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Short communication

Application of hydrophobic palladium nanoparticles for the development of electrochemical glucose biosensor

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ABSTRACT

An amperometric glucose biosensor based on an *n*-alkylamine-stabilized palladium nanoparticles (PdNPs)-glucose oxidase (GOx) modified glassy carbon (GC) electrode has been successfully fabricated. PdNPs were initially synthesized by a biphase mixture of water and toluene method using *n*-alkylamines (dodecylamine, C_{12}-NH_2 and octadecylamine, C_{18}-NH_2) as stabilizing ligands. The performance of the PdNPs-GOx/GC biosensor was studied by cyclic voltammetry. The optimum working potential for amperometric measurement of glucose in pH 7.0 phosphate buffer solution is −0.02 V (*vs.* Ag/AgCl). The analytical performance of the biosensor prepared from C_{18}-PdNPs-GOx is better than that of C_{12}-PdNPs-GOx. The C_{18}-PdNPs-GOx/GC biosensor exhibits a fast response time of *ca.* 3 s, a detection limit of 3.0 μM (S/N = 3) and a linear range of 3.0 μM–8.0 mM. The linear dependence of current density with glucose concentration is 70.8 μA cm^{-2} mM^{-1}. The biosensor shows good stability, repeatability and reproducibility. It has been successfully applied to determine the glucose content in human blood serum samples.

1. Introduction

It is well recognized that amperometric enzyme electrodes based on glucose oxidase (GOx) play a leading role for the determination of glucose, attributing to their high sensitivity, repeatability, stability and simple operation. Since the first development of glucose biosensor, strategy striving to improve the response performance of enzyme electrodes has been the main focus in biosensor research (Dong et al., 2010; Wei et al., 2010). The key issue in developing a successful amperometric biosensor mainly relies on the effectiveness and stability of the immobilized enzymes such as oxidase or dehydrogenase on the electrode surface. Among the various enzyme immobilization approaches, the enzyme monomolecular-level modification of the electrode surface has become a method of choice since this method can retain the biological recognition properties and stability of the immobilized enzymes. Immobilized enzymes have many operational advantages over free enzymes such as possibility of continuous operation, modulation of catalytic properties, lower cost of operation, reusability, good stability and wider working concentration range of analytes (Song et al., 2010).

In the past decade, nanostructured materials have been used as substrates or platforms for immobilizing enzymes because of their favorable intriguing properties such as large surface-to-volume ratio, high catalytic efficiency, and high surface reaction activity (Deng et al., 2010; Pandey and Singh, 2008; Rosi and Mirkin, 2005; Katz and Willner, 2004). Direct electron transfer of redox biomolecule to nanoparticles (NPs) is thought to occur when nanomaterial is used to modify the surfaces of working electrodes. Many kinds of nanomaterials have been used in biosensor fabrication (Zheng et al., 2010, 2011; Wang et al., 2010; Zhang et al., 2010; Rakhi et al., 2009; Shan et al., 2009). However, most of these nanomaterials require cross-linking reagents or Nafion film to prevent enzyme leaching from electrode surface (Kausaite-Minkstimiene et al., 2010; Wu et al., 2007). Moreover, electrochemical sensors using nano-metal materials for non-enzymatic glucose detection are prone to chloride ion interference which is abundant in nature, particularly in physiological fluids (Chen et al., 2009; Taylor et al., 1997; Lei et al., 1995). Chloride ion can completely suppress glucose adsorption, resulting in total loss of enzyme activity. As such, the search for new nanomaterials and methods for immobilizing enzymes is still an important subject in developing more sensitive and stable biosensors.

Palladium (Pd) is the preferred metal for electrocatalysis because of its various advantageous properties. Different methodologies have been attempted to incorporate Pd particles into

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polymer matrices for fabrication of Pd modified electrodes (Willner et al., 2007). In this work, we explore the use of *n*-alkylamine-stabilized PdNPs for immobilizing GOx and its application in glucose biosensing. The biosensing mechanism is based on the enzymatic oxidation of glucose to produce hydrogen peroxide (H_2O_2) which can subsequently enhance the reduction current of palladium(II) oxide (PdO) on a PdNPs-GOx modified glassy carbon (GC) electrode (*vide infra*). To our knowledge, this is the first report on using hydrophobic nanomaterial membrane to immobilize GOx to achieve higher enzyme activity, better detection sensitivity and stability. The effect of pH, loading of GOx and PdNPs on the response of the glucose biosensor was studied in detail. Our proposed glucose biosensor has been successfully applied for the determination of glucose in human serum samples.

