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REKERTAITĖ

Formation and characterization of Prussian blue, polypyrrole and glucose oxidase modified electrodes for amperometric detection of glucose

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REKERTAITĖ

Elektrodų, modifikuotų Berlyno mėlynuoju, polipirolu ir gliukozės oksidaze, skirtų amperometriniam gliukozės nustatymui, kūrimas ir tyrimas

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List of original papers by PhD candidate

Paper 1 A. Ramanavicius, A. I. Rekertaitė, R. Valiūnas, A. Valiūnienė, Single-Step Procedure for the Modification of Graphite Electrode by Composite Layer Based on Polypyrrole, Prussian Blue and Glucose Oxidase. Sensors and Actuators B-Chemical 2017, 240, 220–223.

Paper 2 A. Valiūnienė, A.I. Rekertaitė, A. Ramanavičienė, L. Mikoliūnaitė, A. Ramanavičius, Fast Fourier transformation electrochemical impedance spectroscopy for the investigation of inactivation of glucose biosensor based on graphite electrode modified by Prussian blue, polypyrrole and glucose oxidase. Colloids and Surfaces A: Physicochemical Aspects 2017, 532, 165-171.

Paper 3 A. Valiūnienė, P. Virbickas, A. Rekertaitė, and A. Ramanavičius, Amperometric Glucose Biosensor Based on Titanium Electrode Modified with Prussian Blue Layer and Immobilized Glucose Oxidase. Journal of The Electrochemical Society 2017, 164 (14) B781-B784.

Paper 4 A. I. Rekertaitė, A. Valiūnienė, A. Ramanavicius, Physicochemical Behavior of Polypyrrole/(Glucose oxidase)/(Prussian Blue)-based Biosensor Modified with Ni- and Co-hexacyanoferrates. Electroanalysis. Electroanalysis 2018, 30, 1–9.

Author's contribution to the original papers

Paper 1 Developed a method for modifying a graphite electrode with a composite of bioselective layer of Prussian blue, polypyrrole and glucose oxidase (PB-Ppy-GOx) using a one-step method. Optimal conditions for the immobilization of glucose oxidase were chosen to achieve an effective biosensor function. Prepared the literary part, wrote the introduction of the article, evaluated the results, actively contributed to the preparation of the manuscript for the press.

Paper 2 Planned and performed experiments. Performed FFT-EIS studies, prepared samples for bioselective layer fragments by atomic force microscope. Summarized the results, actively participated in preparing the manuscript of the article.

Paper 3 Planned most of the experiments, performed metallurgical titanium surface preparation, selected optimal conditions for titanium electrode coating and immobilization of glucose oxidase in different ways. Prepared a literary part, actively contributed for a press release.

Paper 4 Designed and carried out experimental work, Prussian blue layer additionally was modified with Ni-hexacyanoferrate (NiHCF) and with Co-hexacyanoferrate (CoHCF) and Ppy-GOx-PB-NiHCF and Ppy-GOx-PB-CoHCF electrodes were designed, respectively. Evaluated the effect of temperature on analytical performance of the Ppy-Gox-PB-NiHCF based biosensor assessing the physico-chemical properties of the bioselective layer at different temperatures. Summarized the data, prepared the introduction, the literary part, prepared the manuscript for the press.

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LIST OF ABBREVIATIONS

AFM	Atomic force microscopy
CPE	Constant phase element
DNA	Deoxyribonucleic acid
E_a	Activation energy
EIS	Electrochemical impedance spectroscopy
FFT	Fast Fourier transformation
GE	Graphite electrode
GOx	Glucose oxidase
HCF	Hexacyanoferrate
K_M	Michaelis-Menten constant
NIST	National Institute of Standards and Technology
PB	Prussian blue
Ppy	Polypyrrole
RMS	Root mean square
SDD	Silicon drift detector
SEM	Scanning electron microscope
v_{max}	Maximal reaction rate

V_{ET} heterogeneous electron transfer rate

k_{ET} heterogeneous electron transfer rate constant

INTRODUCTION

Diabetes mellitus is a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by the ineffectiveness of the insulin produced. Such a deficiency results in increased concentrations of glucose (polyhydroxil alcohol) in the blood, which in turn damage many of the body's systems, in particular the blood vessels and nerves.

Type 1 diabetes is known as insulin-dependent. In this case the pancreas fails to produce the insulin which is essential for survival. This form develops most frequently in children and adolescents, but is being increasingly noted later in life.

Type 2 diabetes is known as insulin-independent due to the inability of insulin receptors to respond to the action of secreted insulin in the pancreas. This usually occurs in adults, but is also seen in adolescents.

Blood glucose monitoring has been established as a valuable tool in the management of diabetes. Since maintaining normal blood glucose levels is recommended, a series of suitable glucose biosensors have been developed. During the last 50 years, glucose biosensor technology, continuous glucose monitoring systems has been significantly improved. Electrochemical sensors are very promising analytical device because they can be modified to increase their sensitivity and selectivity. Amperometric biosensors consisting of glucose oxidase and Prussian blue have attracted great attention because Prussian blue and other metal hexacyanoferrates (such as cobalt (II), nickel (II), etc.) potentially can be used as an electrocatalytic properties promoting material in development of glucose biosensors. There is a lot of information in scientific literature concerning development of glucose biosensors, the challenge to find the most suitable substrate for bioselective layer formation and to simplify the electrode modification procedure as much as possible optimizing the immobilization of enzymes, still remains topical. For these reasons, great attention was devoted to solving these problems in our research.

The aim of the study:

To form and investigate amperometric glucose biosensor by modifying graphite and titanium electrodes with composite layer of Prussian blue, Polypyrrole and Glucose Oxidase (PB-Ppy-GOx).

The objectives of the study:

1. Modify graphite electrode by "single step" procedure with composite layer of Prussian blue, Polypyrrole and Glucose Oxidase (PB-Ppy-GOx) and to form glucose biosensor based on this bioselective composite layer by using the method of cyclic voltammetry.
2. Determine the required pyrrole concentration in the electrodeposition solution for the optimal sensivity of glucose biosensor.
3. Investigate the surface properties of a graphite electrode modified by a bioselective PB-Ppy-GOx layer using the AFM method.
4. Determine the optimum value of the biosensor potential for recording the amperometric signal.
5. Evaluate the electrochemical characteristics of the bioselective PB-Ppy-GOx layer by using the method of Fast Fourier Transformation Electrochemical Impedance Spectroscopy (FFT-EIS).
6. Evaluate inactivation of GE-PB-Ppy-GOx electrode during the course of storage and measurements of glucose concentrations.
7. Modify Ti electrode by using spontaneous or/and electrochemical deposition of PB and immobilization of GOx in various ways. Evaluate the operational parameters of the obtained biosensors.
8. Entrap ions of Ni^{2+} or Co^{2+} into PB-Ppy-GOx layer by using "single step" procedure and to form glucose biosensors based on Ppy-GOx-PB-NiHCF or Ppy-GOx-PB-CoHCF bioselective layer on the graphite electrode and to investigate physicochemical properties of the obtained biosensors.

Scientific novelty:

The Prussian blue electrocatalytic ability to reduce hydrogen peroxide is widely used in the development of biosensors for determination of glucose concentration. Among the various types of glucose biosensors, the electrochemical glucose biosensors have exceptional properties such as a

low detection limit, fast response, longer service life and lower cost. However, these electrochemical biosensors have encountered few problems for selecting the optimal conditions for deposition of the bioelectrocatalytic layer and determining the appropriate electrochemical parameters for effective biosensor functioning. With respect to the aforementioned problems, the major contributions of this thesis are:

1. An amperometric glucose biosensor was developed, using simplified "single-step" electrochemical method, where composite layers consisting of Prussian Blue, Polypyrol and Glucose Oxidase (PB-Ppy-GOx) were electrochemically deposited on the graphite electrode.
2. For the first time it was demonstrated that the proposed composite layer (PB-Ppy-GOx) could be deposited using the "single-step" method not only on the surface of graphite, but also on the surface of the titanium electrode, thus expanding the application of biosensor due to unique properties of titanium such as good chemical stability, non-toxicity and, above all, bio-compatibility, which is required for development of medical devices and/or implants.
3. The composite PB-Ppy-GOx layer is suitable for the registration of amperometric response towards glucose at a relatively low electrode potential of 0.05 V *vs* Ag|AgCl|$KCl_{sat.}$, thus avoiding the influence of the oxidative current of other electrochemically active materials which can be oxidized and their oxidation current can affect the results of analysis.
4. The electrochemical properties of the glucose biosensor formed on the graphite electrode based on the composite layer of PB-Ppy-GOx were investigated by using the method of fast Fourier transform electrochemical impedance spectroscopy (FFT-EIS). The charge transfer rate and diffusion limiting parameters before and after modification with composite layer were determined and analysed.

Statements for defense:

1. Using „single step“ procedure the modification of graphite electrode by polypyrrole, Prussian blue and glucose oxidase based composite layer (Ppy-PB-GOx) can be applied in the design of glucose biosensor.
2. Optimal potential for the registration of amperometric response towards glucose of the designed Ppy-PB-Gox layer-based electrodes is at +0.05 V *vs* Ag|AgCl|$KCl_{sat.}$.
3. Electrochemical parameters of glucose biosensor based on PB-Ppy-GOx bioselective layer can be determined by using FFT-EIS method and used for evaluating electrocatalytic activity of biosensor toward glucose detection.
4. The titanium electrode can be used to form glucose biosensors by spontaneous and electrochemical deposition of PB and immobilization of GOx by both during electrochemical polypyrrole synthesis and using glutaraldehyde vapor deposition.

1. LITERATURE REVIEW

Glucose biosensors

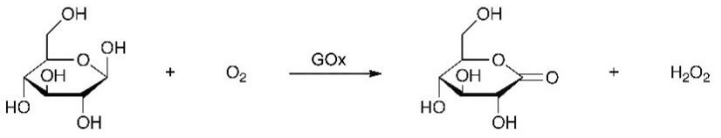
Diabetes mellitus is a group of diseases, characterized by disorders of insulin secretion [1]. Nowadays the amount of people who have diabetes mellitus is growing and it is prognosticated that by 2035 more than 592 million people in the world will have this disease [2]. The early diagnosis of diabetes mellitus is an important way that can help to protect people who have this disease from complication of diabetes mellitus [2,3,4]. By the diagnosis of diabetes mellitus and in controlling the course of this disease the concentration of glucose is determined in patient blood sample [5,6,7]. Glucose biosensors are progressive instruments for analysing glucose concentration and they are hoped to be developed to achieve better accuracy, specificity and reliability in glucose sample analysis [8].

A biosensor is an analytical device that consists of confident biological component in conjunction with a transducer device that converts a biochemical signal into an amplified electrical signal [9,10,11]. Recently biosensors play an important role in the greatly expanding area of clinical diagnostics. Enzymatic biosensors belong to one of most frequently used type of biosensors, which are based on immobilized enzymes [12]. Out of six classes of enzymes the calls of oxido-reductases is the most suitable for the design of oxidation-reduction reaction based electrochemical biosensors. Oxidases belongs to one of the most important subtype of oxido-reductases, – these enzymes are oxidizing their specific substrates in the presence of molecular oxygen and are leading to the formation of hydrogen peroxide as a reaction product [13,14].

Various biosensor devices, including electrochemical, optical/visual, silica, glass and nanomaterials-based biosensors, were developed for specific purposes [15,16,17,18,19,20,21]. Among the numerous processes and methodologies for creating new glucose biosensors, electrochemical techniques show lower detection limit, faster response time, better long term stability and inexpensiveness [22,23,24]. Electrochemical biosensors are complex analytical devices based on a combination of a specific biological element, and a transducer, which translates this biorecognition signal into an appropriate electrical signal [25,26]. An amperometric biosensor is a type of electrochemical biosensor, which measures the current that arises on a

working electrode by direct electrochemical oxidation or reduction of products formed during the biochemical reaction [27,28,29]. These biosensors are widely used in clinical diagnostics, environment monitoring, food analysis, drug detection and industrial purposes because they are reliable, highly sensitive and comparably cheap [30]. Electrochemical glucose biosensors are typically prepared by modifying the surface of metal and carbon electrodes using biomaterials, such as enzyme glucose oxidase (GOx). Challenging problem in the development of glucose sensors is selection of the most suitable substrate for the formation of bio-electroactive layer. The main efforts of investigators are concentrated on the selection of the electrode material, which should ensure high electrochemical activity in parallel to chemical and mechanical stability. In recent years, titanium oxide [31,32] has been widely used in the formation of biosensors for the determination of glucose [33,34,35,36,37], cholesterol [38,39], DNA [40, 41].

One of the major groups of electrochemical biosensors is based on the principles of amperometric measurements [42]. Most of the electrochemical glucose biosensors are based on the glucose oxidase (GOx) enzyme, which catalyzes the oxidation of glucose to gluconolactone and hydrogen peroxide (Eq. 1):



(1)

β-D-Glucose D-Glucono-1,5-lactone

The most popular way for the quantification of glucose is based on electrochemical determination of enzymatically formed H_2O_2 concentration [43, 44, 45].

The common problem in the application of amperometric glucose biosensors is the potential at which biosensors are operating [46]. In some biosensors the determination of glucose concentration in the sample is based on the registration of oxidational current of hydrogen peroxide, and that required relatively high potential of working electrode (0.7 V *vs* $Ag|AgCl|KCl_{sat.}$). At such high potentials some other electrochemically

active materials can be also oxidized and their oxidation current can affect the results of analysis [14,47,48]. In order to avoid these interferences glucose biosensor could be covered with some polymers layers, for example: nafion layer [49,50]. Nafion layer protect biosensor from electrochemically active anionic molecules, for example; uric acid and ascorbic acid – these molecules cannot reach the electrode and be oxidized [51]. Despite that, the potential of biosensor coated with nafion still should be relatively high, whenever it is known that low potential kept during analysis shows advantageous effect for cells proliferation [52].

Hydrogen peroxide, which is product of oxidase-catalysed reactions, can be easily determined electrochemically, but such determination on not-modified electrodes is possible only at relatively high positive electrode potentials in the range of +400 to +900 mV. Such high potential reduces the selectivity of biosensors due to direct oxidation of some interfering compounds, which is induced at higher positive potentials. Therefore, the modification of electrodes by redox mediators and some redox-able compounds, which are capable to reduce or oxidize formed hydrogen peroxide at low potential, is suitable way for the solution or at least reduction of this problem.

Prussian blue as redox mediator

In oxidase-based biosensing systems metal-ion-coordinated hexacyanoferrates have been widely used as redox mediators [29]. Among many such compounds ferric hexacyanoferrate – Prussian blue (PB) – has very attractive redox properties. It was determined that the PB forms electroactive layers after electrochemical or chemical deposition onto the electrode surface, it has opened a new area in application of this compound in oxidase-based amperometric biosensors [53,54,55,56,57,58,59], e.g. PB and glucose oxidase (GOx) based modified electrodes were successfully applied in the design of flow-injection biosensors, which were used for the determination of residual glucose concentrations in wines [60]. Therefore the ability of Prussian blue, to reduce hydrogen peroxide very sensitively and selectively by selective reduction hydrogen peroxide in the presence of oxygen is of very high importance in design of amperometric oxidase-based biosensors [61]. The redox activity of Prussian blue (PB), towards hydrogen peroxide, which is reduced by PB is of high importance in

bioelectroanalytical chemistry [61,62,63]. Due to redox and catalytic activity PB and some its analogues have found a number of applications in electro-analytical devices and bioelectrochemical systems [14], e.g.: very stable hydrogen peroxide transducers were made by sequential deposition of the iron- and nickel-hexacyanoferrate layers; The layers based on both compounds demonstrated superior chemical and mechanical stability, therefore they are suitable for the development for glucose sensors [61]. PB is mostly used in the forms of electroactive layers, which could be chemically or electrochemically deposited onto the electrode surface [14]. Electrochemical deposition of PB on conductive surfaces is more efficient since it could be easily controlled by switching on/off electrical current. This procedure is usually carried out in aqueous solutions containing a mixture of Fe^{3+} and ferricyanide ($[Fe(CN)_6]^{3-}$) ions [64], either spontaneously at open-circuit regime or by applying a reductive electrochemical driving force [64,65,66]. Moreover, the electrochemical formation of PB and homologous compounds allows the modification of microelectrodes and in this way some most promising structures consisting of alternating pattern of three layers of PB and three layers of Ni-HCF were applied for the modification of ultra-micro electrodes, which were used as sensors in scanning electrochemical microscopy based imaging of local hydrogen peroxide evolution in oxidase-based systems [67].

