Asian Journal of Chemistry; Vol. 24, No. 6 (2012), 2703-2706

ASIAN JOURNAL OF CHEMISTRY

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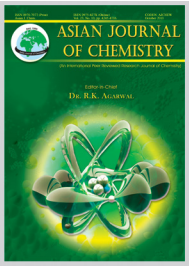

Electrochemical Characterization of Immobilized Glucose Oxidase on Matrix of Graphene-Ion Liquid Composite

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(*Received*: 18 June 2011; *Accepted*: 17 January 2012) AJC-10977

The graphene-ion liquid nanocomposite (IL-GNs), characterized by good conductivity, high stability and good biocompatibility, provides a suitable biosensing matrix. Enzymes can be firmly incorporated into the matrix without the aid of other cross-linking reagents. Based on this, the glucose oxidase (GOD) is entrapped in the composite of IL-GNs and the direct electron transfer reaction between glucose oxidase and electrode takes place. The electron transfer rate of glucose oxidase is greatly enhanced to 6.7 s^{-1} in the system. It demonstrates that the conformational change of glucose oxidase in the microenvironment enables the accessibility of active site for glucose oxidase to the electrode. In addition, a third-generation glucose biosensor has been developed. The amperometric response of this sensor to glucose is observed with a linear range between 0.2 mM to 5 mM and a detection limit of 30 μM. The facile procedure of immobsilizing glucose oxidase will promote the developments of electrochemical research for protein, biosensors and other bio-electrochemical devices.

Key Words: Graphene, Ionic liquid, Glucose oxidase, Direct electron transfer, Biosensor.

INTRODUCTION

Geim *et al.*[1] in the Manchester University of UK discovered graphene. Graphene is a 2D single layer of carbon atoms with the hexagonal packed structure. It also has some good properties of both graphite and carbon nanotubes, such as high thermal conductivity and high mechanical strength. More importantly, it has unique electronic structure and electrical properties[2,3]. Unmodified grapheme has very poor solubility in water and other common organic solvents. Many exploratory studies have demonstrated that functional graphene not only improves the solubility, but also provides graphene with new physical and chemical properties due to the characteristics of modifier[4-6].

Recently, many bio-active molecules in biochemistry have been used for covalently binding to the graphene surface and the results have shown that grapheme-biological molecules nano-composite materials are expected to become a very useful tool in the biochemical field[7]. Glucose oxidase (GOD) with molecular weights of 150-180 kDa is a flavin enzyme and its glucose-catalytic properties has been widely used to monitor blood glucose in patients with diabetes[8]. However, the active center of glucose oxidase is buried in the protective shell of protein, so it is extremely difficult to achieve the direct electrochemistry of glucose oxidase. Direct contact of redox proteins with the naked metal surface usually leads to the changes in structure and function of proteins to lose its biological activity. The ideal interface properties can be fulfilled by the modification of electrode and proteins.

In recent years, room temperature ionic liquids (IL), which has been increasingly concerned in the fields of biology and bio-electrochemistry, can maintain and promote the activity of protein[9]. Ionic liquids is an environmental protected reagent. At room temperature, it is entirely composed of ions and has a certain viscosity and the unique physical and chemical properties, such as high thermal stability, low vapour pressure and relatively high ionic conductivity. Due to the good electrochemical stability and the ability to maintain or even elevate the biological activity of the enzyme, ionic liquids has wide application prospects in the fields of biology and bio-electrochemistry.

In this paper, graphene can be easily dispersedly distributed by simple grinding of graphene and ionic liquids. Ionic liquids-graphene nano-composite (IL-GNs) is a new type of biological platform for biocompatible enzyme immobilization. We use a simple embedding method for glucose oxidase immobilization in the composite surface, achieve the direct electron transfer of glucose oxidase and maintain the glucose-catalytic activity of glucose oxidase. Thus the third generation of glucose sensors is successfully prepared.

