Glucose Biosensor Based on Chitosan-Gold and Prussian Blue-Gold Nanoparticles

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Abstract: Amperometric glucose biosensors were constructed by codeposition of glucose oxidase (GOx) with chitosan-gold (chitosan-AuNP) on gold-Prussian Blue (Au-PB) nanoparticles modified glassy carbon electrodes (GCE). The high stability of the biosensors was achieved using electrochemically controllable in situ Au-PB film preparation following the film covering with GOx layer encrusted with chitosan-AuNP. The biosensors showed 95 % response time up to 10 s, sensitivity up to 9.5 μA M^{-1} cm^{-2} and linear calibration graph up to 3 mM of glucose.

Keywords: Nanoparticle, gold, glucose, glucose oxidase, chitosan, Prussian Blue.

1. INTRODUCTION

Recent advances in medicine biochemistry stimulate a demand for highly sensitive and precise analytical methods of glucose determination. The biosensors based on the glucose oxidase (GOx) catalyzed glucose oxidation have generated considerable interest. The detection of glucose may be accomplished by monitoring the consumption of an electroactive enzyme cosubstrate, such as oxygen, or the formation of an electroactive product, such as hydrogen peroxide [1,2]. However, the high positive working potential required for anodic hydrogen peroxide (H_2O_2) detection leads to interferences from reducing species such as ascorbic acid, uric acid and acetaminophen [3]. This disadvantage can be eliminated with the use of electrocatalysts that allows H_2O_2 monitoring at lower potentials [4].

Prussian Blue (PB), called as "artificial peroxidase", has a great potential to be used as electrocatalyst in hydrogen peroxide sensing. It was shown that PB has the structure $Fe_4^{III}[Fe^{II}(CN)_6]_3$ and could be prepared by chemical, electrochemical and sonochemical methods [5].

Chemical synthesis of PB can be accomplished by mixing Fe^{II} or Fe^{III} salts with hexacyanoferrate ions containing iron atoms in a different oxidation state [6]. The electrosynthesis of PB film involves potentiodynamic cycling between potential limits of the working electrode in a supporting electrolyte containing both the metal ion (M^{n+}; M=Fe) and ferricyanide species.

The PB may be deposited on glassy carbon (GC), graphite (PG), platinum (Pt), or gold (Au) [7].

Recognizing the role of PB in the electrochemical catalysis PB has been extensively investigated in the form of thin polycrystalline electrodeposited films [7]. It is now realized that the use of nanoparticles can improve the analytical performance of PB as electrocatalyst of H_2O_2 reduction. Nanoparticles have unique physical and chemical properties, often showing very interesting attributes unmatched by their bulk counterparts. The large surface to volume ratio and the increased surface activity of nanoparticles, when compared to those of bulk materials, enable their use in a catalysis and a sensing [8]. Nanoparticles of PB have been prepared by using stabilizators such as polyvinylpyrrolidone (PVP) or surfactants [9]. Karyakin *et al.* have produced nanostructured thin films of PB for electrochemical detection of H_2O_2 and for biosensors production [10]. Au-PB nanocomposite film, could also be prepared using ferricyanide as precursor and $AuCl_4^-$ acting as an oxidizer [7]. These films responded to H_2O_2, but they have not been used for biosensors construction.

It has been reported that PB modified electrodes are unstable at neutral pH and would be disrupted after a few scans, which limited they use in biosensor fabrication [11]. The use of protective layers such as Nafion or chitosan may effect the operational stability of PB [12,13]. However, manual coating of PB films doesn't allow to control the films thickness that causes poor reproducibility and slow response of the sensors. Furthermore, partially or even full loss of an enzyme incorporated in the layers could be observed.

The aim of this work was to fabricate and characterize the glucose biosensors constructed by codeposition of GOx with chitosan-AuNP on Au-PB nanoparticles modified glassy carbon electrode.

2. MATERIALS AND METHODOLOGY

2.1. Reagents

Chloroauric acid ($HAuCl_4$) from Aldrich, potassium ferricyanide, glucose, potassium nitrate, hydrochloric acid, sulphuric acid, sodium hydroxide from Reachim and hydrogen

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peroxide from P.O.Ch. were reagent grade and were used as obtained from suppliers. Glucose oxidase, GOx (E. C. 1.1.3.4) from *Aspergillus niger*, having activity of 210 IU/mg, was from Sigma. Solutions were prepared using doubly distilled and subsequently deionized (18 MΩ Millipore Milli-Q) water.

