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Enzymatically synthesized polyaniline layer for extension of linear detection region of amperometric glucose biosensor

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ABSTRACT

In this article a new method for fabrication of enzymatic electrodes suitable for design of amperometric glucose biosensor and/or anode of biofuel cell powered by glucose is presented. Glucose oxidase (GOx) E.C. 1.1.3.4. from *Penicillium vitale* was immobilized on the carbon rod electrode by cross-linking it with glutaraldehyde (GOx-electrode). Catalytic activity of immobilized GOx was exploited for polymerisation of aniline by taking a high concentration of hydrogen peroxide produced during the catalytic action of immobilized GOx and locally lowered pH due to the formation of gluconic acid; it created optimal conditions for the polymerisation of aniline. The GOx layer was self-encapsulated within formed polyaniline (PANI) matrix (GOx/PANI-electrode). Properties of the GOx/PANI-electrode have been studied and results were compared with GOx-electrode. The results show that the upper detection limit of glucose using GOx-electrode was dramatically changed by the formation of PANI layer. An increase in the upper detection limit, optimal pH region for operation and stability of GOx based electrode modified by PANI was detected when comparing that of an unmodified GOx-electrode.

1. Introduction

Since the development of the first glucose biosensor (Clark and Lyons, 1962) vast numbers of research studies have been devoted for fabrication of enzyme electrodes reliable for generation of bioelectricity (Ramanavicius and Ramanaviciene, 2009) and/or measurement of glucose concentrations (Kakehi et al., 2007). Recently biosensors played an important role in the improvement of public health, food and beverage industry, environmental monitoring. This is all due to its rapid detection, high sensitivity, small size, and specificity. Among other biosensors, amperometric biosensors are prevalent because of their high selectivity and simple fabrication methods (Ramanavicius et al., 2004). Many advantages and new possibilities to detect biologically active compounds provide amperometric biosensors based on conducting polymers (Ramanavicius et al., 2005; Rahman et al., 2008; Ekanayake et al., 2008), because some of these polymers offer good biocompatibility *in vivo* (Ramanaviciene et al., 2007) and can be served as versatile immobilization matrix (Ramanaviciene and Ramanavicius, 2004). Proper immobilization of enzymes is very important procedure in biosensor design. Researchers constantly try to improve the sensitivity, stability, and the range of determinable concentrations and reproducibility of glucose biosensors by different techniques of immobilization of an enzyme (glucose oxidase (GOx) or glucose hydrogenase) on suitable electrodes with supportive materials (Kaimori et al., 2006). The immobilization of enzymes can be carried out using many different procedures such as physical adsorption, entrapment, chemical cross-linking and other methods, while retaining the biological recognition properties of enzymes. Characteristics of immobilized enzyme based electrodes depend on factors such as the immobilization method, thickness and stability of the layer or membrane formed during immobilization (Malhotra et al., 2005). Successfully immobilized enzymes have many operational advantages over free enzymes such as continuous operational mode, possible modulation of the catalytic properties and easier prevention of microbial growth, reduced cost of operation, reusability, enhanced stability and extended range of determinable concentrations.

Conducting polymers (CPs) are especially suitable for immobilization of various enzymes (Ramanavicius et al., 2006; Davis and Higson, 2007). The most widely used CPs for enzyme immobilization is polyaniline (PANI), polypyrrole and polythiophene. In comparison to other enzyme immobilization matrices, CPs have attracted attention due to their ability to bind oppositely charged complex entities in their oxidized conducting state and to release them in their neutral insulating state (Gaikwad et al., 2006). They provide stable and porous matrix for the immobilization and also

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facilitate the electron transfer process (Gaikwad et al., 2007). Out of many other conducting polymers, PANI is the well-known synthetic organic polymer firstly reported by Letheby (1862). PANI becomes very attractive due to its high conductivity (Kang et al., 2004), good environmental (MacDiarmid et al., 2001), thermal (Wang et al., 1995), electrochemical stability (Chiang and MacDiarmid, 1986), interesting electrochemical, electronic, optical and electro-optical properties (Heeger, 2001), and strong biomolecular interactions. Furthermore, due to its excellent conductivity and electroactivity, PANI can act as a redox mediator for enzyme-modified electrodes. It can accept electrons directly from the enzyme active site and transfer them to the electrode (Luo and Do, 2004; Shi et al., 2004; Luo et al., 2006; Michira et al., 2007). PANI is compatible to most enzymes and can be easily synthesized from aniline monomer in an aqueous solution. Because of the mentioned advantages PANI has found application as immobilization matrix in the design of conductometric (Ajay and Srivastava, 2007), potentiometric (Qaisar and Adeloju, 2009) and amperometric (Xu et al., 2009) biosensors. In the designing of amperometric biosensors PANI has been used as a matrix for covalent enzyme immobilization (Singh et al., 2006). Amperometric enzyme biosensors based on PANI nanoparticles have also been reported (Morrin et al., 2005). However, one of the simplest and the most widely used methods for immobilization of enzymes at the electrode surface is entrapment into PANI or other CPs films. Such entrapment provides a possibility to improve selectivity by preventing the active parts of the biosensors from disruptive materials and biocompatibility of biosensors prevents the enzyme from being leached out. At the same time it maintains the accessibility of the catalytic sites due to the permeability of the film to analytes (Ho et al., 2000). Moreover, after enzyme immobilization within conducting polymers due to increasing diffusion limitations such parameter as apparent Michaelis constant ($K_{M(apparent)}$) can be changed significantly thus causing an increase in the upper detection limit of the created biosensors and an extension of determinable concentration range (Persson et al., 1993; Baronas et al., 2003; Ramanavicius et al., 2008) or even reduction of influence of enzyme inactivation (Baronas et al., 2010). Similar diffusion-based effects of conducting (Gorton, 1995) and non-conducting (Somasundrum and Aoki, 2002) matrixes used in amperometric biosensors were reported.