Besides Prussian blue (PB), which is indeed ferric hexacyanoferrate, deposits of different metal hexacyanoferrates (such as copper (II), cobalt (II), nickel (II), etc.) potentially can be also used as an electrocatalytic properties promoting material in development of chemical sensors and biosensors [14,68,69,70,71,72,73,74,75]. However, the mechanism of hydrogen peroxide reduction catalysis by transition metals hexacyanoferrates is not discovered yet. Moreover, it is claimed that the electrocatalytic activity of non–iron transition metals hexacyanoferrates occurs due to presence of some Prussian blue in the crystal lattice of these hexacyanoferrates [76,77,61]. Recently Sitnikova et. al. has developed hydrogen peroxide sensor in which Prussian blue was deposited together with Ni hexacyanoferate by using layer-by-layer deposition of Fe and Ni ion based hexacyanoferrates [78]. This multi-layered structure exhibits significantly improved tolerance to alkaline conditions and has increased stability in time, comparing with the bare Prussian blue layer.

Therefore, the application of different metal hexacyanoferrates in modified electrodes has been extensively studied [76,77,78] and these materials are becoming used as redox mediators in biosensors based on oxidase-type enzymes as the biological recognition element.

Immobilization of enzymes

During the development of continuously operating biosensors based on GOx this biological recognition component is mostly immobilised on the surface of signal transducer. The most appropriate way to immobilize redox enzymes in electrochemical biosensors is based on their immobilization in such manner, which would allow efficient electron transfer between the redox centre of enzyme and the electrode [79, 80]. To increase the electrochemical properties of electrodes, various conducting polymers have been used [81]. It has been reported that composite films based on Au or Pd nanoparticles [82], Pt [83], metal oxides [84,85,86,87], carbon nanotubes [88,89] in combination with polymers are of high interest due to their exceptional applicability in areas such as catalysis, batteries, sensors, etc. Many different polymers such as polypyrrole (Ppy), polyaniline, polyphenol, polyvinyl alcohol etc. have been used for the electrochemical entrapment of GOx [90,91]. In many biosensors the polymer polypyrrole has been used as a matrix for the immobilization of GOx [92,93,94,95,96,97,98] because this polymer has some advantages such as good stability under ambient conditions, some electric conductivity, which allows applying it as ‘an electron relay’. Moreover it is possible to deposit Ppy layer from aqueous solutions of pyrrole monomers at neutral pH and at ambient ‘chemically mild’ conditions [99].

The challenging problem in the development of bioelectrochemical systems is proper immobilization of biocatalysts [100]. However, this procedure is not always successful due to very different natures of electrode material and protein-based biocatalysts, which are selected for the immobilization. Conducting polymers could be applied in the design of biosensors as a matrixes for the immobilization of enzymes and by this method electrocatalytic properties of the enzymes immobilized within layer of conducting polymers could be tuned and adjusted according to the requirements of electroanalytical system [101,102,103]. Moreover conducting polymers, such as polypyrrole (Ppy), allows co-entrapment of

additional redox compounds and/or various nano-structures (e.g. gold nanoparticles, carbon nanotubes, etc.) that are facilitating the development of electroanalytical signal [104,105]. Recent attempts to create Ppy-PB composites demonstrated that such composites are very suitable for the reduction of hydrogen peroxide [106,107,108,109]. Ppy-PB composite could be very promising for oxidase-based biosensor design, because such biosensors are based on generation/detection of hydrogen peroxide. Among hundreds of recently used oxidases the glucose oxidase (GOx) is mostly used as a model enzyme, which could be applied in bioanalytical systems and biosensors [110].

2. MATERIALS AND METHODS

Chemicals

Glucose oxidase (360 U mg^{-1}), glucose and all inorganic salts - $FeCl_2 \cdot 4H_2O$, $FeCl_3 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $K_4[Fe(CN)_6]$, $K_3[Fe(CN)_6]$ of 'highest purity' were purchased from ROTH (Germany). Pyrrole was purchased at Alfa Aesar (Germany) and it was purified as described in previous researches [101,104]. The buffer solution consisting of 0.1 M NaCl and 0.01 M NaH_2PO_4, pH 7.3, was used in electrochemical experiments. All experiments were carried out using distilled and deionized water (Milli Q-plus-Millipore system).

Modification of graphite electrode with bioselective layer based on Prussian blue, polypyrrole and glucose oxidase

The graphite rods of 6 mm diameter and of 0.28 cm^2 geometric areas were electrochemically modified with Prussian blue, polypyrrole and glucose oxidase bioselective layer (PB-Ppy-GOx) using single-step based procedure. Before the modification with PB-Ppy-GOx layer the surface of the graphite rods was mechanically polished until a 'mirror finish' was observed and then electrodes were treated by ultrasound in an ultrasonic bath for 3 min. After this the graphite rods were placed in electrochemical cell filled by solution containing 1 mM of $FeCl_2$, 1 mM of $K_4[Fe(CN)_6]$, 1 mg mL^{-1} of glucose oxidase and pyrrole, which concentration was varying in the range of 10 – 40 mM. From this solution the composite layer of PB-Ppy-GOx was electrochemically deposited on the graphite electrode while applying 20 cycles of potential in the range from +400 to +800 mV *vs* $Ag|AgCl|KCl_{sat}$. at sweep rate of 40 mV s^{-1}.

In order to obtain PB-NiHCF-Ppy-GOx or PB-CoHCF-Ppy-GOx layers, the graphite rods were placed in an electrochemical cell filled by solution containing 1 mM of $FeCl_2$, 1 mM $CoCl_2 \cdot 6H_2O$ or 1 mM $NiCl_2 \cdot 6H_2O$, 2 mM of $K_4[Fe(CN)_6]$, 1 mg mL^{-1} of glucose oxidase and 30 mM of pyrrole. The electrolysis in these solutions resulted in the formation of the corresponding composite layers of PB-NiHCF-Ppy-GOx or PB-CoHCF-Ppy-GOx. The mentioned composite layers were electrochemically

deposited by 20 potential cycles in the range from +400 to +800 mV at potential sweep rate of 40 mV s^{-1} *vs* Ag|AgCl|KCl_{sat}. In case of PB-NiHCF-Ppy-GOx and PB-CoHCF-Ppy-GOx layers the response of biosensors to a different glucose concentration was studied within the temperature range from 15 ℃ to 30 ℃. The temperature was controlled in water bath from JULABO (Germany).

Electrochemical equipment and registration of electrochemical signals

Potentiostat/galvanostat μAUTOLAB from ECO-Chemie (Utrecht, The Netherlands) was used for the modification of graphite electrodes by electrochemical deposition of PB-Ppy-GOx, PB-NiHCF-Ppy-GOx, PB-CoHCF-Ppy-GOx based layers and for all chronoamperometric investigations. All experiments were performed in the electrochemical cell in three electrode based mode, where: (i) a graphite rod was used as working electrode, (ii) a platinum wire was served as an auxiliary electrode; (iii) Ag|AgCl|$KCl_{sat.}$ electrode was used as a reference electrode, which was placed as close as possible to the working electrode using 'Luggin capillary' connector. Amperometric responses of PB-Ppy-GOx-modified working electrode and PB-NiHCF-Ppy-GOx or PB-CoHCF-Ppy-GOx-modified electrodes were registered at 0.05 V *vs* Ag|AgCl|$KCl_{sat.}$ in electrochemical cell filled by 0.1 M phosphate buffer, pH 7.3. Before each electrochemical measurement electrochemical system was allowed to reach stable value of background current and only then calculated amounts of 1 M glucose solution were added into the cell, which was stirred by magnetic stirrer, in order to get inside the cell glucose solution of required concentration. Then the resulting current of working electrode was recorded and the difference of registered current from initial current, which was observed before any addition of glucose, was evaluated as analytical signal of biosensor.

In order to evaluate the stability of the GE-PB-Ppy-GOx electrodes the amperometric measurements were repeated three times: (i) immediately after the preparation of electrode, (ii) after 24 hours and (iii) after 5 days. Between the measurements the electrode was stored above the phosphate buffer, pH 7.3 at 4℃.

AFM-based imaging of electrode surface

The roughness of pristine and modified graphite electrodes was evaluated using atomic force microscope BioscopeII/Catalyst from Veeko (Santa Barbara, USA). The Scan Asyst based on Peak Force Tapping mode equipped with the conventional silicon Scan Asyst Air AFM tip (resonance frequency 70 kHz, spring constant 0.4 N m^{-1}) was used for AFM-based imaging. Surface root mean square (RMS) values were calculated using NanoScope Analysis program provided by Veeko (Santa Barbara, USA).

FFT-EIS based evaluation of electrodes

All electrochemical impedance spectra were recorded under the potentiostatic setting using a fast Fourier transformation based electrochemical impedance spectrometer EIS-128/16 constructed by prof. G. Popkirov (University of Kiel, Germany) [111]. The peak-to-peak amplitude of sinusoidal perturbation voltage was 10 mV. The obtained impedance spectra were validated by comparing the power spectra of perturbation and response as it is described previously [112]. Such method enables to reduce the duration of collection of impedance spectra 5–20 times when compared with conventional EIS equipment. The overall measurement duration using FFT-EIS is limited only by the lowest perturbation frequency of the spectrum and by the data transfer to the computer. In these FFT-EIS experiments we have chosen the frequency range between 1.5 Hz and 25 kHz ensuring minimal distortions of response signal. Thus, the duration of one electrochemical impedance spectrum registration lasted for 1.3 s. EIS data were evaluated and measured data fitted with that calculated applying the most appropriate equivalent circuits using for all these procedures specialized data acquisition program “Zview”.

Modification of titanium plate based electrodes with bioselective layer

Titanium plates of 2 mm thickness and of 1 cm^2 geometric area were modified by bioselective layer using four different techniques of PB layer deposition and GOx immobilization. Before the modification the surface of Ti plates was mechanically polished until a ‘mirror finish’ was observed and then electrodes were treated by sonication in deionized water for 3 min.

After this, the titanium plates were modified in the following sequence using four techniques:

1st titanium electrode modification method applied for the formation of $GOx_{(C\text{-}Link)}$-$PB_{(SP)}$/Ti electrode. The titanium plate was immersed into a solution containing 1 mM of $FeCl_3$ and 1 mM of $K_3[Fe(CN)_6]$ for 40 min and PB deposition was performed spontaneously (non-electroplating method). After PB deposition, thermal treatment was used: PB/Ti electrode was heated at 100 °C for one hour, and then cooled to the room temperature and washed with millipore water. GOx was immobilized by dropping 10 μl of GOx solution (40 g l-1 glucose oxidase solution made in pH 7.3 phosphate buffer) on the surface of PB coated titanium plate, drying the electrode while having it attached above a solution of glutaraldehyde (25 %) for 15 min.

2nd titanium electrode modification method applied for the formation of $GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti electrode. The titanium plate was placed in electrochemical cell filled with solution containing 1 mM of $FeCl_3$ and 1 mM of $K_3[Fe(CN)_6]$, and electrochemical deposition of PB was carried out by potential cycling and monitored in cycling voltammograms. 40 potential cycles in potential range between +0.4 V and +0.8 V *vs* Ag|AgCl|KCl_{sat} at a scan rate of 40.0 mV s^{-1} were applied. Electrochemically coated with PB layer titanium electrode was thermally treated and modified, immobilizing GOx with vapour of glutaraldehyde ($GOx_{(C\text{-}Link)}$) as described in the *1st method*.

3rd titanium electrode modification method applied for the formation of Ppy-GOx-$PB_{(SP)}$/Ti electrode. The titanium plate was coated with PB layer spontaneously and thermal treatment was used as described in the *1st method*. Then immobilization of GOx by electrochemical deposition of polypirrole (Ppy) was performed. The composite layer of Ppy, PB and GOx was deposited by cyclic voltammetry in solution containing 1 mM of $FeCl_3$, 1 mM of $K_3[Fe(CN)_6]$, 30 mM of pyrrole and 1 mg ml^{-1} of GOx. 20 cycles in potential range between +0.4 V and +0.8 V *vs* Ag|AgCl|$KCl_{sat.}$ at a scan rate of 40.0 mV s^{-1} were applied in order to create the most optimal layer of Ppy-GOx-PB.

4th titanium electrode modification method applied for the formation of Ppy-GOx-$PB_{(ElCh)}$/Ti electrode. The titanium plate was coated with PB layer electrochemically as described in the *2nd method*, and thermal treatment was

also applied as it is described in the above techniques. Immobilization of GOx by electrochemical deposition of polypyrrole was performed as described in the *3rd method*.

Scanning electron microscopy

Samples of modified electrodes were examined using Hitachi SEM TM3000 scanning electron microscope equipped with tungsten filament and silicon drift detector (SDD). Three types of graphite electrodes covered with bioselective composite layers (i) PB-Ppy-GOx, (ii) PB-NiHCF-Ppy-GOx or (ii) PB-CoHCF-Ppy-GOx were fixed on the sample holder with carbon conductive adhesive tape. Energy-dispersive X-ray spectroscopy technique was used to determine elemental composition of the samples. To stimulate the emission of characteristic X-rays from a specimen, a high-energy beam of electrons was focused in an area of 4 mm^2 of the sample Constituting elements were identified and quantified by respective K_α, K_β lines.

3. RESULTS AND DISCUSSION

Single-step based modification of graphite electrode by PB-Ppy-GOx layer

PB-Ppy-GOx composite layer was deposited by one-step based electrochemical procedure (Fig. 1). By this procedure PB was formed directly within growing layer conducting polymer – Ppy – and contemporaneously the enzyme was immobilized within formed pyrrole. The deposition of PB on various conductive surfaces is usually carried out from aqueous solutions containing a mixture of iron(III) (Fe^{3+}) and ferricyanide ($[FeIII(CN)_6]^{3-}$) ions [14]. However, in order to keep higher activity of immobilized GOx the solution of $FeCl_3$ could not be used because it is stable only at relatively low pH, which is harmful for GOx. Therefore during here proposed deposition in initial solution we have replaced both in synthesis of PB traditionally used $FeCl_3$ and $K_3[Fe(CN)_6]$ by $FeCl_2$ and $K_4[Fe(CN)_6]$, respectively. Therefore in here proposed PB synthesis protocol the Fe^{3+} ions, which are required for the synthesis of PB, were formed in close proximity to the electrode by oxidation of $FeCl_2$ when sufficiently high electrode potential was reached during the potential cycling. PB was produced according to this reaction:

$$4Fe^{3+} + 3[Fe(CN)_6]^{4-} \rightarrow Fe_4[Fe(CN)_6]_3 \quad (2)$$

Simultaneously to the formation of PB pyrrole monomers also started to form the Ppy layer at higher oxidative potentials and under chemical polymerization induced by formed Fe^{3+} ions. Moreover the GOx become entrapped within formed Ppy layer in close proximity to the PB formed on the electrode surface. For the PB it is very beneficiary to be in close range to immobilized GOx, because then higher concentrations of hydrogen peroxide, which is formed in catalytic action of GOx, are reaching the surface of PB particles and significantly higher surface area of PB is capable to be involved into catalytic action.

