Fabrication of an Amperometric Glucose Biosensor Based on a Prussian Blue/Carbon Nanotube/Ionic Liquid Modified Glassy Carbon Electrode

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Abstract

An amperometric glucose biosensor was developed based on synergistic contributions of prussian blue (PB) and a bucky gel (BG) consisting of carbon nanotubes (CNTs) and ionic liquid (IL). The PB nanoparticles were first deposited onto the surface of a BG modified glassy carbon (GC) electrode (BG/GC). Then, the Ni^{2+} ions were electrochemically inserted into the PB lattice to improve its stability at physiological pH. Afterwards, Glucose oxidase (GOx) was immobilized on the BG/GC electrode using a cross-linking method. Amperometric measurements of glucose were performed at -0.05 V vs. Ag/AgCl in 0.05 M phosphate buffer solution at pH 7.4. The glucose biosensor exhibited a sensitivity of 45.03 µA/(mM.cm^2) with a detection limit of 5×10^{-7} M. The amperometric response was linear in the range of 5×10^{-7} to 8.3×10^{-4} M.

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Introduction

Reliable and sensitive detection of glucose is of great importance in a variety of fields, such as clinical diagnosis [1, 2], diabetes control [3–7], food industry and environmental monitoring [2]. Among various analytical methods for glucose detection, the enzyme-based amperometric biosensors have received considerable attention due to their selectivity, sensitivity, simplicity and low cost [8, 9]. Most of these biosensors are based on the enzymatic reaction of glucose oxidase (GOx). This enzyme catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide (H_2O_2) using molecular oxygen as an electron acceptor (reaction 1) [10].

$$\text{Glucose} + O_2 \xrightarrow{\text{GOx}} \text{gluconic acid} + H_2O_2 \quad (1)$$
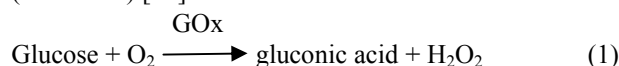
Accordingly, glucose concentration can be determined by amperometric monitoring of the liberated H_2O_2 [3]. A great drawback of this approach is the high overpotential required for H_2O_2 oxidation, which may cause considerable interferences from other electroactive species frequently present in real samples (i.e., ascorbate, urate, acetaminophen, etc.) [11]. One of the most common ways to overcome this problem is introducing electrochemical inorganic mediators [12]. Prussian blue (PB, ferric hexacyanoferrate) is considered as an "artificial peroxidase" due to its high catalytic activity toward H_2O_2 reduction [13]. In addition to the ease of preparation, PB allows selective

detection of H_2O_2 at low potentials [14]. So, it is widely used in the development of electrochemical sensors for H_2O_2 detection [15]. However, PB exhibits poor cycling stability at physiological pH, due to the attack of hydroxide ions, which limits its application in biosensors [12]. Recently, numerous studies have been performed to improve the operational stability of PB, in which PB hybrid composites are more frequent [16–18].

Carbon nanotubes (CNTs) have been extensively used in electrochemical biosensors due to their remarkable chemical, physical and mechanical properties [19, 20]. Because of their good electrical conductivity and the property of being particle carriers, CNTs are regarded as potential mediators for PB-modified electrodes [21]. PB/CNTs hybrids exhibit better electrochemical stability and improved catalytic activity for H_2O_2 reduction [17]. Besides, these nanocomposites form a highly porous network that is suitable for immobilizing large amounts of an enzyme [22].

Ionic liquids (ILs) are salts, which are composed of large ions [23] and can't easily form an ordered crystal and thus remain in a liquid state at or near room temperature [24]. They have many unique properties, such as wide liquid-phase range, negligible vapor pressure, chemical and thermal stability, high conductivity and wide electrochemical potential window [24, 25]. It is proven that ILs not only increase the PB deposition on the electrode but also enhance the catalytic activity of PB-modified electrodes for H_2O_2 reduction and improve their electrochemical sta-

bility [26, 27]. Moreover, it is found that ILs and CNTs can interact to form a homogeneous bucky gel (BG) [28]. CNTs/IL based hybrids offer the opportunity to improve the sensor performance by combining the beneficial properties of its components [29]. Recently, BG-modified electrodes have been demonstrated as ideal candidates for the fabrication of PB-based biosensors due to the synergistic amplification effects of CNTs and IL on the electrocatalytic properties of PB [30]. On the other hand, IL show good biocompatibility and promotion effect on the activity and stability of enzymes [31].