Voltammetric and amperometric experiments were carried out using electroanalytical system model CS-1090 (Cypress Systems, Inc. Lawrence, Kansas, USA) and home made chronoamperometric system [14], respectively. Standard three-electrode cell was used for preparation of composite films and for all electrochemical measurements. The working electrode was a glassy carbon (GC) disk (3 mm, diameter) and the counter electrode was made from Pt wire. All potentials were expressed versus saturated KCl calomel (SCE) reference electrode. Before modification, glassy carbon electrode was polished successively with fine grade aqueous alumna slurries (size 5 ± 0.5 μm) on a polishing cloth and treated ultrasonically in water. Prior to any electrochemical measurements, the electrode surface was examined by cyclic voltammetry in potential range from 0.0 to 1.0 V. The biosensors response of was carried out in thermostated electrochemical cell at 25 ± 0.1 °C.

2.2. Glassy Carbon Electrodes Modification with Au-PB

For the formation of Au-PB films on the surface of GCE one-step electrochemical procedure was used. In short, the cell was filled with 4 ml solution containing 1mM $HAuCl_4$, 1mM potassium ferricyanide and 0.1M KNO_3. The GC electrode potential was scanned between 0.0 and 1.0 V at a scan rate 50 mV/s for 35-50 times. According to literature [7] the gold-PB nanocomposites form on the surface of the electrode spherically shaped aggregates with dimensions in range of 50 to 300 nm. Due to instability of PB at neutral pH voltammetric response of film-covered GCE was examined using blank solution pH 3.2 containing 0.1 M KNO_3. To protect gold-PB layers design for biosensors preparation it was covered with chitosan-AuNP film having incorporated GOx molecules (method is described in section 2.4).

2.3. Synthesis of Chitosan-AuNP

In a typical experiment 100 μl of 0.125 M concentrated aqueous solution of $HAuCl_4$ was added by heating into 100 ml of chitosan solution prepared in 1 % acetic acid. Chitosan concentration was 0.05, 0.01, 0.1, 0.2, 0.5 and 1 % w/v. The solution was heated at 70°C during 15 min and a ruby-red colour appeared at all chitosan concentrations. The most stable chitosan-AuNPs were prepared at chitosan concentration 0.2 %. These nanoparticles were used in further experiments. They showed surface plasmon band [15] at 523 nm, and absence of visible aggregation during 6 months.

2.4. Immobilization of GOx on the Au-PB Modified Electrode

Two different methods were used for GOx immobilization on modified with Au-PB GCE electrode.

Following the first method 5 μl of 20 mg/ml GOx was dropped onto modified electrode, and dried for 1 hour at room temperature. The biosensor was further marked as the I-type biosensor.

Following the second method GOx was deposed electrochemically together with chitosan-AuNP. For this purpose a mixture of GOx and chitosan–AuNP solution was prepared by dissolving 2.5 mg of glucose oxidase in 3.5 ml of 2.5 nM chitosan–AuNP solution. The GC electrodes were immersed into the mixture, and a constant potential of -1.5 V was applied for 200 s. This biosensor was further marked as II-type biosensor. The biosensors were washed with water and stored in buffer solution pH=7.2 at 4 °C until the use.

The response of the biosensors was measured in 10 ml thermostated and well mixed buffer solution.

3. RESULTS AND DISCUSSION

3.1. The Formation *in situ* of Au-PB Nanocomposite Film

The deposition of PB on a GCE at low pH was fulfilled by adding of chloroauric acid in ferricyanide solution. In absence of chloroauric acid the PB was formed in the bulk solution and GCE has not been modified. Following the investigations of Kumar *et al.* [7] it is possible to conclude that modification of the electrode starts with Au nuclei formation on GCE surface. These Au nuclei acts as centres for the *in situ* synthesis of PB nanoparticles. The change of voltammograms during the synthesis is shown in Fig. (**1**). The electrochemical behaviour of GCE modified with Au-PB can be explained by two sets of voltammetric peaks that originate from PB reactions; the oxidation of PB to iron (III) hexacyanoferrate (III) (Prussian Yellow) progresses at 0.9V, and the reduction PB to iron(II) hexacyanoferrate (II) (Prussian white) proceeds at about 0.2 V (Fig. **2**).

