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POLYANILINE AND POLYPYRROLE, GOLD NANOPARTICLES AND DIFFERENT ELECTRON TRANSFER MEDIATORS APPLICATION IN GLUCOSE BIOSENSORS DESIGN

Summary of doctoral dissertation

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VILNIAUS UNIVERSITETAS

FIZINIŲ IR TECHNOLOGIJOS MOKSLŲ CENTRO CHEMIJOS INSTITUTAS

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INTRODUCTION

Diabetes mellitus, or simply diabetes, is a group of metabolic diseases characterized by high blood glucose levels that result from defects in the body's ability to produce and/or use insulin. Diabetes is a worldwide public health problem, because it is one of the leading causes of death and disability in the world. The diagnosis of this disease requires a precise detection of blood glucose concentration. Electrochemical biosensing systems are the most suitable for the determination of analytes in complicated and complex samples including blood, blood serum, etc. Among electrochemical biosensing systems enzymatic amperometric glucose biosensors are the most common devices commercially available, and have been widely studied over the last few decades. These biosensors are usually based on the two enzyme families, glucose oxidase (GOx) and glucose-1-dehydrogenase (GDH). Because of a relatively higher selectivity for glucose over other blood sugars and stability, more simple purification procedure and lower price, better resistance in extremes pH, ionic strength and temperature than many other enzymes], GOx is the most popular enzyme for the modelling of biosensors. GOx is a dimmeric protein containing one tightly bound flavin adenine dinucleotide (FAD) per monomer as cofactor. FAD functions as a coenzyme because of its ability to undergo reversible redox reactions. The redox active centre of the FAD is the isoalloxazine ring system. The basic concept of the glucose biosensor is based on the fact that the immobilized flavoprotein GOx catalyses the oxidation of β-D-glucose to D-glucono-δ-lactone, which is non-enzymatically hydrolyzed to β-D-gluconic acid, and hydrogen peroxide using molecular oxygen as an electron acceptor.

Immobilization of enzymes and establishment of electron transfer (ET) are the most challenging and important steps in the development of amperometric biosensors. Immobilized enzymes have many operational advantages if compared with dissolved enzymes including possible reusability, continuous operational mode, easy separation from the reaction mixture, and possible modulation of the catalytic properties. Fast, simple and low-cost detection of biologically active analytes is the major advantage of biosensing systems. In some cases a combination of nanomaterials and nanotechnological approaches resolve challenging bioanalytical problems, including specificity, stability and sensitivity.

Some conjugated polymers provide an effective immobilization patterning for enzymes on surfaces of different electrodes and in some cases facilitate ET from enzymes to electronically conductive electrodes and improve biosensor sensitivity. Composite nanomaterials exhibit improved physical and chemical properties over their single-component counterparts, and hence they are potentially useful in a wide range of applications including analytical, bioanalytical systems and biofuel cells. Conducting polymer-based layers on the electrode surface are traditionally synthesized by electrochemical or chemical synthesis. Both, electrochemical and chemical synthesis, require high concentrations of monomers and usually are performed at extremely low pH values. In traditional chemical synthesis of conducting polymers, usually toxic catalysts or strong oxidizing agents are used. Therefore enzymatic synthesis of conducting polymers is applied as an alternative method suitable for the formation of composite materials based on conducting polymers such as polyaniline (PANI) and polypyrrole (PPY).

Despite of numerous scientific papers and other informational issues related to improvement of biosensor performance using advanced redox mediators, the most significant part of this information is not showing clear comparison between different mediators influence in the biosensor response to substrate. Consequently, one of the objectives of this study was to evaluate the effect of few different mediators on the response of GOx-based amperometric biosensor.

The aim of the work:

Apply π-π conjugated polymers polyaniline and polypyrrole, gold nanoparticles and different electron transfer mediators in amperometric glucose biosensor

Main tasks of the work:

- Investigate the formation of polyaniline and polypirole and encapsulate glucose oxsidase immobilized on electrode surface within the polymer layer;
- Determine and compare the influence of polyaniline and polypirole layer to the characteristics of amperometric glucose biosensor;

- Synthesize composite particles of glucose oxsidase encapsulated within polyaniline layer and glucose oxsidase with gold nanoparticles encapsulated within polyaniline layer and determine the influence of gold nanoparticles on polyaniline formation rate;
- Compare the influence of synthesized composite structures on amperometric glucose biosensor analytic characteristics;
- Investigate the influence of different electron transfer mediators immobilized on carbon electrode surface on amperometric glucose biosensor analytic characteristics;

Statements to be defended:

Polypyrrole and polyaniline layer formed on graphite electrode surface modified by glucose oxidase, extends concentration range of linear dependence of analytical signal for glucose biosensors, increases stability and improves the repeatability of analytical signal;

Gold nanoparticles not only increases the enzymatic aniline polymerization reaction rate, but also being as a part of glucose oxidase and polyaniline nanocomposite structures, during the enzymatic oxidation of glucose, ensure efficient transport of electrons from the glucose oxidase redox center flavine adenine dinucleotide to the electrode, and thus increases the biosensor analytical signal;

Electron transfer mediators tetrathiafulvalene, phenazine methosulfate, 5,6-diamin-1,10-phenanthroline, tetrathiafulvalene - tetracyanoquinodimethane complex, methylene blue, toluidine blue and potassium ferrocyanide immobilized on the surface of the graphite electrode enables efficient transport of electrons from the glucose oxidase redox center to the electrode surface . The most effective electron transfer has tetrathiafulvalene and tetrathiafulvalene - tetracyanoquinodimethane complex.

EXPERIMENTAL

Electrode pre-treatment prior to modification

Graphite rod electrodes 3mm in diameter, 150mm 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 0.7mm^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 size0.1µm), followed by rinsing the electrode surface with ethanol and distilled water. Electrodes were dried at room temperature before coating them with enzymes.

Electrode modification by GOx

During the preparation of GOx-modified electrodes (CR/GOx electrodes), 3 µl of 40 mg/ml of enzyme solution in water were deposited on the electrode and later water was evaporated at room

temperature. Then electrodes were stored for 24 h over the 5% solution of glutar aldehyde at 4 ˚C in the closed vessel. The lateral surface of the electrode was isolated with silicone tube to prevent the contact of electrode side surface with the solution and working surface area of modified electrodes was 7.065 mm^2. Prior to electrochemical measurements CR/GOx electrodes were 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 A-PBS buffer, pH 6.0.

Modification of GOx coated electrode by PANI or PPY layer

For the covering of CR/GOx electrodes by polymer layer these electrodes were immersed into A-PBS buffer, pH 6.0,containing 20 mM of glucose and 200 mM of aniline (CR/GOx/PANI electrodes) or 200 mM of pyrrole (CR/GOx/PPY electrodes) at 4 ◦C, for a defined period, lasting from 0 to 272 h. Prepared electrodes were thoroughly washed with distilled water and were stored at 4 ◦C in a closed vessel hanging over a drop of A-PBS buffer, pH 6.0, until they were used in experiments.

Investigation of enzymatic polymerisation of aniline and pyrrole in solution

Polymerisation was performed in A-PBS buffer, pH 6.0, containing 1 mg/ml of glucose oxidase, 20 mM of glucose and 200 mM of suitable monomer. Synthesis was carried out at room temperature in darkness. The UV–Vis spectra of solutions were recorded after 48 h from the beginning of polymerization process. UV–vis spectrophotometer Perkin-Elmer LAMBDA 25 (Shelton, USA) was usedfor monitoring of polymers formation.

Formation of GOx/PANI and GOx/Au-NPs/PANI nanoparticles

A number of different polymerization solutions were prepared for the selection of the optimal polyaniline formation pHs. For this, A-PBS buffers were prepared at pH values of 4.0; 4.5; 5.0; 6.0; 7.0; 8.0. In order to get PANI-based (GOx)PANI and (GOx/AuNP)PANI nanoparticles, enzymatic formation of PANI was applied. To obtainthe (GOx)PANI nanoparticles, the polymerization bulk solution was consisted of 200 mM aniline, 1 mg/mL GOx and 20 mM glucose. In order to form (GOx/AuNP)PANI nanocomposite, AuNP were added into the polymerization solution. A fixed concentration of 7.5 mg/mL of different size (3.5 nm; 6 nm; 13 nm) gold nanoparticles was used in the polymerization solution with a total volume of 1.0 mL. The polymerization reaction was carried out at +4◦C in the dark. After 24 h reaction time, the solution was centrifuged, and all formed nanocomposites were separated from the polymerization solution and transferred into 100 µL volume of buffer for additional washing procedures. After a second centrifugation procedure, the washing buffer solution was removed and all synthesized particles were mixed thoroughly in 100 µL of buffer. Prepared (GOx)PANI particle solution was applied in the design of the CR/(GOx)PANI electrodes and (GOx/AuNP)PANI nanoparticle solution for the formation of the (GOx/AuNP)PANI electrodes.

Electrode modification by GOx and mediators

Two methods were used for preparation of modified electrodes. During preparation by the first method, 3.0 µL of mediator solution were dropped and distributed on the electrode surface and solvent evaporated at room temperature by intensive ventilation. Then 3.0 µL of GOx solution were deposited and solvent evaporated, and finally, after complete drying, the electrode was left in a closed vessel at 2 cm above the 5 % GA solution at +4°C for 24 h. Then the modified electrode (CR/M/GOx) was thoroughly washed with distilled water to remove non-cross-linked enzyme. Prepared electrodes were stored in closed test-tubes over a drop of A-PBS, pH 6.0, at +4°C between electrochemical measurements. In order to get modified electrodes by the second method (CR/M-GOx), solutions of enzyme and mediator were mixed in a ratio or 1:1 and 3.0 µL of prepared solution were dropped and distributed on the electrode surface twice with evaporating between each depositing. Then the electrodes were incubated in glutar aldehyde vapor and stored as previously described.

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 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 0.05M sodium acetate and sodium/potassium phosphate (A-PBS) buffer solution, pH 6.0 containing 0.1M KCl. Solution in electrochemical cell was mixed at 120 rpm. Electrochemical detection of analytical signal was performed at room temperature at +300mV vs. Ag/AgCl in presence of 10mM of phenasine methoslulphate (PMS) and different concentrations of glucose.

Special conditions used for determination of pH dependence

Modified electrodes were tested in A-PBS buffer solution contained 0.1M of KCl, with fixed pH value. Electrochemical detection of analytical signal was performed similarly as it is presented in previous section.

Special conditions in lifetime and stability test

Modified electrodes were stored between the measurements at 4 ◦C in closed vessel hanging over A-PBS buffer solutions, pH 6.0. Electrochemical detection of analytical signal was performed similarly as it is presented in previous section.

Special conditions in temperature influence test

For this experiment modified electrodes were prepared by polymerization lasting for 24, 48 and 72 h. The effect of temperature variations on the enzyme activity of modified electrodes were tested by heating of these electrodes at particular temperature for 5 min in laboratory incubator “incucell 55-standart” from “Blue Line” (MMM Medcenter Einrichtungen GmbH, Germany) in air atmosphere and in A-PBS buffer solutions, pH 6.0.