The aim of this study was to develop a novel glucose biosensor based on the synergistic contributions of PB and BG. For this purpose, PB was first electrodeposited on the BG-modified electrodes including 1-Butyl-3-methylimidazolium hexafluorophosphate (BMIM-PF6). Then, Ni^{2+} ions were incorporated into the structure of PB to improve its stability in physiological pH. Subsequently, the GOx enzyme was immobilized through a cross-linking method employing glutaraldehyde (GA) and Nafion®. After optimization of some of the experimental conditions, the analytical response of the developed biosensor toward glucose was evaluated.

Materials and Methods
Reagents
Bamboo-like multi-walled CNTs (BCNTs, purity >95%, diameter 30 ± 10 nm, length 1–5 μm) were purchased from NanoLab Inc. (Brighton, MA, USA). GOx (E.C. 1.1.3.4, 150,000 U/g, from Aspergillus niger), D-(+)-glucose (Glu) monohydrate, Nafion® (5 wt % ethanol solution), bovine serum albumin (BSA) and BMIM-PF6 (>97%), as a typical IL, were obtained from Sigma (St. Louis, MO, USA). N,N-dimethylformamide (DMF), Hydrochloric acid (HCl, 37%), potassium chloride (KCl), potassium phosphate (KH_2PO_4, K_2HPO_4), nickel dichloride ($NiCl_2$), potassium ferricyanide ($K_3[Fe(CN)_6]$), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and glutaraldehyde (GA) (50%, solution in water) were all of analytical grade and obtained from Merck (Darmstadt, Germany).

Apparatus and measurements
The electrochemical experiments were done with a Potentiostat-Galvanostat (model 302N, Autolab B.V., Utrecht, The Netherlands) connected to a personal computer and controlled by NOVA software, version 1.7. All experiments were performed at room temperature in a three-electrode cell. A glassy carbon electrode (GC, 2.0 mm in diameter, Metrohm) was employed as the working electrode. A silver/silver chloride (Ag/AgCl, 3.0 M KCl) and a platinum electrode from Metrohm were used as the reference and auxiliary electrodes, respectively. The surface morphologies of the modified electrodes were observed by a field emission scanning electron microscope (FE-SEM, model S-4160, Hitachi, Tokyo, Japan). Energy dispersive X-ray spectroscopy (EDX, Tescan, VEGA-3 LMU VPSEM, Czech Republic) was applied to characterize the chemical composition of the modified electrodes after incorporation of Ni^{2+}.

Preparation of BG
The BG nanocomposite was prepared according to the method described earlier [32]. Briefly, 2.0 mg of BCNTs was mixed with 1.0 ml of BMIM-PF6 by grinding in an agate mortar, until a homogenized black gel was obtained. The mixture was then centrifuged at 18,000 rpm for 30 minutes. After that, the transparent liquid phase (pure IL) was removed and the gel phase was isolated. The prepared BG was dispersed in 1.0 ml of DMF with the aid of sonication.

Fabrication of the modified electrodes
BG/GC electrodes
Prior to the surface modification, the GC electrodes were first polished with 1.0, 0.3 and 0.05 μm alumina slurry, successively. Then, the electrodes were rinsed with double-distilled water. Finally, 2 μl of the BG suspension was dropped on the surface of the GC electrode and dried at room temperature.

