VILNIUS UNIVERSITY
CENTER FOR PHYSICAL SCIENCES AND TECHNOLOGY

Laura
GLUMBOKAITĖ

Synthesis and Application of Polypyrrole-2-Carboxylic Acid in Glucose Biosensors

SUMMARY OF DOCTORAL DISSERTATION

Natural sciences,
Chemistry N 003

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Academic supervisor
Assoc. Prof. Dr. Asta Kaušaitė-Minkštimienė (Vilnius University, Natural Sciences, Chemistry N 003)

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Chairman – **Prof. Dr. Stasys Tautkus** (Vilnius University, Natural Sciences, Chemistry N 003).
Members:
Assoc. Prof. Dr. Piotras Cimmperman (Vilnius Gediminas Technical University, Natural Sciences, Physics, N 002);
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Prof. Habil. Dr. Aivaras Kareiva (Vilnius University, Natural Sciences, Chemistry N 003);
Assoc. Prof. Dr. Germanas Peleckis (Australian Institute for Innovative Materials, University of Wollongong, Technology, Materials Engineering, T 008).

The dissertation shall be defended at a public meeting of the Dissertation Defence Panel at 14:00 on 20th March 2020 in Room 141 of the Faculty of Chemistry and Geosciences Institute of Chemistry (Vilnius University).
Address: Naugarduko st. 24., Room No. 141, Vilnius, Lithuania
Tel. +370 (5) 219 3105; e-mail: info@chgf.vu.lt
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Laura
GLUMBOKAITĖ

Poli(pirol-2-karboksirūgšties) sintezė ir taikymas gliukozės biologiniuose jutikliuose

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Mokslinė vadovė:
Doc. dr. Asta Kaušaitė-Minkštimienė (Vilniaus universitetas, gamtos mokslai, chemija, N 003)

Gynimo taryba:
Pirmininkas – **prof. dr. Stasys Tautkus** (Vilniaus universitetas, gamtos mokslai, chemija, N 003).
Nariai:
Doc. dr. Piotras Cimmperman (Vilniaus Gedimino technikos universitetas, gamtos mokslai, fizika, N 002);
Doc. dr. Vitalijus Karabanovas (Nacionalinis vėžio institutas, gamtos mokslai, biofizika, N 011);
Prof. habil. dr. Aivaras Kareiva (Vilniaus universitetas, gamtos mokslai, chemija, N 003);
Doc. dr. Germanas Peleckis (Australijos novatoriškų medžiagų institutas, Volongongo universitetas, technologijos mokslai, medžiagų inžinerija, T 008).

Disertacija ginama viešame Gynimo tarybos posėdyje 2020 m. kovo mėn. 20 d. 14 val. Vilniaus Universiteto Chemijos ir geomokslų fakulteto Chemijos instituto Neorganinės chemijos auditorijoje. Adresas: Naugarduko g. 24, Vilnius, Lietuva, tel. +370 (5) 219 3105; el. paštas info@chgf.vu.lt.

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INTRODUCTION

Analysis of complex biological materials is one of the most important chemistry tasks. In many cases there are not one but quite a lot of components in samples. These components can influence results of analysis. Analysing crude samples saves a lot of time and expenses. Biosensors are analytical devices that can measure analite concentration in complex matrix. Biosensor contains biological element that recognizes specific analite. There are many biological materials that are used as recognition elements. One of them – enzymes. Glucose oxidase is an enzyme which specifically oxidizes β-D-glucose. Biosensor with immobilized glucose oxidase detect glucose in various samples. Glucose biosensors are used in medicine, food and drinks industry. They have to be sensitive, fast, reliable, portable, low-cost and so forth. Consequently, glucose biosensors are still widely studied.

One of the most popular are amperometric glucose biosensors. Their response time is short, cost – relatively low. Although they are quite simple, the possibilities of applying various component for enhancing biosensor properties are endless. Most common materials are conjugated polymers, nanomaterials and quantum dots. Additional substances increase analytical signal stability and reproducibility, sensitivity, improves response time and other analytical characteristics. These materials can facilitate electron transfer, create stable matrix for enzyme immobilization, isolate electrode surface from interfering materials.

Conjugated polymer polypyrrole is often used in developing biosensors. This is due to its facile synthesis, good stability and biocompatibility. However poly(pyrrole-2-carboxylic acid) can be the key for stable and long lasting enzyme immobilization, because of its ability to form strong covalent bond with biologically active molecule. Covalent linkage ensures preservation of enzyme in

polymeric material and in such way improves stability of biosensor response. Nanomaterials also provide advantages in biosensors development. Gold nanoparticles improve sensitivity, stability and response time, increase conductivity. Combination of materials can be even better solution for improving biosensors.

Hence the development of simple, sensitive, selective, reliable, and low-cost electrochemical glucose biosensors is important research area. Until finally this analytical devise will precisely, quickly and inexpensively determine glucose concentration in food products, drinks and most importantly in patient's blood.

The aim of the work

Enzymatically, chemically and electrochemically synthesize poly(pyrrole-2-carboxylic acid) and apply it in glucose biosensors.

Main tasks of the work

1. Investigate and compare poly(pyrrole-2-carboxylic acid) particles synthesized by enzymatic polymerization and chemical polymerization.
2. Chemically polymerized poly(pyrrole-2-carboxylic acid) particles modify by glucose oxidase and apply it in glucose biosensor, using mediator phenazine methosulfate; determine main analytical characteristics of biosensor, test its ability to detect glucose in real samples.
3. Electrochemically polymerized poly(pyrrole-2-carboxylic acid) modify by glucose oxidase and apply it in reagentless glucose biosensor, using mediator 1,10-phenantroline-5,6-dione and gold nanoparticles; determine main analytical characteristics of biosensor, test its ability to detect glucose in real samples.

Statements to be defended

1. Poly(pyrrole-2-carboxylic acid) particles can be synthesized by chemical polymerization method, using hydrogen peroxide as initiator, and by enzymatic polymerization, using glucose oxidase and glucose.
2. Biosensor based on chemically polymerized poly(pyrrole-2-carboxylic acid) particles and glucose oxidase biocomposite can be used to detect glucose in real samples.
3. Reagentlesss biosensor based on electrochemically polymerized poly(pyrrole-2-carboxylic acid) and glucose oxidase can be used to detect glucose in real samples.

RESULTS AND DISCUSSION

Enzymatic and chemical synthesis of poly(pyrrole-2-carboxylic acid)

Poly(pyrrole-2-carboxylic acid) (PPCA) particles were synthesized by enzymatic catalysis and by chemical polymerization. Both synthesis were compared, evaluated and characterized.

First synthesis was initiated by oxidant hydrogen peroxide resulting from the redox enzyme glucose oxidase (GOx) catalysed glucose oxidation reaction. GOx catalyses oxidation of β-D-glucose to D-glucono-1,5-lactone and H_2O_2 using molecular oxygen as an electron acceptor. Enzyme catalysed polymerization solutions consisted of four compounds: PCA – monomer, GOx – H_2O_2 producing enzyme, glucose – reducing substrate of GOx, and dissolved oxygen – oxidizing substrate of GOx. The spectra are shown in Fig. 1A for reaction at pH 3.0, and in Fig. 1B for reaction at pH 5.0. Initially all polymerization solutions were slightly yellowish and only two low intensity absorption peaks of GOx at 359 and 445 nm were present in the absorption spectra. H_2O_2 produced during catalytic GOx reaction was able to oxidize PCA in the initiation step resulting in chemically active monomer cation radicals.

Polymerization can be observed by increase of absorption peak at 465 nm indicating the presence of the pyrrole ring in the formed PCA oligomers or soluble isolated nanoparticles of the PPCA. As can be seen in Fig. 1, different absorption spectra for solutions of different pH were observed. Only one absorption peak at 465 nm was observed for the PPCA synthesized at pH 2.0 and two peaks at pH 5.0. The absorption peak at 345 nm corresponds to the π–π* transition of the C=C double bond resulting from the formation of PPCA. Absorption peak at 465 nm corresponds to bipolaron transition, a characteristic feature for the oxidized state of PPCA. In

addition, the absorption peak at 465 nm can be attributed to the higher conjugation length of PPCA.

