Highly stable biosensor based on glucose oxidase immobilized in chitosan film for diagnosis of diabetes

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Abstract
In this work, a highly stable electrochemical biosensor was fabricated by biomaterials for determination of glucose in the aqueous phase. Chitosan (CHIT) was used for immobilization of glucose oxidase (GOx) on the surface of platinum (Pt) electrode. The performance of the fabricated biosensor was assessed by cyclic voltammetry and amperometry. The modified electrode showed an excellent performance for glucose detection with a short response time of 5 s, high sensitivity of 38.5 µA mM$^{-1}$ cm$^{-2}$, the linear range of 2×10$^{-4}$-9.1×10$^{-3}$ M and detection limit of 7.4 µM glucose at a signal to noise ratio of 3. The low value (11.3 mM) of the apparent Michaelis–Menten constant (K_M) indicated that the biosensor had a high affinity to glucose. The effect of temperature on the amperometric response was investigated to evaluate the thermal stability of the biosensor. The study of the long-term stability of biosensor revealed that the modified electrode retained 97% of its initial current response after 30 days. The obtained results showed that CHIT enhanced anti-interference ability, and the thermal and long-term stability of the biosensor.

Keywords: Glucose biosensor, Glucose oxidase, Chitosan, Electrochemical biosensor, Amperometry

1. Introduction
Biosensors are essential analytical tools in the food industry, environmental monitoring and especially in medical and health applications[1-3]. One of the healthcare measurements is glucose analysis which is vital for diabetic patients [4]. Based on the transducer, different types of biosensor have been used for glucose detection such as electrochemical, piezoelectric, optical and thermal biosensors [5, 6]. Among these methods, electrochemical biosensors have attracted considerable attention because of their simple procedure and also accurate and fast response [7, 8].

Enzymes as biological elements have been widely employed in biosensors due to their high selectivity, quick and specific responses to analytes. For glucose detection, glucose oxidase (GOx), found in various fungal sources, with high activity in a wide range of temperature and pH is used. GOx is a glycoprotein consisting of two identical polypeptide chains which covalently joined together by disulfide bonds [4, 9]. GOx catalyzes the oxidation of glucose to gluconolactone (which can be hydrolyzed to gluconic acid) and H$_2$O$_2$ in the presence of oxygen. The electrochemical changes resulted from the enzymatic reaction and generated H$_2$O$_2$ are often monitored for determination of glucose [10, 11]. Immobilization of enzyme plays an important role in the performance of biosensors. The various techniques based on physical (adsorption, entrapment, encapsulation) and chemical (cross-linking, covalent binding) methods
are commonly used for enzyme immobilization [12-14]. Coating with biopolymers is an efficient method for immobilization of enzyme on the surface of electrode [15]. Chitosan (CHIT) is a polysaccharide derived by deacetylation of chitin found in fungal cell walls and the exoskeleton of arthropods including insects, arachnids, and crustaceans (lobsters, crabs, and shrimps) [16, 17]. Increasingly over the last decade, CHIT has been used for enzyme immobilization in biosensors because it provides a biocompatible environment for the enzyme, has no inhibitory effect on the enzyme activity and maintains enzymatic activity [18, 19]. Also, CHIT with excellent adhesion and film-forming ability prevents enzyme leakage from the support and simultaneously allows the analyte to pass through its porous matrix [19, 20].

The aim of this work is to present the development of a simple but highly stable biosensor for determination of glucose. CHIT was selected as a support for immobilization of GOx on the electrode surface. The analytical performance of the biosensor was evaluated by cyclic voltammetry and amperometry. The thermal and long-term stability of the biosensor were investigated. Also, the apparent Michaelis–Menten constant and activation energy were determined.