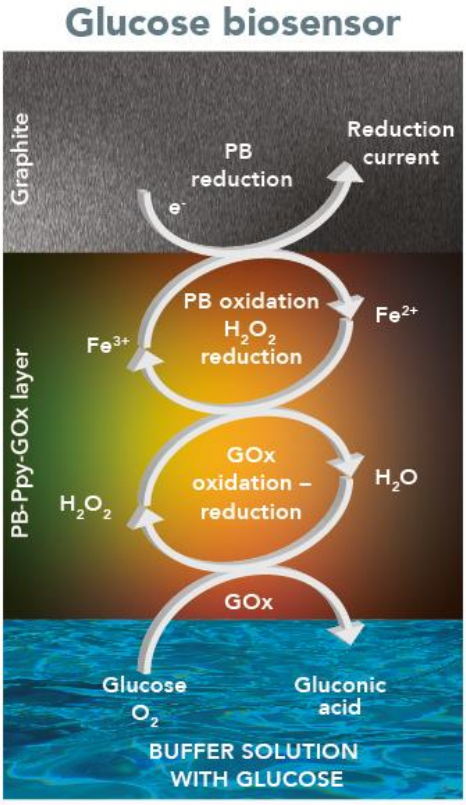


Fig. 1. Schematic representation of single-step based modification of graphite electrode by PB-Ppy-GOx layer.

The determination of optimal electrode potential for the registration of analytical signal

After the modification of graphite electrodes by PB-Ppy-GOx layer cathodic polarization curves were registered in order to determine optimal electrode potential for further amperometric measurements. The polarization curves were registered in buffer solutions: (i) without glucose (Fig. 2, curve 1) and (ii) with 2 mM of glucose (Fig. 2, curve 2). Figure 2 shows that relatively low anodic potentials, which start from +0.15 V *vs* Ag|AgCl|$KCl_{sat.}$ can be chosen for the determination of analytical signal

towards glucose. However it is known that during the determination of glucose in real samples the interfering effects by some materials (e.g., ascorbic acid, uric acid), which are mostly present in body fluids at varying concentrations, are more distinct at higher positive potentials [113]. Due to this fact the much lower potential value of +0.05 V *vs* Ag|AgCl|$KCl_{sat.}$ was chosen as the most optimal potential for further experiments by PB-Ppy-GOx-modified graphite electrode. The variation of cathodic polarization curves (Fig. 2, curve 1-2) shows that selected electrode potential of +0.05 V *vs* Ag|AgCl|$KCl_{sat.}$ is well suitable for this kind of electro-analytical processes. However, lover potentials were not suitable due to much lower stability of PB at electrode potentials below 0 V *vs* Ag|AgCl|$KCl_{sat.}$ [14,114].

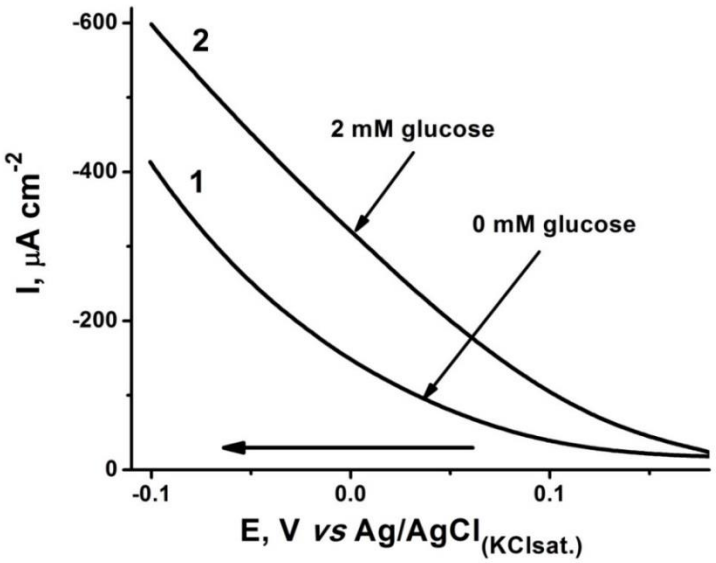


Fig. 2. Cathodic polarization curves of graphite electrode modified by PB-Ppy-GOx layer registered in 0.1 M phosphate buffer solution, pH 7.3, without glucose (curve 1) and with 2 mM of glucose (curve 2). Potential sweep rate was 0.002 V s^{-1}. PB-Ppy-GOx layer was formed on the graphite electrode by potential cycling in solution containing 1 mM of $FeCl_2$, 1 mM of $K_4[Fe(CN)_6]$, 1 mg mL^{-1} of glucose oxidase, and 20 mM of pyrrole.

The effect of initial pyrrole concentration on the sensitivity of PB-Ppy-GOx-modified graphite electrodes

In order to investigate the influence of ratio between formed PPy and entrapped GOx in the PB-Ppy-GOx composite, several different PB-Ppy-GOx layers were formed from solutions with different initial pyrrole concentrations.

Four different initial concentrations (10; 20; 30; 40 mM) of pyrolle were used for the modification of graphite electrodes by PB-Ppy-GOx layer. From amperometric responses of PB-Ppy-GOx - modified graphite electrodes towards different glucose concentrations the calibration curves were derived (Fig. 3 A). The sensitivity of the biosensor was calculated as a function of current density response towards different glucose concentration, and it was found to be in a range of 1.0 - 1.9 μA cm^{-2} mM^{-1}. Kinetic parameters such as Michaelis constant (K_M) and maximal reaction rate (v_{max}), which is a direct function of maximal electrode current (I_{max}) for the PB-Ppy-GOx -modified graphite electrodes were calculated using Lineweaver-Burk plot (Fig. 3 B) or hyperbolic Michaelis-Menten function (Eq. 3):

$$v = (v_{max} \cdot [S])/(K_M + [S]) \qquad (3)$$

where [S] is the concentration of a substrate, v_{max} is maximal reaction rate achieved in the system, which is directly proportional to maximal electrode current (I_{max}), at maximal substrate concentration, K_M is Michaelis constant.

Determined K_M values were in line with that reported for electrodes based on GOx immobilized within polymeric layers, e.g. the K_M for GOx based electrode, in which the GOx was entrapped within polyaniline-polyvinylsulfonate film the K_M was reported as 0.186 mM [43]. Moreover, K_M values of GOx were 18.0, 11.9, 9.34 mM reported, respectively [115,116,117].

Results represented in figure 3 shows that the highest observed response of PB-Ppy-GOx layer towards glucose was formed from the solution containing 30 mM of pyrrole, because at lower pyrrole concentration the thickness of layer and therefore the amount of immobilized enzyme was

lower in comparison with that immobilized when 30 mM of pyrrole was present in the solution, which was used for the polymerization. The response current densities of such biosensor showed a 1.9 μA cm^{-2} mM^{-1} sensitivity in a linear range of the calibration curve (Fig. 3 A, curve 3). However further increase of pyrrole concentration and therefore increased thickness of formed Ppy layer created additional diffusional limitations for glucose that has significantly influenced the decrease of registered electrode current.

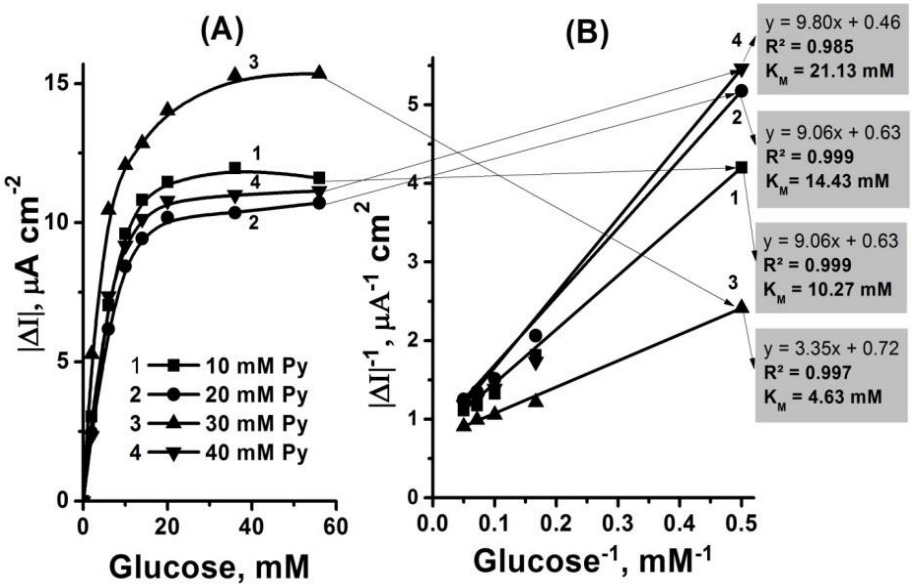


Fig. 3. Amperometric responses of PB-Ppy-GOx -modified graphite electrodes towards glucose registered at 0.05 V *vs* Ag|AgCl|$KCl_{sat.}$ in phosphate buffer, pH 7.3, at 25 °C. (**A**) - ΔI *vs* concentration plot for graphite electrodes modified in presence of (■) 10 mM (curve 1), (●) 20 mM (curve 2), (▲) 30 mM (curve 3), (▼) 40 mM (curve 4) of pyrrole. (**B**) – the same data in reciprocal coordinates (Lineweaver-Burk plot).

Surface roughness evaluation of PB-Ppy-GOx modified graphite electrode

Atomic force microscopy is a material characterization technique, which can operate in a liquid conditions, allowing its use for the investigation of the mechanical properties of biological materials and plays an important role

for the evaluation of topology and materials properties [118,119,120]. Registered AFM images of pristine graphite electrode and modified with bioselective layer of PB-Ppy-GOx are depicted in figure 4 and the calculated values of RMS are summarized in table 1. PB-Ppy-GOx-modified electrodes were investigated before and after amperometric determination of glucose concentration by GE/PB-Ppy-GOx electrode. As it could be seen from the results (Table 1), the polished pristine GE surface is mostly smooth in comparison to other here investigated surfaces (the RMS value of pristine GE surface was around 4 nm). After the modification of the electrode with composite consisting of PB, Ppy and GOx, the surface becomes rougher (RMS was around 15-19 nm). Up to 30 mM of initial pyrrole concentration the surface roughness did not show much change due to the increase of the monomer concentration. However, using 40 mM of pyrrole solution for the modification of electrodes the roughness and RMS has increased almost up to 32 nm. This might be because the higher concentration of pyrrole resulted in the formation of larger micelles of the monomer in the solution and the larger polymer aggregates on the surface of the electrode [97,121]. After amperometric measurement of glucose concentration by GE/PB-Ppy-GOx electrode the surface became smoother for almost all tested electrodes. Only for the electrode modified by PB-Ppy-GOx layer in the presence of 20 mM of pyrrole the minor increase of roughness was observed. This indicates that during the amperometric measurements of glucose concentration by GE/PB-Ppy-GOx based electrode some parts of the PB-Ppy-GOx layer that are not strongly attached either are washed away or are rearranging into smoother structures. Therefore, for further experiments 30 mM of initial pyrrole concentration was used for the modification of graphite electrodes.

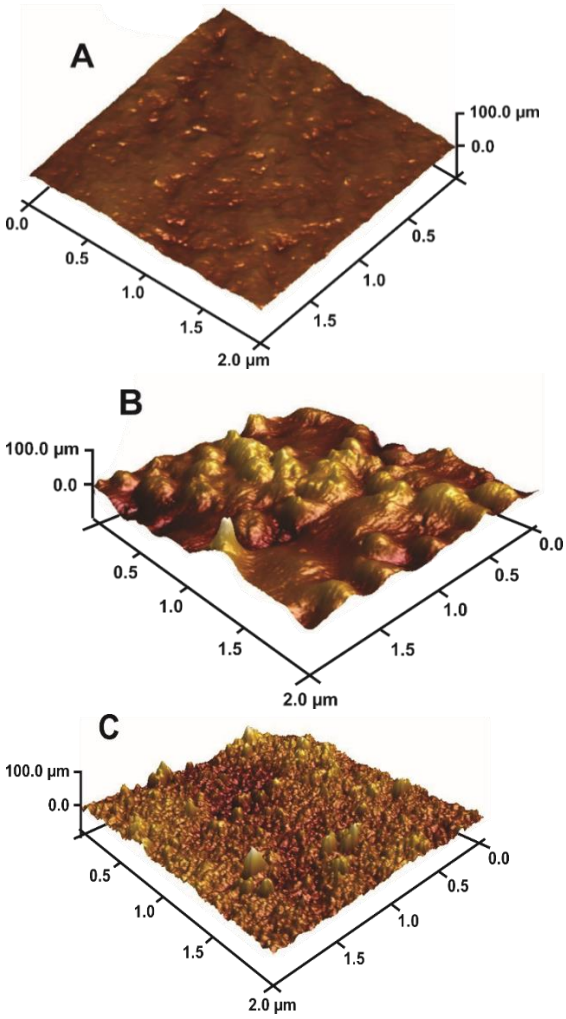


Figure 4. Surface topography of: **A** – polished graphite rod electrode; Graphite electrode modified by PB-Ppy-GOx layer in a solution containing 1 mM of $FeCl_2$, 1 mM of $K_4[Fe(CN)_6]$, 1 mg mL^{-1} of glucose oxidase, and 30 mM of pyrrole; **B** – before and **C** – after amperometric measurements of glucose concentration by GE/PB-Ppy-GOx based electrode.

Table 1. The RMS values of pristine and modified graphite electrodes before and after amperometric measurements of glucose concentration by GE/PB-Ppy-GOx based electrode.

Sample	Concentration of pyrrole used in electrochemical modification of graphite electrode, mM	RMS, nm	
		Before amperometric measurements	After amperometric measurements
Graphite electrode modified with PB-Ppy-Gox layer	10	19.4	14.0
	20	19.1	21.6
	30	15.2	12.8
	40	31.8	21.5
Pristine graphite electrode	--	3.9	-

FFT-EIS-based evaluation of PB-Ppy-GOx modified graphite electrode

The interfacial characteristics of the pristine graphite electrode and PB-Ppy-GOx modified graphite electrodes (GE/PB-Ppy-GOx) before and after amperometric determination of glucose concentrations were studied by FFT-EIS. The experiments were carried out at operating potential of the biosensor of +0.05 V *vs* Ag|AgCl|$KCl_{sat.}$ (see section 3.1). The spectra recorded in phosphate buffer, pH 7.3, are shown in figure 5.

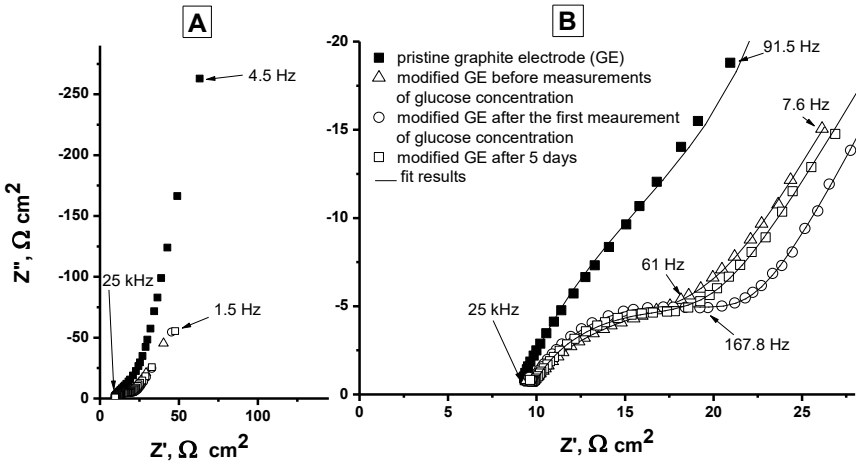


Fig.5. A – comparison of Nyquist plots in buffer solution obtained at +0.05 V potential *vs* Ag|AgCl|KCl_{sat} for pristine graphite electrode (filled squares) and for GE modified with PB-Ppy-GOx layer in a solution containing 1 mM of $FeCl_2$, 1 mM of $K_4[Fe(CN)_6]$, 1 mg mL^{-1} of glucose oxidase, and 30 mM of pyrrole before measurements of glucose concentration, i.e. immediately after the preparation (open triangles); after the first measurement of glucose concentration (open circles), and after storing the electrode above the buffer solution at 4°C for 5 days and measuring glucose concentration (open squares). **B** – extension of selected (high frequency) region.

The experimental FFT-EIS data were fitted using equivalent circuit (Fig. 6B) based on the Randle's model where polarization is based on a combination of both kinetics and diffusion limited processes. This equivalent electrical circuit, used for fitting the recorded electrochemical impedance spectra obtained for both pristine and modified GE, is in agreement with the literature data [122,123,124,125].

