Slovak Society of Chemical Engineering

Institute of Chemical and Environmental Engineering

Slovak University of Technology in Bratislava

PROCEEDINGS

38th International Conference of Slovak Society of Chemical Engineering

Hotel Hutník

Tatranské Matliare, Slovakia

May 23 – 27, 2011

Editor: J. Markoš

Knezevic-Jugovic, Z., Jugovic, B., Jakovetic, S., Bezbradica, D., Antov, M., Ali Saied, O., Gvozdenovic, M.: Design of a polyaniline based biosensor electrode for glucose: a comparative study of two immobilized systems, Editor: Markoš, J., In *Proceedings of the 38th International Conference of Slovak Society of Chemical Engineering*, Tatranské Matliare, Slovakia, 1519–1525, 2011.

Design of a polyaniline based biosensor electrode for glucose: A comparative study of two immobilized systems

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Key words: polyaniline based biosensor; polyaniline film doped with *m*-aminobenzoate acid; enzyme cross-linking; covalent immobilization

Introduction

There has been considerable interest in the design of biosensors specific for glucose [1]. Biosensors for glucose determination have been developed based on potentiometric and amperometric methods. Among these, amperometric glucose biosensors have attracted substantial interest due to good sensitivity and low detection limit. However, upon applying a high polarizing voltage (0.6–0.8 V) interfering substances such as ascorbic acid and uric acid, which are commonly present in biological fluids are also oxidized, leading to nonspecific signals.

The successful commercial development of a biosensor depends on a number of factors which can be directly influenced by the choice of an immobilization system. Various matrices have been used for the immobilization of enzymes such as polymeric films, membranes, gels, carbon graphite, silica etc. in the preparation of biosensors. Several studies have been reported so far on the development of immobilized glucose oxidase systems based on conducting polymers among which the use of polyaniline (PANI) as a support has drawn considerable interest [2].

PANI and its derivatives possess an array of interesting properties, including good electrical conductivity, biocompatibility, stability, ability to bond covalently with enzymes and ease of processing and functionalization [3,4]. PANI can be obtained using both chemical and electrochemical oxidative synthesis. However, electrochemical synthesis is favorable, permitting the synthesis without oxidizing agent together with doping by organic and inorganic ions in a single step while resulting polymer is obtained directly in its conductive state.

In this study, an electroconducting polymer, polyaniline (PANI), obtained by electrochemical polymerization was selected as the support for immobilization of glucose oxidase (GOx). Since glucose oxidase (GOx) is well-studied, inexpensive, stable and practically applied in clinical and chemical analyses, it was used as a model enzyme. Two different methods of immobilization and two types of electrochemically synthesized polyaniline based supports for attachment of GOx were tested for design a potentiometric glucose biosensor. The results of two different methods employed are compared with respect to enzyme loadings, biosensing efficiency and potential stability.

2. Experimental

2.1. Materials

Glucose oxidase (GOx; β-D-glucose: oxygen 1-oxidoreductase, E.C 1.1.3.4, Type II-S: from *Aspergillus niger*, 21200 U/g solid, 2.36 g solid) and D-glucose were purchased from Sigma-Aldrich Chem. Aniline (p.a. Fluka) was distilled in argon atmosphere prior to use. All other chemicals were analytical grade.

2.2. Electrochemical polymerization of polyaniline based polymers

Before polymerization, graphite electrode was first mechanically polished with fine emery papers (2/0, 3/0 and 4/0 respectively) and than with polishing alumina (1 μm, Banner Scientific Ltd.) on polishing cloths (Buehler Ltd.), the traces of the polishing alumina were ultrasonically (Bandelin Sonorex) removed from the electrode surface during 5 min. Two polyaniline based polymers have been electrochemically synthesized on the electrode surface and used as supports for GOx immobilization: polyaniline (PANI) and self-doped PANI with *m*-aminobenzoate acid (*m*-ABA/PANI)-a carboxy group functionalized polyaniline. Electrochemical polymerization of PANI on graphite electrode ($S = 0.64$ cm^2) was performed at constant current density of 2.0 mA/cm^2 from aqueous solution of 1.0 mol/dm^3 HCl (p.a. Merck) containing 0.25 mol/dm^3 aniline. The electrolyte used for polymerization of *m*-ABA/PANI consisted of 0.5 M HCl with addition of monomer, *m* - aminobenzoic acid.