The imaging by scanning electron microscopy

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.

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 Tapping Mode were used for all measurements.

RESULTS AND DISCUSSION

Glucose oxidase (GOx) is a FAD-dependent enzyme that catalyzes oxidation of ˇ-d-glucose by molecular oxygen to hydrogenperoxide and d-glucono-1,5-lactone (1) which subsequently hydrolyzes spontaneously to gluconic acid. In this work section study catalytic activity of GOx immobilized on graphite electrode by cross-linking with glutar aldehyde was exploited for polymerisation of aniline. The main precursors for initiation of polymerization reaction was hydrogen peroxide as an initiator of polymerization reaction (3) and gluconic acid as a medium what reduced pH towards the acidic pH suitable for template polymerization of aniline. All mentioned precursors were produced during the catalytic action of immobilized GOx (1) and following hydrolization of reaction products. Since aniline has a pKa of 4.63, whereas GOx has a pI of 4.2 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.

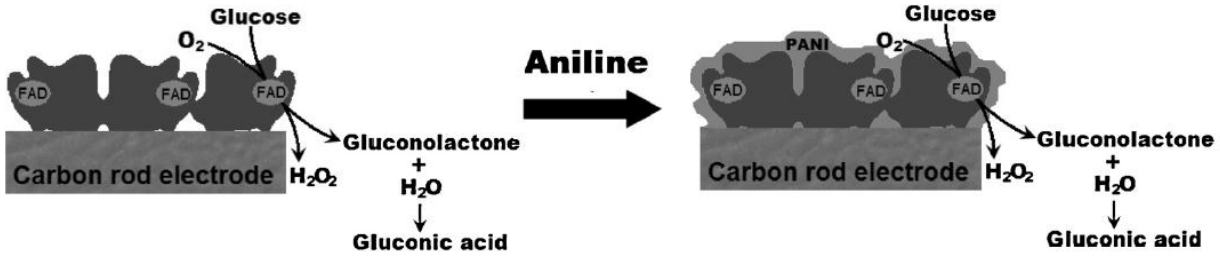

Fig. 1 Principle scheme illustrating modification of GOx coated electrode by PANI.

It is presumptive that locally lowered pH (4.3–4.5) and high concentration of oxidator (H_2O_2) created optimal conditions for the polymerizations of aniline and increased the probability of GOx encapsulation within formed PANI to form CR/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.

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

For confirmation of insight that under such favourable conditions the polymerizations of aniline might be initiated 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 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) free diffusing and/or not encapsulated enzyme might be washed out fromthe 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.

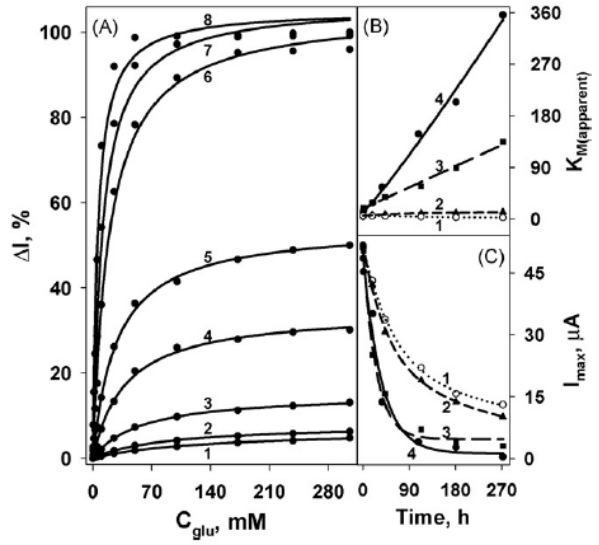

Fig. 2. (A) Glucose calibration curves obtained with a GOx modified graphite electrode, before treatment (curve 8) and after treatment with 200 mM aniline and 20 mM glucose solution at 4 ˚C temperature (curve 7–10 min, 6–70 min, 5–18 h,4–43 h, 3–113 h, 2–181 h and 1–272 h). Calculated $K_{M(apparent)}$ (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 polymerization solution at 4 ◦C (curve 3) and at 18 ˚C (curve 4). Experiments presented in plot 'A' were performed at +300mV vs. Ag/AgCl in A-PBS buffer solution, pH 6.0, contained 100 mM of KCl and 10 mM of PMS

The peculiarity of the present work section is that formation of PANI layer on GOx modified electrodes occurs only in the case if the GOx modified electrode is immersed in polymerization solution containing both glucose and aniline. The main evidence of polymerization 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 culations presented in Fig. 2B and Table 1 CR/GOx and CR/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 Imax were around 5.75mM and 51.72µA, respectively. While the $K_{M(apparent)}$ for the CR/GOx/PANI modified electrode might be extended up to 134.90mM, if polymerization was carried out at 4 ◦C temperature (Fig. 2B curve 3) and up to 356.00mM, if polymerisation is carried out at 18 ◦C temperature (Fig. 2B curve 4). An opposite effect was obtained for the Imax values. The results presented in Fig. 2C demonstrate than the I_{max} can decrease from 51.72µA (CR/GOx) up to 3.06 µA or 0.42µA, if polymerization is carried out at 4 ◦C or 18◦C temperature, respectively. The comparison of kinetic parameters of cross-linked CR/GOx and CR/GOx/PANI based electrodes let us to state that in the case of CR/GOx/PANI 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 polymerization). Such significant increase of $K_{M(apparent)}$ and decrease of Imax are demonstrating that diffusion limitations in this case of CR/GOx/PANI are playing a significant role. An increment of $K_{M(apparent)}$ by over 10 times might be exploited as the evidence that the immobilized on graphite 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 CR/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 CR/GOx/PANI based electrode because of hindered diffusion of both: glucose and redox mediator phenasine methoslulphate (PMS). Detected thickness of the PANI layer was 0,4µm, 0.8µm and 1.1µm after 18 h, 113 h and 181 h of polymerization period correspondingly. No linear dependence between polymerization 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.

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

Time (h)	CR/GOx/PANI		Conntrol 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 .58	51.72±1.81	5.80±0.14	51.81±1.35	5.78±0.21	51.68±1.04
0.17	11.28±1.25	51.53±1.23	-	-	-	-
1.17	19.38±1.54	50.32±2.49	-	-	-	-
18	28.30±1.48	25.11±0.97	5.63±0.17	43.11±1.12	7.09±0.23	42.11±0.99
43	38.62±2.82	15.69±0.63	5.47±0.19	33.69±0.93	8.56±0.39	30.69±0.83
113	57.05±2.14	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	134.91±11.84	3.06±0.01	2.64±0.07	13.06±0.61	12.09±0.53	10.06±0.43

The data presented in Fig. 2A shows that the polymerization 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 CR/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 polymerization reaction; 2nd solution (control solution II)—without glucose, which is crucial for formation of hydrogen peroxide initiator of aniline polymerization. To obtain control electrodes 1 and 2 CR/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 CR/GOx/PANI-electrode when polymerization 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 CR/GOx/PANI electrode 5 and 51 times, respectively (Table 1). Additionally in contrast to the results represented above for control electrode 2 and CR/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. 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.

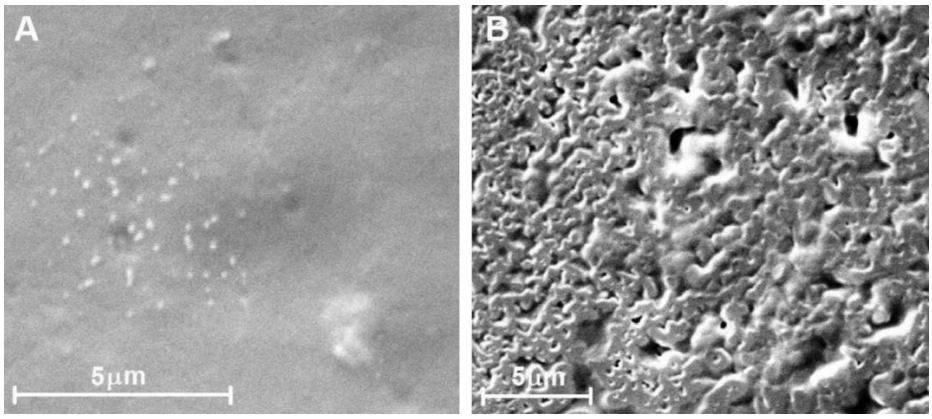

Fig. 3 SEM images of GOx (A) and GOx/PANI (B) modified graphite electrode

The SEM images of CR/GOx (A), CR/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).

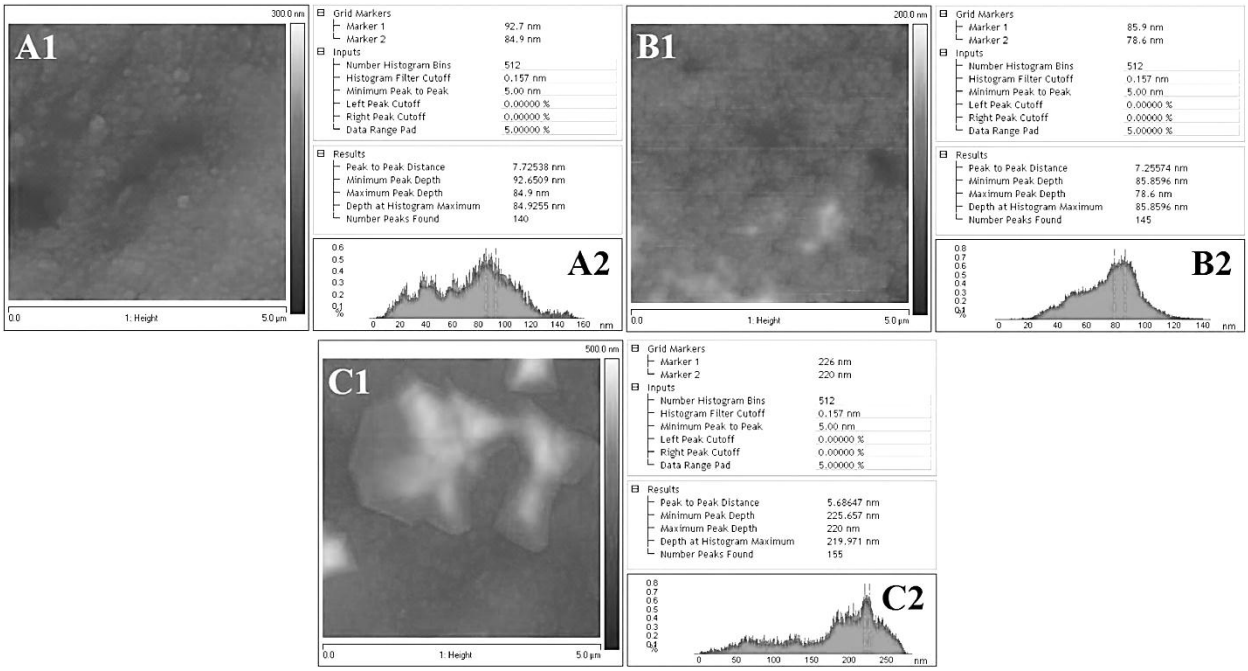

Fig. 4 AFM images and height distribution diagrams of: AFM image (A1) and height distribution diagram (A2) of unmodified graphite electrode, AFM image (B1) and height distribution diagram (B2) of CR/GOx modified graphite electrode, and AFM image (C1) and height distribution diagram (C2) of CR/GOx/PANI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

The value of pH of the polymerization reaction medium allows an efficient entrapment of the enzyme. It also prevents the loss of the enzyme activity under polymerization conditions. Therefore, biosensor response depends on the working pH of the sample solution. The effect of pH on the catalytic activity of enzyme in CR/GOx and CR/GOx/PANI modified electrodes was investigated in A-PBS buffer solutions contained 0.1M of KCl over the range of pH 3.0–10.0 in the presence of 20 mM glucose (Fig. 5). The CR/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. As it can be seen from the figure below pH4 and above pH8, 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. At pH 3, even 0.1M KCl completely inhibits the enzyme activity. The pH optimum was detected at pH= 6.5 (Fig. 5, solid line) for CR/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 CR/GOx/PANI electrode became broader and shifted towards higher pH values than that of CR/GOx electrode. This broader shift of 0.5 pH units pH profile was detected because of the influence of PANI matrix.

