VILNIUS UNIVERSITY

CPST INSTITUTE OF CHEMISTRY

JAROSLAV VORONOVIČ

GOLD NANOPARTICLES AND π-π CONJUGATED POLYMER POLYPYRROLE FOR GLUCOSE BIOSENSORS DESIGN

Summary of doctoral dissertation

Physical sciences, chemistry (03 P)

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The research was carried out in Department of Analytical and Environmental Chemistry, Faculty of Chemistry, Vilnius University, in the period of 2009 – 2013.

Scientific supervisors:

Doc. dr. Almira Ramanavičienė (Vilnius University, Physical Sciences, Chemistry – 03 P) (2010-11-23 – 2013-09-30)

Prof. habil. dr. Arūnas Ramanavičius (Vilnius University, Physical Sciences, Chemistry – 03 P) (2009-10-01 – 2010-11-22)

The defence council:

Prof. dr. Stasys Tautkus (Vilnius University, Physical Sciences, Chemistry – 03P)

Members:

Prof. dr. Vida Vičkačkaitė (Vilnius University, Physical Sciences, Chemistry – 03P)

Doc. dr. Jolanta Sereikaitė (Vilnius Gediminas Technical University, Technological Sciences, Chemical Engineering – 05T).

Prof. habil. dr. Ričardas Rotomskis (Vilnius University, Biomedical Sciences, Biophysics – 02B)

Dr. Igoris Prosyčevas (Kaunas University of Technology, Physical Sciences, Chemistry – 03P)

Official opponents:

Prof. habil. dr. Aniolas Sruoga (Vytautas Magnus University, Biomedical Sciences, Biology 01B)

Prof. habil. dr. Albertas Malinauskas (Center for Physical Sciences and Technology, Institute of Chemistry, Physical Sciences, Chemistry – 03P)

The defense of the dissertation will be held at … p.m. on 29th of September 2014 at the public meeting of the council at the Auditorium of Physical Chemistry at the Faculty of Chemistry of Vilnius University. Address: Naugarduko 24, LT-03225, Vilnius, Lithuania.

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VILNIAUS UNIVERSITETAS

FIZINIŲ IR TECHNOLOGIJOS MOKSLŲ CENTRO

CHEMIJOS INSTITUTAS

JAROSLAV VORONOVIČ

AUKSO NANODALELIŲ IR π-π KONJUGUOTO POLIMERO POLIPIROLO TAIKYMAS GLIUKOZĖS BIOLOGINIUOSE JUTIKLIUOSE

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Moksliniai vadovai:

doc. dr. Almira Ramanavičienė (Vilniaus universitetas, fizikiniai mokslai, chemija – 03 P) (2010-11-23 – 2013-09-30)

prof. habil. dr. Arūnas Ramanavičius (Vilniaus universitetas, fizikiniai mokslai, chemija – 03 P) (2009-10-01 – 2010-11-22)

Disertacija ginama Vilniaus universiteto Chemijos mokslo krypties taryboje:

Pirmininkas:

Prof. dr. Stasys Tautkus (Vilnius universitetas, fiziniai mokslai, chemija − 03P)

Nariai:

Prof. dr. Vida Vičkačkaitė (Vilniaus universitetas, fiziniai mokslai, chemija – 03P)

Doc. dr. Jolanta Sereikaitė (Vilniaus Gedimino technikos universitetas, technologijos mokslai, chemijos inžinerija – 05T).

Prof. habil. dr. Ričardas Rotomskis (Vilniaus universitetas, biomedicinos mokslai, biofizika – 02B)

Dr. Igoris Prosyčevas (Kauno technologijos universitetas, fiziniai mokslai, chemija – 03P)

Oponentai:

Prof. habil. dr. Aniolas Sruoga (Vytauto Didžiojo universitetas, biomedicinos mokslai, biologija 01B)

Prof. habil. dr. Albertas Malinauskas (Fizinių ir technologijos mokslų centro Chemijos institutas, fiziniai mokslai, chemija – 03P)

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INTRODUCTION

The field of electrochemical glucose biosensors has grown rapidly in past decades. Fast, simple and low-cost detection of biologically active analytes are the major advantages of biosensors. The amperometric and voltammetric biosensors, based on gold nanoparticles, were designed and applied for biochemical, clinical and environment applications, including cancer diagnostic, detection of infections, determination of vitamins, amino acids and sugars. Electrochemical biosensors are very selective, sensitive and fast.

Gold, silver, platinum and SiO_2 particles in the range of 1–100 nm often provide an ideal remedy for immobilized enzymes with minimal diffusion limitations, promotion of electrochemical reaction, high surface-to-volume ratio and possible achievement of enzymes direct wiring to electrode surface. Gold nanoparticles (AuNPs) increase retention and enzymatic activity, because the smaller gold nanoparticles would bind directly with enzymes without disrupting its biological recognition properties. Also, nanoparticles increase electron transfer rate between enzyme and an electrode surface.

Materials based on $\pi - \pi$ conjugated polymers are often used as electrocatalysts or immobilisation matrixes for biomolecules. Conjugated polymers provide effective immobilisation patterning for biomolecules on different substrates. Moreover in some cases $\pi - \pi$ conjugated polymers facilitate electron transfer from enzymes to electrically conductive electrodes. Most promising $\pi - \pi$ conjugated polymers, e.g. polyaniline, polypyrrole (PPy) are chemically stabile on different substrates. Also, conducting polymers are easily synthesised by electrochemical, chemical and enzymatic oxidative polymerisation techniques. Insoluble, stable in ambient conditions PPy films usually are prepared electrochemically by the oxidation of commercially available pyrrole monomers.

Glucose oxidase (GOx) has an extensive over 50-year-long application record in amperometric glucose biosensor design. However catalytic action of GOx was thoroughly investigated for GOx from *Aspergillus niger*. Glucose oxidases purified

from various strains of *Aspergillus niger* are also used in fabrication of the majority commercial glucose sensors. Given expanding market demand, the studies in many research centers around the globe have been focused on search and further selection of novel hyperactive strains, which are producing this enzyme.

The aim of the work:

To evaluate the applicability of gold nanoparticles of different size, several glucose oxidases and π-π conjugated polymer polypyrrole for the modification of graphite electrode and development of amperometric glucose biosensors.

Main tasks of the work:

- ✓ Investigation of differently designed electrodes, which are based on gold nanoparticles of different diameters, for the amperometric detection of glucose.
- ✓ Evaluation of different concentrations of gold nanoparticles colloidal solution on glucose biosensing and performance of enzymatic biosensors in the presence and absence of electron transfer mediator.
- ✓ Investigation of the influence of π-π conjugated polymer polypyrrole on glucose biosensing and determination of the analytical characteristics of enzymatic biosensors modified with different size gold nanoparticles.
- ✓ Comparison of analytical signal and stability of electrodes modified with glucose oxidases from *Penicillium adametzii*, *Penicillium funiculosum*, *Aspergilus niger* and different electron transfer mediators.

Statements to be defended:

1. Gold nanoparticles immobilized on the surface of graphite electrode together with soluble electron transfer mediator provide more efficient electron transfer from glucose oxidase to electrode and analytical signal of this system is higher when compared to system without gold nanoparticles.
2. The low concentration of colloidal gold nanoparticles in the solution (in the range from 0.01 to 0.60 nmol L^{-1}) together with electron transfer mediator increases the rate of mediated electron transfer, and this process does not depend on size of gold nanoparticles (3.5, 6.0 and 13.0 nm).

3. The polypyrrole layer formed on electrode modified with different size gold nanoparticles and GOx extends the linear detection range of glucose. Biosensors based on smaller gold nanoparticles exhibit higher amperometric responses at same concentration of glucose before and after polypyrrole layer formation.
4. Glucose oxidases from *Penicillium adametzii* and *Penicillium funiculosum* immobilized on graphite electrode are more stable than that from *Aspergillus niger*. The highest analytical signals are registered with glucose oxidase from *P. funiculosum* and immobilized electron transfer mediators - ferrocenecarboxylic acid and α–methylferrocenemethanol.