Electrochemical characterization of the electroactive polymers was carried out on the surface of a graphite disk electrode by cyclic voltammetry. It appears that polymer layers are relatively easy to prepare and result in thick and homogenous coverage of the metal. These electroactive polymers were then successfully applied for GOx immobilization.

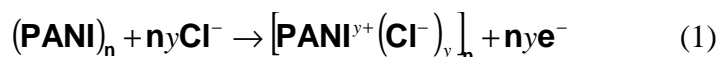### 2.3. Preparation and properties of GOx electrodes

Two different methods presented in Figs. 2 and 5 were studied to evaluate their suitability for immobilization of GOx onto polyaniline based supports: 1) enzyme cross-linking using glutaraldehyde as a bifunctional agent; and 2) covalent immobilization of enzyme by amide bond using carbodiimide as a coupling agent. The amount proteins before and after immobilisation was determined spectrophotometrically $\lambda = 595$ nm (Ultrospec 330 pro, Amersham Biosciences) using Bradford standard procedure [5].

Enzyme electrode was investigated at constant current density of 10 μA cm^{-2}, in solution containing different glucose concentration. Before measurements glucose stock solution (D-(+) glucose monohydrate for biochemistry) was left overnight in order to compete mutarotation. All electrochemical experiments were performed in standard three compartment electrochemical cell at ambient temperature. Saturated calomel (SCE) electrode was used as reference, while Pt wire served as a counter electrode. The measurements were carried out using PAR 273A potentiostat/galvanostat interfaced to PC.

3. Results and discussion

3.1. Electrochemical polymerization of PANI

Electrochemical polymerization of aniline on graphite electrode from 1.0 mol dm^{-3} HCl and 0.25 mol dm^{-3} aniline at constant current density of 2.0 mA cm^{-2} during 1080 is given in Fig. 1. Electrochemical polymerization of aniline occurs together with insertion of chloride ions (doping) according to equation:

$$(\mathbf{PANI})_\mathbf{n} + \mathbf{n}y\mathbf{Cl}^- \rightarrow \left[\mathbf{PANI}^{y+}\left(\mathbf{Cl}^-\right)_y\right]_\mathbf{n} + \mathbf{n}y\mathbf{e}^- \qquad (1)$$

where y is the doping degree, defined as ratio between the number of charges in the polymer and the number of monomer units. Theoretical polymerized mass of the of the PANI on graphite electrode was calculated to be 0.64 mg (1.0 mg cm^{-2}) assuming that the current efficiency of the polymerization process was 100% as previously described [6]. It appears that the polymer layer is relatively easy to prepare and results in thick and homogenous coverage of the metal. The electroactive polymer was then successfully applied for GOx immobilization.
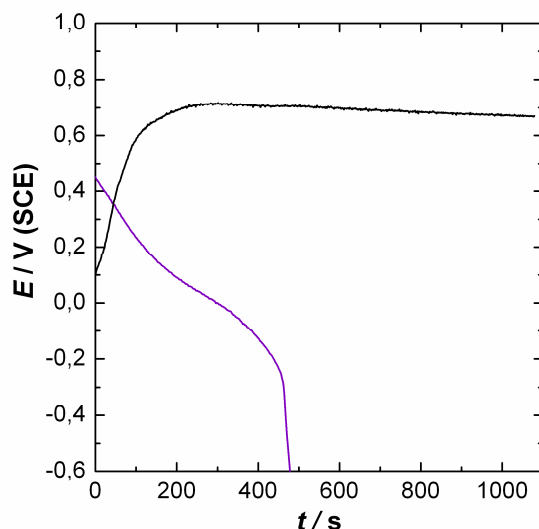

Figure 1. Electrochemical polymerization of aniline at graphite electrode in 1.0 mol dm^{-3} HCl and 0.25 mol dm^{-3} aniline, $j = 2.0$ mA cm^{-2}.