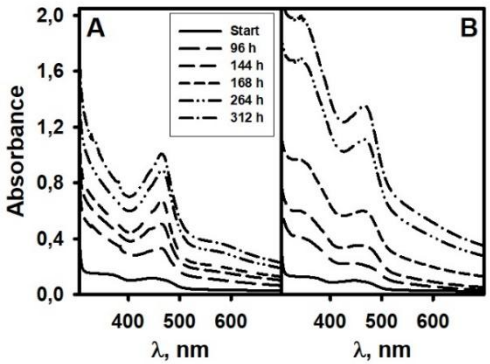

Fig. 1. Absorption spectra of PPCA oligomers in A-PBS–ethanol solution pH 3.0 (A) and pH 5.0 (B) containing 200 mmol/L PCA, 1 mg/mL GOx and 200 mmol/L glucose.

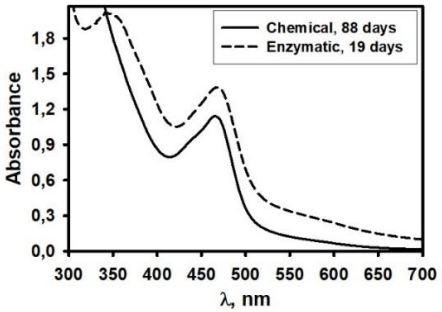

Fig. 2. Absorption spectra of PCA oligomers or soluble isolated nanoparticles of PPCA in A-PBS–ethanol solution pH 5.0. Initial concentrations: 200 mmol/L of PCA and 300 mmol/L of H_2O_2 (chemical polymerization) and 200 mmol/L of PCA, 200 mmol/L of glucose and 1 mg/mL of GOx (enzyme catalysed polymerization).

Second synthesis was initiated by H_2O_2 added to the polymerization solution. GOx catalysed PCA polymerization was

compared with the chemical polymerization by recording the UV-Vis absorption spectrum between 300 and 800 nm with increasing reaction time. The experimental data presented in Fig. 2 illustrates the absorption spectra recorded in polymerization solutions with pH 5.0.

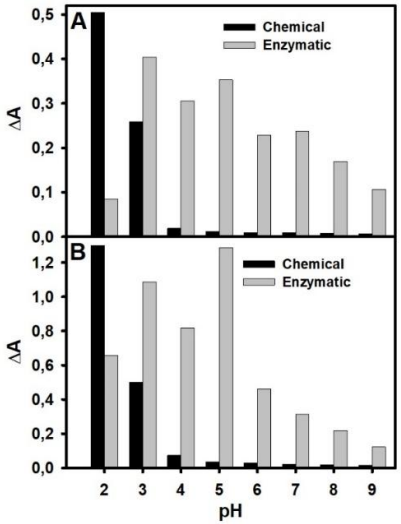

Fig. 3. Absorbance of PCA oligomers or soluble isolated PPCA nanoparticles at 465 nm vs. pH of polymerization solution. Initial concentrations of chemical polymerization solutions were: 200 mmol/L of PCA and 300 mmol/L of H_2O_2. Initial concentrations of enzyme catalysed polymerization solutions were: 200 mmol/L of PCA, 200 mmol/L of glucose and 1 mg/mL of GOx. Duration of polymerization was 6 (A) and 19 days (B).

PPCA particles formation rate synthesized by GOx catalysed polymerization was compared with formation rate of the particles synthesized by chemical polymerization. Results presented in Fig. 3 show relationship between absorbance of the PCA oligomers or soluble isolated nanoparticles of PPCA and the pH value of

polymerization solution. It is clearly seen that the best medium for chemical PCA polymerization was strongly acidic medium. The highest ΔA value was observed at pH 2.0. Increase in medium pH resulted in a decrease in PPCA formation rate, and polymer formation was almost undetectable when the pH of polymerization solution exceeded pH 6.0.

According to results obtained for GOx catalysed polymerization, formation of PPCA was observed in the entire investigated pH range. Highest ΔA values at six-day period were observed at pH 3.0 (Fig. 3). However, in a subsequent polymerization period the highest ΔA values were recorded at pH 5.0. Results can be explained by the facts that the polymerization is faster in acidic medium, because free glucose oxidase from Aspergillus niger type X-S exhibits maximal activity at pH 5.5.

The polymerization of PCA was confirmed using FTIR spectroscopy. PPCA particles were separated from the polymerization solution and freeze dried. PPCA powder was used for FTIR spectroscopy. Fig. 4 shows FTIR spectra of PPCA prepared by chemical (C) and enzyme catalysed polymerization at pH 2.0 (D) and pH 5.0 (E). For comparison, PPy FTIR spectrum is shown in Fig. 4A. PCA FTIR spectrum is shown in Fig. 4B. FTIR spectra of PCA (Fig. 4B) and PPCA (Fig. 4C–E) show infrared absorption peaks corresponding to the C=O and O–H groups that are absent in the PPy spectrum.

Table 1. The infrared absorption peaks of C=O and O–H groups and their respective assignments.

	Infrared absorption peak position, wavenumber (cm^{-1})			
	PCA	PPCA, chemical	PPCA, enzymatic, pH 2.0	PPCA, enzymatic, pH 5.0
C=O	1664	1674	1652	1652
O=H	2912	2970	2926	2945

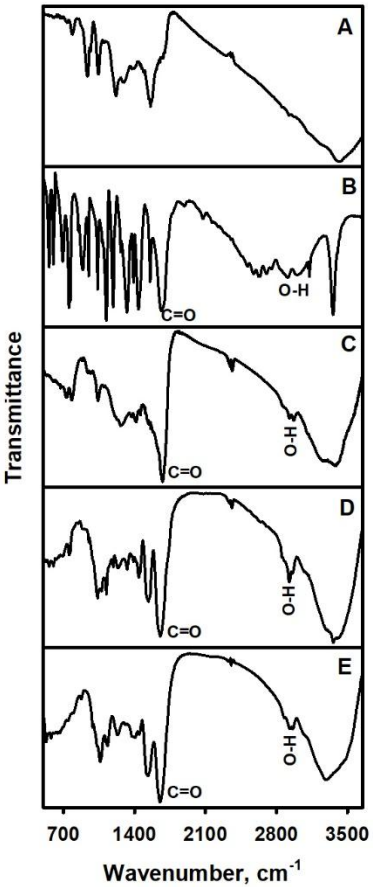

Fig. 4. FTIR spectra of PPy (A), PCA (B) and PPCA prepared by chemical polymerization at pH 2.0 (C) and enzyme catalysed polymerization at pH 2.0 (D) and pH 5.0 (E).

Potentiometric back-titration was performed in order to validate the presence of carboxylic groups in the formed PPCA particles. 100 mg of freeze-dried PPCA particles were mixed with $NaHCO_3$ solution and titrated with HCl solution. Titration curve is

shown in Fig. 5 (solid line) and control titration of known amount of $NaHCO_3$ solution with the HCl solution is shown in Fig. 5 (dashed line). HCl addition decrease pH of solution. Equivalent points were defined by decrease of solution pH with the addition of about 6.90 mL (solid line) and 7.15 mL (dashed line) of HCl. HCl volumes difference at equivalent points between two titration curves is evidenced of carboxylic groups in PPCA particles.

