

Polypyrrole-based Enzyme Electrode with Immobilized Glucose Oxidase for Electrochemical Determination of Glucose

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Polypyrrole enzyme electrode was formed by immobilization of glucose oxidase via glutaraldehyde into electrochemically synthesized polypyrrole on glassy carbon electrode. Electrochemical synthesis was performed in 0.5 mol dm⁻³ HCl and 0.2 mol dm⁻³ pyrrole at constant current density of 2 mA cm⁻². Chronopotentiometric curves of polypyrrole enzyme electrode were recorded at current density of 42 nA cm⁻² for different glucose concentrations. The determined value of the apparent Michaelis-Menten constant was 0.045 mmol dm⁻³ which is significantly lower than that of free enzyme indicating enhanced enzyme efficiency when it is immobilized into electroconducting polymer matrix.

Keywords: conducting polymers; electrochemistry; glucose oxidase; material testing; sensors.

1. INTRODUCTION

There is an increasing tendency for use of biosensors in food industry due to their low cost, simplicity and short duration of analysis in comparison to conventional methods. Biosensors found wide application in detection of calcium in milk [1], evaluation of antioxidant capacity of tea [2] and orange juices [3], determination of sulfite in food and beverages [4], for analysis of ethanol, glucose and lactate in wine [5], determination of polyphenols in beers [6]. The determination of glucose is a well-known application of biosensors, and is very popular for research because of the high importance

of glucose in the human metabolic process. Diabetes mellitus is a widespread disease, characterized by blood glucose concentration higher or lower than normal range of 8-12 mg dm⁻³ due to the body's inability to produce or properly respond to insulin, the hormone that signals cells to take up and use glucose [7]. Diabetics represent about 6.4% of the world's population, therefore it is socially important to develop low cost, sensitive, reliable, selective glucose sensors [8].

Biosensor has three major components - bioreceptor (enzyme, antibody, DNA) for the recognition of an analyte, immobilization surface (conducting polymers [9], nanomaterials [10], sol-gel films [11] for the immobilization of biomolecule and transducer unit for conversion of biochemical reaction product into a recognizable signal [7]. Electrochemical biosensor is based on converting the analyte concentration into an electrochemical signal. Depending on what is measured it can be potentiometric, amperometric or conductometric. Because of their simplicity, selectivity and short response times, amperometric glucose biosensors are the most common used for glucose detection [12]. Amperometric biosensors based on conducting polymers provide many advantages and new possibilities to detect biologically active compounds [13, 14].

Hence, to have a stable and sensitive response, the surface of the electrode should be modified [15]. That can be achieved by conductive polymers because of a number of favorable characteristics among which is direct and easy deposition on the sensor electrode by electrochemical oxidation of the monomer [16]. They provide stable and porous matrix for the immobilization of the biocomponent and act as transducers to convert a chemical signal into an electrical one [7,17]. Conducting polymers are also known for their ability to be compatible with biological molecules in neutral aqueous solutions [9]. Biosensors, prepared using a conducting polymer as a support material, have a fast response time with a high storage and operational stability [18]. They can be obtained using both chemical and electrochemical synthesis, but the latter is favorable.

The advantage of electrochemical synthesis is that the doping reaction occurs simultaneously with the growth of the polymer chain, hence the polymer is obtained in its conducting form. This can be achieved by electrochemical techniques such as: potentiostatic, galvanostatic technique, cyclic voltammetry. Galvanostatic method has the advantage, because film thickness can be easily controlled by polymerization time.

Among the conducting polyheterocyclic polymers polypyrrole (PPy) is of particular interest because the relatively low oxidation potential of the monomer enables films to be grown from aqueous solutions that are compatible with most of biological elements [19]. It acts as an immobilization platform and electron mediator in the same time.

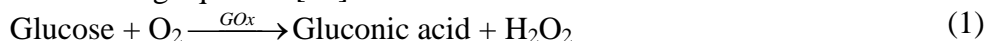
General requirement in electrochemical biosensors is to reproducibly immobilize the biomolecules onto the biosensor while keeping their biological activity. Activity, reusability and sustainability of enzyme can also be enhanced using conducting nanofibers as immobilization matrix. The porosity of conducting polymer is an important factor for the facile immobilization of an enzyme [18,19].