PB/BG/GC electrodes
The electrodeposition of PB nanoparticles on the surface of the BG/GC electrodes was done by potential cycling (between -0.2 and 1.0 V) at a scan rate of 0.1 V/s. The electrolyte solution consisted of 2.0 mM $FeCl_3$, 2.0 mM $K_3Fe(CN)_6$, 0.1 M KCl and 0.1 M HCl. Then, in order to improve the pH stability of PB-modified electrodes in mild alkaline medium, the Ni^{2+} ions were incorporated into the PB structure [33]. For this purpose, the PB/BG/GC electrodes were successively cycled (15 cycles, between −0.15 and 0.5 V), in a solution containing 0.1 M KCl, 0.1 M HCl, and 0.01 M $NiCl_2$. Finally, the obtained PB/BG/GC electrodes were cycled in 0.1 M KCl and 0.1 M HCl solution in the same potential range for 10 cycles.
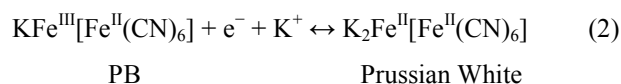
GOx/PB/BG/GC electrodes
GOx was immobilized onto the surface of the PB/BG/GC modified electrodes by cross-linking with GA. To optimize the GOx concentration, a series of enzyme solutions were prepared by dissolving different amounts of GOx (1, 3, 5, 7, 10, 15, 20 and 25 mg/ml) in 0.05 M phosphate buffer solution (PBS, 0.1 M KCl), pH 7.4. During the experiment, the total protein content was kept constant (at 35 mg/ml) by adding BSA. Subsequently, 20 μl of each enzyme solution was mixed with 5 μl Nafion®. After dropping 3 μl of the mixtures on the PB/BG/GC electrodes' surfaces, 1 μl GA (0.2%, v/v) was added to each drop and the electrodes were left to dry in air. Then, the electrodes were immersed in 0.1 M glycine to saturate the free aldehyde groups. For optimizing the concentration of GA, the same procedure was used at a fixed amount of GOx (5 mg/ml). When not in use, the GOx/PB/BG/GC electrodes were stored in PBS (pH 7.4) at $4°C$.
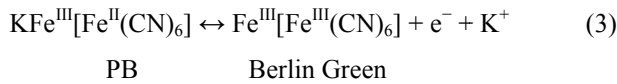
Results and Discussion
PB electrodeposition
Fig. 1 illustrates the typical cyclic voltammograms (CVs) recorded during the deposition of PB onto the BG/GC electrode. The representative redox peaks of PB were obtained at ~0.2 and ~0.8 V, which corresponds to PB reduction to Prussian White and its oxidation to Berlin Green, respectively (See reactions "2" and "3") [26].

$$KFe^{III}[Fe^{II}(CN)_6] + e^- + K^+ \leftrightarrow K_2Fe^{II}[Fe^{II}(CN)_6] \qquad (2)$$

PB   Prussian White

$$KFe^{III}[Fe^{II}(CN)_6] \leftrightarrow Fe^{III}[Fe^{III}(CN)_6] + e^- + K^+ \qquad (3)$$

PB   Berlin Green

As seen in Fig. 1, the redox peak currents were increased with the increase of the cycle number, which reveals the continuous growth of the PB film onto the BG/GC modified electrode. Then, to increase the stability of PB in physiological pH, Ni^{2+} ions were incorporated into the crystal structure of PB by the successive potential cycling of the PB/BG/GC electrodes in a solution containing 0.1 M HCl, 0.1 M KCl, and 0.01 M $NiCl_2$ [33]. The incorporation of Ni^{2+} ions into the PB structure was demonstrated by EDX spectroscopy (Fig. 2), which showed the characteristic NiLα peak at 0.85 keV.
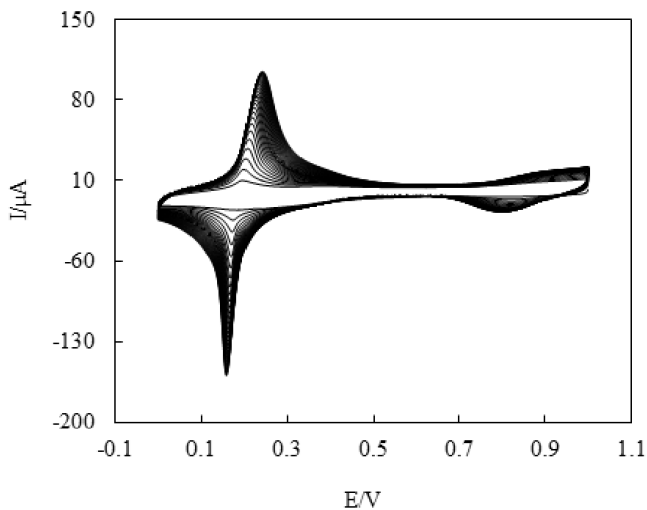

Figure 1. 30 successive CV cycles recorded during electrodeposition of PB on the BG/GC modified electrode at a scan rate of 0.1 V/s. The electrolyte solution contained 2.0 mM $K_3Fe(CN)_6$, 2.0 mM $FeCl_3$, 0.1 M KCl and 0.1 M HCl.

