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Reagent-less amperometric glucose biosensor based on a graphite rod electrode layer-by-layer modified with 1,10-phenanthroline-5,6-dione and glucose oxidase

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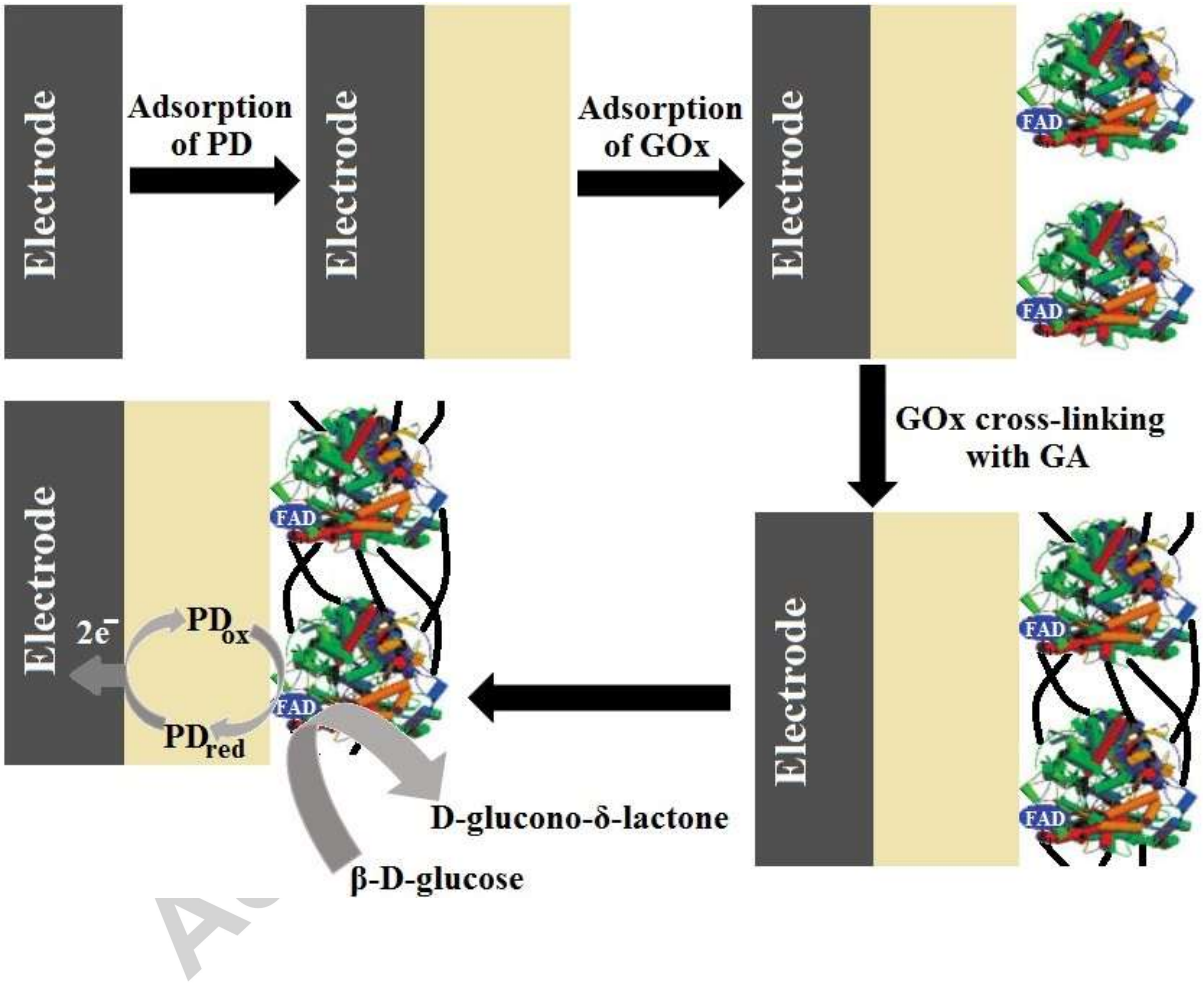
Abstract

A reagent-less amperometric glucose biosensor operating in not-stirred sample solution was developed. A working electrode of the designed biosensor was based on a graphite rod (GR) electrode, which was modified with 1,10-phenanthroline-5,6-dione (PD) and glucose oxidase (GOx). The PD and the GOx were layer-by-layer adsorbed on the GR electrode surface with subsequent drying followed by chemical cross-linking of the adsorbed GOx with glutaraldehyde (GA). Optimal preparation conditions of the working electrode (GR/PD/GOx) were achieved with 12.6 μg and 0.24 mg loading amount of PD and GOx, respectively and 25 min lasting cross-linking of the GOx with GA. A current response to glucose of the GR/PD/GOx electrode was measured at +200 mV potential *vs* Ag/AgCl reference electrode. Maximum current response was registered when the pH of the buffer solution was 6.0. The registered current response to glucose was linear in the concentration range of 0.1 – 76 mmol L^{-1} (R^2 = 0.9985) and a detection limit was 0.025 mmol L^{-1}. The GR/PD/GOx

electrode demonstrated good reproducibility and repeatability with the relative standard deviation of 6.2 and 1.8 % (at 4.0 mmol L^{-1} of glucose), respectively, high anti-interference ability to uric and ascorbic acids. It was highly selective to glucose and demonstrated good accuracy in the analysis of human serum samples.

Graphical abstract



Keywords: amperometric glucose biosensor, glucose oxidase, 1,10-phenanthroline-5,6-dione, redox mediator.

Introduction

Diabetes is a serious health disorder causing disability or even death. According to International Diabetes Federation 415 million adults worldwide have diabetes and it is expected that by 2040 this number will rise to 642 million.[1] In addition, it is supposedly that more than 200 million people have still undiagnosed diabetes. Therefore, frequent monitoring of the blood glucose levels is greatly important. Among the other available analytical tools, electrochemical biosensors are the best choice for clinical patients who have to monitor blood glucose levels on a daily basis. Glucose biosensors also can be useful in providing real time information for adjusting medications, dietary uptake and physical activities in order to achieve optimal level of blood glucose. Determination of blood glucose concentration by electrochemical glucose biosensors the mostly is associated with invasive blood sampling. It is a routine clinical procedure. However, this procedure is painful and inconvenient. Saliva, tears, urine and sweat are the other body fluids where glucose levels can be monitored.[2] Estimation of glucose in these body fluids is more convenient than painful and damaging invasive taking of blood from fingertip or vein. For this reason, non-invasive optical[3] and electrochemical glucose biosensors have been investigated[4,5,6] and among the electrochemical glucose biosensors amperometric biosensors are the most widely studied. Electrochemical glucose biosensors are usually based on the two enzyme sub-classes, namely glucose oxidase (GOx)[7,8,9,10] and glucose dehydrogenase (GDH). Glucose 6-phosphate dehydrogenase[11], glucose-1-dehydrogenase[12], quinoprotein glucose dehydrogenase[13] and flavine adenine dinucleotide (FAD) dependent glucose dehydrogenase[14] are four different glucose dehydrogenases that have been used in glucose biosensors. The GOx has a relatively high selectivity for glucose in comparison to that for other blood sugars and stability[15], simple purification procedure and lower price and good resistance towards extreme environment pHs, ionic strengths and temperatures. The biosensors are based on immobilized GOx usually have good electroactivity, biocompatibility and permeability. Because of mentioned facts, such type of biosensors is becoming very attractive.[16]