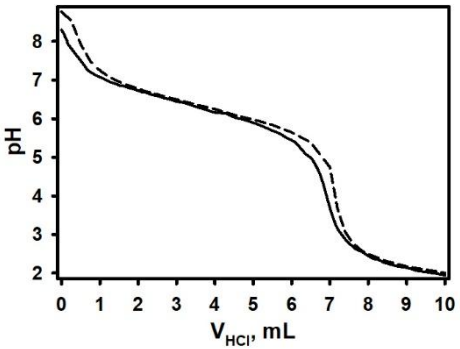

Fig. 5. Titration curve (solid line) of PPCA with 50.0 mmol/L HCl solution after the addition of 10 mL 50.0 mmol/L of $NaHCO_3$. The control titration of 10 mL 50.0 mmol/L solution of $NaHCO_3$ with the same HCl solution (dashed line).

The morphology of PPCA particles was characterized by SEM. Images show aggregates composed of smaller PPCA particles. As can be seen in Fig. 6A, aggregates of chemically synthesized PPCA particles are several microns in diameter and consist of smaller particles with a globular morphology and approximately of 200−1000 nm in diameter. PPCA particles synthesized by enzyme catalysed polymerization (Fig. 6B and C) are formed by globules of smaller diameter (50–150 nm).

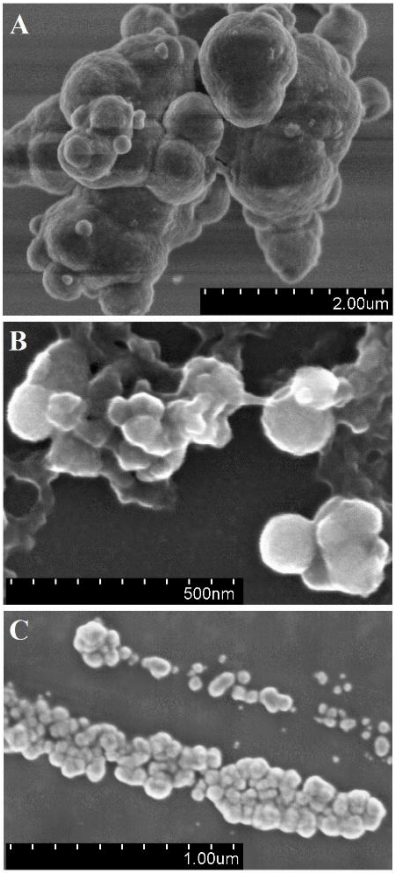

Fig. 6. SEM images of PPCA particles synthesized by chemical polymerization at pH 2.0 (A) and enzyme catalysed polymerization at pH 2.0 (B) and pH 5.0 (C). Conditions for image (A): duration of polymerization – 10 days, magnification – 20 000 times; accelerating voltage – 2000 V; emission current – 30 000 nA; Conditions for the image (B): duration of polymerization – 12 days, magnification – 100 000 times; accelerating voltage – 5000 V; emission current – 28 000 nA; for image (C): duration of polymerization – 70 days, magnification – 50 000 times; accelerating voltage – 2000 V; emission current – 30 000 nA.

Glucose biosensor based on chemically synthesized poly(pyrrole-2-carboxylic acid)

Glucose biosensor based on $PPCA_{CH}$-GOx biocomposite was designed. PPCA particles were synthesized by chemical polymerization. PPCA was modified with GOx by direct covalent amide coupling with a mixture of EDC and NHS. $PPCA_{CH}$-GOx particles were mixed with A-PBS buffer containing BSA. Schematical representation is shown in Fig. 7. Amperometric response of biosensor was generated due to the oxidation of β-D-glucose into D-glucono-δ-lactone, which non-enzymatically was hydrolysed into gluconic acid, catalysed by GOx immobilized on PPCA modified surface of working electrode (Fig. 8). During enzymatic reaction, 2 electrons is carried from glucose to enzyme cofactor flavine adenine dinucleotide (FAD). Then 2 electrons are accepted by PMS, which is used as electron transfer mediator, and during reoxidation of $PMSH_2$ is transferred to GR/$PPCA_{CH}$-GOx electrode. During transfer of electrons, amperometric signal proportional to the glucose concentration is generated.

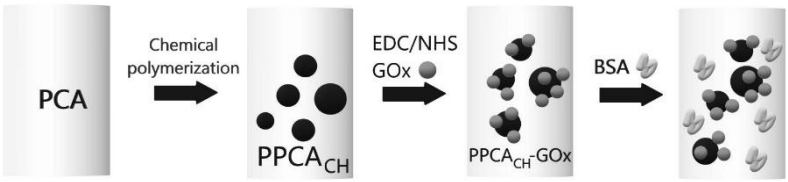

Fig. 7. Schematical representation of $PPCA_{CH}$ polymerization, activation of polymer carboxylic groups, GOx attachment and final $PPCA_{CH}$-GOx in BSA solution.

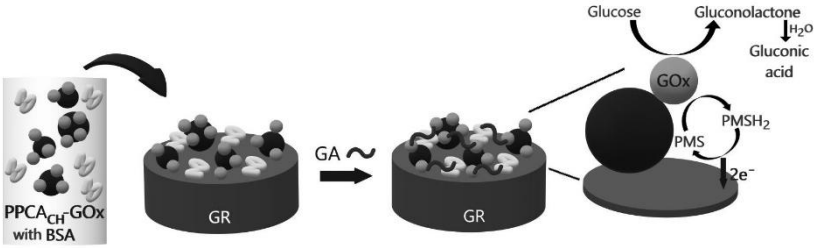

Fig. 8. Illustration of preparation and operation of designed amperometric glucose biosensor based on the $PPCA_{CH}$-GOx biocomposite.

Characterization of GR/$PPCA_{CH}$-GOx electrode was performed by cyclic voltammetry. Fig. 9 shows cyclic voltammograms (CV) registered in a pure A-PBS-KCl buffer solution (curve 1) and in A-PBS-KCl buffer solution containing 5.0 mmol/L glucose (curve 2), 10.0 mmol/L PMS (curve 3) or 5.0 mmol/L glucose and 10.0 mmol/L PMS (curve 4). Oxidation-reduction current peaks are not observed in a pure A-PBS-KCl buffer solution or A-PBS-KCl buffer solution containing only glucose. Well-defined current peaks are observed in solution with PMS. Its oxidation is seen at a potential of +91.3 mV vs Ag/AgCl, whereas reduction at -165.8 mV vs Ag/AgCl and difference between anodic and cathodic peaks was 257.1 mV. Increase in peak currents of $PMSH_2$/PMS redox couple is observed by adding glucose into solution.

Value of an operation potential was selected for amperometric measurements. Amperometric current change (ΔI) achieved maximum value at +300 mV vs Ag/AgCl.

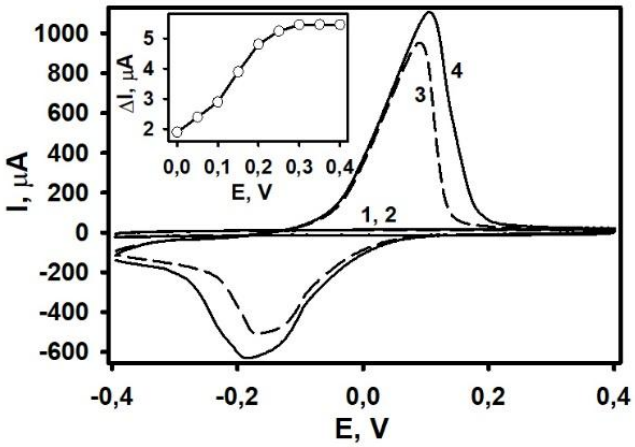

Fig. 9. Cyclic voltammograms of GR/PPCA$_{CH}$-GOx electrode registered in a pure A-PBS-KCl pH 6.0 (curve 1), in a presence of 5.0 mmol/L glucose (curve 2), 10.0 mmol/L PMS (curve 3) or 5.0 mmol/L glucose and 10.0 mmol/L PMS (curve 4). Intercept shows an effect of operating potential on the amperometric response of GR/PPCA$_{CH}$-GOx electrode in A-PBS-KCl buffer solution, pH 6.0, in the presence of 5.0 mmol/L glucose. Electrode preparation conditions: duration of PPCA particles synthesis 48 hours; 6 mL of the PPCA$_{CH}$-GOx.