FE-SEM characterization

Fig. 3 shows the surface morphologies of the modified electrodes. The FE-SEM image of the BG/GC electrode showed that the BCNTs-IL are untangled and uniformly cover the surface of the GC electrode (Fig. 3A). After electrodeposition of PB, many small cubic PB particles were observed on the surface of the BG/GC modified electrode (Fig. 3B). The insertion of Ni^{2+} ions did not change the morphology of PB (data not shown).

Fig. 3C shows the surface morphology of GOx/PB/BG/GC electrode. As seen, the surface of the PB/BG/GC electrode was coated with a compact film of Nafion®, containing GOx, BSA, and GA.

Optimization of experimental variables
Optimization of GOx concentration

The current response of the biosensor strongly depends on the amount of the immobilized enzyme on the electrode surface. To optimize the GOx concentration, eight electrodes with different enzyme loadings (1, 3, 5, 7, 10, 15, 20 and 25 mg/ml) were prepared at a fixed concentration of GA (0.2% (v/v)). The dependence of the biosensor response (toward 0.7 mM glucose) on the GOx concentration is shown in Fig. 4. As seen, the response of the biosensor increased with increasing GOx concentration, up to an optimal concentration of 5 mg/ml. Increase the enzyme amount above this concentration led to a decrease in the current response, most likely because of steric hindrance of the enzymes.

Optimization of GA concentration

Fig. 5 shows the effect of GA concentration on the biosensor response (toward 0.7 mM glucose) at the optimal concentration of GOx (5 mg/ml). It can be seen that the best response was achieved at 0.2% GA. At lower GA concentration, the biosensor response decreased probably because of the leakage of enzyme from the electrode surface. However, at higher GA concentrations, forming a stronger diffusion barrier led to a decrease in the biosensor response. So, 0.2% GA was chosen for further experiments.
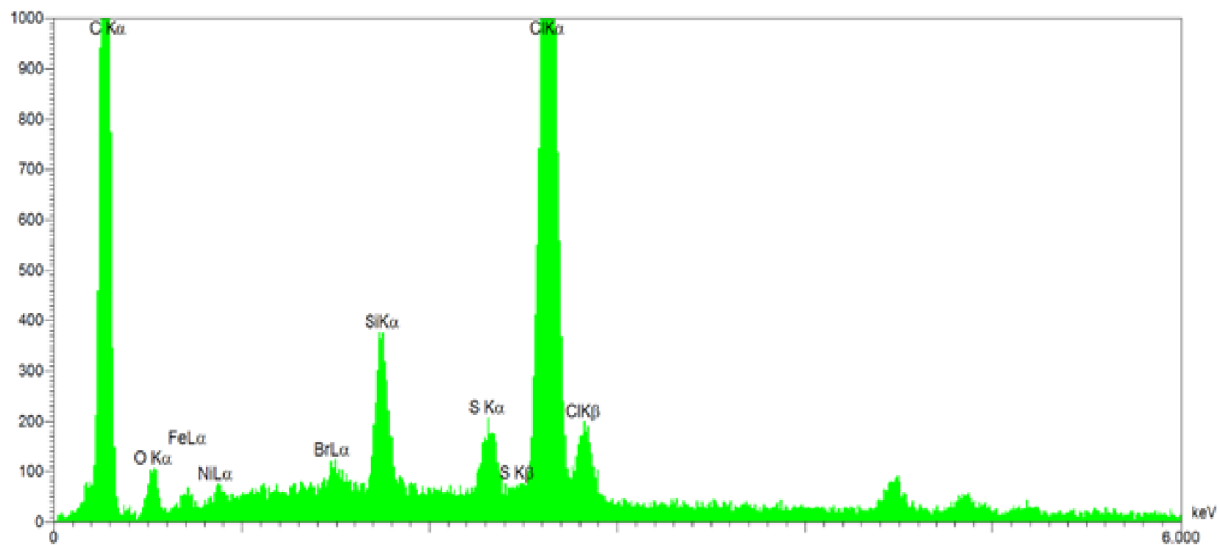