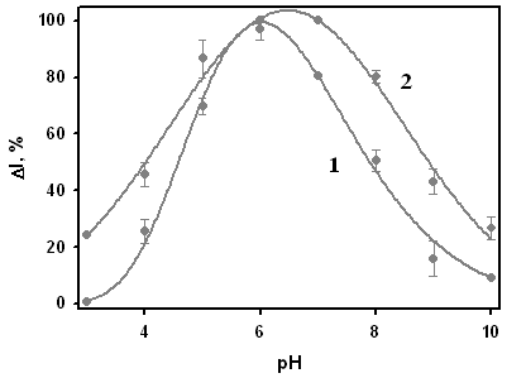

Fig. 5 Amperometric signal vs. pH of solution: CR/GOx (dashed line) and CR/GOx/PANI(solid line)modified graphite electrode. Detection of analytical signal was performed in 50 mM sodium acetate and sodium/potassium phosphate buffer contained 100 mM of KCl at room temperature in presence of 10 mM of PMS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

One of the most important characteristics is the stability of the electrode current response over a period of time. The stability of CR/GOx and CR/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 A-PBS buffer solution,pH6.0. As seen from the results presented the current response of the CR/GOx electrode to glucose fell below 65% of the initial its value after the first measurement (Fig. 6, curve 1). However, after this downfall, significantly less decrement of current response was registered. It has been found that CR/GOx electrode retained about 44% of GOx activity after 86 days. The current response downfall of CR/GOx/PANI modified electrodes (Fig. 6, curves 2, 3 and 4) was much less than CR/GOx modified electrode. Furthermore, this current response downfall decreased with elongation of polymerization 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. 6 show that CR/GOx/PANI modified electrodes retained 54.31%, 59.2% and 68.5% of GOx activity after an 86-day period in case of polymerization period 22 h, 48 h and 69 h, respectively.

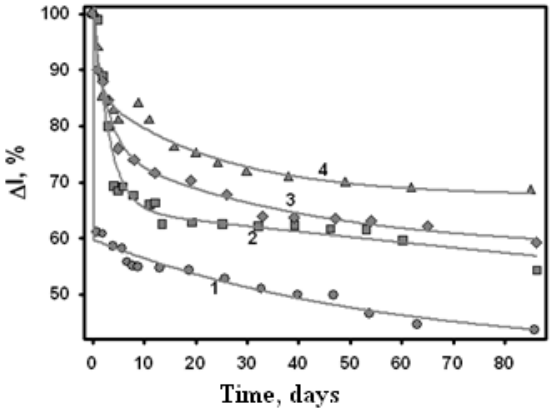

Fig. 6 Stability of GOx modified electrodes: electrochemical signal vs. time: CR/GOx electrode (1), CR/GOx/PANI electrode (polymerization time: 22 h (2), 48 h (3) and 69 h (4)). Experiments were performed at room temperature in 5A-PBS buffer, pH 6.0, and containing 100 mM of KCl and 10 mM of PMS, 20 mM of glucose was added for each measurement; working electrode potential was +300mV vs. Ag/AgCl

Enzymes are known to be sensitive to the changes of temperature and they display maximal activity at a temperature known as“optimal-temperature”. In the range between 40 ˚C and 70 ˚C the most of enzymes get denaturized and they lose their activity. Thermal denaturation of the GOx is mainly influenced by the destabilization of ionic and hydrophobic interactions and by the dissociation of hydrogen bonds, Van der Waal’s forces and ionic interactions; since all here mentioned factors lead to a conformational change in the tertiary structure of the enzyme and render it inactive. The effect of temperature on the activity of immobilized GOx was examined (Fig. 7). Before amperometric measurements the CR/GOx and CR/GOx/PANI electrodes (formed by polymerisation lasting for 24, 48 and 72 h) were heated at indicated temperature for 5 min in air atmosphere (Fig. 7A) or in A-PBS buffer, pH 6.0 (Fig. 7B). As it is shown in Fig. 7, current responses of CR/GOx and CR/GOx/PANI electrodes (formed by polymerization lasting for 24 and 48 h) to 20 mM of glucose in both cases slowly decreases by the increase of temperature, but this decrease was lower for CR/GOx/PANI electrodes. Quite different result was observed for the CR/GOx/PANI-electrode formed by longest duration (72 h) of polymerization. The current-response of this electrode increases gradually with the temperature while reaching a maximal value at 40 ◦C, and then it decreases sharply as the temperature is further increased. Studies on thermal stability of GOx show that the entrapment of GOx into PANI matrix stabilizes the enzyme. The decrease of current-response at over 40 ◦C can be attributed to the loss of enzymatic activity what is caused by the denaturation of GOx and/or some degradation of polymer matrix.

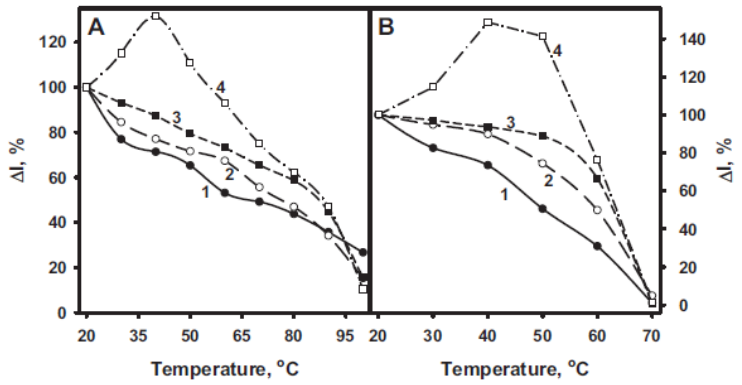

Fig. 7 Effect of temperature variations on the activity of GOx obtained with a GOx modified graphite electrode before (curve 1) and after treatment with 200 mM aniline and 20 mM glucose solution (curve 2–24 h, 3–48 h and 4–72 h). Experiments presented in A plot were carried out by heating electrodes at particular temperature for 5 min in incubator in air atmosphere and presented in B plot in 0.05 M sodium acetate buffer solutions, pH 6.0. Detection of analytical signal was performed in A-PBS buffer at room temperature at +300 mV vs. Ag/AgCl in presence of 10 mM of PMS and 20 mM of glucose.

The glucose detection may be interfered by some materials including ascorbic and uric acid. To evaluate the effect of polymer film on these interfering substances, a series of experiments were performed by GOx- and GOx/PANI-electrodes. In these experiments the influence of ascorbic and uric acid were analyzed within the concentration range of 0.05–1.33 mM, which is the most probable range of both compounds in real samples. The data presented in Fig. 8 shows that the current-responses to 1.33 mM ascorbic and uric acid registered by the CR/GOx electrode were as 14.29 µA (Fig. 8A, curve 1) and 0.76 µA (Fig. 8B, curve 1), respectively. The current responses to the same concentration of ascorbic and uric acid registered by the CR/GOx/PANI electrode were as 12.47 µA (Fig. 8A, curve 2) and 0.70 µA (Fig. 8B, curve 2), respectively. Experimental data shows that the presence of PANI film on the CR/GOx/PANI electrode slows down the diffusion of ascorbic and uric acid by 12.74 and 7.89%, respectively if compared to the GOx electrode. Thus the PANI film on the electrode surface acts as a diffusional barrier, which limits the diffusion of some electro active compounds.

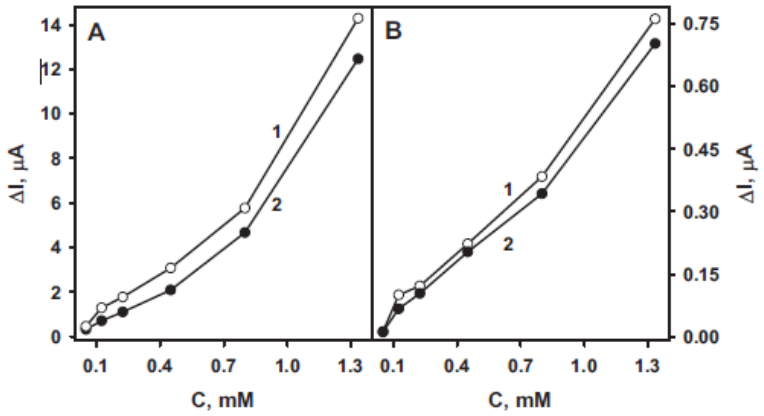

Fig. 8 Response to ascorbic (A) and uric (B) acid for both GOx-electrode (1) and GOx/PANI-electrode (2). Experiments presented in plots were performed at room temperature at +300 mV vs. Ag/AgCl in A-PBS buffer containing 10 mM of PMS.

In this work section, 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 polymerization of PANI was induced by GOx catalyzed reaction. Our continuing works will demonstrate that similar immobilization route is suitable for development of biofuel cells based on oxidases.

In order to compare PANI and PPY application in newly created glucose biosensor, CR/GOx/PANI and CR/GOx/PPY were prepared and thoroughly investigated. the kinetic properties of GOx, which is acting as a biocatalyst in the CR/GOx, CR/GOx/PANI and CR/GOx/PPY electrodes, were compared.