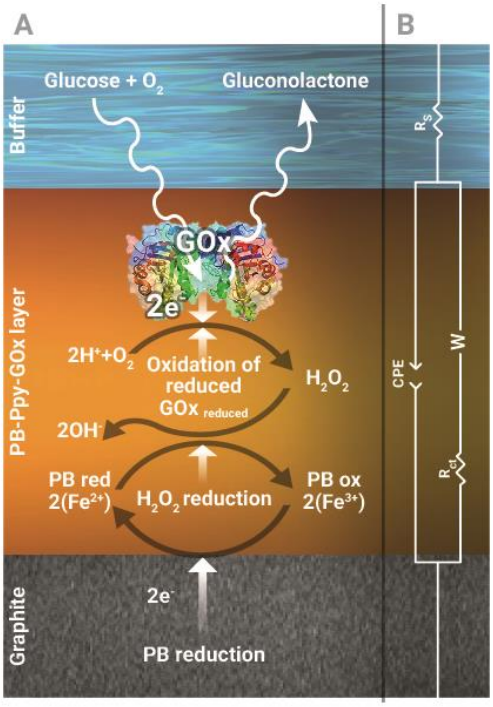


Fig. 6. A - schematic representation of glucose biosensor based on graphite electrode modified by Prussian blue, polypyrrole and glucose oxidase. **B** - equivalent electrical circuit used for fitting of EIS data obtained for pristine graphite and PB-Ppy-GOx modified graphite electrode: $\boldsymbol{R_s}$ – electrolyte solution resistance; ***CPE*** – constant phase element; $\boldsymbol{R_{ct}}$ – charge-transfer resistance; W – Warburg impedance.

The charge transfer resistance, $\boldsymbol{R_{ct}}$, in parallel with a constant phase element, ***CPE***, depends on the distribution of insulating and dielectric features at the electrode/electrolyte interface, respectively. Constant phase element, ***CPE***, in the equivalent circuit represents non-ideal capacitor, which is most probably based on the inhomogeneity of the interfaces. According to reference [126], the impedance of ***CPE*** can be defined as:

$$\boldsymbol{Z_{CPE} = A^{-1} (j\omega)^{-n}} \qquad (4)$$

where: $\boldsymbol{A}$ is the proportionality factor of the constant phase element and its physical meaning corresponds to the average capacitance of double layer; $\boldsymbol{n}$ is the exponential number, which shows the phase shift of impedance *vs* applied perturbation. When the electrode surface is homogenous, then $\boldsymbol{n}$ equals to 1 and the equation (4) can be transformed into simple double layer capacitor impedance equation:

$$\boldsymbol{Z_{CPE}} = (\boldsymbol{C_D j\omega})^{-1} \qquad (5)$$

We have got satisfactory coincidence of experimental data (Fig.5, symbols) with results calculated using equivalent circuit (Fig.5, line). Values of electrolyte solution resistance, $\boldsymbol{R_s}$, calculated by the fitting of equivalent circuit, are varying from 9.1 to 9.6 Ω cm^2 and values of other parameters are presented in table 2.

As can be seen from table 2 the calculated CPE capacitance of pristine GE is equal to 149.6 μF cm^{-2} with exponential number of 0.79. Meanwhile, capacitance of GE/PB-Ppy-GOx electrode immediately after preparation (before measurements of glucose concentration) in comparison with pristine GE surface, increases to 860.7 μF cm^{-2} with decrease in exponential number, $\boldsymbol{n}$, to 0.64, as expected for the modified surface of high heterogeneity. Contrary to expectations, strong decrease in $\boldsymbol{CPE}$ value (152.5 μF cm^{-2}) was obtained for GE/PB-Ppy-GOx electrode after first amperometric measurement of glucose concentration. This effect can be related to significant changes in homogeneity of the electrode surface during the enzymatic oxidation of glucose, which was followed by electrochemical reduction of formed hydrogen peroxide. During these processes the rearrangement of some parts of the PB-Ppy-GOx layer into smoother structures is observed, it coincides with the decrease of RMS registered by AFM for the GE/PB-Ppy-GOx electrode formed using 30 mM of pyrrole containing solution. When GE/PB-Ppy-GOx electrode was stored at 4°C for 5 days and then applied for determination of glucose concentration (Fig. 5, open squares), then calculated CPE value increased just very slightly – up to 194.3 μF cm^{-2}. This result may be explained by the absence of significant morphological changes in the bioselective layer of GE/PB-Ppy-GOx electrode during storage period of 5 days.

Table 2. Electrochemical parameters obtained by fitting FFT-EIS data to equivalent electrical circuit, which is presented in figure 6 B.

Sample	CPE, $\mu F\ cm^{-2}$	n	R_{ct}, $\Omega\ cm^2$	W_R, $k\Omega\ cm^2$	W_T, $F\ cm^{-2}$	W_p
Pristine graphite electrode (GE)	149.6	0.79	42.8	232.2	5.7	0.95
Modified GE before measurements of glucose concentration	860.7	0.64	14.4	0.27	2.9	0.71
Modified GE after the first measurement of glucose concentration	152.5	0.76	13.5	0.27	2.9	0.71
Modified GE after 5 days and after measurements of glucose concentration	194.3	0.77	11.3	0.28	3.2	0.67

After the modification of GE with PB-Ppy-GOx layer, an increase in capacitance of freshly formed GE/PB-Ppy-GOx-modified electrode in comparison with that of pristine GE electrode was determined, with corresponding significant decrease in charge transfer resistance, $\boldsymbol{R_{ct}}$, (from 42.8 to 14.4 $\Omega\ cm^2$). This decrease of charge transfer resistance, $\boldsymbol{R_{ct}}$, denotes an increase of the interface surface area of GE/PB-Ppy-GOx -modified electrode at the interphase with the buffer solution [111]. With respect to GE/PB-Ppy-GOx -modified electrodes after their application for the determination of glucose concentration, values of $\boldsymbol{R_{ct}}$ were very similar to the values, which were calculated for GE/PB-Ppy-GOx -modified electrode, before amperometric determination of glucose concentrations. The reason for such similarity of determined $\boldsymbol{R_{ct}}$ values is related to much easier charge

transfer, which can be attributed to the lack of any tendency of degradation of PB-Ppy-GOx layer [101].

The Warburg element, $\boldsymbol{W}$, represents the impedance of diffusion. As can be seen from figure 5, the different shapes of FFT-EIS spectra were obtained for pristine GE and for modified GE/PB-Ppy-GOx electrodes before and after determination of glucose concentration. In this case, an additional explanation of differences in calculated parameters of Warburg element should be added. Namely, for pristine graphite electrode the diffusion based resistance, $\boldsymbol{W_R}$, was calculated as 232.2 kΩ cm^2 with exponential number $\boldsymbol{n}$=0.95, which is showing clear electric capacitance related nature of $\boldsymbol{W_R}$ parameter. In the case of GE/PB-Ppy-GOx electrode the calculated values of $\boldsymbol{W_R}$ significantly decreases to 0.27 kΩ cm^2 indicating conditions suitable for faster diffusion, however the Warburg exponent was calculated as 0.7 instead of 0.5, which is characteristic for linear diffusion. This indicates that diffusing ions does not follow the ordinary linear law [127]. It should be noted that interpretation of diffusion of electrochemically active ions trough PB-Ppy-GOx layer by Warburg element is complicated because the surface of electrode is not very smooth, and diffusion of a few different electrochemically active ions occurs in the system. In this case, $\boldsymbol{W}$ represents diffusion of the ions inside the bulk of PB-Ppy-GOx layer. Despite the complicate nature of diffusion features at the interface of electrode/electrolyte, the modelling of EIS data by equivalent circuit, which contains Warburg element, $\boldsymbol{W}$, is proposed in previous research [128]. The most obvious finding of these experiments, which is deducted from the evaluation of FFT-EIS data, is that the modification of GE with PB-Ppy-GOx layer causes faster charge transfer at the electrode surface with limitations of nonlinear diffusion. One unanticipated finding was that only a small variation in charge transfer resistance was observed while applying GE/PB-Ppy-GOx electrode for amperometric determination of glucose concentration. This finding preliminary suggests that electrochemical properties of GE/PB-Ppy-GOx - modified electrode do not change significantly during the use of this electrode for the determination of glucose concentrations.

Evaluation of GE/PB-Ppy-GOx electrode inactivation during the course of storage and measurements of glucose concentrations

Consequently, further experiments of amperometric responses of GE/PB-Ppy-GOx electrode towards different glucose concentration were carried out in order to evaluate the stability of this electrode and preliminary determine inactivation mechanism. Amperometric responses of GE/PB-Ppy-GOx -modified electrode towards different glucose concentration immediately after the modification of electrode (Fig. 7, curve 1), after 24 hours (Fig. 7, curve 2) and after 5 days (Fig. 7, curve 3) shows good hyperbolic behaviour, which is characteristic of enzymatic reactions. In the case if GE/PB-Ppy-GOx -based electrode will be applied in glucose biosensors the part of hyperbolas up to 5 mM can be treated as 'pseudo-linear part of this biosensor.

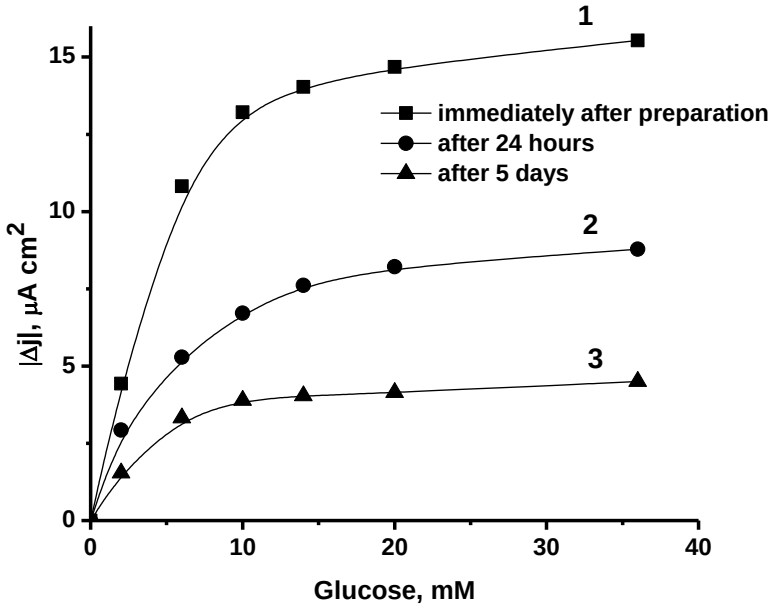


Fig. 7. Amperometric responses of GE/PB-Ppy-GOx -electrode versus different glucose concentration registered at 0.05 V *vs* Ag|AgCl|$KCl_{sat.}$ in phosphate buffer, pH 7.3 - (1) immediately after preparation, (2) after 24 hours, (3) after 5 days.

Hyperbolic dependences of GE/PB-Ppy-GOx-modified electrode responses are well confirmed by the transformation of response *vs* substrate concentration plot into reciprocal plot, which is represented in coordinates of 1/response *vs* 1/(substrate concentration) (Fig. 8). Averaged convergation area of the lines is below the X axis (Fig. 8) what indicates that inactivation of GE/PB-Ppy-GOx electrode is determined by the mixed-type of inhibition based on uncompetitive and non-competitive inhibition of GOx. This type of mixed inhibition is the characteristic of such inactivation based on both (i) complete inactivation of some fraction of enzyme and (ii) significant changes in active site of enzyme, which lead towards significant slow-down in the formation of enzyme-substrate complex and further transformation of this complex into reaction products.

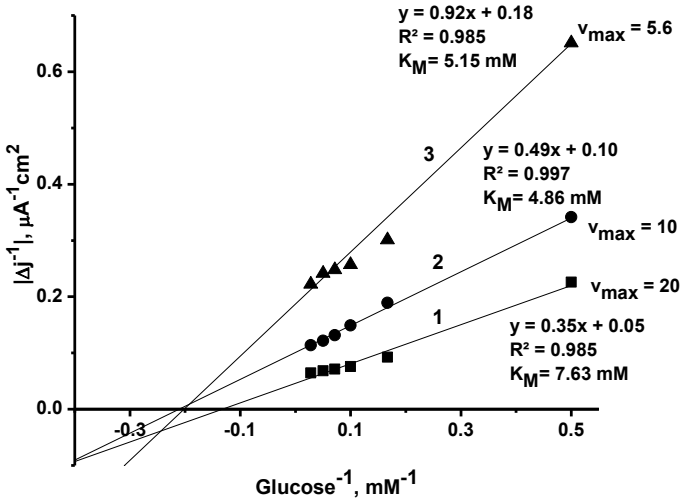


Fig. 8. Transformation of amperometric responses of GE/PB-Ppy-GOx electrode towards different glucose concentration presented in reciprocal coordinates (1) immediately after preparation, (2) after 24 hours, (3) after 5 days.

Figure 8 illustrates that both Michaelis-Menten constant, K_M, and maximum velocity of GE/PB-Ppy-GOx electrode action, v_{max}, decreases thus the inhibitor binds preferentially to the enzyme-substrate complex. It is determined by such changes of enzyme structure, which are related to deformation/denaturation/destruction of protein-based structure of enzyme,

which leads towards significant deformations/changes of active site of enzyme. This effect is slowing down the action of enzyme. Therefore these evaluations of amperometric response kinetics let us suggest that the decrease of GE/PB-Ppy-GOx electrode based biosensor signal is determined by mixed-type (uncompetitive and non-competitive) inhibition of GOx, which indicates that inhibition is based on two main factors: complete inactivation of significant fraction of the enzyme and significant changes of active site of enzyme molecules that are still remaining in action.

In order to calculate heterogeneous electron transfer rate (and heterogeneous electron transfer rate constant) from graphite electrode during the operation of PB-Ppy-GOx -based electrode in the presence of glucose, at linear range of biosensor action we have applied expression of first order heterogeneous reaction kinetics (Eq. 6):

$$V_{ET}=k_{ET}[\text{Gluc}]\ (\text{mol cm}^{-2}\,\text{s}^{-1}) \tag{6}$$

From which the heterogeneous electron transfer rate constant (k_{ET}) can be calculated in simple way:

$$k_{ET}=V_{ET}/[\text{Gluc}]\ (\text{cm s}^{-1}) \tag{7}$$

Then the data from figure 7 were recalculated in such way: (i) we have taken into account that during action of electrode registered differences of current (ΔI) can be expressed in the number of coulombs passing through the electrode during one second C/s (C s^{-1}); (ii) Then the registered current density (ΔJ), which is passing through 1 cm^2 is expressed in C s^{-1} cm^{-2}; (iii) In such way we have calculated how many moles of electrons are passing through cm^2 area of electrode during one second:

$$V_{ET}=k_{ET}[\text{Gluc}]=\Delta J\times 96500^{-1}(\text{mol cm}^{-2}\,\text{s}^{-1}) \tag{8}$$

From equation 6:

$$k_{ET}=V_{ET}/[\text{Gluc}]=\Delta J\times 96500^{-1}\times 10^{-3}\,(\text{cm s}^{-1}) \tag{9}$$

Calculated heterogeneous electron transfer rate constants (k_{ET}) for PB-Ppy-GOx-based electrode were 2.29×10^{-5}, 1.52×10^{-5}, 7.96×10^{-5} (cm s^{-1}) on the 1st, 2nd, 6th day, respectively.

Formation and evaluation of bioselective layer on metallurgical titanium plates

Modification of titanium plate based electrodes with bioselective layer is described in section 2 (Materials and Methods) of this disertation. Four different types of glucose biosensors $GOx_{(C\text{-}Link)}$-$PB_{(SP)}$/Ti, $GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti, Ppy-GOx-$PB_{(SP)}$/Ti, Ppy-GOx-$PB_{(ElCh)}$/Ti were designed by modifying metallurgical titanium plates with PB layer and subsequently immobilizing GOx. PB layer was deposited either electrochemically or spontaneously (non-electroplating method), and GOx was immobilized either electrochemically within synthesis of conducting Ppy polymer layer or using cross linking with glutaraldehyde by incubation in the vapour of glutaraldehyde [129]. Schematic representation of glucose biosensor based on titanium electrode modified by Prussian Blue, polypyrrole and glucose oxidase is presented in figure 9.