2. Materials and Methods

Materials

Glucose oxidase (GOx) (E.C.1.1.3.4, type X-S, 153100 Ug⁻¹) from Aspergillus niger and chitosan (CHIT) (85% deacetylated) were supplied by Sigma-Aldrich (USA). All other chemicals were purchased from Merck (Germany). A 0.1 M phosphate buffer (PB) (pH 7) solution was prepared using K₂HPO₄ and KH₂PO₄. A sufficient amount of KCl was added to PB solution to obtain 0.1 M concentration for preventing charged electroactive species from migrating in the electric field gradient. For adjusting pH, 0.1 N solution of KOH was gradually added to the supporting electrode until the pH reached to 7. Glucose stock solution (0.1 M) was prepared and stored at room temperature for 24 h to ensure mutarotation equilibrium. CHIT solution (0.6 wt%) was prepared by dissolving CHIT powder in 1 wt% acetic acid solution and stirred at room temperature for 30 min.

Electrochemical measurements

All electrochemical experiments were carried out using a potentiostat/galvanostat (Ivium, A08085, Netherlands). A conventional three-electrode system consisting of a modified platinum (Pt) (2 mm diameter) working electrode, a Pt wire auxiliary electrode and an Ag/AgCl reference electrode was used. All electrodes were obtained from Azar-Electrode (Iran). Electrochemical measurements were conducted in an electrochemical cell containing 10 mL supporting electrolyte. All potentials were reported versus the Ag/AgCl electrode. A heated stirrer (Velp, Scientifica, Italy) equipped with thermoregulator was used for adjusting the temperature of the electrolyte. Amperometric measurements were performed at 150 rpm using a stirring bar to ensure convective transport.

Electrode preparation

Prior to each experiment, the Pt working electrode was initially cleaned in boiling 6 M HNO₃ solution and polished using 0.05 m alumina slurry [21], and then washed with double distilled water. The electrode was ultrasonicated in double distilled water and allowed to dry at room temperature. Dropping method was used to deposit GOx on the surface of the electrode. Firstly, 5µL of GOx solution (5 mgmL⁻¹) was dropped onto the Pt electrode surface and dried in air for 2 h. Then 10 µL of CHIT solution (0.6 wt%) was dropped on the surface of GOx/ and dried in air for 12 h. The modified electrode was maintained at 4°C in a dry state when not in use.
3. Results and Discussions

Electroactive surface area of the modified electrode

The electroactive surface area of the modified electrode was assessed by cyclic voltammetry at different scan rates. In this method, potassium ferricyanide was employed as a probe to evaluate the performance of the electrode. The cyclic voltammetric experiment was performed from −0.3 to 0.85 V at scan rates of 10-90 mV s\(^{-1}\) in 10 mL of 1 M KCl solution containing 5 mM K\(_3\)[Fe(CN)\(_6\)] (Fig. 1).

![Fig. 1. CVs of modified electrode at different scan rates of 10-90 mV s\(^{-1}\) in 1 M KCl solution containing 5 mM K\(_3\)[Fe(CN)\(_6\)]. Inset: the plot of peak current versus the square root of scan rate.](image1)

The peak current for a reversible electrochemical reaction is given by the Randles-Sevcik equation as follows [22]:

\[
i_p = 0.4463 \left( \frac{F^3}{RT} \right)^{\frac{1}{2}} n^{\frac{3}{2}} D^{\frac{1}{2}} A C v \]

(1)

where \(i_p (A)\) is the peak current of the CV, \(n\) is the number of electrons transferred in redox reaction, \(A\) (cm\(^2\)) is the electroactive surface area of the electrode, \(D\) (cm\(^2\) s\(^{-1}\)) is the diffusion coefficient of redox probe (K\(_3\)[Fe(CN)\(_6\)]) in bulk solution (KCl), \(C\) (mol cm\(^{-3}\)) is the concentration of redox probe, \(v\) (V s\(^{-1}\)) is the scan rate, \(R\) is the universal gas constant, \(T\) is the temperature in Kelvin and \(F\) is Faraday’s constant. As shown in the inset of Fig. 1, the plot of peak current versus the square root of scan rate was relatively linear (correlation coefficient of 0.98), which shows a diffusion controlled process [23]. By considering that \(N\) for this redox reaction is equal to 1, \(D\) for 5 mM K\(_3\)[Fe(CN)\(_6\)] in 1 M KCl solution is 7.60 \times 10^{-6} \text{ cm}^2\text{s}^{-1} [24], and from the slope of the linear plot, the electroactive surface area of the CHIT/GOx/Pt electrode was calculated to be 1.18 \times 10^{-2} \text{ cm}^2.

