GRAPHENE MODIFIED ELECTROSPUN PVA NANOFIBROUS MEMBRANES FOR GLUCOSE OXIDASE IMMOBILIZATION

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Abstract
In this study, PVA/Glucose oxidase (GOx)/graphene biocomposite membranes were prepared by electrospinning technique and applied for enzyme immobilization successfully. The morphology of PVA/GOx/graphene membrane was examined by SEM and TEM. The Electrochemical sensitivities of PVA/GOx/graphene membrane were studied by Chronoamperometry. Kinetic parameters were determined for understanding the role of graphene for enzyme immobilization. Spectrophotometric assay was used to determine the amount of active enzyme. The results indicated that the presence of graphene may contribute to stabilize the conformation of enzyme, facilitate the catalytic reaction and increase the survivability of the enzyme.

1 Introduction
Diabetes mellitus is a worldwide public health problem. This metabolic disorder results from insulin deficiency and hyperglycemia and is reflected by blood glucose concentrations higher or lower than the normal range of 80-120mg/dL (4.4 - 6.6 mM) [1]. The diagnosis and management of diabetes mellitus requires careful monitoring of blood glucose levels. The glucose biosensor has thus become one of the most important and popular device, accounting for about 85% of the entire biosensor market [1].
Biosensor is a device which is combined immobilized biorecognition element with transducer. It can monitor chemical substances on the inside or outside of organism by translating them into a transducer signal after a coupling of the biochemical and transducer type reaction [2]. Glucose biosensor is based on electrochemical principles and employed enzymes as biological components for molecular recognition. Then, the performance of biosensors strongly depends on the influence imposed on enzyme molecules by immobilization. The immobilization method and the host molecule used can influence the sensitivity of the biosensor. A number of techniques have been used for the immobilization of enzymes on different substrates, such as poly(vinyl alcohol) (PVA) [3], poly(ethylene oxide) (PEO) [4], Chitosan [5], polymethylmethacrylate (PMMA) [6] and poly(vinyl pyrrolidone) (PVP) [7], to improve the enzymatic activity and stability. PVA has been used as an idea immobilization matrix because of its inherent non-toxicity, high thermal stability, good biocompatibility and desirable physical properties such as elastic nature, good film forming property, high degree of swelling in aqueous solutions and that is considered an appropriate matrix for enzyme immobilization [8]. Different methods, such as cross-linking of PVA [9], freeze-thawed PVA [10],

encapsulation of enzymes in PVA/silicate hybrid materials [3], have employed to immobilize the enzyme molecules successfully. However, because of the compaction and low-conductivity of the PVA membrane, it is adverse for the substrate to infiltrate into the enzyme membrane and for the electrons to transfer between the enzyme membrane and the electrode [11].

Electrospinning has been proved to be an efficient method for generating nanofibrous membranes with large surface area to volume ration and high porosity. Electrospun nanofibrous membranes could immobilize biological molecules with higher loading [12]. The enzyme activity can be enhanced through decreasing the diffusion resistance of substrate. In addition, nanofibrous membranes can be easy prepared in situ by means of electrospinning; the enzyme-immobilized samples therefore show great potential in development of biosensor.

Electrochemical biosensors using nanomaterials have recently attracted great attentions. Many nanomaterials, such as carbon nanotubes [5,13], gold nanoparticles [14], and metal oxides [15] have been used in biosensors. A new class of carbon material, graphene, which is a two-dimensional sheet of carbon atoms, has attracted increasing attention for phtoelectronic devices, supercapacitors, sensors and nanocomposites applications [16]. Shan [17] and Kang [6] first reported the direct electrochemistry of GOx on graphene. Their results demonstrated that grapheme possesses excellent electrochemical catalytic activity and electron transfer promoting ability which makes grapheme extremely attractive for enzyme-based biosensor.

In our previous study, the PVA/GOx electrospun membranes have demonstrated promising enzyme immobilization capability compared to the casted membranes because of its porous structure and large specific surface area. In order to further increase the enzyme immobilization efficiency, graphene was introduced to modify the PVA/GOx electrospun nanofibrous membrane. In order to understand the interaction between graphene and GOx, the effect of graphene adding content on the sensitivity, enzyme kinetics and activity of electrospun nanofibrous membranes were studied.