One of the key steps in biosensor construction process is enzyme immobilization. There are many methods for enzyme immobilization: physical entrapment, adsorption [19], covalent binding [20], crosslinking and doping [21]. The weak point of many immobilization methods is enzyme

leakage. However, this problem can be significantly overcome by using the chemical cross-linking method of immobilization via glutaraldehyde [16, 17, 21].

Glucose oxidase (GOx) is the most widely used enzyme in the field of biosensors because of its high specificity for a commercially important analyte, high turnover number and high stability [22]. Therefore, glucose biosensors with immobilized GOx are probably the most investigated biosensors.

The principle of the glucose biosensor is based on the reaction between GOx and glucose shown in the following equation [23]:



The level of hydrogen peroxide, directly proportional to the amount of glucose, can be electrochemically determined at an anode according to equation:



The aim of this paper was to investigate the possibility of glucose determination using enzyme electrode obtained by immobilization of GOx into Ppy electrode. PPy electrochemically polymerized Scanning electron micrographs of PPy on glassy carbon electrode were taken in order to reveal characteristics of its surface. Relevant performances regarding kinetic parameters and storage stability of prepared biosensor were determined.

2. MATERIALS AND EXPERIMENT

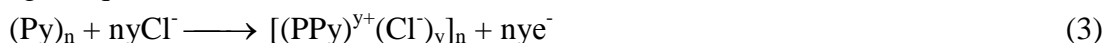
2.1. Materials and physicochemical characterization

Electrochemical measurements were performed in standard three compartment electrochemical cell at ambient temperature (22 °C). Cylindrically shaped glassy carbon electrode was used as working, saturated calomel electrode (SCE) as reference and platinum wire as counter electrode. PAR 273A potentiostat/galvanostat connected to PC was used for electrochemical measurements.

Before polymerization glassy carbon electrode was mechanically polished with fine emery papers (2/0, 3/0 and 4/0 respectively) and then with polishing alumina (1 μm, Banner Scientific Ltd.) on polishing cloths (Buehler Ltd.). The surface of the electrode was cleaned ultrasonically (Bandelin Sonorex) for 5 minutes to remove traces of the polishing alumina.

2.2. Preparation of PPy electrode

Electrochemical polymerization of PPy on glassy carbon electrode was performed at constant current density of 2.0 mA cm⁻² from aqueous solution of 0.5 M HCl (p.a. Merck) containing 0.2 M pyrrole (p.a. Fluka). Doping by chloride ions occurred simultaneously with the polymerization process according to equation:



Where y refers to doping degree, defined as ratio between the number of charges in the polymer and the number of monomer units.

2.3. Immobilization of GOx on Ppy electrode

Immobilization of GOx from *Aspergillus niger* (Sigma–Aldrich) was performed via glutaraldehyde (1.2% (w w⁻¹), Fluka). PPy electrode was first left in glutaraldehyde during 1 h, and then immersed in phosphate buffer solution (pH 5.5; 0.100 M) containing 15 mg cm⁻³ GOx during 24 h.

Determination of enzyme amount before and after immobilization was performed by measurement of protein concentration according to Bradford method [24] with BSA as standard.

Enzyme electrode was investigated at constant current density of 42 nA cm⁻² in solutions containing different glucose concentration. Before that glucose stock solution (D-(+) glucose monohydrate for biochemistry, Merck) was left for 24 h in order to complete mutarotation.

2.4. SEM micrographs of PPy electrode

Scanning electron microscopy (SEM) micrograph of PPy electrode surface was taken using MIRA3 TESCAN by field emission SEM at 10 kV.

3. RESULTS AND DISCUSSION

3.1. Electrochemical polymerization of PPy

Electrochemical polymerization of PPy on glassy carbon electrode from aqueous solution of 0.5 M HCl containing 0.2 M pyrrole at constant current density of 2.0 mA/cm² during 1200 s is given in Figure 1.

