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The use of different glucose oxidases for the development of an amperometric reagentless glucose biosensor based on gold nanoparticles covered by polypyrrole

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ABSTRACT

The amperometric glucose biosensors based on adsorbed electron transfer mediator (ETM) tetrathiafulvalene (TTF) or 1,10-phenanthroline-5,6-dione (PD) and glucose oxidase (GOx) from *Aspergillus niger* ($GOx_{A.niger}$), *Penicillium adametzii* ($GOx_{P.adametzii}$) or *Penicillium funiculosum* ($GOx_{P.funiculosum}$) cross-linked with glutaraldehyde were investigated. ETM and enzyme were immobilized layer by layer on bare graphite rod electrode (GR) premodified with gold nanoparticles (AuNP) of (i) 3.5 nm (GOx/ETM/$AuNP_{3.5}$/GR), (ii) 6.0 nm (GOx/ETM/$AuNP_{6.0}$/GR) and (iii) 13.0 nm (GOx/ETM/$AuNP_{13.0}$/GR) size. The amperometric signals for all the developed biosensors were higher using PD in comparison with TTF. The biosensor based on $GOx_{P.funiculosum}$ showed higher analytical signal to glucose in a comparison to biosensors based on $GOx_{A.niger}$ and $GOx_{P.adametzii}$. The registered current to glucose using $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode was linear in the glucose range from 0.1 to 10.0 mmol L^{-1} and the limit of detection was 0.024 mmol L^{-1}. Enzymatical synthesis of polypyrrole (Ppy) layer on the electrode was applied in order to expand the linear glucose detection range. After 22 h of polymerization the amperometric signal was linear in the glucose concentration range from 0.1 to 25.0 mmol L^{-1}, while after 69 h this rage was increased up to 50.0 mmol L^{-1}. Additionally Ppy layer on the electrode surface reduced the influence of interfering species on the amperometric signal. The performance of developed biosensor was investigated in human serum samples.

1. INTRODUCTION

Glucose biosensor-related research has tremendous interest from the introduction of the first glucose biosensor based on glucose oxidase (GOx) in 1962 [1]. The electrochemical GOx-based biosensors are still among the most widely used, although many improvements have been added since the 1960's. Currently, these biosensors are applied in different areas such as food and pharmaceutical industry and particularly in clinical diagnostics because diabetes is a worldwide public health problem [2–5]. A glucose biosensor with a commercial success is likely to be small, not expensive and portable to meet the interest of millions of diabetic patients which daily need to perform glucose test in a simple way and everywhere. The biosensor should display high storage and operational stability, enjoy a simple and stable calibration. Real challenge is to minimize blood volume and design an alternative system to avoid painful sampling [6].

Over the last ten years glucose biosensors based on nanomaterials with enhanced performance were widely designed. The application of nanomaterials in biosensor design allows developing a biosensor with a commercial success, such as sensitivity, stability, miniaturisation, continuous and *in situ* monitoring in a complex matrix [2]. High biocompatibility, nanometric scale, long shelf-life, perfect optical properties and simple preparation make gold nanoparticles (AuNP) one of the most attractive and widely studied nanomaterial [7–9]. AuNP provide many advantages and

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new possibilities for enzymes immobilization on the surface of electrode [10]. Various size AuNP have been used in glucose biosensors design, but small nanoparticles appeared the most suitable for enzyme immobilization, because they could bind with enzymes without unfolding of their structure [11,12].

A lot of methods are used for the deposition of AuNP on the surface of electrode, but non-electrochemical methods are the most attractive and usable [13–15]. Chemically immobilized AuNP thin film is stable [10] and it improves conductivity, analytical sensitivity and selectivity of the electrode, as well as facilitates the electron transfer [16–19]. Novel amperometric glucose biosensor based on the immobilization of GOx and 12.0 nm AuNP on a glassy carbon electrode by a Nafion film was developed by Zhao et al. [20]. Hoshi et al. [21] proposed glucose sensors based on multilayers consisting of layer by layer deposited GOx and AuNP (5.0, 10.0, or 50.0 nm) on sensor substrates, such as a platinum electrode and a quartz glass plate. Another group of scientists developed a novel way to fabricate glucose biosensor by covalent attachment of GOx to a 2.6 nm size AuNP monolayer modified gold electrode [22]. Liu and Ju described the direct electrochemistry of GOx adsorbed on a 24.0 nm AuNP modified carbon paste electrode [23]. A group of scientists proposed a novel method to fabricate glucose biosensor by immobilization of GOx on 11.0 nm AuNP, which had self-assembled on gold electrode modified with thiol-containing three-dimensional network of silica gel [24]. A feasible approach to constructing multilayer films of GOx and 12.0 nm AuNP on gold electrode surfaces using cysteamine as a covalent cross-linker was described by Yang et al. [25]. Due to large specific surface area and high surface free energy AuNP can adsorb biomolecules strongly. The AuNP are biocompatible and can retain biological activity of adsorbed biomolecules. That plays an important role in the immobilization of biomolecules and construction of biosensors [12].

Many advantages and new possibilities are provided by π–π conjugated polymers. In designing biosensors they can be used as a matrix for physical adsorption or covalent enzyme immobilization. Due to excellent conductivity and electroactivity, these polymers can act as mediators and facilitate the electron transfer to the electrode. One of the most widely used π–π conjugated polymer is polypyrrole (Ppy). Ppy is chemically stable on various substrate materials and can be synthesized by different electrochemical, chemical oxidative and enzymatic polymerization techniques [26–28]. Ppy is often used as a matrix for the incorporation of metal particles and for the immobilization of enzymes [29,30].

The aim of this research was to evaluate the efficiency and the applicability of GOx from *Aspergillus niger* ($GOx_{A.niger}$), *Penicillium adametzii* ($GOx_{P.adametzii}$) and *Penicillium funiculosum* ($GOx_{P.funiculosum}$), and AuNP of different size for amperometric glucose biosensors design. Tetrathiafulvalene (TTF) and 1,10-phenanthroline-5,6-dione (PD) immobilized on the electrode were used as electron transfer mediators (ETM). The influence of enzymatically synthesized Ppy layer on the linear glucose detection range was tested. The performance of the developed biosensor and influence of interfering species on the amperometric signal were investigates in human serum samples.