Cyclic voltammetric characterization

Cyclic voltammetry was conducted at bare Pt, CHIT/Pt, and CHIT/GOx/Pt electrodes to study the biosensor response to glucose. Cyclic voltammetric experiments were carried out in the absence and presence of 5.6 mM (100 mg dL\(^{-1}\)) glucose in 0.1 M PB solution by scanning the potential from 0.2 to 1.0 V at a scan rate of 100 mV s\(^{-1}\). Featureless cyclic voltammograms were obtained at Pt and CHIT/Pt electrodes, whereas the peak current of 7.5 \mu A was in the CV of the CHIT/GOx/Pt electrode (Fig. 2). Therefore, this peak most likely results from the oxidation of glucose at the surface of modified electrode.
Fig. 3 shows the CVs of CHIT/GOx/Pt electrode at different glucose concentrations from 0.2 to 12.28 mM. It can be observed that distinct peak currents were obtained with increasing glucose concentration even at glucose concentrations lower than 990 µM. Therefore, the fabricated biosensor can be used to detect glucose clearly.

**Effect of pH on the biosensor response**

The effect of pH on the biosensor response to 5.6 mM glucose solution in the range of pH 5-8 using amperometric method applied the potential of 0.75 V was investigated. The maximum current response was obtained at pH 7 (see Fig. 4); therefore, this pH was selected as optimum pH and used for further studies.

**Effect of temperature**

The biosensor response to 5.6 mM glucose solution was studied within the temperature range from 5 to 75°C (Fig. 5). As shown, the current response increased with increasing the temperature and reached a maximum value at 60°C and then decreased at higher temperature due to enzyme denaturation. An increase in temperature provides the required activation energy for the enzymatic reaction; in contrary at any temperature higher than 60°C, the enzyme active sites are deteriorated, and enzyme loses its activity. This obtained optimum temperature value was higher than that reported by J. RUBIO & al. (45°C) [25], J. YU & al. (52°C) [26], and D. SHAN & al. (55°C) [27]. An increase in the optimum temperature indicated that CHIT enhanced thermal stability of the immobilized GOx.

The relation between current response and temperature can be presented by Arrhenius equation ($i = i_0 \exp(-E_a/RT)$). The plot of $lni$ versus 1000/T is depicted in the inset of Fig. 5. From the slope of the straight line, the activation energy was found to be 34.6 kJ mol$^{-1}$ which is consistent with other glucose biosensors reported in the literature (22.4–50 kJ mol$^{-1}$) [26, 28].

**Amperometric performance of the biosensor**

Amperometric measurements were carried out at an applied potential of +0.75 V at 25±0.2°C in the electrochemical cell containing 10 mL PB solution (0.1 M). The background current was allowed to decay to a steady state value, and then aliquots of glucose stock solution were added to the supporting electrolyte. Since the short response time and the steady-state...
current were critical parameters in the performance of biosensors, the effect of GOx and CHIT concentrations on current response was evaluated to find optimum condition. The amperometric response of biosensor for different concentrations of enzyme and CHIT was carried out. The current responses for adding 0.9 mM glucose into 4.7 mM glucose (5.6 M) solution are compared in Fig. 6.

Fig. 5. The effect of temperature on the current response of the biosensor to 5.6 mM glucose in 0.1 M PB, (Inset: the plot of ln i versus (1/T)×10^3 for determination of activation energy).