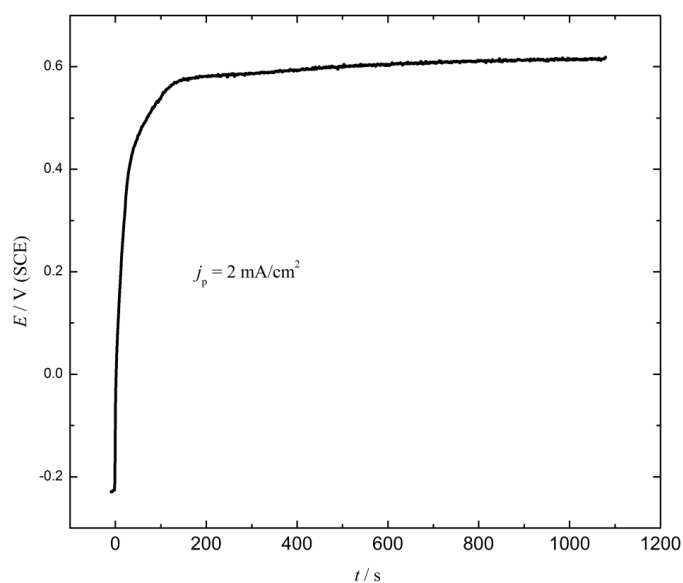


Figure 1. Electrochemical polymerization of PPy from 0.5 mol dm⁻³ HCl and 0.2 mol dm⁻³

As it can be seen from Figure 1, during first 150 s the electrode potential increases rapidly as a consequence of electrode surface being coated by PPy film. This is followed by a period in which potential is practically constant (about 0.6 V (SCE)) corresponding to further polymerization and deposition of the PPy on the initially formed film.

Micrograph of the PPy electrode is shown in Figure 2, it could be seen that electrochemically formed PPy was uniformly deposited onto electrode, with highly developed surface suitable for both heterogeneous electron transport and biomolecule immobilization [25].

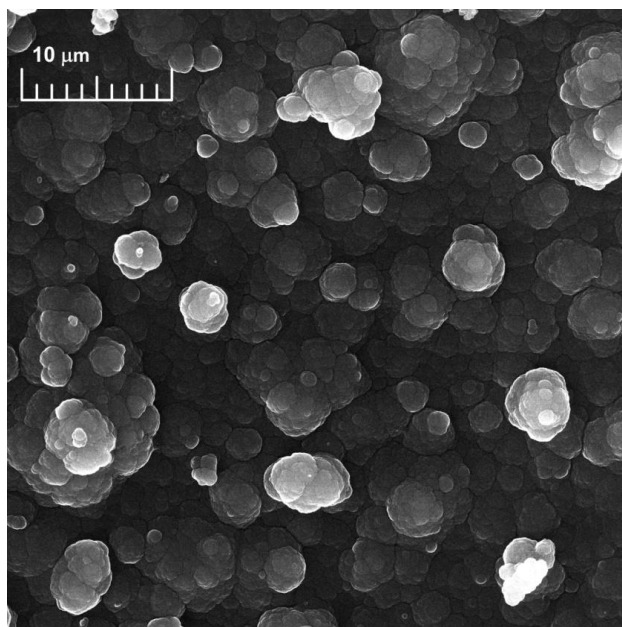


Figure 2. SEM micrograph of electrochemically polymerized PPy on glassy carbon electrode.

3.2. Determination of the immobilized enzyme

The amount of the immobilized enzyme in the PPy electrode was estimated from the difference of the amount of proteins in solution before and after the immobilization. The amount of proteins in the solution before immobilization was 1.73 mg cm^{-3} . It was estimated that after immobilization there was 0.12 mg cm^{-3} of proteins left in the solution. That means that 1.61 mg cm^{-3} of the proteins were immobilized in the PPy electrode, which is 93%. Volume of the immobilization solution was 3.0 cm^3 , so it could be estimated that 5.19 mg (10.38 mg cm^{-2}) was immobilized into the PPy electrode.

In a previous study GOx was immobilized in chitosan-SiO₂ gel by crosslinking via formaldehyde and immobilization yield was 97% [26]. While in polyaniline enzyme electrode, immobilization yield for GOx electrode was around 29% [25].