2. Experimental

2.1. Chemicals

$GOx_{A.niger}$, type VII was purchased from Sigma–Aldrich (Buchs, Switzerland). TTF and PD were purchased from Sigma–Aldrich (Buchs, Switzerland). D-(+)-glucose, tetrachloroauric acid ($HAuCl_4 \times 3H_2O$) and tannic acid were obtained from Carl Roth GmbH&Co (Karlsruhe, Germany), $NaH_2PO_4 \times 2H_2O$ and $Na_2PO_4 \times 12H_2O$ – from Reachim (Saint Petersburg, Russia). CH_3COONa was purchased from Penta (Praha, Czech Republic), HCl – from Acta Medica (Hradec Kralove, Czech Republic), KCl – from Lachema (Neratovice, Czech Republic), acetonitrile – from Carl Roth GmbH&Co (Karlsruhe, Germany), pyrrole (Py) – from Sigma–Aldrich (Steinheim, Germany), glutaraldehyde – from Fluka Chemie GmbH (Buchs, Switzerland). All other chemicals used in the present study were either analytical pure or of highest quality.

$GOx_{P.adametzii}$ and $GOx_{P.funiculosum}$ were received from Institute of Microbiology, National Academy of Science (Minsk, Belarus). Enzyme preparations from active strain-producer of extracellular $GOx_{P.adametzii}$ LF F-2044.1 [31] and GOx $_{P.funiculosum}$ 46.1 [32] were produced by sequential ultrafiltration [33,34]. The method for the determination of GOx activity is based on enzymatic conversion of 1.4-benzoquinone into hydroquinone and the measurement of hydroquinone formation rate at 290 nm [35–37]. The unit of GOx activity (U) was defined as the amount of enzyme sufficient to catalyze transformation of 1 mmol L^{-1} 1.4-benzoquinone into hydroquinone during 1 min at 25 °C. Enzyme activity is expressed in U mL^{-1}. Glucose oxidases derived from *Penicillium* fungi are distinguished by lower specific activities, but their glucose binding efficiencies are higher [33,34].

Pyrrole was purified by passing 1.5 mL aliquots through a neutral Al_2O_3 column (5.0 cm length and 0.4 cm diameter) to remove all coloured components. Before investigations glucose solution was allowed to stay overnight to reach equilibrium of the α and β optical isomers. 0.05 mol L^{-1} sodium acetate-phosphate buffer solution (A-PBS) pH 6.0 with 0.1 mol L^{-1} KCl and other solutions were prepared in deionised water purified with water purification system Millipore S.A. (Molsheim, France). Graphite rods (3.0 mm diameter, 150 mm length, 99.999% purity, low density) were purchased from Sigma–Aldrich (St. Louis, USA).

2.2. Synthesis of AuNPs

AuNP of different size (3.5, 6.0 and 13.0 nm) were synthesized reducing $HAuCl_4 \times 3H_2O$ by sodium citrate in the presence of tannic acid. An aqueous solution of tetrachloroauric acid (81 mL of 0.01% [w/v] $HAuCl_4 \times 3H_2O$) was brought to boiling in an Erlenmeyer flask on a magnetic stirrer with electric heating. Sodium citrate solution (4 mL of 1% [w/v]) and tannic acid in deionised water (5.0, 0.5 and 0.05 mL of 1% [w/v] solution for 3.5, 6.0 and 13.0 nm AuNP, respectively) were added to the flask and heated up to 60 °C stirring rapidly. After preheating solutions were mixed, heated up to 98 °C and kept at this temperature for 3 min to yield solution of AuNP. The reaction duration was 10 min and the mixing speed 1000 rpm. Then 5 mL of 25 mmol L^{-1} Na_2CO_3 was added for the neutralization of the solution containing 3.5 nm size AuNP. The size of AuNP was measured by atomic force microscope (AFM). The average size of AuNP distributed on the surface of 8 Å SiO_2 substrate received from AIXTRON AG (Aachen, Germany) was evaluated from the height-distribution histograms of AFM images. Formed AuNP are nearly monodispersed since distribution in diameter of 13.0, 6.0 and 3.5 nm AuNPs is narrow, within the range of 12–16, 5–7 and 2–5 nm, respectively [29]. The initial concentration of gold in all used solutions was the same – 0.058 mg mL^{-1}. Solutions of AuNP were stored in dark glass flasks at +4 °C [38].

2.3. Pre-treatment of the working electrodes

Graphite rod was cut and polished on fine emery paper. After this the surface of electrodes was rinsed with distilled water, dried at 20 ± 2 °C and sealed into silicone tube in order to prevent contact of the electrode side surface with the solution. The working surface area of GR electrodes was 0.071 cm^2.

2.4. Electrode premodification by AuNP

Colloid solutions of 3.5, 6.0 and 13.0 nm AuNP were used. During premodification of GR electrodes, 3 μL AuNP of a certain size were deposited on the electrode (AuNP/GR) and then water was evaporated at room temperature by intensive ventilation ($AuNP_{3.5}$/GR, $AuNP_{6.0}$/GR and $AuNP_{13.0}$/GR).