Fig. 6. The amperometric current of modified electrodes (a) 1 mg ml⁻¹ GOx, 0.6 wt% CHIT (b) 15 mg ml⁻¹ GOx, 0.6 wt% CHIT (c) 5 mg ml⁻¹ GOx, 0.6 wt% CHIT (d) 5 mg ml⁻¹ GOx, 1.2 wt% CHIT (e) 5 mg ml⁻¹ GOx, 0.2 wt% CHIT, for adding 0.9 mM glucose into 4.7 mM glucose (5.6 mM), and (f) 5 mg ml⁻¹ GOx, 1.2 wt% CHIT, for adding 0.8 mM glucose into 9.1 mM glucose (9.9 mM).

For low concentration of GOx (1 mg mL⁻¹) the steady state response with a low current of 1.9 µA was obtained (Fig. 6a). For 15 mg mL⁻¹ GOx, the current has not achieved to steady state response because at high concentration of GOx, the thickness of enzyme layer increased and glucose molecules slowly diffused into the active sites of the internal layer of GOx (Fig. 6b). For a low concentration of CHIT (0.2 wt%), after adding glucose the current response was increased, but it was noisy and tended to decrease; this phenomenon might be due to enzyme leakage from the thin layer of CHIT (Fig. 6e). For 1.2 wt% CHIT the current response was almost closed to steady state and slightly increased; this increase was probably attributed to increasing of the thickness of CHIT layer which hinders the diffusion of glucose into the enzyme active sites (Fig. 6d). However, at high concentration of glucose (9.9 mM) the noisy current was observed which might be explained because of the accumulation of product molecules within the thick layer of CHIT. For 5 mg mL⁻¹ GOx and 0.6 wt% CHIT, the steady state response with a current of 7.2 µA and short response time (within 5 s) were obtained.
Therefore, 5 mg mL\(^{-1}\) GOx and 0.6 wt% CHIT were selected as optimum concentrations fabrication of biosensor. The amperometric response of fabricated biosensor is shown in Fig. 7a.

![Fig. 7. (a) The amperometric response of the biosensor to successive addition of glucose in 0.1 M PB (pH 7) at 25°C and applied the potential of 0.75 V. (b) The current response versus glucose concentration, Inset: the plot of 1/i versus 1/C for determination of Michaelis–Menten constant.](image)

The plot of current response versus glucose concentration is depicted in Fig. 7b. As shown, the calibration plot was linear in the glucose concentration range of 0.2–9.1 mM (equivalent to 0.9-164 mg/dL) which is suitable coverage range of blood glucose (70-110 mg/dL). The modified electrode showed an excellent performance for glucose detection with a high sensitivity of 38.5 µA mM\(^{-1}\) cm\(^{-2}\) and the detection limit of 7.4 µM glucose (S/N=3). The performances of fabricated biosensor and other glucose biosensors reported in the literature are summarized in Table 1.

<table>
<thead>
<tr>
<th>Glucose biosensor</th>
<th>Linear range (mM)</th>
<th>Sensitivity (µA mM(^{-1}) cm(^{-2}))</th>
<th>Detection limit (µM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHIT/GOx/Pt</td>
<td>0.02-9.1</td>
<td>38.5</td>
<td>7.4</td>
<td>Present work</td>
</tr>
<tr>
<td>GOx/silica gel-MWNT*-polyacrylonitrile/Pt</td>
<td>Up to 2</td>
<td>16.52</td>
<td>1</td>
<td>R. NENKOVA &amp; al. [29]</td>
</tr>
<tr>
<td>CHIT-GOx/gold</td>
<td>Up to 4.9</td>
<td>1.85</td>
<td>10</td>
<td>Y. TAN &amp; al. [19]</td>
</tr>
<tr>
<td>IO(_4)-oxidized-GOx/gold NPs(^\ddagger)-SBA-15(^\ddagger)/gold</td>
<td>0.02–14</td>
<td>6.1</td>
<td>15</td>
<td>Y. BAI &amp; al. [2]</td>
</tr>
<tr>
<td>polydopamine-GOx-graphene/gold</td>
<td>Up to 4.7</td>
<td>28.4</td>
<td>0.1</td>
<td>C. Ruan &amp; al. [30]</td>
</tr>
<tr>
<td>GOx-(o)-phenylenediamine / Pt NPs/polyvinyl ferrocenium perchlorate/Pt</td>
<td>0.06-9.64</td>
<td>17.4</td>
<td>18</td>
<td>E. TURKMEN &amp; al. [31]</td>
</tr>
</tbody>
</table>