2. Experimental

2.1. Materials and reagents

n-Dodecylamine, D(+)-glucose (99.5%), *n*-octadecylamine, palladium(II) chloride and sodium borohydride were obtained from Aldrich (Milwaukee, WI, USA). Sodium dihydrogen phosphate dihydrate and disodium hydrogen orthophosphate dihydrate were from Fluka (Buchs, Switzerland). Glucose oxidase (EC 1.1.3.4. from *Aspergillus niger*) with a specific activity of 235 units per milligram of solid was from Sigma (St. Louis, MO, USA). Acetaminophen, ascorbic acid (AA), glutamate (GA), H_2O_2 solution (30 wt% aqueous) and uric acid (UA) were from Beijing Chemical Reagent Company (Beijing, China). Blood serum samples were kindly provided by the Shanxi University Hospital (Taiyuan, China). All other reagents of analytical-reagent grade were used without further purification. Standard glucose solutions were prepared in 0.10 M pH 7.0 phosphate buffer solution (PBS) and were allowed to mutarotate for 24 h at room temperature before use.

2.2. Immobilization of PdNPs-GOx on GC electrode

The synthesis of *n*-alkylamine-stabilized PdNPs is based on our previous work using a biphase mixture of water and toluene (Li et al., 2010). Two types of PdNPs stabilized with *n*-dodecylamine (C_{12}-NH_2) and *n*-octadecylamine (C_{18}-NH_2) ligands, *i.e.*, C_{12}-NH_2-PdNPs and C_{18}-NH_2-PdNPs, were used to prepare glucose biosensors. First, a 0.40% (w/v) enzyme solution was prepared by dissolving 4.0 mg GOx in 1.0 mL PBS. Second, a known quantity of C_{18}-NH_2-PdNPs (or C_{12}-NH_2-PdNPs) was dissolved in toluene and various volumes of this PdNPs solution were used to prepare the C_{18}-NH_2-PdNPs-GOx (or C_{12}-NH_2-PdNPs-GOx) modified GC electrodes. The diameter and surface area of the GC working electrode were 3.0 mm and 0.07065 cm^2, respectively. The procedure followed the layer-by-layer deposition method. In a typical C_{18}-NH_2-PdNPs-GOx modified GC electrode preparation, a 4.0-μL aliquot of 0.40% (w/v) GOx solution was dropped onto the surface of a GC electrode, left for 5 min at room temperature, and then 6.0 μL of a 0.10% (w/v) C_{18}-NH_2-PdNPs toluene solution was added onto the GC electrode. The whole C_{18}-NH_2-PdNPs-GOx modified GC electrode was left overnight in ambient conditions to complete dryness. Fig. 1 illustrates the structure of a C_n-NH_2-PdNPs-GOx/GC modified electrode, where $n = 12$ or 18.

2.3. Instrumentation

All electrochemical studies were conducted on an Electrochemical Workstation CHI 660B (CH Instruments, Austin, TX, USA). A 15-mL electrolytic cell, an Ag/AgCl (saturated KCl) reference electrode, a platinum foil auxiliary electrode, and a C_n-NH_2-PdNPs-GOx modified GC working electrode were used for electrochemical measurements. Before glucose analysis, the background response of the C_n-NH_2-PdNPs-GOx modified GC working electrode was allowed to decay to a steady state with stirring.

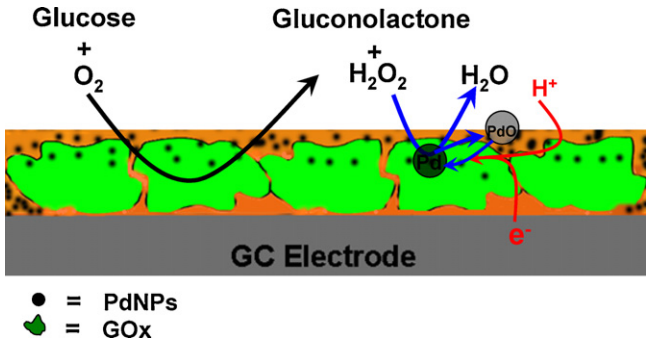

Fig. 1. Schematic diagram of *n*-alkylamine-stabilized PdNPs-based glucose biosensor (not in scale).

