Influence of the immobilization method on enzyme loading and performance of glucose oxidase biosensor

Ruska Nenkova, Jiamin Wu, Yahong Zhang, Tzonka Godjevargova

Abstract: *Seven glucose biosensors were prepared by entrapment and layer-by-layer assembly technique to study the the enzyme loading and electrochemical characteristics of the constructed biosensors. The highest enzyme concentration on the electrode surface corresponded to the electrodes prepared by layer-by-layer method and that is in accordance with the highest sensitivity of these biosensors: from -1.423 to -1.315 μA.mV^{-1}.s and from 0.673 to 0.570 μA.mV^{-1}.s. Amperometric measurements of the seven glucose oxidase electrodes were carried out. The layer-by-layer assembly Pt/(PDDA/NZ)$_5$/GOD electrode is characterized with the highest sensitivity - 1.4733 μAmM^{-1} and quick response (4 s).*

Keywords: *biosensor, glucose oxidase, layer-by-layer, electropolymerization*

IINTRODUCTION

The effective coupling of enzymes to electrodes is an important issue in the biosensor fabrication. There are a variety of methods used to immobilize enzymes. Two of the most common ones are entrapment and layer-by-layer assembly technique. The layer-by-layer technique is a facile method to engineer surface with targeted properties for the construction of biofunctional films [4]. The procedure is based on the alternative deposition of the oppositely charged polyelectrolyte [2], proteins [1], ceramics, or charged nanoparticles [9] on a charged surface by attractive electrostatic force. Biomolecules embedded into such multilayer film could keep a secondary structure close to their native form, which is crucial to biological analysis [7].

Electrochemical entrapment methods induced by electropolymerization of the monomer in the presence of the enzyme are simple and can be used to localize the biomaterials. Amperometric biosensors involving the physical entrapment of glucose oxidase (GOD) within different conducting polymers such as polypyrrole [6], polyaniline [5], and poly-3,4-ethylenedioxythiophene [3] by electropolymerization have been reported.

The aim of this study was to investigate the influence of two immobilization methods on the enzyme loading and electrochemical characteristics of the constructed biosensors - the layer-by-layer assembly technique using nanozeolites and polydiallyldimethyl-ammonium (PDDA) for enzyme adsorption and the entrapment of the biocatalyst and nanozeolites in polypyrrole film by electropolymerization.

MATERIALS AND METHODS

Reagents

Nanozeolites Silicalite-1 after detemplation (S-1-DT) was used as enzyme carrier. Glucose oxidase from *Aspergillus niger* with specific activity of 157 U.mg^{-1} (Fluka). Glucose stock solutions were allowed to rotate at room temperature overnight before use. Phosphate buffer solution (0.1 M, pH 6.2) was used as the supporting electrolyte in all measurements. Pyrrole (Py), 98% from Sigma-Aldrich, USA. All the other reagents used for analyses were reagent grade. All solutions were prepared using deionized water from PURELAB Ultra- system.

Instrumentation

Cyclic voltammetric (CV), amperometric measurements and electropolymerization of Py monomer on the working electrode surface were carried out with the PalmSens Electrochemical Instrument (Palm Instruments BV, Netherlands) and three-electrode electrochemical cell: a platinum plate electrode (1 cm^2 area) as a working electrode, platinum wire as a counter electrode and saturated calomel (SCE) electrode as a reference electrode were used both in the cyclic voltammetric and amperometric

measurements. Measurements of peroxide formation as product of enzymatic reaction are commonly carried out on a platinum electrode [8].

Cleaning of the working electrode surface

The working Pt electrode was mechanically polished with 0.3 and 0.05 μm alumina, rinsed with distilled water, acetone and once again with water. Then, it was cleaned electrochemically in 1 M H_2SO_4 by potential cycling between −0.25 and +1.45 V versus SCE at a scan rate of 0.075 $V.s^{-1}$ for 10 - 15 min.

Preparation of the Pt/(PDDA/NZ)n/GOD biosensor

Nanozeolite modified Pt/(PDDA/NZ)n/GOD electrodes were prepared by LbL assembly method: the surface of the working electrode was coated with cationic PDDA film. Afterwards, the electrode was alternately incubated in negatively charged nanozeolite solution (0.6 $mg.ml^{-1}$) and positively charged PDDA solution (3 $mg.ml^{-1}$) for 30 min. When this cycle procedure was repeated N times (3, 5 and 7), the Pt/(PDDA/NZ)n electrode was obtained. Glucose oxidase immobilization was achieved by immersing this modified electrode in GOD solution (0.1 % solution, 0.1 M phosphate buffer pH 5.8) at 4˚C for 2h.