Traditionally, PANI is synthesized *via* electrochemical and chemical methods (Huang et al., 1986). The electrochemical polymerisation usually yields a thin polymeric film at the electrode surface; while by chemical polymerisation the polymer is obtained as an amorphous suspension precipitated in bulk of the solution. During the electrochemical PANI polymerisation process negatively charged enzyme molecules could be easily entrapped into the positively charged backbone of the polymer (Skinner and Hall, 1997; Trojanowicz et al., 1995). The advantages of this method are: one-step, direct, easy and controlled localization of biologically active molecules in a defined area on the electrode surface, control of the film thickness and redox conductivity (Ahuja et al., 2007; Cosnier, 2003). However, the electrochemical PANI polymerisation has drawbacks as a necessity to use low-pH acidic aqueous solutions, high concentration of monomer and biomolecules (Cosnier, 2003). The PANI polymerisation drawbacks are: (i) extremely low-pH of polymerisation solution, (ii) application of toxic catalysts and/or oxidants, (iii) and formation of undesirable by-products. These limitations significantly reduce the applicability of PANI based systems in the design of amperometric biosensor.

An alternative approach for enzyme entrapment into electrochemical synthesised PANI films might be enzymatic PANI polymerisation (Cruz-Silva et al., 2004). This simple, one-step and environmentally friendly process does not require strong acidic media, it is free of oxidation by-products and uses catalysts derived from renewable resources (Xu et al., 2006). Studies of the enzymatic polymerisation of aniline using horseradish peroxidase (Nabid and Entezami, 2005), laccase (Karamyshev et al., 2003) or palm tree peroxidase (Sakharov et al., 2004) have been reported. Our previous studies demonstrated that glucose oxidase (GOx) dissolved in polymerisation bulk might be encapsulated within PANI by formation of colloid-particles (GOx/PANI-nanoparticles), which exhibits significantly different catalysed reaction kinetics if compared with the native enzyme (Kausaite et al., 2009). Therefore GOx/PANI-nanoparticles can be applied in the design of amperometric biosensors that allows the detection of glucose in a wider concentration interval when compared with biosensors based on native GOx immobilized in the similar way (Ramanavicius et al., 2005, 2008). These studies encouraged us to apply this GOx self-encapsulation into the PANI matrix method for increasing the glucose detection limits.

Thus the main aim of this study was to modify cross-linked GOx based graphite electrode by self-encapsulation of GOx within the formed PANI layer in order to show a possibility to increase the upper detection limit. Along with this an optimal pH region for operation and stability of created biosensor were investigated.

2. Experimental

2.1. Chemicals

GOx (E.C. 1.1.3.4.) from *Penicillium vitale* (130 units/mg) was received from BIOTUL (Kiev, Ukraine). Aniline monomer, glucose, phenasine methoslulphate (PMS) and other chemicals of analytical-reagent grade or better were purchased from SIGMA–ALDRICH CHEMIE GmbH (Steinheim, Germany). All aqueous solutions were prepared in triply distilled water. The solutions of glucose were prepared at least 24 h before use to allow glucose to mutarotate and to reach equilibrium between α- and β-forms. When needed the GOx solution was freshly prepared from powder of the enzyme. Aniline was purified by passing 1.5 mL aliquots through a neutral Al_2O_3 column (5 cm length and 0.4 cm diameter) to remove all coloured components. Sodium/potassium phosphate buffers of different pH were prepared by mixing sodium hydrophosphate with potassium dihydrophosphate.

2.2. Electrode pre-treatment prior to modification by GOx and PANI

Graphite rod electrodes 3 mm in diameter, 150 mm in length, 99.999% pure and low density were obtained from SIGMA–ALDRICH, Inc. (St. Louis, MO, USA). Graphite rod electrodes were sealed into epoxy to prevent the contact of electrode side surface with the solution. Working surface area of graphite electrodes was 0.7 mm^2. Before the formation graphite electrodes were prepared as follows: first, rods of graphite were cut and polished on a fine emery paper and then polished by Al_2O_3 slurry (grain size 0.1 μm), followed by rinsing the electrode surface with ethanol and distilled water. Electrodes were dried at room temperature before coating them with enzymes.

2.3. Electrode modification by GOx

During preparation of GOx coated electrodes (GOx-electrodes), 3 μl of 40 mg/ml of enzyme solution were deposited on the electrode and later water was evaporated at room temperature by intensive ventilation. Then electrodes were stored for 24 h over the 5% solution of glutaraldehyde at 4 °C in the closed vessel as it was described previously (Ramanavicius et al., 2008). Prior to electrochemical measurements, the electrodes where thoroughly washed with distilled water to remove non-cross-linked enzyme and stored

at 4 °C in the closed vessel hanging over a drop of 0.05 M sodium acetate buffer solution, pH 6.0.