PPCA$_{CH}$-GOx preparation consists of two stages – PPCA particles synthesis and covalent linking of GOx. As shown in Figure 10, an amperometric response is greatly affected by GOx solution pH which was used for PPCA particles modification. Highest current change after glucose addition is registered when the GOx solution of pH 4.0 is used (Figure 10, solid line).

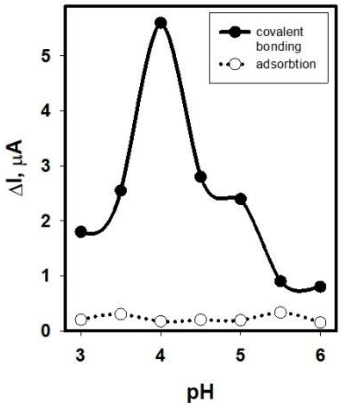

Fig. 10. GR/PPCA$_{CH}$-GOx electrode amperometric response dependence on pH of GOx solution used for PPCA particles modification. Conditions: duration of PPCA particles synthesis 144 hours; 3 mL of PPCA$_{CH}$-GOx; applied potential +300.0 mV; A-PBS-KCl pH 6.0; 20.0 mmol/L glucose; 10.0 mmol/L PMS.

To validate covalent bonding between enzyme and polymeric particle, control was prepared. PPCA modification was carried out without EDC and NHS. Experiment showed the current response of electrodes prepared with PPCA particles with adsorbed GOx was insignificant (Fig. 10, dashed line).

Polymerization duration is important in PPCA particles formation. Synthesis of PPCA particles was carried out for 24, 48, 96 and 144 hours. Particles that were polymerized for 24 hours were too small to completely separate from polymerization solution so no experiments were performed with them. The rest were separated from polymerization solution and modified with GOx. Fig. 11 shows that current response to glucose decreases with increasing polymerization duration. According to the results 48 hours polymerization duration was used for PPCA synthesis.

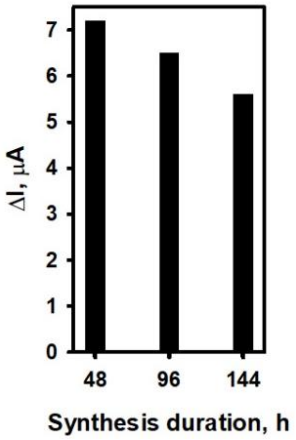

Fig. 11. GR/PPCA$_{CH}$-GOx electrode amperometric response dependence on PPCA polymerization duration. Conditions: 3 mL PPCA$_{CH}$-GOx; applied potential +300.0 mV; A-PBS-KCl pH 6.0; 20.0 mmol/L glucose; 10.0 mmol/L PMS.

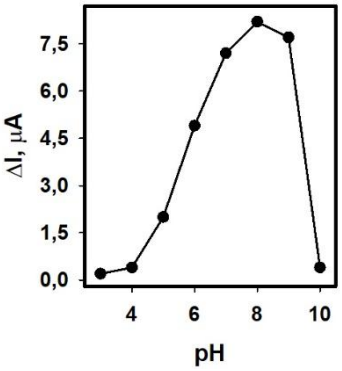

Fig. 12. GR/PPCA$_{CH}$-GOx electrode amperometric response dependence on pH of A-PBS-KCl buffer solution. Conditions: duration of PPCA particles synthesis 48 hours; 3 mL PPCA$_{CH}$-GOx; applied potential +300.0 mV; 10.0 mmol/L glucose; 10.0 mmol/L PMS.

Because pH influences GOx biocatalytic activity and electrochemical behaviour of electron transfer mediator, sample solution pH effect on current response was investigated. A-PBS-KCl buffer solutions were prepared in a pH range of 3.0–10.0. Current response optimum was achieved at pH 8.0 (Fig. 12).

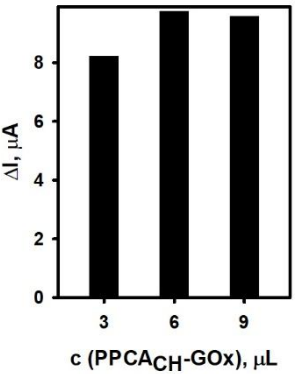

Fig. 13. GR/PPCA$_{CH}$-GOx electrode amperometric response dependence on loading amount of PPCA$_{CH}$-GOx suspension. Conditions: duration of PPCA particles synthesis 48 hours; applied potential +300.0 mV; A-PBS-KCl pH 8.0; 10.0 mmol/L glucose; 10.0 mmol/L PMS.

Important step is to find out perfect amount of PPCA$_{CH}$-GOx particles on electrode. The amount is expressed as volume of particles suspension in 250 mL of A-PBS buffer containing 5.0 mg/mL BSA. Results are shown in Figure 13. The highest current response was measured when 6.0 mL of suspension were used for the modification of electrode.

Dependence between GR/PPCA$_{CH}$-GOx electrode current response and glucose concentration is shown in Figure 14. It was studied under optimized experimental conditions, in the glucose concentration range of 0.1–150.0 mmol/L. Dependence between glucose concentration and current response was linear up to

15.0 mmol/L. The detection limit value was determined as 0.039 mmol/L (N:5).

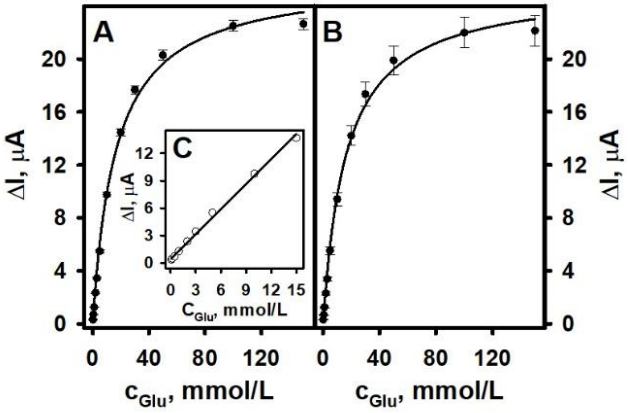

Fig. 14. Amperometric signal vs glucose concentration: A – one GR/$PPCA_{CH}$ -GOx electrode; B – five GR/$PPCA_{CH}$-GOx electrodes. C – calibration curve of GR/$PPCA_{CH}$-GOx electrode. Conditions: $PPCA_{CH}$ particles synthesis duration 48 hours; 6 mL of $PPCA_{CH}$-GOx; applied potential +300.0 mV; A-PBS-KCl pH 8.0; 10.0 mmol/L PMS.

To evaluate repeatability of analytical signal, GR/$PPCA_{CH}$-GOx electrode was prepared under optimized operating conditions. Then five calibration curves were recorded by detecting its current response to glucose in concentration range 0.1–150.0 mmol/L. Error bars correspond to the standard deviation for five independent measurements (Fig. 14A). Standard deviation and relative standard deviation was found to be 0.10 and 1.83 %, respectively. To evaluate reproducibility five individual GR/$PPCA_{CH}$-GOx electrodes were prepared. Standard deviation and relative standard deviation was found to be 0.29 and 5.21 %, respectively.