2 Materials and testing methods

2.1 Material

Poly (vinyl Alcohol) (PVA) with 98~99% hydrolyzed, and a molecular weight of 146,000-186,000 was purchased from Sigma-Aldrich. Graphene oxide aqueous solution (50wt%, N002-PS) was purchased from Angstron Material Inc., U.S.A. Glucose oxidase (153,100 units/G, G7141, Sigma-Aldrich) was used in the form of powder as received. Disodium hydrogen phosphate 12-water ($Na_2HPO_4 \cdot 12H_2O$, Showa Chemical Industry Co.) and sodium hydrogen phosphate dehydrate ($NaH_2PO_4 \cdot 2H_2O$, Showa Chemical Industry Co., Japan) were used for a phosphate buffer solution (PBS, 0.1M and pH 6.8). Dextrose Anhydrous (D(+)-Glucose) was purchased from Showa Chemical Industry Co., Japan. Glutaraldehyde (GA) solution with 25% concentration and 4-Aminoanipyrine (4-AA), peroxidase (POD) with 150 U/mg and N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (EHSPT) were purchased from Sigma-Aldrich. A Pt electrode with a length of 10 mm, width of 10 mm and thickness of 0.5 mm was purchased from Leesan Precious Metal CO., LTD, Taiwan.

2.2 Sample Preparations

2.2.1 Preparation of the Electrospinning Solution

A 7 wt% PVA solution was prepared by adding 7 g of PVA powder into 93 g of distilled water. Then, the solution was heated and stirred at a temperature of approximately 80℃ for 3 hours, after which, the well-dissolved solution was kept at room temperature.

The 7 %(w/v) PVA solutions were mixed with 14 mg of GOx powder and different volumes of graphene oxide solution, creating the electrospinning solution. The contents of graphene in the mixed solutions were 0, 5, 10 and 20 ppm.

2.2.2 Preparation of the PVA/GOx electrospun membranes

A self-assembled electrospinning device with an injection spinneret (0.008ml/min) powered by a syringe pump (KDS 101 Series, Kd scientific, USA) was used for the PVA/GOx electrospun membrane preparation. The syringe pump was connected to a Teflon tube which was attached to a stainless steel needle with a 0.31 mm internal diameter that acted as the spinneret. A copper grid covered by construction paper acted as the collector. An electrostatic controller (LGC-300 series, Taiwell, Taiwan) connected to the collector, and a ground wire connected to the spinneret; together, these were referred to as the collector charging system. The PVA/GOx electrospun nanofibrous membranes were directly electrospun and deposited onto the Pt electrode (with area: 10 mm^2) under the electrospinning conditions: applied voltage: 25 kV, spinning time; 10 min, and the working distance: 18 cm at a 40 % relative humidity (RH). Based on the experimental results, the addition of graphene did not influence the electrospinning behavior.

Aqueous solutions of glutaraldehyde and sulfuric acid were prepared for the purpose of PVA nanofibrous membranes cross-linking. The concentrations of glutaraldehyde and sulfuric acid were 5% and 2.5%, respectively. PVA nanofibrous membranes were treated with the vapor of the cross-linking solutions for 15 min. Then the sample was rinsed in a PBS solution for 3 times (5 min/time).

2.3 Electrochemical Measurements

In this study, the electrochemical measurement of glucose biosensor was three-electrode system with potentiostat (CH Instruments Electrochemical Analyzer). The working potential was 0.8V. The PBS (phosphate buffered solution, 0.1M and PH6.8) is a buffer solution containing disodium hydrogen phosphate 12-water and sodium hydrogen phosphate dehydrate. All electrochemical reactions were in the solution.

2.4 Evaluation of Enzyme Activity

To study the activity of the immobilized enzyme in the PVA/GOx electrospun nanofibrous membranes, a simple, spectrophotometric assay was used. The enzyme activity for GOx at 30°C was measured using a UV-Vis Spectroscopy spectrophotometer (Chrom Tech, Singapore) and Beer-Lambert Law.

First, we prepared a working solution (25 ml), containing 0.1 M phosphate buffer (pH 5.7), 15% glucose solution, 0.5% 4-Aminoanipyrine, 40 mM EHSPT solution ,and 5 U/ml GOD. The samples, each within the range of 0.5 mg to 1.7 mg, were immersed in a working solution and mixed by gentle inversion, respectively. Then, we record the increase in absorbance of the quinoneimine dye formed, as shown by Equation 1~4, at 555 nm against water per 30 sec for 8 times in a spectrophotometer thermostatic at 30℃ and calculated the absorbance per minute from the initial linear portion of the curve. The reaction in the working solution was as follows:

$$\text{B-D-Glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{D-Glucono-}\delta\text{-lactone} + H_2O_2 \quad (1)$$

$$2\,H_2O_2 + \text{4-AA} + \text{EHSPT} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4\,H_2O \quad (2)$$