2.4. Modification of GOx coated electrode by PANI layer

For preparation of GOx coated electrodes modified by PANI layer (GOx/PANI-electrodes), electrodes coated with GOx were immersed into 0.05 M sodium acetate buffer solutions, pH 6.0 contained 20 mM of glucose and 200 mM of aniline at 4 °C or 18 °C temperature, for a definite period. Prior to electrochemical measurements the GOx/PANI-electrodes were thoroughly washed with distilled water. GOx/PANI-electrodes for the lifetime and stability investigations after a period of polymerisation were stored at 4 °C in the closed vessel above a drop of 0.05 M sodium acetate buffer solution, pH 6.0.

2.5. Preparation of control electrodes

For this reason two types of "inadequate" 0.05 M sodium acetate buffer solutions, pH 6.0 not containing one of the chemicals crucial for polymerisation (glucose or aniline) were used to prepare two control electrodes and to investigate the influence of these chemicals on kinetic parameters ($K_{M(apparent)}$ and I_{max}) of these electrodes. For preparation of first control electrode (*control electrode 1*) GOx-electrode was immersed into 0.05 M sodium acetate buffer solution, pH 6.0 containing only 20 mM of glucose (*control solution I*) for a definite period. While for preparation of second control electrode (*control electrode 2*) 0.05 M sodium acetate buffer solution, pH 6.0 containing only 200 mM of aniline (*control solution II*) was used. The first "inadequate" control solution (*control solution I*) does not contain glucose and the second was free of aniline. Prior to electrochemical measurements the electrodes were thoroughly washed with distilled water

2.6. Electrochemical detection of analytical signal

All electrochemical measurements were performed by potentiostat-galvanostat PGSTAT 30 with GPES3 v3,2 software ECO-Chemie/Autolab (Utrech, Netherlands). For the registration of amperometric signal three-electrode circuits were applied. The GOx or GOx/PANI modified graphite electrode was switched as the working one, Ag/AgCl electrode as reference and 2 cm^2 Pt electrode was used as an auxiliary one. Electrochemical signals were detected in 0.05 M sodium acetate and sodium/potassium phosphate buffer solution, pH 6.0 containing 0.1 M KCl. Solution in electrochemical cell was mixed at 120 rpm. Electrochemical detection of analytical signal was performed at room temperature at +300 mV *vs.* Ag/AgCl in presence of 10 mM of PMS and different concentrations of glucose.

2.7. Special conditions used for determination of pH dependence

GOx and GOx/PANI modified (duration of polymerisation 24 h) electrodes were tested in 0.05 M sodium acetate and sodium/potassium phosphate buffer solution contained 0.1 M of KCl, with fixed pH value. Electrochemical detection of analytical signal was performed similarly as it is presented in previous section.

2.8. Special conditions in lifetime and stability test

GOx and GOx/PANI modified (duration of polymerisation 24, 48 and 72 h) electrodes were stored between the measurements at 4 °C in closed vessel hanging over 0.05 M sodium acetate buffer solutions, pH 6.0. Electrochemical detection of analytical signal was performed similarly as it is presented in previous section.

2.9. The imaging by scanning electron microscopy

The GOx and GOx/PANI modified graphite electrodes were examined using JEOL JSM-7600F scanning electron microscope. Prior to investigations the modified electrodes were thoroughly washed with distilled water and then dried at room temperature.

2.10. The imaging by atomic force microscopy

Tapping mode atomic force microscopy was used for the imaging of differently modified graphite electrode surfaces. The BioScope II, Veeco Instruments Ltd. (Santa Barbara, USA) was used for all AFM experiments. For all AFM Images 200 Images 200 × 200 pixels image resolution was applied, scanning rate 10 μm/s. Experimental data were processed by diNanoScope 7.30 and Gwyddion 2.10 NT-MDT Nova programs. Sharp silicon probes ideal for TappingMode were used for all measurements.

3. Results and discussion

Glucose oxidase (GOx) is a FAD-dependent enzyme that catalyzes oxidation of β-D-glucose by molecular oxygen to hydrogen peroxide and D-glucono-1,5-lactone (1) which subsequently hydrolyzes spontaneously to β-D-gluconic acid (2). The formation of PANI in aqueous media induced by GOx dissolved in polymerisation bulk solution consisting of glucose and aniline monomer was described in detail in our previous paper (Kausaite et al., 2009). In the recent study catalytic activity of GOx immobilized on graphite electrode by cross-linking with glutaraldehyde (GOx-electrode) was exploited for polymerisation of aniline. Referring to our investigations the main precursors for initiation of polymerisation reaction was hydrogen peroxide as an initiator of polymerisation reaction (3) and gluconic acid as a medium what reduced pH towards the acidic pH suitable for template polymerisation of aniline. All mentioned precursors were produced during the catalytic action of immobilized GOx (1) and following hydrolization of reaction products (2). Since aniline has a p*K*a of 4.63 (Lide, 1993), whereas GOx has a p*I* of 4.2 (Ramanaviciene et al., 2009) at a pH lower than 4.63 aniline is positively charged and GOx at a pH higher than 4.2 is negatively charged. In the presence of glucose and dissolved oxygen immobilized on graphite electrodes GOx generated hydrogen peroxide and gluconolactone, which hydrolyzes to gluconic acid (Fig. 1).