All pH measurements were taken by a combined glass electrode and a pHS-3C digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China). A JEOL JEM-1010 transmission electron microscope (Tokyo, Japan) operating at an accelerating voltage of 100 kV in bright field mode was used to observe the appearance and morphology of C_n-NH_2-PdNPs and C_n-NH_2-PdNPs-GOx samples. The sample was prepared by casting and evaporating an aqueous suspension of C_n-NH_2-PdNPs or C_n-NH_2-PdNPs-GOx droplet onto a carbon-coated copper grid (400 mesh). Purified water from a Milli-Q-RO4 water purification system (Millipore, Bedford, MA, USA) with a resistivity higher than 18 MΩ cm was used to prepare all solutions.

3. Results and discussion

3.1. TEM images of C_n-NH_2-PdNPs and C_n-NH_2-PdNPs-GOx

TEM technique is a powerful technique to determine the morphology, size and dispersity of metal NPs. Fig. S1 depicts the TEM images of C_n-NH_2-PdNPs and C_n-NH_2-PdNPs-GOx. It is clearly observed that small spherical C_n-NH_2-PdNPs were evenly dispersed on the carbon-coated copper grids. Particle analysis revealed that the average sizes of C_{18}-NH_2-PdNPs (Fig. S1A) and C_{12}-NH_2-PdNPs (Fig. S1B) were 5.6 ± 0.8 and 6.8 ± 0.8 nm, respectively. Fig. S1C and D shows the TEM images of C_{18}-NH_2-PdNPs-GOx and C_{12}-NH_2-PdNPs-GOx, respectively. Some light grey patches of enzyme GOx on the carbon-coated copper grids are observed. Black dots of spherical C_n-NH_2-PdNPs are densely adhered to the enzyme patches. The sizes of the enzyme immobilized C_n-NH_2-PdNPs are more or less the same as the free C_n-NH_2-PdNPs (Fig. S1A and B), indicating no aggregation of C_n-NH_2-PdNPs in C_n-NH_2-PdNPs-GOx hybrid material. It is interesting to note that all the C_n-NH_2-PdNPs were readily attached to the enzyme molecules. This can be explained by the fact that the enzyme molecule consisting of amino acids such as aspartic acid, glutamic acid and serine residues (O'Malley and Weaver, 1972) can bind to the C_n-NH_2-PdNPs via the linkage of –COHRN :→ Pd (Li et al., 2010). Excess C_{18}-NH_2-PdNPs or C_{12}-NH_2-PdNPs will form a thin hydrophobic layer on the enzyme, resulting in a stable nanohybrid material of C_n-NH_2-PdNPs-GOx, ready to be employed for biosensing in our subsequent work. Collectively, the TEM results demonstrate that the spherical C_n-NH_2-PdNPs were successfully adhered to the enzyme molecules and retained their morphology without aggregation. It is also observed that a clear thin C_n-NH_2-PdNPs film was formed on the GC electrode which could keep the enzyme molecules on