2.5. Immobilization of ETM and GOx

Two types of working electrodes were used: (i) premodified with AuNP and (ii) bare GR electrodes. During adsorption of ETM 4.5 μL of 0.048 mol L^{-1} TTF or PD solution in acetonitrile were deposited on the electrode surface (ETM/GR, ETM/$AuNP_{3.5}$/GR, ETM/$AuNP_{6.0}$/GR and ETM/$AuNP_{13.0}$/GR). Solvent was evaporated at room temperature. Then 2.09 μL of $GOx_{P.funiculosum}$ solution (initial activity of enzyme solution 2105.1 U mL^{-1}), 3.02 μL of $GOx_{A.niger}$ solution (1455.6 U mL^{-1}) or 2.76 μL of $GOx_{P.adametzii}$ solution (1594.9 U mL^{-1}) were deposited on above-mentioned electrodes ($GOx_{P.funiculosum}$/ETM/GR, $GOx_{A.niger}$/ETM/GR, $GOx_{P.adametzii}$/ETM/GR, $GOx_{P.funiculosum}$/ETM/AuNP/GR, $GOx_{A.niger}$/ETM/AuNP/GR, $GOx_{P.adametzii}$/ETM/AuNP/GR) in order to achieve the same amount of active enzyme on the surface of electrode (4.4 U). After water evaporation all modified electrodes were incubated for 15 min over 25% solution of glutaraldehyde at room temperature in a closed vessel. The development and optimization of this immobilization procedure was described in detail previously [39]. Prior to electrochemical measurements, the electrodes were thoroughly washed with distilled water to remove non-cross-linked enzyme and/or AuNP. All modified electrodes were stored in a closed vessel hanging over a drop of A-PBS pH 6.0 at +4 °C to maintain constant humidity until needed in the experiment.

2.6. Modification of $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode by layer of Ppy

Polymerization of pyrrole over $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode was carried out by immersing the electrode into A-PBS pH 6.0 containing 0.05 mol L^{-1} glucose and 0.2 mol L^{-1} pyrrole (polymerization solution) at +4 °C for a definite period of time (22, 43 and 69 h). Prior to electrochemical measurements the electrodes were thoroughly washed with distilled water. Between measurements electrodes were stored in a closed vessel hanging over a drop of A-PBS pH 6.0 at +4 °C.

2.7. Electrochemical measurements

All electrochemical measurements were performed using a computerized potentiostat PGSTAT 30/Autolab (EcoChemie, Netherlands) with GPES 4.9 software in a constant potential amperometry mode at +0.3 V *vs.* Ag/AgCl in 3 mol L^{-1} KCl (Ag/AgCl/KCl). A conventional three-electrode system comprising a modified working graphite electrode, 2 cm^2 platinum as an auxiliary electrode and Ag/AgCl/KCl Metrhom (Herisau, Switzerland) as a reference was employed for all electrochemical experiments. All experiments were performed at room temperature in stirred (120 rpm) A-PBS pH 6.0 with 0.1 mol L^{-1} KCl. Electrochemical detection of the analytical signal was performed at different concentration of glucose. The results of all electrochemical measurements are reported as the mean value of three independent experiments.

2.8. Calculations

Amperometric signals showed hyperbolic dependence on glucose concentration and it was in agreement with Michaelis–Menten kinetics. The kinetic parameters, in particular maximal current generated during enzyme-catalyzed reaction (I_{max}) and apparent Michaelis constant ($K_{Mapp.}$), were correspondingly *a* and *b* parameters of hyperbolic function $y = ax/(b + x)$, which was used for approximation of results. The kinetic parameters of the enzyme-catalyzed reaction were calculated using SigmaPlot software.

Calibration curves of all investigations were obtained by measuring triplicate, and calibration curve parameters (slope, intercept, correlation coefficient) were calculated. The limit of detection (LOD) as the lowest concentration of analyte, which gives an analytical signal greater than the background value plus 3 δ was estimated.

3. Results

The principle of electrochemical biosensors is usually based on the monitoring of current associated with oxidation or reduction of an electroactive species involved in the recognition process [40]. In our research, redox mediators TTF or PD were adsorbed on bare GR or AuNP/GR electrode surface and then corresponding GOx (from *Aspergillus niger*,*Penicillium adametzii* or *Penicillium funiculosum*) was immobilized onto the physically modified graphite surface by cross-linking with glutaraldehyde (GOx/ETM/GR and GOx/ETM/AuNP/GR electrodes). In the presence of glucose and dissolved oxygen immobilized GOx generated hydrogen peroxide and gluconolactone, which was non-enzymatically hydrolysed to gluconic acid. During GOx catalysed enzymatic reaction electrons were transferred towards positively charged electrode and steady-state current was registered (Fig. 1). The redox mediator and AuNP mediated electron transfer between the FAD/$FADH_2$ and electrode surface [39]. This results in the increase of electrocatalytic current [8,41], therefore the sensitivity of electrochemical biosensors becomes higher. According to our previous published research

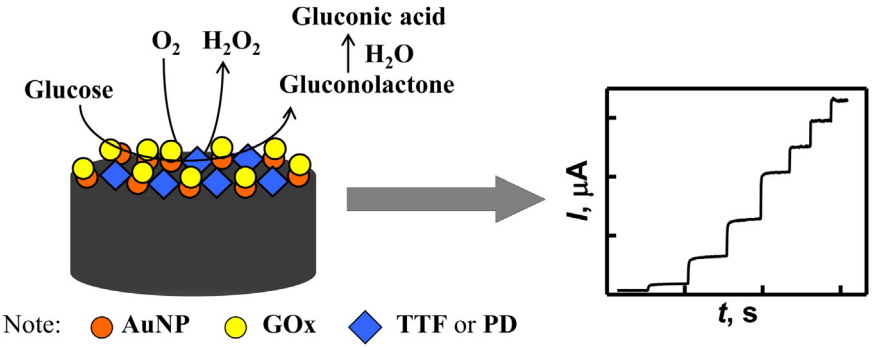

Fig. 1. Schematic illustration of glucose amperometric biosensor based on GR electrode premodified with AuNP, immobilized ETM and GOx.