The GOx is a dimeric protein, which per monomer unit contains one tightly bound FAD molecule as a redox-active centre insulated within a protective protein shell. The basic concept of amperometric glucose biosensors with immobilized GOx is based on the fact that the GOx catalyses the oxidation of β-D-glucose into D-glucono-δ-lactone, which is non-enzymatically hydrolysed to β-D-gluconic acid, and hydrogen peroxide, which is formed in the action of molecular oxygen as an electron acceptor. The biocatalytic reaction at a working

electrode of biosensor involves the reduction of the FAD by reaction with β-D-glucose. This process results the formation of the reduced form of the enzyme (GOx-$FADH_2$):

$$GOx\text{-}FAD + \beta\text{-D-glucose} \rightarrow GOx\text{-}FADH_2 + \text{D-glucono-}\delta\text{-lactone} \quad (1)$$

Then the oxidation of formed GOx-$FADH_2$ with molecular oxygen is occuring. During this process, the reduced form – GOx-$FADH_2$ – is regenerated back into oxidized form – GOx-FAD, and in addition the H_2O_2 is formed:[17]

$$GOx\text{-}FADH_2 + O_2 \rightarrow GOx\text{-}FAD + H_2O_2 \quad (2)$$

Taking into account the reaction equation *2*, the monitoring of glucose levels with the 1st generation of electrochemical biosensors constructed by the immobilization of GOx can be based either on the determination of oxygen consumption or on hydrogen peroxide generation. In the 2nd generation of electrochemical biosensors artificial electron acceptors, which are called – redox mediator, are applied. These redox mediators are able to replace natural electron acceptor – oxygen and to transfer electrons from FAD cofactor to the working electrode as it is depicted in following reactions:

$$GOx\text{-}FADH_2 + 2M_{ox} \rightarrow GOx\text{-}FAD + 2M_{red} + 2H^+ \quad (3)$$

$$2M_{red} \rightarrow 2M_{ox} + 2e^- \quad (4)$$

The reduced form of the redox mediator (M_{red}) is reoxidized back to its oxidized form (M_{ox}) at the surface of electrode. Due to this, an amperometric signal, which is proportional to the glucose concentration, is generated.[18] Here mentioned set-up is the most widely applied in the design of amperometric glucose biosensors. The most important advantage of such set-up is that the analytical signal of mediated biosensor is not dependent on the partial pressure of oxygen, and therefore the concentration of glucose can be determined at lower potentials that do not provoke interfering reactions with coexisting electroactive species presenting in biological fluids. Furthermore, this type of biosensors has much faster electron transfer, which provides larger linear dynamic range and higher sensitivity.[19] Various compounds such as ferrocene and its derivatives[20], 9,10-phenanthrenequinone[21], ferricyanide[22], thionine[23], tetrathiafulvalene[24], phenazine methosulfate[25] and many other have been used to improve the performance of amperometric glucose biosensors. Recently it has been shown that 1,10-phenanthroline-5,6-dione (PD) layer on the graphite rod (GR) electrode surface exhibits excellent electrocatalytic activity and can be applied to facilitate electron transfer between redox enzyme GOx and GR electrode.[26]

In order to prevent the depletion of substrate near working electrode, an analytical signal of amperometric biosensor mostly is recorded in vigorously stirred sample solution. Differently from mentioned configuration, Arakawa et al.[27] proposed non-invasive biosensor,

which was dedicated for the monitoring of glucose in saliva, operating at not-stirred conditions. Glucose determination at not-stirred sample conditions is very important in the development of cheap, disposable and/or attachable amperometric glucose biosensors. This fact encouraged us to design and to investigate an amperometric glucose biosensor operating at not-stirred conditions. The biosensor was based on a graphite rod (GR) working electrode modified with a redox mediator PD and enzyme GOx. The PD and the GOx was layer-by-layer adsorbed on the GR electrode surface with subsequent airing and chemical cross-linking of the adsorbed GOx with glutaraldehyde (GA). The impact of loaded amount of the immobilized GOx and PD, an optimal pH region for the operation, reproducibility, repeatability, stability and other characteristics of designed biosensor were investigated in detail. Finally, the developed biosensor was used for the determination of glucose in real human serum samples.

Experimental

Materials and reagents

GOx type X-S from *Aspergillus niger* (EC.1.1.3.4.) of 117200 U/g enzymatic activity, PD, 25 % GA, and L-ascorbic acid (AA) were purchased from Sigma-Aldrich chemie GmbH (Steinheim, Germany). Sodium acetate (CH_3COONa), potassium chloride (KCl) and uric acid (UA) were purchased from AppliChem GmbH (Darmstadt, Germany). Potassium dihydrogen phosphate monohydrate ($KH_2PO_4 \times H_2O$) was obtained from Fluka Feinchemikalien GmbH (Neu-Ulm, Germany). Potassium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \times 12\ H_2O$), D-(+)-glucose (Glu) monohydrate, D-(+)-galactose, D-(+)-xylose, D-(+)-mannose and D-(–)-fructose were purchased from Carl Roth GmbH&Co (Karlsruhe, Germany). 100 % ethanol was obtained from MERCK KGaA (Darmstadt, Germany). Human serum was provided by the National Blood Centre (Vilnius, Lithuania).