The specific activity, expressed in μmol oxidized substrate/min/mg enzyme preparation, was calculated by the use of the Beer-Lambert Law:

$$\text{Abs.} = \varepsilon \times b \times c \quad (3)$$

where Abs. is the measured absorbance per minute, b is the light path length (in this case 1 cm), and c is the concentration (μmol/mL) of the measured substance (quinoneimine dye) at the absorbance concerned. The Millimolar extinction coefficient ε for the quinoneimine dye under the assay conditions is 32.8 (cm^2/μmol). Because one U causes the formation of one micromole of hydrogen peroxide (half a micromole of quinoneimine dye) per minute, the activity of the immobilized enzyme in solution (25mL) is 2*25c. When analyzing solid and

semi-solid samples, which are weighed out for sample preparation, the activity (U/mg) is calculated from the amount weighed as follows:

$$\text{GOx activity (U/mg of preparation)} = \frac{\text{GOx activity (U/mL sample solution)}}{\text{Weight}_{\text{sample}}\text{(mg/mL sample solution)}} \quad (4)$$

$\text{Weight}_{\text{sample}}$ ~ the weight of sample (mg).

3 Results and Discussion

3.1 The Morphology of PVA/GOx electrospun nanofibrous Membranes

Figure1 shows the deposited configuration of the PVA/GOx electrospun membrane on Pt electrode. The electrospun membrane was well-distributed on the Pt electrode in semitransparent appearance. Figure 2 shows the SEM image of the electrospun nanofibrous membrane after cross-linked. A porous structure with partial dissolving was found in the cross-linked PVA electrospun nanofibrous membrane. This proves that a larger reaction area between glucose and GOx was provided. The dispersion of graphene within the electrospun PVA nanofibers was examined by TEM and was shown in Figure 3. The graphene layers were folded and randomly distributed without overlapping within the electrospun PVA nanofibers.

Figure 1. The deposited configuration of the electrospun membrane on the Pt electrode.

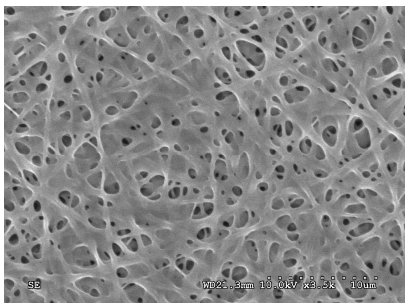

Figure 2. SEM image of the electrospun nanofibrous membrane after cross-linking.

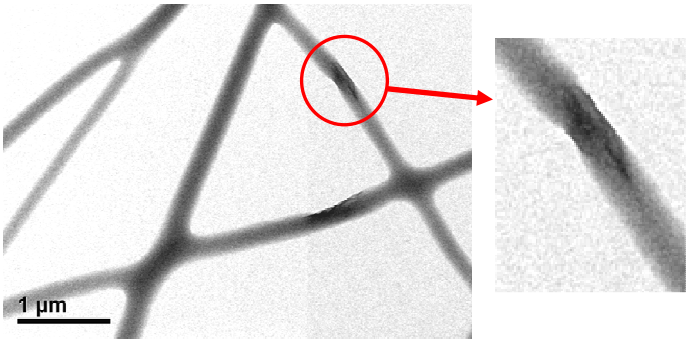

Figure 3. TEM image of the electrospun nanofibrous membrane modified by graphene

3.2 Electrochemical Characterization

3.2.1 Sensitivity analysis

Figure 4 displays the current-time (i-t) curves of different PVA membrane samples containing various graphene contents obtained by the chronoamperometric method. The glucose concentration was successive added from 1 to 34 mM. The plot of current vs. glucose concentration in Figure 4 generated the calibration curves of PVA samples containing different content of graphene as shown in Figure 5. A linear response to current is noticed for the glucose concentration range from 0 mM to 10 mM. The sensitivity of the PVA/GOx electrospun membrane was determined by the slope of the linear least-squares calibration plot

over the range 0 mM to 10 mM, and listed in Table 1. The sensitivity of the PVA/GOx electrospun membrane increased with the increasing of graphene concentration. The highest sensitivity was 38.7 mA/mM for PVA/GOx sample with 20 ppm graphene added, which exhibited 109 % increase compared to the PVA/GOx sample without graphene addition with sensitivity value of 18.5 mA/mM. The sensitivity increase with increasing of the graphene addition could be attributed to the effects of graphene on stabilize the enzyme conformation, facilitate the electrocatalytic reaction and increase the survivability of the enzyme. A plateau in current response was observed for a glucose concentration beyond 10 mM. This signifies the operation of the Michaelis-Menten kinetic mechanism for the enzyme-catalyzed process and will be discussed further in the next section.