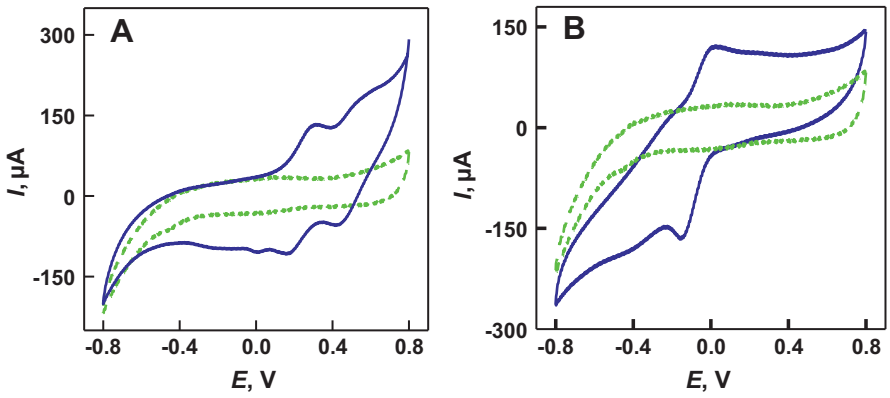

Fig. 2. Cyclic voltammograms registered using electrodes without and with redox mediator TTF or PD. Scan rate: 100 mV s^{-1} in A-PBS. Solid line – cyclic voltammograms with redox mediator, while dashed line – cyclic voltammograms without redox mediator.

using GOx/AuNP$_{13.0}$/GR electrode and soluble redox mediator 1.8 times higher amperometric signal was registered when compared with AuNP non-modified electrodes [39]. Unique chemical and physical properties of AuNP could make them suitable for designing new highly sensitive electrochemical sensors and biosensors.

The electrochemical behaviour of adsorbed electron transfer mediators TTF and PD was determined by cyclic voltammetry. Fig. 2 shows the cyclic voltammograms of GOx/GR and GOx/ETM/GR electrodes registered in A-PBS pH 6.0 with 0.1 mol L^{-1} KCl. Anodic peaks at +0.307 and +0.566 V were observed for the GOx/TTF/GR electrode (Fig. 2A, solid line). The anodic peak for the GOx/PD/GR electrode was at +0.010 V (Fig. 2B, solid line). From these cyclic voltammograms it is obvious that the constant potential of +0.3 V *vs.* Ag/AgCl/KCl is suitable for both TTF and PD. Hence, all electrochemical measurements were performed at a constant potential of +0.3 V in order to make a correct comparison of registered currents. The anodic peak at +0.3 V has been reported earlier for a GOx/TTF/GR electrode [42]. Cyclic voltammogram of PD adsorbed on a glassy carbon electrode modified with multi-walled carbon nanotubes [43] is very similar to resent our results.

For GOx/ETM/GR and GOx/ETM/AuNP/GR electrodes the hyperbolic dependences of analytical signals on the concentration of glucose in the range from 0.1 to 250 mmol L^{-1} in A-PBS pH 6.0 with 0.1 mol L^{-1} KCl were registered. Hyperbolic dependences were in agreement with Michaelis–Menten kinetics. The kinetic parameters maximal current generated during enzyme-catalyzed reaction (I_{max}) and apparent Michaelis constant ($K_{Mapp.}$) are correspondingly *a* and *b* parameters of hyperbolic function $y = ax/(b+x)$. In accordance with the experimental data and the calculations made, GOx/ETM/GR and GOx/ETM/AuNP/GR electrodes exhibited different I_{max} and $K_{Mapp.}$ values. In addition, these kinetic parameters depended on the type of mediator and GOx used. As mentioned above, two types of modified GR electrodes were developed and studied. One type was based on GR electrodes modified with ETM (TTF or PD) and different types of GOx (from *Aspergillus niger*, *Penicillium adametzii* or *Penicillium funiculosum*). Another type was based on GR electrodes premodified with AuNP of different size (3.5, 6.0 and 13.0 nm), ETM and different types of GOx. As can be seen from results presented in Table 1, the I_{max} obtained using GOx$_{P.adametzii}$/PD/AuNP$_{3.5}$/GR electrodewas 1.56, 1.51 and 1.20 times higher in comparison with GOx$_{P.adametzii}$/PD/GR, GOx$_{P.adametzii}$/PD/AuNP$_{13.0}$/GR and GOx$_{P.adametzii}$/PD/AuNP$_{6.0}$/GR electrode, respectively. A similar effect was observed with other types of GOx used. I_{max} value achieved using GOx$_{A.niger}$/PD/AuNP$_{3.5}$/GR electrode was 1.31, 1.16 and 1.09 times higher in comparison to GOx$_{A.niger}$/PD/GR, GOx$_{A.niger}$/PD/AuNP$_{13.0}$/GR and GOx$_{A.niger}$/PD/AuNP$_{6.0}$/GR electrodes, respectively. The highest I_{max} value was achieved using GOx$_{P.funiculosum}$/PD/AuNP$_{3.5}$/GR electrode and it was 1.63, 1.59 and 1.33 times higher in comparison to GOx$_{P.funiculosum}$/PD/GR, GOx$_{P.funiculosum}$/PD/AuNP$_{13.0}$/GR and GOx$_{P.funiculosum}$/PD/AuNP$_{6.0}$/GR electrode, respectively. It could be related to significantly increased electron transfer to GR electrode, increased effective surface area of electrode, as well as to retained enzyme activity after immobilization on smaller AuNP [39].

According to calculations presented in Tables 1–2, the currents of developed glucose biosensors in A-PBS pH 6.0 with 0.1 mol L^{-1} KCl depended not only on the AuNP particle size, but also on the type of mediator and GOx used. PD increased the response to glucose more significantly than TTF. I_{max} values calculated for GOx$_{A.niger}$/PD/AuNP$_{3.5}$/GR, GOx$_{P.adametzii}$/PD/AuNP$_{3.5}$/GR and GOx$_{P.funiculosum}$/PD/AuNP$_{3.5}$/GR electrodes were 1.01, 1.91 and 1.84 times higher in comparison to GOx$_{A.niger}$/TTF/AuNP$_{3.5}$/GR, GOx$_{P.adametzii}$/TTF/AuNP$_{3.5}$/GR and GOx$_{P.funiculosum}$/TTF/AuNP$_{3.5}$/GR electrodes, respectively. The similar tendency was observed for GR electrodes based on immobilized GOx and redox mediator. I_{max} values calculated for GOx$_{A.niger}$/PD/GR, GOx$_{P.adametzii}$/PD/GR and GOx$_{P.funiculosum}$/PD/GR electrodes were 1.72, 4.00 and 2.52 times higher in a comparison to GOx$_{A.niger}$/TTF/GR, GOx$_{P.adametzii}$/TTF/GR and GOx$_{P.funiculosum}$/TTF/GR electrodes. As can be seen from presented results, biosensor based on 3.5 nm AuNP and GOx from *P. funiculosum* showed higher I_{max} value to glucose in a comparison to biosensors based on GOx from *A. niger* and *P. adametzii*. I_{max}

Table 1
Calculated I_{max} and $K_{Mapp.}$ values for different types of electrodes using PD as electron transfer mediator.