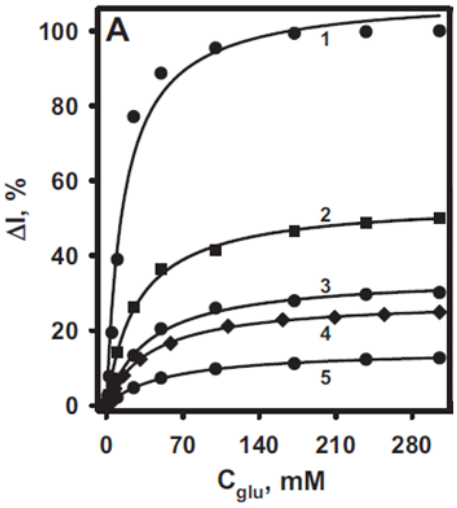

Fig. 9 Glucose calibration curves obtained with a CR/GOx modified graphite electrode: (i) before treatment (curve 1) and (ii) after treatment (curve 2 after treatment for 18 h, curve 3 after 43 h) with solution containing 200 mM of aniline and 20 mM of glucose and (iii) after treatment with 200 mM of pyrrole and 20 mM of glucose solution (curve 4 after treatment for 18 h, curve 5 after treatment for 43 h)

For this the modified electrodes were analyzed at room temperature in the presence of 0.05–300 mM of glucose. The amperometric signals after the addition of various glucose concentrations showed hyperbolic dependence on the glucose concentration (Fig. 9A) and this was in agreement with Michaelis–Menten kinetics. According to calculations presented in Fig. 10A, CR/GOx/PANI and CR/GOx/PPY electrodes exhibit significantly different $K_{M(app.)}$ values. Besides the $K_{M(app.)}$) depends on the duration of polymerisation reaction. The calculated $K_{M(app.)}$ value for the GOx-electrode was 5.75 mM. The data presented in Fig. 10A shows that after 272 h duration of polymerization the $K_{M(app.)}$ might be extended up to 134.90 and 241.96 mM that allows to increase the $K_{M(app.)}$ by 23.5 and 42.1 times for the CR/GOx/PANI (Fig. 10A, curve 1) and GOx/PPY electrode (Fig. 10A, curve 2), respectively. An opposite effect was obtained for the Imax values, what is in agreement with the diffusion-based kinetics of enzymatic reactions. The results presented in Fig. 10B demonstrate than the I_{max} can decrease down from nearly 52 μA for CR/GOx electrode up to 3.06 for CR/GOx/PANI electrode and 1.34 μA for CR/GOx/PPY electrode. Thus the comparison of the I_{max} of CR/GOx, CR/GOx/PANI and CR/GOx/PPY electrodes illustrates that the I_{max} of CR/GOx/PANI electrode and CR/GOx/PPY electrode has decreased by 16.9 and 38.9 times, respectively. From these results, it is evident that the CR/GOx electrode behaves differently in comparison to CR/GOx/PANI and CR/GOx/PPY electrodes even if the same substrate and redox mediator are used. In the case of CR/GOx/PANI and CR/GOx/PPY electrodes the diffusional limitations are playing a significant role and therefore they are operating in the diffusion-controlled mode, while the CR/GOx electrode is operating in the kinetic-controlled mode since the diffusion limitations in this case are minimal. Increased diffusional limitations caused the decrease of I_{max}, which is generated by CR/GOx/polymer electrodes because of hindered diffusion of both glucose and redox

mediator phenasine methosulphate (PMS). An increment of $K_{M(app.)}$, which exceeded 10 times, was exploited as an evidence for the entrapment of GOx within formed polymer layer. On the other hand, the $K_{M(app.)}$ has increased due to diffusional limitations and it caused significant increase in the linear-range of CR/GOx/PANI and CR/GOx/PPY electrodes based analytical systems when compared with CR/GOx electrode based system. Moreover, the polymer layer can prevent the GOx molecules from leaking out from the electrode surface.

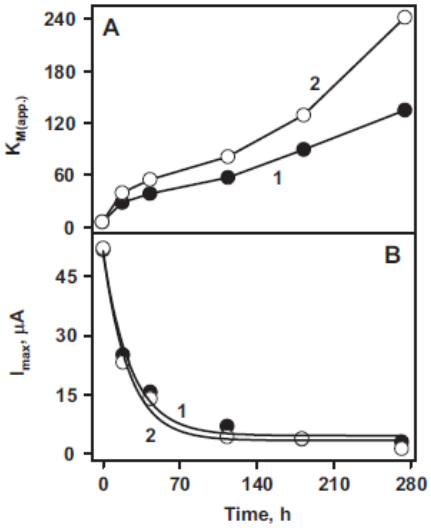

Fig. 10 Calculated $K_{M(app.)}$ (A) and I_{max} (B) values for glucose vs. duration of incubation in the polymerization solution, which contained aniline (curve 1) and pyrrole (curve 2).

The results presented in Fig. 10A demonstrate that the $K_{M(app.)}$ value of CR/GOx/PPY electrode after polymerization period lasting for 272 h is almost 2 times higher if compared with that for CR/GOx/PANI electrode after the same polymerization period. The difference of $K_{M(app.)}$ for CR/GOx/PANI electrode and CR/GOx/PPY electrode might be related to different pyrrole and aniline polymerization rates and different thickness and/or density of formed polymer film. Analysis of polymerization rate indicates that pyrrole polymerization rate is higher than aniline polymerization rate; therefore the formed PPY layer is thicker and/or denser than PANI layer. Here presented research results are in principle agreement with theoretical studies, which by the evaluation of limitations in the diffusion of the analyte and reaction products in/out from the modified electrode have predicted that after

entrapment of enzyme into conducting polymer film, the thickness of enzymatically active layer is typically 200–400 nm.

The influence of pH on the catalytic activity of glucose oxidase in CR/GOx, CR/GOx/PANI and CR/GOx/PPY electrodes were investigated in A-PBS buffer within the pH range of 3.0–10.0, at room temperature in the presence of 10 mM of PMS and 20 mM of glucose. As it is illustrated in Fig. 11, the current response of modified electrodes vs. pH of sample solution shows well-expressed "bell-shaped" curve. The maximal change of current-response registered by CR/GOx electrode was observed at pH 6.0 (Fig. 11, curve 1), it is in agreement with an optimal pH value reported for the native GOx. As it can be seen from the figure below the pH 4 and above the pH 9, the catalytic activity of the GOx and current, which is generated by the CR/GOx electrode, decreases significantly, e.g., at pH 10 only about 10% of the initial GOx activity remains. The pH optimum was determined at pH 6.5 for CR/GOx/PANI electrode (Fig. 11, curve 2) and at pH 7 for CR/GOx/PPY electrode (Fig. 11, curve 3). This not significant shift of pH-optimum indicates that the GOx entrapped into PANI and PPY layer retained the native structure and therefore major enzymatic characteristics retained unchanged. The pH profile of the GOx activity in the case of CR/GOx/PANI and CR/GOx/PPY electrodes became broader and it is shifted towards higher pH-values than that of CR/GOx electrode. This not significant shift of pH-profile was determined by some interactions of GOx with polymer matrix. It should be noted,that the catalytic activity of enzymes depends on the presence of a native conformational structure in the folded polypeptide chain and even minor alterations in the tertiary structure results in the loss of their biocatalytic activity; these alterations and even denaturation can inspired by several major factors such as temperature, pH, unsuitable solvent composition and long term storage. Consequently, the response of CR/GOx modified electrodes depends on these factors. Whereas the enzyme activity depends: (i) on the ionization state of amino acids in the active site; (ii) on the pH value of the sample solution, which is usually regarded as the most important factor in the performance of the enzyme and its sensitivity for substrate.

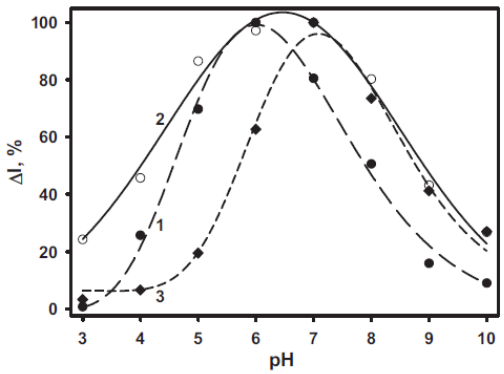

Fig. 11 Amperometric signal vs. pH of solution: CR/GOx (curve 1), CR/GOx/PANI (curve2) and CR/GOx/PPY (curve 3) modified graphite electrode. Detection of analytical signal was performed in A-PBS buffer at room temperature at +300 mV vs. Ag/AgCl in presence of 10 mM of PMS and 20 mM of glucose.

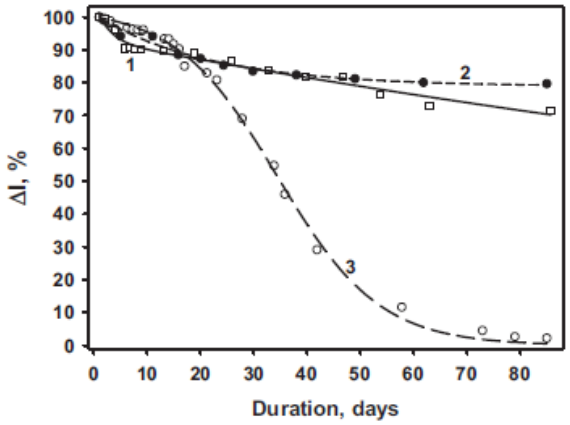

Fig. 12 Electrochemical signal vs. time: CR/GOx electrode (1), CR/GOx/PANI-electrode (2) and CR/GOx/PPY electrode, polymerization duration was 69 h. Experiments were performed at room temperature at +300 mV vs. Ag/AgCl in A-PBS buffer containing 10 mM of PMS and 20 mM of glucose.

Other important characteristic of biosensor is the stability of the current-response within actual time-frame. Material utilized for the fabrication of bioelectrode, the morphology of fabricated film and the method of biomolecule immobilization are among the most important factors that significantly affect the stability of the biosensor. The operational stability of the CRGOx, CR/GOx/PANI and CR/GOx/PPY electrodes were investigated by

the repeated measurementsof analytical signals towards 20 mM of glucose within 86-days. Three consecutive measurements of 20 mM of glucose were performed to get each point indicated in Fig. 12. Between measurements electrodes were stored at 4 ˚C in the closed vessel above a drop of A-PBS buffer solution, pH 6.0. The results obtained revealed that the current response of the CR/GOx electrode to glucose decreased below 65% of the initial value after the first measurement (this value is not shown in the graph). But after this significant downfall the analytical signal appeared more stable and it was found that the CR/GOx electrode retained about 72% of its initial activity even after 86 days lasting investigations (Fig. 12, curve 1). The decrease of analytical signals generated by CR/GOx/PANI and CR/GOx/PPY electrodes after the first measurement was much lower than that for the CR/GOx electrode. Such stabilization of current-response could be attributed to the decreased leakage of immobilized GOx due to efficient encapsulation of GOx within polymer layer. The results represented in Fig. 12 show that the CR/GOx/PPY electrode was more stable than other two types of electrodes while tested within 22-days. However, after this time-frame an opposite effect was discovered. Other stability tests reveal that the CR/GOx/PANI electrode retains almost 80% of initial activity after 86 days (Fig. 12, curve 2), while the CR/GOx/PPY electrode retains only about 2% of its initial activity (Fig. 12, curve 3) after the same period.