40.0 mg mL^{-1} stock solution of GOx was freshly prepared from lyophilized enzyme powder in a buffer solution composed of 50.0 mmol L^{-1} CH_3COONa, 50.0 mmol L^{-1} Na_2HPO_4, 50.0 mmol L^{-1} NaH_2PO_4 and 100.0 mmol L^{-1} KCl (A-PBS-KCl), pH 6.0. 2.1 mg/mL^{-1} stock solution of PD was prepared in ethanol. 1.0 mol L^{-1} stock solutions of glucose, galactose, xylose, mannose or fructose and 4.0 mol L^{-1} stock solutions of uric acid or ascorbic acid were prepared in water. In order to reach an equilibrium between glucose isomers, glucose solution was made 24 hours before use. All aqueous solutions were prepared in ultra-high quality (UHQ) water purified by DEMIWA rosa 5 water purification system (WATEK, Czech Republic).

Electrode pre-treatment prior to modification by PD and GOx

Graphite rods (3 mm in diameter, 150 mm in length, 99.999 % pure, low density) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Primarily, these rods were cut and then polished using a coarse grit 130 (P500 according to ISO/FEPA Grit designation), followed by a fine 131 grit (P1500, P2000 and P2500) emery paper and finally polished to specular reflection using Al_2O_3 slurry (grain size 0.1 μm). Polished rods were washed with ethanol and three times with water and then dried at ambient temperature. In order to avoid a contact of lateral surface with solution a lateral surface of the graphite rod electrodes was isolated with silicone tube (GR). Geometric area of the GR electrodes surface prepared in the following way was 7.065 mm^2.

Electrode modification by the PD and GOx

In order to obtain one layer of the PD on the GR electrode, 3.0 μL of 2.1 mg/mL^{-1} PD solution were distributed on its surface and the solvent was evaporated at ambient temperature by intensive ventilation (GR/PD). In order to obtain the second layer of redox mediator, the PD distribution on electrode surface and the solvent evaporation procedure was repeated. Then 3.0 μL of 40.0 mg mL^{-1} GOx solution (used for the formation of one layer) were distributed on the PD modified electrode and the solvent was evaporated. In order to obtain the second layer of the enzyme on the electrode surface, the GOx distribution and the solvent evaporation procedure was repeated. After complete evaporation of the solvent, PD and GOx modified electrode (GR/PD/GOx) was hanged in a closed test-tubes at 2 cm distance over the 25 % GA solution at ambient temperature for 20 minutes. Finally, in order to remove non-cross-linked enzyme, the modified electrode was thoroughly washed with UHQ water. The GR/PD/GOx electrodes were prepared in the following way were stored in closed test-tubes above a drop of A-PBS-KCl, pH 6.0, at +4°C temperature. All conditions (loading amount of the GOx and PD, immobilization time) were optimized in order to achieve maximal sensitivity of GR/PD/GOx electrodes. For control experiments the GR electrode, which was modified by adsorbed and then chemically cross-linked GOx, (GR/GOx) was designed.

Electrochemical measurements

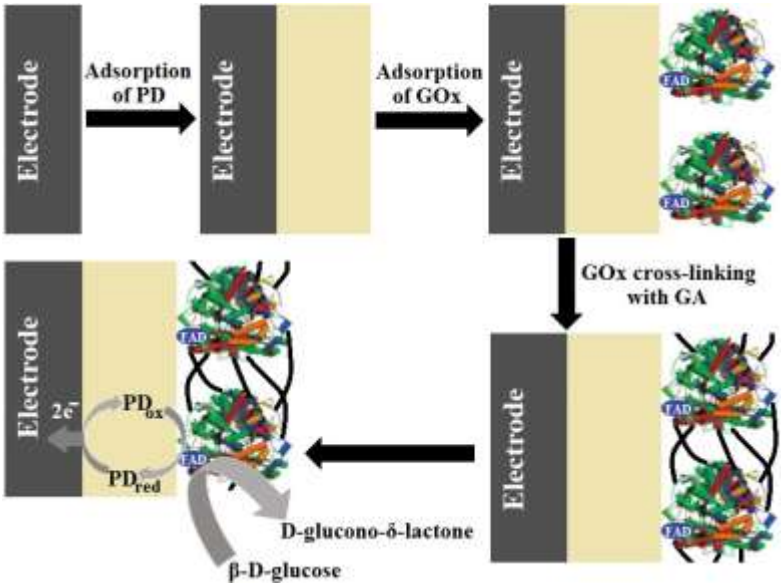
All electrochemical measurements were performed with potentiostat/galvanostat Autolab PGSTAT 30 (Eco Chemie, Netherlands). The three-electrode electrochemical cell consisting

of selected GR-based working electrode, Pt counter electrode and Ag/AgCl in 3.0 mol L^{-1} solution of KCl reference electrode (Ag/AgCl) was used. All experiments were carried out in A-PBS-KCl buffer solution with fixed pH value inside a Faraday-cage at ambient temperature. Cyclic voltammograms were recorded in the potential range from –400 to +400 mV at scan rate of 100 mV s^{-1} and it is necessary in the presence of 100 mmol L^{-1} glucose. An anodic current response to glucose of the GR/PD/GOx electrode was measured at constant potential of +200 mV. In order to measure this response, A-PBS-KCl buffer solution was firstly pipetted into the electrochemical cell and left in the cell for some time to allow stabilization of the background current. Then a solution of glucose was injected by increasing its concentration in the electrochemical cell. In order to get homogenous medium, a solution in the electrochemical cell was quickly stirred immediately after each addition of glucose, while the anodic current was recorded without stirring of a sample solution. The change of electrode current (ΔI) after the addition of glucose was dependent on the glucose concentration in the electrochemical cell. The results of all amperometric measurements were represented as a mean value of three independent measurements.

Results and discussions

Principle action of a reagent-less amperometric glucose biosensor

In this study, reagent-less amperometric glucose biosensor was prepared by layer-by-layer adsorbing a redox mediator PD and an enzyme GOx on a GR electrode surface and chemical cross-linking of the adsorbed GOx with GA as described in experimental section. The biosensor preparation procedure and its operation is displayed in scheme 1. Such biosensor design due to non-free-diffusionally mediated electron transfer between FAD and electrode surface and elimination of additional injecting of a redox mediator into sample solution before each measurement is advantageous for disposable and/or attachable amperometric glucose biosensors. The operation principle of the designed biosensor is based on the enzymatic oxidation of β-D-glucose catalysed by immobilized GOx as it is represented in *equation 1* and scheme 1. During the oxidation of glucose, two electrons are carried from glucose to the GOx. These electrons are accepted by cofactor of GOx – FAD. Then the oxidized form of the PD (PD_{ox}) accepts electrons from the reduced GOx, and the PD_{ox} is turning into the reduced form of PD (PD_{red}) and in such way, oxidized form of GOx is regenerated. Finally, the electrons are transferred from the PD_{red} to the GR electrode and an oxidation current proportional to the glucose concentration is generated when PD turns back into its oxidized form.