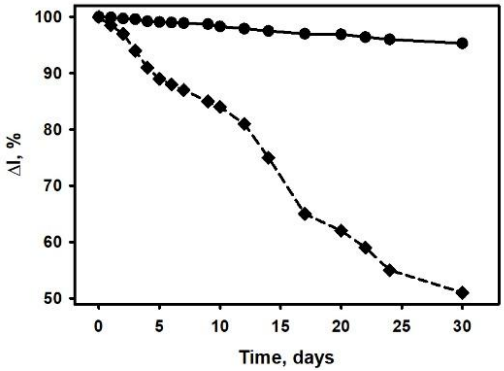

Fig. 15. GR/PPCA$_{CH}$-GOx (solid line) and GR/GOx (dash line) amperometric response vs time. GR/PPCA$_{CH}$-GOx electrode conditions: PPCA$_{CH}$ particles synthesis duration 48 hours; 6 mL PPCA$_{CH}$-GOx; applied potential +300.0 mV; A-PBS-KCl pH 8.0; 10.0 mmol/L glucose; 10.0 mmol/L PMS. GR/GOx electrode conditions: 3 mL 40 mg/mL GOx; 10 min over 25% solution of GA; applied potential +300.0 mV; A-PBS-KCl pH 6.0; 10.0 mmol/L glucose; 10.0 mmol/L PMS.

GR/PPCA$_{CH}$-GOx electrode was monitored periodically during one-month period. As can be seen, from experimental results presented in Fig. 15, after one-month electrode still retained 95.3 % of its initial current response, revealing good long-term stability. For comparison, GR electrode modified with GOx, chemically cross-linked with GA vapour (GR/GOx), retains only about 50 % of its initial activity.

In order to investigate selectivity, current response of GR/PPCA$_{CH}$-GOx electrode to galactose, mannose, fructose, xylose and saccharose was investigated. Results (Fig. 16) show electrode is only selective to glucose because no change in the current response was observed to other sugars.

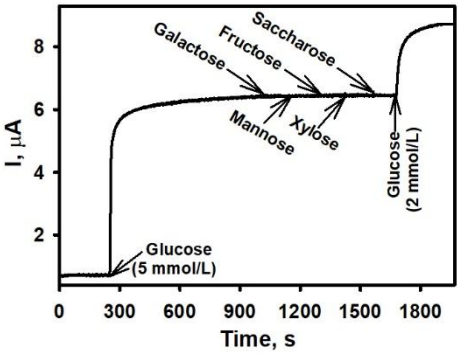

Fig. 16. GR/PPCA$_{CH}$-GOx amperometric response to glucose, galactose, mannose, fructose, xylose and saccharose. Conditions: PPCA$_{CH}$ particles synthesis duration 48 hours; 6 mL of PPCA$_{CH}$-GOx; applied potential +300.0 mV; A-PBS-KCl pH 8.0; 10.0 mmol/L PMS; concentration of other sugars 5.0 mmol/L.

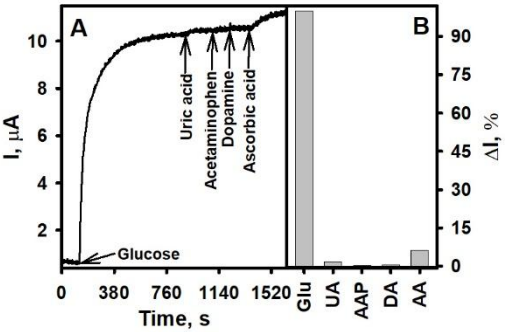

Fig. 17. GR/PPCA$_{CH}$-GOx amperometric response to glucose (Glu), uric acid (UA), acetaminophen (AAP), dopamine (DA) and ascorbic acid (AA). Conditions: PPCA$_{CH}$ particles synthesis duration 48 hours; 6 mL PPCA$_{CH}$-GOx; applied potential +300.0 mV; A-PBS-KCl pH 8.0; 10.0 mmol/L PMS; 10.0 mmol/L Glu; 0.30 mmol/L UA; 0.10 mmol/L AAP; 0.07 mmol/L DA; 0.10 mmol/L AA.

The interference effect was studied using 10.0 mmol/L of glucose, 0.3 mmol/L of uric acid (UA), 0.1 mmol/L of acetaminophen (AAP), 0.07 mmol/L of dopamine (DA) and 0.1 mmol/L of ascorbic acid (AA). As shown in Fig. 17 B, the current change caused by UA, AAP, DA and AA was 1.75, 0.21, 0.52 and 6.19 % of the current response to 10.0 mmol/L of glucose, respectively.

Practical applicability of biosensor was tested by detecting glucose in human serum samples 10 times diluted with A-PBS-KCl buffer solution (pH 8.0). Serum samples were supplemented with a known concentration of glucose within the linear range described above: 2.0, 6.0 and 12.0 mmol/L. Results presented in Table 1. Results demonstrate good accuracy and indicate that newly developed glucose biosensor can be used in clinical analysis for the monitoring of glucose concentrations in blood serum samples of diabetic patients.

Table 2. Determination of glucose in human serum samples.

Added concentration of glucose, mmol/L	Detected concentration of glucose (n=3), mmol/L	Recovery, %
2,0	2,12	106,0
6,0	6,1	101,7
12,0	11,9	99,17

Glucose biosensor based on poly(1,10-phenanthroline-5,6-dione), poly(pyrrole-2-carboxylic acid), gold nanoparticles and glucose oxidase

Reagent-less amperometric glucose biosensor based on nanobiocomposite consisting of poly(1,10-phenanthroline-5,6-dione) (PPD), poly(pyrrole-2-carboxylic acid), gold nanoparticles (AuNP) and glucose oxidase was designed (Fig. 18). First, 1,10-phenanthroline-5,6-dione (PD) was adsorbed on graphite rod electrode, then immersed into electrochemical cell filled with buffer solution containing pyrrole-2-carboxylic acid and colloidal AuNP. Electrochemical polymerization of PD and PCA was performed by CV. During polymerization layers of PPD and poly(pyrrole-2-carboxylic acid) ($PPCA_{EL}$) with encapsulated AuNP were developed. Carboxylic groups of PPCA enabled covalent immobilization of GOx and GR/PPD/(AuNP)$PPCA_{EL}$-GOx electrode was prepared.

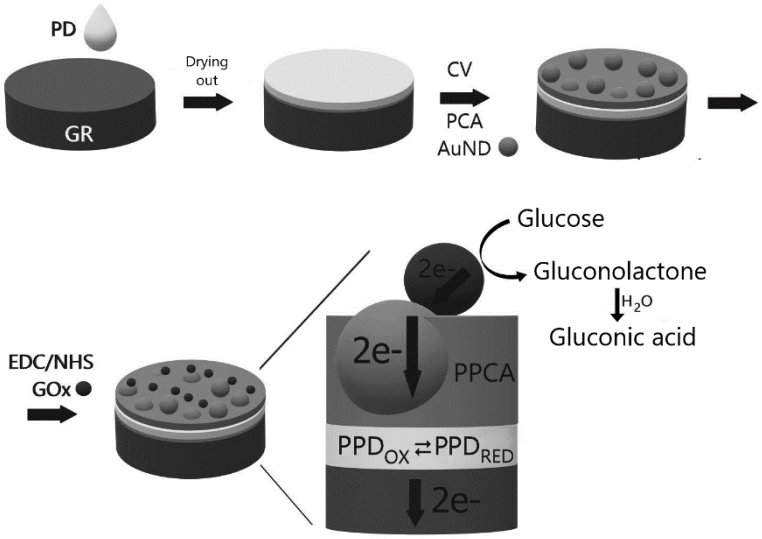

Fig. 18. Illustration of working GR/PPD/(AuNP)$PPCA_{EL}$-GOx electrode preparation and operation of the designed biosensor.