3.2. General method for GOx immobilization on PANI electrode by cross-linking (method I)

PANI enzyme electrode was formed by immobilization of GOx onto electrochemically synthesized PANI on graphite. The conventional method for GOx immobilization on the polymer involves the enzyme cross-linking via glutaraldehyde as a bifunctional agent (Fig. 2).
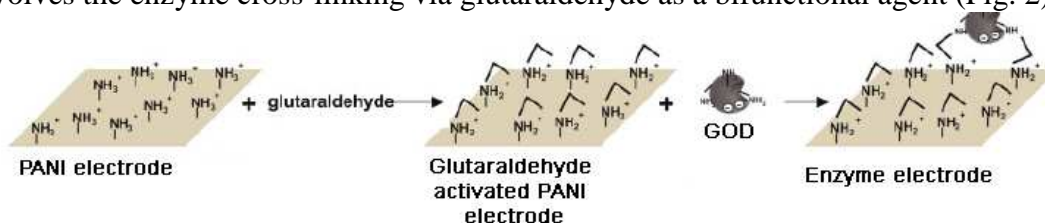

Figure 2. Schematic illustration of the GOx immobilization on PANI electrode by cross-linking via glutaraldehyde as a bifunctional agent (method I).

The amount of the immobilized proteins in the PANI enzyme electrode was estimated spectrophotometrically as a difference of the amount of proteins in solution before and after immobilization. The amount of immobilized proteins was estimated to be 3.1 mg cm^{-2}, representing around 29% of the initial enzyme amount.

The potential dependence was obtained from hronopotentiometric curves, given in the insert of Fig. 3, of PANI enzyme electrode, obtained in different glucose concentration at constant current density of 10 µA cm^{-2} (for simplicity, potentiometric curves are given for only five different glucose concentration, as marked on Fig. 3).
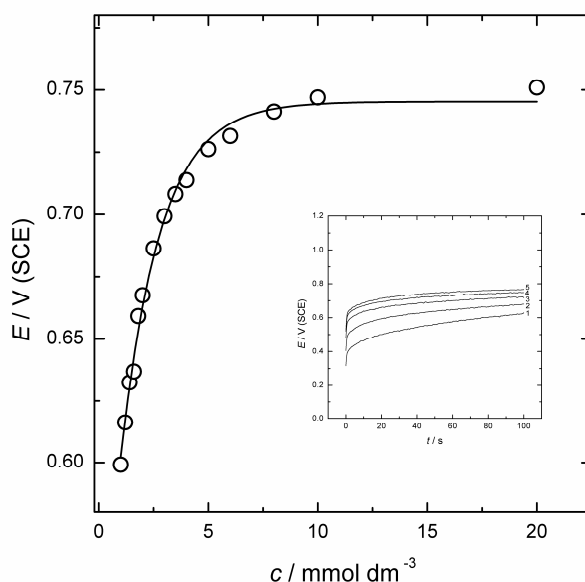
Figure 3. Dependence of the PANI enzyme electrode potential on glucose concentration. Insert: hronopotentiometric curves of the PANI enzyme electrode in: 1–1.0; 2– 2.0; 3–5.0; 4–10 and 5–20 mM glucose solution.

The potential dependence of the PANI enzyme electrode on glucose concentration had the shape of a rectangular hyperbola, typical for the enzyme kinetics. The maximal reaction rate in terms of potential was $v_m = 0.78$ V (determined as reciprocal value of the intercept of the dependence in Fig. 4), while the apparent Michaelis–Menten constant was $K_m` = 30$ mM (determined as ratio between the intercept and the slope). The $K_m`$ constant is drastically lower than previously reported result for free GOx from *A. niger*, implying that enzyme exhibits even higher affinity towards substrate after immobilization [7].
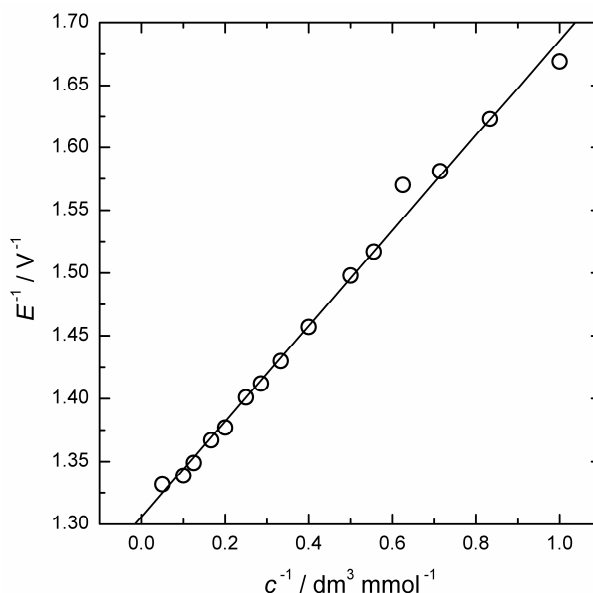

Figure 4. Linearized *Michaelis-Menten* model for PANI electrode in order to determination of apparent Michaelis–Menten parameters.