EXPERIMENTAL

Glucose oxidase from *Aspergillus niger* was purchased from Sigma. Polylysine was purchased from Aldrich and used

without purification. Graphite powder, hydrazine, potassium permanganate, sulfuric acid, aqueous ammonia and glucose were purchased from Beijing Chemical Reagent Company, China. The glucose solution was placed at least 24 h prior to use to allow a balance between the different isomers. Other reagents were of analytical grade. Phosphate buffer saline (PBS) with different pH value, as support electrolytes, was composed of 0.1 M potassium dihydrogen phosphate and disodium hydrogen phosphate. Millipore Milli-Q ultrapure water was used in each experiment.

Graphite powder was used as raw material and Hummers' method for synthesis of graphite oxide was performed through liquid phase oxidation. In brief, 23 mL of concentrated sulfuric acid was cooled to 0 ºC and then 1 g of graphite powder was added, followed with stirring to obtain solution A. A certain amount of $KMnO_4$ was slowly added with stirring to solution A, followed with the reaction in 35 ºC water bath for 2 h. A certain amount of deionized water was slowly added for dilution and the solution was not boiling during the process. The dilution was added to 30 % H_2O_2, followed with filtration when the solution was hot. The filter cake (graphite oxide) was dried in 100 ºC oven and stored for use.

Graphite oxide was dissolved in PBS to prepare 1 mg mL^{-1} solution. 3.5 µL of hydrazine and 40.0 µL of aqueous ammonia were added to 5 mL of graphite oxide solution, stirring a few minutes and incubating in 95 ºC oil bath for 1 h. After cooling to room temperature, graphene was acquired by filtration. 20 mg of graphene and 0.2 mL of ionic liquids were mixed and grinded in a mortar for about 20 min to prepare black viscous IL-GNs. Under the optical microscope, the appropriate amount of IL-GNs was carefully coated on the surface of the glassy carbon (GC) working electrode to prepare IL-GNs/GC electrode. The procedure for the preparation of glucose oxidase/IL-GNs/GC electrode was similar to the preparation of IL-GNs/GC electrode, but glucose oxidase was dissolved in the ionic liquids.

Detection method: Atomic force microscopy (AFM) was performed by the SPA-400 instruments equipped with the SPI-3800 software (Seiko Instruments Industry Co., Tokyo, Japan). Cyclic voltammetry and chronoamperometry were performed by CHI660B Electrochemical Instrument (USA); during the process, the conventional three-electrode system was used, the modified and unmodified glassy carbon electrodes served as the working electrodes, spiral-shaped platinum wire was used as a counter electrode and Ag/AgCl electrode (saturated KCl solution) served as a reference electrode. Prior to the experiment, high-purity nitrogen or high-purity oxygen were input at least 30 min or 15 min respectively to prepare the nitrogen- or oxygen-saturated solution. Chronoamperometry was performed with the conditions of oxygen-saturated solution, constant potential and continuous stirring. All experiments were conducted at room temperature.

RESULTS AND DISCUSSION

Characterization of IL-GNs membrane: Fig. 1 showed the surface morphology of IL-GNs membrane determined by atomic force microscopy. The single layer IL-GNs membrane was 1.8 nm in the thickness, which was apparently thicker when compared with non-functional graphene with a thickness of 0.5 nm, indicating that ionic liquids modification to the graphene surface was successful.