In this study, we demonstrated a new way for the creation of amperometric biosensors based on GOx, which was self-encapsulated within conducting polymers PANI and PPY matrixes. The polymerization of PANI and PPY was induced by catalytic activity of GOx cross-linked on electrode surface. Self-encapsulated GOx exhibited significantly different kinetic constants of catalyzed reaction due to increased diffusional limitations if compared with that of the GOx electrode. Major advantages of proposed biosensors are: (i) the layer of PPy or PANI at some extent increase the selectivity of here proposed glucose biosensors, because both polymeric layers are acting as discrimination membranes against ascorbic acid and uric acid (both of them are usually present in actual glucose samples); (ii) the layer of PPy or PANI at some extent are increasing stability of proposed biosensors; (iii) the layer of PPy or PANI increase the $K_{M(app.)}$ it means that the linear range of proposed

biosensors is increasing and it allows determination of glucose concentrations in undiluted samples, here mentioned effect is observed because self-encapsulated GOx exhibited significantly different kinetic constants of catalyzed reaction due to increased diffusional limitations if compared with that of the CR/GOx electrode. Despite worse performance of CR/GOx/PPY electrodes in comparison to CR/GOx/PANI electrodes, here reported GOx/polymer electrodes displayed significantly wider linear range of glucose detection, much broader pH-profile of the GOx activity, better thermal and operational stabilities and increased reproducibility in comparison with those parameters of CR/GOx electrode. The proposed self-encapsulation of GOx opens a new avenue for the development of enzymatic biosensors and biofuel cells due to mild polymerization reaction conditions, which are very suitable for enzyme immobilization. It is expected that other oxidases could have been immobilized and/or modified by here-evaluated approach.

In order to compare some parameters of two different nanocomposite materials, enzymatic polymerization of polyaniline was performed in two types of polymerization solution, which differed by the presence/absence of AuNPs. The first aniline polymerization solution was made up of four main components (aniline, glucose oxidase, glucose, oxygen) dissolved in a buffer. In such a solution the GOx-catalyzed the formation of the strong oxidizing agent, hydrogen peroxide, which initiated the formation of polyaniline and encapsulation, or at least partial coverage, of GOx with the formed polyaniline layer (Fig. 13A).

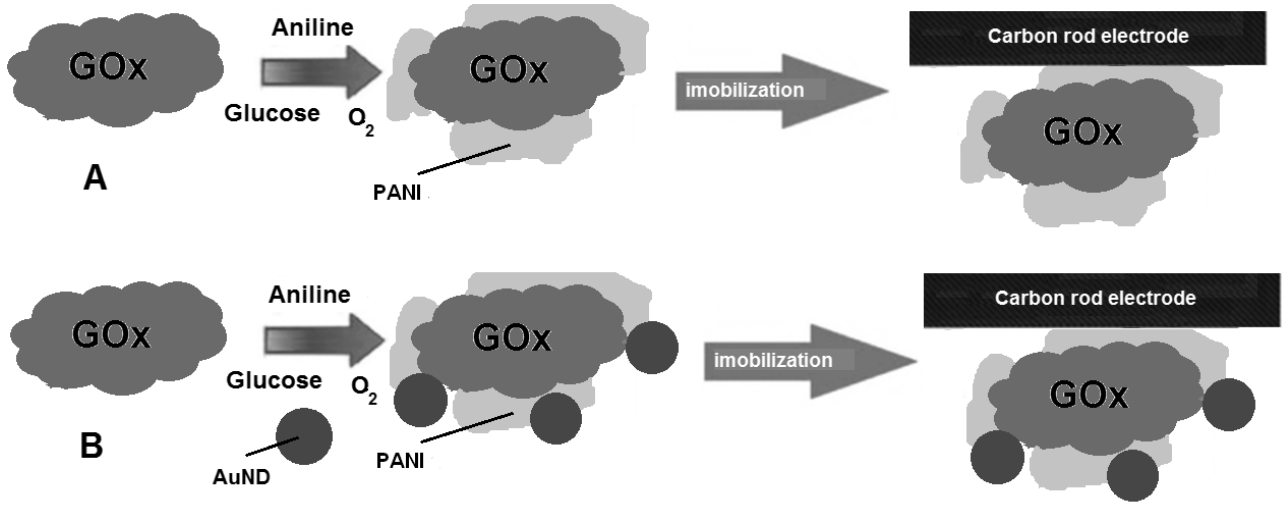

Fig. 13 Schemes of: (A) (GOx)PANI particle formation and carbon rod electrode modification, (B) (GOx/AuNP)PANI 'nanoparticle' formation and modification of electrode

The next type of polymerization solution contained Au-NPs, and it was observed that in the presence of AuNPs the formation of PANI (Fig. 13B) is taking place and is faster in comparison with that in the absence of AuNPs (Fig. 14). In order to determine the influence of gold nanoparticles on the kinetics of polyaniline formation, polymerization solutions were supplemented by 7.5 mg/mL of different size (3.5, 6 and 13 nm) AuNPs. In all evaluated polymerization solutions, absorption increased with time (Fig. 13 A), indicating the formation of polyaniline. In Fig. 13 B polyaniline absorption spectra after polymerization for 116 h are presented. Two absorption maxima were observed: one at λ_1= 360 nm and another at λ_2= 450 nm. The absorption peak, which was observed at around λ_1= 360 nm, indicates that small molecular weight PANI oligomers are formed in bulk of polymerization solution. The other absorption peak observed around λ_2= 450 nm is related to the formation of polyaniline with branched structure. The experimental data indicates that the presence of gold nanoparticles in the polymerization solution increased the concentration of the formed polyaniline after 300 h and the highest polyaniline concentration was observed in the solution supplemented by 3.5 nm gold nanoparticles. Therefore particles of this size were chosen for further research (Fig. 14A).

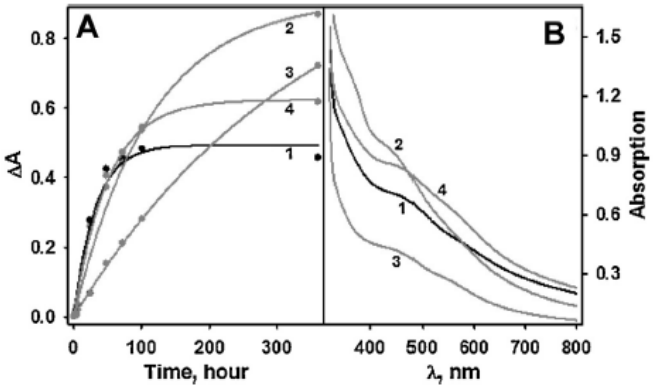

Fig. 14 (A) Polyaniline absorption at different periods of the polymerization process; the measurement points are determined as changes in absorption (ΔA) atλ_2= 450 nm vs. duration of polymerization (time), in the presence of 1 mg/ml GOx,20 mM glucose and 200

mM aniline and different size AuNP: 1 – without AuNP, 2 – with 3.5 nm AuNP, 3 – with 6 nm AuNP, 4 – with 13 nm AuNP; (B) Polymerization solution spectra after 116 h: 1 – without AuNP; 2 – with 3.5 nm AuNP, 3– with 6 nm AuNP, 4 – with 13 nm AuNP.

In order to determine the effect of pH on polyaniline formation two sets of mixtures of A-PBS buffers of different pHs (4.0–8.0.) were investigated. One of those sets contained 200 mM of aniline,1 mg/mL of GOx and 20 mM of glucose (Fig. 14A). The other polymerization solution set was enriched by 7.5 mg/mL of 3.5 nm gold nanoparticles (Fig. 14B). The polymerization reaction course overtime was monitored spectrophotometrically by recording the absorption over the wavelength range of 300–800 nm. Absorption peak (at λ_2= 450 nm) dependence on the duration of polymerization is presented in Fig. 15.

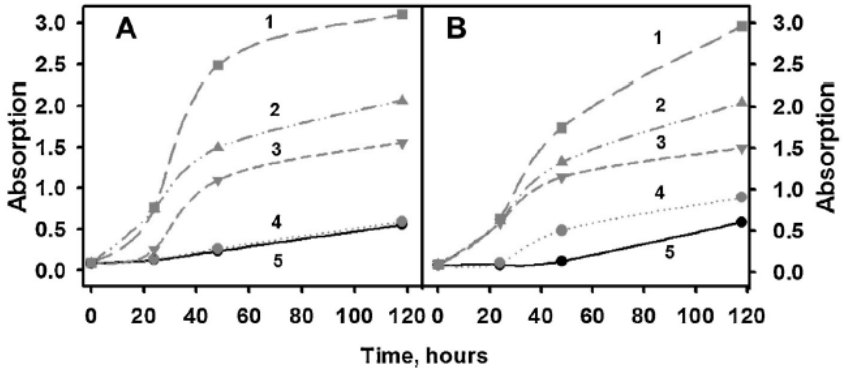

Fig. 15 Polyaniline absorption maxima (λ_2= 450 nm) propagation within time: (A) without AuNP (B) with 7.5 mg/mL of 3.5 nm AuNP. All polymerization reactions were performed in polymerization bulk solution consisting of 1 mg/ml GOx, 20 mM glucose and 200 mM aniline at different pH values: 1 – pH 4.0; 2 – pH 5.0; 3 – pH 6.0; 4 – pH7.0; 5 – pH 8.0.

The data illustrates that with or without the gold nanoparticles, faster polymerization occurs in acidic media than in neutral or alkaline one. The fastest polymerization reaction is observed at pH 4.0. More acidic and more alkali medias were not evaluated because according to our previous experience the activity of GOx decreases significantly and irreversibly outside of this pH region, thus leading to a reduction of formed PANI.

The aim of this part of the research was to evaluate the kinetic parameters of the formed (GOx)PANI and (GOx/AuNP)PANI nanocomposite materials. Amperometric

biosensor design was chosen for the evaluation of enzymatic activity of GOx, which was entrapped within (GOx)PANI and (GOx/AuNP)PANI nanocomposites, due to some advantages of this technique: (i) the same electrode modified by (GOx)PANI and (GOx/AuNP)PANI nanocomposites can be used for a number of measurements, therefore it allows reuse of these materials; (ii) for such investigations, just a very small amount of (GOx)PANI and (GOx/AuNP)PANI nanocomposites is required; (iii) electrode modifications can be performed very easily; (iv) and experimental conditions are reproducible. In order to get a reproducible system for evaluation carbon rod electrodes were prepared as described and were modified by (GOx)PANI or (GOx/AuNP)PANI nanocomposites synthesized at different pH. Investigations of the catalytic activity of (GOx)PANI and (GOx/AuNP)PANI nanocomposites demonstrated that GOx retained its catalytic activity (Fig. 16). The results revealed that the highest amperometric signal was observed for the electrode modified by (GOx)PANI nanocomposites that were synthesized in the range of pH 4.0 – 4.5 and for (GOx/AuNP)PANI nanocomposites synthesized at pH 4.5. The influence of pH on the efficiency of (GOx)PANI and (GOx/AuNP)PANI nanocomposites formation is related to pKa value of aniline, which is 4.63, while the pI value of GOx is 4.2. At pH values lower than 4.63 the aniline is positively charged, while GOx at pH greater than 4.2 is negatively charged. Therefore at pH 4.5, electrostatic interaction of positively charged aniline monomer with negatively charged GOx results in PANI formation in close proximity to the GOx/solution interface.