EXPERIMENTAL

Electrochemical measurements

All electrochemical measurements using gold nanoparticles were performed with a computerized potentiostat PGSTAT 30/Autolab (EcoChemie, The Netherlands) with GPES 4.9 software in amperometry modes (potential + 0.3V or + 0.6 V *vs* $Ag/AgCl/KCl_{3M}$). A conventional three-electrode system comprising a prepared working graphite electrode (GR), 2 cm^2 platinum as an auxiliary electrode and $Ag/AgCl/KCl_{3M}$ Metrohm (Switzerland) as a reference electrode was employed for all electrochemical experiments. Between measurements all electrodes were stored at + 4°C in a closed vessel hanging over the solution of buffer to maintain constant humidity. The results of all electrochemical measurements are reported as the mean value of three independent experiments.

Synthesis of gold nanoparticles

Gold nanoparticles of several different diameters (3.5, 6.0 and 13.0 nm) were synthesized while reducing $HAuCl_4 \cdot 3H_2O$ by sodium citrate in the presence of tannic acid. The average diameter of AuNPs distributed on surface of 8 Å SiO_2 substrate was investigated by atomic force microscopy (AFM) using tapping mode.

Modification of electrodes by gold nanoparticles, glucose oxidase and the polypyrrole layer

The working surface area of the graphite electrode was 0.071 cm^2. Graphite rods of spectroscopic graphite were cut and polished on fine emery paper and then polished by slurry of alfa alumina powder containing 0.3 micron grains of Al_2O_3. After this electrode surface was rinsed with distilled water and dried at room temperature at 20±2°C. Then electrodes were sealed into silicone tube to prevent contact of the electrode side surface with the solution.

During the preparation of graphite electrode modified with glucose oxidase (GR/GOx) 3 μL of solution containing 40 mg mL^{-1} GOx were deposited on the electrode and water was evaporated at room temperature. For the preparation of GR/GOx/AuNPs electrode additionally 3 μL of AuNPs colloid solution were deposited on the GOx-electrode. For the preparation of GR/AuND/GOx electrodes 3 μL of gold nanoparticle colloid solution were deposited on the working electrode and after the evaporation of water additionally 3 μl of solution containing 40 mg mL^{-1} GOx were deposited. After water evaporation all electrodes were stored for 15 min in a closed vessel over 25% solution of glutaraldehyde at room temperature. Prior to all electrochemical measurements, working electrodes were thoroughly washed with distilled water to remove non-cross-linked enzyme and/or gold nanoparticles. All working electrodes were stored in a closed vessel over the solution of buffer at + 4°C until used in the experiment.

Chemical polymerisation of pyrrole over modified graphite electrodes was performed in sodium acetate buffer, pH 6.0, containing 0.05 mol L^{-1} glucose and 0.5 mol L^{-1} pyrrole. Electrochemical polypyrrole polymerisation on the platinum electrode was performed with pulsed potential based voltamperometry in sodium acetate buffer, pH 6.0, containing 0.1 mol L^{-1} KCl and 0.5 mol L^{-1} pyrrole.

During GR modification process 3.0 μL solution (10.0 mg mL^{-1}) of redox mediator in acetonitrile were dropped on the electrode surface and solvent was evaporated; this step was repeated for three times. After evaporation of solvent the electrode was additionally treated with appropriate solution of GOx, each electrode

was modified with the same amount of active enzyme measured in units of enzyme activity (U). During the modification of electrode surface, each following drop of mediator or GOx solution was added after drying of previous drop at room temperature. Then electrodes were stored for 20 h over a 5 % solution of glutaraldehyde at + 4 ºC in a closed vessel. Prior to all electrochemical measurements, working electrodes were thoroughly washed with deionised water in order to remove non-cross-linked enzyme. Then electrodes were sealed into silicone tube to prevent contact of the electrode side surface with the solution.

The imaging of differently modified electrodes by AFM

AFM working in tapping mode was used for the imaging of differently modified surfaces. The BioScope II, Veeco Instruments Ltd. (Santa Barbara, USA) and TESP cantilevers (Veeco, USA) were used for all AFM experiments. Experimental data were processed by "diNanoScope 7.30" and "Gwyddion 2.10" NT-MDT Nova" programs.

Calculations

The kinetic parameters: the maximal analytical signal (I_{max}) (corresponds to maximal rate of the reaction according to Michaelis-Menten kinetics) and the apparent Michaelis constant ($K_{M(app.)}$) are correspondingly *a* and *b* parameters of hyperbolic function $y = ax/(b+x)$ used for approximation of results.

RESULTS AND DISCUSSION

Glucose biosensors based on graphite electrodes modified with glucose oxidase and gold nanoparticles

The influence of 13.0 nm diameter AuNP on analytical signal of glucose biosensor

The aim of this research was to evaluate the efficiency and the applicability of 13 nm gold nanoparticles in the improvement of amperometric glucose biosensors based on GOx. In this biosensor electrons were transferred towards + 0.3 V *vs.* $Ag/AgCl/KCl_{3M}$ charged electrode and steady-state currents were registered. The analytical signal in this biosensor was the difference of steady-state currents before and after addition of glucose (ΔI).

Two types of amperometric biosensors were developed and compared: graphite electrode based on immobilized GOx and graphite electrode based on immobilized GOx and AuNPs (Fig. 1) in to different ways: GR/AuND/GOx and GR/GOx/AuND. Amperometric measurements were performed with both types of modified electrodes using soluble redox mediator N-methylphenazonium methyl sulphate (PMS).

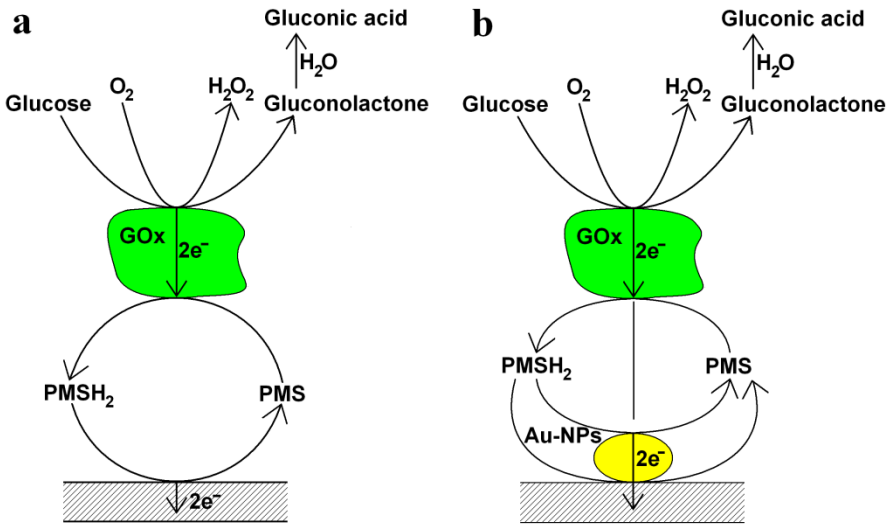

Fig. 1 Electrocatalytic oxidation of glucose in the presence of glucose oxidase (a) or glucose oxidase and gold nanoparticles with mediator – N-methylphenazonium methyl sulphate (PMS).

The GOx immobilized on graphite electrode in the first type of biosensor in the presence of glucose and dissolved oxygen generated hydrogen peroxide and gluconolactone, which was hydrolyzed to gluconic acid (Fig. 1a). PMS is reoxidizing the active site of GOx and then electrons *via* reduced PMS are transferred towards graphite electrode. The reactions that take place in GR/AuNPs/GOx electrodes are presented in figure 1b. In this case the electron transfer *via* PMS could follow by two ways: directly to the graphite electrode and through AuNPs what is in line with other studies.

The major advantage of GR/AuNPs electrode is that AuNPs are chemically and electrochemically more active and has a higher surface energy when compared with non-modified graphite electrode. This positively influences the analytical signal and the sensitivity of biosensors becomes higher. The AuNPs offers advanced electrically

active surface, enhance charge transport towards graphite electrode and it accelerates electrocatalytic reactions.

The hyperbolic dependences of amperometric signals on the concentration of glucose in the range from 0.1 to 100 mmol L^{-1} (Fig. 2) were observed by all three types (GR/GOx, GR/AuNPs/GOx and GR/GOx/AuNPs) of electrodes. All presented hyperbolic dependences were in agreement with Michaelis-Menten kinetics and are presented in Table 1.

