly with the addition of maltose concentration from the 0.5 mM to 5 mM with a correlation coefficient of R^2 value of 0.9975. The linear graph displayed positive correlation between x and y. Hence, there was a good linear correlation between maltose concentration and sensor response. The sensitivity of the GOx/GD biosensor obtained from the slope of graph was approximately 29.14 $\mu AmM^{-1}cm^{-2}$ was found to be much higher than 15.2 $\mu AmM^{-1}cm^{-2}$ for PB/MWNTs-GOx-CS-ICPTES-GCE [22] and 10 $\mu AmM^{-1}cm^{-2}$ for CS-Fc/GO/GOx [23]. Limit of detection (LOD) of the sensor was 2.4 $\times 10^{-2}$ mM, which was comparable with the previous result [2].

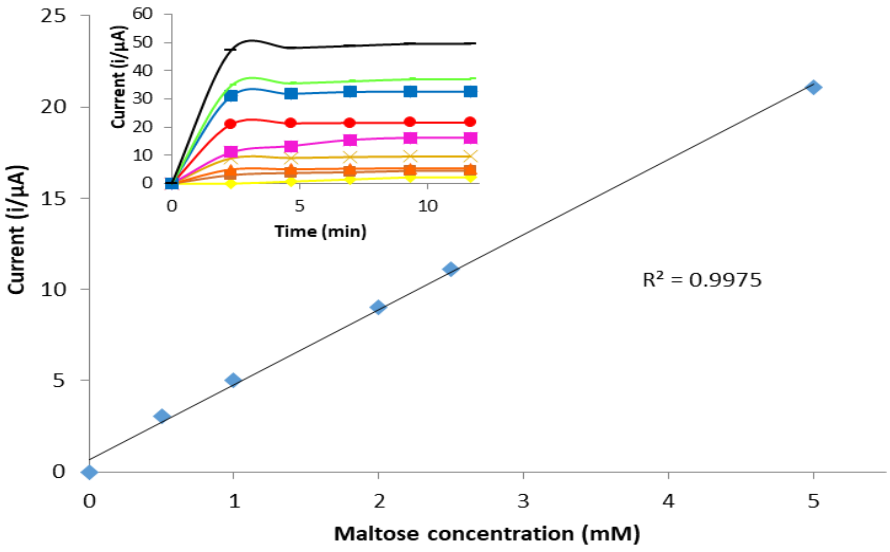


Figure 7. Amperometric response of the GOx/GD biosensor versus time with various concentrations of maltose

2.3 Enzyme Kinetic Studies

The kinetic approach explained the rate at which the reactions occurred and associated with the activation energy. The rate of reaction affected by factors such as temperature, pH, and the presence of catalyst. Kinetic study was used to determine the enzyme activity using Michaelis-Menten constant K_m which is an indication of both enzymatic affinity and the ratio of microscopic kinetic constant. Five different concentrations of maltose were chosen as substrate to study the enzyme kinetics. The Michaelis-Menten constant K_m was calculated using equation 1. The K_m value increased, indicating that the enzymes, GOx and GD activities decreased [18]. The K_m was calculated to be 12.72 mM. Superior enzyme loading was achieved with a boosted performance by thermo and pH-stability, reusability, and lower K_m (Michaelis-Menten constant of enzyme). In another work, Roth *et al.* developed an electrostatic assembly of cellulase and silica-coated magnetic nanoparticles [24].

2.4 Starch Hydrolysis

The measurement of α-amylase was carried out using starch in 0.1 M PBS and 0.1 M KCl supporting electrolyte at room temperature. Background current signal caused by starch present in PBS and α-amylase was recorded at the reaction times 5, 10, 15, 20, 25, and 30 minutes (Figure 9).