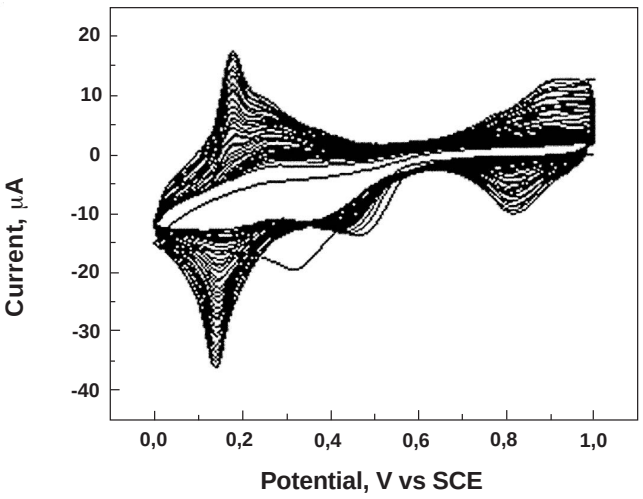

Fig. (1). The voltammetry of GCE during cyclic potential scan from 1.0 to 0.0 V. Scan rate was 0.05 V/s. 0.1M KNO_3 solution contained 1 mM $HAuCl_4$ and 1 mM potassium ferricyanide.

3.2. The properties of chitosan-AuNP.

The disadvantage of PB modified electrodes is a low stability at neutral pH that is typical for enzyme reactions. To increase the stability of the electrode the modification of GCE was performed by electroplating chitosan-gold nanoparticles, which shows electronic conductivity and electrophoretic mobility [8]. The chitosan-AuNP was prepared as described and showed absorbance with the maximum at 526 nm (Fig. **3**).

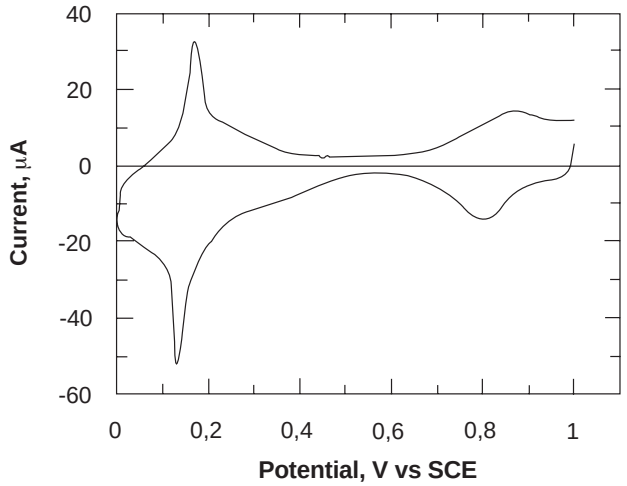

Fig. (2). The **c**yclic voltammogram of the Au-PB modified Au electrode in 0.1 M KNO_3, pH 3.2 at scan rate 0.05 V s^{-1}.

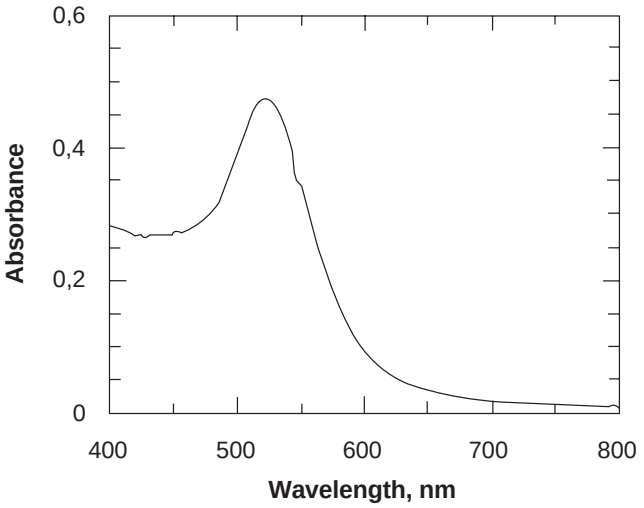

Fig. (3). The absorption spectrum of gold nanoparticles synthesized using 0.2 % (w/v) chitosan.