Scheme 1. The principle scheme of preparation and operation of the biosensor developed in this research.

Cyclic voltammetric investigation of the electrocatalytic activity of the PD

The electrocatalytic activity of the PD was investigated by cyclic voltammetry. Figure 1 shows the cyclic voltammograms (CV) of pristine-GR, GR/PD, GR/PD/GOx, GR/GOx electrodes registered in A-PBS-KCl buffer solution, pH 6.0, at ambient temperature and in the absence or presence of 100 mmol L^{-1} glucose. As it can be seen, oxidation-reduction current peaks are not observed for the pristine-GR and the GR/GOx electrodes in the potential range from –400 to +400 mV *vs* Ag/AgCl neither in the absence (Fig. 1, curves 1 and 7) nor in the presence of glucose (Fig. 1, curves 2 and 8). The width of CVs registered by GR/GOx electrode was considerably greater in comparison to that registered by pristine-GR, due to additional electrochemical capacitance of immobilized enzyme layer. A pair of well-defined current peaks was observed in the CV registered by GR/PD electrode. Anodic peaks at +44 mV and cathodic peaks at –112 mV were registered in both CVs: (i) in the absence (Fig. 1, curve 3), and (ii) in the presence of 100 mmol L^{-1} of glucose (Fig. 1, curve 4). The difference between anodic and cathodic peaks was 156 mV. These peaks are attributed to the reversible transformation between oxidized and reduced form of the PD. The GR/PD/GOx electrode due to immobilized enzyme displayed a noticeably different electrochemical behaviour from that of the GR/PD electrode and, as can be seen from obtained results (Fig. 1, curves 5 and 6) an increase of registered CV current was observed after the addition of 100 mmol L^{-1} of glucose (Fig. 1, curve 6). Oxidation peak in CV registered by GR/PD/GOx electrode (Fig. 1, curves 5 and 6) was shifted towards more

positive potentials and the difference between oxidation and reduction peaks was lower than that registered by GR/PD electrode (Fig. 1, curves 3 and 4). Anodic peak in CV registered by GR/PD/GOx electrode in the absence of glucose was registered at +64 mV *vs* Ag/AgCl (Fig. 1, curve 5). An increase of the anodic current was observed when glucose was added into the solution. These experimental results demonstrate that the PD is transferring electrons from FAD to the electrode surface.

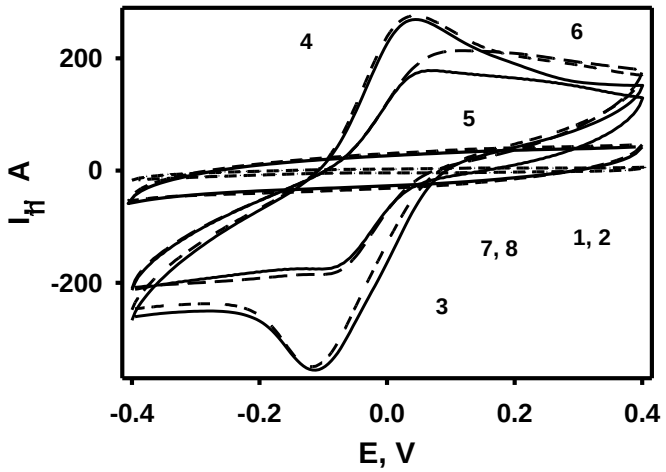


Fig. 1. Cyclic voltammograms of pristine-GR (curves 1 and 2), GR/PD (curves 3 and 4), GR/PD/GOx (curves 5 and 6) and GR/GOx electrode (curves 7 and 8) registered in A-PBS-KCl buffer solution, pH 6.0, in the absence (curve 1, 3, 5 and 7) or presence 100.0 mmol L^{-1} of glucose (curve 2, 4, 6 and 8) at a scan rate of 100 mV s^{-1}. Electrode preparation conditions: 12.6 μg of PD; 0.24 mg of GOx.

Optimization of the GR/PD/GOx electrode operation potential

The selection of operation potential of the working electrode plays a significant role for amperometric determination of glucose. An increase of operation potential increases driving force between the immobilized enzyme and the redox mediator, which increases amperometric response and sensitivity of glucose biosensor. However, high operation potential can result a strong electrochemical interference by oxidizable compounds such as ascorbic and uric acids, which are present in the biological samples and reduce an accuracy of biosensor. Electrochemical interference is a serious problem in practical application of amperometric biosensors with an operation potential of +400.0 mV *vs* Ag/AgCl or higher.[28] While low operation potential greatly reduces or even completely eliminates the influence of potentially interfering compounds. Therefore, in generally, the choice of an operation potential has to be a compromise between sensitivity and accuracy. Considering all of the

above, the effect of operation potential on the amperometric response of designed biosensor was investigated. For this purpose, an amperometric signal of the GR/PD/GOx electrode was measured in A-PBS-KCl buffer, pH 6.0, at ambient temperature and constant potential in the potential range from 0 to +400 mV *vs* Ag/AgCl. Firstly A-PBS-KCl buffer solution was pipetted into the electrochemical cell. When a background current reached steady-state equilibrium then 40 mmol L^{-1} of glucose was injected into the solution. After the addition of glucose a solution in the electrochemical cell was stirred immediately with a magnetic stirrer, then magnetic stirrer was switched off and the anodic current change was recorded without any stirring of the sample until the current response of the GR/PD/GOx electrode reached constant steady-state value. Amperometric current change (ΔI), which was registered after the addition of glucose, was plotted *vs* applied potential. As it is shown in figure S1 (see Supplementary Information), the current response of the GR/PD/GOx electrode to glucose increased gradually with the increase of operation potential. It reached a maximum value of 5.13 μA at +250 mV *vs* Ag/AgCl and it slightly decreased when more positive potential was applied. The current response of 5.10 μA was registered at +200 mV and it was slightly lower than that at +250 mV. Taking into account these results and previously discussed interference at higher potential, an operation electrode potential of +200 mV *vs* Ag/AgCl was selected for the subsequent amperometric measurements in order to attain the highest sensitivity of the designed biosensor. It should be noted that this potential is significantly lower than the electrode potentials, which has been used in previous published studies on amperometric glucose biosensors based on graphite rod electrode modified with the PD and GOx.[21,26,29] Moreover, the amperometric signal was registered without stirring of the sample solution.