3.3. Covalent immobilization of GOx on m-ABA/PANI electrode by amide bond using carbodiimide as a coupling agent (method II)

m-ABA/PANI enzyme electrode was formed by covalent immobilization of GOx *via* peptide bond onto electrochemically synthesized *m*-ABA/PANI on graphite (Fig. 5). The amount of

the immobilized proteins on the *m*-ABA/PANI electrode was estimated to be 5.45 mg cm^{-2}, representing around 51% of the initial enzyme amount.
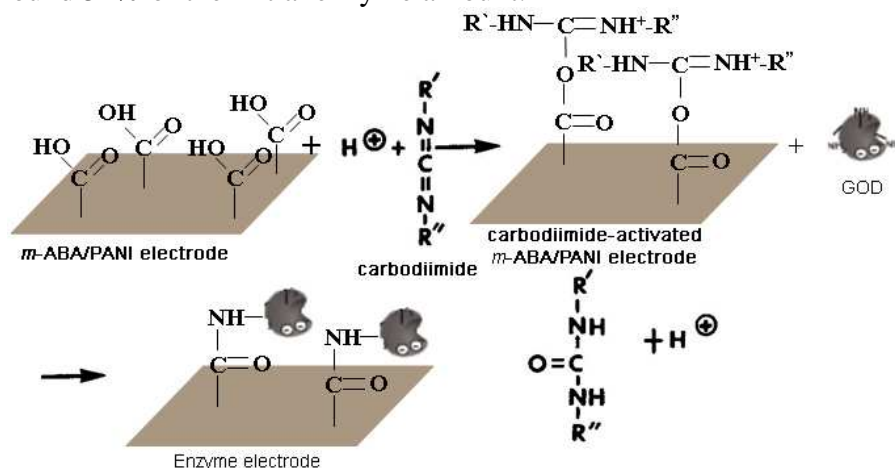
Figure 5. Schematic illustration of the GOx immobilization on *m*-ABA/PANI electrode by covalent coupling. The covalent coupling of enzyme to the surface of polymer seems to ensure immobilization without leaching of the enzyme from the polymer surface.

The potential dependence was obtained from hronopotentiometric curves, given in the insert of Fig. 6, of *m*-ABA/PANI enzyme electrode, obtained in different glucose concentration at constant current density of 10 μA cm^{-2}. Linearization of the *Michaelis-Menten* curve allowed the estimation of apparent kinetics parameters as given in Fig. 7. The maximal reaction rate in terms of potential was $v_m = 0.47$ V, while the apparent Michaelis–Menten constant was $K_m` = 48$ mM.
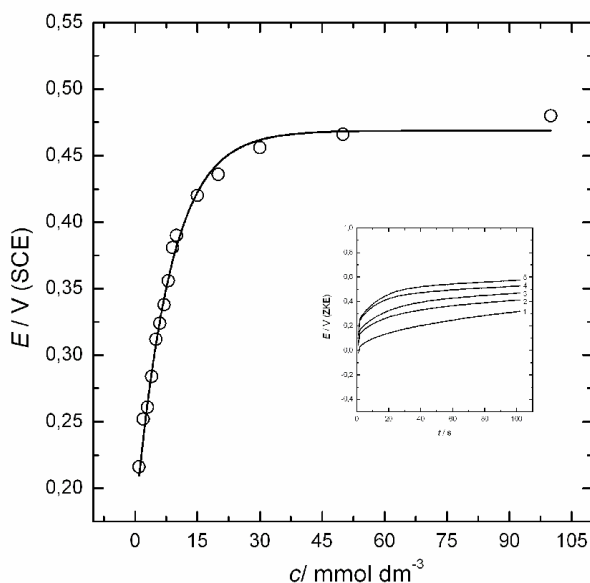
Figure 6. Dependence of the *m*-ABA/PANI enzyme electrode potential on glucose concentration. Insert: Hronopotentiometric curves of the *m*-ABA/PANI enzyme electrode in: 1–1.0; 2– 2.0; 3–5.0; 4–10 and 5–20 mM glucose solution.
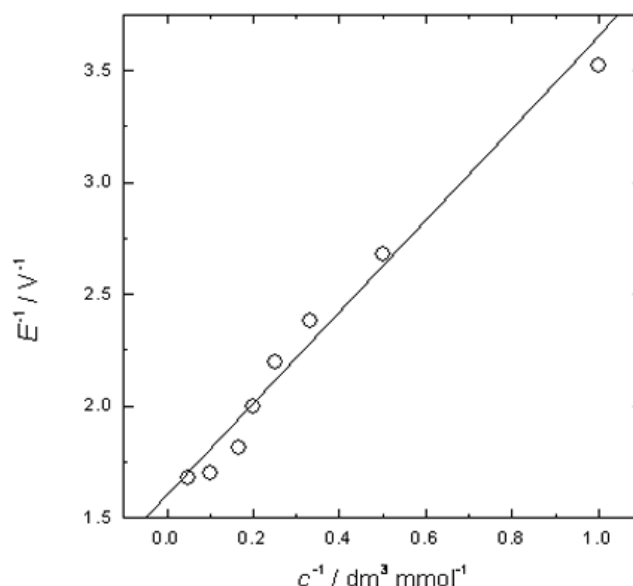
Figure 7. Determination of apparent Michaelis–Menten parameters.