The dimensions of chitosan-AuNP nanoparticles calculated using Mie theory [16] are from 10 nm to 50 nm; the largest amount of nanoparticles has 20 nm diameter. The concentration of NPs that was determined using molar absorbance for the plasmon resonance 2.33×10^8 $M^{-1}cm^{-1}$ [17] varied between 2.3-2.7 nM. Prepared chitosan-AuNPs were stable at least for six months in contrast to nanoparticles formed by simple mixing of chitosan and AuNPs solution that aggregated during some days.

3.3. Electrodeposition of Chitosan-AuNP on the Au-PB Modified Electrode

Chitosan become insoluble when pH is higher than 6.3. This permits to deposit chitosan hydrogel containing GOx and AuNP onto electrodes by increasing local pH at close proximity to the electrode. The electrode potential of –1.5 V gave the most stable GOx chitosan-Au layer. The more positive or more negative potentials resulted in too slow generation or(and) labile chitosan-AuNP and GOx film formation [4]. The change of electrode current during electrodeposition is shown in Fig. (**4**). The increase of current during deposition of NP reached steady-state after 200 s indicating the finishing of the process. The deposition time of 200 s was chosen for further preparation of biosensors.

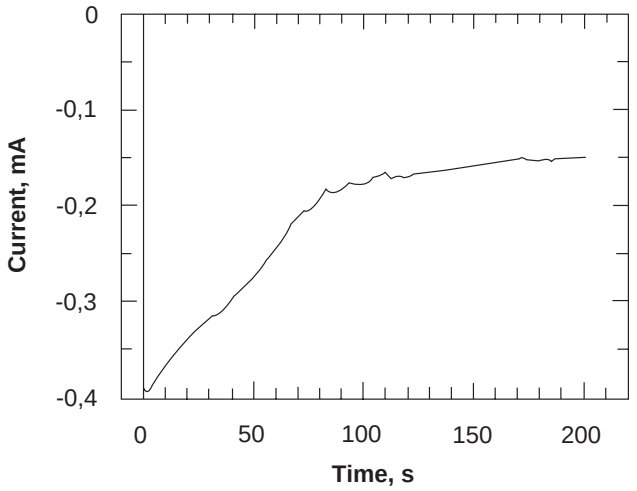

Fig. (4). The amperometric response of the Au-PB modified GCE during electrodeposition of chitosan-AuNP and GOx. Electrode potential was –1.5V vs. SCE.

3.4. Glucose Biosensor Action

The reduced form of PB (Prussian White) catalyzes reduction of H_2O_2 at electrode potential of - 0.15 V [4]. Therefore, the application of GCE modified with Au-PB allows to construct glucose biosensors acting at low potential. In this work two types of biosensors with different procedures of PB formation were prepared.

The dependence of biosensors response on electrode potential was studied between -0.2V and +0.2V. The results show that the first and the second type biosensor responded in a different manner (Fig. **5**).

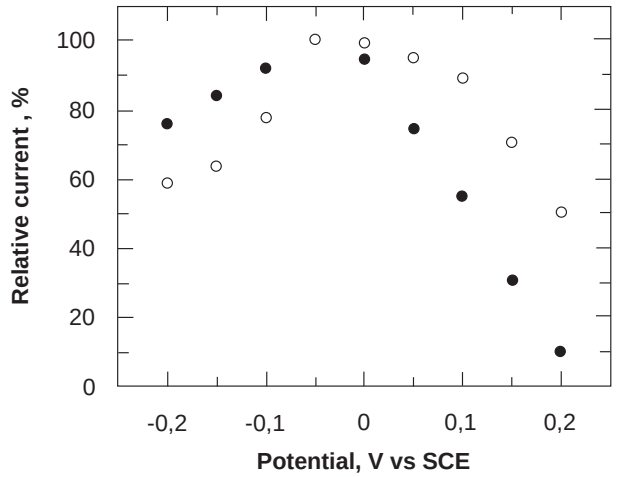

Fig. (5). The dependence of biosensor response on electrode potential. Na-phosphate buffer solution pH 7.2 contained 1 mM glucose and 0.1 M KCl. Unfilled circles represent data of I-type biosensor. Filled circles represent experimental data of II-type biosensor.