Preparation of the Pt/PPy/GOD biosensor

Before electropolymerization, the monomer Py solution was purged with high-purity nitrogen gas for at least 10 min in order to remove dissolved oxygen. An inert environment was maintained in the electrochemical cell during the polymerization by purging the cell atmosphere with a flow of nitrogen. The electropolymerization of Py was carried out in 0.1 M KCl as supporting electrolyte, containing 0.1 M NaCl and 0.4 M Py monomer solution [42]. The final concentration of glucose oxidase in this solution was 0.1%. The working electrode potential was cycled in the potential range from −1.0 to +0.7 V vs SCE, at a scan rate of 0.05 $V.s^{-1}$, 10, 20 and 40 cycles.

Cyclic voltammetric and amperometric measurements of glucose oxidase electrodes.

Cyclic voltammetric studies of the seven enzyme electrodes were carried out in an electrochemical cell containing phosphate buffer solution (0.1 M, pH 6.2) at 25°C. The experiments were performed at potentials from -0.3 to 1 V at a scan rate of 0.1 $V.s^{-1}$. Kinetic studies were carried out on the glucose oxidase electrodes by cyclic voltammograms (CVs) as a function of scan rates varying from 0.01 to 0.2 $V.s^{-1}$.

The amperometric measurements of the seven enzyme electrodes were conducted in an electrochemical cell containing 0.1 M PBS (pH 6.2) solution under stirring at 25°C. A potential of 0.8 V was applied to the working electrode and the electrochemical current was awaited to become stationary. The glucose concentrations were changed in a controlled manner by adding aliquots of 0.1 M glucose solution. After each measurement, the biosensor was washed with buffer solution to stabilize the base signal.

RESULTS AND DISCUSSION

Kinetic studies of the glucose oxidase biosensors obtained by the two different immobilization techniques

The effect of the scan rate on the current of the seven glucose oxidase biosensors ($Pt/(PDDA/NZ)_3$/GOD, $Pt/(PDDA/NZ)_5$/GOD $Pt/(PDDA/NZ)_7$/GOD, Pt/PPy/GOD(10 cycles), Pt/PPy/GOD(20 cycles), Pt/PPy/NZ/GOD(20 cycles), Pt/PPy/GOD(40 cycles)) obtained by the two immobilization methods was studied. The scan rate varied from 0.01 to 0.2 $V.s^{-1}$. The current at 0.05 and 0.8 V decreased and increased linearly respectively with increasing the scan rate. The response was stable, with no changes observed, after 20 potential cycles. The equations of these linear dependences are presented in Table 1.

Table 1

Characteristics of cyclic voltammograms of enzyme electrodes obtained by the two methods of immobilization

Electrode	I*, mol.cm^{-2}	Linear equation between peak current (y) at 0.05V and potential scan rate (x)	Linear equation between shift current (y) at 0.8V and potential scan rate (x)
Pt/(PDDA/NZ)$_3$/GOD	0.381	y = -1.429x – 43.76	y = 0.673x + 15.287
Pt/(PDDA/NZ)$_5$/GOD	0.352	y = -1.321x – 53.98	y = 0.604x + 11.168
Pt/(PDDA/NZ)$_7$/GOD	0.350	y = -1.315x – 53.939	y = 0.570x + 11.106
Pt/PPy/GOD(10 cycles)	0.266	y = -0.998x – 36.149	y = 0.755x + 25.703
Pt/PPy/GOD(20 cycles)	0.173	y = -0.652x – 30.83	y = 0.723x + 25.151
Pt/PPy/GOD(40 cycles)	0.127	y = -0.478x – 15.703	y = 0.408x + 29.111
Pt/PPy/NZ/GOD(20cycles)	0.254	y = -0.953x – 38.758	y = 0.582x + 17.912

As can be seen, the Pt/(PDDA/NZ)$_3$/GOD, Pt/(PDDA/NZ)$_5$/GOD and Pt/(PDDA/NZ)$_7$/GOD electrodes are the most sensitive due to the greatest slope of the linear interval between the current and the potential scan rate. The sensitivity of the biosensors obtained by the layer-by-layer assembly technique is the highest: from -1.423 to -1.315 μA.mV^{-1}.s and from 0.673 to 0.570 μA.mV^{-1}.s. The sensitivity of the biosensors obtained by the enzyme electrodeposition method is the lowest - from -0.478 to -0.998 μA.mV^{-1}.s and from 0.408 to 0.755 μA.mV^{-1}.s, (Table 1).