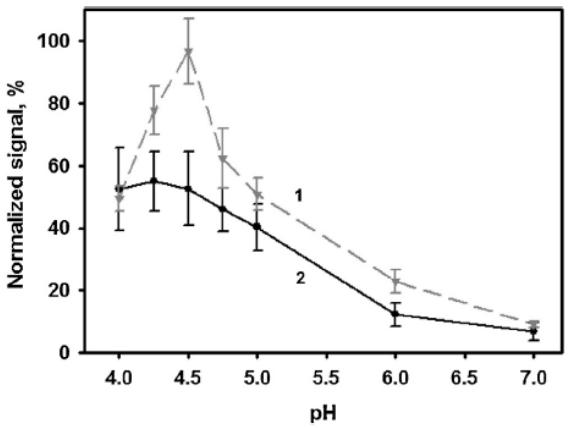

Fig. 16 The dependence of amperometric signal on the pH of the polymerization solution: 1 – electrodes modified by (GOx/AuNP)PANI nanocomposites, 2 – electrodes modified by (GOx)PANI nanocomposites

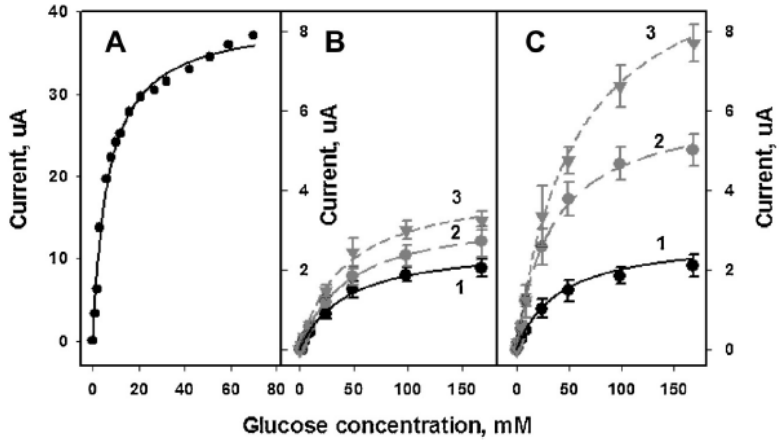

Fig. 17 Current dependence on the amount of the synthesized nanocomposite immobilized on working electrode surface: (A) GOx control electrode; $K_{M(app.)}$ was 6.7 ± 1.1 mM.(B) Electrodes modified by (GOx)PANI particles: 1 – 40 mg/cm^2, 2 – 120 mg/cm^2, 3 – 360 mg/cm^2; $K_{M(app.)}$ for electrodes 1, 2 and 3 evaluated were 42.2, 43.7 and 40.7 mM, respectively. (C) Electrodes modified by (GOx/AuNP)PANI particles: 1 – 40 mg/cm^2, 2 – 120 mg/cm^2, 3 – 360 mg/cm^2; $K_{M(app.)}$ for electrodes 1, 2 and 3 evaluated were 36.6, 33.5 and 35.1 mM, respectively.

The herein proposed GOx encapsulation method is partially based on electrostatic interaction between the positively charged aniline monomer and negatively charged GOx. It reduces the formation of branched polymer structure and promotes linear growth of PANI chains. In addition, the more enzyme was covered with a PANI layer to form nanoparticles, the greater amount of the enzyme entrapped within PANI nanoparticles that was separated from the polymerization solution by centrifugation. Furthermore, the data presented in Fig. 16 also shows that the working electrode modified by (GOx/AuNP)PANI nanoparticles synthesized at pH 4.5 exhibits a current strength twice that of the electrode modified with (GOx)PANI nanoparticles synthesized under the same conditions. This effect can be explained by the fact that the gold nanoparticles not only enhance the GOx activity and

positively affect PANI formation, but they also may have the properties of an ET agent, biomolecule protective agent or catalytic properties in an electrochemical reaction. It is in agreement with previously publishedresearch where the influence of gold nanoparticles in ET efficiency from GOx to some organic dyes and electrodes was evaluated. Amperometric signals of electrodes modified by GOx and AuNP layer were investigated and it was found that gold nanoparticles have a positive effect on the amperometric signals of such electrodes. This might be explained by a significantly increased ET rate from GOx to the graphite electrode, where AuNP increased the effective surface area of the electrodes and/or played the role of a redox mediator. Prior to the present study, the best amperometric signals were obtained when gold nanoparticles were deposited as an interlayer between the electrode surface and GOx. This electrode design was used in the evaluation of the influence of diameter of Au-NPs and it was found that highest ET rate was achieved if 3.5 nm AuNP were applied in the system.

From increased amperometric signals it could be predicted that some AuNP were close to the redox site of GOx (Fig. 16). Moreover it should be noted that AuNP containing composites are more conductive. Hyperbolic dependencies of amperometric signals on the concentration of glucose in the range from 0.1 to 150 mM were observed for both types (GOx)PANI and (GOx/AuNP)PANI of electrodes (Fig. 17 B and C) and they obeyed Michaelis-Menten kinetics, which is in agreement with research where CR/GOx modified electrodes were enzymatically covered by Ppy [1,6,14] and PANI [1,14].From the data presented in Fig. 17, the apparent Michaelis constants of the CR/GOx, CR/(GOx)PANI and CR/(GOx/AuNP)PANI electrodes were calculated as 6.7 ± 1.1, 42.2 ± 1.7 and 35.1 ± 1.6 mM, respectively. The preservation of catalytic properties of GOx in (GOx)PANI and (GOx/AuNP)PANI nanocomposites and a significant increase of $K_{M(app.)}$ is proof that GOx was encapsulated within the polymer layer. Incrementally greater $K_{M(app.)}$ values of (GOx)PANI (Fig. 17 B) and (GOx/AuNP)PANI (Fig. 17 C) based electrodes if com-pared with that for a control electrode based only on bare GOx (Fig. 17 A) is in agreement with previous studies reporting that KM of immobilized GOx significantly increases if a GOx layer is coveredby PPY or PANI.

During more detailed examination of CR/(GOx)PANI and CR/(GOx/AuNP)PANI modified electrodes, three different surface concentrations (40 mg/cm^2; 120 mg/cm^2; 360 mg/cm^2) of (GOx)PANI or (GOx/AuNP)PANI nanocomposites synthesized at pH 4.5 were deposited on the surface of the electrodes. The evaluation of amperometric signals (Fig. 17) indicates that the increase of AuNP concentration positively influences the recorded electrocatalytic currents. This can be explained by the higher enzyme concentration and increased conductivity of nanocomposite material containing higher AuNP concentration. In addition, the electrode modified by (GOx/AuNP)PANI nanocomposites generated current twice as high compared to that obtained with electrodes modified by the same amount of (GOx)PANI nanocomposites. AuNP in combination with the enzymatically formed PANI (Fig. 13 B) layer offered some advantages for the design of electrochemical biosensors as also reported by other researchers based on other conducting polymers e.g. PPY.

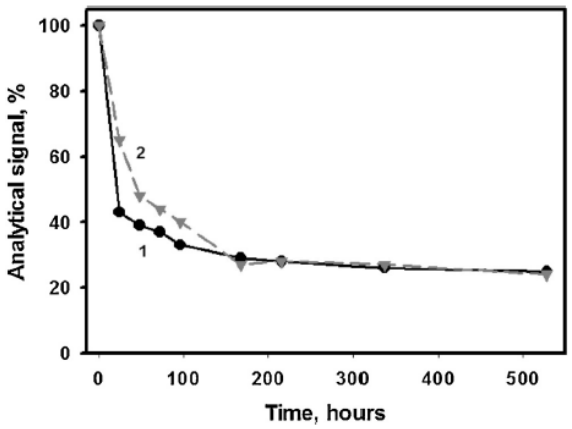

Fig. 18 Evaluation of amperometric signal with time: 1 – electrode modified by (GOx)PANI; 2 – electrode modified by (GOx/AuNP)PANI. Both (GOx)PANI and (GOx/AuNP)PANI nanocomposites were synthesized at pH 4.5

Stability of the amperometric signals of (GOx)PANI and (GOx/AuNP)PANI modified electrodes was tested over 538 h (Fig. 18). It was determined that current generated by the (GOx)PANI nanocomposites modified electrode sharply drops to 43.5% of its initial value within the first 24 h while the (GOx/AuNP)PANI modified electrode was at least slightly more stable because the signal from this electrode during the same time period decreased to only 64% of its initial value. Such decrease of amperometric signal can be

explained by the partial disengagement of particles from the electrode surface during the initial investigation phase. After the sharp initial decrease of amperometric signal, a significant stabilization of amperometric signal was observed; even after 538 h, the (GOx)PANI modified electrode retained about 23%, while the (GOx/AuNP)PANI modified electrode retained 20% of its initial activity. This phase of decrease of amperometric signal could be mostly related to the inactivation of encapsulated enzyme.

In this work section the applicability of (GOx)PANI and (GOx/AuNP)PANI nanocomposites in the design of amperometric glucose sensors was demonstrated. It was found that AuNP included in (GOx/AuNP)PANI nanocomposites significantly increases the amperometric signal compared with that of (GOx)PANI.

Glucose oxidase catalyzes the oxidation of glucose to hydrogen peroxide and gluconolactone using molecular oxygen as an electron acceptor. During this process gluconolactone is non-enzymatically hydrolyzed to gluconic acid. GOx does not directly transfer electrons to conventional electrodes because its redox-active centre flavin adenine dinucleotide is enclosed by a thick protein layer, which determines a spatial separation of the electron donor-acceptor pair and an intrinsic barrier to direct electron transfer. Therefore the transfer of electrons between the FAD and the electrode surface is limiting factor in the operation of amperometric glucose biosensors. In order to establish electrical contact between the FAD and electrode surface it is useful to use artificial mediators that are able to transfer electrons between the active site of GOx and the electrode surface. Such action of mediators is displayed in Figure 1, where M_{ox} and M_{red} are the oxidized and reduced forms of the mediator. The M_{red} is reoxidized at the electrode, generating a current signal proportional to the glucose concentration.