In consequence of gluconolactone hydrolysis it might be predicted that the pH decreased locally because of the formation of gluconic acid, while hydrogen peroxide concentration increased close to the immobilized GOx active site and the interfaces between electrode/enzyme/solution. It is presumptive that locally lowered pH (4.3–4.5) and high concentration of oxidator (H2O2) created optimal conditions for the polymerisation of aniline and increased the probability of GOx encapsulation within formed PANI to form GOx/PANI-electrode. Thus, our proposed GOx-encapsulation method was at least partially based on electrostatic alignment of aniline monomer onto anionic immobilized GOx template to minimize branching and promote linear PANI chains growth (Kumar et al., 2003).

$$\beta\text{-D-glucose} + O_2 \xrightarrow{GOx} \text{D-glucono-1,5-lactone} + H_2O_2 \quad (1)$$

$$\text{D-glucono-1,5-lactone} + H_2O \rightarrow \beta\text{-D-gluconic acid} \quad (2)$$

$$\text{Aniline} \xrightarrow{H_2O_2} \text{Polyaniline} \quad (3)$$

For confirmation of insight that under such favourable conditions the polymerisation of aniline might be initiated (3) a basic amperometric biosensor design was selected. In our opinion it was the most appropriate way for this kind of estimation: (i) it needs just

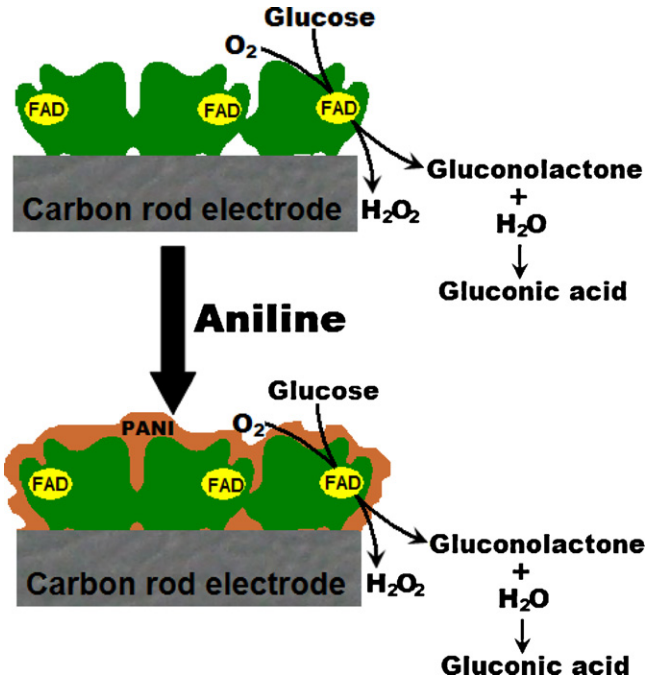

Fig. 1. Principle scheme illustrating modification of GOx coated electrode by PANI.

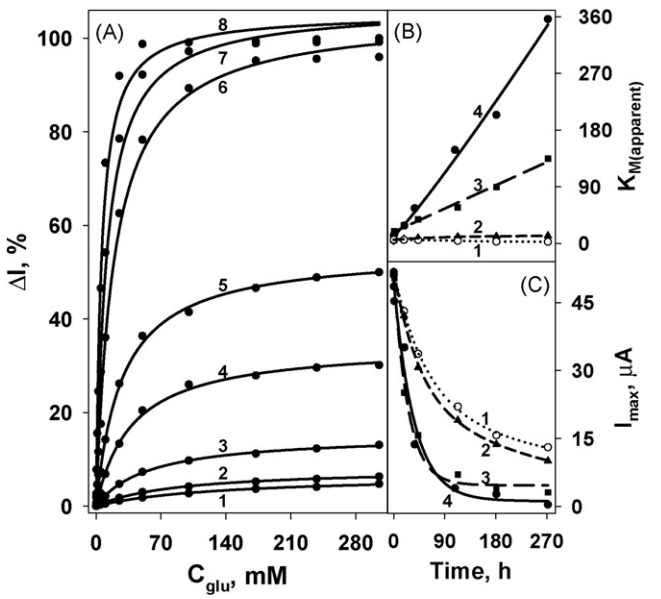

Fig. 2. **(A)** Glucose calibration curves obtained with a GOx modified graphite electrode, before treatment (curve 8) and after treatment with 200 mM aniline and 20 mM glucose solution at 4 °C temperature (curve 7–10 min, 6–70 min, 5–18 h, 4–43 h, 3–113 h, 2–181 h and 1–272 h). Calculated $K_{M(apparent)}$ **(B)** and I_{max} **(C)** values for glucose *vs.* duration of incubation in control solution *1* at 4 °C (curve 1), control solution *2* at 4 °C (curve 2) and polymerisation solution at 4 °C (curve 3) and at 18 °C (curve 4). Experiments presented in plot '**A**' were performed at +300 mV *vs.* Ag/AgCl in 50 mM sodium acetate and sodium potassium phosphate buffer solution, pH 6.0, contained 100 mM of KCl and 10 mM of PMS.

a very small amount of materials; (ii) the same electrode might be applied for several measurements; (iii) modification of electrodes might be performed at similar conditions; (iv) and free diffusing and/or not encapsulated enzyme might be washed out from the sensor surface. The cross-linking GOx by glutaraldehyde was applied since it can stabilize the steric structure of enzyme and to avoid the denaturation of it under relatively severe conditions and it is a typical procedure for immobilization of oxidases used in biosensorics (Ramanavicius, 2007).