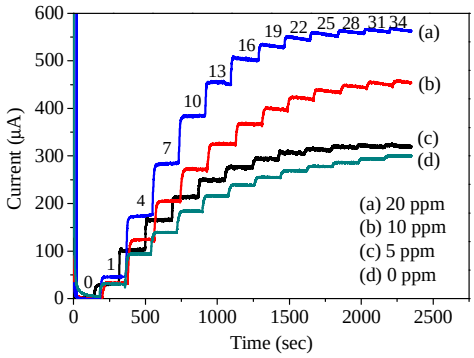

Figure 4. The current-time (i-t) curves of samples containing different content of graphene

Figure 6 shows the amperometric response to successive addition of 0 mM and 1 mM glucose on the PVA/GOx samples containing different contents of graphene. It can be seen that the PVA/GOx membranes responds rapidly to the injection of glucose, and reaching steady-state current within 12-20 sec. after each injection. The fast response occurred for the sample with 20 ppm graphene added, could be attributed to fast electron transfer and good electrocatalytic property provided by graphene and the close contact between GOx and graphene layers. The close contact between GOx and graphene layers allows the glucose oxidation induced oxygen concentration change being quickly monitored, and the signal can be transferred to electrode through numerous electron transfer pathways provided by graphene layer.
Compared to our previous study results on the best performance of gold nanoparticles added in PVA/GOx membranes, which had a sensitivity of 37.7 μA/mM and response time of 5.6 sec, graphene had a better efficiency on the sensitivity, but longer response time.

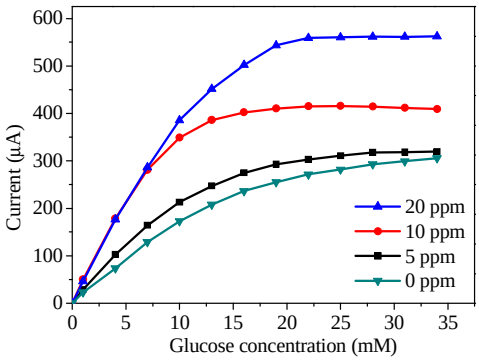

Figure 5. The resulting calibration curves of samples containing different content of graphene

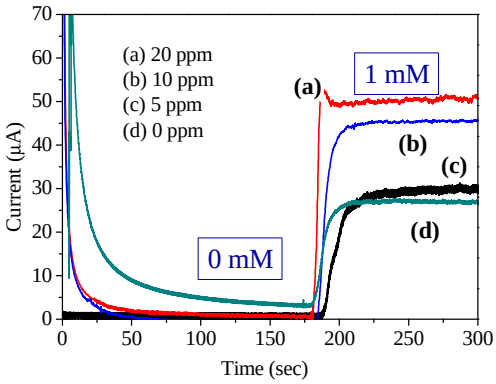

Figure 6. Amperometric response of PVA samples to successive addition of 0-1mM glucose

Content (ppm)	0	5	10	20
Sensitivity (μA/mM)	18.5	21.4	35.3	38.7
Response time (sec)	18.9	16.0	19.7	12.0
K_m^{app} (mM)	13.2	4.2	3.2	1.1
I_{max} (μA)	434.8	357.1	476.2	588.2

Table 1. The amperometric response of PVA/GOx samples modified by graphene

3.2.2 Enzyme Kinetics

As shown in Figures 5, when the glucose concentration was high, the response current had a slow and non-linear increase. These results reveal a Michaelis–Menten dynamic characteristic. The apparent Michaelis-Menten constant (K_m^{app}) can be calculated from the Hanes-Woolf regression [3] as follow:

$$\frac{S}{I} = \frac{1}{I_{max}} S + \frac{K_m^{qpp}}{I_{max}} \quad (5)$$

$$I_{max} = \frac{1}{\text{Slope}} \quad (6)$$

$$K_m^{app} = \text{y - intercept} \times I_{max} \quad (6)$$

where S is glucose concentration, I is the steady-state current, and Imax is the maximum current measured under saturated substrate condition.