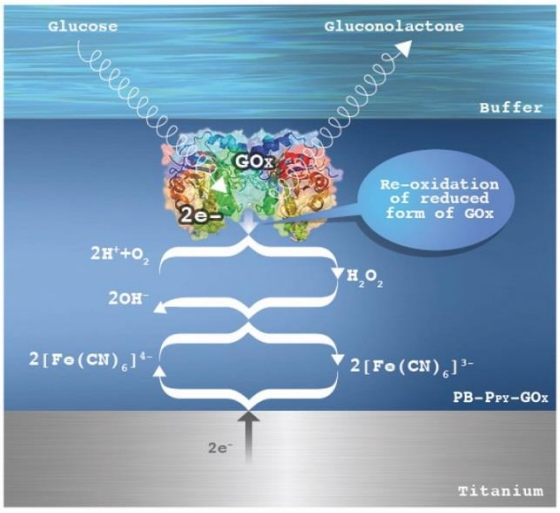


Fig. 9. Schematic representation of glucose biosensor based on titanium electrode modified by Prussian Blue, polypyrrole and glucose oxidase.

Amperometric responses of titanium-based glucose biosensors prepared in four different ways: $GOx_{(C\text{-}Link)}$-$PB_{(SP)}$/Ti, $GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti, Ppy-GOx-$PB_{(SP)}$/Ti, Ppy-GOx-$PB_{(ElCh)}$/Ti are shown in figure 10.

From figure 10 (curves 1 and 2) it is seen that cathodic current densities generated by using electrodes with spontaneously deposited PB layer (Ppy-GOx-$PB_{(SP)}$/Ti and $GOx_{(C\text{-}Link)}$-$PB_{(SP)}$/Ti) have reached the highest values of -32 and -34 μA cm^{-2} respectively. However, current densities generated by using electrodes with electrochemically deposited PB layer (Ppy-GOx-$PB_{(ElCh)}$/Ti and $GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti) have reached moderately high (-21 μA cm^{-2}) or lower (-13 μA cm^{-2}) values (Fig. 10, curves 3 and 4).

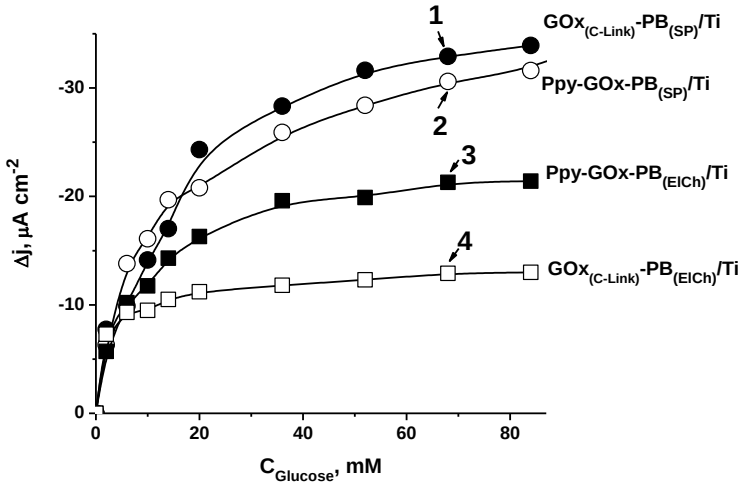


Fig. 10. Amperometric responses of (●) $GOx_{(C\text{-}Link)}$-$PB_{(SP)}$/Ti (curve 1), (○) Ppy-GOx-$PB_{(SP)}$/Ti (curve 2), (■) Ppy-GOx-$PB_{(ElCh)}$/Ti (curve 3) and (□) $GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti (curve 4) electrodes to glucose concentration in phosphate buffer, pH 7.3. Measurements were carried out at a potential of 0.05 V *vs* $Ag|AgCl|KCl_{sat.}$

It was observed that Ppy-GOx-$PB_{(SP)}$/Ti and $GOx_{(C\text{-}Link)}$-$PB_{(SP)}$/Ti electrodes showed sensitivity towards glucose in a range from 0.47 to 3.87 μA cm^{-2} mM^{-1} (Fig. 11, black and white columns). However, for Ppy-GOx-$PB_{(ElCh)}$/Ti and $GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti biosensors a lower sensitivity of 0.19 to 3.65 μA cm^{-2} mM^{-1} was obtained (Fig. 11, medium and sparse columns).

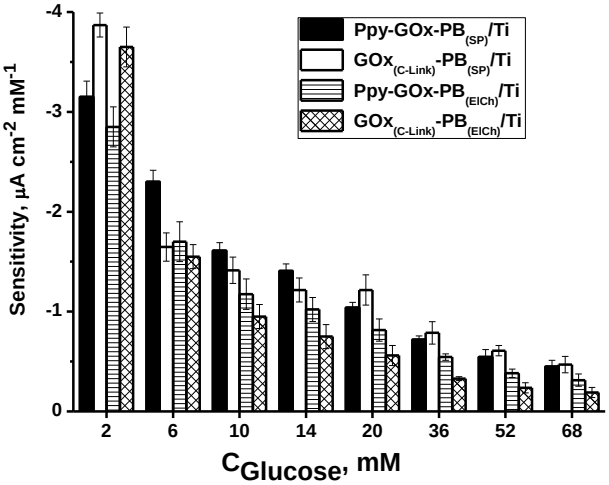


Fig. 11. Dependencies of sensitivity of Ppy-$PB_{(SP)}$/Ti (black columns), $GOx_{(C\text{-}Link)}$-$PB_{(SP)}$/Ti (white columns), Ppy-GOx-$PB_{(ElCh)}$/Ti (medium pattern columns), and $GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti (sparse pattern columns) electrodes on the glucose concentration in buffer solution.

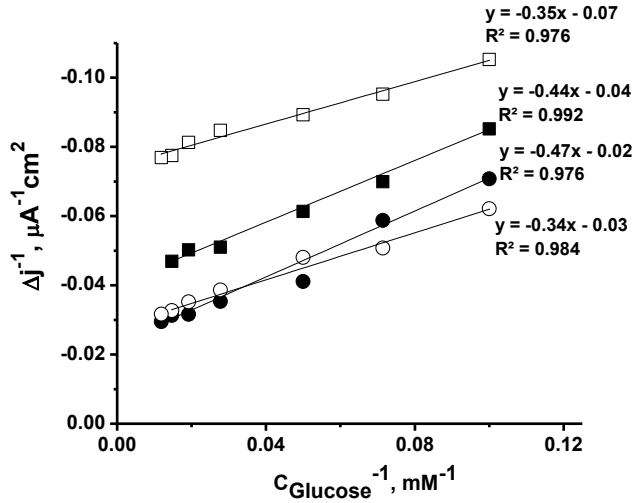


Fig. 12. Data of amperometric analysis expressed in double reciprocal plot (Lineweaver-Burk plot) for (●) $GOx_{(C\text{-}Link)}$-$PB_{(SP)}$/Ti, (○) Ppy-GOx-$PB_{(SP)}$/Ti, (■) Ppy-GOx-$PB_{(ElCh)}$/Ti and (□) $GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti electrodes.

Kinetic parameters such as Michaelis constant (K_M) and maximal reaction rate (v_{max}) were calculated using Lineweaver-Burk plot (Fig. 12) derived from experimental results (Fig. 10) approximated by hyperbolic Michaelis-Menten function [130] .

As could be expected, each type of the investigated electrode showed different values of K_M and v_{max}, (Table 3) because both may be influenced by the charge and conformation of the protein and substrates.

Table 3. Calculated values of Michaelis constant (K_M) and maximal reaction rate (v_{max}) for different electrodes.

Electrode type	K_M, mM	v_{max}, μA cm^{-2}
$GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti	4.74	13.66
Ppy-GOx-$PB_{(ElCh)}$/Ti	10.87	24.87
Ppy-GOx-$PB_{(SP)}$/Ti	11.76	34.96
$GOx_{(C\text{-}Link)}$-$PB_{(SP)}$/Ti	20.84	44.05

Of all the electrodes investigated in this work, the lowest value of Michaelis constant (4.74 mM) was found for $GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti electrode indicating the greatest extent of binding between the enzyme and its substrate for a given substrate concentration. This result allows one to conclude indirectly that $GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti electrode could be found more stable than other electrode designed in this work. Further experiments were carried out to evaluate the stability of the designed electrodes. Amperometrically detected cathodic currents of glucose oxidation were registered (i) immediately after the preparation of electrode, (ii) after 24 hours, (iii) after 7 days, (iv) after 16 days and (v) after 28 days. Between the measurements the electrodes were stored above the phosphate buffer solution, pH 7.3 at 4°C.

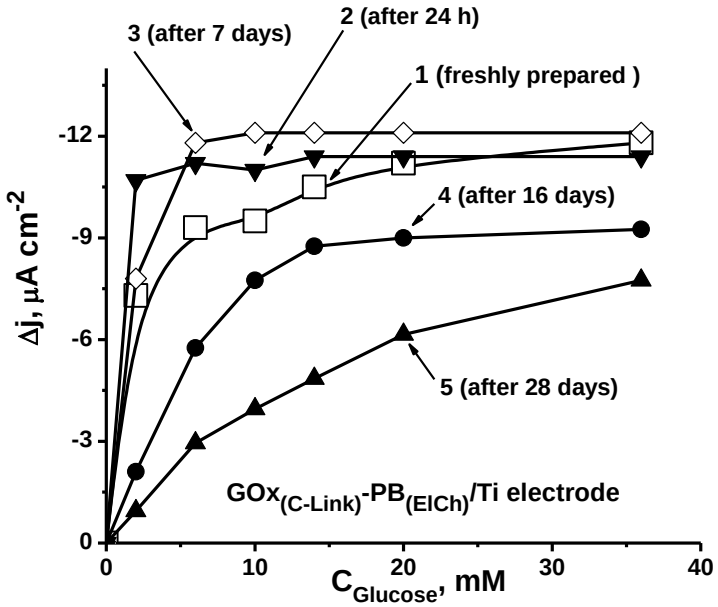


Fig. 13. Amperometric responses of $GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti electrode towards glucose registered at a potential of 0.05 V *vs* Ag|AgCl|$KCl_{sat.}$ (□) immediately after the preparation of electrode, (▼) after 24 hours, (◊) after 7 days, (●) after 16 days and (▲) after 28 days.

A significant decrease in amperometric responses towards different glucose concentration was obtained for both titanium electrodes with spontaneously deposited PB layer ($GOx_{(C\text{-}Link)}$-$PB_{(SP)}$/Ti and Ppy-GOx-$PB_{(SP)}$/Ti) and also for Ppy-GOx-$PB_{(ElCh)}$/Ti electrode (data not presented). Only by using titanium electrode with electrochemically deposited PB layer and GOx entrapped within vapours of glutaraldehyde ($GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti biosensor), generated cathodic current densities do not change significantly, registering amperometric responses immediately after the preparation of electrode, after 24 hours and after 7 days (Fig. 13, curves 1, 2, 3). Good hyperbolic behaviour, which is characteristic of enzymatic reactions, was obtained registering amperometric signals of $GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti electrode (reaching 9 and 8 μA cm^{-2} respectively) even after 16 and 28 days (Fig. 13, curve 5 and 6).

Table 4. Comparison between the kinetic parameters of amperometric glucose biosensors reported in literature.

Biosensor type	E, V *vs* Ag|AgCl|KCl$_{sat.}$	K_M, mM	v_{max}, µA cm^{-2}	Linear detection range of glucose, mM	Sensitivity to glucose, µA cm^{-2} mM^{-1}
Pt/PB/GOD–PAn [48]	0	10.3	38	–	–
Pt/GOD/Fe_3O_4/Cs/nafion [50]	0.4	0.611	–	0.006 – 2.2	11.54
TiO_2 nanotube arrays unhybridized [35]	−0.45	–	–	0.05 – 0.65	199.6
GOx/n-TiO_2/PANI/GCE [37]	−0.45*	0.122	–	0.02 – 6.0	6.31
GOx/Au-NPs/CR [104]	0.3	19.7 - 48.1	43.5– 107.3	–	–
Biosensors presented in this study					
GOx$_{(C\text{-}Link)}$-PB$_{(ElCh)}$/Ti	0.05	4.74	13.66	2 - 68	0.15 – 3.65
Ppy-GOx-PB$_{(ElCh)}$/Ti	0.05	10.87	24.87	2 - 52	0.31 – 2.85
Ppy-GOx-PB$_{(SP)}$/Ti	0.05	11.76	34.96	2 - 68	0.47 – 3.87
GOx$_{(C\text{-}Link)}$-PB$_{(SP)}$/Ti	0.05	20.84	44.05	2 - 68	0.22 – 3.15
PPy-PB-GOx-modified graphite electrodes	0.05	4.6– 21.13	1.39 – 2.17	– 20	1.0 – 1.9

Amperometric behavior of PB-Ppy-GOx, PB-NiHCF-Ppy-GOx and PB-CoHCF-Ppy-GOx based electrodes

In this part of work amperometric glucose biosensors based on graphite electrode, modified with different bioselective layer either of PB-Ppy-GOx, PB-NiHCF-Ppy-GOx or PB-CoHCF-Ppy-GOx were designed and investigated. The composite layers were deposited by single-step based procedure in cyclic voltammetric conditions as explained in section 3.1. Entrapment of nickel and cobalt hexacyanoferrates was carried out while modifying graphite electrode with bioactive layer, expecting to explore the electrocatalytic characteristics of PB-NiHCF-Ppy-GOx and PB-CoHCF-Ppy-GOx layers, deposited by single-step based procedure. The deposition of PB-Ppy-GOx, PB-NiHCF-Ppy-GOx and PB-CoHCF-Ppy-GOx composite layers is based on the entrapment of hexacyanoferrates of transition metals and GOx in the growing polypyrrole film, which polymerizes due to pyrrole oxidation occurring at the potentials equal or higher than 0.55 V *vs* Ag|AgCl|KCl_{sat} [131]. It is also known that Fe^{2+}, Ni^{2+} and Co^{2+} ions react with $[Fe(CN)_6]^{4-}$ ions to form metal hexacyanoferrates [132,133,134]. During the deposition of PB-NiHCF-Ppy-GOx and PB-CoHCF-Ppy-GOx layers, ions of Fe^{2+} (1 mM) were mixed with $[Fe(CN)_6]^{4-}$ (2 mM) and either Ni^{2+} (1 mM) or Co^{2+} (1 mM) to get both Prussian blue (FeHCF) and either NiHCF or CoHCF, to ensure that both forms of hexacyanoferrates (Prussian blue + NiHCF or Prussian blue + CoHCF) will coexist in the deposition solution.

According to single-step based modification of graphite electrode with PB-Ppy-GOx layer, relatively low anodic potential of +0.05 V *vs* Ag|AgCl|KCl_{sat} can be used for the determination of analytical signal towards glucose. It is in agreement with investigations claiming that when PB is used in electrochemical glucose biosensor, the optimal potential range is around 0 V *vs* Ag|AgCl|KCl_{sat} [76]. It should be noted, that at the standard conditions hydrogen peroxide is not being reduced on bare graphite electrode until at least -0.45 V *vs* Ag|AgCl|KCl_{sat} [135], however, during electrochemical experiments, which are described in recent paper, we observed the reduction of H_2O_2 at +0.05 V *vs* Ag|AgCl|$KCl_{sat.}$ Therefore, we can conclude that during electrodeposition of PB-Ppy-GOx composite layer, Prussian blue and Co- or Ni-hexacyanoferrates are entrapped within formed Ppy layer. Otherwise, the reduction flux of hydrogen peroxide could not be

registered by modified electrodes. Furthermore, scanning electron microscopy (SEM) indicates the presence of Ni and Co ions in composite layers of PB-NiHCF-Ppy-GOx and PB-CoHCF-Ppy-GOx, respectively (Fig.14 A, B, C).