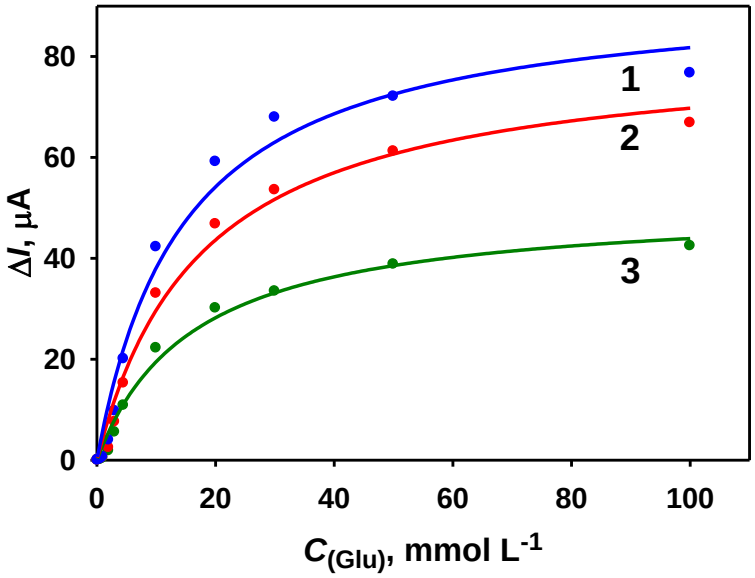

Fig. 2 Calibration plots of GR/AuNPs/GOx (1), GR/GOx/AuNPs (2) and GR/GOx (3) electrodes. In 0.05 mol L^{-1} sodium acetate buffer, pH 6.0, containing 2 mmol L^{-1} PMS; at +0.3V *vs* Ag/AgCl/KCl_{3M}. The mean values of three independent measurements by three electrodes of the same type are presented.

Results presented in figure 2 and table 1 show that the application of 13 nm AuNPs increase amperometric signals. It could be related to significantly increased electron transfer rate from GOx to graphite electrode, where AuNPs increases effective surface area of electrode and/or plays a role of redox mediator.

In table 1 presented results illustrate that for GR/GOx electrode in 0.05 mol L^{-1} sodium acetate buffer analytical signal is 1.8 times lower, when compared with that of GR/AuNPs/GOx electrode and 1.6 times lower, if compared with analytical signal of

GR/GOx/AuNPs electrode. It can be concluded, that the highest analytical signal was registered using biosensor based on GR/AuNPs/GOx electrode.

For all types of here studied electrodes calculated $K_{M(app.)}$ constants are approximately from two to four times lower if compared with $K_{M(app.)}$ = 33 mmol L^{-1} of non-immobilized GOx in the solution (this parameter depends on the nature and properties of the buffer solution) (Table 1).

Table 1 Parameters calculated from Michaelis-Menten equation. (All conditions are the same as presented in Fig. 2)

Type of electrode	$K_{M(app.)}$, mmol L^{-1}	I_{max}, μA	R^2
GR/GOx	16.2	51.0	0.9928
GR/GOx/AuNPs	17.6	82.0	0.9896
GR/AuNPs/GOx	14.6	93.7	0.9911

Such significant differences in $K_{M(app.)}$ may be related to the application of different materials as working electrodes in systems that are presented in literature and different pH of bulk solutions. The $K_{M(app.)}$ of GR/GOx/AuND was 17.6 mmol L^{-1}. The optimal combination of high analytical signal and $K_{M(app.)}$ for the GR/AuNPs/GOx electrode was found in 0.05 mol L^{-1} sodium acetate buffer, pH 6.0, containing 2 mmol L^{-1} PMS, thereby this solution was used for further studies. Amplification of electrochemical signal by AuNPs is in-line with results reported in other studies.

The same dependences of kinetic parameters were observed and with 0.05 mol L^{-1} sodium phosphate buffer, pH 6.0. However, analytical signals are lower: for GR/GOx analytical signal was 49.8 μA, for GR/GOx/AuND – 66.0 μA and for GR/AuND/GOx – 70.6 μA.

AFM imaging of electrodes modified by 13 nm diameter gold nanoparticles and glucose oxidase

On GR/AuNPs/GOx electrode surface registered features appeared to be 16.5 ± 6 nm in height (Fig. 3a,b), these features can be expected as clusters of gold nanoparticles and enzyme. It was detected that the surface of bare graphite electrode is fairly rough,

the high-distribution is in the quite wide range within 5 – 25 nm (Fig. 3c,d). After the modification of electrode surface with 13 nm AuNPs, the high-distribution was within 10 – 15 nm (Fig. 3f,e), but according to high-distribution diagram the number of 13.5 nm diameter nanoparticles dominates among numbers of nanoparticles of another diameters. From differences in height-distribution diagrams it could be predicted that gold nanoparticles penetrates into microgaps of graphite electrode, AuNPs cover the surface of graphite forming continuous layer and for this reason the electrode surface becomes much smoother. Additional modification of the same surface with enzyme shows that the heights of most nano-features slightly increased until ~ 16.5 nm.

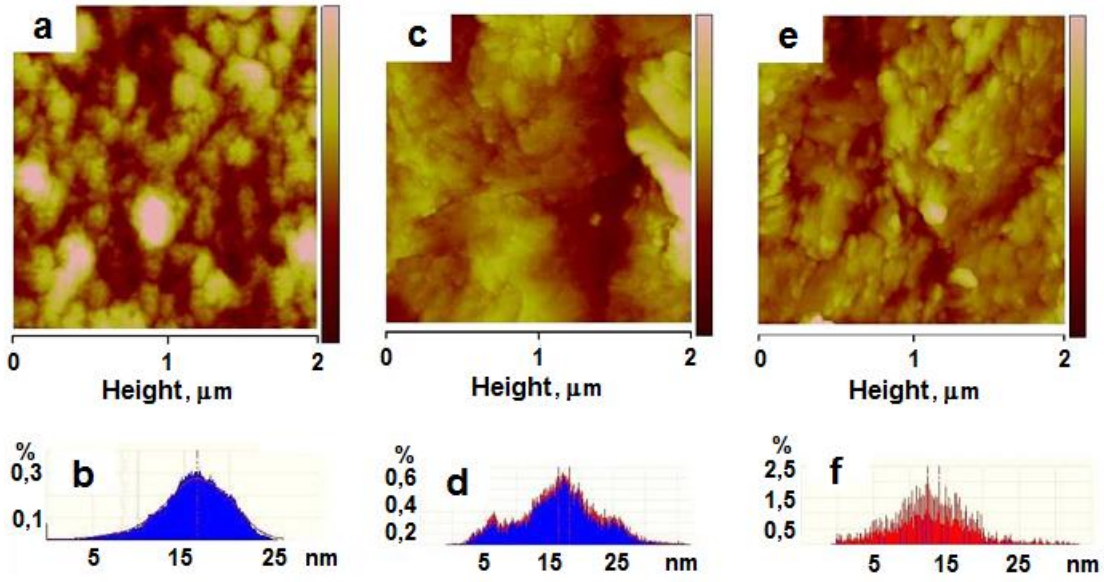

Fig. 3 AFM images of GR/AuNPs/GOx (a), bare graphite (c) and GR/AuNPs (e) electrodes; (b, d, f) height-distribution diagrams of AFM images of GR/AuNPs/GOx, bare graphite and GR/AuNPs electrodes, respectively.

At the same time the presence of colloidal gold gives more freedom for immobilized enzyme molecules in orientation. Amperometric results let us predict that some amount of AuNPs are close to the redox site of GOx. This reduces the insulating properties of the enzyme shell, thereby the active sites of the enzyme could be closer to the electrically conducting surface of graphite/gold, what decreases formal ohmic resistance of electrochemical system, reduces diffusion distance for oxidized/reduced forms of PMS and facilitates electron transfer between enzyme and electrode. These factors positively affect the sensitivity of the AuNPs-based amperometric sensors.

Stability characteristics of gold nanoparticles and glucose oxidase modified glucose biosensors

In the next stage of this work the storage stability of GR/GOx and GR/AuNPs/GOx electrodes were examined. Steady-state currents were registered after the addition of 10 mmol L^{-1} glucose. The $\tau_{1/2}$ for GR/GOx electrode was 49.3 days and for GR/AuNPs/GOx electrode it was 19.5 days when the electrodes were stored at + 4°C. The stability of electrodes was tested during a 66-day period; 43% and 22% of the initial current response for GR/GOx and GR/AuNPs/GOx electrodes were retained.