The relatively less response of I-type biosensor at smaller potential could be ascribed to instability of PB, resulting in decreasing the biosensor sensitivity. However, the maximal response for both biosensors was at -0.05 V. This potential

was selected for further biosensor parameters measurements. This low working potential is significant less in comparison to other types of glucose biosensors in which high positive potentials are used [18,19].

For stability test both types of biosensors were kept in buffer solution. The results show that the use of the chitosan–AuNP hybrid film enhances the stability of the II-type biosensor. It has been reported that the PB layer would be disrupted after a few scans at neutral and alkaline pH solutions [11]. We also observed that in absence of chitosan-AuNP layer (I-type biosensor) the PB desorbs from the electrode during 10 min at -0.05 V. Furthermore, holding of I-type biosensor at neutral pH between experiments for 30 min resulted in doubly lowered sensitivity, indicating low stability of Au-PB film (Fig. **6**).

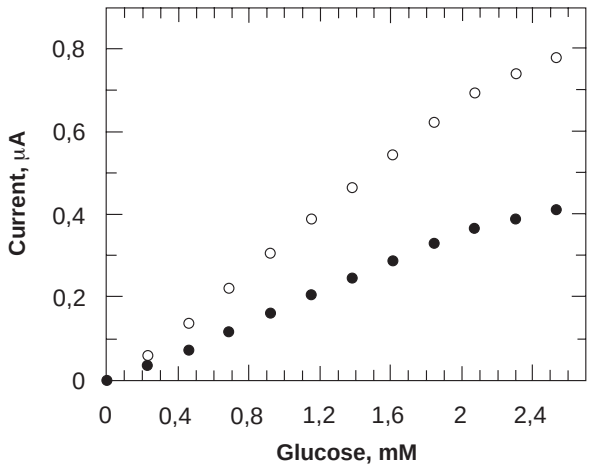

Fig. (6). The calibration curves of the I-type biosensor in Na-phosphate buffer pH 7.2, containing 0.1 M KCl. Unfilled circles represent calibration curve just after biosensor preparation. Filled circles represent calibration curve when biosensor was kept for 30 min in buffer solution.

When the PB modified electrode was protected by the chitosan-AuNP layer, having incorporated GOx, it became more stable. Even after continuous functioning for more than 2 h the biosensor response was similar to initial response (Fig. **7**).

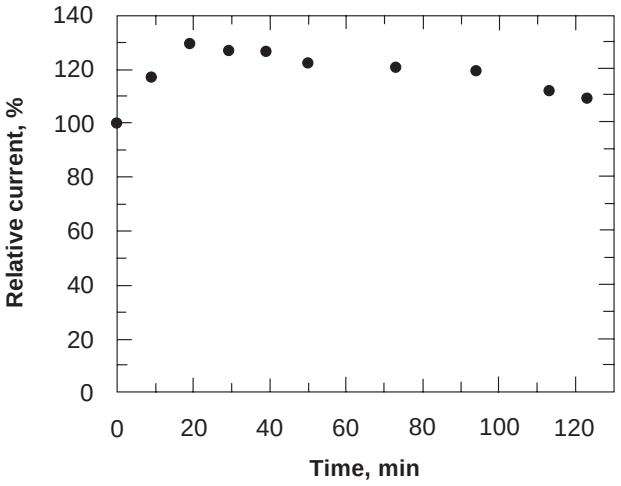

Fig. (7). The response of the II-type biosensor to 1 mM glucose. The initial response was taken as 100 %.

Long term analysis revealed that biosensor remains 83 % of its initial response at 24 h, and it kept 34 % at 96 h (data not shown).

The use of chitosan-AuNP layer resulted in stable biosensor preparation. The typical response of II-type biosensor to consecutive addition of glucose is presented in Fig. (**8**). The response time of biosensor determined as time when 95 % of steady state response was achieved was about 10 seconds.