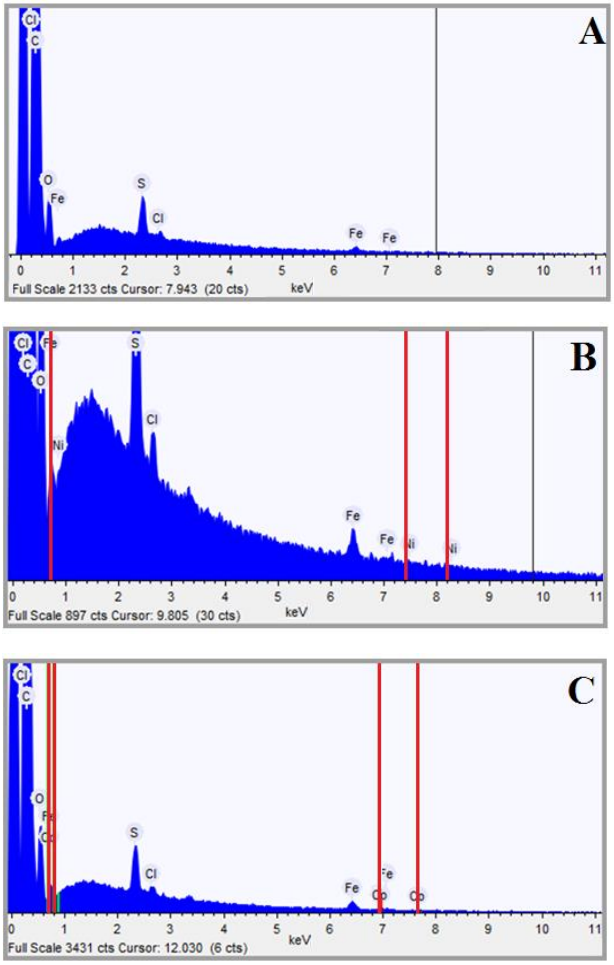


Fig. 14. Energy-dispersive X-ray spectroscopy analysis of: **A** – PB-Ppy-GOx, **B** – PB-NiHCF-Ppy-GOx, **C** – PB-CoHCF-Ppy-GOx composites.

From data presented in table 5, it is seen that the highest amount of composition is based on carbon, which is obvious because carbon atoms are forming a core of all materials that are present in deposited composites. In PB-NiHCF-Ppy-GOx the presence of Ni was determined. In this composite

the ratio between Fe and Ni atoms was 8:1 while in PB-CoHCF-Ppy-GOx the presence of Co was determined and in this composite the ratio between Fe and Co atoms was 9:1.

Table 5. Elemental composition (%) of PB-Ppy-GOx, PB-NiHCF-Ppy-GOx and PB-CoHCF-Ppy-GOx layers. Data was calculated from the results of Energy-dispersive X-ray spectroscopy.

Composite layer type	Elemental composition (%)						
	C	O	S	Cl	Fe	C	Ni
PB-Ppy-GOx	93.263	6.346	0.265	0.044	0.082	-	-
Ppy-GOx-PB-NiHCF	92.460	7.100	0.302	0.046	0.083	-	0.010
Ppy-GOx-PB-CoHCF	92.264	7.282	0.254	0.046	0.138	0.015	-

After the deposition of bioselective layers of PB-Ppy-GOx, PB-NiHCF-Ppy-GOx and PB-CoHCF-Ppy-GOx, amperometric responses of modified graphite electrodes towards different glucose concentration were registered and are presented in figure 15 A. These signals for PB-CoHCF-Ppy-GOx and PB-NiHCF-Ppy-GOx electrodes were lower comparing to those registered for PB-Ppy-GOx electrode without the presence of CoHCF and NiHCF in the structure of bioselective layer. This result corresponds to the finding reported by other authors, who proposed that electrocatalytic activity of Ni- and Co-hexacyanoferrates is at least 2 times lower compared to that of Prussian blue [61]. However, despite the reduced amperometric signal, the PB-NiHCF-Ppy-GOx electrode showed wider linear range of the calibration curve indicating this type of electrode being more sensitive to glucose concentration up to 100 mM of glucose. Meanwhile, for PB-Ppy-GOx and PB-CoHCF-Ppy-GOx electrodes, the registered linear range of the calibration curve was much narrower and did not exceeded 20 mM of glucose.

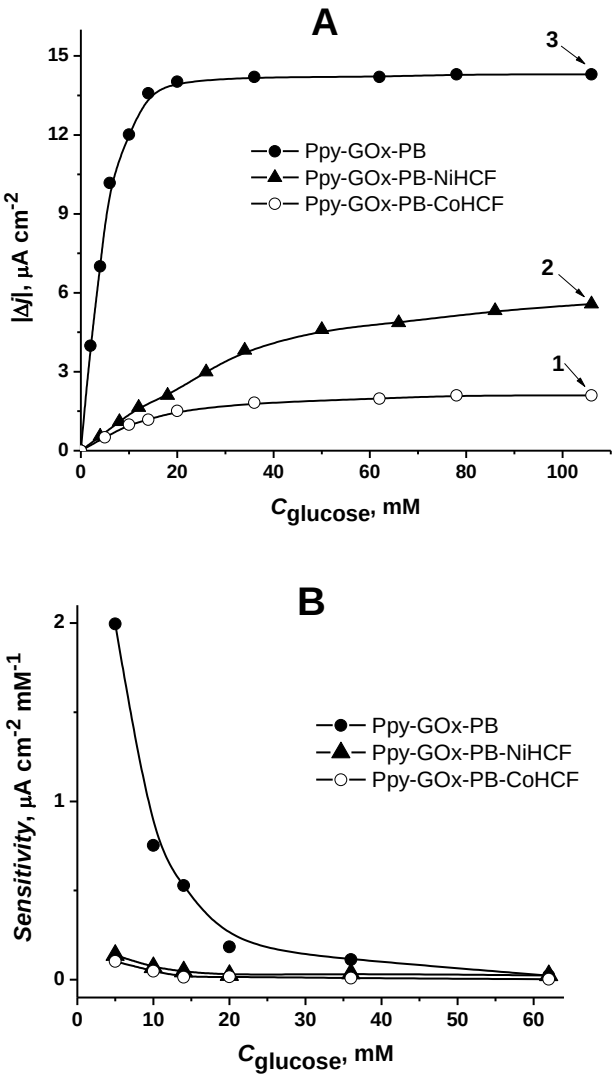


Fig. 15. A - amperometric responses of graphite electrode modified with (1) – PB-CoHCF-Ppy-GOx, (2) – PB-NiHCF-Ppy-GOx, (3) – PB-Ppy-GOx layer versus different glucose concentration registered at 0.05 V *vs* Ag|AgCl|$KCl_{sat.}$ in phosphate buffer, pH 7.3. **B** - dependencies of sensitivity of PB-CoHCF-Ppy-GOx, PB-NiHCF-Ppy-GOx and PB-Ppy-GOx electrodes towards glucose concentration in buffer solution.

The best registered sensitivity of PB-Ppy-GOx biosensor was equal to 2 μA cm^{-2} mM^{-1} in the dynamic range from 0 to 2 mM of glucose. Best sensitivities of the electrodes modified with PB-NiHCF-Ppy-GOx and PB-CoHCF-Ppy-GOx layers were 0.14 μA cm^{-2} mM^{-1} (in the dynamic range from 0 to 12 mM) and 0.1 μA cm^{-2} mM^{-1} (in the dynamic range from 0 to 10 mM), respectively (Fig. 15 B).

For the better understanding of kinetics of the biochemical oxidation of glucose, the hyperbolic behaviour of amperometric responses was plotted in reciprocal coordinates proposed by Lineweaver-Burk where $1/|\Delta j|$ *vs* $1/C_{glucose}$ is outlined (Fig. 16). These plots were found linear, thus being useful for further calculations of maximum velocity of the reaction (v_{max}) and apparent Michaelis constant ($K_{M(app.)}$). In all cases the slopes of the curves varied dependently on the composition of bioselective layer.

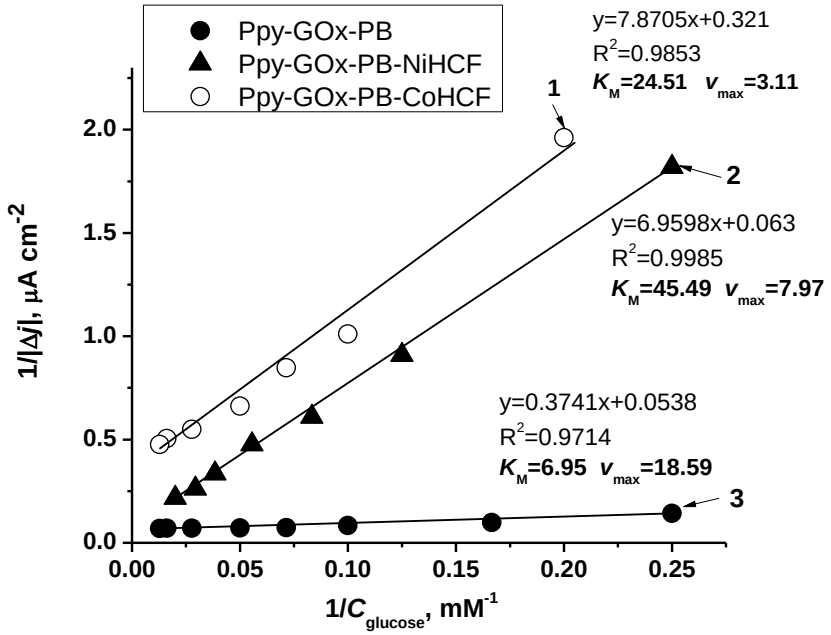


Fig. 16. Transformation of amperometric responses of graphite electrode modified with (1) – PB-CoHCF-Ppy-GOx, (2) – PB-NiHCF-Ppy-GOx, (3) – PB-Ppy-GOx towards different glucose concentration presented in reciprocal coordinates.

The maximum velocity of the reaction of glucose oxidation, v_{max}, was calculated to be equal to 18.59 μA cm^{-2} for the electrode modified with PB-Ppy-GOx bioselective layer and decreases down to 7.97 and 3.11 μA cm^{-2} for the electrodes modified with PB-NiHCF-Ppy-GOx and PB-CoHCF-Ppy-GOx layer, respectively. The decrease in v_{max} corresponds to the increase in $K_{M(app.)}$, suggesting that the modification of bioselective layer with NiHCF and CoHCF results in mixed type of inhibition when the affinity of the substrate for the active site is reduced. The highest value of $K_{M(app.)}$ (45.49 mM) was calculated for the electrode modified with PB-NiHCF-Ppy-GOx layer. This behaviour could be interpreted as a lower analytical signal, which is proportional to the registered current, nevertheless, the modification of graphite electrode by PB-NiHCF-Ppy-GOx layer enabled to increase significantly the detection range for glucose towards higher concentrations (Fig. 15), thus this type of electrode was chosen for further experiments presented in this work. The limit of detection (LOD) of PB-NiHCF-Ppy-GOx electrode for glucose was determined as 0.15 mM.

Effects of temperature on amperometric responses of PB-NiHCF-Ppy-GOx based biosensor

It should be pointed out that kinetics of enzymatic reactions is strongly influenced by experimental conditions, particularly by the temperature at which the reaction is carried out. Therefore, it is desirable to measure the dependence of amperometric response of enzyme reaction on temperature. The experimental data at the temperature range from 15 ℃ to 30 ℃ are shown in figure 17.

From amperometric responses (Fig. 17 A) it is seen that the variation of temperature leads to significant changes of enzymatic reaction rate, which is observed as a different registered current density. This effect is very obvious at high glucose concentrations, which are above 20 mM. Amperometric signals increase with the increase of temperature from 15 ℃ to 20 °C (Fig. 17 B), however further temperature increase causes the decrease of current densities generated by enzymatic glucose oxidation.

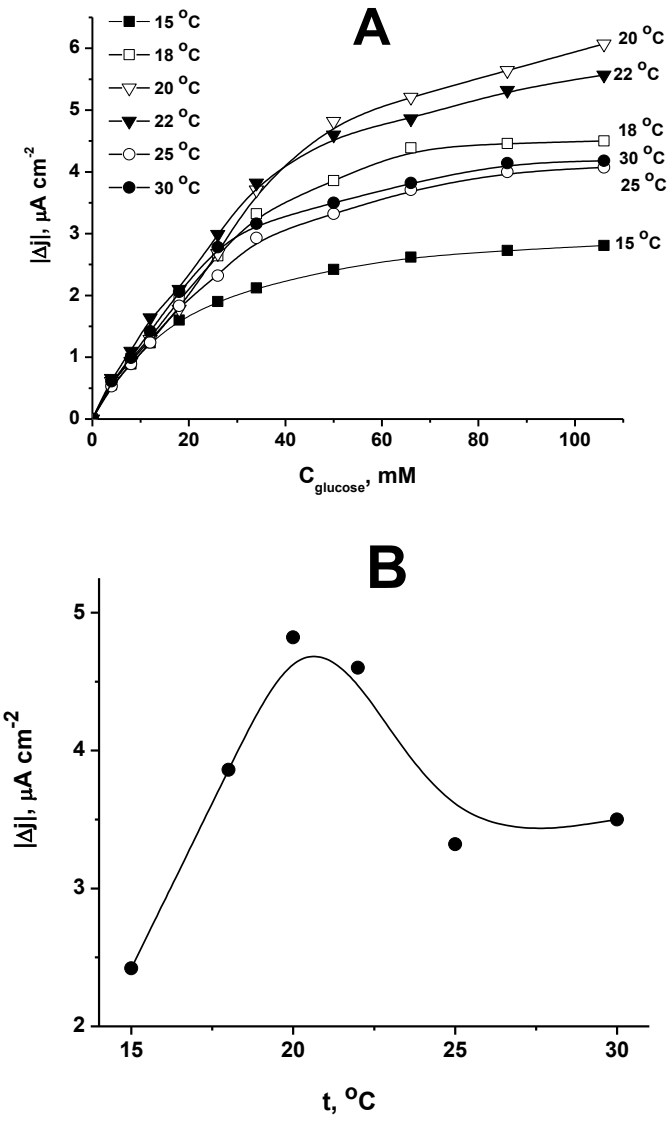


Fig. 17. A – Amperometric responses of PB-NiHCF-Ppy-GOx based biosensor *versus* different glucose concentration registered at different temperatures in phosphate buffer, pH 7.3 (E = 0.05 V *vs* Ag|AgCl|$KCl_{sat.}$). **B** – Effects of temperature on the current density of PB-NiHCF-Ppy-GOx - modified electrode at a glucose concentration of 50 mM.

It should be noted that room temperature conditions, defined by National Institute of Standards and Technology (NIST) as 20 ℃ are the most optimal for the operation of PB-NiHCF-Ppy-GOx based glucose biosensor. From here presented experimental data we can to predict that temperature range from 20 ℃ until 22 ℃ is convenient for the investigations based on glucose oxidase catalysed glucose oxidation reaction. This suggestion can be explained assuming that the glucose diffusion and the rate of enzymatic reaction becomes slow at the temperatures below 20 ℃ but at the higher than at 25 ℃ temperatures some inhibitory effect of glucose oxidase takes place. In this case the optimal temperatures for the operation of PB-NiHCF-Ppy-GOx based biosensor are in the range between 20 ℃ and 22 ℃.

Once the amperometric responses of PB-NiHCF-Ppy-GOx based biosensor showed good hyperbolic behaviour at all temperatures studied, the transformation of analytical response *versus* glucose concentration was plotted into reciprocal plot (Fig. 18) and kinetic parameters of v_{max} and $K_{M(app.)}$ were calculated (Table 6) from linear dependencies.