The surface concentration (I*) of the absorbed electroactive species could therefore be estimated from the plot of I_p versus v, in accordance with the Brown Anson model [42] using the equation:

$$I_p = n^2 F^2 I^* A v / 4 R$$

where I_p represents the cathodic peak current at a different scan rate, A is the surface area of the electrode (1 cm^2), v is the scan rate (V.s^{-1}), I* is the surface concentration of the absorbed electro-active species, R is the gas constant (8.314 J.(mol. K)$^{-1}$), T is the absolute temperature (298 K) of the system, F is the Faraday constant (96.584 C.mol^{-1}), n - is the number of electrons transferred in the process (2). The surface concentration of electroactive species was calculated on the base of the CVs of the seven glucose oxidase electrodes and was presented in Table 1.

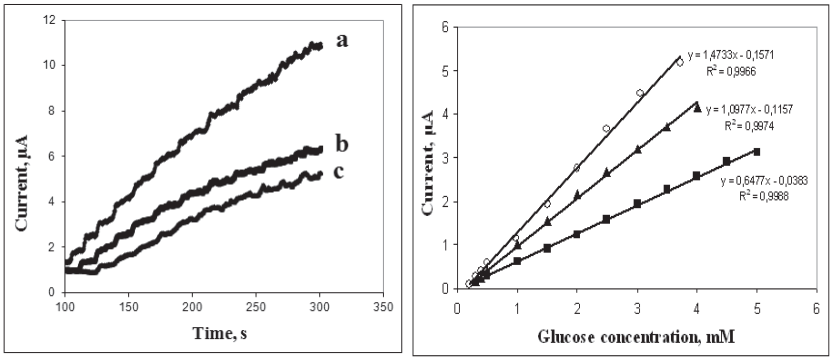
The highest concentration (I*) of the enzyme on the electrode surface corresponded to the electrodes prepared by the layer-by-layer assembly technique (Pt Pt/(PDDA/NZ)$_3$/GOD, Pt/(PDDA/NZ)$_5$/GOD, Pt/(PDDA/NZ)$_7$/GOD) and that is in accordance with the highest sensitivity of these biosensors (Table 1).

Amperometric response of the obtained glucose biosensors

The effect of the applied potential on the response current of the glucose biosensors was studied. The applied potential was changed from 0.4V to 1.0 V, and the corresponding response current to 0.1 M glucose was measured (date is not shown). The oxidation of the enzymatically formed H_2O_2 started at potential of 0.4 V for the fabricated enzyme electrodes. The response current increased rapidly with increasing the applied potential till the potential value 0.8V. This indicated that the response of the enzyme electrodes was controlled by the electrochemical oxidation of H_2O_2. The current response was constant at higher potential than 0.8V. The constant value of the current response was attributed to the rate-limiting process of enzymatic kinetics and depended on the electrode nature. The potential of 0.8V was selected as the operational potential for all amperometric measurements.

The current time response of the three glucose biosensors obtained by layer-by-layer assembly technique is presented on Fig. 1A. From the results presented for these three enzyme electrodes, the Pt/(PDDA/NZ)$_5$/GOD electrode is characterized with the