3.4. Comparison of parameters of PANI and m-ABA/PANI electrodes

Since k_{cat} is directly proportional to the maximum voltage response, v_m, attainable for a biosensor, it can be used as an index of sensor sensitivity. The values of the biosensor efficiency factor, $k_{cat}/K_m`$, show that the PANI biosensor exhibits greater efficiency for glucose detection than *m*-ABA/PANI. However, GOx/*m*-ABA/PANI immobilized system is more stable than PANI electrode, as a result of the fact that enzyme is chemically attached, and the polymer itself is more stable at given pH.

Table 1. Comparison of parameters of PANI and *m*-ABA/PANI electrodes

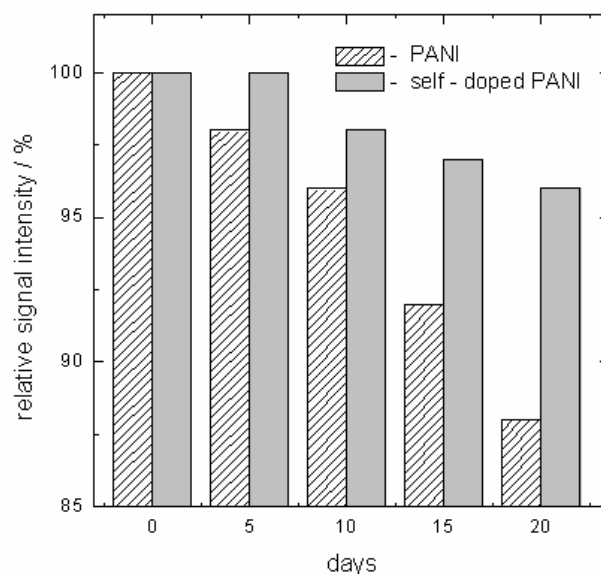| Electrode	P_g, mg/cm^2	K_m, mM	v_m, V	$k_{cat}/K_m`$ /10^5 V/cm^2	$t_{1/2}$, dani
GOx/PANI electrode	3.1	30	0.78	5.03	109.3
GOx/*m*-ABA/PANI electrode	5.4	48	0.47	3.30	277.2

Figure 8. Comparison of the storage stabilities of PANI and *m*-ABA/PANI enzyme electrodes.

Conclusion

The present study compares the results of two different methods employed for preparation of polyaniline based glucose biosensor with respect to enzyme loading, biosensing efficiency and potential stability. Kinetic analysis of the potentiometric data for two enzyme immobilized electrode systems show that the GOx/PANI electrode is suitable for assaying samples with low analyte concentrations, whereas the GOx/m-ABA/PANI electrode system exhibits a better potential stability. It may therefore be possible to achieve high level of biosensing efficiency by chemical modeling and synthesis combined with careful selection of the immobilization method.

Acknowledgement

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