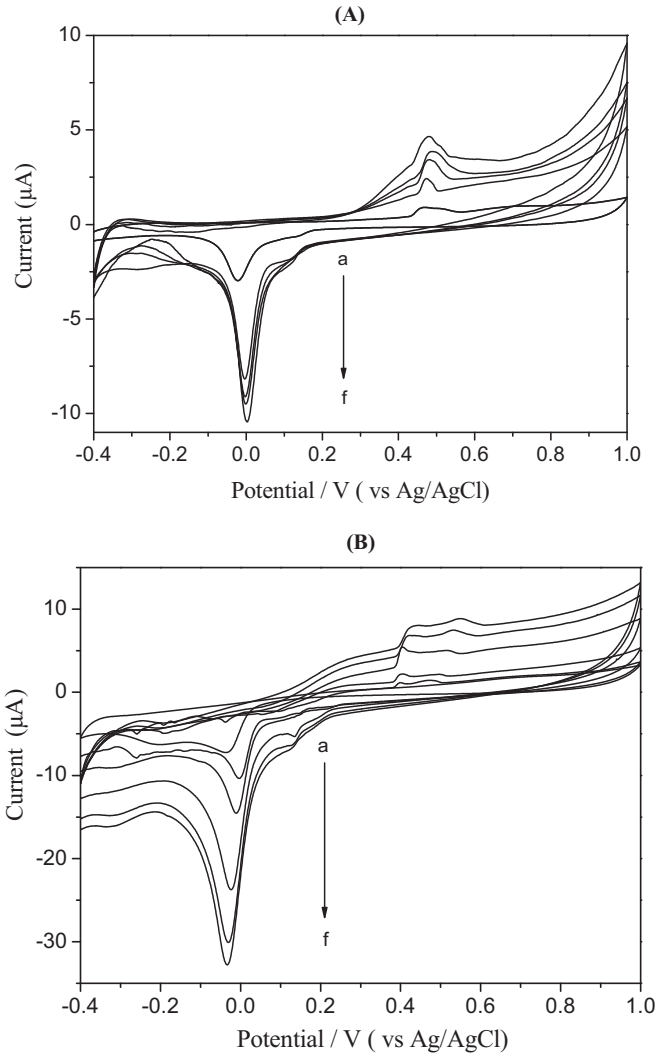

Fig. 2. Cyclic voltammetric measurements at the (A) C_{12}-NH_2-PdNPs-GOx and (B) C_{18}-NH_2-PdNPs-GOx modified GC electrodes in various concentrations of glucose PBS solutions (0.10 M, pH 7.0): (a) 0.00, (b) 0.050, (c) 0.20, (d) 0.50, (e) 1.00, and (f) 1.50 mM. The scan rate is 20 mV/s.

the electrode surface; thus preventing their leakage to the solution.

3.2. Response mechanisms of glucose

Fig. 2 displays the cyclic voltammograms (CVs) of (A) C_{12}-NH_2-PdNPs-GOx and (B) C_{18}-NH_2-PdNPs-GOx modified GC electrodes in 0.10 M pH 7.0 PBS containing various concentrations of glucose at a scan rate of 20 mV/s. In the absence of glucose (Fig. 2A(a) and B(a)), both C_{12}-NH_2-PdNPs-GOx and C_{18}-NH_2-PdNPs-GOx modified GC electrodes exhibit CVs with well defined reversible redox waves in both forward and reverse scans at *ca.* +0.2–0.6 and −0.05 to 0.0 V (*vs.* Ag/AgCl) respectively (see also Fig. S2A and B of Supplementary data). In the forward scan, the current starts to rise at a potential of *ca.* +0.2 V due to the oxidation of Pd to form Pd–OH and then rapidly increases at above +0.4 V corresponding to the formation of Pd oxides (*i.e.*, $Pd \rightarrow Pd^{2+} + 2e^-$). In the reverse scan, a large cathodic peak at −0.03 to 0.00 V appeared which is related to the reduction of Pd oxides (*i.e.*, $Pd^{2+} + 2e^- \rightarrow Pd$) (Jia et al., 2009). On the addition of glucose to the PBS, both anodic and cathodic currents increase with the increase in glucose concentration as shown in Fig. 2A(b–f) and B(b–f). It is well known that glucose is enzymatically oxidized to gluconic acid with a concomitant release of H_2O_2:

$$\text{D-Glucose} + O_2 + H_2O \xrightarrow{GOx} \text{D-Gluconic acid} + H_2O_2 \quad (1)$$

The generated H_2O_2 can be oxidized on the C_{12}-NH_2-PdNPs and C_{18}-NH_2-PdNPs modified GC electrodes as depicted in Fig. S2 (Supplementary data), producing high anodic current. Thus, the anodic peaks are broad attributing to the electro-oxidation of both H_2O_2 and PdNPs on the C_{12}-NH_2-PdNPs-GOx and C_{18}-NH_2-PdNPs-GOx modified GC electrodes. However, the oxidation potentials for H_2O_2 and PdNPs are so close that they cannot be resolved or identified accurately.

On the reverse scan, the PdO is electrochemically reduced back to PdNPs as follows:

$$PdO + 2e^- + 2H^+ \rightarrow Pd + H_2O \quad (2)$$

It has been reported that the cathodic current of a PdNPs/GC modified electrode is enhanced in the presence of H_2O_2, attributing to the electrocatalytic effect of PdNPs on H_2O_2 at the GC electrode (Liu et al., 2010). In this work, the cathodic peak is enhanced possibly due to the presence of residual H_2O_2. To compare and verify the effect of H_2O_2, a set of experiments was performed to determine the electrochemical response of the C_n-NH_2-PdNPs/GC modified electrode in 0.10 M PBS (pH 7.0) with and without H_2O_2 as depicted in Fig. S2 (Supplementary data). It was found that H_2O_2 is very effective in enhancing the cathodic and anodic currents of C_{12}-NH_2-PdNPs and C_{18}-NH_2-PdNPs modified GC electrodes.