The electro-catalytic activity of the GR/PD electrode towards the electro-oxidation of hydrogen peroxide

The electro-catalytic activity of the *GR/PD electrode* in the presence of various concentrations of hydrogen peroxide was evaluated at +200 mV *vs* Ag/AgCl and experimental results are presented in figure S2. When a background current in A-PBS-KCl buffer solution reached constant steady-state value, then 5.0 mmol L^{-1} of hydrogen peroxide was injected into the solution. After the addition of hydrogen peroxide a solution in the electrochemical cell was stirred immediately with a magnetic stirrer, then the magnetic stirrer was switched off and the anodic current change (ΔI) was recorded without any stirring of a sample solution. Such injection of hydrogen peroxide was repeated five times.

Arrows in the figure S2 (see Supplementary Information) indicate a moment of hydrogen peroxide injection into the electrochemical cell. It is seen, that no current response was observed at +200 mV *vs* Ag/AgCl after injection of hydrogen peroxide. A slight decrease in anodic current after injection of hydrogen peroxide can be explained by the fact that the background current in A-PBS-KCl buffer solution did not reached a constant value. Taking into account these results can be concluded that GR/PD electrode does not have any significant electro-catalytic activity towards hydrogen peroxide.

The effect of pH of medium on the sensitivity of the GR/PD/GOx electrode

Whereas pH of environment influences both the biocatalytic activity of the GOx and electrochemical behaviour of redox mediator, the pH of sample solution is usually regarded as the most important factor in the performance of the enzyme electrode and its sensitivity for substrate. It has been reported that extreme pH values may significantly influence the kinetics of glucose measurements.[30] The pH value of optimal activity of dissolved GOx type X-S, which was isolated from *Aspergillus niger*, was found to be at 5.5[31], but it can be influenced by the method used for the immobilization[32]. Therefore, in order to increase the sensitivity, the effect of sample solution pH on the GR/PD/GOx electrode response was investigated. The experiment was performed in not-stirred A-PBS-KCl buffer solution in the range of pH from 2.0 to 9.0 at ambient temperature in the presence of 100 mmol L^{-1} glucose. As shown in figure S3 (see Supplementary Information), the GR/PD/GOx electrode exhibited an optimum response at pH 6.0. Thus, the pH value for the optimal activity for the immobilised GOx was slightly shifted to higher pH values. It was similar to that reported previously.[31,33] It should also be noted that the current response of the GR/PD/GOx electrode was very small when exposed to strong acidic or alkaline environments. At pH 2.0 the biocatalytic activity of GOx and the current generated by the GR/PD/GOx electrode was almost undetectable. Taking into account these results, the pH 6.0 was chosen for further experiments.

The effect of immobilized amount of GOx and PD

The loading amount of immobilized enzyme and redox mediator during the biosensor fabrication process strongly affects the biosensor performance in terms of sensitivity and dynamic range. For this reason, the influence of loading amount of GOx and PD on the magnitude of analytical signal registered with the GR/PD/GOx electrode was examined in not-stirred A-PBS-KCl buffer solution, pH 6.0, at ambient temperature in the presence of 100.0 mmol L^{-1} glucose. Firstly, the effect of the PD was studied in the range of 6.3 –

25.2 μg. In this experiment, the loading amount of the GOx was fixed at 0.24 mg. The experimental data presented in figure 2 A shows that current response of the GR/PD/GOx electrode reached a maximum when 12.6 μg of PD was deposited on the electrode surface and then it decreased when higher amount of PD was deposited. These results can be explained by the fact that higher amount of loaded redox mediator had a negative effect on permeability of substrate, glucose and during the reaction formed product – gluconolactone.[9] Therefore, it was concluded that the mediating efficiency of the PD reached the maximum when 12.6 μg of the PD were used. Therefore, in order to maximize the sensitivity of the biosensor this amount of PD was applied for the design of the GR/PD/GOx electrodes.

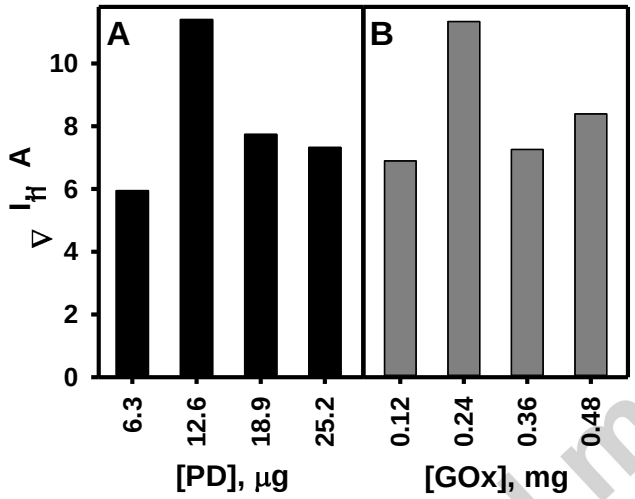


Fig. 2. Amperometric signal of GRE/PD/GOx electrodes *vs* loading amount of the PD (A) and the GOx (B). Conditions: applied potential +200 mV; A-PBS-KCl buffer solution, pH 6.0; 100 mmol L^{-1} of glucose. Electrode preparation conditions: 0.24 mg of GOx (A); 12.6 μg of PD (B).

The dependence of current response of the GR/PD/GOx electrode on the amount of deposited GOx in the range of 0.12 – 0.48 mg is shown in figure 2 B. According to the presented results, analytical signal of the GR/PD/GOx electrode increased with increasing amount of deposited enzyme and the highest analytical signal was detected when 0.24 mg of the GOx was immobilized on each electrode. While signal decrease was recorded at higher amount of immobilized GOx. The same effect has been reported in other papers describing relationship between the amount of enzyme and the magnitude of current response.[34,35] These papers state that when the amount of immobilized GOx is increased up to a certain level an amperometric signal of biosensor starts to decrease. This is explained by the insulating nature

of the GOx, which reduces the electron exchange rate between the sensing layer and electrode and impedes the electron exchange pathways between redox mediator and electrode when too thick layer of GOx is immobilized on the electrode surface.[9] Taking into account results obtained, 0.24 mg of the GOx was used for the modification of electrodes applied in all subsequent experiments in order to attain an optimum sensitivity of biosensor.