The increase in current was observed for different reaction time of α-amylase from 5 minutes to 30 minutes and reached maximum current of 28.94 µA in 30 minutes. In this duration, starch was completely hydrolyzed by α-amylase and generated the maximum amount of maltose which was then hydrolyzed by GD to α-D-glucose and β-D-glucose. Phosphate ions were used to enhance the mutarotation [1]. β-D-glucose was hydrolysed by GOx to gluconic acid and hydrogen

$$Activity\ (IU\ mg^{-1}) = \frac{released\ maltose\ (\mu mol))}{amount\ of\ \alpha\text{–amylase (mg)x Time (minutes)}} \quad [2]$$

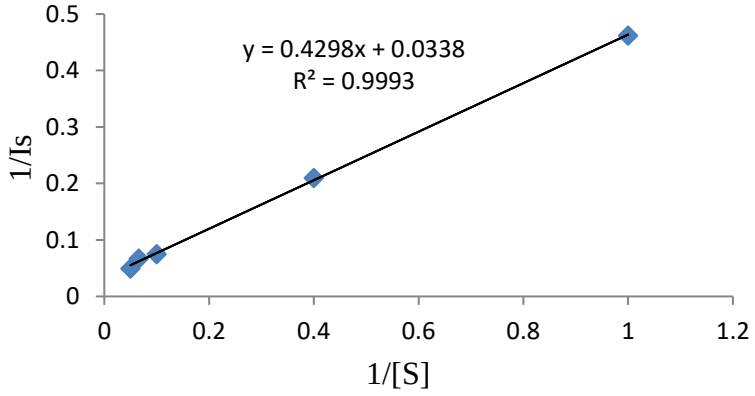


Figure 8. Lineweaver-Burk plot of the GOx/GD biosensor

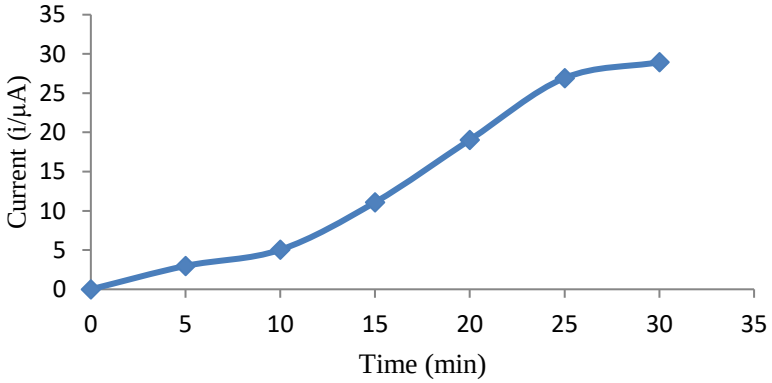


Figure 9. Current measurement of the GOx/GD biosensor to starch hydrolysis in the presence of α-amylase

peroxide. This series of reactions allowed the determination of glucose obtained from starch hydrolysis by α-amylase. α-amylase activity was calculated according to the following equation [2]:

CONCLUSION

A hybrid sol-gel modified nanocomposite GOx/GD biosensor has been used as an efficient platform to immobilize enzymes for biosensing applications. MTMOS was used as precursor for the synthesis of SiO_2-based hybrid sol-gel. MWNTs were tightly held by the sol-gel to form a rigid matrix which facilitated the electron transfer to enhance the electrode conductivity. The presence of amine groups in APTMOS controlled the covalent bonding sites for the enzymes and cross linked with the sol-gel. Fc was used as mediator for electron transfer between a redox enzyme and an electrode. This had exhibited significant effects on the biosensor performance.

The bi-enzymatic biosensor was applied for maltose analysis and exhibited optimal performance with immobilization of 20 U/mL GOx and 10 U/mL GD on the surface of the electrode. A good linearity of the biosensor response was obtained in the maltose concentration of 0.5 mM to 5 mM with R^2 value of 0.9975 and sensitivity of 29.14 $\mu AmM^{-1}cm^{-2}$. The detection limit of the sensor was 2.4×10^{-2} mM. High sensitivity of the biosensor was achieved with the presence of sol-gel/Fc/MWNTs composite which allowed superior conductivity for the electron transfer. The enzyme biosensor displayed rapid response and good reproducibility. With low K_m value, it showed that the biosensor possessed higher biological affinity to maltose.

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