In essence, well-defined and larger cathodic and broad anodic glucose responses were obtained at the C_{18}-NH_2-PdNPs-GOx biosensor at potentials of +0.45 and −0.02 V, respectively (Fig. 2B). Unfortunately, it is found that the C_{18}-NH_2-PdNPs-GOx biosensor also responds to acetaminophen, AA and UA at the working potential of +0.45 V since these interferents are also electrochemically oxidized. A lower operating potential should greatly reduce this problem (Lin et al., 2004). As such, we chose the operating potential of −0.02 V to minimize interferences from easily oxidizable species such as AA and UA (*vide infra*). In essence, the detection of glucose by our C_{18}-NH_2-PdNPs-GOx/GC electrode can be realized at a very low working potential (−0.02 V *vs.* Ag/AgCl) and the resulting current should be proportional to the level of glucose in sample solutions.

3.3. Optimization of fabrication of C_{18}-PdNPs-GOx glucose biosensor

PdNPs and GOx play important roles in the response of PdNPs-GOx modified GC electrode to glucose. In this work, C_{18}-NH_2-PdNPs-GOx modified GC electrode was chosen to optimize the fabrication condition of PdNPs-GOx in order that more stable and higher sensitive glucose biosensor can be produced. The effect of enzyme concentration (1.0–6.0 mg/mL) in the PdNPs-GOx on the current response of C_{18}-NH_2-PdNPs-GOx/GC electrode at −0.02 V to 1.0 mM glucose in PBS (pH 7.0) was investigated and depicted in Fig. S3A. The current increases with the increase in enzyme loading until its maximum at 4.0 mg/mL. It is obvious that increasing the amount of GOx will increase the sensitivity of glucose detection. However, the current drops slightly after 4.0 mg/mL attributing to the thicker enzyme layer of excess GOx. As such, 4.0 mg/mL of GOx was chosen as the optimal enzyme concentration for preparing the C_{18}-NH_2-PdNPs-GOx modified GC electrode in the subsequent work.

The influence of the amount of C_{18}-NH_2-PdNPs on the response of C_{18}-NH_2-PdNPs-GOx modified GC to glucose was investigated (Fig. S3B). When the amount of C_{18}-NH_2-PdNPs is insufficient to cover and bind with the enzyme molecules, the enzyme will leach.

By contrast, when the amount of C_{18}-NH_2-PdNPs is too large, a thicker layer of C_{18}-NH_2-PdNPs will be formed and thus block or slow down the contact between glucose and enzyme, resulting in a decrease of current. As a result, 4.0 μL of 0.40% (w/v) GOx solution and 6.0 μL of 0.10% (w/v) C_{18}-NH_2-PdNPs solution were used to prepare the C_{18}-NH_2-PdNPs/GOx modified GC electrode.

3.4. Effect of pH on the response to glucose

The activity of the immobilized GOx is closely related to the pH of the buffer solution. The effect of pH (5.0–9.0) on the catalytic activity of GOx in C_{18}-NH_2-PdNPs-GOx/GC modified electrode was investigated in 0.10 M PBS containing 0.10 M KCl and 1.0 mM glucose (Fig. S4). When the pH is lower, the sensitivity is lower. The highest sensitivity is obtained at pH 7.0. When the pH is above 7.0, the sensitivity drops slightly. As a result, 7.0 was chosen as the optimal pH in this study.

