Graphene modified electrospun poly(vinyl alcohol) nanofibrous membranes for glucose oxidase immobilization

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Received 10 February 2014 ; accepted in revised form 18 April 2014

Abstract. In this study, poly(vinyl alcohol) (PVA)/Glucose oxidase (GOx)/graphene biocomposite membranes were prepared using an electrospinning technique and used for enzyme immobilization. The PVA/GOx/graphene membrane's morphology was examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM), while its electrochemical sensitivity was studied by chronoamperometry. Kinetic parameters were determined to clarify the role of graphene in enzyme immobilization, while a spectrophotometric assay was used to quantify the active enzyme. The results indicated that the presence of graphene helps to stabilize the enzyme's conformation, facilitate the catalytic reaction, and increase the survivability of the enzyme.

Keywords: polymer membranes, electrospun, glucose biosensor, immobilization, graphene

1. Introduction

Diabetes is an increasingly important worldwide public health problem. The metabolic disorder results from either insulin deficiency, in type 1 diabetic patients, or from insulin resistance and its complications in type 2 patients. Both conditions can result in hyperglycemia leading to blood glucose concentrations higher than the normal range 80–120 mg/dL (4.4–6.6 mM) [1]. The diagnosis and management of diabetes requires the careful monitoring of blood glucose levels. The glucose biosensor has thus become one of the most important physiological monitoring devices, accounting for about 85% of the entire biosensor market [1].

A biosensor is a device which combines an immobilized bio-recognition element with a transducer. It can monitor chemical substances on the inside or outside of an organism by generating a signal representative of the analyte's concentration after cou-

pling the biochemical and transducer reactions [2]. Glucose biosensors are based on electrochemical principles and frequently employ enzymes as the biological recognition element. The biosensor's performance and sensitivity often strongly depends on the influence imposed on the enzyme's structure by immobilization. Such influences result from the choice of host molecule and the immobilization method used. In attempts to retain enzymatic activity and stability various molecules have been used to immobilize enzymes on different substrates, e.g. poly(vinyl alcohol) (PVA) [3], poly(ethylene oxide) (PEO) [4], chitosan [5], polymethylmethacrylate (PMMA) [6], poly(vinyl pyrrolidone) (PVP) [7] and polyurethane (PU) [8]. PVA is often used as an immobilization matrix because of its inherent non-toxicity, high thermal stability, good biocompatibility and its desirable physical properties such as its elastic nature, good film forming properties, and its

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high degree of swelling in aqueous solutions – all of which contribute to making it a good matrix for enzyme immobilization [9]. Different methods, such as the cross-linking of PVA [10], freeze-thawed PVA [11], enzyme encapsulation in PVA/silicate hybrid materials [3], have been successfully employed to immobilize the enzyme molecules in various membranes. However, because of the compaction and low-conductivity of the PVA membrane, substrate infiltration and electron transfer between the enzyme membrane and the electrode remains problematic [12].

Electrospinning has proved to be an efficient method for generating nanofibrous membranes with large surface area to volume ratios and high porosities, because electrospun nanofibrous membranes can immobilize biological molecules with higher loadings [13–15], while enzyme activity can be enhanced by decreasing the diffusion resistance of the substrate. In addition, nanofibrous membranes can be easily prepared *in situ* by electrospinning; the enzyme-immobilized materials therefore show great potential for biosensor development [16, 17].

Electrochemical biosensors using nanomaterials have recently attracted much attention. Many nanomaterials, such as carbon nanotubes [5, 18], gold nanoparticles [19, 20], and metal oxides [21, 22] have been used in biosensors. More recently a new class of carbon material, graphene, which is a two-dimensional sheet of carbon atoms, has attracted increasing attention with respect to its potential applications in photoelectronic devices, supercapacitors, sensors and nanocomposites applications [6, 23–32]. Due to their planar morphology and thus larger accessible surface area, graphene may perform better than any other carbon-based materials for an electrode sensor fabrication. Kang *et al.* [6], reported the direct electrochemistry of GOx on graphene. Their results demonstrated that graphene possesses both an excellent electrochemical catalytic activity and an electron transfer promoting ability making it extremely attractive for applications in enzyme-based biosensors.