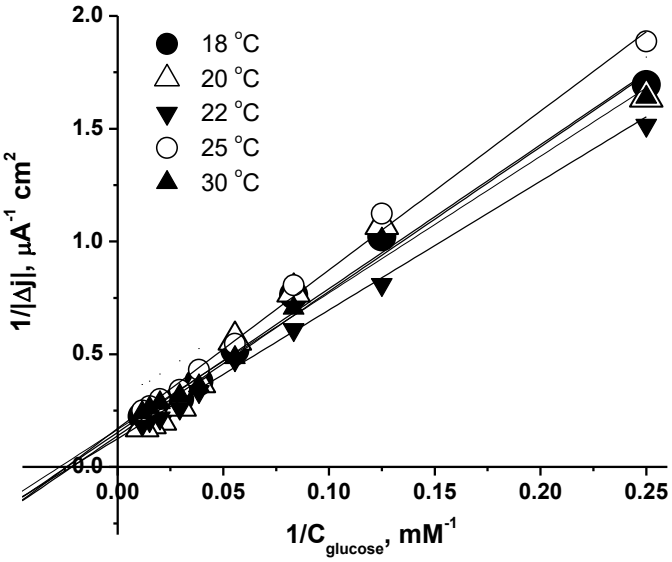


Fig. 18. The plot of $1/|\Delta j|$ *versus* $1/C_{glucose}$, which was applied for the determination of apparent Michaelis constant ($K_{M(app.)}$), and maximum velocity of the reaction, v_{max}.

The plot area (Fig. 18) where the most lines, which were derived from data presented in figure 17, are crossing to each other is what indicates that the inactivation of PB-NiHCF-Ppy-GOx-based glucose electrode is at highest extent determined by noncompetitive inhibition of GOx. This type of inhibition is the characteristic one for the temperature induced inactivation based on conformational changes of enzyme, which significantly change the geometry of active site of enzyme.

Table 6 illustrates that both apparent Michaelis constant ($K_{M(app.)}$) and maximum velocity (v_{max}) of PB-NiHCF-Ppy-GOx based glucose biosensor action, reaches the maximal values at the temperature of 22 ℃. Actually, in usual enzymatic systems at fixed temperature both these processes (the variation of $K_{M(app.)}$ and v_{max}) mostly are reciprocal to each-other: by the increase of $K_{M(app.)}$ the value of v_{max} – decreases. But in this particular case, it should be taken into account that by the increasing temperature several main processes are taking place: (i) from one hand reaction rates are increasing what positively affects maximum velocity of enzymatic reaction (v_{max}) and rates of other chemical and diffusional process; (ii) from another hand conformational changes of active site at higher temperatures significantly decrease the affinity of active site of enzyme towards glucose what naturally increases the apparent Michaelis constant ($K_{M(app.)}$). However at temperatures higher than 25 ℃ maximal velocity (v_{max}) already tends to decrease.

Table 6. Calculated values of apparent Michaelis constant ($K_{M(app.)}$) and maximal reaction rate (v_{max}) for PB-NiHCF-Ppy-GOx based glucose biosensor investigated at different temperatures.

T, K	*t*, ℃	$K_{M(app.)}$, mM	v_{max}, μA cm^{-2}
288	15	21.49	3.43
291	18	41.59	6.52
293	20	43.19	6.80
295	22	45.49	7.97
298	25	41.32	5.80
303	30	36.42	6.01

The registered temperature dependencies were transformed into an Arrhenius plot, which in the case of heterogeneous electrochemical reaction is the natural logarithm of current densities *vs* reciprocal values of the absolute temperature, clearly illustrate the behaviour of enzymatic glucose oxidation (Fig. 19).

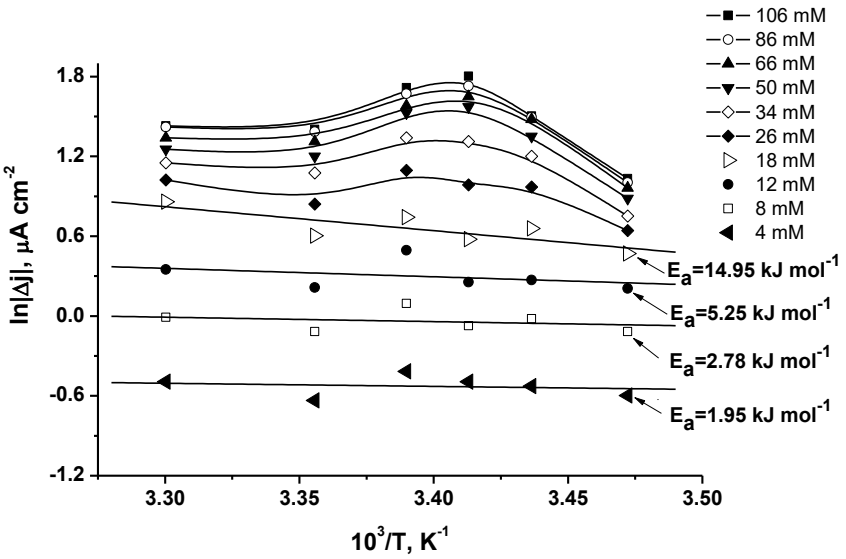


Fig. 19. Arrhenius plots of the data presented in figure 17A, plot of ln|Δj| *versus* 10^3/T.

The temperature-dependent activation energy (E_a) calculated from linear Arrhenius plot is related to the rate of elementary chemical reaction [136], whereas in this work non-linear plots were obtained indicating that the investigated enzymatic reaction has a complex multi-step mechanism. Nevertheless, almost linear dependencies were observed at glucose concentrations from 4 mM to 18 mM providing the basis for the determination of E_a. By using the straight lines with a slope of $-E_a R^{-1}$ (R is the gas constant) the activation energies were found to be decreasing from 14.95 to 1.95 kJ mol^{-1} at glucose concentration of 18 mM and 4 mM, respectively. At glucose concentration of 18 mM, calculated activation energy is slightly lower than that reported for different glucose sensors by other authors (approx. 22 – 50 kJ mol^{-1}) [137,138,139,140,141,142]. At

glucose concentrations higher than 18 mM Arrhenius plots are becoming non-linear and not well suitable for the interpretation of activation energy E_a. Such nonlinear Arrhenius plots are formed because different steps of catalytic process catalysed by enzymes are differently affected by temperature (the same is demonstrated by not coherent influence of temperature on apparent Michaelis constant ($K_{M(app.)}$) and maximal velocity (v_{max}) and at different temperatures different steps of catalysed reaction are limiting. Therefore, the summary effect, which is reflected in Arrhenius plots, becomes non-linear. At glucose concentrations higher than 18 mM and at temperatures in the range from 15 ℃ until 22 ℃ the slope of Arrhenius plot increases what indicates that generalized activation energy (E_a) of evaluated complex bio-catalytic and electro-catalytic process, which occurs at PB-NiHCF-Ppy-GOx electrodes, significantly increases up to the values, which are reported in other researches [137,138,139,140,141,142]. Interestingly, the temperature dependencies are significantly decreasing the slope of lines in Arrhenius plot at glucose concentration lower than 18 mM, denoting relatively low values of activation energy. Low activation energies can be attributed to well establish multi-step catalytic processes [143]. In our case, the lowest activation energy was observed for relatively low glucose concentration of 4 mM.

GENERAL CONCLUSIONS

1. Simple, single-step based procedure for the modification of graphite electrode by PB-Ppy-GOx layer was developed. The PB-Ppy-GOx - modified graphite electrode displayed 1.0 - 1.9 μA cm^{-2} mM^{-1} sensitivity towards glucose in the range of 0.1 - 20 mM.
2. The PB-Ppy-GOx-modified graphite and/or Ti electrodes can be applied in the design of glucose biosensors, and optimal potential for the registration of amperometric response towards glucose is at +0.05 V *vs* $Ag|AgCl|KCl_{sat.}$
3. Electrochemical characteristics of bioselective PB-Ppy-GOx layer detected by FFT-EIS method do not vary significantly during the use of the GE-PB-Ppy-GOx electrode in the detection of glucose.
4. The amperometric signals of the GE-PB-Ppy-GOx electrodes decrease during the measurements due to the mixed-type (uncompetitive and non-competitive) inhibition of GOx, as it was determined by the evaluation of cathodic currents vs glucose concentrations.
5. Using titanium as a substrate, an electrochemically active layer of Prussian blue can be deposited spontaneously and/or electrochemically. Subsequently, GOx can be immobilized within synthesis of Ppy layer or within vapours of glutaraldehyde. The designed biosensors exhibited good amperometric responses to glucose concentration (from 2 mM to 68 mM) and displayed sensitivity of glucose detection in the range from 0.19 to 3.87 μA cm^{-2} mM^{-1}.

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SANTRAUKA

Įvadas

Tinkama gliukozės koncentracija (3,3 – 5,5 mmol/l) kraujyje - labai svarbus veiksnys siekiant efektyviai gydyti cukrinį diabetą ir išvengti daugelio šios ligos komplikacijų. Šiam tikslui plačiai naudojami biojutikliai – nebrangūs, patikimi prietaisai, kurių gamybos technologijos intensyviai vystomos pastaruosius 50 metų. Elektrocheminiai biojutikliai yra labai perspektyvūs analitiniai prietaisai, kurie nuolat tobulinami, gerinant jų jautrumą ir selektyvumą.

Kuriant amperometrinius gliukozės biojutiklius, iki šiol susiduriama su problema, parenkant potencialą, kuriam esant veikia biojutiklis. Biojutikliuose, kuriuose gliukozės koncentracijos nustatymas paremtas H_2O_2 oksidacinės srovės registravimu, reikalingas santykinai didelis darbinio elektrodo potencialas (0,7 V *vs* Ag|AgCl|$KCl_{sat.}$). Esant tokiam dideliam potencialui, gali būti oksiduotos kitos elektrochemiškai aktyvios medžiagos, esančios tiriamajame mėginyje, ir jų oksidacinės srovės gali turėti neigiamos įtakos tyrimų rezultatams.

Kita svarbi bioelektrocheminių sistemų kūrimo problema yra tinkamas biokatalizatorių imobilizavimas. Ši procedūra ne visada sėkminga dėl labai skirtingos biokatalizatorių, kurie yra baltyminės medžiagos, ir jų imobilizavimui naudojamų medžiagų prigimties. Kaip matrica fermento imobilizavimui gali būti naudojami laidūs polimerai. Tokiu būdu imobilizavus fermentus, neprarandamos biojutiklio elektrokatalizinės savybės.

Kad amperometrinis biojutiklis efektyviai veiktų, svarbu parinkti tinkamą substratą bioselektyvaus sluoksnio formavimui. Vis dar aktualu sukurti gliukozės biojutiklį, tinkamą implantavimui žmogaus organizme, o šiuo atveju biojutiklio pagrindas turėtų pasižymėti geru elektriniu laidumu, ilgaamžiškumu ir, svarbiausia, biosuderinamumu, kuris reikalingas mediciniinių prietaisų ir/ar implantų kūrimui.

Šioje disertacijoje pateikti eksperimentiniai tyrimai buvo skirti aukščiau minėtų problemų sprendimui. Tokiu būdu, buvo siekiama maksimaliai supaprastinti elektrodų modifikavimo procedūrą ir optimizuoti fermentų

imobilizavimą bei sukurti elektrocheminę sistemą, gebančią tiksliai registruoti elektrocheminį atsaką, esant žemam potencialui, taip išvengiant pašalinių elektrochemiškai aktyvių medžiagų oksidacinės srovės įtakos fiksuojamam amperometriniam signalui.

Darbo tikslas:

Suformuoti ir ištirti amperometrinį gliukozės biojutiklį, modifikuojant grafito ir titano elektrodus kompozicine Berlyno mėlynojo, polipirolo ir gliukozės oksidazės (PB-Ppy-GOx) danga.

Darbo uždaviniai:

1. Ciklinės voltamperometrijos būdu modifikuoti grafito elektrodą kompozicine PB-Ppy-GOx danga taikant "vieno žingsnio" metodą ir šios dangos pagrindu suformuoti biojutiklį gliukozės koncentracijos nustatymui.
2. Nustatyti reikiamą pirolo koncentraciją kompozicinės dangos elektronusodinimo tirpale, siekiant optimalaus gliukozės biojutiklio jautrumo.
3. Ištirti grafito elektrodo, modifikuoto bioselektyviu PB-Ppy-GOx sluoksniu, paviršines savybes atominės jėgos mikroskopijos metodu.
4. Nustatyti optimalią biojutiklio potencialo vertę amperometrinio signalo registravimui.
5. Greitosios Fourier transformacijos elektrocheminio impedanso spektroskopijos (FFT-EIS) metodu įvertinti bioselektyvaus PB-Ppy-GOx sluoksnio elektrochemines charakteristikas.
6. Įvertinti PB-Ppy-Gox kompozicine danga modifikuoto grafito elektrodo inaktyvaciją gliukozės koncentracijos matavimo metu.
7. Modifikuoti Ti elektrodą chemiškai ir elektrochemiškai nusodinant PB ir skirtingais būdais imobilizuojant GOx. Įvertinti gautų biojutiklių veikimo ypatumus.
8. Suformuoti gliukozės biojutiklius "vieno žingsnio" metodu ant grafito elektrodo nusodinant PB-Ppy-GOx dangas ir įterpiant į jas Ni^{2+} ar Co^{2+} jonus, taip suformuojant Ppy-GOx-PB-NiHCF ar Ppy-GOx-PB-CoHCF bioselektyvius sluoksnius, bei ištirti gautų biojutiklių fizikochemines savybes.

Mokslinis naujumas:

Berlyno mėlynojo elektrokatalizinis gebėjimas redukuoti vandenilio peroksidą plačiai taikomas kuriant biojutiklius gliukozės kiekio nustatymui. Tarp įvairaus tipo gliukozės biojutiklių išskirtinėmis savybėmis pasižymi elektrocheminiai gliukozės biojutikliai, pasižymintys žema detekcijos riba, greitu atsaku, ilgesniu eksploatavimo laiku ir žemesne savikaina. Tačiau iki šiol susiduriama su problemomis, parenkant optimalias sąlygas bioelektrokatalizinio sluoksnio nusodinimui bei nustatant tinkamus elektrocheminius parametrus efektyviam biojutiklio veikimo užtikrinimui. Šio darbo metu atlikti eksperimentiniai tyrimai buvo skirti minėtų problemų sprendimui. Analizuojant gautus rezultatus, galima išskirti keletą šio darbo mokslinio naujumo elementų:

1. Sukurtas amperometrinis gliukozės biojutiklis, pritaikant supaprastintą „vieno žingsnio“ elektrocheminį metodą, kurio metu ant grafito elektrodo nusodinta kompozicinė danga, sudaryta iš Berlyno Mėlynojo, polipirolo ir gliukozės oksidazės (PB-Ppy-GOx).
2. Pademonstruota, kad pasiūlyta kompozicinė danga (PB-Ppy-GOx) „vieno žingsnio“ metodu gali būti nusodinama ne tik ant grafito, bet ir ant titano elektrodo paviršiaus, tokiu būdu praplečiant kuriamo biojutiklio taikymo galimybes dėl unikalių titano savybių, tokių kaip geras cheminis stabilumas, netoksiškumas ir, svarbiausia, - biologinis suderinamumas, reikalingas medicininių prietaisų ir/ar implantų kūrimui.
3. Nustatyta, jog PB-Ppy-GOx kompozicinė danga yra tinkama amperometrinio gliukozės oksidacijos signalo registravimui, esant santykinai žemam darbinio elektrodo potencialui 0,05 V *vs* $Ag|AgCl|KCl_{sat.}$. Naudojant tokį potencialą, išvengta pašalinių elektrochemiškai aktyvių medžiagų oksidacinės srovės įtakos fiksuojamam amperometriniam signalui.
4. Gliukozės biojutiklio, suformuoto ant grafito elektrodo PB-Ppy-GOx kompozicinės dangos pagrindu, elektrocheminės savybės ištirtos taikant greitosios Fourier transformacijos elektrocheminio impedanso spektroskopijos (FFT-EIS) metodą ir nustatytas krūvio pernašos greitis bei difuziją ribojantys parametrai prieš elektrodo modifikavimą kompozicine danga ir po modifikavimo bei panaudojimo jį gliukozės koncentracijos nustatymui.