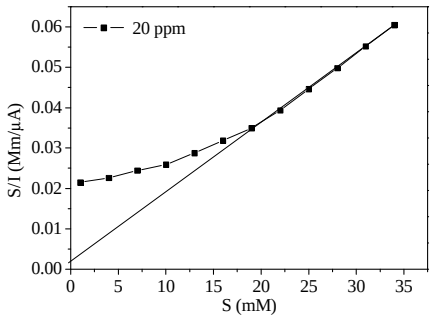

Figure 7. Hanes-Woolf Curve for PVA/GOx electrospun membrane with 20 ppm graphene content

The K_m^{app}, which gives an indication of enzyme substrate kinetics for the biosensor, was estimated based on the data obtained from the S/I vs. S curve, as shown in Figure 7. The K_m^{app} values for different PVA/GOx samples were thus determined and listed in Table 1. The K_m^{app} value decreased with the graphene concentration increased. The K_m^{app} value for PVA/GOx sample without graphene was 13.2 mM, whereas a much small value: 1.1 mM was obtained

for sample with 20 ppm of graphene addition. The lower value of K_m^{app} proved that the addition of graphene could facilitate electrocatalytic reaction between GOx and glucose and increase a higher biological affinity of immobilized enzymes to glucose. The I_{max} value increased with the graphene concentration increased. The results also support the electrocatalytic effect which exhibited higher rate of enzyme reaction at high graphene loading (20 ppm).

3.3 Enzyme Activity

To study the activity of the immobilized enzymes in the PVA/GOx electrospun membranes, spectrophotometric assay was used. Figure 8 shows the linearity of the reaction curves of the PVA/GOx electrospun membranes modified by graphene. The specific enzyme activity was calculated by the Beer-Lambert law. The enzyme activity of the immobilized enzymes in the electrospun nanofibrous membrane without graphene addition was 0.56±0.12 U/mg, whereas for the PVA/GOx sample with 20 ppm of graphene addition was 0.93±0.31 U/mg, which exhibits 66% increase compared to the sample without graphene added. This result indicated graphene may stabilize the conformation of enzyme and increase the survivability of the enzyme. Besides, graphene may also facilitate the catalytic reaction because of its high surface area and surface energy. Nanomaterial likes graphene with excellent electrical property; they display high affinity of immobilized enzymes, which improves the transfer of electrons between the active redox center of the enzyme and O_2 in the bulk solution during the redox process. Moreover, graphene may act as direct-electron-transferring channels from the active center of GOx to O_2 and facilitate the transfer of electrons. Those effects accelerate the regeneration of GOx and thus increase the relative activity of the enzymes. An schematic illustration reveals the interaction between graphene and enzyme within PVA/GOx electrospun nanofibrous membranes was thus proposed as shown in Figure 9.

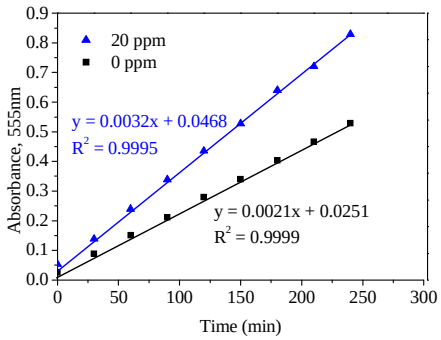

Figure 8. The linearity of the reaction curves of samples

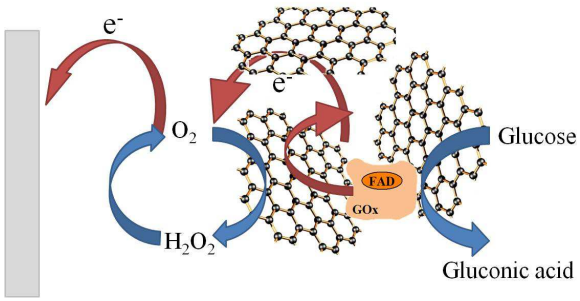

Figure 9. Schematic illustration reveals the interaction between graphene and enzyme

5. Conclusion

In this study, graphene modified PVA/GOx electrospun membranes were prepared to examine the immobilization mechanism between graphene and GOx. TEM image revealed that

graphene nanoplates were well exfoliated and dispersed within the nanofibers. The results of electrochemical measurement show that the sensitivities increased with the increasing of the graphene concentration, and tended to a constant at graphene concentration above 20 ppm. The highest sensitivity for PVA/GOx membrane with 20 ppm graphene addition was 38.7 (μA/mM), with 110% increase compared to the membrane without graphene. The K_m^{app} for 20 ppm graphene added sample was 1.12mM, which was much lower than the membrane without grapheme (13.2 mM). The lower K_m^{app} value could be attributed to the facilitation effect on catalytic reaction between GOx and glucose and higher biological affinity between the immobilized enzyme and glucose. The activities of immobilized enzyme in PVA/GOx membrane with and without grapheme added were 0.93 and 0.56 U/mg, respectively. This result indicated that the presence of graphene may contribute to stabilize the conformation of enzyme, facilitate the catalytic reaction and increase the survivability of the enzyme.

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