According to presented results the GR/AuNPs/GOx electrode gives higher amperometric signals what is in line with the results presented by other researchers. However in this study reported GR/AuNPs/GOx electrode is less stable in comparison with GR/GOx electrode system. Such decrease in stability of GR/AuNPs/GOx electrode could be explained by leakage of some AuNPs modified with enzyme from electrode surface during repetitive electrochemical measurements. On the other hand, even in automated commercial systems electrodes should be exchanged every week and should be calibrated several times a day.

The influence of different size gold nanoparticles on amperometric glucose biosensing

In amperometric biosensors the sensitivity of electrochemical established method depends on the diameter of AuNPs immobilized on the surface of graphite rod electrode. In this part of research we have evaluated gold nanoparticles of 3.5, 6.0 and 13.0 nm diameter.

The AuNPs were synthesised according to the protocol presented in experimental part and dry samples were investigated by AFM using tapping mode. AFM images illustrate the size distribution of AuNPs of different sizes (Fig. 4). Shape of height histogram of AuNPs shows that formed AuNPs 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 nm, 5 – 7 nm and 2 – 5 nm, respectively (Fig. 4).

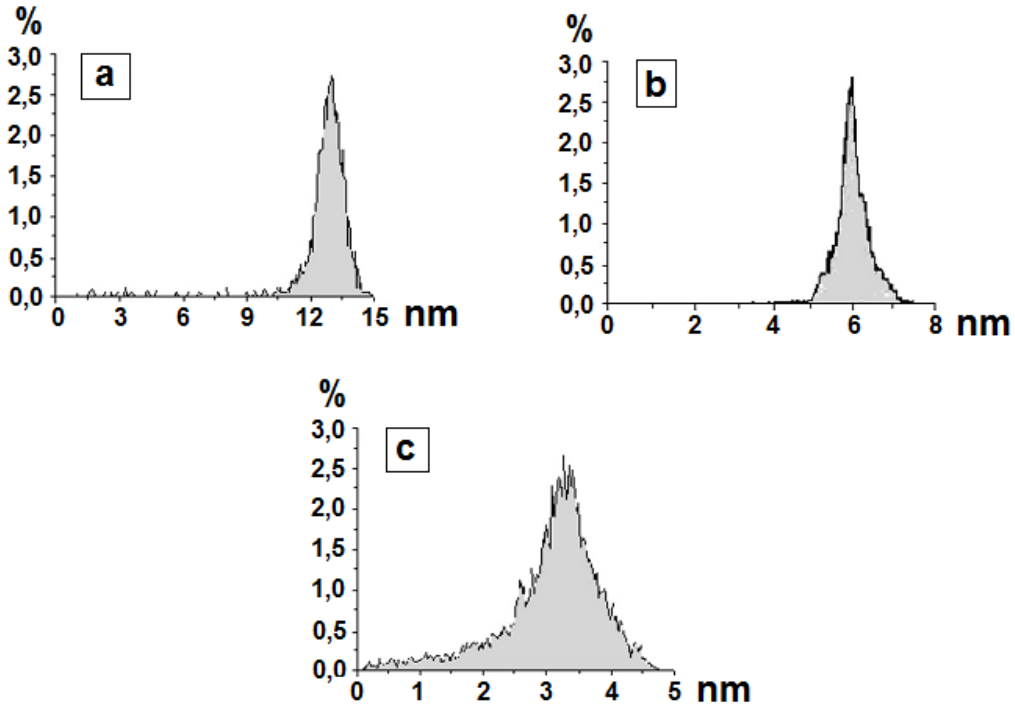

Fig 4 Size distribution histograms of gold nanoparticles registered by AFM. a – 13.0 nm, b – 6.0 nm and c – 3.5 nm AuNPs. Conditions: AFM tapping mode; all data were summarized with programs "diNanoScope 7.30" and "Gwyddion 2.10 NT-MDT Nova".

We observed a hyperbolic dependence of oxidation currents to concentration of glucose in the range from 0.1 to 100 mmol L^{-1} in 0.05 mol L^{-1} sodium acetate buffer, pH 6.0, containing 2 mmol L^{-1} PMS (Fig. 5). The current of developed biosensors increased along with analyte concentration. It is seen that GR/AuNPs/GOx electrodes based on smaller diameter (3.5 and 6.0 nm) AuNPs had a higher electrocatalytic activity, compared with that of larger diameter (13.0 nm) AuNPs.

The AuNPs have large specific surface areas and it is possible to load more enzyme molecules. On the other hand AuNPs facilitate electron transfer between enzyme and electrode surface, therefore the presence of AuNPs facilitates the indirect electron transfer through the conducting circuit based on AuNPs (Fig. 1b).

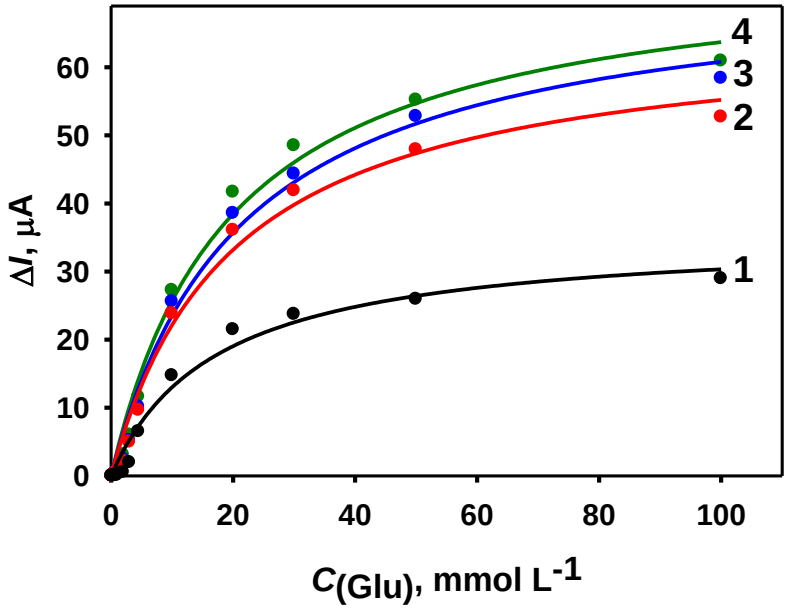

Fig. 5 Calibration plots of GR/AuNPs/GOx electrodes based on different diameter of gold nanoparticles as a function of glucose concentrations. 1 – GR/GOx, 2 – GR/AuNPs$_{13nm}$/GOx, 3 – GR/AuNPs$_{6nm}$/GOx, 4 – GR/AuNPs$_{3.5nm}$/GOx electrodes, tested in 0.05 mol L^{-1} sodium acetate buffer, pH 6.0, containing 2 mmol L^{-1} PMS, at + 0.3 V *vs.* Ag/AgCl/KCl$_{3M}$. The means of three independent measurements by different electrodes are presented.

From the presented data (Fig. 5 and Table 2) it can be concluded that electrodes based on smaller AuNPs show the highest analytical responses. The increase of the maximal analytical signal by 2.14, 2.07 and 1.84 times for electrodes based on 3.5, 6.0 and 13.0 nm AuNPs respectively was registered in comparison with electrode based only on GOx. Insignificant difference of $K_{M(app.)}$ (1.1 times) with decreasing of AuNPs size was observed and could be explained by the density changes of AuNPs layer. The layer of smaller AuNPs is denser, and the transfer of electrons between GOx and graphite electrode is faster when compared with that based on larger AuNPs. For all electrodes based on AuNPs maximal current was about two times higher if compared with the same parameter calculated for GR/GOx electrode.

Table 2. Parameters calculated from Michaelis-Menten equation. (All conditions are the same as presented in Fig. 6)

Type of electrode	$K_{M(app.)}$, mmol L^{-1}	I_{max}, μA	R^2
GR/$AuND_{3.5nm}$/GOx	19.7	76.2	0.9939
GR/$AuND_{6nm}$/GOx	21.3	73.8	0.9941
GR/$AuND_{13nm}$/GOx	19.9	66.2	0.9931
GR/GOx	17.4	35.6	0.9875

The linear response range of GR/GOx and GR/AuNPs/GOx electrodes for glucose can be extended at least until 10 mmol L^{-1}, limits of detection of these sensors were determined as 0.1 mmol L^{-1} and 0.08 mmol L^{-1} respectively, at a signal to noise ratio of 3. Also, newly designed GOx and AuNPs based electrodes shows relatively good reproducibility.