The peculiarity of the present work is that formation of PANI layer on GOx modified electrodes occurs only in the case if the GOx modified electrode is immersed in polymerisation solution containing both glucose and aniline. The main evidence of polymerisation process on GOx modified electrodes was an increase of apparent Michaelis–Menten constant ($K_{M(apparent)}$) and decrease in maximum current measured under saturated analyte (glucose) conditions (I_{max}). Kinetic properties of GOx acting as a biocatalyst in the GOx-electrode and GOx/PANI-electrode were analyzed at room temperature using different concentrations of glucose (0.05–305 mM). The results obtained are shown in Fig. 2 and Table 1. The amperometric signals showed hyperbolic dependence on glucose concentration (Fig. 2A), this was in agreement with Michaelis–Menten kinetics. The kinetic parameters I_{max} (correspond to V_{max}) and $K_{M(apparent)}$ are correspondingly *a* and *b* parameters of hyperbolic function $y = ax/(b+x)$. According to calculations presented in Fig. 2B and Table 1 GOx and GOx/PANI modified electrodes exhibit significantly different $K_{M(apparent)}$ values. As seen from the Table 1 for electrode based on cross-linked GOx calculated values of $K_{M(apparent)}$ and I_{max} were around 5.75 mM and 51.72 μA, respectively. While the $K_{M(apparent)}$ for the GOx/PANI modified electrode might be extended up to 134.90 mM, if polymerisation was carried out at 4 °C temperature (Fig. 2B curve 3) and up to 356.00 mM, if polymerisation is carried out at 18 °C temperature (Fig. 2B curve 4). An opposite effect was obtained for the I_{max} values. The results presented in Fig. 2C demonstrate than the I_{max} can decrease from 51.72 μA (GOx-electrode) up to 3.06 μA or 0.42 μA, if polymerisation is carried out at 4 °C or 18 °C temperature, respectively. The comparison of kinetic parameters of cross-linked GOx and GOx/PANI based electrodes let us to state that in the case of GOx/PANI-electrode I_{max} decreased by 16.69 or 121.59 times and at the same time $K_{M(apparent)}$ increased by 23.46 or 61.91 times (after 270 h of polymerisation). Such significant increase of $K_{M(apparent)}$ and decrease of I_{max} are demonstrating that diffusion limitations in this case of GOx/PANI-electrode are playing a significant role. An increment of $K_{M(apparent)}$ by over 10 times (Ramanavicius et al., 2005; Ramanavicius et al., 2008) might be exploited as the evidence that the immobilized on graphite

Table 1
$K_{M(app.)}$ and I_{max} calculated for GOx/PANI modified electrode, Control electrode 1 and Control electrode 2.

Time(h)	GOx/PANI-electrode		Control electrode 1		Control electrode 2	
	$K_{M(app.)}$(mM)	I_{max}(μA)	$K_{M(app.)}$(mM)	I_{max}(μA)	$K_{M(app.)}$(mM)	I_{max}(μA)
0	5.75 ± 0.13	51.72 ± 1.81	5.80 ± 0.14	51.81 ± 1.35	5.78 ± 0.21	51.68 ± 1.04
0.17	11.28 ± 0.67	51.53 ± 1.23	–	–	–	–
1.17	19.38 ± 0.98	50.32 ± 2.49	–	–	–	–
18	28.3 ± 1.84	25.11 ± 0.97	5.63 ± 0.17	43.11 ± 1.12	7.09 ± 0.23	42.11 ± 0.99
43	38.62 ± 1.36	15.69 ± 0.63	5.47 ± 0.19	33.69 ± 0.93	8.56 ± 0.39	30.69 ± 0.83
113	57.05 ± 2.44	7.03 ± 0.11	4.19 ± 0.14	22.03 ± 0.29	10.39 ± 0.54	19.03 ± 1.12
181	89.34 ± 3.07	3.72 ± 0.04	2.94 ± 0.09	15.72 ± 0.33	11.26 ± 0.49	13.72 ± 0.64
272	134.90 ± 8.84	3.06 ± 0.01	2.64 ± 0.07	13.06 ± 0.61	12.09 ± 0.53	10.06 ± 0.43

electrode surface GOx was entrapped within formed PANI layer. Moreover, due to diffusion limitations increased $K_{M(apparent)}$ caused significant increases in linear range of GOx/PANI based analytical system if it is compared with cross-linked GOx based system. Such significant extensions of analyte detection intervals are especially relevant for detection of glucose concentration in food and beverage samples. On the other hand, increased diffusion limitations caused the decrease in maximal current generated by GOx/PANI based electrode because of hindered diffusion of both: glucose and redox mediator phenasine methoslulphate (PMS). Detected thickness of the PANI layer was 0,4 μm, 0.8 μm and 1.1 μm after 18 h, 113 h and 181 h of polymerisation period correspondingly. No linear dependence between polymerisation period and/or thickness of film and/or $K_{M(apparent)}$ as well as with I_{max} was determined. It means that morphology of PANI film has changed and/or internal density of formed polymer layer has increased during here reported formation of PANI film. The results are in principle agreement with theoretical studies, which have predicted that after embedment of enzyme into conducting polymer film, the thickness of enzymatically active layer is typically 200–400 nm due to limitations in the diffusion of the analyte and reaction products in/out from the film (Oh et al., 2002).