Figure 2. EDX spectrum of the Ni-PB/BG/GC electrode after cycling of the PB/BG/GC modified electrode in an electrolyte solution of 0.1 M KCl, 0.1 M HCl and 0.01 M $NiCl_2$.
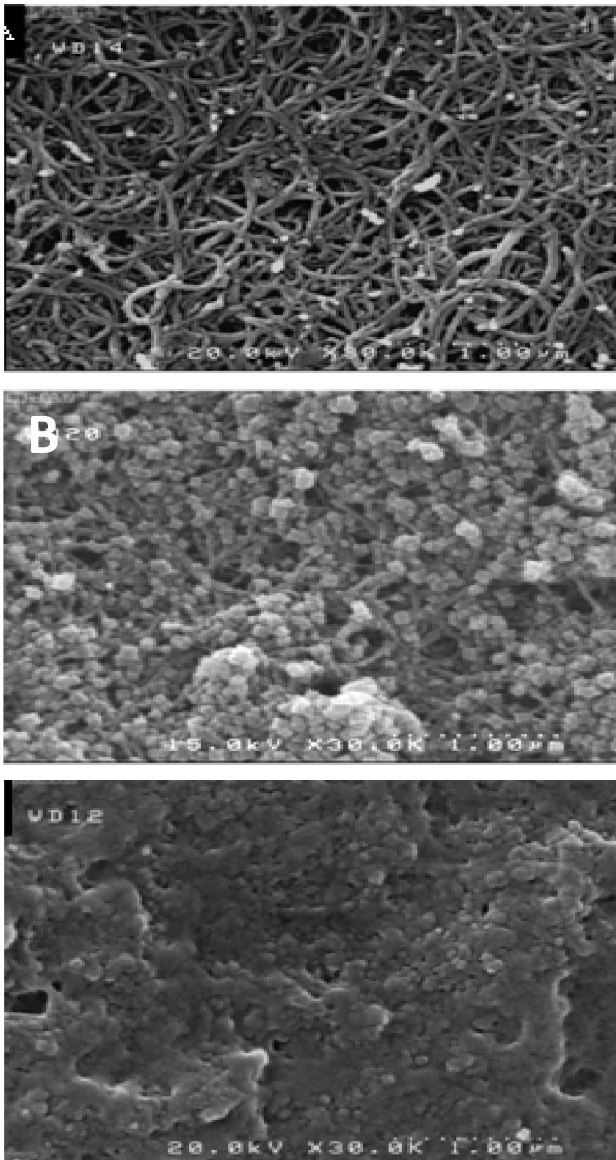
Figure 3. FE-SEM images of BG/GC (A), PB/BG/GC (B) and GOx/Ni-PB/BG/GC (C) electrodes.
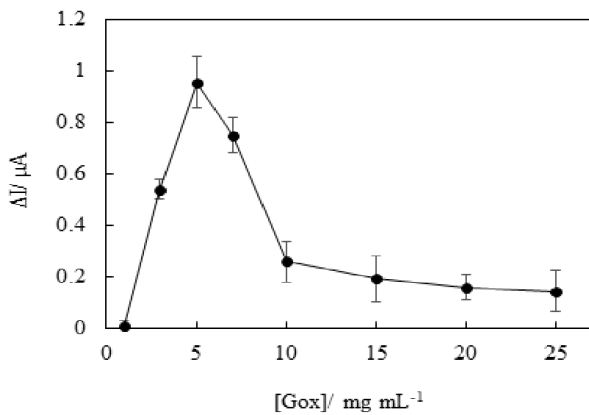

Figure 4. Dependence of the biosensor response toward 0.7 mM glucose on the GOx concentration in PBS (0.05 M + 0.1 M KCl, pH 7.4) at −0.05 V vs. Ag/AgCl. The concentrations of GA were fixed at 0.2% (v/v). The points are mean values of three different measurements.