Type of electrode	The type of GOx					
	Aspergillus niger		*Penicillium adametzii*		*Penicillium funiculosum*	
	I_{max}, μA	$K_{Mapp.}$, mmol L^{-1}	I_{max}, μA	$K_{Mapp.}$, mmol L^{-1}	I_{max}, μA	$K_{Mapp.}$, mmol L^{-1}
GOx/PD/AuNP$_{3.5}$/GR	86.7	120.0	69.4	101.0	118.0	32.3
GOx/PD/AuNP$_{6.0}$/GR	79.4	128.1	57.8	78.4	88.4	32.1
GOx/PD/AuNP$_{13.0}$/GR	74.9	131.5	46.1	81.0	74.3	36.0
GOx/PD/GR	66.4	121.9	44.4	96.2	72.2	45.1

Table 2
Calculated I_{max} and $K_{Mapp.}$ values for different types of electrodes using TTF as electron transfer mediator.

The type of electrode	The type of GOx					
	Aspergillus niger		*Penicillium adametzii*		*Penicillium funiculosum*	
	I_{max}, μA	$K_{Mapp.}$, mmol L^{-1}	I_{max}, μA	$K_{Mapp.}$, mmol L^{-1}	I_{max}, μA	$K_{Mapp.}$, mmol L^{-1}
GOx/TTF/$AuNP_{3.5}$/GR	85.7	26.9	36.4	11.3	64.2	26.5
GOx/TTF/$AuNP_{6.0}$/GR	71.2	34.0	25.2	12.1	60.6	33.2
GOx/TTF/$AuNP_{13.0}$/GR	65.6	29.9	15.8	20.4	40.2	21.6
GOx/TTF/GR	38.5	26.4	11.1	17.5	28.6	16.4

value for $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode was 1.36 and 1.70 times higher than for electrodes based on GOx from *A. niger* and *P. adametzii*.

The immobilization of the enzyme is followed by relatively low $K_{Mapp.}$, which could indicate that the enzyme immobilized on the surface of graphite rod retains its activity with relatively low diffusion barrier [44]. Coinciding with our calculations presented in Table 1, $K_{Mapp.}$ value (45.1 mmol L^{-1}) obtained for $GOx_{P.funiculosum}$/PD/GR electrode was 1.40 times higher, if compared with $K_{Mapp.}$ value obtained for $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR and $GOx_{P.funiculosum}$/PD/$AuNP_{6.0}$/GR electrode (32.3 and 32.1 mmol L^{-1}) and 1.25 times higher if compared to $GOx_{P.funiculosum}$/PD/$AuNP_{13.0}$/GR electrode (36.0 mmol L^{-1}). High apparent Michaelis constant value provides a broad linear range of analytical system. It is particularly relevant for the detection of glucose concentrations in food, beverages and blood. But the magnitude of analytical signal is also highly important factor that influences the accuracy of the analysis. Therefore, $GOx_{P.funiculosum}$/PD/AuNP/GR electrodes were chosen for next investigations. These electrodes were characterized by sufficient $K_{Mapp.}$ (32.3, 32.1 and 36.0 mmol L^{-1} using 3.5, 6.0 and 13.0 nm AuNP, respectively) and the highest I_{max} value (118.0 μA using 3.5 nm AuNP).

Hyperbolic dependences of the analytical signal on the concentration of glucose in the range from 0.1 to 250 mmol L^{-1} are presented in Fig. 3A for $GOx_{P.funiculosum}$/PD/AuNP/GR electrodes. According to experimental results and calculations presented in Table 1, the application of 3.5 nm AuNP for the electrode construction significantly increased the registered current. I_{max} values calculated for $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrodes were 1.33 and 1.59 times higher in comparison to electrodes modified with 6.0 and 13.0 nm AuNP.

The registered current at 10 mmol L^{-1} concentration of glucose (ΔI = 28.8 μA) using $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode (Fig. 3B curve 4) was compared with analytical signals (registered at the same glucose concentration) of other published analytical systems where AuNP were applied to amplify the electrochemical signal. The registered current of glucose biosensor based on immobilized GOx in thin films of chitosan containing graphene and 7–16 nm AuNP at a gold electrode was about 11 μA [13]. The current of glucose biosensor based on the immobilized GOx and 12.0 nm AuNP on a glassy carbon electrode by a Nafion film was slightly more than 4.5 μA [20], while the current of about 3.6 μA value was registered after covalent attachment of GOx on the 2.6 nm AuNP monolayer formed on the gold electrode [22]. For the glucose biosensor fabricated by immobilization of GOx on 11.0 nm AuNP which had self-assembled on gold electrode modified with thiol-containing three-dimensional network of silica gel registered analytical signal to 10 mmol L^{-1} of glucose was 2.6 μA [24]. The current of about 2.4 μA was registered for biosensor based on multilayer films of GOx and 12.0 nm AuNP formed on the gold electrode surface using a cysteamine [25]. The current of glucose biosensor based on multilayer membranes formed by the layer by layer deposition of GOx and 10 nm AuNP on a platinum electrode was about 1.0 μA [21]. Significant differences in values of current registered with $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode and described in scientific papers could be explained by different measurement's methodology, size of AuNP, electrode surface modification protocol and choice of proper ETM. AuNP together with PD increased electron transfer from redox centre of GOx to the surface of GR electrode and it was reflected in higher I_{max}. Therefore, it can be stated that in the present research designed $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode is characterized by higher registered current at the same analyte concentration in comparison to other discussed glucose biosensors.