The data presented in Fig. 2A shows that the polymerisation reaction takes over 270 h. In this reaction using glucose and aniline as well as formed hydrogen peroxide and gluconic acid theoretically could have significant influence on the kinetic parameters of GOx coated electrode. These are the several reasons: (i) possible reaction initial materials (glucose and aniline) as well as formed products (hydrogen peroxide and gluconic acid) with protein shell and active centre of enzyme; (ii) fouling and electrochemical inactivation of electrode surface; and (iii) detachment of immobilized enzyme from the surface of electrode. To study the influence of such effects on kinetic parameters control electrodes based on immobilized GOx were requested. For this reason two theoretically possible types of "incomplete" solutions were prepared: 1st solution (*control solution I*)—without aniline, which is essential for polymerisation reaction; 2nd solution (*control solution II*)—without glucose, which is crucial for formation of hydrogen peroxide initiator of aniline polymerisation. To obtain control electrodes 1 and 2 GOx-electrodes for a definite period were immersed into *control solution I* and *control solution II* respectively. The presence of aniline in the absence of glucose in incubation solution (*control solution II*) resulted in an increase of $K_{M(apparent)}$ for control electrode 2, but in this case the increase of $K_{M(apparent)}$ was over 11 times (Fig. 2B curve 2, Table 1) lower at 4 °C (Fig. 2B curve 3 and Table 1) and almost 30 times lower at 18 °C temperature (Fig. 2B curve 4) when compared with that obtained using both components (glucose and aniline). The decrease of I_{max} for the control electrode 2 was 3.29 times smaller (Fig. 2C curve 2 and Table 1) if compared with that obtained for GOx/PANI-electrode when polymerisation was carried out at 4 °C (Fig. 2C curve 3, Table 1). If glucose was present and aniline was absent in incubation solution (*control solution I*) the $K_{M(apparent)}$ calculated for control electrode 1 after 272 h of incubation period was smaller if compared with $K_{M(apparent)}$ calculated for control electrode 2 and GOx/PANI-electrode 5 and 51 times, respectively (Table 1). Additionally in contrast to the results represented above for control electrode 2 and GOx/PANI-electrode during incubation the $K_{M(apparent)}$ calculated for control electrode 1 has decreased three-times. It can be explained by slow degradation of GOx layer, which became thinner and diffusion through it become faster. Similar results were achieved when bare buffer solution without any aniline or glucose was used (data not shown). After 272 h of incubation the calculated $K_{M(apparent)}$ value was 2.64 mM (Fig. 2C curve 1, Table 1) which is close to K_M of native GOx. It means that the GOx layer became so thin that sensor started to act in kinetically controlled mode instead of previously detected diffusion controlled mode.

The SEM images of GOx (A), GOx/PANI (B) modified graphite electrodes are shown in Fig. 3. Fig. 3A demonstrates that before formation of PANI the surface of the electrode was doughy due to non-conducting GOx layer, which scattered the electrons. Fig. 3B shows that the morphology and conductivity of electrode surface has been changed significantly after the formation of PANI layer. Different electron scattering in Fig. 3A and B shows that surface of PANI/GOx-electrode is more conducting if compared with the surface of GOx-electrode. The morphology PANI/GOx-electrode clearly shows that some new polymeric structures appeared on the electrode surface.

AFM images (Fig. 4A1–C1) and from these images derived height distribution diagrams (Fig. 4A2–C2) clearly demonstrates changes in morphology during immobilization of GOx (Fig. 4A and B) as well as during formation of the PANI layer (Fig. 4B and C). Presented AFM figures as well as height histograms (Fig. 4C) demonstrates increase of surface roughness what is in agreement with previously presented SEM image (Fig. 3B) and can be attributed to the formation of PANI layer, which is not smooth if compared with immobilized GOx layer (Figs. 3A and 4B).

The value of pH of the polymerisation reaction medium allows an efficient entrapment of the enzyme. It also prevents the loss of the enzyme activity under polymerisation conditions (Fabiano et al., 2000). Therefore, biosensor response depends on the working pH of the sample solution. The effect of pH on the catalytic activity of

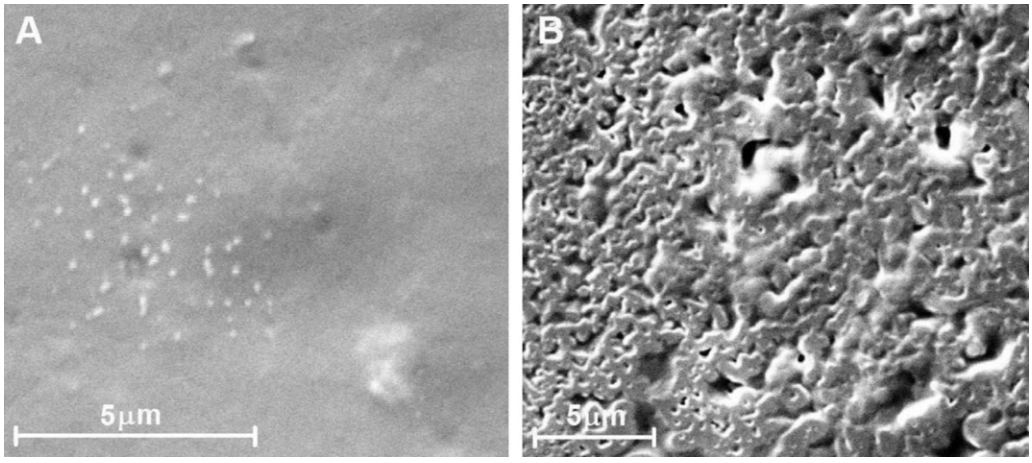

Fig. 3. SEM images of GOx **(A)** and GOx/PANI **(B)** modified graphite electrode.