\*MWNT: Multiwall carbon nanotube

\(^\ddagger\)NPs: Nanoparticles

\(^\ddagger\)SBA-15: Mesoporous silica SBA-15
The enzyme–substrate kinetic of the fabricated biosensor was characterized by Lineweaver–Burk equation given as follows [32]:

\[
\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_M}{I_{max}} \frac{1}{C}
\]  

(2)

where \( I_{ss} \) is the steady state current response after each step of substrate addition, \( I_{max} \) is the maximum current response under saturated substrate condition, \( C \) is the substrate concentration in the bulk solution and \( K_M \) is the apparent Michaelis–Menten constant. To determine \( K_M \) value of enzyme electrode from equation 2, the reciprocal of steady state current response versus the reciprocal of glucose concentration was plotted in the inset of Fig. 7b. From the slope and intercept of the Lineweaver–Burk plot, the \( K_M \) value was found to be 11.3 mM, which is lower than those reported by R. NENKOVA & al. (13.9 mM) [29], T. KONG & al. (19 mM) [33] and Y. ZOU & al. (14.4 mM) [34]. The low value of \( K_M \) indicated that the CHIT film provided a biocompatible environment for GOx and assisted immobilized GOx to retain high enzymatic activity; therefore the CHIT/GOx/Pt electrode exhibited a high affinity to glucose.

**Interference analysis**

Uric acid (UA) and ascorbic acid (AA) are common electroactive species which have a undesired effect on the accuracy of glucose detection. In human blood, the concentration range of UA is 2.5-8.0 mgdL\(^{-1}\) [35] (1 mg dL\(^{-1}\) of UA equivalent to 59.48 µM). The upper level of AA in blood is about 1.3 mg dL\(^{-1}\) [36] (1 mg dL\(^{-1}\) of AA equivalent to 56.78 µM). Generally, AA is commonly used as supplements in food industries.

In this work, the influence of these two electroactive interferents on the amperometric response was investigated. For this purpose, 0.1 mM AA and 0.5 mM UA were consecutive added into 4.7 mM glucose solution. In order to obtain a proper response to the final glucose concentration of 5.6 mM, a 0.9 mM glucose was added to the primary solution as discussed above. Fig. 8 demonstrates the current responses to AA (0.1 mM) and UA (0.5 mM) were negligible (in compare to reference state, 4.7 mM glucose). The current response to 5.6 mM glucose was 7.5 µA, which shows a slight increase (4.2%) in compare to steady state current response (7.2 µA) through amperometric measurement. Based on obtained results, the fabricated biosensor showed a good anti-interference ability.

The long-term stability of the biosensor

The long-term stability of the fabricated biosensor was explored by measuring current response to 5.6 mM glucose. The result demonstrated that the biosensor retained 97% of its initial response after 30 days which was higher than other glucose biosensor reported by J. YU & al., T. KONG & al. and L. LUO & al. [33, 37, 38]. This high long-term stability of biosensor can be attributed to the biocompatible environment provided by CHIT and the point that no extra material was used in the fabrication of biosensor.

4. Conclusions

In this work, a highly stable biosensor was fabricated for glucose detection. CHIT as a coating material was used to immobilize GOx on the electrode surface, and no extra material was added to the electrode to prevent denaturation of GOx. The fabricated biosensor showed accurate, fast and reliable responses with a high sensitivity of 38.5 µA mM\(^{-1}\) cm\(^{-2}\). The low apparent Michaelis–Menten of 11.3 mM showed high affinity of the biosensor to glucose. According to the thermal stability study of the biosensor, the optimum temperature was determined to be 60°C which was significantly high. Also, the biosensor exhibited a good anti-interference ability and high long-term stability (97% of initial response after 30 days). The results revealed that the Pt electrode modified with biomaterials (GOx and CHIT) had high performance for glucose determination.

References

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