Optimization of the GOx immobilization based on cross-linking by vapour of GA

The GOx was immobilized on the GR electrode by physical adsorption and further chemical cross-linking of the enzyme by GA. Cross-linking by GA severely reduces the leaching of enzyme, however, it can lead to heavily altered tertiary and secondary structures and reduced stability and activity of the enzyme.[36] Therefore, duration of cross-linking with vapour of GA (immobilization time) also plays an important role on the activity of sensing layer formed on the electrode surface. In order to enhance the performance of the biosensor, the duration of enzyme cross-linking with GA was optimized and results of the experiment are represented in figure S4 (see Supplementary Information). In this experiment, the surface of electrodes was modified by the loading of 12.6 μg of PD and 0.24 mg of GOx. Then the GOx was cross-linked by GA as it is described in the experimental part. As it can be seen from results (Fig. S4), the current response of GR/PD/GOx electrode increased with an increase of cross-linking time and it was maximal if GOx cross-linking lasted for 25 min and decreased when the duration of cross-linking was longer. This can be explained by the fact that longer duration of cross-linking increased diffusional resistance, which was caused by excessive cross-linking of protein chains by GA. Furthermore, longer than 25 min cross-linking time could also adversely affect the activity of the enzyme due to conformation changes induced by cross-linking of vital residues, which are present in the structure of the enzyme.[37] Therefore, 25 min cross-linking duration was chosen in the further experiments in order to prevent high diffusional resistance and the decrease of enzyme activity and to maximize the sensitivity of the biosensor.

Reproducibility and repeatability of the biosensor signal

Reproducibility and repeatability of analytical signal are among the most important characteristics of the biosensor. Therefore, these characteristics of the GR/PD/GOx electrodes, which were prepared under optimal conditions obtained above, were also studied and evaluated. For this reason, five calibration curves with the same GR/PD/GOx electrode

or with five individual electrodes prepared under the same conditions (one curve per electrode) were recorded at the same day by detecting the current response to glucose in the concentration range from 0.1 to 255 mmol L^{-1} in not-stirred A-PBS-KCl buffer solution, pH 6.0, at ambient temperature. If one GR/PD/GOx electrode was employed to construct five calibration curves the biosensor was rinsed with A-PBS-KCl buffer solution between each calibration curve. The results of this experiment are presented in figure 3 A–B. The error bars illustrate the standard deviation (STDEV) within five independent measurements. As can be seen, in both cases electrodes show similar current responses for the same amount of glucose. A typical glucose concentration for healthy adults is between 3.3 and 5.5 mmol L^{-1} in capillary blood, 4.1 and 5.9 mmol L^{-1} in venous blood, 4.25 and 6.4 mmol L^{-1} in blood serum. While for patients, which are suffering from diabetes mellitus, glucose concentrations are higher than the normal range and can reach 11 mmol L^{-1} or even higher concentrations in venous blood. The STDEV calculated for the same GR/PD/GOx electrode in 4.0 and 12 mmol L^{-1} solution of glucose was found to be 0.0076 and 0.027, respectively. The relative standard deviation (RSD) by successive detection performed for five times at these concentrations was 1.81 and 1.80 %, respectively. The STDEV of the amperometric responses calculated for five different GR/PD/GOx electrodes was a little higher and it was found to be 0.026 and 0.099 in 4.0 and 12 mmol L^{-1} solution of glucose, respectively. The RSD for the same concentrations was found to be 6.2 and 6.6 %. These results demonstrated that the designed biosensor exhibited a good reproducibility and repeatability of the analytical signal. Inaccuracy between the different electrodes might be caused by slightly varying surface-concentration of immobilized enzyme and mediator, loss of enzymatic activity during the preparation of electrodes, which differ for different electrodes, and unequal distribution of enzyme on the carbon rod electrode surface.

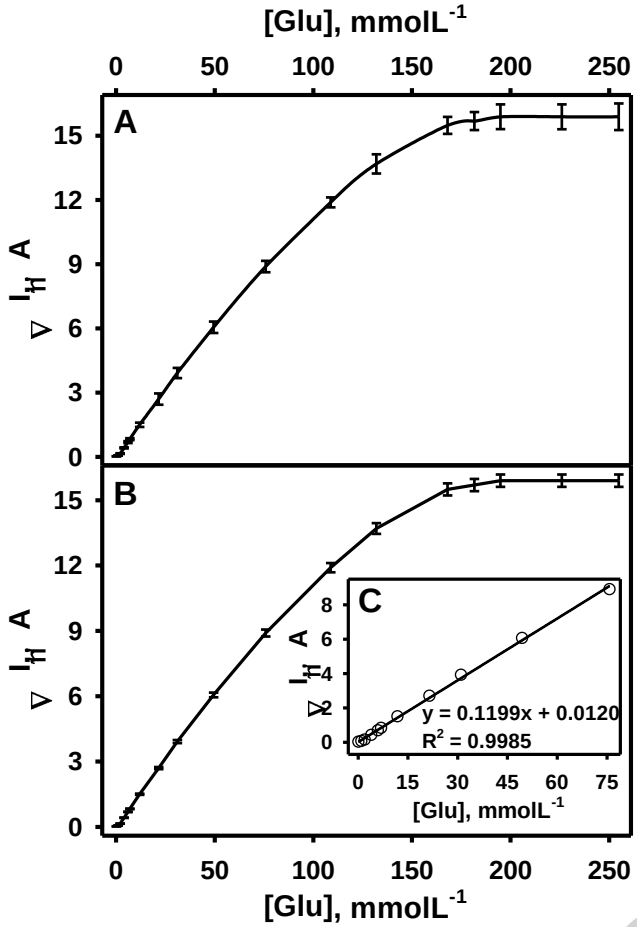


Fig. 3. Amperometric signal *vs* concentration of glucose: A – for five GR/PD/GOx electrodes; B – for the same GR/PD/GOx electrode. C – calibration curve of the GR/PD/GOx electrode. Conditions: applied potential +200 mV; A-PBS-KCl buffer solution, pH 6.0. Electrode preparation conditions: 12.6 μg of PD; 0.24 mg of GOx.

Analytical performance of biosensor

It was found that under the optimized experimental conditions the increase of glucose concentration influenced the proportional increase of the analytical signal of the GR/PD/GOx electrode in the range of 0.1 – 75.8 mmol L^{-1} where the change of current strength had a good linear correlation ($R^2 = 0.9985$) with glucose concentration (Fig. 3 C). This linear dynamic range of the biosensor is sufficient to quantify glucose concentrations for both healthy and diabetic patients. The current strength achieved the saturation level when the concentration of glucose was higher than 195 mmol L^{-1} (Fig. 3 A–B). This can be explained by the saturation of active sites of the sensing layer since a certain amount of GOx is loaded on the electrode and any further increase in glucose concentration can not generate any appreciable increase in the current response of the GR/PD/GOx electrode. The detection limit

was estimated as concentration, which generated three times higher signal in comparison with the standard deviation of the background, it was found to be 0.025 mmol L^{-1}. The wide linear range and low detection limit provides an opportunity to apply the designed biosensor for the determination of glucose. Table 1 lists the analytical parameters of the designed biosensor compared with other GOx based glucose biosensors. The result illustrates that the GR/PD/GOx electrode exhibits better performance than some other electrodes applied for determination of glucose.