3.3. Determination of kinetic parameters

PPy enzyme electrode was formed by immobilization of GOx, via glutaraldehyde, on PPy, previously electrochemically synthesized on glassy carbon electrode. Chronopotentiometric curves for

different glucose concentration made at current density of 42 nA cm^{-2} are given in Figure 3. Based on that data extracted from Figure 3, the dependence of electrode potential on glucose concentration was determined and is shown in Figure 4.

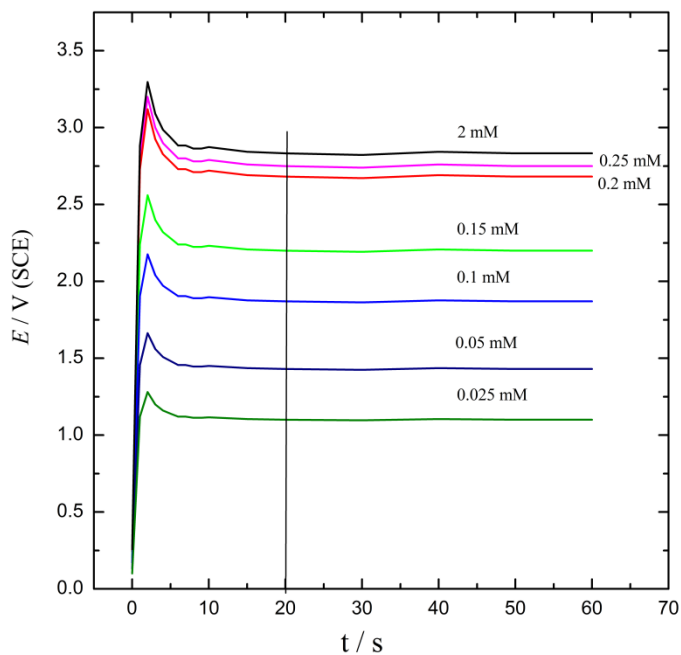


Figure 3. Chronopotentiometric curves of PPy enzyme electrode in glucose solution of 0.025, 0.05, 0.1, 0.15, 0.2, 0.25 and 2 mmol dm^{-3} .

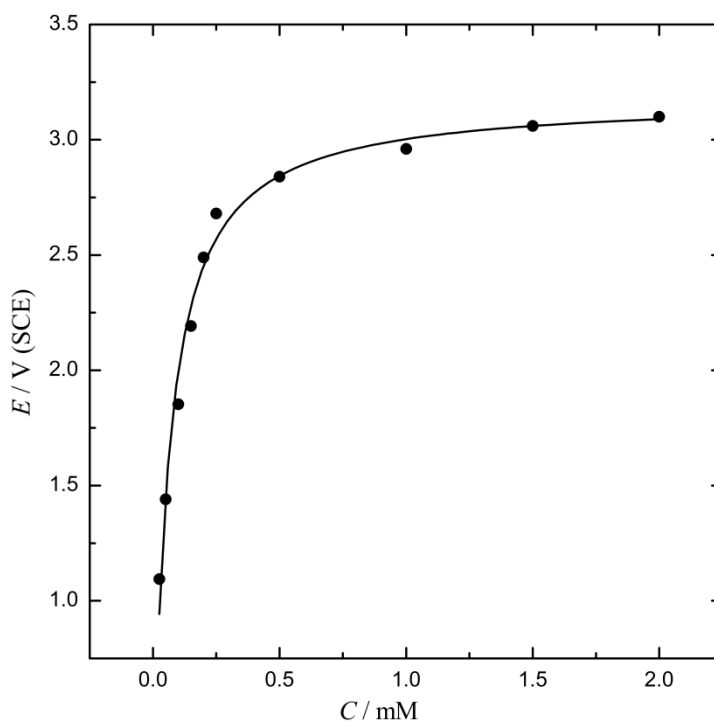


Figure 4. Dependence of PPy enzyme electrode potential on glucose concentration.

As can be seen in Figure 4, the electrode potential increases with the increase in glucose concentration in the entire range of examined glucose concentration, while the linearity region is observed between 0.06 and 0.25 mM. The observed dependence of the electrode potential on glucose concentration is typical for enzyme kinetics. If it is assumed that the enzyme had been uniformly immobilized into PPy film, for lower glucose concentration the reaction would occur on the polymer surface, corresponding to the linearity region in Figure 5. For higher glucose concentrations, delay of the response time is observed, as a result of diffusion limitation. Further increasing in glucose concentration results in constant electrode potential.