Shan *et al.* [25] prepared graphene sheets protected by polyvinylpyrrolidone that, exhibited high electrocatalytic activity towards the reduction of O_2 and H_2O_2 , which could be stably dispersed in water. Because of their favorable electronic properties and biocompatibility, graphene-based composites accomplished the direct electron transfer of redox enzyme

while maintaining a good level of bioactivity. The authors claimed direct electron transfer from the GOx via the graphene sheets. Shan *et al.* [26] also constructed graphene/Au/chitosan nanocomposite electrodes and suggested that the synergistic effect of graphene and Au nanoparticles might promote electrocatalysis towards H_2O_2 . The high sensitivity and good stability of such a modified electrode contributed to the construction of a practical glucose biosensor. Su *et al.* [27] reported the synergy mechanism of electrospun PVA/chitosan nanofibers and graphene nanosheets for efficient glucose biosensing. These results indicate graphene shows significant potential for fabricating an electrochemical sensing interface. The graphene based enzyme sensor exhibited excellent sensitivity and long-term stability for measuring glucose. Song *et al.* [28] showed that graphene possesses intrinsic peroxidase catalytic activity due to the presence of carboxylic groups at the edges of the graphene sheets, so there is no need for the GOx enzyme; additionally, such a biosensor exhibits a longer stable shelf-life, as it does not contain any biomolecule within the matrix.

In our previous study [33, 34], PVA/GOx electrospun membranes, with porous structures and large specific surface areas, were demonstrated to have promising enzyme immobilization capabilities compared to cast membranes. In this work we have attempted to understand the interactions between graphene and GOx: here the effects of adding graphene on sensitivity, enzyme kinetics and the activity of electrospun nanofibrous membranes are presented.

2. Materials and testing methods

2.1. Material

Poly(vinyl alcohol) (PVA) 98~99% hydrolyzed, molecular weight 146 000–186 000 was purchased from Sigma-Aldrich. Graphene oxide aqueous solution (50 wt%, N002-PS) was purchased from Angstrom Material Inc., U.S.A. Glucose oxidase (153 100 units/G, G7141, Sigma-Aldrich) was used in the form of powder as received. Disodium phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$, Showa Chemical Industry Co.) and sodium hydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$, Showa Chemical Industry Co., Japan) were used for preparing the phosphate buffer solution (PBS, 0.1M and pH 6.8). Dextrose Anhydrous (D(+)-Glucose) was pur-

chased 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% (w/v) PVA solution was prepared by adding 7 g of PVA powder into 93 mL of distilled water. Then, the solution was heated and stirred at $\sim 80^\circ\text{C}$ for 3 hours, after which, the solution was kept at room temperature. The 7% (w/v) PVA solution was mixed with 14 mg of GOx powder and graphene oxide solution, creating the electrospinning solution. Solutions were prepared to have final contents equivalent to 0, 5, 10, 20 and 40 ppm graphene.

2.2.2. Preparation of the PVA/GOx electrospun membranes

A self-assembled electrospinning device with an injection spinneret (0.008 mL/min) powered by a syringe pump (KDS 101 Series, Kd scientific, USA) was used for the PVA/GOx membrane preparation. The syringe pump was connected to a Teflon tube attached to a stainless steel needle (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 acted as the collector charging system. The PVA/GOx electrospun nanofibrous membranes were directly electrospun and deposited onto the Pt electrode (area 10 mm^2) under the following electrospinning conditions: applied voltage 14 kV, spinning time 10 min, and working distance 18 cm at 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 PVA nanofibrous membrane 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 3 times (5 min/each time).

2.3. Electrochemical measurements

In this study, the glucose biosensor's electrochemical measurements were made using a three-electrode system with a potentiostat (CH Instruments Electrochemical Analyzer) [3]. The auxiliary electrode was a Pt wire, and an Ag/AgCl electrode was used as a reference. The working potential was 0.8 V. Amperometric measurements were carried out under stirring, and the response current was marked with the change value (Δi) between the steady-state current and background current. All experiments were performed at room temperature.

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).