Table 1. Comparison of analytical performances of different amperometric biosensors for the determination of glucose.

Glucose biosensor	Linear range, mmol L^{-1}	Detection limit, mmol L^{-1}	Ref. No
GR/PD/GOx	0.1 – 75.79	0.025	Current work
Paper-based/PB-SPCE	0.25 – 2.0	0.01	33
GCE/Ru-RP/GOx	0 – 10.0	0.29	9
GOx/PD/CNTs/GR	0.0 – 50.0	8.0	29
GOx/ZnO-NWs/graphite	0.03 – 1.52	0.003 – 0.013	38
Ppy/GOx/AuNPs/GR	0.99 – 19.9	0.20	39
PPy-GOx/PPy-Cl	0.5 – 24	0.0269	40
MWCNT/GO/GOx/GCE	0.1 – 19.82	0.028	41

Note: PB-SPCE – Prussian blue modified screen-printed carbon e electrode; Ru-RP – ruthenium complex-tethered redox polymer; GCE – glassy carbon electrode; CNTs – carbon nanotubes; MWCNT – multi-walled carbon nanotubes; NWs – nanowires; Ppy – polypyrrole; AuNPs – gold nanoparticles; GO – graphene oxide.

Stability of the biosensor

Stability of the analytic signal over a period of time is highly important parameter of analytical system. The stability of current generated by the GR/PD/GOx electrode was evaluated by repeated measurements of the current strength in not-stirred A-PBS-KCl buffer solution, pH 6.0, containing 100 mmol L^{-1} of glucose at room temperature during a 16-day period (Fig. 4). Between each subsequent measurement the electrode was stored at 4°C in the closed vessel above a drop of A-PBS-KCl, pH 6.0. Although five measurements performed at the same day revealed nearly identical current response (Fig. 3 B), as can be seen from the

stability test results presented in figure 4, the GR/PD/GOx electrode retained only 23 % of its initial current response towards the same concentration (100 mmol L^{-1}) of glucose after 16 days. Long term instability of electrodes could be attributed to dissociation of the GOx and PD from the electrode surface and/or inactivation of the GOx.

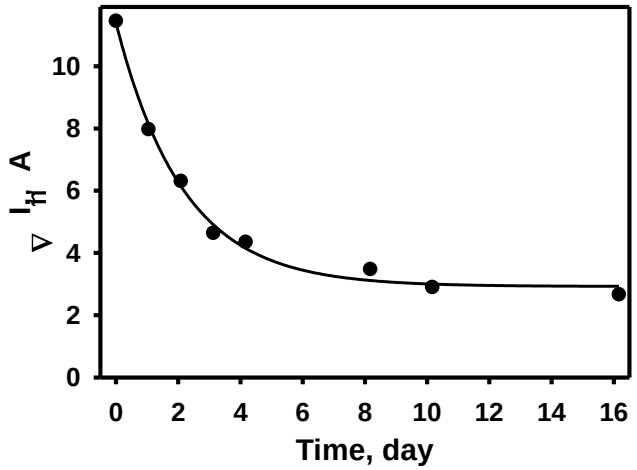


Fig. 4. Amperometric signal *vs* time. Conditions: applied potential +200 mV; A-PBS-KCl buffer solution, pH 6.0; 100 mmol L^{-1} of glucose. Electrode preparation conditions: 12.6 μg of PD; 0.24 mg of GOx.

Interference study

The oxidizable substances such as ascorbic acid and uric acid are usually co-existing in real samples, which are containing glucose. The current contribution from these undesirable interfering materials reduces the accuracy of the biosensor. Therefore, one of the main challenges in glucose analysis by electrochemical biosensors is the elimination of interfering signals. One way to eliminate interference is to construct biosensor operating at relatively low potential, which will not to initiate interfering reactions from the interfering materials. In this research, the ability to reduce the influence of interfering materials was investigated. For this purpose, the influence of ascorbic and uric acids on the analytical signal of the GR/PD/GOx electrode was studied by adding glucose-containing aliquots intercalated with known amount of ascorbic acid and uric acid. This was done by the first injection of 5.0 mmol L^{-1} of glucose into the electrochemical cell filled with A-PBS-KCl buffer solution, pH 6.0 (Fig. 5 A). When the current response of the GR/PD/GOx electrode reached nearly constant value, 0.05 mmol L^{-1} of uric acid was injected, what was followed by injection of 0.05 mmol L^{-1} of ascorbic acid (Fig. 5 A). Finally, 5.0 mmol L^{-1} of glucose was injected again (Fig. 5 A). In order to get homogenous medium a solution in the electrochemical cell was stirred

immediately after the addition of each substance, then the stirring was switched off and the current response of the GR/PD/GOx electrode was recorded without any stirring of a sample solution. As can be seen from the research results, which are presented in Fig. 5 A, only 4.50 and 2.95 % increase in current generated by the GR/PD/GOx electrode was registered (Fig. 5 B) when 0.05 mmol L^{-1} of uric acid and ascorbic acid, respectively were injected into glucose solution (Fig. 5 A). Therefore, it can be stated that the glucose biosensor evaluated in this research has relatively high anti-interference ability, this effect can be attributed to relatively low applied electrode potential of +200 mV, which eliminated the contribution to GR/PD/GOx electrode current induced by uric acid and ascorbic acids.[7]

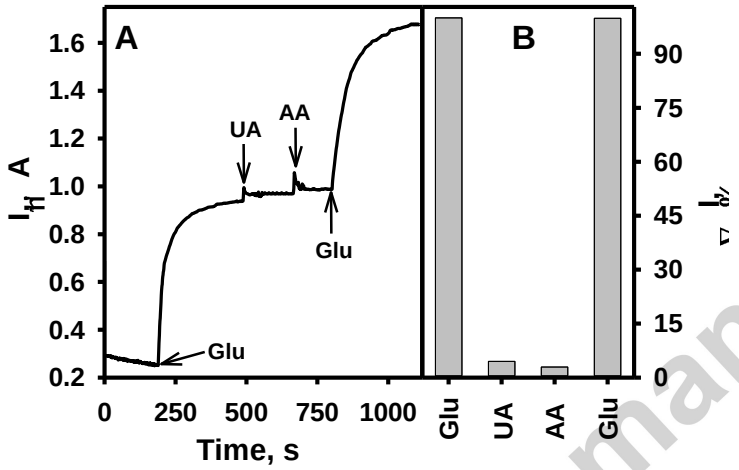


Fig. 5. Amperometric response of the GR/PD/GOx electrode to glucose, uric acid and ascorbic acid. Glu – glucose, UA – uric acid, AA – ascorbic acid. Conditions: applied potential +200 mV; A-PBS-KCl buffer solution, pH 6.0; 5.0 mmol L^{-1} of glucose; 0.05 mmol L^{-1} of ascorbic acid and uric acid. Electrode preparation conditions: 12.6 μg of PD; 0.24 mg of GOx.