The effect of colloidal solutions of gold nanoparticles on performance of electrochemical glucose biosensor

The aim of this study was to investigate redox mediating properties of dissolved gold nanoparticles on the performance of electrochemical glucose biosensor where glucose oxidase is immobilized on the surface of working electrode. The principle of amperometric biosensors is based on monitoring of current associated with oxidation or reduction of an electroactive species involved in the process of biological recognition. The schematic diagram for the reactions, which occur on GR/GOx, is presented in Fig. 6.

The GOx in the presence of glucose and oxygen dissolved in water generate hydrogen peroxide and gluconolactone, which is hydrolysed to gluconic acid (Fig. 6). Electrons from redox centre of enzyme are transferred towards positively (+ 0.3 V *vs.* Ag/AgCl/KCl_{3M}) charged electrode *via* dissolved PMS and registered steady-state current is proportional to the concentration of glucose in the sample.

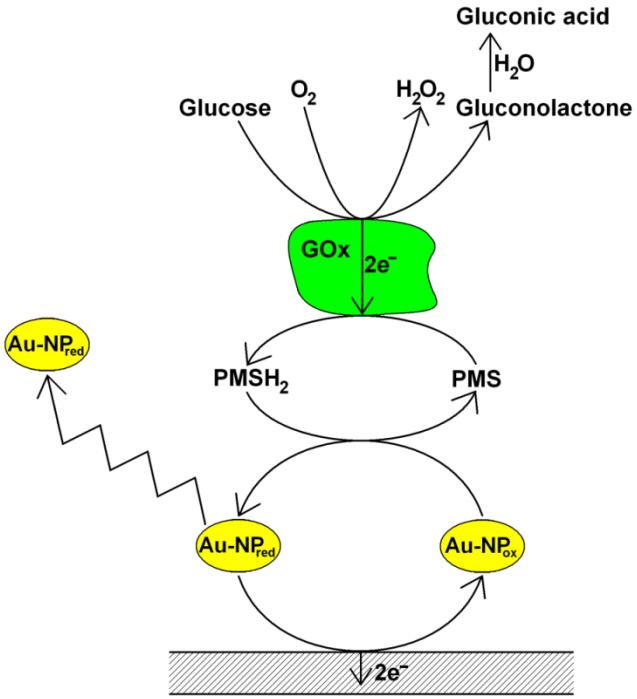

Fig. 6 Principle scheme of electron-transfer process during oxidation of glucose on GR/GOx electrode in the presence of gold nanoparticles and PMS as redox mediators.

As in previous works, the choice of the working potential of +0.3 V for presented investigations was determined by the fact, that this value is the formal redox potential of the mediator – PMS. Amperometric measurements show that catalytic activity of GOx remained after immobilization procedure and after continuous measurements, what is in line with the investigations demonstrated in other studies based on application of other redox enzymes.

Direct electron-transfer between GOx and electrode surface and the combination of the catalytic properties of nanoparticles could give the possibility for the development of ‘reagent-less’ biosensors. The electron transfer from GOx towards graphite electrode in the presence of AuNPs in the solution was tested in present study. Using GR/GOx electrodes hyperbolic dependences of amperometric signal on glucose concentration in the range of 0.1 – 100 mmol L^{-1} (Fig. 7) in sodium acetate buffer solution, pH 6.0, with PMS in presence and absence of 13.0 nm gold nanoparticles were observed.

Results presented in figure 7 and in table 3 illustrate that in the presence of PMS the analytical signal of GR/GOx electrode depends on the concentration of 13.0 nm AuNPs. Thereby using 1.5 nmol L^{-1} and 0.60 nmol L^{-1} of AuNPs in the solution of sodium acetate buffer, pH 6.0, the maximal analytical signal slightly increases (up to 11%) in the comparison with system not-containing AuNPs. No changes of analytical signal were observed in the absence of electron mediator – PMS – and values of analytical signal in this case were similar. These results could be explained by relatively low mobility of 13.0 nm gold nanoparticles and the rate, what establishes relatively low electron transfer from GOx to working electrode. Here presented results demonstrate that in all cases the PMS increases the rate of electron transfer from GOx to the electrode.

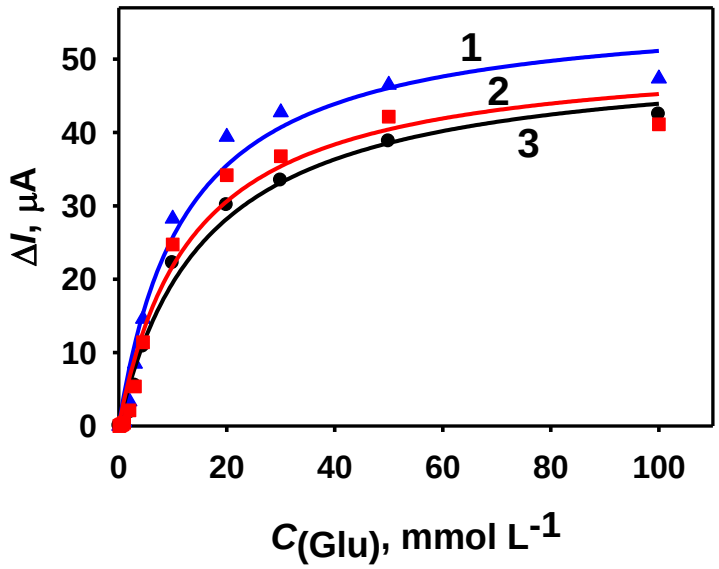

Fig. 7 Calibration plots of graphite electrodes based on glucose oxidase in the presence of 13.0 nm gold nanoparticles of different concentration. 1 - 0.60 nmol L^{-1}, 2 - 1.5 nmol L^{-1} and 3 - absence of gold nanoparticles. Changes in anodic current are presented as a function of glucose concentrations in 0.05 mol L^{-1} sodium acetate buffer, pH 6.0, containing 2 mmol L^{-1} PMS at + 0.3 V *vs.* $Ag/AgCl/KCl_{3M}$.

We compared the analytical signal of sensors with different concentrations of 13.0 nm diameter AuNPs in buffer solution in the absence and in the presence 2 mmol L^{-1} of PMS (Table 3). The maximal analytical signal increased by 6.4 times using 1.5 nmol L^{-1} concentration of 13.0 nm AuNPs and PMS. Similarly, the increase

by 7.2 times was observed using 0.60 nmol L^{-1} concentration of 13.0 nm AuNPs and PMS. The direct electron transfer from redox centre of GOx to electrode using only AuNPs (without PMS) was less efficient. Analytical signal in the system with 2 mmol L^{-1} of PMS depends on the concentration of AuNPs and at 0.60 nmol L^{-1} concentration of 13.0 nm AuNPs was higher than the signal at 1.5 nmol L^{-1} concentration of AuNPs.

The values of $K_{M(app.)}$ were determined by analysis of corresponding slope and intercept for the plot in the reciprocal-coordinates of the steady-state current versus glucose concentration. Michaelis constants of GR/GOx electrode in sodium acetate buffer solution, pH 6.0, with 2 mmol L^{-1} PMS and in the presence of 1.5 nmol L^{-1} or 0.60 nmol L^{-1} 13.0 nm AuNPs (Table 3) were 1.2 and 1.3 times lower, respectively, if compared with the same parameter for the system without gold nanoparticles. If PMS was not used in these systems, $K_{M(app.)}$ was 1.1 and 1.9 times lower, respectively, in comparison with the system without AuNPs.

Table 3 Parameters calculated from Michaelis-Menten equation. (All conditions are the same as presented in Fig. 7)

Type of electrode	with PMS		without PMS	
	$K_{M(app.)}$, mmol L^{-1}	I_{max}, μA	$K_{M(app.)}$, mmol L^{-1}	I_{max}, μA
GR/GOx without AuNPs	16.2	51.0	6.16	0.008
GR/GOx with 0.6 mmol L^{-1} AuNPs	12.3	57.4	3.20	0.008
GR/GOx with 1.5 mmol L^{-1} AuNPs	13.6	51.4	5.72	0.008

However we don't observe any positive effect on the extension of the linear detection ranges of glucose. Also during presented investigations no clear electrochemical response was observed in the absence of soluble redox mediator –

PMS. The $K_{M(app.)}$ in the presence of gold nanoparticles in the solution was calculated to be 3.20 – 5.72 mmol L^{-1} without and 12.3 – 13.6 mmol L^{-1} with mediator (Table 3). It well correlates with $K_{M(app.)}$ values reported by some other authors in the different systems without mediator. The calculated $K_{M(app.)}$ values with PMS and AuNPs (12.3 – 13.6 mmol L^{-1}) is slightly lower if compared with the previous results: 17.6 mmol L^{-1} for the glucose biosensor based on immobilized GOx on carbon rod modified with gold nanoparticles.