enzyme in GOx and GOx/PANI modified electrodes was investigated in 0.05 M sodium acetate and sodium/potassium phosphate buffer solutions contained 0.1 M of KCl over the range of pH 3.0–10.0 in the presence of 20 mM glucose (Fig. 5). The GOx modified electrode exhibited pH optimum at pH = 6.0 (Fig. 5 dash and line), which is in agreement with the pH value reported for the native GOx (Kozhukharova et al., 1988; Kulys et al., 1978). As it can be seen from the figure below pH 4 and above pH 8, the catalytic activity of GOx is rapidly lost. For example, at pH 10 only about 10% of the initial GOx activity remains. At a low-pH, GOx is inhibited by anions (Rogers

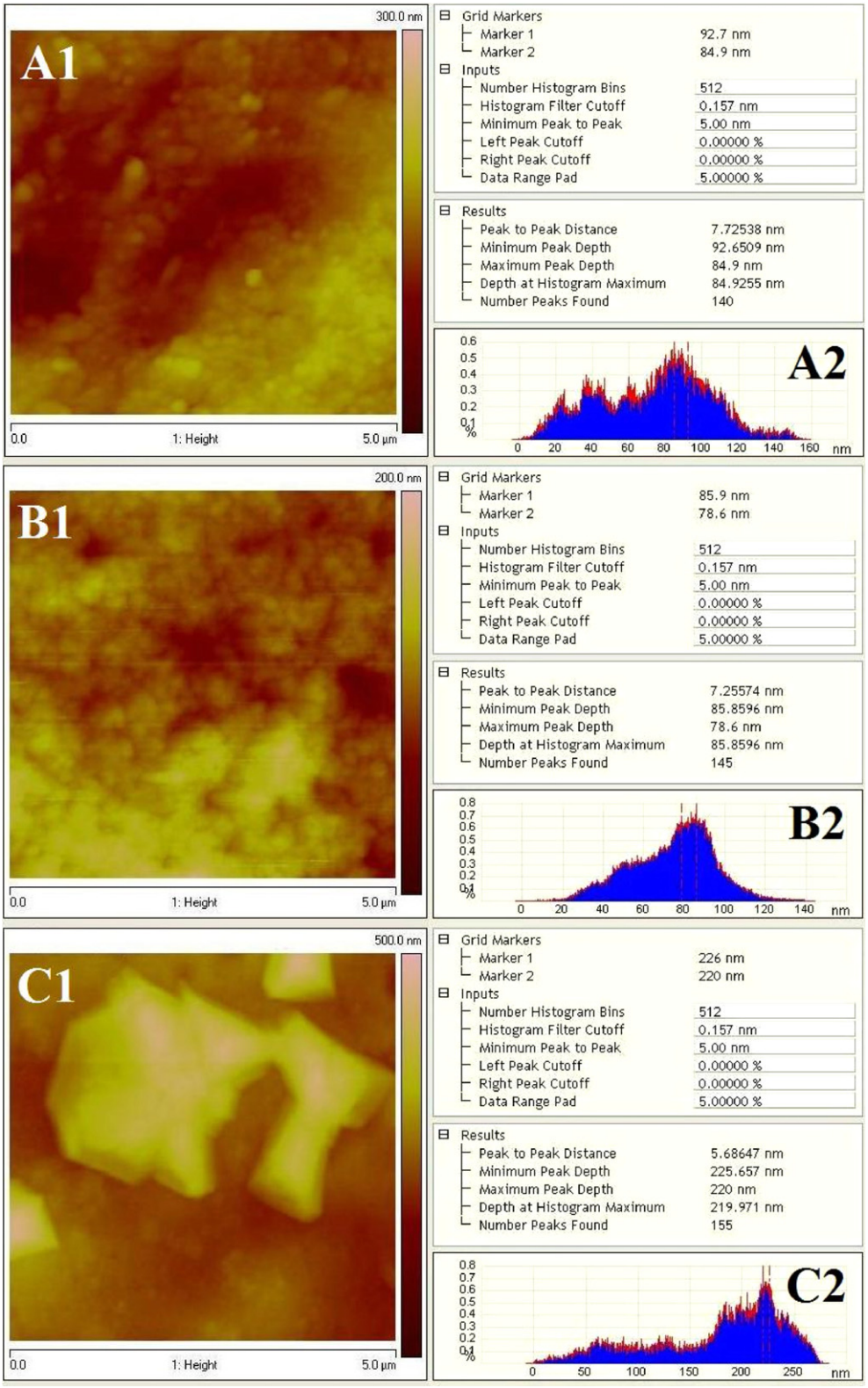

Fig. 4. AFM images and height distribution diagrams of: AFM image **(A1)** and height distribution diagram **(A2)** of unmodified graphite electrode, AFM image **(B1)** and height distribution diagram **(B2)** of GOx modified graphite electrode, and AFM image **(C1)** and height distribution diagram **(C2)** of GOx/PANI modified graphite electrode.