Selectivity of the biosensor

The selectivity of the glucose biosensor designed in this research was studied by adding aliquots of fructose, mannose, xylose and galactose that intercalated with glucose in solution. This was done by the first injection 5.0 mmol L^{-1} of glucose into the electrochemical cell filled with A-PBS-KCl buffer solution, pH 6.0. When the current response of the GR/PD/GOx electrode reached saturation level, then 5.0 mmol L^{-1} of fructose was added, it was followed by addition of 5.0 mmol L^{-1} of mannose, then by 5.0 mmol L^{-1} of xylose and then by 5.0 mmol L^{-1} of galactose. Finally, 5.0 mmol L^{-1} of glucose was added again (Fig. 6). In order to get homogenous medium a solution in the electrochemical cell was

stirred immediately after the addition of each substance, then the stirring was switched off and the current response of the GR/PD/GOx electrode was recorded without stirring of a sample solution. According to the presented results, no changes in the current response due to fructose, mannose, xylose and galactose were observed. Therefore, it can be stated that the glucose biosensor fabricated in this study is significantly more selective to glucose if compared to the selectivity towards fructose, mannose, xylose and galactose.

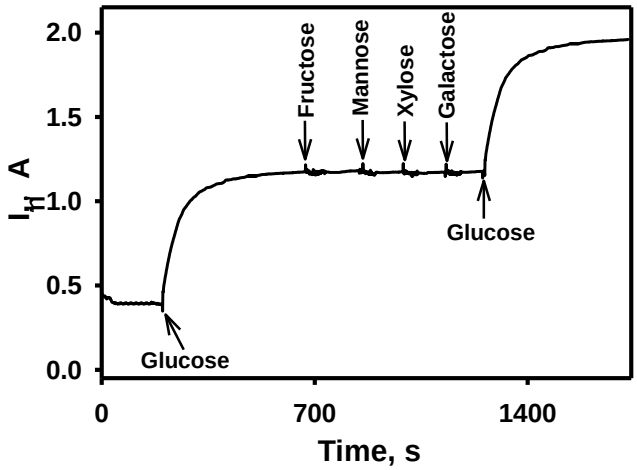


Fig. 6. Amperometric response of the GR/PD/GOx electrode to glucose, fructose, mannose, xylose and galactose. Conditions: applied potential +200 mV; A-PBS-KCl buffer solution, pH 6.0; 5.0 mmol L^{-1} of glucose; followed by addition of 5.0 mmol L^{-1} of fructose, 5.0 mmol L^{-1} mannose, 5.0 mmol L^{-1} xylose, 5.0 mmol L^{-1} galactose and again 5.0 mmol L^{-1} of glucose. Electrode preparation conditions: 12.6 μg of PD; 0.24 mg of GOx;

As can be seen from Fig. 5 A and Fig. 6, the GR/PD/GOx electrode takes 6 – 7 minutes to reach a steady-state value after addition of glucose. This can be explained by relatively slow diffusion of glucose because the current response of the GR/PD/GOx electrode was recorded without stirring of a sample.

Determination of glucose concentrations in human serum

The main requirement for the development of biosensor is the ability to adapt it to the analysis of real samples (urine, saliva, blood, serum, beverages and food). Therefore, the designed biosensor was used to detect glucose concentrations in human serum. In this experiment different concentrations of glucose in the range from 2.0 to 12.0 mmol L^{-1} were added into human serum, which was ten times diluted with A-PBS-KCl buffer solution, pH 6.0, and concentration of glucose was determined using the GR/PD/GOx electrode based

biosensing system, which was preliminary calibrated using solutions with known concentrations of glucose (Fig. 3 C). A sample solution in the electrochemical cell was stirred immediately after each addition of glucose, while the anodic current was recorded without stirring of a sample solution. It was found that the recoveries of the GR/PD/GOx electrode based biosensing system were in the range of 95.0 – 100.0 % (Table 2). Taking into account the results of the experiment, the designed glucose biosensor can be applied for the monitoring of glucose in the serum of diabetic patients.

Table 2. Recovery of glucose in human serum sample using the designed biosensor based on GR/PD/GOx working electrode (n – number of measurements).

Added concentration of glucose, mmol L^{-1}	Detected concentration of glucose (n = 3), mmol L^{-1}	Recovery, %
2.0	2,0	100.0
4.0	4,0	100.0
6.0	5,7	95.0
7.0	7,0	100.0
12.0	11,7	97.5

Conclusions

In the present study, an amperometric biosensor suitable for the determination of glucose in not-stirred sample was developed. Not-stirred sample conditions are very important in the development of cheap disposable and/or attachable amperometric glucose biosensors. A working electrode of the designed biosensor was based on the GR electrode modified by the PD and GOx, which were layer-by-layer adsorbed on the GR electrode surface with subsequent evaporation of solvent and chemical cross-linking of the adsorbed GOx with GA. Such biosensor design is advantageous due to not soluble redox mediator based electron transfer between redox centre of the enzyme and electrode surface. The proposed biosensor has a linear range from 0.1 to 75.8 mmol L^{-1} and a detection limit of 0.025 mmol L^{-1} of glucose, it is highly selective to glucose and exhibit anti-interference ability towards uric acid and ascorbic acid. In addition, here characterized biosensor has a good reproducibility and repeatability of analytical signal. It has been tested for the detection of glucose in human serum sample with good accuracy. These attractive characteristics of GR/PD/GOx electrode could open new opportunities in the development of non-invasive biosensors.

Acknowledgements

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Highlights

- An amperometric glucose biosensor based on redox mediator, 1,10-phenanthroline-5,6-dione (PD), and enzyme, glucose oxidase (GOx) was developed
- The PD and GOx were layer-by-layer adsorbed on the GR electrode surface with subsequent cross-linking of the adsorbed GOx
- Optimal biosensing conditions of the prepared layered GR/PD/GOx electrode were determined
- Analytical performance of GR/PD/GOx electrode based biosensor was demonstrated in the analysis of human serum samples