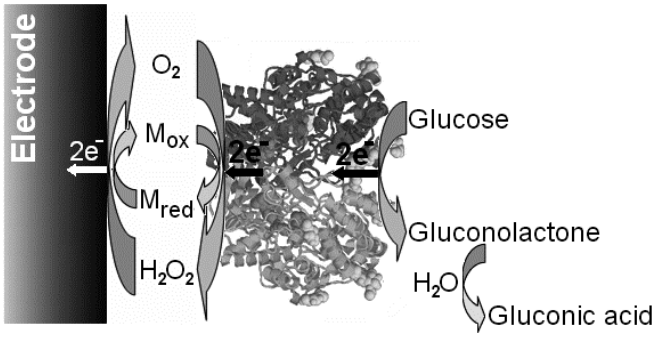

Fig. 19 Schematic representation of the amperometric glucose biosensor operating with mediators.

In the recent study TTF, TTF-TCNQ, PMS, 5,6-DAP, $K_4[Fe(CN)_6]$, MB and TB were investigated in order to evaluate their ability to act as electron carriers between the FAD and graphite rod electrode surface and to compare their influence in the designed amperometric biosensor response to glucose. The selected mediator and GOx were adsorbed at graphite electrode surface and then enzyme cross-linked by glutaraldehyde as described in experimental section. Non-free-diffusionally mediated electrical communication between redox centre of the GOx and an electrode surface provides the significant advantage of eliminating the need for addition of soluble mediator before each measurement. Kinetic properties of GOx acting as a biocatalyst in the CR/M/GOx and CR/M-GOx electrodes were analyzed in A-PBS pH 6.0 at ambient temperature in the range of 0.6 – 290 mM concentrations of glucose. The amperometric signals after addition of glucose showed hyperbolic dependence on glucose concentration (Figure 2) and this was in agreement with Michaelis–Menten kinetics. The kinetic parameters maximal current intensity (I_{max}) (correspond to the maximal limiting rate velocity (V_{max})) and apparent Michaelis constant ($K_{M(app.)}$) are correspondingly a and b parameters of hyperbolic function $y = ax/(b + x)$. According to experimental results and calculations presented in Figure 20 and Table 2 respectively, CR/M/GOx and CR/M-GOx modified electrodes exhibit slightly different $K_{M(app.)}$ and I_{max} values. Besides kinetic parameters depends on the used mediator. The reported values for the Michaelis constant of the native *Aspergillus niger* GOx for β-D-glucose varies from 33 to 110 mM at pH 5.6. Coinciding with our calculations, the maximum $K_{M(app.)}$ (72.9 ± 8.2 mM) was calculated for CR/MB/GOx electrode, whereas the least for CR/TB-GOx electrode (13.9 ± 1.8 mM). An opposite effect was obtained for the I_{max} values. As seen from the Table 1, the highest I_{max} values were obtained in the case of TTF (59.9 ± 1.0 and 55.9 ± 0.9 µA for CR/TTF/GOx and CR/TTF-GOx electrodes respectively), whereas the least in the case of TB (2.7 ± 0.1 and 1.4 ± 0.0 µA for CR/TB/GOx and CR/TB-GOx electrodes respectively). The next most intensive amperometric signal was observed by using TTF-TCNQ as mediator (36.3 ± 0.8 and

34.3 ± 0.4 µA for CR/TTF-TCNQ/GOx and CR/TTF-TCNQ-GOx electrodes respectively). High Michaelis constant value provides a broad linear range of analytical system. It is particularly relevant for the detection of glucose concentrations in food and beverages samples. But the magnitude of analytical signal is also highly important factor that influences the accuracy of an analysis. As a result, CR/TTF/GOx and CR/TTF-TCNQ/GOx electrodes were chosen for rest investigations, though the highest $K_{M(app.)}$ was calculated for CR/MB/GOx electrode. These modified electrodes allowed us to have sufficient Michaelis constant and significant anodic current values.

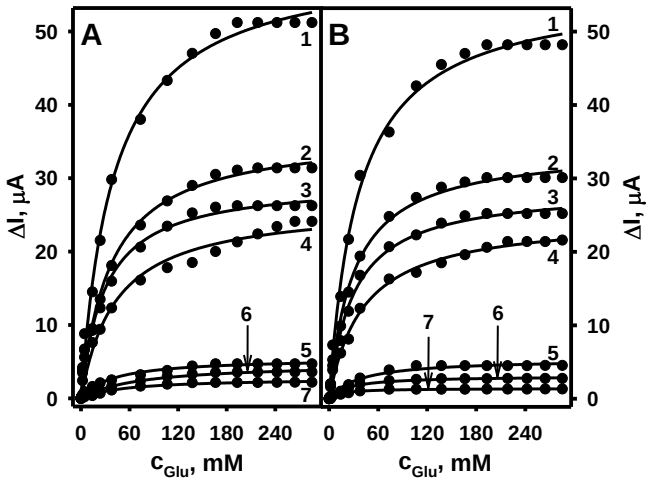

Fig. 20 Glucose calibration curves obtained in A-PBS, pH 6.0, with the CR/M/GOx (**A**) and CR/M-GOx (**B**) electrodes where M is: TTF (1), TTF-TCNQ (2), PMS (3), 5,6-DAP (4), $K_4[Fe(CN)_6]$ (5), MB (6) and TB (7). Conditions: for TTF, TTF-TCNQ, 5,6-DAP or $K_4[Fe(CN)_6]$ working electrode potential was +300 mV, for FMS – +200 mV, for MB – +50 mV and for TB – 0 mV. All potentials are referred *vs* Ag/AgCl/KCl.

Table 2: Calculated K_M(app.) and I_{max} values.

Mediator	CR/M/GOx		CR/M-GOx	
	$K_{M(app.)}$, mM	I_{max}, µA	$K_{M(app.)}$, mM	I_{max}, µA
TTF	39.8±2.6	59.9±1.0	35.9±2.3	55.9±0.9
TTF-TCNQ	36.7±3.2	36.3±0.8	30.1±1.7	34.3±0.4
PMS	29.4±3.2	29.7±0.7	30.3±2.0	28.7±0.4
5,6-DAP	43.0±6.6	26.5±1.0	41.7±3.3	24.8±0.5
$K_4[Fe(CN)_6]$	39.9±4.6	5.5±0.2	37.9±5.7	5.4±0.2
MB	72.9±8.2	4.8±0.2	24.2±2.3	3.1±0.1
TB	55.3±7.2	2.7±0.1	13.9±1.8	1.4±0.0

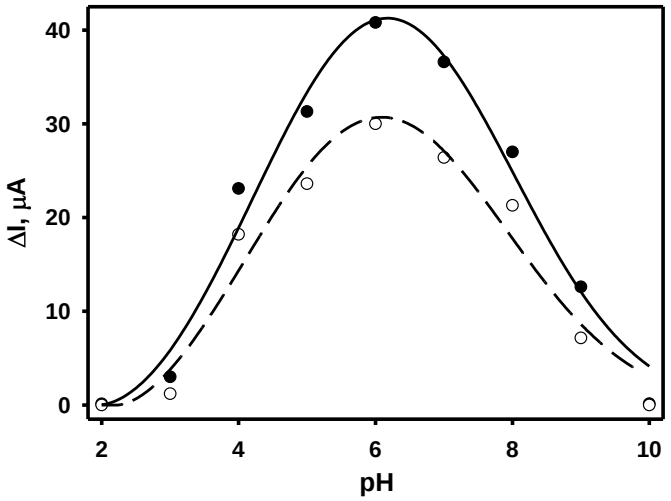

Fig. 21 Amperometric signal of CR/TTF/GOx (solid line) and CR/TTF-TCNQ/GOx (long dash line) electrodes *vs* pH of solution. Detection of analytical signal was performed at +300 mV *vs* Ag/AgCl/KCl in A-PBS with fixed pH value contained 100 mM of glucose.

Several factors, such as pH value of sample solution and time, cause a loss in biocatalytic activity of enzymes. Consequently, response of GOx-modified electrodes depends on these factors. Whereas enzyme activity depends on the ionization state of the amino acids in the active site, the pH value of the sample solution is usually regarded as the most important factor in the performance of the enzyme electrode and its sensitivity for substrate. Therefore, the effect of working pH of the sample solution on the catalytic activity of enzyme in CR/TTF/GOx and CR/TTF-TCNQ/GOx electrodes was investigated. The experimental data measured in A-PBS buffer solution in the range of pH 2.0 to 10.0 at

ambient temperature in the presence of 100 mM glucose is shown in Figure 21. As can be seen, the response of modified electrodes versus sample solution pH shows a bell-shaped curve. More than 50 % of the maximum response current change value was observed between pH 4.0 − 8.0 for both modified electrodes. An optimum was observed at pH 6.0, which is in agreement with the pH value reported for the native GOx from *Aspergilus Niger*. Below the pH 4.0 and above the pH 9.0, the catalytic activity of GOx and current generated by the modified electrodes are rapidly decreasing and at pH 10 nearly 0 % of the initial GOx activity remains. At a low-pH, GOx is inhibited by anions. At pH 3.0, even 0.1 M KCl almost completely inhibits the enzyme activity.

The influence of enzyme concentration on the magnitude of CR/TTF/GOx and CR/TTF-TCNQ/GOx electrodes analytical signal was examined. The experimental data measured in A-PBS buffer solution pH 6.0 at ambient temperature in the presence of 100 mM glucose is shown in Figure 22. The results illustrate that higher amount of GOx produces higher biosensor signal. According to the presented results, the highest amperometric signal (65.9 and 35.4 μA for CR/TTF/GOx and CR/TTF-TCNQ/GOx electrode respectively) was detected in the case of the maximal evaluated concentration of GOx (0.36 mg), whereas the least (43.3 and 27.8 μA for CR/TTF/GOx and CR/TTF-TCNQ/GOx electrode respectively) was detected in the case of the minimal examined concentration of GOx (0.12 mg). Thus, experimental results show that by using three times higher surface concentration of enzyme 1.52 and 1.28 times higher amperometric signal of the CR/TTF/GOx and CR/TTF-TCNQ/GOx electrode respectively was registered.

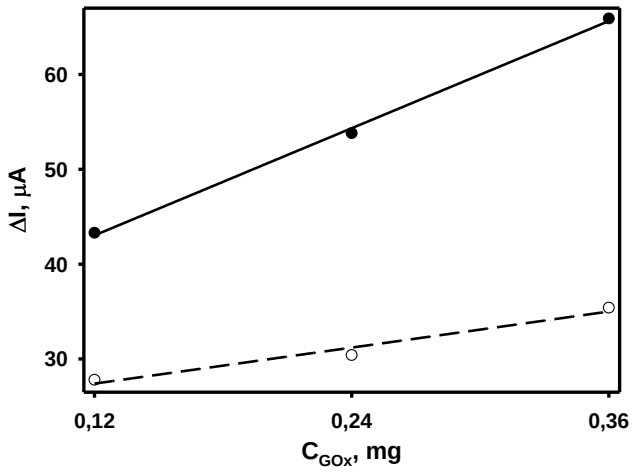

Fig. 22 Amperometric signal of CR/TTF/GOx (solid line) and CR/TTF-TCNQ/GOx (long dash line) electrodes *vs* concentration of glucose oxidase. Detection of analytical signal was performed at +300 mV *vs* Ag/AgCl/KCl in A-PBS, pH 6.0, contained 100 mM of glucose.