The dependences of analytical signal of $GOx_{P.funiculosum}$/PD/AuNP/GR (3.5, 6.0 and 13.0 nm of AuNP) electrodes (Fig. 3B) in comparison to the $GOx_{P.funiculosum}$/PD/GR at low glucose concentrations (from 0.1 to 10 mmol L^{-1}) were close to linear without the intercepts on *x*- or *y*-axis and with correlation coefficients of 0.9931, 0.9904, 0.9970 and 0.9784, respectively. The linear response range for $GOx_{P.funiculosum}$/PD/AuNP/GR and $GOx_{P.funiculosum}$/PD/GR electrodes was very similar (up to 10.0 mmol L^{-1}) to those presented for other above discussed glucose biosensors with AuNP modified electrodes [13,20,22,24]. The calculated sensitivity of $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR and $GOx_{P.funiculosum}$/PD/GR electrodes at 5 mmol L^{-1} glucose was 52.1 and 28.5 μA cm^{-2} (mmol L^{-1})$^{-1}$, respectively. The sensitivity of $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode was higher if compared to the sensitivity of other analytical systems [22–25].

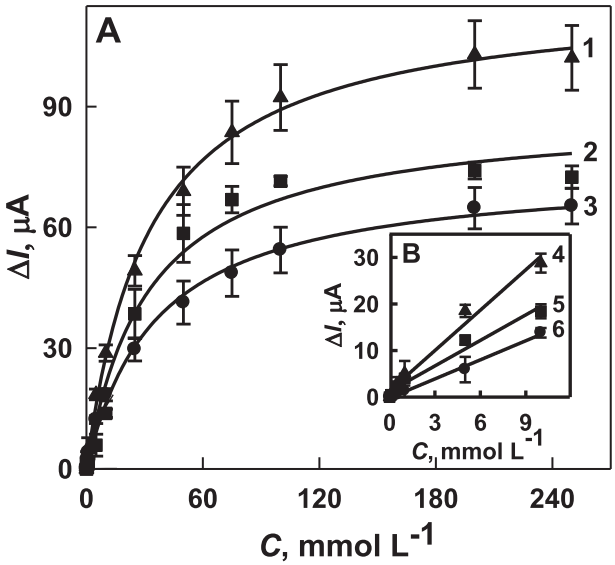

Fig. 3. Calibration plots of $GOx_{P.funiculosum}$/PD/AuNP/GR electrodes modified with AuNP of different size for 0.1–250 mmol L^{-1} (A) and 0.1–10.0 mmol L^{-1} (B) glucose concentrations. 1, 4 curves – 3.5 nm AuNP, 2, 5 curves – 6.0 nm AuNP and 3, 6 curves – 13.0 nm AuNP. Conditions: 0.05 mol L^{-1} A-PBS pH 6.0, with 0.1 mol L^{-1} KCl; +0.3 V *vs.* Ag/AgCl/KCl.

Analytical signals of developed electrochemical biosensors were reproducible. The relative standard deviation (RSD) estimated for different $GOx_{P.funiculosum}/PD/AuNP_{3.5}/GR$ and $GOx_{P.funiculosum}/PD/GR$ electrodes using 4.37 mmol L^{-1} of glucose was found to be 7.5 and 8.5% respectively. Relatively good reproducibility of designed electrodes may be explained by the fact that the amount of assembled AuNP and GOx were consistent and molecules of enzyme are firmly adsorbed on the surface of the nanoparticles [22]. Smaller nanoparticles resulted in a higher biosensor response to glucose and higher sensitivity to the analyte.

The LOD evaluated for $GOx_{P.funiculosum}/PD/AuNP_{3.5}/GR$ electrode was 0.024 mmol L^{-1} and it was 3.96 times lower than for $GOx_{P.funiculosum}/PD/GR$ electrode (0.095 mmol L^{-1}). The LOD depends on the type of enzyme and the size of nanoparticles. The LOD for $GOx_{P.funiculosum}/PD/AuNP_{3.5}/GR$ electrode was lower if compared with some of the previously published glucose biosensors, where different GOx and AuNP immobilization methods and electrodes were used. For biosensor based on GOx immobilized in chitosan films containing nanocomposites of graphene and AuNP (7–16 nm) on Au electrode LOD was 0.18 mmol L^{-1} [13], after immobilization of GOx/AuNP (12 nm) on glassy carbon electrode by Nafion film LOD was 0.034 mmol L^{-1}, but linear glucose detection range up to 6.0 mmol L^{-1} [20]. Using GOx immobilized on AuNP (13 nm) modified GR electrode LOD was 0.080 mmol L^{-1} [39]. The same LOD was obtained using biosensor based on GOx immobilized on AuNP electrochemically predeposited on the GR electrode [45].

The major advantages of newly developed amperometric glucose biosensor based on 3.5 nm AuNP, immobilized PD and GOx from *P. funiculosum* are simple, quick (within 5 s) and reagentless detection of glucose, high sensitivity, linear glucose detection range up to 10 mmol L^{-1} and low limit of analyte detection.

For the extension of linear glucose detection range additional layer of polypyrrole was enzymatically synthetized on the developed electrode surface. The influence of polymer layer on the analytical parameters of $Ppy/GOx_{P.funiculosum}/PD/AuNP_{3.5}/GR$ electrodes was investigated (Fig. 4). During the enzymatic polymerization of pyrrole in the presence of glucose $GOx_{P.funiculosum}/PD/AuNP_{3.5}/GR$ electrode surface was covered with Ppy layer. In A-PBS pH 6.0 GOx is negatively charged, while polymer chains are positively charged, and electrostatic interaction takes place on the modified electrode surface [26]. The measurements performed using electrodes modified with small AuNP showed higher amperometric responses before and after the polymer layer formation if compared with electrodes modified with larger AuNP.