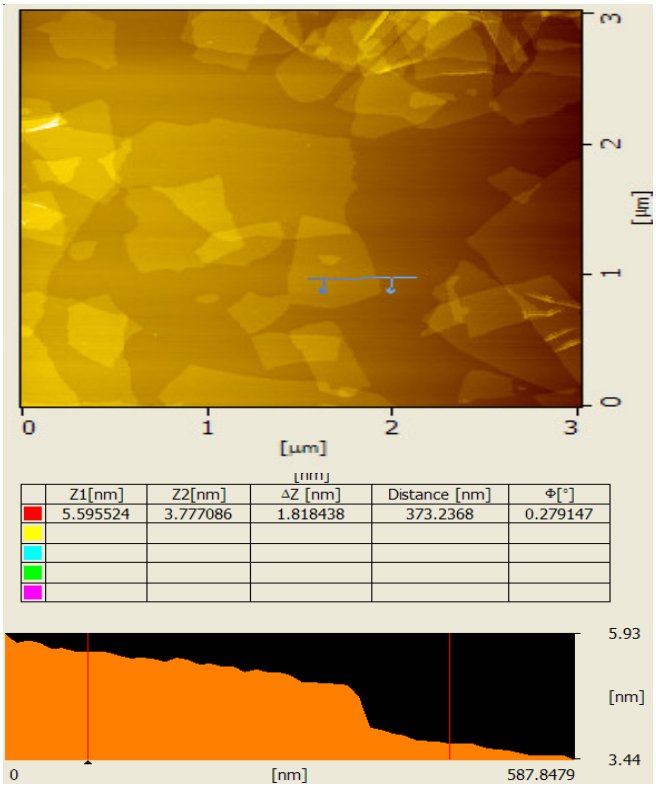

Fig. 1. Atomic force microscopy of ionic liquid-graphene composite on glassy carbon surface

Direct electrochemistry of glucose oxidase (GOD): Fig. 2A showed the cyclic voltammogram of the GOD/IL-GNs/GC electrodes reacted in nitrogen-saturated solution. A pair of distinct, nearly symmetrical and reversible redox peaks appeared at -0.46 V. The peak position and the control experiment demonstrated that this pair of peaks came from glucose oxidase[10]. The value of peak-peak difference (ΔE_p) was 26 mV as the scanning speed was 10 mV s^{-1}. Glucose oxidase in the range of the scanning speed showed a reversible surface redox electrochemistry. As shown in Fig. 2B, the oxidation current and the reduction current had good linear relationships with the scanning speed in the range of 400 mV s^{-1} respectively; its over potential was almost independent of the scanning speed and the oxidation/reduction peak current ratio did not change in the range of the scanning speed. These results demonstrated that the direct electrochemical behaviour of glucose oxidase immobilized on IL-GNs surface was the surface-controlled electron transfer process. When the potential scanning speed was greater than 600 mV s^{-1}, the redox peaks of the electrodes had a serious distortion, indicating that the electrochemical reaction of the electrodes at high scanning speed became into an irreversible process. For the irreversible electrode reaction system, the relationship between the peak potential and the potential scanning speed can be described by Laviron's equation[11].

$$E_{pc} = E^{o'} + \frac{RT}{\alpha nF}\ln\frac{RTK_s}{\alpha nF} - \frac{RT}{\alpha nF}\ln\nu \quad (1)$$

$$E_{pa} = E^{o'} + \frac{RT}{(1-\alpha)nF}\ln[\frac{RT}{(1-\alpha)nF}] + \frac{RT}{(1-\alpha)nF}\ln\nu \quad (2)$$

From the formula, α is the electronic transfer coefficient, K_s is the standard speed constant in the heterogeneous surface reaction, ν is the scanning speed, $E^{o'}$ is the formal potential. Based on the above heterogeneous surface reaction dynamics of the electrodes, the kinetic constants of reaction was calculated: the electron transfer rate of glucose oxidase on the modified electrode was 6.7 s^{-1}, which was higher than 1.7 s^{-1} when the glucose oxidase adsorbed on carbon nanotubes[8] and was higher than 1.6 s^{-1} when glucose oxidase adsorbed on the GC electrode[9]. These results further demonstrated that the unique microenvironment of IL-GNs was conducive to promoting the electron transfer between the active center of glucose oxidase and the electrode surfaces.

Fig. 2C showed the cyclic voltammogram of the GOD/IL-GNs/GC electrodes in PBS with different pH values. As shown in Fig. 2D, the over potential of glucose oxidase and the pH value had a linear relationship and the slope was about -51 mV pH^{-1}, indicating that glucose oxidase occurred the reversible equimolar proton-electron coupling process during the electron transfer. According to Laviron's theory[12], when the adsorption phenomena accords with Langmuir's isothermal formula, the peak current and the scanning speed have a linear relationship:

$$I_p = \frac{n^2F^2A\nu\Gamma}{4RT} = \frac{nFQ\nu}{4RT} \quad (3)$$

From the formula, Q = nFAΓ is the peak area (electric quantity, C), n is the number of electrons involved in the reaction, ν is the potential scanning speed, G is the adsorption capacity of the electrode surface, other symbols are of universal physical meaning. Based on this formula, the calculated number of electron transfer was approximately 2.14, indicating that the process was the coupling of two electrons with two protons. This finding is consistent with the results reported in the literature[7].