By next investigation step the influence of gold nanoparticles concentration on amperometric response was evaluated. The 6.0 and 13.0 nm AuNPs dissolved in sodium acetate buffer solution, pH 6.0, were used to facilitate electron transfer between immobilized GOx and electrode surface. Whereas the analytical signals without PMS were very low.

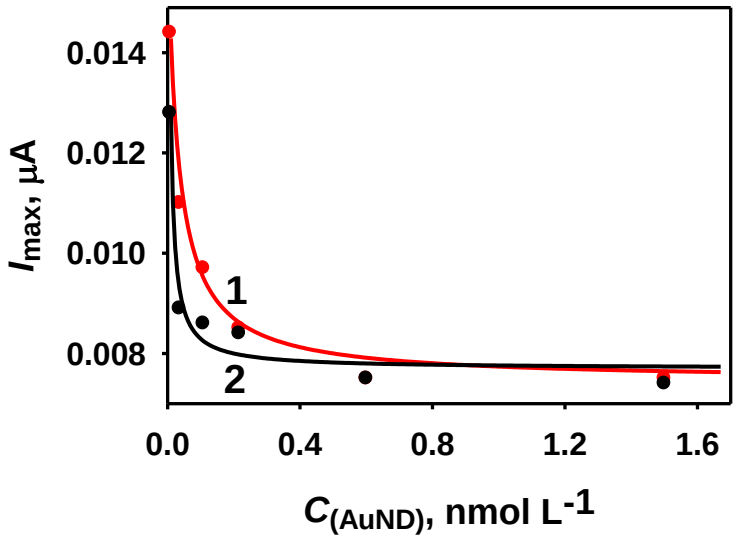

Fig. 8 The dependence of the maximal analytical signal on the concentration of 13.0 nm (1) and 6.0 nm (2) AuNPs in 0.05 mol L^{-1} sodium acetate buffer, pH 6.0, without PMS at +0.3 V vs. $Ag/AgCl/KCl_{3M}$.

Hyperbolic decrease of amperometric signal was detected by increasing the concentration of AuNPs from 0.01 nmol L^{-1} to 1.5 nmol L^{-1} in the buffer solution without PMS (Fig. 8). Electron transfer from enzyme to the AuNPs present in the solution could explain these results. The ratio of AuNPs(red.)/AuNPs(ox.) decreases by significant increase of AuNPs(ox.) concentration, because only limited amount of

AuNPs(ox.) can be utilized by immobilized GOx (in this case it is over 0.01 nmol L^{-1}). It means that by increase of concentration of AuNPs(ox.) significant part of electrons is transferred to AuNPs(red.) that remains in colloid solution and are not re-oxidized on the electrode.

As it is seen from figure 8 and table 3, for all analytical systems based on colloidal solution of 6.0 and 13.0 nm AuNPs, maximal analytical signals were similar for maximal concentration of AuNPs in the solution (1.5 nmol L^{-1}) and about 1.8 times higher for minimal concentration of AuNPs in the solution (0.01 nmol L^{-1}), if compared with the same parameter calculated for the GR/GOx electrode in the absence of AuNPs.

However, higher concentrations of AuNPs in colloidal solution made the electron transfer in the same system less efficient if compared with some lower AuNPs concentrations. As it was shown in the figure 6, AuNPs are also intending to accumulate some electrons generated by GOx and to transfer toward solution instead of being transferred to the electrode.

Table 4 Parameters of Michaelis-Menten kinetics for electrochemical biosensors using different concentration of AuNPs without PMS. (All conditions are the same as presented in figure 8)

AuNPs, nm	Parameters	Concentration of AuNPs, nmol L^{-1}						
		0	0.01	0.04	0.11	0.22	0.60	1.5
6.0	$K_{M(app.)}$, mmol L^{-1}	1.69	7.08	4.26	2.12	2.91	5.80	3.04
	I_{max}, μA	0.007	0.013	0.009	0.009	0.008	0.008	0.007
13.0	$K_{M(app.)}$, mmol L^{-1}	6.16	2.13	1.01	1.74	8.41	3.20	5.72
	I_{max}, μA	0.008	0.014	0.011	0.010	0.009	0.008	0.008

As it seen from table 4, in the systems containing 6.0 and 13.0 nm gold nanoparticles without PMS the higher sensitivity (I_{max} are of 0.013 and 0.014 μA, respectively) was found in sodium acetate buffer solution, pH 6.0, with 0.01 nmol L^{-1} concentration of AuNPs (the lowest concentration of AuNPs tested in our investigations). For future experiments to achieve the higher analytical signals it is recommended to use small concentration of AuNPs in the solution of buffer containing redox mediator.

In the system with 0.60 nmol L^{-1} of AuNPs and 2 mmol L^{-1} of PMS the linear response range of GR/GOx electrode to the concentration of glucose was 10 mmol L^{-1}, the limit of detection for this sensor was determined as 0.05 mmol L^{-1}, at a signal to noise ratio of 3. The reproducibility of analytical responses for developed electrodes close to detection limit was 27 %, because noise at lowest detectable glucose concentration is close to value of detection limit. The major advantages of newly developed biosensing system (GOx/CR electrode in the system with 0.60 nmol L^{-1} of AuNPs and 2 mmol L^{-1} of PMS) are simple and low-cost detection of biologically active analytes, wide linear range of analytical signal *vs* glucose concentration and low detection limit of analyte.

Glucose biosensor based on glucose oxidase and gold nanoparticles of different sizes covered by polypyrrole layer

Four types of amperometric biosensors based on differently modified working electrodes were designed and compared during the chemical polymerisation of pyrrole. One type of working electrode was a graphite electrode with immobilised GOx, other three types of electrodes were modified with AuNPs of different size (3.5, 6.0 or 13.0 nm) and GOx. Amperometric measurements clearly demonstrated that the biological activity of GOx was retained during the immobilisation procedure; it is in line with the results of other investigations where the same immobilisation procedure was used.

During enzymatic reaction electrons are transferred towards the positively charged electrode and steady-state currents are registered. The reactions and electron

transfer routes, which take place because of the catalytic action of GR/AuNPs/GOx electrodes, are presented in figure 9a. Here PMS serves as a redox mediator and re-oxidases the active site of GOx. In this case electron transfer *via* a reduced form of mediator ($PMSH_2$) may occur in two ways: directly to the electrode (I way) or in combination of $PMSH_2$ with AuNPs (II way) as shown in other studies. The major advantage of GR/AuNPs/GOx electrode is advanced electrochemical activity due to AuNPs compared with that of a non-modified carbon electrode. This results the increase of electrocatalytic current, therefore the sensitivity of amperometric biosensors becomes higher. According to previous results GR/AuNPs/GOx electrode gives 1.8 times higher amperometric signal when compared with GR/GOx electrode.

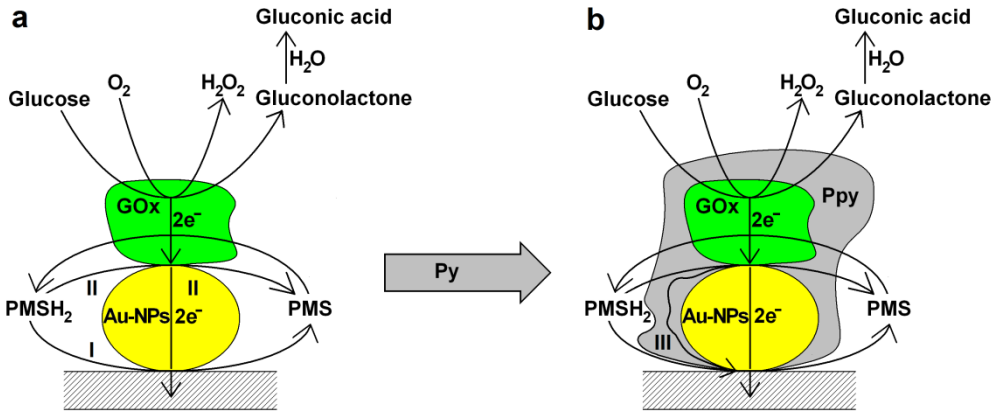

Fig. 9 Schematic representation of polypyrrole-coated gold nanoparticles and glucose oxidase in N-methylphenazonium methyl sulphate (PMS) mediated biosensor design.