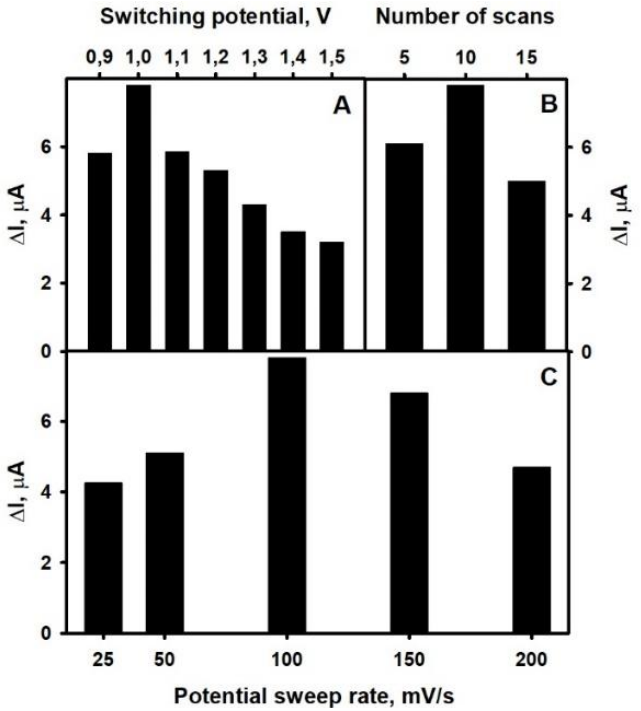

Fig. 19. GR/PPD/PPCA$_{EL}$-GOx electrode current response dependence on switching potential (A), number of scans (B) and potential scan rate (C). Measurements conditions: applied potential +200.0 mV; A-PBS-KCl, pH 6.0; 100.0 mmol/L glucose. Electrode preparation conditions: 8.45 μg/cm^2 PD; 0.1 mol/L PCA; 10 scans (A and C); switching potential 1.0 V (B and C); scan rate 100 mV/s (A and B); 40 mg/mL GOx; EDC/NHS activation duration 30 min; GOx coupling duration 30 min.

Optimization of GR/PPD/(AuNP)PPCA$_{EL}$-GOx electrode started with selecting optimum conditions for electrochemical polymerization. A switching potential, a potential sweep rate and a number of potential scan cycles were optimized. The highest analytical signal was obtained when switching potential was 1.0 V, potential sweep rate – 100 mV/s and 10 potential scan cycles were applied (Fig. 19).

Studies on the AuNP ability to facilitate electron transfer have shown that AuNP amplifies analytical signal of the biosensor (Fig. 20 A). The highest analytical signal in the presence of 100.0 mmol/L glucose was registered when 0.15 nmol/L of AuNP was used.

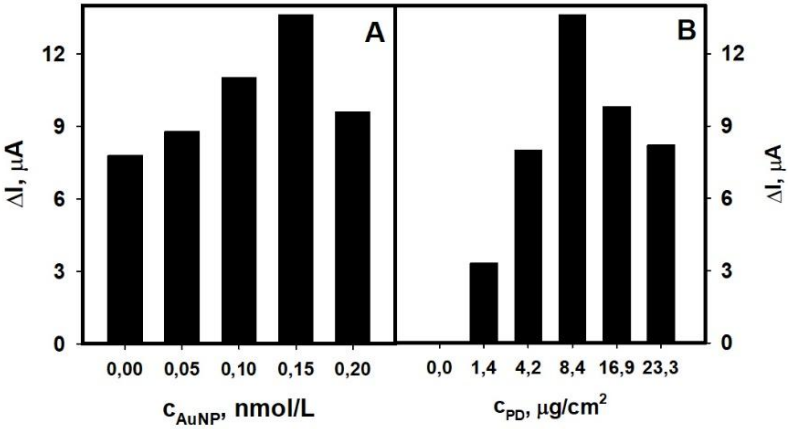

Fig. 20. GR/PPD/(AuNP)PPCA$_{EL}$-GOx electrode current response dependence on AuNP concentration in polymerization solution (A) and surface concentration of PD (B). Measurements conditions: applied potential +200.0 mV; A-PBS-KCl, pH 6.0; 100.0 mmol/L glucose. Electrode preparation conditions: 8.45 μg/cm^2 PD (A); 0.15 nmol/L AuNP (B); 0.1 mmol/L PCA; 10 scans; switching potential 1.0 V; scan rate 100 mV/s; 40 mg/mL GOx; EDC/NHS activation duration 30 min; GOx coupling duration 30 min.

Mediating ability to transfer electrons from GOx active site to GR electrode surface of electrochemically synthesized PPD was investigated. Different surface concentration of PD (0.00, 1.41, 4.23, 8.45, 16.90 and 25.35 μg/cm^2) were adsorbed on GR electrode surface. Figure 20 B shows influence of adsorbed PD on the current response of GR/PPD/(AuNP)PPCA$_{EL}$-GOx electrode. In the absence

of PD, no analytical signal was recorded. Highest value was recorded with 8.45 μg/cm^2 PD amount.

Applied potential effect on GR/PPD/(AuNP)PPCA$_{EL}$-GOx electrode current response was studied. It showed that current response increased with increasing the applied potential up to 200 mV and only a slight increase in current response was observed over 200 mV (Fig. 21). The applied potential value of 200 mV was selected as optimal for amperometric measurements.

Characterization of pristine-GR, GR/PPD, GR/PPCA$_{EL}$, GR/PPD/(AuNP)PPCA$_{EL}$ and GR/PPD/(AuNP)PPCA$_{EL}$-GOx modified electrodes was performed by CV (Fig. 21). No oxidation-reduction current peaks are observed in a voltammogram recorded for the pristine-GR electrode. In the first anodic potential scan, oxidation process starts formation of the PPD and PPCA layers on a surface of GR electrodes. Oxidation-reduction current peaks are not observed for GR/PPCA$_{EL}$ modified electrode either. However, during following scans increase in voltammograms width and decrease in current value at the switching potential were observed. Increase in voltammograms width in the second and following scans is associated with a gradual deposition of PPCA layer on electrode surface and thickening of the polymer layer.

Cyclic voltammogram of GR/PPD electrode, recorded during the first scan, showed well-defined monomer oxidation and reduction current peaks. During second and following scans, decrease of current peaks and appearance and gradual increase of a new current peak at about 200 mV was observed. This can be explained by the formation of polymer and the decrease in a surface concentration of monomer during the formation of polymer.

A shift of PD and PPD current peaks towards positive potential values and decrease of peaks current were observed for GR/PPD/(AuNP)PPCA$_{EL}$ electrode. But the width of voltammogram was higher than that registered for the GR/PPD electrode. This indicates an additional layer of the PPCA with encapsulated AuNP.

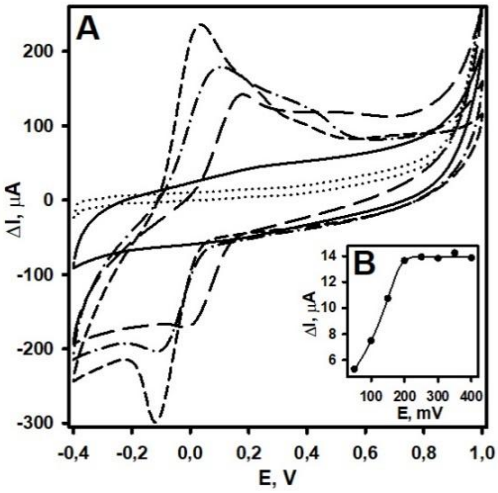

Fig. 21. (A) Cyclic voltammograms of pristine-GR (·····), GR/PPD (- - -), GR/PPCA$_{EL}$ (—), GR/PPD/(AuNP)PPCA$_{EL}$ (— —) and GR/PPD/(AuNP)PPCA$_{EL}$-GOx (— · —) modified electrodes registered in a pure APBS-KCl pH 6.0 (GR and GR/PPD) and in a presence 0.1 mol/L of PCA (GR/PPCA) or 0.1 mol/L of PCA and 0.15 nmol/L of AuNP (GR/PPD/(AuNP)PPCA$_{EL}$) at a scan rate of 100 mV/s. (B) Dependence of current response of GR/PPD/(AuNP)PPCA$_{EL}$-GOx electrode on applied potential. Measurements conditions: A-PBS-KCl pH 6.0; 100 mmol/L glucose. Electrode preparation conditions: 8.45 μg/cm^2 PD; 0.15 nmol/L AuNP; 100 mmol/L PCA; 10 scans; switching potential 1.0 V; scan rate 100 mV/s; 40 mg/mL GOx; EDC/NHS activation duration 30 min; GOx coupling duration 30 min.