Linearization of potential dependence on glucose concentration is given in Figure 5 and it was used for estimation of the apparent Michaelis-Menten kinetics parameters.

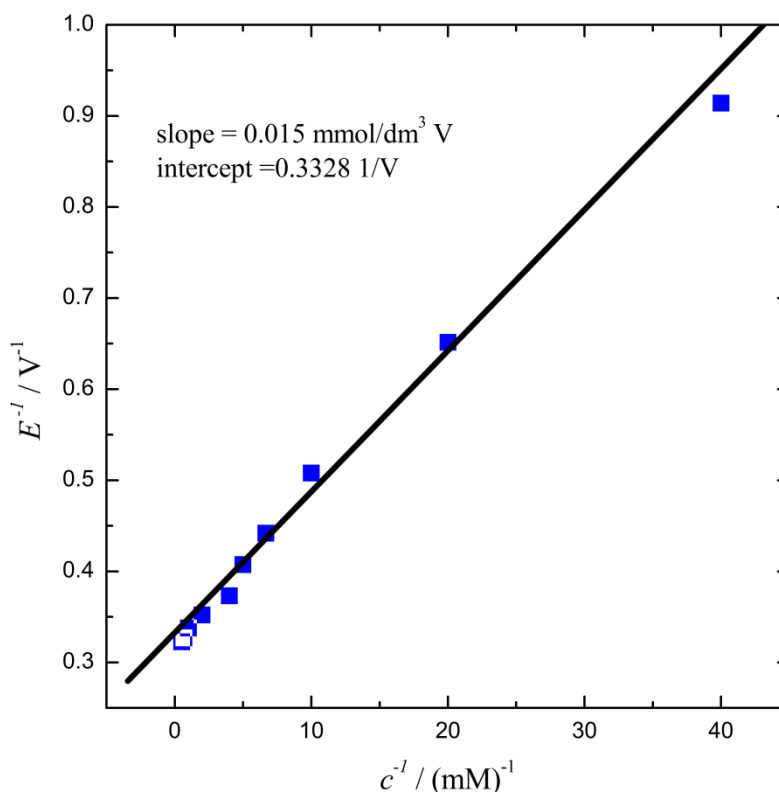


Figure 5. Determination of Michaelis-Menten kinetics parameters.

Maximum reaction rate was determined as a reciprocal value of the intercept of the dependence from Figure 5, and it was 3 V. Apparent Michaelis-Menten constant, K_m , was determined as ratio between the interception and the slope and it was 0.045 mM. That value is significantly lower than results for free GOx from *Aspergillus niger* [27], which means that enzyme shows higher affinity for substrate after immobilization. Also, K_m from this experiment is considerably lower than results from studies such as for gold nanoparticles [28, 29], ZnO nanotubes [30], chitosan-SiO₂ [26], CoFe₂O₄/SiO₂ [31]. More importantly, it was significantly lower than K_m from other studies in which GOx was entrapped in electrochemically synthesized Ppy apparent Michaelis-Menten constant varied in the range from 6.5 to 43.3 mM [22, 32-34].

Furthermore, in the research which used template process for formation of the PANI/GOx biosensor K_m was 11.09 mM, still much higher than the value reported in this paper [23].

This low value for apparent Michaelis-Menten constant is probably the result of excellent three-dimensional structure of PPy that prevents diffusion limitations and allows favorable orientation of bound enzyme and, because of that, high accessibility to substrate.

3.4. Determination of the storage stability

In order to determine storage stability of PPy enzyme electrode, that is essential for the practical analytical application, the electrode was left in phosphate buffer (0.1 M, pH 5.5) at 8 °C. Potential-time curves for glucose concentration of 0.1 mM were recorded after 5, 10, 15 and 20 days. Results are given in Figure 6 in terms of the relative signal intensity.