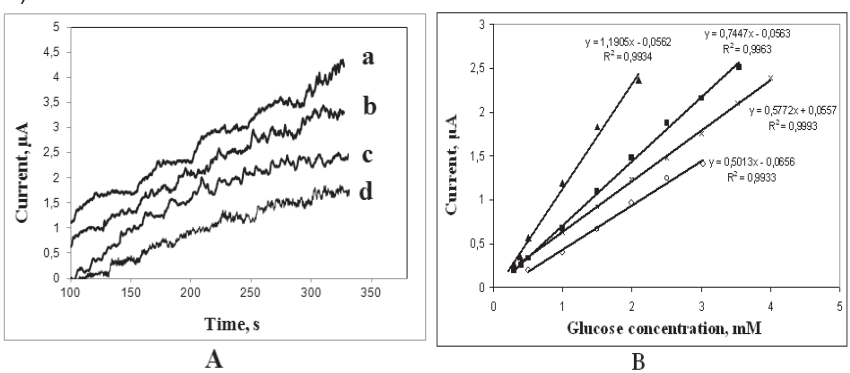
highest sensitivity - 1.4733 μAmM^{-1} and quick response (4 s), (Fig. 1B). The Pt/(PDDA/NZ)$_3$/GOD electrode is characterized with the largest linear range of glucose concentratons (0.5-5 mM) contrary to its lower sensitivity (0.6477 μAmM^{-1}). The amperometric response of this kind of biosensors increases linearly up to 4-5 mM glucose with increasing the glucose concentration.



A B

Fig. 1. A) Current time response of the three glucose biosensors obtained by layer-by-layer assembly technique (a) Pt/(PDDA/NZ)$_5$/GOD, (b) Pt/(PDDA/NZ)$_7$/GOD, (c) Pt/(PDDA/NZ)$_3$/GOD in 0.1 M phosphate buffer at applied potential of 0.8 V B) Calibration lines: ■ - Pt/(PDDA/NZ)$_3$/GOD; ○ - Pt/(PDDA/NZ)$_5$/GOD; ▲ - Pt/(PDDA/NZ)$_7$/GOD electrodes.

The enzyme electrodes prepared by electrochemical deposition of polypyrrole and glucose oxidase on the electrode surface are distinguished with the lowest sensitivity among the seven studied biosensors (Pt/PPy/GOD(10 cycles) - 0.5772 μAmM^{-1}, Pt/PPy/GOD(20 cycles) - 0.7447 μAmM^{-1}, Pt/PPy/GOD(40 cycles) - 0.5013 μAmM^{-1}), (Fig.2).



A B

Fig.2. A) Current time responses of the four glucose biosensors prepared by electrochemical deposition of polypyrrole and glucose oxidase on the electrode surface (a) Pt/PPy/GOD(20 cycles), (b) Pt/PPy/NZ/GOD(20 cycles), (c) Pt/PPy/GOD(10 cycles), (d) Pt/PPy/GOD(40 cycles) in 0.1 M phosphate buffer at applied potential of 0.8 V: B) Calibration lines: x - Pt/PPy/GOD(10 cycles); ■ - Pt/PPy/GOD(20 cycles); ▲ - Pt/PPy/NZ/GOD(20 cycles), ◊ - Pt/PPy/GOD(40 cycles) electrodes.

The results show that only the Pt/PPy/NZ/GOD (20 cycles) electrode, including nanozeolite particles in the polymer matrix, is characterized with a relatively high sensitivity - 1.1905 μAmM^{-1} and response (21 s). Such good performance of that biosensor might be

attributed to the fact that the nanozeolite particles provide the biocompatible microenvironment for enzyme immobilization, which is an important issue for the sensitive biosensor. The amperometric response of this kind of electrodes obtained by electrochemical deposition of polypyrrole increased linearly up to 2-4 mM glucose with increasing the glucose concentration.

CONCLUSION

The results showed that the enzyme loading and the performance of the biosensor was influenced by the immobilization method (layer-by-layer assembly technique and electrochemical deposition of polypyrrole and glucose oxidase). The layer-by-layer method causes two times higher biosensor sensitivity and also higher amount of immobilized enzyme than the other method.

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About the authors:

Ruska Nenkova, Department of Biotechnology, University “Prof. d-r Assen Zlatarov”, Prof. Yakimov Street 1, Burgas 8010, Bulgaria, email: rdnenkova@abv.bg

Prof. Tzonka Godjevargova, Department of Biotechnology, University “Prof. d-r Assen Zlatarov”, Prof. Yakimov Street 1, Burgas 8010, Bulgaria, email**:** godjevargova@yahoo.com

Jiamin Wu, Department of Chemistry and Shanghai Key Laboratory of Molecular Catalysis and Innovative Materials, Fudan University, Shanghai, P. R. China

Yahong Zhang, Department of Chemistry and Shanghai Key Laboratory of Molecular Catalysis and Innovative Materials, Fudan University, Shanghai, P. R. China

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