The morphology of GR/PPCA$_{EL}$ and GR/PPD/(AuNP)PPCA$_{EL}$ modified electrodes surfaces was studied by SEM. GR/PPCA$_{EL}$ electrode had smooth surface with nodular morphology (Fig. 22 A). GR/PPD/(AuNP)PPCA$_{EL}$ modified electrode showed a similar morphology with spherical bright spots distributed in the polymer layer (Fig. 22 B).

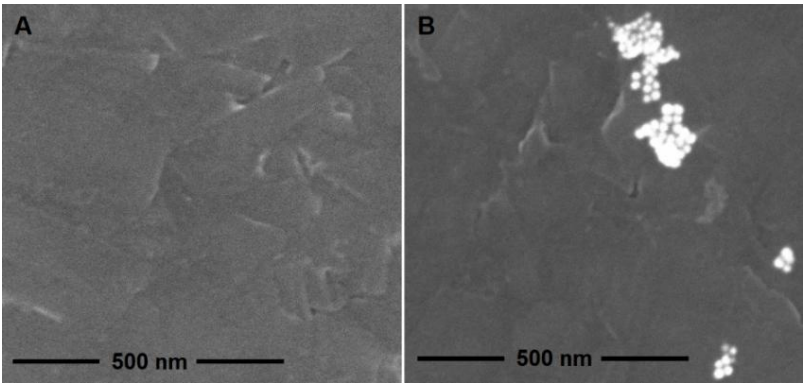

Fig. 22. SEM images of GR/PPCA$_{EL}$ (A) and GR/PPD/(AuNP)PPCA$_{EL}$ (B) modified electrodes. Conditions for images: magnification – 65 000 times; accelerating voltage – 5.0 kV; current – 0.4 nA.

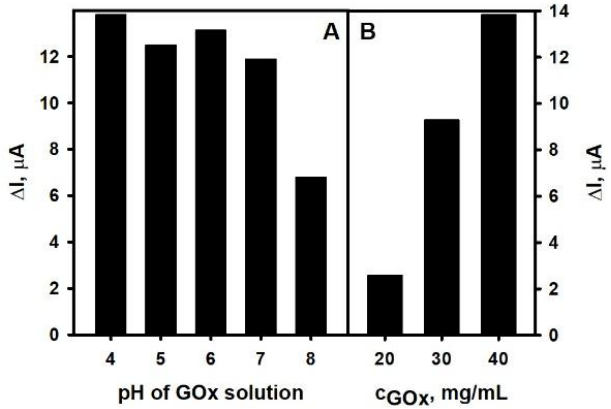

Fig. 23. GR/PPD/(AuNP)PPCA$_{EL}$-GOx electrode current response dependence on pH of GOx solution **(A)**, GOx concentration **(B).** Measurements conditions: applied potential +200.0 mV; A-PBS-KCl pH 6.0; 100 mmol/L glucose. Electrode preparation conditions: 8.45 μg/cm^2 PD; 0.15 nmol/L AuNP; 0.1 mol/L PCA; 10 scans; switching potential 1.0 V; scan rate 100 mV/s; 40 mg/mL GOx (A); pH 4.0 GOx solution (B); EDC/NHS activation duration 30 min; GOx coupling duration 30 min.

GOx solution pH, GOx concentration, duration of activation and duration of coupling were optimized. To find most suitable pH for GOx covalent linking, GR/PPD/(AuNP)PPCA$_{EL}$ electrodes after activation of carboxyl groups were submerged into GOx solutions prepared in A-PBS buffer of different pH. Highest current signal 13.80 μA was registered at pH 4.0 (Fig. 23 A). The most suitable GOx concentration appeared to be 40 mg/mL (Fig. 23 B).

For the duration of activation and duration of enzyme coupling studies, GR/PPD/(AuNP)PPCA$_{EL}$ electrodes at activation and enzyme coupling stages were submerged for 10, 20, 30 or 40 minutes into EDC/NHS mixture or GOx solution, respectively. Results show current response reached a maximal value at 30 minutes for both stages of modification (Fig. 24).

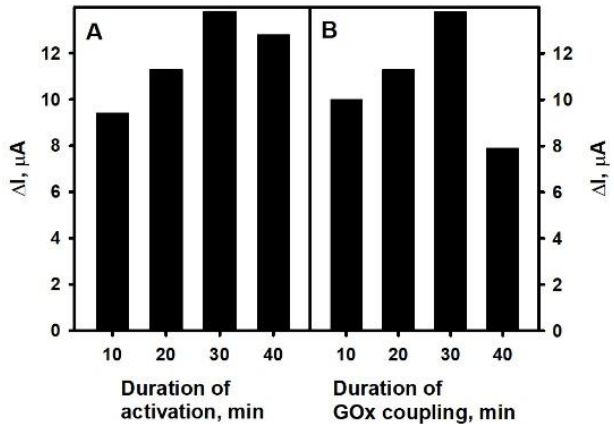

Fig. 24. GR/PPD/(AuNP)PPCA$_{EL}$-GOx electrode current response dependence on duration of activation **(A)** and duration of GOx coupling **(B)**. Measurements conditions: applied potential +200.0 mV; A-PBS-KCl pH 6.0; 100 mmol/L glucose. Electrode preparation conditions: 8.45 μg/cm^2 PD; 0.15 nmol/L AuNP; 0.1 mol/L PCA; 10 scans; switching potential 1.0 V; scan rate 100 mV/s; 40 mg/mL GOx; pH 4.0 GOx solution; EDC/NHS activation duration 30 min (B); GOx coupling duration 30 min (A).

After optimizing GR/PPD/(AuNP)PPCA$_{EL}$-GOx electrode preparation procedure, the influence of glucose concentration on the

analytical signal was investigated. Figure 25 shows a hyperbolic relationship between glucose concentration and GR/PPD/(AuNP)PPCA$_{EL}$-GOx electrode current response in the glucose concentration range from 0.20 to 500.0 mmol/L. A linear relationship with $R^2 = 0.999$ (N:3) was observed up to 150.0 mmol/L (Fig. 25B). The detection limit was estimated to be 0.08 mmol/L. The sensitivity of the biosensor was found to be 0.135 μA/mmol/L.

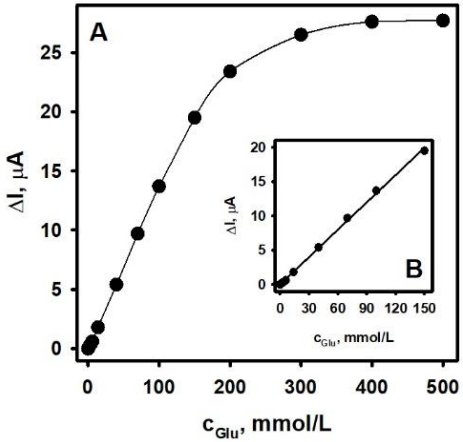

Fig. 25. GR/PPD/(AuNP)PPCA$_{EL}$-GOx electrode current response concentration of glucose **(A)** and calibration curve **(B)**. Electrode preparation conditions: 8.45 μg/cm^2 PD; 0.15 nmol/L AuNP; 0.1 mmol/L PCA; 10 scans; switching potential 1.0 V; scan rate 100 mV/s; 40 mg/mL GOx; EDC/NHS activation duration 30 min; GOx coupling duration 30 min.

Repeatability and reproducibility of the designed biosensor were also investigated. One GR/PPD/(AuNP)PPCA$_{EL}$-GOx electrode was prepared under optimized preparation conditions and its current responses to 5.0, 50.0 and 100.0 mmol/L glucose were measured three times. Relative standard deviation (RSD) calculated for three independent measurements were 4.22, 2.34 and 1.67 %, respectively for 5.0, 50.0 and 100.0 mmol/L glucose. During the reproducibility experiment, three identical GR/PPD/(AuNP)PPCA$_{EL}$-GOx electrodes were prepared and their current responses to glucose were measured.