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 to 1.7 mg, were immersed in a working solution and mixed by gentle inversion. Then, we recorded the increase in absorbance of the quinoneimine dye formed, as shown by Equations (1)–(4), at 555 nm against water for 30 sec (8 times) in a spectrophotometer, thermostatically controlled at 30°C . The absorbance (per minute) was calculated from the initial linear portion of the curve. The reaction in the working solution was as follows (Equations (1) and (2)):

The specific activity, expressed in μmol oxidized substrate/min/mg enzyme preparation, was calculated by the use of the Beer-Lambert law (Equations (3)):

$$\text{Abs.} = \epsilon \cdot b \cdot c \quad (3)$$

where $Abs.$ is the measured absorbance per minute, b is the light path length (in this case 1 cm), and c is the concentration [$\mu\text{mol/mL}$] of the measured substance (quinoneimine dye) at the absorbance concerned. The Millimolar extinction coefficient ε for the quinoneimine dye under the assay conditions is $32.8 \text{ cm}^2/\mu\text{mol}$. Because 1 U (enzyme activity) results in the formation of one micromole of hydrogen peroxide (half a micromole of quinoneimine dye) per minute, the activity of the immobilized enzyme in solution (25 mL) 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 Equation (4):

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

$\text{Weight}_{\text{sample}} \sim$ the weight of sample [mg].

3. Results and discussion

3.1. The morphology of PVA/GOx electrospun nanofibrous membranes

Figure 1 shows the deposited configuration of the PVA/GOx electrospun membrane on the Pt electrode. The electrospun membrane was well-distributed on the Pt electrode and had a semitransparent appearance. Figure 2 shows the SEM image of the electrospun nanofibrous membrane after cross-linking. A partially dissolved porous structure was found in the cross-linked PVA electrospun nanofibrous membrane proving the existence of a large reaction area between glucose and GOx. The dispersion of

graphene within the electrospun PVA nanofibers was examined by TEM and is shown in Figure 3. The graphene layers were folded and randomly distributed within the electrospun PVA nanofibers. The successful loading of graphene in PVA nanofibers may play an important role in the immobilization of

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 using different magnifications

enzyme molecules and the access of enzymes to the substrate.

3.2. Electrochemical characterization

3.2.1. Sensitivity analysis

Figure 4 shows 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 increased, by adding the analyte, 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 apparent for glucose concentrations from 0 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 to 10 mM, see Table 1. The sensitivity of the PVA/GOx electrospun membrane increased with increasing graphene concentrations up to 20 ppm, but decreased at 40 ppm. The highest sensitivity

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

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

was 38.7 mA/mM, found for the PVA/GOx sample with 20 ppm graphene, representing a 109% increase compared to the PVA/GOx sample without graphene addition (sensitivity: 18.5 mA/mM). The sensitivity increase with increasing graphene additions is thought to result from enhanced enzyme conformational stabilization due to the presence of graphene, facilitating the electrocatalytic reaction while simultaneously increasing the enzyme's survivability. However, the sensitivity decrease at high graphene addition (40 ppm) may be attributed to the aggregation of graphene within the electrospun PVA nanofibers. A plateau in current response was observed for glucose concentrations beyond 10 mM, resulting from operation of Michaelis-Menten kinetics and will be discussed further in the next section.

Figure 6 shows the amperometric response to successive additions of 1 and 4 mM glucose on the PVA/GOx samples with different graphene contents. It can be seen that the PVA/GOx membranes respond rapidly to the injection of glucose, and reach a steady-state current within 10–14 sec. after each injection. The fast response apparent with 20 ppm graphene is attributed to the fast electron transfer and good electrocatalytic properties provided by graphene and to the close contact between the GOx and the graphene layers. The close contact between the GOx and the graphene layers allows the glucose concentration change to be quickly monitored with the signal being transferred to the electrode through numerous electron transfer pathways provided by the graphene layer.