Reproducibility of the analytical signal is one of the most important parameters of any analytical system. Therefore the reproducibility of the CR/TTF/GOx and CR/TTF-TCNQ/GOx electrodes was also studied. Reproducibility was evaluated in two ways by the comparison of amperometric signals at different concentrations of glucose (i) using the same electrode and (ii) by three individual electrodes prepared under the same conditions. All experiments were performed in A-PBS buffer solution, pH 6.0, at ambient temperature. The results represented in Figure 23 show that the shift of current strength depends on the concentrations of glucose in the sample. The error bars illustrate the standard deviation (STDEV) for three replicated measurements. The increase of glucose concentration influenced the proportional increase of the analytical signal of CR/TTF/GOx and CR/TTF-TCNQ/GOx electrodes in the range of 0.6 – 38.0 mM where the current strength shift was linearly dependent on the concentration of glucose. The current strength shift achieved saturation level and did not change with an increase of analyte concentration, when the concentration of glucose was higher than 195.0 mM.

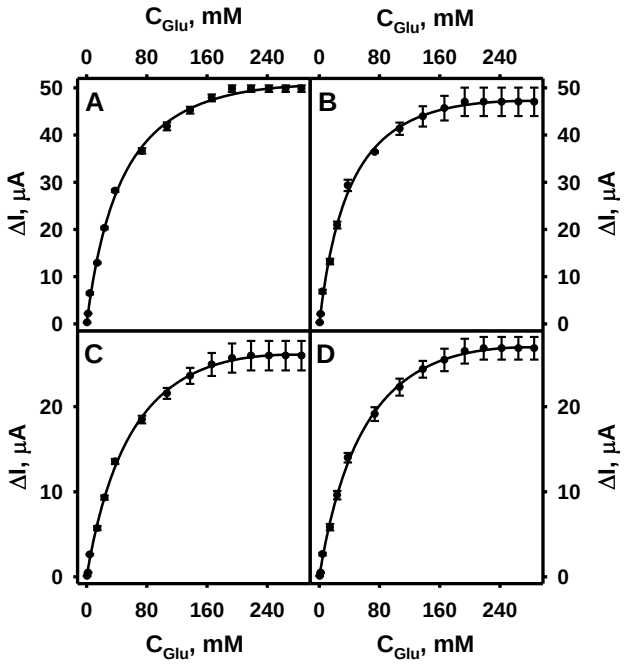

Fig. 23 Amperometric signal *vs* concentration of glucose: A – for the same CR/TTF/GOx electrode; B – for three CR/TTF/GOx electrodes; C – for the same CR/TTF-TCNQ/GOx electrode; D – for three CR/TTF-TCNQ/GOx electrodes. Detection of analytical signal was performed at +300 mV *vs* Ag/AgCl/KCl in A-PBS, pH 6.0.

The results presented in Figure 23 revealed that CR/TTF/GOx and CR/TTF-TCNQ/GOx electrodes have a sufficient reproducibility. In both cases the same electrode and three electrodes prepared under the same conditions show similar current responses for the same amount of glucose. Normal glucose level for adults is 3.3 – 5.5 mM in capillary blood, 4.1 – 5.9 mM in venous blood and 4.25 – 6.4 mM in blood serum. The STDEV calculated for the same CR/TTF/GOx and CR/TTF-TCNQ/GOx electrode amperometric signals in 4.37 mM of glucose, was found to be 0.24 and 0.08 respectively. Relative standard deviation (RSD) was found to be 3.68 and 2.92 %, yielding a measurement repeatability of 96.32 and 97.08 %. The STDEV of the amperometric responses calculated for three similarly prepared CR/TTF/GOx and CR/TTF-TCNQ/GOx electrodes in 4.37 mM of glucose was a little higher. It was found to be 0.40 and 0.15 for CR/TTF/GOx and CR/TTF-TCNQ/GOx electrodes respectively. The RSD was found to be 5.81 and 5.73 %,

yielding the repeatability of measurement of 94.19 and 94.27 %. Insignificant inaccuracy between the different electrodes might be caused by slightly varying surface-concentration of immobilized enzyme and mediator, loss of enzymatic activity during preparation of electrodes, which differ for different electrodes, and unequal distribution of enzyme on the carbon rod electrode surface.

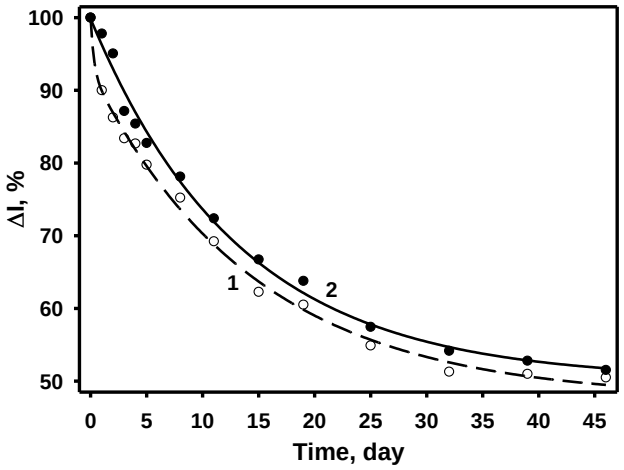

Fig. 24 Amperometric signal *vs* time: 1 – CR/TTF/GOx electrode, 2 – CR/TTF-TCNQ/GOx electrode. Detection of analytical signal was performed at +300 mV *vs* Ag/AgCl/KCl in A-PBS, pH 6.0, containing 100 mM of glucose.

Stability of the analytic signal over a period of time is also highly important parameter of analytical system. The stability of current generated by CR/TTF/GOx and CR/TTF-TCNQ/GOx electrodes was tested in A-PBS buffer solution, pH 6.0, containing 100 mM of glucose at room temperature during a 46-day period. Repeated detections of current strength were performed 14 times. Between measurements the electrodes were stored at 4°C in the closed vessel above a drop of A-PBS buffer solution, pH 6.0. As seen from the results presented in Figure 6, both modified electrodes retained more than 50 % of their initial current response to glucose after continuous measurements for over 46 days. The stability of the CR/TTF-TCNQ/GOx electrode (Figure 24, curve 2) was slightly better than that for the CR/TTF/GOx electrode (Figure 24, curve 1). Long term instability of electrodes

could be attributed to dissociation of enzyme and mediator from the electrode surface and/or inactivation of enzyme.

This work demonstrates the comparison of influence of immobilized redox mediators TTF, TTF-TCNQ, PMS, MB, 5,6-DAP, $K_4[Fe(CN)_6]$ and TB on the GOx-based biosensor electrochemical response to the different concentrations of glucose. According to our knowledge, this is the first scientific paper wherein is present such comparison. All investigated mediators were capable to transfer electrons between the active site of GOx and the graphite rod electrode surface, but the highest amperometric signals were observed by using TTF and TTF-TCNQ. Here investigated biosensors have several advantages, such as: (i) simple procedure for the preparation of the working electrode, (ii) basic application because no addition of soluble mediator before each measurement was required, and (iii) sufficient reproducibility of the analytic signal. Due to these advantages, the designed biosensor is expected to be applicable for the determination of glucose.

Conclusions

- Absorption spectra of synthesized polypyrrole and polyaniline are identical using enzymatic and chemical oxidation synthesis methods. Appropriate pH value of the polymerization solution is the main difference of these synthesis methods. In case of chemical synthesis faster polymerization reaction takes place in more acidic media, in an alkaline medium polymerization will not occur. Enzymatic synthesis is preferably carried out in the pH range in which the enzyme is most active. In addition, the synthesis will occur in alkaline conditions. The presence of gold nanoparticles in polymerization solution increases rate of polyaniline formation. The greatest impact has a 3.5 nm-sized gold nanoparticles.
- Polymer layer formed on graphite electrode surface modified by glucose oxidase, extends concentration range of linear dependence of analytical signal for glucose biosensors, increases stability and improves the repeatability of analytical signal. The electrodes modified by polypyrrole layer are less stable compared to the electrodes modified by polyaniline layer.
- Gold nanoparticles being as a part of glucose oxidase and polyaniline nanocomposite structures ensure efficient transport of electrons from the glucose oxidase redox center to the electrode. Best catalytic properties have nanocomposite structures synthesized at pH 4.5 polymerization solution. These nanocomposite structures twice increases registered analytical signal of biosensors
- Electron transfer mediators tetrathiafulvalene, phenazine methosulfate, 5,6-diamin-1,10-phenanthroline, tetrathiafulvalene - tetracyanoquinodimethane complex, methylene blue, toluidine blue and potassium ferrocyanide immobilized on the surface of the graphite electrode has an effective electron transfer. The kinetic parameters of glucose oxidase depend not only on immobilized electron transfer mediator in nature, but also on electrode preparation method. The most effective electron transfer has tetrathiafulvalene and tetrathiafulvalene - tetracyanoquinodimethane complex. Electrodes modified by these electron transfer mediators have good analytical characteristics.

List of publications by the author included into dissertation thesis:

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3. **V. Mazeiko**, A. Kausaite-Minkstimiene, A. Ramanaviciene, Z. Balevicius, A. Ramanavicius, Gold Nanoparticle and Conducting Polymer – Polyaniline – Based Nanocomposites for Glucose Biosensor Design, *Sensors and Actuators B* 189 (2013) 187 - 193
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5. A. Kausaite-Minkstimiene, **V. Mazeiko**, A. Ramanaviciene, A. Ramanavicius, Evaluation of chemical synthesis of polypyrrole particles, *Colloids and Surfaces A: Physicochemical and Engineering Aspects* (2015)

List of conference thesis:

1. **V. Mazeiko**, A Kausaite – Minkstimiene. Enzyme/Polymeric and Enzyme/Gold/Polymeric nanoparticles for amperometric glucose biosensor design. The 13-th International Conference-School Advanced Materials and Technologies, August 27-31, 2011, Palanga, Lithuania.

2. **V. Mazeiko**, A Kausaite – Minkstimiene. Amperometric Glucose Biosensor based on Glucose Oxidase encapsulated within Enzymatically Synthesized Conducting Polymers. 5-th International Conference The Vital Nature Sign, May 19-21, 2011, Kaunas, Lithuania.
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6. **V. Mazeiko**, A Kausaite – Minkstimiene. Polyaniline synthesis and application in bioanalytical systems. 15-th International Conference-School Advanced Materials and Technologies, August 27-31, 2013, Palanga, Lithuania.

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