Determination of glucose by GOD/IL-GNs/GC electrode: Glucose oxidase adsorbed on the electrode surface has the electro-catalytic response to the reduction of dissolved oxygen according to the eqn. (4)[5]:

$$\begin{aligned} GOD(FAD) + 2e^- + 2H^+ &\rightarrow GOD(FADH_2) \\ GOD(FADH_2) + O_2 &\rightarrow GOD(FAD) + H_2O_2 \end{aligned} \quad (4)$$

Glucose oxidase (GOD) can catalyze the reduction of dissolved oxygen and apparently elevate the reduction peak current of the direct electrochemistry of glucose oxidase. After addition of glucose (the substrate of glucose oxidase) solution to air saturated PBS, glucose oxidase immobilized on the electrode surface performed the enzyme-catalytic reactions according to the eqn. (5):

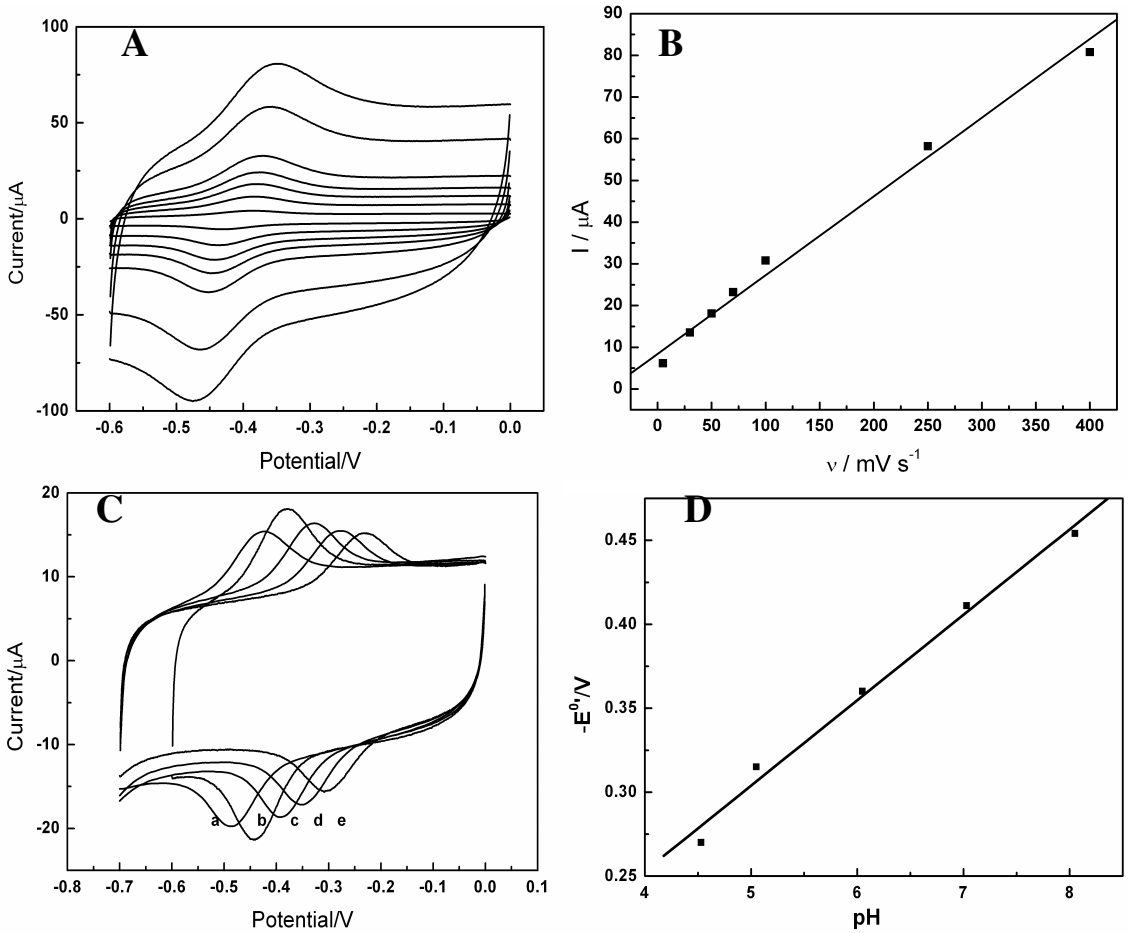

Fig. 2. (A) Cyclic voltammograms of the GOD/IL-GNs/GC electrode in PBS with N_2-saturated at various scan rate. (B) The plots of peaks vs. scan rates. (C) Cyclic voltammograms of the GOD/IL-GNs/GC electrode in PBS with N_2-saturated at various pH, from a to e the pH value is 4.5, 5.0, 6.0, 7.0 and 8.0. (D) The plots of peaks *vs*. pH value

$$\text{Glucose} + \text{GOD(FAD)} \longrightarrow \text{gluconolactone} + \text{GOD(FADH}_2\text{)} \quad (5)$$

This restricted the electro-catalytic reaction of glucose oxidase on the dissolved oxygen and reduced the direct electrochemical reduction peak current of glucose oxidase. Therefore, with the increase in the concentration of glucose, the direct electrochemical reduced current response of glucose oxidase for the GOD/IL-GNs/GC electrode was decreased. Accordingly, a third-generation of glucose sensor was constructed.