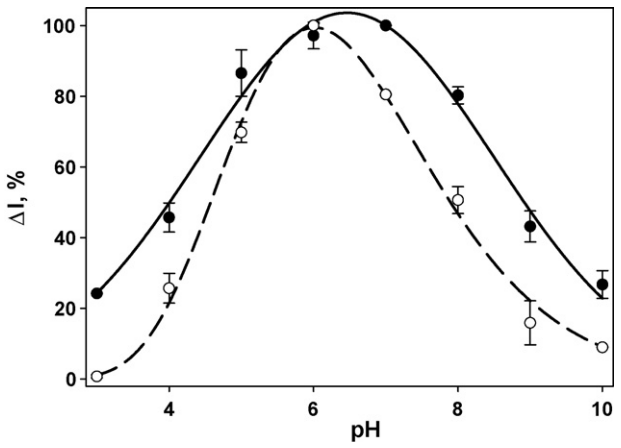

Fig. 5. Amperometric signal *vs.* pH of solution: GOx (dashed line) and GOx/PANI (solid line) modified graphite electrode. Detection of analytical signal was performed in 50 mM sodium acetate and sodium/potassium phosphate buffer contained 100 mM of KCl at room temperature in presence of 10 mM of PMS and 20 mM of glucose; Working electrode potential was +300 mV *vs.* Ag/AgCl.

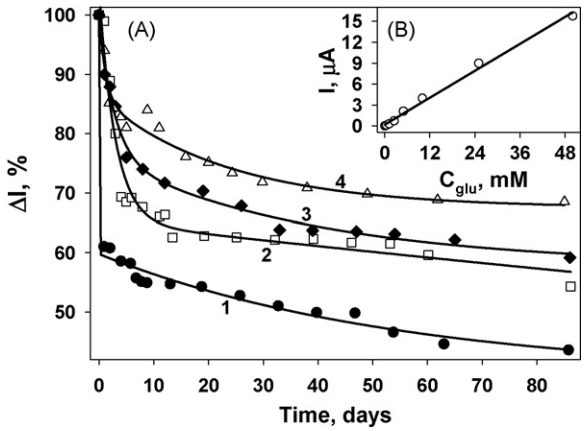

Fig. 6. (**A**) Stability of GOx modified electrodes: electrochemical signal *vs.* time: GOx-electrode (1), GOx/PANI-electrode (polymerisation time: 22 h (2), 48 h (3) and 69 h (4)). Experiments were performed at room temperature in 50 mM sodium acetate and sodium/potassium phosphate buffer, pH 6.0, and containing 100 mM of KCl and 10 mM of PMS. 20 mM of glucose was added for each measurement; working electrode potential was +300 mV *vs.* Ag/AgCl; (**B**) Calibration curve: linear dependence of amperometric signal *vs.* glucose concentration in the rage between 0 and 50 mM, $y = (0.19 \pm 0.11) + (0.32 \pm 0.01)^{*}x$, $r^2 = 0.9934$.

and Brandt, 1971). At pH 3, even 0.1 M KCl completely inhibits the enzyme activity (Weibel and Bright, 1971). The pH optimum was detected at pH = 6.5 (Fig. 5, solid line) for GOx/PANI modified electrodes. This indicates that the GOx entrapment into PANI layer procedure kept the native characteristics of glucose oxidase. The pH profile of the GOx activity in case of GOx/PANI-electrode became broader and shifted towards higher pH values than that of GOx-electrode. This broader shift of 0.5 pH units pH profile was detected because of the influence of PANI matrix.

One of the most important characteristics is the stability of the electrode current response over a period of time. The stability of GOx and GOx/PANI modified electrodes were studied by measuring immobilized GOx activity during an 86-day period. Between measurements electrodes were stored at 4 °C in the closed vessel above a drop of 0.05 M sodium acetate buffer solution, pH 6.0. As seen from the results presented the current response of the GOx-electrode to glucose fell below 65% of the initial its value after the first measurement (Fig. 6A, curve 1). However, after this downfall, significantly less decrement of current response was registered. It has been found that GOx-electrode retained about 44% of GOx activity after 86 days. The current response downfall of GOx/PANI modified electrodes (Fig. 6A, curves 2, 3 and 4) was much less than GOx modified electrode. Furthermore, this current response downfall decreased with elongation of polymerisation period. Such current response reduction of first derivative could be attributed to decreased leaking of immobilized GOx due to encapsulation of GOx within PANI layer. The results represented in Fig. 6A show that GOx/PANI modified electrodes retained 54.31%, 59.2% and 68.5% of GOx activity after an 86-day period in case of polymerisation period 22 h, 48 h and 69 h, respectively. The most suitable for application GOx/PANI-electrode (formed after 48 h of incubation in polymerisation solution) was evaluated (Fig. 6B) it shows significantly extended region of liner dependence of analytical signal *vs.* analyte concentration with very good correlation of results.

4. Conclusions

In this study, we demonstrated a new way for the construction of amperometric glucose biosensor based on glucose oxidase self-encapsulated within polyaniline matrix. Proposed self-encapsulation opens a new venue for biosensor designing. It is presumed that in this study proposed mild conditions for self-encapsulation of immobilized GOx will provide a promising route for the fabrication of biosensors based on other enzymes. Here reported glucose biosensor displayed a significantly wider linear detection range, broader pH profile of the GOx activity and better stability in comparison with conventional glucose biosensor based on cross-linked GOx. The most important point in this study is that polymerisation of PANI was induced by GOx catalysed reaction. Our continuing works will demonstrate that similar immobilization route is suitable for development of biofuel cells based on oxidases.

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