In order to extend the linear glucose detection range Ppy layer was synthetized on the electrode surface for 22, 43 and 69 h. In the presence of glucose and dissolved oxygen immobilized GOx generated hydrogen peroxide and gluconolactone, which was hydrolysed to gluconic acid. The result of this reaction is the decreased pH and increased hydrogen peroxide concentration close to GOx. These conditions are excellent for the polymerization and covering the $GOx_{P.funiculosum}/PD/AuNP_{3.5}/GR$ electrode by Ppy layer [29]. Hyperbolic dependences of amperometric signals on the concentration of glucose in the range from 0.1 to 250 mmol L^{-1} were observed for $Ppy/GOx_{P.funiculosum}/PD/AuNP_{3.5}/GR$ electrodes at different stages of Ppy layer formation (Fig. 4A). The duration of polymerization had a significant influence on the current and linear glucose detection range. For $Ppy/GOx_{P.funiculosum}/PD/AuNP_{3.5}/GR$ electrodes I_{max} decreased 1.71, 1.95 and 2.79 times after 22, 43 and 69 h of the polymerization if compared with electrode without polymer layer (Fig. 4B). On the contrary, $K_{Mapp.}$ increased and it was 37.93, 47.36 and 55.41 mmol L^{-1}, respectively. The Michaelis constant for native glucose oxidase from *P. funiculosum* 46.1 is 12.9–15.4 mmol L^{-1} in the pH range within 5.0–7.0 for phosphate, citrate and universal buffers [33].

It was determined that the linear glucose detection range of $Ppy/GOx_{P.funiculosum}/PD/AuNP_{3.5}/GR$ electrodes increased together with the time of the polymerization. For electrodes that were kept in the polymerization solution for 22 h the linear glucose detection range was extended up to 25 mmol L^{-1} (2.5 times in comparison with electrode without the Ppy layer), while after 69 h it can be extended up to 50 mmol L^{-1} of glucose (5 times in comparison with electrode without the Ppy layer). The same tendency after Ppy layer formation was observed by electrodes modified with 13 nm AuNP, $GOx_{A.niger}$ and using soluble electron transfer mediator PMS [29]. Extension of the linear glucose detection range could be useful for the determination of glucose in real samples where glucose concentration might be in the range of 0.1–25 mmol L^{-1}.

The performance of developed amperometric glucose biosensor using $Ppy/GOx_{P.funiculosum}/PD/AuNP_{3.5}/GR$ electrode after 22 h of

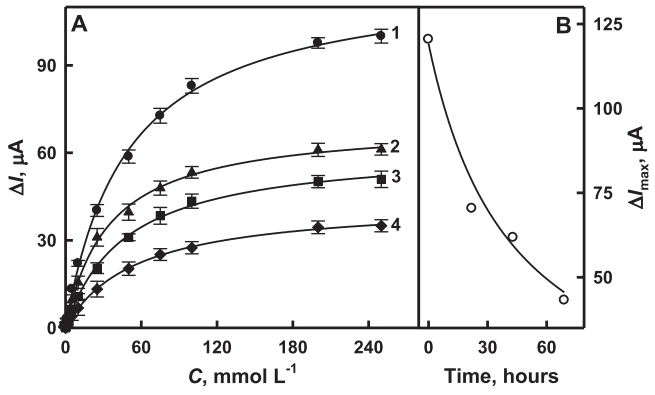

Fig. 4. (A) Calibration plots registered for $GOx_{P.funiculosum}/PD/AuNP_{3.5}/GR$ (curve 1) and $Ppy/GOx_{P.funiculosum}/PD/AuNP_{3.5}/GR$ electrodes covered with different thickness Ppy layer (curves 2–4 – after 22, 43 and 69 h of polymerization) and (B) changes of maximal current. Conditions: 0.05 mol L^{-1} A-PBS pH 6.0 with 0.1 mol L^{-1} KCl; +0.3 V *vs.* Ag/AgCl/KCl.

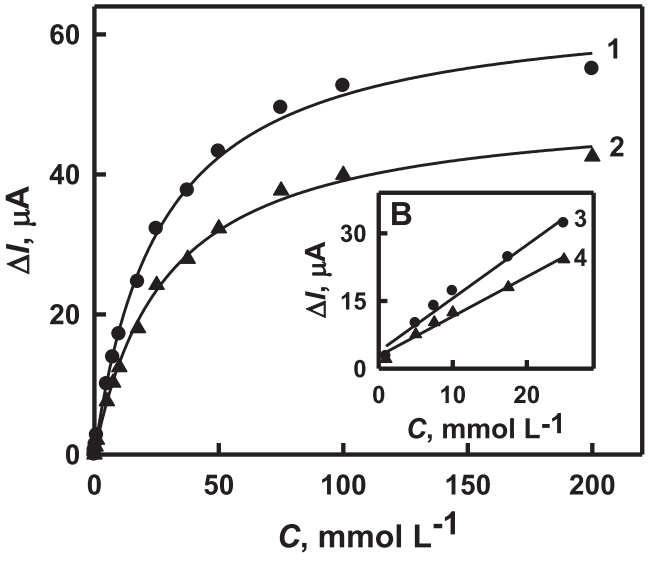

Fig. 5. Calibration plots of $Ppy/GOx_{P.funiculosum}/PD/AuNP_{3.5}/GR$ electrode after 22 h of the polymerization in the buffer and diluted serum from 0.1 to 200 mmol L^{-1} (A) and from 0.1 to 24.8 mmol L^{-1} of glucose (B). 1, 3 curves –0.05 mol L^{-1} A-PBS pH 6.0 containing 0.1 mol L^{-1} KCl; 2, 4 curves – human serum diluted 10 times with A-PBS pH 6.0 containing 0.1 mol L^{-1} KCl. Amperometric signal was recorded at +0.3 V *vs.* Ag/AgCl/KCl.