Fig. 3A showed a typical time current response curve applied potential, -0.5 V) of the GOD/IL-GNs/GC electrode in response to the successive addition of glucose. The linear range of glucose detected was 0.2-5 mM and the minimum detection limit of the GOD/IL-GNs/GC electrode was 30 μM (Fig. 3B). The reproducibility of the multi-layer membrane modified electrode was also investigated in this paper. Six electrodes prepared under the same conditions were used to detect 2 mM glucose and the relative standard deviation of the electrochemical signals was 5.5 %. The long-term stability of the modified electrodes was investigated. The modified electrodes were stored in 4 ºC and were taken out for use in the detection of glucose each day. The results showed that the electrochemical signals could still remain 90.1 % after 30 d.

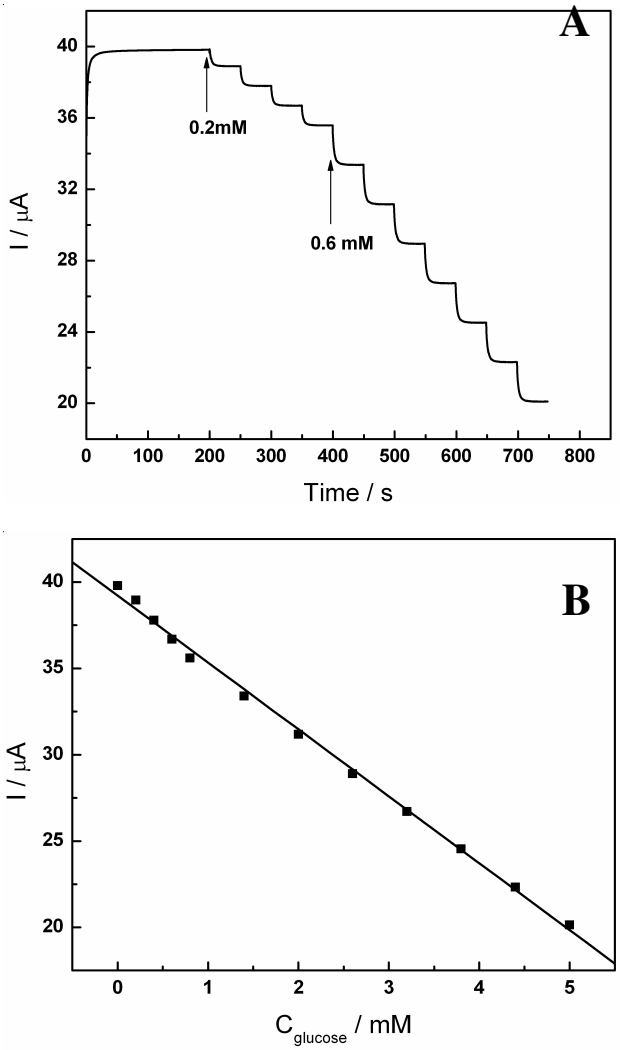

Fig. 3. (A) Typical steady-state current response of the biosensor on successive addition of glucose. Applied potential, -0.5 V. (B) Calibration curve of the electrocatalytic current on the concentration of glucose

Determination of glucose in serum: The concentration of glucose in serum samples was determined by standard addition method. In the range of 8.0×10^{-4}-3.2×10^{-3} mol L^{-1} of glucose, the recovery rates for eight consecutive measurements were in the range of 95.3-106.2 %. Uric acid and ascorbic acid did not affect the determination of serum samples.