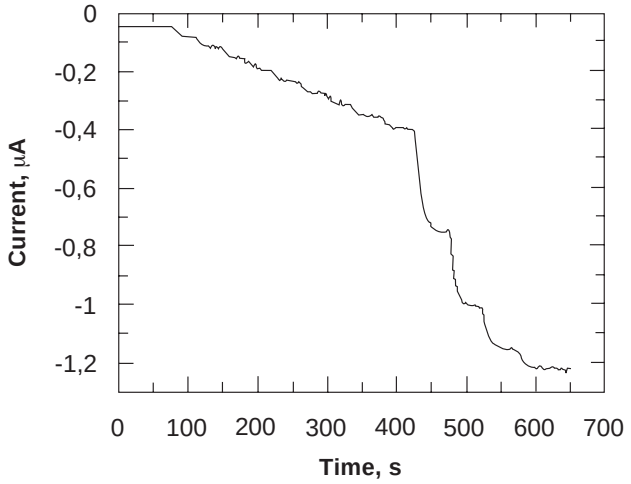

Fig. (8). The response of the second type biosensor to glucose. Glucose concentrations: 0.15, 0.3, 0.45, 0.6, 0.75, 0.9, 1.05, 1.2, 1.35, 2.35, 3.35, 4.35, 5.35, 6.35 mM in Na-phosphate buffer pH 7.2 solution containing 0.1M KCl. Electrode potential -0.05 V vs. SCE.

The calibration curve, calculated from the current/time dependence is presented in Fig. (**9**).

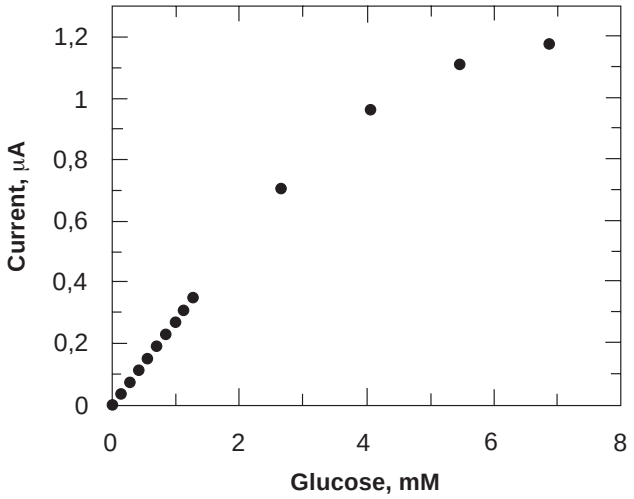

Fig. (9). The calibration curve of the response of the II-type biosensor in stirring Na-phosphate buffer pH 7.2 solution containing 0.1M KCl. Operating potential: -0.05 V vs. SCE.

The sensitivity of the II-type biosensor determined as a slope at linear part of calibration curve was 9.54 μA M^{-1} cm^{-2}. The current was linearly dependent on glucose concentration up to $3 \cdot 10^{-3}$ M of glucose (R = 0.9998, n = 10). This interval was larger than other PB containing biosensors [4].

In contrast, linear range of I-type biosensor was lower; the calibration started to saturate at 1.9×10^{-3} M of glucose.

The scheme of biosensors action is shown in Fig. (**10**). It includes glucose diffusion from bulk solution into GOx film where it is oxidized with H_2O_2 generation. The produced H_2O_2 is catalytically reduced with PW (reduced PB). The reduced PB is generated at electrode potential of -0.05 V.

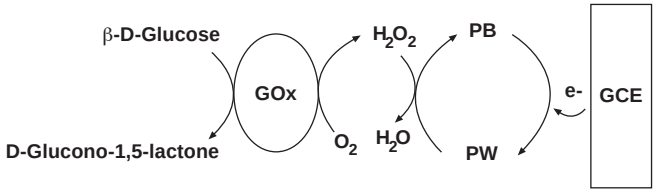

Fig. (10). The scheme of biosensor action.

CONCLUSION

A novel glucose biosensor showing high stability of response was prepared by simultaneous electrochemical deposition of GOx with chitosan-AuNP on Au-PB modified glassy carbon electrodes. The presence of gold nanoparticles and chitosan increases the stability of the biosensor. The application of this novel method of electrocatalytical layer preparation permits to produce the biosensor exhibiting wide linear range, fast response time and satisfactory operational stability.

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