During the oxidation of glucose the pH locally decreased close to the GOx active site, while hydrogen peroxide concentration locally increased. Therefore low pH value and high concentration of oxidator near the enzyme created optimal conditions for the polymerisation of polypyrrole. During the enzymatic polymerisation GOx is covered by polymer (Fig. 9b), negatively charged GOx at pH 6.0 and positively charged polymer chains electrostatic interaction takes place. Strong interaction between PPy chains enables aggregation of newly composed chains with previously formed ones. After the formation of PPy layer the influence of this layer on the sensitivity of modified electrodes was investigated. In this case III-rd additional way of electron transfer *via* π - π conjugated polymer becomes possible (Fig. 9b).

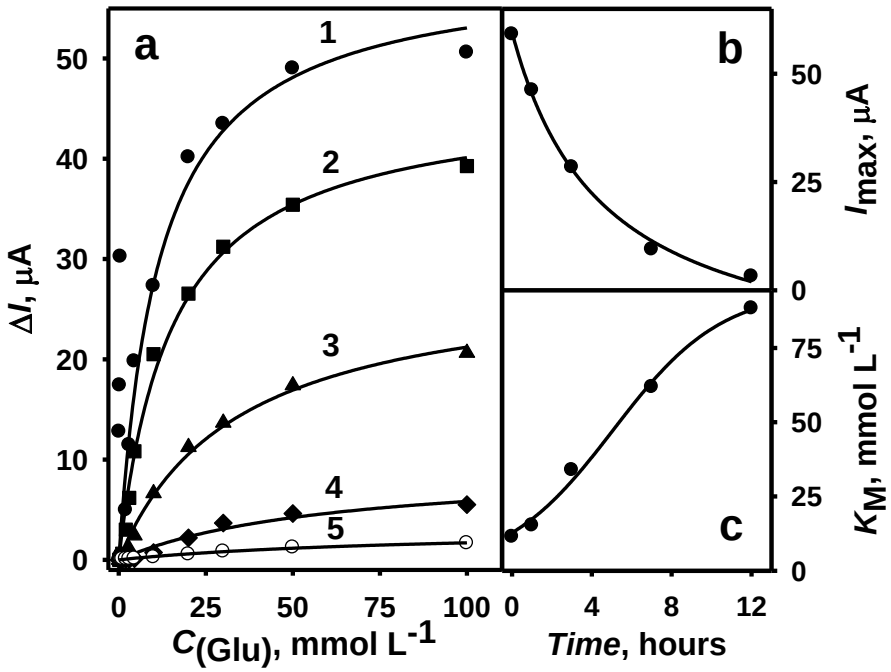

Fig. 10 Calibration plots of GR/GOx/PPy electrode registered at different stages of polypyrrole layer formation (a); changes of analytical signal (b) and the apparent Michaelis constant (c) (1 curve – amperometric signals registered before the start of polymerisation; 2 – 5 curves – after 1, 3, 7 and 12 h of polymerisation, respectively, in 0.05 mol L^{-1} acetate buffer, pH 6.0, with 0.05 mol L^{-1} glucose and 0.5 mol L^{-1} pyrrole).

The polymerisation duration had a significant influence on the sensitivity of electrodes. Hyperbolic dependences of amperometric signals on the concentration of glucose in the range from 0.1 to 100 mmol L^{-1} were observed at GR/GOx/PPy (Fig. 10) and GR/AuNPs$_{13.0nm}$/GOx/PPy electrodes (Fig. 11) at different stages of PPy layer formation. To form PPy layer electrodes were kept in polymerisation solution for 1, 3, 7 and 12 h. The analytical signal generated by the enzymatic reaction during different stages of polymer layer formation and the apparent Michaelis constant are presented in figures 10b,c and 11b,c for GR/GOx/PPy and GR/AuNPs$_{13.0nm}$/GOx/PPy electrodes respectively. During the chemical polymerisation both electrodes were covered by a layer of polypyrrole. After the 12 h polymerisation I_{max} decreased by 18.5 and 8.9 times while $K_{M(app.)}$ increased by 7.75 and 7.37 times for GR/GOx/PPy and GR/AuNPs$_{13.0nm}$/GOx/PPy electrodes, respectively.

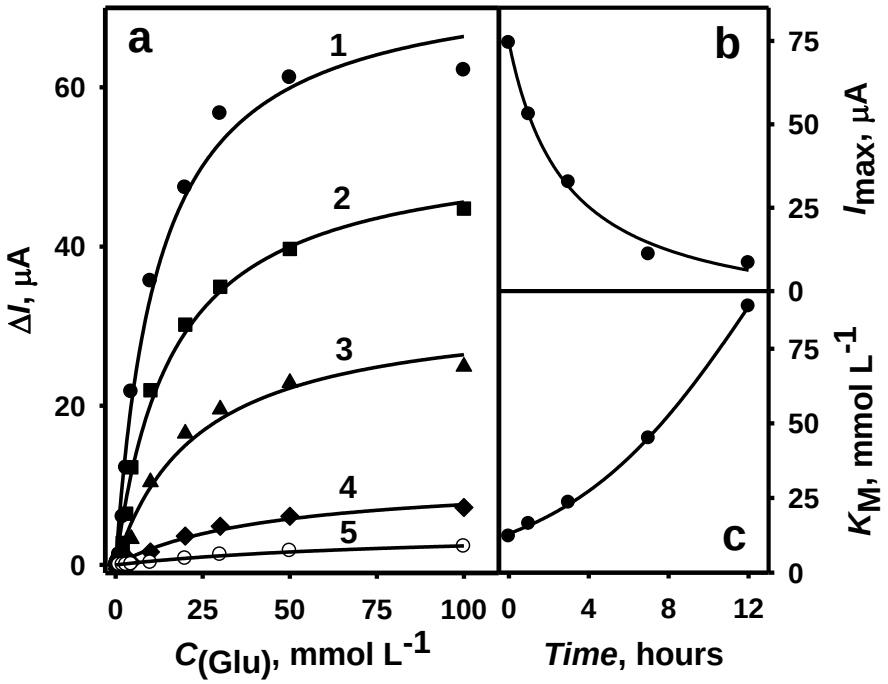

Fig. 11 Calibration plots of GR/AuNPs$_{13.0nm}$/GOx electrode registered at different stages of polypyrrole layer formation (a); changes of analytical signal (b) and the apparent Michaelis constant (c) (1 curve – amperometric signals registered before the initiation of polymerisation; 2 – 5 curves – after 1, 3, 7 and 12 h of polymerisation, respectively, in 0.05 mol L^{-1} acetate buffer, pH 6.0, with 0.05 mol L^{-1} glucose and 0.5 mol L^{-1} pyrrole).

The increment of $K_{M(app.)}$ by more than 7 times after the polymerisation might be considered as an evidence that the GOx immobilised on the carbon rod electrode surface was wrapped within the formed polypyrrole layer. However, these results illustrate that gold nanoparticles have only minor influence on the Michaelis constant after polymer layer formation.

The duration of polymerisation process influences the linear detection range of PPy modified electrodes, e.g., it was determined that GR/GOx/PPy and GR/AuNPs$_{13.0nm}$/GOx/PPy electrodes linear ranges of detection increased along with extended polymerisation duration. The linear response range of GR/GOx/PPy and GR/AuNPs$_{13.0nm}$/GOx/PPy electrodes modified by 3 h PPy formation can be extended to not less than 20 mmol L^{-1} of glucose, which is at least twice higher than that of

unmodified electrodes (10 mmol L^{-1}). The extension of linear range towards higher concentrations of glucose usually is related to decrease of sensitivity. Estimated I_{max} (Figs. 10b and 11b) and $K_{M(app.)}$ values (Figs. 10c and 11c) illustrate that the increase of $K_{M(app.)}$ up to 50 mmol L^{-1} for GR/GOx/PPy electrode was followed by decrease of sensitivity for 7.8 times, while for GR/AuNPs$_{13.0nm}$/GOx/PPy electrode the same increase of $K_{M(app.)}$ was followed by decrease of sensitivity by 6.2 times. Such significant increase of $K_{M(app.)}$ for both GR/GOx/PPy and GR/AuNPs$_{13.0nm}$/GOx/PPy electrodes is followed by increase of linear analyte detection intervals and such modified electrodes might be applied for the detection of glucose concentration in real samples where glucose concentration is in the range of 1.5 – 20 mmol L^{-1}.