Conclusion

The ionic liquid-graphene nano-composite sol (IL-GNs) was used to modify the glassy carbon electrode surface, forming a stable modified layer. The IL-GNs had the dual nature of graphene and ionic liquids. Particularly, the synergistic effect of graphene and ionic liquids greatly improved the conductivity and biocompatibility of the electrodes and thus the modified electrodes could serve as a good bio-electrochemical platform. Glucose oxidase could be stably coated on the modified electrodes and perform the direct electrochemistry. Graphene and ionic liquids had the synergistic effect on promoting the enzyme's electron transfer and made more reversible electron transfer behaviour of glucose oxidase. A high electron transfer speed of 6.7 s^{-1} may be because glucose oxidase under the specific micro-environment had the conformation change that was more conducive to the active center approaching to the electrode surface. In addition, glucose oxidase in the system could better maintain the catalytic activity on glucose. Based on this, a third-generation of glucose sensor was constructed. The linear range for the detection of glucose was 0.2-5 mM and the minimum detection limit of the modified electrodes was 30 μM. The traits, such as good catalytic ability and facile preparation, indicated that ionic liquid-graphene nanocomposites could greatly contribute to the biosensors and other bio-electrochemical fields.

ACKNOWLEDGEMENTS

This work was supported by China postdoctoral scientific fund (No.20090451122); Jilin Science and Technology Commission fund (No. 20090184).

REFERENCES

1. K.S. Novoselov, A.K. Geim, S.V. Morozov, D. Jiang, Y. Zhang, S.V. Dubonos, I.V. Grigorieva and A.A. Firsov, *Science*, **306**, 666 (2004).
2. A.A. Balandin, S. Ghosh, W.Z. Bao, I. Calizo, D. Teweldebrhan and F. Miao and C.N. Lau, *Nano Lett.*, **8**, 902 (2008).
3. K.S. Noselov, Z. Jiang, Y. Zhang, H.L. Stormer, U. Zeitler, J.C. Maan, G.S. Boebinger, P. Kim and A.K. Geim, *Science*, **315**, 1379 (2007).
4. A. Varykhalov, J. Sánchez-Barriga, A. M. Shikin, C. Biswas, E. Vescovo, A. Rybkin, D. Marchenko and O. Rader, *Phys. Rev. Lett.*, **101**, 157601 (2008).
5. A. Tiberj, J.-R. Huntzinger, J. Camassel, F. Hiebel, A. Mahmood, P. Mallet, C. Naud and J.-Y. Veuillen, *Nanoscale Res. Lett.*, **6**, 171 (2011).
6. D.-C. Wei and Y.-Q. Liu, *Adv. Mater.*, **22**, 3225 (2010).
7. C.S. Shan, H.F. Yang, J.F. Song, D.X. Han, A. Ivaska and L. Niu, *Anal. Chem.*, **81**, 2378 (2009).
8. A. Guiseppi-Elie, C. Lei and R.H. Baughman, *Nanotechnology*, **13**, 559 (2002).
9. X.B. Lu, Q. Zhang, L. Zhang and J.H. Li, *Electrochem. Commun.*, **8**, 874 (2006).
10. E.J. Laviron, *Electroanal. Chem.*, **101**, 19 (1979).
11. J. Bard and L.R. Faulkner, Electrochemical Methods:Fundamentals and Applications, John Wiley & Sons, Inc., New York, edn. 2, p. 75 (2001).
12. P. De Taxis, Du Poet, S. Miyamoto, T. Murakami, J. Kimura and I. Karube, *Anal. Chim. Acta*, **235**, 255 (1990).