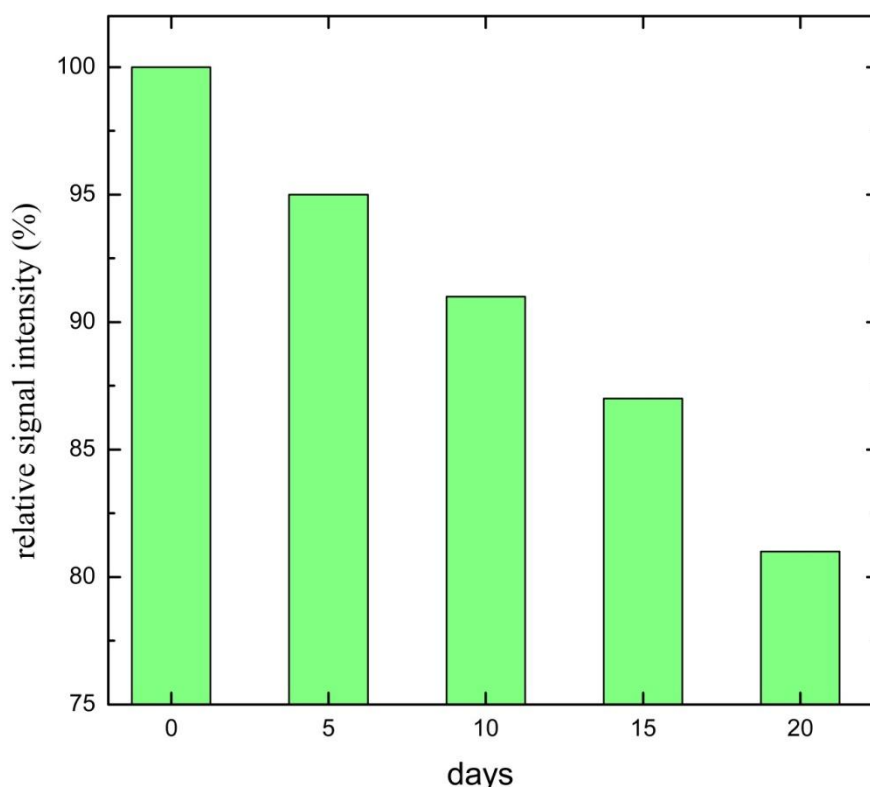


Figure 6. Storage stability of PPy/GOx electrode.

Uang and Chou [35] examined the stability of PPy/GOx biosensor and determined that after 14 days signal decreased by more than 50%. In another paper the stability for two step process and template process were compared for PANI/GOx biosensor. It was determined that after 30 days the signal intensity from the biosensor obtained by the template process dropped only 4.6% while the signal from the electrode synthesized by the two step process decreased by 55.4% after the same period of time [24].

Rauf et al. [36] examined storage stability of GOD immobilized on celluloseacetate-polymethylmethacrylate (CA-PPMMA) membrane. Immobilized enzyme retained 94% of its activity

after one month. Storage stability of immobilized GOD on chitosan-SiO₂ gel was examined in one more study and it was determined that 86 % of signal was retained after 10 and 56 % after 15 days [25]. Wang et al. immobilized GOD using CoFe₂O₄/SiO₂ as carrier and after examination of storage stability it was determined that 87% of the initial activity was maintained after 28 days [31].

After 5 days the PPy enzyme electrode lost 5% of its signal and after 20 days the electrode kept 82% of its initial signal. The loss of the signal intensity could be result of enzyme leaking and PPy degradation during storage, the enzyme leaking can be probably overcome to a certain extent by application of different immobilization techniques that can provide chemical binding of the enzyme and polypyrrole.

4. CONCLUSIONS

PPy enzyme electrode was formed by immobilization of GOx in PPy, electrochemically polymerized on glassy carbon electrode. It was estimated that 93% of proteins were immobilized in PPy enzyme electrode. The apparent Michaelis-Menten constant of 0.045 mM was determined from potential-glucose concentration dependence. The obtained value of 0.045 mM is lower than the value for the free enzyme implying that higher affinity was achieved by immobilization. On the other Apparent Michaelis-Menten constant is also significantly lower than values obtained from similar systems. This improvement can be related to three dimensional PPy structure, which allowed favorable orientation of the bounded enzyme and led to high accessibility to the substrate. Loss of the electrode signal could be related to enzyme leaking and PPy degradation during storage which led to lost in polymer conductivity.

ACKNOWLEDGEMENTS

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