Ginamieji teiginiai:

1. Gliukozės biojutiklio formavimui gali būti taikomas „vieno žingsnio“ metodas, kurio pagalba grafito gali būti modifikuojamas elektrochemiškai nusodinant Berlyno Mėlynojo, polipirolo ir gliukozės oksidazės (PB-Ppy-GOx) bioselektyvųjį sluoksnį.
2. Naudojant gliukozės biojutiklio formavimui bioselektyvųjį PB-Ppy-GOx sluoksnį, amperometrinio gliukozės oksidacijos signalo registravimui yra tinkamas žemas darbinio elektrodo potencialas 0,05 V *vs* Ag|AgCl|$KCl_{sat.}$ įgalinantis išvengti pašalinių elektrochemiškai aktyvių medžiagų oksidacinės srovės įtakos analiziniam signalui.
3. Gliukozės biojutiklio, sukurto kompozicinės PB-Ppy-GOx dangos pagrindu, elektrocheminiai parametrai gali būti nustatyti FFT-EIS metodu ir naudojami gliukozės biojutiklio elektrokataliziniam aktyvumui įvertinti.
4. Titano elektrodas gali būti naudojamas formuojant biojutiklius, chemiškai ir elektrochemiškai nusodinant PB ir imobilizuojant Gox, tiek elektrocheminės polipirolo sintezės metu, tiek naudojant gliutaraldehido garus.

Gliukozės biojutikliai

Cukrinis diabetas (CD) yra medžiagų apykaitos liga; kuri gali būti įgimta arba įgyta. CD priežastis yra absoliutus arba santykinis hormono insulino deficitas, t.y. visiškas jo nebuvimas arba trūkumas. Pasaulinės Sveikatos Organizacijos duomenimis nuo šios ligos kenčia daugiau kaip 190 milijonų pasaulio gyventojų. Prognozuojama, kad iki 2035 metų sergančiųjų bus daugiau nei 592 milijonai. Ankstyva CD diagnostika, tiksli ir patikima gliukozės koncentracijos kontrolė kraujyje bei tinkamas gydymas leidžia išvengti daugelio ligos komplikacijų ir kontroliuoti ligos eigą. Gliukozės koncentracijos kontrolei plačiai naudojami gliukozės biojutikliai, kurių pagalba galima tiksliai ir patikimai nustatyti gliukozės koncentraciją ligonių kraujyje.

Biojutiklis - tai analitinis prietaisas, kurį sudaro biologinis komponentas kartu su signalo keitikliu, konvertuojančiu biocheminį signalą į elektros signalą. Vieni iš dažniausiai naudojamų biojutiklių yra fermentiniai jutikliai,

kuriuose esantys fermentai, oksidazės, oksiduoja savo specifinius substratus, ko pasekoje susidaro vandenilio peroksidas.

Elektrocheminiai biojutikliai – tai biojutikliai, turintys elektrocheminį signalo vertiklį. Amperometriniai biojutikliai atlieka elektros srovės, susijusios su analitės redokso reakcijomis, matavimus. Šio tipo biojutikliai plačiai taikomi klinikinėje diagnostikoje, aplinkosaugoje, maisto analizėje, narkotikų tyrimuose, nes jie patikimi, labai jautrūs ir palyginti pigūs.

Grafito elektrodo modifikavimas PB-Ppy-GOx danga, taikant "vieno žingsnio metodą"

Šiame darbe PB-Ppy-GOx kompozicinis sluoksnis nusodintas taikant "vieno žingsnio"elektrocheminę procedūrą, naudojant tirpalą, sudarytą iš 1 mM $FeCl_2$, 1 mM $K_4[Fe(CN)_6]$, 1 mg mL^{-1} GOx bei pirolo, atliekant 20 potencialo skleidimo ciklų +400 - +800 mV intervale, esant 40 mV s^{-1} skleidimo greičiui. Fe^{3+} jonai, reikalingi PB sintezei susidarė oksiduojant $FeCl_2$ tiesiogiai prie elektrodo paviršiaus, kai ciklinimo metu buvo pasiektas pakankamai aukštas elektrodo potencialas:

$$4Fe^{3+} + 3[Fe(CN)_6]^{4-} \rightarrow Fe_4[Fe(CN)_6]_3 \quad (1)$$

Susidarant PB, pirolo monomerai pradeda formuoti PPy sluoksnį, esant aukštesniems oksidacijos potencialams. Vykstant pirolo polimerizacijai, GOx imobilizuojama PPy sluoksnyje kartu su PB, kuris formuojasi ant grafito elektrodo paviršiaus. PB buvimas visai greta imobilizuotos GOx yra labai naudingas, nes leidžia didesnėms vadenilio peroksido koncentracijoms, susidarančioms dėl katalizinio GOx veikimo, pasiekti PB paviršiaus daleles ir tuo būdu žymiai padidinti PB paviršiaus plotą, galintį dalyvauti kataliziniame procese.

Optimalaus elektrodo potencialo nustatymas analitinio signalo registravimui

Grafito elektrodus modifikavus PB-PPy-GOx sluoksniu, buvo registruojamos katodinės poliarizacinės kreivės, siekiant nustatyti tinkamiausią elektrodo potencialą būsimiems amperometriniams

matavimams. Yra žinoma, kad matuojant gliukozę realiuose pavyzdžiuose, kai kurios medžiagos (pvz., askorbo rūgštis, šlapimo rūgštis), dažniausiai esančios kūno skysčiuose, gali oksiduotis, esant aukštesnėms teigiamų potencialų reikšmėms. Dėl to, tinkamiausios yra galimai minimalios teigiamų potencialų reikšmės. Katodinių poliarizacinių kreivių skirtumai tirpaluose su gliukoze ir be gliukozės parodė, kad +0.05 V *vs* $Ag|AgCl|KCl_{sat.}$ elektrodo potencialo reikšmė geriausiai tinka PB-Ppy-GOx - modifikuotų grafitinių elektrodų taikymui gliukozės koncentracijos nustatymo procesams. Žemesnės potencialo reikšmės nebuvo pasirinktos siekiant užtikrinti PB stabilumą.

Pirolo koncentracijos elektronusodinimo tirpale įtaka PB-Ppy-GOx - modifikuotų grafitinių elektrodų jautrumui

Modifikuojant grafito elektrodą PB-Ppy-GOx sluoksniu buvo naudoti tirpalai su skirtingomis pirolo koncentracijomis (10; 20; 30; 40 mM). Bandymų rezultatai parodė, kad optimaliausia yra naudoti tirpalą, kuriame yra 30 mM pirolo. Esant žemesnėms pirolo koncentracijoms, Ppy sluoksnio storis gaunasi mažesnis, taip pat mažesnis ir imobilizuoto fermento kiekis. Pasitvirtino, kad didinant pirolo koncentraciją daugiau, nei 30 mM ir tuo būdu storinant susidarantį PPy sluoksnį, atsiranda difuziniai gliukozės apribojimai, todėl mažėja registruojami srovės dydžiai.

PB-Ppy-GOx modifikuotų grafito elektrodų amperometrinis atsakas buvo įvertintas kalibracinių kreivių pagalba. Suformuoto biosensoriaus jautrumas buvo skaičiuojamas kaip srovės tankio kitimo funkcija, kintant gliukozės koncentracijoms ir nustatyta, kad kitimo ribos yra 1.0-1.9 $\mu A\ cm^{-2}\ mM^{-1}$.

Modifikuotų grafitinių elektrodų tyrimas FFT-EIS metodu

FFT-EIS tyrimai buvo atlikti siekiant įvertinti švaraus grafito elektrodo ir PB-Ppy-GOx sluoksniu modifikuotų elektrodų (GE/PB-Ppy-GOx) elektrochemines charakteristikas, prieš ir po gliukozės koncentracijos matavimų. FFT-EIS spektrai buvo registruojami fosfatiniame buferyje, pH 7,3 esant +0.05 V *vs* Ag|AgCl|$KCl_{sat.}$ elektrodo potencialo reikšmėms.

Įvertinus FFT-EIS duomenis, nustatyta, kad GE modifikuotas PB-Ppy-Gox sluoksniu pasižymi greitesne krūvio pernaša, kuri vyksta esant netiesinės difuzijos apribojimams. Taip pat nustatyta, kad po GE/PB-Ppy-GOx elektrodo taikymo gliukozės koncentracijos nustatymui, FFT-EIS parametrai keičiasi labai nežymiai. Tai patvirtina prielaidą, kad elektrocheminės GE/PB-Ppy-GOx modifikuoto elektrodo savybės menkai kinta, naudojant jį gliukozės koncentracijoms nustatyti.

Bioselektyvaus sluoksnio ant metalurginio titano plokštelių formavimas ir vertinimas

Sukurti keturių tipų biojutikliai skirtingais būdais modifikuojant Ti plokšteles PB sluoksniu ir imobilizuojant GOx. PB sluoksnis formuotas elektrochemiškai ar chemiškai (be elekronusodinimo metodo), o GOx imobilizuota elektrochemiškai sintetinant laidų Ppy polimero sluoksnį, arba inkubuojant gliutaraldehido garuose.

Kiekvieno tipo elektrodas tyrimuose parodė skirtingas K_M ir v_{max} reikšmes. Iš visų šiame darbe ištirtų elektrodų žemiausia Michaelio konstanta (4,74 mM) nustatyta elektrodui $GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti, kas rodo didžiausią galimą ryšį tarp fermento ir substrato, esant duotai substrato koncentracijai. Šis rezultatas leidžia daryti netiesioginę išvadą, kad $GOx_{(C\text{-}Link)}$-$PB_{(ElCh)}$/Ti elektrodas yra stabiliausias iš visų, ištirtų šiame darbe.

Amperometrinis PB-Ppy-GOx, PB-NiHCF-Ppy-GOx ir PB-CoHCF-Ppy-GOx elektrodų tyrimas

Šioje darbo dalyje buvo sukurti, išbandyti ir ištirti amperometriniai gliukozės biojutikliai, naudojant grafitinius elektrodus, modifikuotus įvairiais bioselektyviais sluoksniais – PB-Ppy-GOx, PB-NiHCF-Ppy-GOx,

PB-CoHCF-Ppy-GOx. Kompozito sluoksnis suformuotas taikant elektronusodinimą ciklinės voltamperometrijos būdu. Nikelio ir kobalto heksacianoferatai įterpti grafito elektrodus modifikuojant, „vieno žingsnio“ procedūros metu. Tikėtasi pagerinti elektrokatalitines PB-NiHCF-Ppy-GOx ir PB-CoHCF-Ppy-GOx savybes. Tačiau elektrodų amperometriniai rodikliai pasirodė prastesni, kai į jų bioselektyviuosius sluoksnius buvo įterpti CoHCF ir NiCHF. Vis dėlto, PB-NiHCF-Ppy-GOx elektrodas pasižymėjo didesne kalibracinės kreivės tiesine sritimi, patvirtinančia, kad šis elektrodas yra jautresnis gliukozės koncentracijoms platesniame intervale (iki 100 mM gliukozės), nei PB-Ppy-GOx ir PB-CoHCF-Ppy-Gox elektrodai, kurių tiesinės kalibracinės kreivės sritys yra daug siauresnės (iki 20 mM gliukozės). Amperometriniai PB-NiHCF-Ppy-GOx rezultatai parodė, kad temperatūros kitimas įtakoja registruojamos srovės tankio dydį. Tai ypač pastebima, kai gliukozės koncentracija viršija 20 mM. Amperometriniai signalai didėja kylant temperatūraiintervale tarp 15 – 20^{o} C. Toliau didinant temperatūrą, srovės tankis mažėja dėl fermentinės gliukozės oksidacijos.

IŠVADOS

1. Sukurta paprasta, “vieno žingsnio” procedūra grafito elektrodo modifikavimui PB-Ppy-GOx danga. Nustatytas, kad PB-Ppy-GOx danga modifikuoto grafito elektrodo jautrumas gliukozei yra 1,0 - 1,9 μA cm^{-2} mM^{-1} gliukozės koncentracijos intervale nuo 0,1 mM iki 20 mM.
2. Ppy-PB-Gox kompozicine danga modifikuoti grafito ir/ar Ti elektrodai gali būti naudojami kuriant biosensorius gliukozės koncentracijai nustatyti, ir optimalus potencialas amperometrinio atsako registravimui yra +0,05 V *vs* Ag|AgCl|KCl$_{sat.}$
3. FFT-EIS metodu nustatyta, kad elektrocheminiai parametrai, charakterizuojantys procesus fazių sąlyčio riboje grafito elektrodas – kompozicinė danga PB-Ppy-GOx kinta nežymiai, naudojant biojutiklį gliukozės koncentracijos nustatymui.
4. Matuojant amperometrines sroves nustatyta, kad amperometrinis GE-PB-Ppy-GOx elektrodų atsakas sumažėja dėl mišrios GOx inhibicijos tipo.

5. Naudojant titaną kaip substratą, elektrochemiškai aktyvus PB sluoksnis gali būti suformuotas chemiškai ir/ar elektrochemiškai. GOx gali būti imobilizuota elektrochemiškai polipirolu arba naudojant glutaraldehido garus. Biojutikliai pasižymi geru amperometriniu atsaku gliukozės koncentarcijų intervale nuo 2 mM iki 68 mM, bei jautrumu gliukozės koncentracijos nustatymui nuo 0.19 iki 3.87 $\mu A\ cm^{-2}\ mM^{-1}$.

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Didžiulis ačiū kolegoms doktorantams Ingai, Tomui, Povilui už gerą ūpą, pokštus, puikią atmosferą laboratorijoje. Be jų būtų buvę liūdna ir daug dalykų būtų atrodę sudėtingesni negu jie yra iš tikrųjų.

Dėkoju dr. Linai Mikoliūnaitei už pagalbą atliekant AFM tyrimus, Tomui Murauskui už kantrybę dirbant vakarais prie SEM‘o. Ačiū visai Fizikinės chemijos katedrai už geranoriškumą.

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Didelis ačiū, VšĮ Respublikinės Vilniaus universitetinės ligoninės, Laboratorinės diagnostikos skyriaus kolektyvui už supratimą ir pagalbą atliekant tyrimus.

Ačiū mielam draugui Vaidui Jankauskui už kantrų darbą ir profesionalią pagalbą ruošiant grafinius paveikslus.

Ir galiausiai ačiū šeimai – dukrai ir vyrui už supratimą, kantrybę, motyvaciją. Ačiū mamai už besalygišką palaikymą, tėveliui už konsultacijas, pagalbą rašant darbą ir moralinę paramą. Ačiū broliui, daug prisidėjusiam, kad šis darbas būtų toks koks yra.

CURRICULUM VITAE

EDUCATION

1. 2015 – 2019 Vilnius University, Faculty of Chemistry and Geoscience, doctoral studies in Chemistry.
2. 1997 – 1998 Vilnius University, Medical Faculty, Clinical laboratory diagnostics.
3. 1983 – 1988 Vilnius University, Faculty of Chemistry, MSc degree in Chemistry, study program Physical Chemistry.

WORK EXPERIENCE:

1. 1991 – until now Vilnius Republican University hospital, senior medical biologist.
2. 1988 – 1991 Institute of Higiena, Laboratory for Labor Conditions, Laboratory doctor.

PROFESSIONAL TRAINING

1. Roche days 2019, Jurmala, Lavia 23 – 24 January 2019.
2. Aspects of Diabetes Type II Study and Amperometric Glucose Assay, Tosoh Bioscience, Tessenderlo, Belgium 28 June – 26 August 2018.
3. XIV Baltic Congress of Laboratory Medicine, Vilnius, 10 – 12 May 2018.
4. Effective monitoring of diabetes mellitus Tosoh Bioscience, Tessenderlo, Belgija, 18 – 22 September 2017.
5. 4th EFLM-BD European Conference on Preanalytical Phase Amsterdam, Netherlands 24 – 25 March 2017.
6. XIII Baltic Congress in Laboratory Medicine Tartu, Estonia 12 – 14 May, 2016.
7. Coagulation Master Class Seminar for Advanced Students Conducted by the Expert Community in Coagulation Dubrovnik, Republic of Croatia, 16 – 18 September 2015.

8. EFLM-BD European Conference on Preanalytical Phase, Porto (Portugal) 20 – 21 March 2015.

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