3.5. Comparison of C_{12}-NH_2-PdNPs-GOx and C_{18}-NH_2-PdNPs-GOx GC electrodes

Two biosensors were fabricated using C_{12}-NH_2-PdNPs-GOx and C_{18}-NH_2-PdNPs-GOx respectively and their responses to glucose were compared. Fig. 3A displays the current response of the C_{18}-NH_2-PdNPs-GOx/GC modified electrode on addition of various concentrations of glucose at a working potential of −0.02 V (*vs.* Ag/AgCl). Upon addition of successive aliquots of glucose to PBS (pH 7.0), an increase in the response current was observed. The biosensor responds well to glucose with a broad linear range of 3.0 μM–8.0 mM with a correlation coefficient of 0.9982 as shown in Fig. 3B. The linear dependence of current density with glucose concentration gives 70.8 $\mu A\,cm^{-2}\,mM^{-1}$. The inset of Fig. 3B displays the calibration curve at the micromolar range and the detection limit is 3.0 μM (S/N = 3). Ten C_{18}-NH_2-PdNPs-GOx/GC electrodes were fabricated using the same procedure and their responses to 0.10 mM glucose were assessed. The relative standard deviation (RSD) of the response was 3.8%, inferring that the fabrication process is very reproducible.

Similarly, the response of C_{12}-NH_2-PdNPs-GOx/GC modified electrode to glucose was investigated (Fig. S5). The current increased during the first 10 injections of glucose (5.0 μM glucose increment) and became unstable when the concentration of glucose reached 6.0 mM. The linear working range is 5.0 μM–6.0 mM with a correlation coefficient of 0.9970 (inset of Fig. S5). The linear dependence of current density with glucose concentration is 225 $\mu A\,cm^{-2}\,mM^{-1}$ and the detection limit (S/N = 3) is 5.0 μM. Although the C_{12}-NH_2-PdNPs-GOx/GC modified electrode shows higher sensitivity than that of the C_{18}-NH_2-PdNPs-GOx/GC modified electrode, it displays narrower dynamic range and is less stable. In addition, the responses of the five C_{12}-NH_2-PdNPs-GOx/GC modified electrodes to 0.10 mM glucose are not so reproducible with a RSD of 8.1%. As such, C_{18}-NH_2-PdNPs-GOx/GC modified electrode was used for most of our work.

3.6. Selectivity and stability

For real applications, the effect of interference has to be considered. In this study, the tolerant ratio for co-existing substances to 0.10 mM glucose was found to be 200-fold for Ba^{2+}, K^+, Na^+, Mg^{2+}, Ca^{2+}, 100-fold for Zn^{2+} and 10-fold for Fe^{3+}. In addition, to affirm the selectivity of the glucose biosensor, investigations on potential interferents were carried out by measurement of 0.10 mM glucose in PBS in the presence of a specified concentration (0.10 mM) of AA, GA and UA as depicted in Fig. S6. GA does not cause observable interference whereas AA and UA produce only 1.2 and 4.6% interferences to the biosensor, respectively. Therefore, the effect of these interferents on the current response of the biosensor is small. These results indicate that low working potential (−0.02 V) would reduce interference effect. Additional experiments were carried out to assess the stability of the biosensor. The C_{18}-NH_2-PdNPs-GOx/GC biosensor exhibits a fast response time of *ca.* 3 s. The biosensor was stored at 4 °C when not in use, and retained about 90% of its original sensitivity after three-month storage, indicating good stability of the biosensor.