Analytical characteristics of the glucose biosensor

Fig. 6 shows the amperometric responses of the proposed biosensor to successive additions of 1 mM glucose to the PBS solution (0.05 M, 0.1 M KCl, pH 7.4) at −0.05 V. The response time, (defined as required time to reach 95% of the steady-state current) was 2 s.
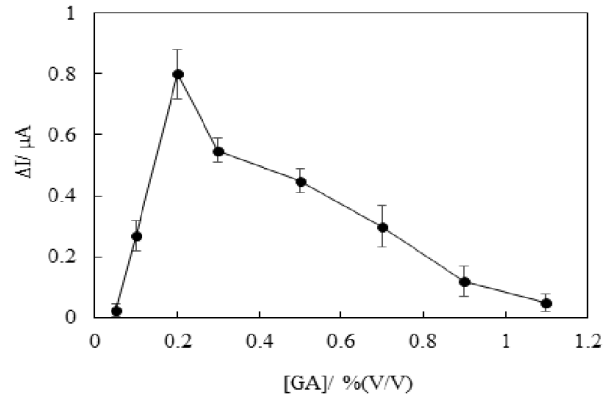

Figure 5. Dependence of the biosensor response toward 0.7 mM glucose on the concentration of GA in PBS (0.05 M + 0.1 M KCl, pH 7.4) at −0.05 V vs. Ag/AgCl. The concentration of GOx was fixed at 5 mg/ml. The points are mean values of three independent experiments.
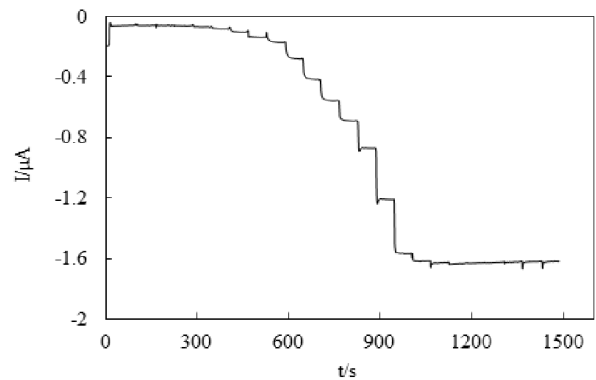

Figure 6. Amperometric response of the GOx/PB/BG/GC electrode toward successive addition of 1 mM glucose to 4 ml of PBS (0.05 M + 0.1 M KCl, pH 7.4) at −0.05 V. The rotation speed was 500 rpm. The injected volumes of glucose were 2, 5, 10, 20, 40, 70, 100, 200, 400, 600, 800 and after that 1000 μl (every 60 s) until saturation.
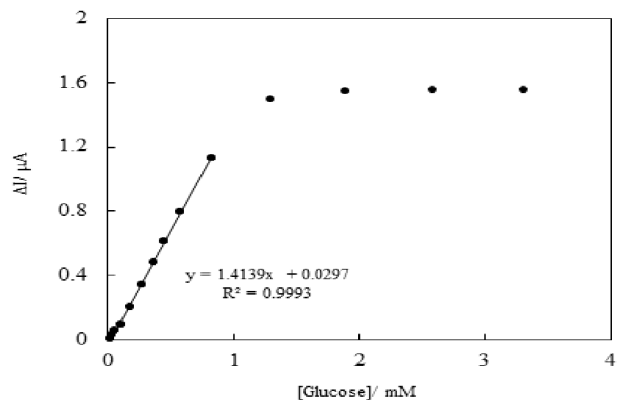

Figure 7. The calibration curve for glucose detection. The electrolyte solution was PBS (0.05 M + 0.1 M KCl, pH 7.4).

Figure 7 shows the resulting calibration curve. As seen, the biosensor displayed a linear range from 5×10^{-7} to 8.3×10^{-4} M, with a sensitivity of 45.03 µA/(mM.cm^2). The detection limit was 5×10^{-7} M (based on a signal-to-noise ratio of 3).

Conclusion

An amperometric glucose biosensor was designed based on the synergistic beneficial roles of PB, CNTs and IL. The results showed that the proposed nanocomposite could be employed as a favorable matrix for detection of glucose. Features such as a relatively long linear range and low detection limit (DL) make the proposed biosensor a promising platform for commercial applications.

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