Calculated RSD were 6.93, 2.23 and 1.17 %, respectively for 5.0, 50.0 and 100.0 mmol/L glucose.

The effect of common interfering substances such as UA, AAP, acetylsalicylic acid (ASA), DP and AA on analytical signal was investigated and evaluated. It showed that UA (0.30 mmol/L), AAP (0.10 mmol/L) and ASA (0.07 mmol/L) had no effect on analytical signal of the biosensor (Fig. 26). AA (0.1 mmol/L) increased analytical signal by 6.45 %. DP (0.1 mmol/L) had the most significant effect and increased the analytical signal by 13.31 %.

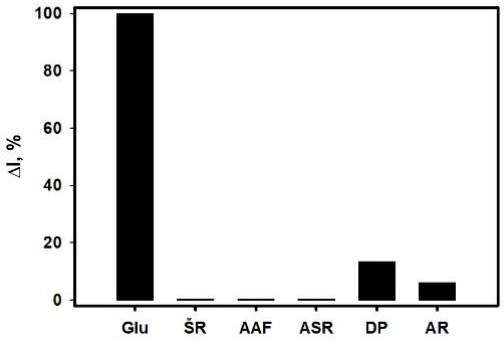

Fig. 26. GR/PPD/(AuNP)PPCA$_{EL}$-GOx electrode current response electrode to glucose (Glu) and interfering substances (uric acid (UA), acetaminophen (AAP), acetylsalicylic acid (ASA), dopamine (DP) and ascorbic acid (AA)) Electrode preparation conditions: 8.45 μg/cm^2 PD; 0.15 nmol/L AuNP; 0.1 mol/L PCA; 10 scans; switching potential 1.0 V; scan rate 100 mV/s; 40 mg/mL GOx; EDC/NHS activation duration 30 min; GOx coupling duration 30 min. Measurements conditions: applied potential +200.0 mV.

GR/PPD/(AuNP)PPCA$_{EL}$-GOx electrode was tested during 14-day period. After 100 mmol/L glucose addition current was gradually decreasing over a period of 14 days and at the last day of experiment it was 3.98 % lower than the initial (Fig. 27). Covalent linking prevents leaking of the enzyme from electrode surface during repeated measurements.

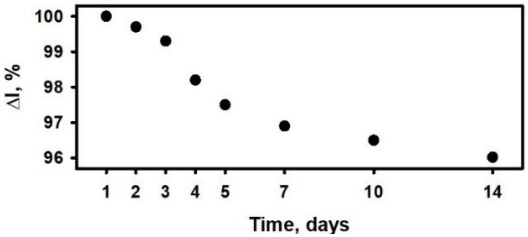

Fig. 27. GR/PPD/(AuNP)PPCA$_{EL}$-GOx electrode current response *vs* time. Electrode preparation conditions: 8.45 μg/cm^2 PD; 0.15 nmol/L AuNP; 0.1 mol/L PCA; 10 scans; switching potential 1.0 V; scan rate 100 mV/s; 40 mg/mL GOx; EDC/NHS activation duration 30 min; GOx coupling duration 30 min. Measurements conditions: applied potential +200.0 mV.

In order to test suitability of glucose biosensor for practical applications, glucose concentration in pharmaceutical preparations Orsalit Drink and Rehydron Optim and human serum was measured. Glucose concentration was determined by a calibration graph made by analysing solutions of known glucose concentration in the range of $0.20 - 150.0$ mmol/L and derived line equation. Results are presented in a table 2. According to the results, developed glucose biosensor based on GR/PPD/(AuNP)PPCA$_{EL}$-GOx working electrode is capable of detecting glucose in real samples with good accuracy.

Table 3. Determination of glucose in real samples.

Preparation	Test No.	Determined concentration, mmol/L	Average	Declared concentration, mmol/L	RE, %
Orsalit Drink	1	75.08	74.37	73.17	1.64
	2	73.92			
	3	74.12			
Rehydron Optim	1	373.36	374.93	374.58	0.09
	2	376.53			
	3	374.91			
Human serum	1	5.50	5.58	5.55	0.54
	2	5.66			
	3	5.58			

CONCLUSIONS

1. PPCA particles was synthesized by chemical polymerization method, using H_2O_2 as initiator, and by enzymatic polymerization, using glucose oxidase and glucose. Enzymatic polymerization occurs in pH 2 – 9, optimal pH is 5. Optimal pH for chemical polymerization is 2. Enzymatic polymerization occurs faster in less acidic media than chemical polymerization, although particles are smaller.
2. Biocomposite consisting of PPCA particles, synthesized by chemical polymerization, and GOx was used in developing glucose biosensor, as mediator using PMS. Optimal preparation conditions: polymerization duration 48 hours, pH 4.0 GOx solution, 5 mg/mL BSA, 6 µl of biocomposite suspension. Optimal conditions for measurements: +300 mV, pH 8.0 sample solution. Linear range of biosensor 0.1 – 15.0 mmol/L, detection limit 0.039 mmol/L. Biosensor can be used for real samples analysis.
3. Nanobiocomposite consisting of electrochemically polymerized PPCA and PPD, AuNP and GOx was used in developing reagent-less glucose biosensor. PPD acts as mediator, AuNP facilitate electron transfer. Optimal preparation conditions: potential sweep from -0.4 V to 1.0 V, 10 cycles, potential sweep rate 100 mV/s, 0.15 nmol/L AuNP, 8.45 µg/cm^2 PD, 40 mg/ml GOx solution in pH 4.0, duration of carboxylic groups activation and GOx coupling 30 min. Optimal conditions for measurements: +200 mV, pH 6.0 sample solution. Linear range of biosensor 0.2 – 150.0 mmol/L, detection limit 0.08 mmol/L. Biosensor can be used for real samples analysis.

LIST OF PUBLICATIONS

1. A. Kausaite-Minkstimiene, **L. Glumbokaite**, A. Ramanaviciene, A. Ramanavicius, Reagent-less amperometric glucose biosensor based on nanobiocomposite consisting of poly(1,10-phenanthroline-5,6-dione), poly(pyrrole-2-carboxylic acid), gold nanoparticles and glucose oxidase. *Microchemical Journal*, 2020, 154, 104665 (IF: 3,206).
2. A. Kausaite-Minkstimiene, **L. Glumbokaite**, A. Ramanaviciene, E. Dauksaite, A. Ramanavicius, An Amperometric Glucose Biosensor Based on Poly (Pyrrole-2-Carboxylic Acid)/Glucose Oxidase Biocomposite. *Electroanalysis*, 2018, 30, 1634-1644 (IF: 2,691).
3. A. Kausaite-Minkstimiene, A. Ramanaviciene, R. Simanaityte, D. Gabrielaitis, **L. Glumbokaite**, A. Ramanavicius. Evaluation of poly(pyrrole-2-carboxylic acid) particles synthesized by enzymatic catalysis. *RSC Advances*, 2015, 5, 105475-105483 (IF: 3,049).

CURRICULUM VITAE

Laura Glumbokaitė

Personal data

1989 09 27
Vilnius
lauraglumb@gmail.com

Education

2015.10 – 2019.09
Vilnius University
Faculty of Chemistry and Geosciences Institute of Chemistry
Doctoral studies

2012.09 – 2014.06
Vilnius University
Faculty of Chemistry
Department of Analytical and Environmental Chemistry
Master studies

2008.09 – 2012.06
Vilnius University
Faculty of Chemistry
Department of Analytical and Environmental Chemistry
Bachelor studies

2004.09 – 2008.06
Vilnius Žemyna gymnasium

NOTES

NOTES

Vilniaus universiteto leidykla
Saulėtekio al. 9, LT-10222 Vilnius
El. p. info@leidykla.vu.lt,
www.leidykla.vu.lt
Tiražas 15 egz.