Compared to our previous study results [19] that showed the best performance of gold nanoparticles, added in PVA/GOx membranes, to have a sensitivity of 37.7 μ A/mM and a response time of 5.7 sec;

Figure 6. Amperometric response of PVA samples to successive additions of 1–4 mM glucose

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

Content [ppm]	0	5	10	20	40
Sensitivity [$\mu\text{A}/\text{mM}$]	18.50	21.40	35.30	38.70	28.50
Response time [s]	13.90	12.70	10.20	9.90	23.80
Reaction rate [$\mu\text{A}/\text{s}$]	3.52	7.92	8.22	9.76	2.43
K_m^{app} [mM]	13.20	4.20	3.20	1.10	13.80
I_{max} [μA]	434.80	357.10	476.20	588.20	454.50

Figure 7. The current-to-voltage response for PVA and nanogold, graphene modified PVA electrospun membranes

membranes with added graphene showed a higher sensitivity, but a longer response time. We know that the layer structure of graphene nanoplates exhibits an electrical barrier leading to reduced conductivity. The current-to-voltage response for pure PVA and nanogold, graphene modified PVA membranes are shown in Figure 7. The determined values are $7.7 \cdot 10^{-5}$, $2.0 \cdot 10^{-4}$ and $4.9 \cdot 10^{-4}$ mhos for PVA, PVA/graphene and PVA/nanogold electrospun membranes respectively. Thus, the response time is influenced by electrical conductivity under similar sensitivity.

3.2.2. Enzyme kinetics

As shown in Figure 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 shown by Equations (5)–(7):

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

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

Figure 8. Hanes-Woolf curve for PVA/GOx electrospun membrane with 20 ppm graphene content

$$K_m^{\text{app}} = y - \text{intercept} \cdot I_{\text{max}} \quad (7)$$

where S is glucose concentration, I is the steady-state current, and I_{max} is the maximum current measured under saturated substrate conditions.

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, shown in Figure 8. The K_m^{app} values for different PVA/GOx samples were determined and are listed in Table 1. The K_m^{app} value decreased with the increase in graphene concentration. The K_m^{app} value for the PVA/GOx sample without graphene was 13.2 mM, whereas a much smaller value: 1.1 mM was obtained for a sample with a 20 ppm graphene addition. The lower value of K_m^{app} proved that the addition of graphene could facilitate the electrocatalytic reaction between GOx and glucose while increasing the biological affinity of immobilized enzymes to glucose. The I_{max} value increased with increases in the graphene concentration. The results also support the notion of an electrocatalytic effect driving higher rates of enzyme reaction at high graphene loadings (20 ppm).

3.3. Enzyme activity

To study the activity of the immobilized enzymes in the PVA/GOx electrospun membranes a spectrophotometric assay was used. Figure 9 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

Figure 9. The linearity of the reaction curves of samples

Figure 10. Schematic illustration reveals the interaction between graphene and enzyme

with 20 ppm of graphene it was 0.93 ± 0.31 U/mg, i.e. a 66% increase compared to the sample without graphene. This result indicated graphene may stabilize the enzyme's conformation and hence increase its survivability. Graphene may also facilitate the catalytic reaction because of its high surface area and surface energy. Nanomaterials like graphene with good electrical properties display high affinity for immobilized enzymes, due to their ability to improve 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 supply direct-electron-transferring channels from the active center of GOx to O_2 , such effects accelerate the regeneration of GOx and thus increase the relative activity of the enzymes. A schematic illustration (Figure 10) reveals the interaction between graphene and enzyme within PVA/GOx electrospun nanofibrous membranes.

5. Conclusions

In this study, graphene modified PVA/GOx electrospun membranes were prepared to examine the immobilization mechanism between graphene and GOx. TEM images revealed that graphene nanoplates were dispersed within the nanofibers. The electro-

chemical measurement results show that the sensitivities increased with increasing graphene concentrations up to 20 ppm. The highest sensitivity recorded $38.7 \mu A/mM$ was for a PVA/GOx membrane with 20 ppm graphene representing a 109% increase over a membrane made without graphene. The K_m^{app} for the 20 ppm graphene content membrane was 1.1 mM, which was much lower than the membrane without graphene (13.2 mM). The lower K_m^{app} value was attributed to the facilitation of the catalytic reaction between GOx and glucose and the higher biological affinity between the immobilized enzyme and glucose. The activities of the immobilized enzyme in the PVA/GOx membrane with and without graphene added were 0.93 and 0.56 U/mg, respectively. These results indicate that the presence of graphene contributes to the maintenance of the enzyme's conformational stability which in turn facilitates the catalytic reaction while extending its working lifespan.

Acknowledgements

Part of this work is financially supported from the National Science Council of Taiwan, ROC, under contract numbers: NSC 101-2221-E-035-013 and NSC 102-2221-E-011 -160.

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