Table 3
Recovery of glucose in human serum sample using an electrochemical biosensor based on Ppy/$GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode after 22 h of polypyrrole synthesis (n – number of measurements).

Added concentration of glucose, mmol L^{-1}	Detected concentration of glucose ($n = 3$), mmol L^{-1}	Recovery, %
4.82	4.62	96
6.47	6.30	97
11.6	11.3	97
20.0	19.5	98

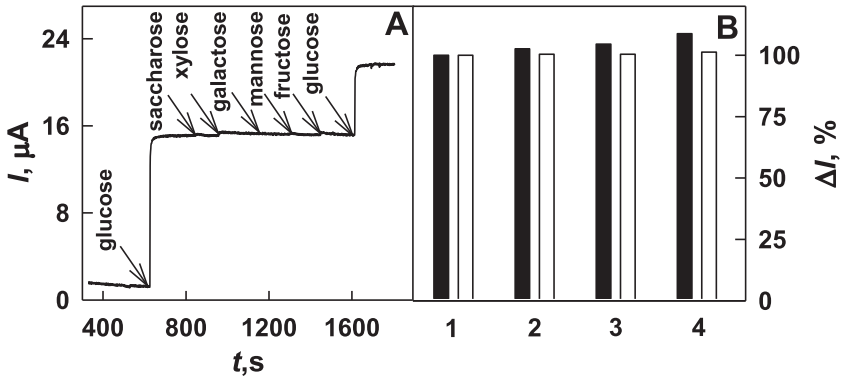

Fig. 6. Effect of interfering species on the amperometric signal of glucose biosensor before and after the Ppy layer formation. (A) – Amperogram registered in a 10 times diluted human serum sample after addition of the 10.0 mmol L^{-1} glucose, 1 mmol L^{-1} of saccharose, xylose, galactose, mannose or fructose. (B) Diagrams of registered currents in human serum after addition of 10.0 mmol L^{-1} glucose (1 column); 10.0 mmol L^{-1} glucose with 0.01 mmol L^{-1} ascorbic acid (2 column); 10.0 mmol L^{-1} glucose with 0.05 mmol L^{-1} ascorbic acid (3 column); 10.0 mmol L^{-1} glucose with 0.1 mmol L^{-1} uric acid (4 column); black columns – results obtained with $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode, white columns – Ppy/$GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode after 22 h of polymerization. Steady-state currents were registered at +0.3 V *vs.* Ag/AgCl/KCl.

polypyrrole synthesis was investigated in real samples. For this reason a human serum was diluted 10 times with A-PBS pH 6.0 containing 0.1 mol L^{-1} KCl and known concentration of glucose was added after stable base line was recorded. Hyperbolic dependence of analytical signals on the concentration of glucose in the range from 0.1 to 200 mmol L^{-1} (Fig. 5A) have the same tendency in the A-PBS and diluted serum, however registered currents in the serum were lower. The linear glucose detection range for Ppy/GOx/PD/$AuNP_{3.5}$/GR electrode in the serum was up to 24.8 mmol L^{-1} of glucose.

In the next step of experiment different concentrations of glucose were added to 10 times diluted human serum and detected using the developed electrode from the linear region of calibration curve (Fig. 5B curve 4). It was found that the recoveries were in the range of 96–98% (Table 3). The better recovery was obtained at higher glucose concentrations. Therefore, the developed electrochemical biosensor based on amperometric detection of glucose is applicable for the monitoring of glucose in the serum of diabetic patients.

The selectivity of Ppy/$GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode based biosensor was investigated in the solution of 10.0 mmol L^{-1} glucose after the addition of 1 mmol L^{-1} of saccharose, xylose, galactose, mannose or fructose (Fig. 6A). It was determined that the addition of these substrates had no effect on the registered current in the presence of glucose.

It is known that some electroactive species (ascorbic acid and uric acid) in human serum may influence the performance of electrochemical biosensors. In order to evaluate the influence of ascorbic acid and uric acid on the determination of glucose amperometric signal was registered in the solution of 10.0 mmol L^{-1} glucose with 0.01 mmol L^{-1} and 0.05 mmol L^{-1} ascorbic acid and in the solution of 10.0 mmol L^{-1} glucose with 0.1 mmol L^{-1} uric acid using unmodified and with Ppy layer modified electrodes (polymerization was performed 22 h). The influence of ascorbic and uric acids on the analytical signal of $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode was higher than using electrode modified with polymer (Fig. 6B). After the addition of glucose with 0.1 mmol L^{-1} uric acid the current increased 1.09 times, while after the addition of glucose with 0.05 and 0.01 mmol L^{-1} ascorbic acid the current increased 1.05 times using Ppy/$GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode, if compared with the current registered to glucose without electroactive species. Thus Ppy layer on the electrode surface reduced the influence of interfering species on the amperometric signal.

4. Conclusions

A comparative study of GOx from *Aspergillus niger*, *Penicillium adametzii*, *Penicillium funiculosum* and AuNP of different size for amperometric glucose detection by reagentless biosensor was performed. Graphite electrode was modified with 3.5, 6.0 or 13.0 nm AuNP, TTF or PD as electron transfer mediator and GOx. The biosensor based on $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode was characterized by quick response, high registered current and sensitivity (52.1 μA cm^{-2} (mmol L^{-1})$^{-1}$, linear glucose detection range up to 10 mmol L^{-1}, good reproducibility and low limit of detection (0.024 mmol L^{-1}). The $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode after 22 h modification by enzymatically formed Ppy layer exhibited 2.5 times higher linear glucose detection range in comparison with the same electrode without polymer layer. After 69 h this range was increased up to 50.0 mmol L^{-1}. Extended analyte detection interval could be useful for the detection of glucose concentrations in real samples. Furthermore, Ppy layer on the electrode surface reduced the influence of interfering species on the amperometric signal. In summary, glucose oxidase from *Penicillium funiculosum*, 3.5 nm gold nanoparticles and 1,10-phenanthroline-5,6-dione were selected as the most suitable for the development of amperometric reagentless glucose biosensor.

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