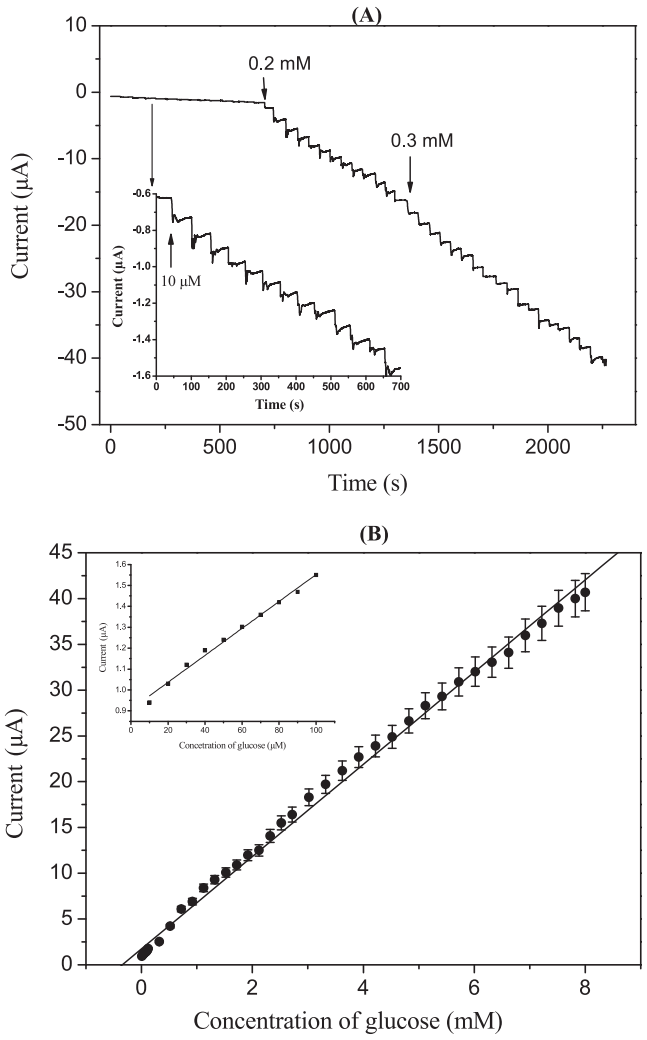

Fig. 3. (A) Current–time curves obtained with C_{18}-NH_2-PdNPs-GOx modified GC electrode upon successive addition of glucose (0.010, 0.20 or 0.30 mM) to 0.10 M PBS solution (pH 7.0) as indicated. (B) Calibration curve of the biosensor as a function of glucose concentration. The working potential and temperature are −0.02 V (*vs.* Ag/AgCl) and 25 °C, respectively. The calibration data are average values from five modified electrodes.

3.7. Application to real samples

The applicability of the biosensor was assessed by determining the glucose concentration in human serum samples. The samples were directly diluted with appropriate volumes of PBS (pH 7.0) and subjected to analysis by the C_{18}-NH_2-PdNPs-GOx modified GC electrode. The results are summarized in Table 1. Our results compare well with the data obtained in the local hospital. In addition, the recovery tests for glucose were performed by adding known amounts of glucose to the blood serum samples, diluted with PBS (pH 7.0) and determined by the C_{18}-NH_2-PdNPs-GOx modified GC electrode. The results are displayed in Table 1. The recoveries are in the range of 97–105%, demonstrating that

Table 1
Determination of glucose in human blood serum samples.

Sample	Added (mM)	Found (mM) (n = 7)	RSD (%)	Recovery (%)	Determined by the local hospital (mM)[a]
	0.00	4.38	1.54	–	4.30
1	0.10	4.48	3.00	100	–
	0.50	4.90	2.65	104	–
	0.00	5.12	1.86	–	5.15
2	0.20	5.33	1.00	105	–
	1.00	6.09	2.12	97	–
	0.00	5.00	1.78	––	5.02
3	0.20	5.21	1.00	105	–
	1.50	6.54	2.12	103	–
	0.00	5.50	1.09	–	5.48
4	0.20	5.71	1.00	105	–
	2.00	7.47	2.12	98.5	–

[a] The analysis was conducted in Shanxi University Hospital using a spectrophotometric method.

our biosensor method offer excellent, accurate and precise results (RSD = 1.00–3.00%, n = 7) for the determination of blood glucose.

4. Conclusion

An enzyme bio-electrode based on the immobilization of GOx in C_{18}-NH_2-PdNPs film has been developed to determine blood glucose. The C_{18}-NH_2-PdNPs film provides a hydrophobic layer to prevent enzyme leaching as well as maintaining the activity of GOx. The major attributes of our C_{18}-NH_2-stabilized PdNPs are: First, PdNPs are especially effective at reduction of H_2O_2 as their smaller size can induce higher chemical reactivity. Second, PdNPs of core diameter 5–7 nm stabilized with a monolayer of long carbon chain C_{18}-NH_2 can provide a large hydrophobic micro-environment for loading and surface-attachment of a thin layer of enzyme molecules. Third, the amino acid residues of enzyme molecules can covalently bind to PdNPs, thus minimizes enzyme leaching and maintains good enzyme activity. The proposed C_{18}-NH_2-PdNPs-GOx/GC biosensor exhibits the attributes of high sensitivity, fast amperometric response, low detection limit, wide linear dynamic range, good anti-interferent ability, good reproducibility and stability. The biosensor has been successfully applied to determine the glucose content in human blood serum samples and the results compared well to a standard spectrophotometric method commonly used in hospitals. It is anticipated that C_{18}-NH_2-stabilized PdNPs could be an attractive nanomaterial for the fabrication of other amperometric biosensors and this work is in progress in our laboratories.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.04.057.

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