The limit of detection of glucose was determined for both types of electrodes and was equal to 0.07 mmol L^{-1} at a signal to noise ratio of 3 standard deviations. Reproducibility of GR/GOx/PPy and GR/AuNPs$_{13.0nm}$/GOx/PPy electrodes at larger values of glucose concentration (20 mmol L^{-1}) was 12.5 and 9.4 % respectively.

AFM imaging of electrodes modified by gold nanoparticles, glucose oxidase and π - π conjugated polymer polypyrrole

Variations in height of 16.5 ± 6 nm appear on GR/AuNPs$_{13.0nm}$/GOx electrode surface and these features can be explained as clusters of gold nanoparticles and enzyme. It was found that gold nanoparticles penetrated into the micro-gaps of carbon rod electrode. 13.0 nm AuNPs covered the surface of carbon forming continuous layer and for this reason the electrode surface became smoother. Additional modification of the same surface with enzyme showed that the height of most nano-features slightly increased to ~16.5 nm (Fig. 12a,b). AFM investigations showed that after 12 h of chemical polymerisation the surface of GR/AuNPs$_{13.0nm}$/GOx electrode was covered by polypyrrole, which was unequally distributed on the surface of the electrode. PPy-based features of about 700 nm in height appeared after the 12 h polymerisation period (Fig. 12c,d).

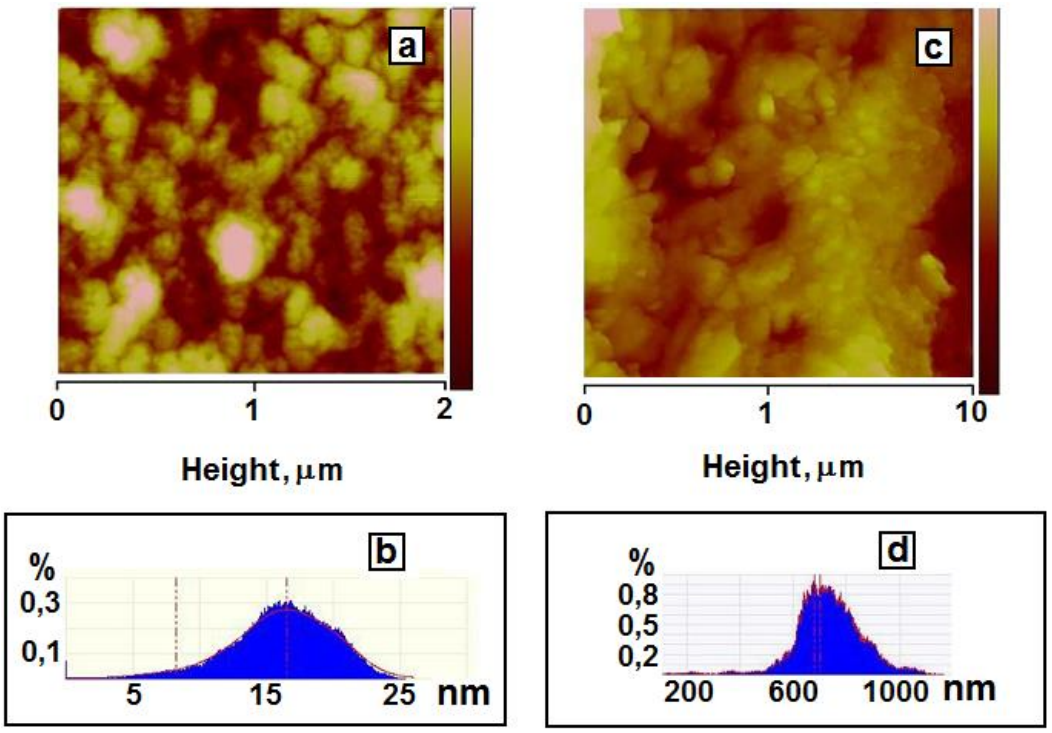

Fig. 12 AFM images (a and c) and height-distribution diagrams (b and d) of GR/AuNPs$_{13.0nm}$/GOx electrode unmodified (a and b) and modified (c and d) with PPy after 12 h polymerisation in 0.05 mol L^{-1} acetate buffer, pH 6.0, with 0.05 mol L^{-1} glucose and 0.5 mol L^{-1} pyrrole.

The effect of different size gold nanoparticles and π - π conjugated polymer polypyrrole on the performance of electrochemical glucose biosensor

It was estimated that the sensitivity of established electrochemical systems depends on the size of gold nanoparticles immobilised on the surface of the electrode: GR/AuNPs/GOx electrode with 3.5 and 6.0 nm of AuNPs showed higher electrocatalytic activity in comparison with 13.0 nm AuNPs. The sublayer based on smaller AuNPs has a larger effective surface area, and the transfer of electrons between GOx and electrode becomes more efficient compared with that observed for electrodes based on larger AuNPs or not modified by AuNPs. Also insignificant differences of $K_{M(app.)}$ were observed with decreasing size of AuNPs (from 13.0 to 3.5 nm).

Table 5 $K_{M(apparent)}$ and I_{max} calculated for GR/AuNPs/GOx electrodes based on adsorbed 3.5, 6.0, 13.0 nm gold nanoparticles and for GR/GOx electrode (glucose concentration ranged from 0.1 to 100 mmol L^{-1}; polymerisation solution – 0.05 mol L^{-1} acetate buffer, pH 6.0, with 0.05 mol L^{-1} glucose and 0.5mol L^{-1} pyrrole).

Type of electrode	Time of polymerisation, hours	I_{max}, μA	$K_{M(app.)}$, mmol L^{-1}	R^2
GR/$AuND_{3.5nm}$/GOx	0	76.2	19.7	0.9877
	13	46.0	43.7	0.9921
GR/$AuND_{6.0nm}$/GOx	0	73.8	21.3	0.9882
	13	35.7	48.1	0.9920
GR/$AuND_{13.0nm}$/GOx	0	66.2	19.9	0.9862
	13	30.9	46.9	0.9922
GR/GOx	0	35.6	17.4	0.9751
	13	23.3	30.3	0.9768

Nonetheless, it was investigated that the size of AuNPs has a significant influence on Michaelis–Menten kinetics after the same duration of PPy layer formation (Table 5). After 13 h of enzymatic polymerisation I_{max} decreased by 1.66, 2.07, 2.14 and 1.53 times for the GR/$AuNPs_{3.5nm}$/GOx/PPy, GR/$AuNPs_{6.0nm}$/GOx/PPy, GR/$AuNPs_{13.0nm}$/GOx/PPy, and GR/GOx/PPy electrodes, respectively, if compared with electrodes not covered by PPy. The decrease of I_{max} depends on the size of AuNPs: smaller AuNPs (3.5 nm) have lower effect if compared with larger AuNPs (13.0 nm). This effect might be explained by increased electrochemically effective area of electrode, which is modified with AuNPs, and surface-area-dependent facilitation of electron transfer efficiency. Electrodes modified with smaller AuNPs showed higher amperometric responses before and after the polymer layer formation if compared with electrodes modified with larger AuNPs. Nevertheless, the analytical signal of electrodes modified with AuNPs of different sizes before and after the

formation of PPy is higher if compared with electrode GR/GOx/PPy, which was designed without AuNPs. The presence of AuNPs influences the value of $K_{M(app.)}$ after the chemical polymerisation of PPy. After 13 h of enzymatic polymerisation performed in order to get GR/AuNPs$_{3.5nm}$/GOx/PPy, GR/AuNPs$_{6.0nm}$/GOx/PPy, GR/AuNPs$_{13.0nm}$/GOx/PPy and GR/GOx/PPy electrodes the $K_{M(apparent)}$ registered by these electrodes increased by 2.22, 2.26, 2.36 and 1.74 times, respectively. Results show that the most significant increase of $K_{M(app.)}$ was registered if AuNPs-modified electrode was covered by polypyrrole.

Application of electrochemically synthetized PPy layer in biosensors

Polypyrrole may be synthesized chemically and electrochemically. The aim of this research was to electrochemically synthesize polypyrrole layer on Pt electrode and apply modified electrode for determination of glucose oxidase or glucose.

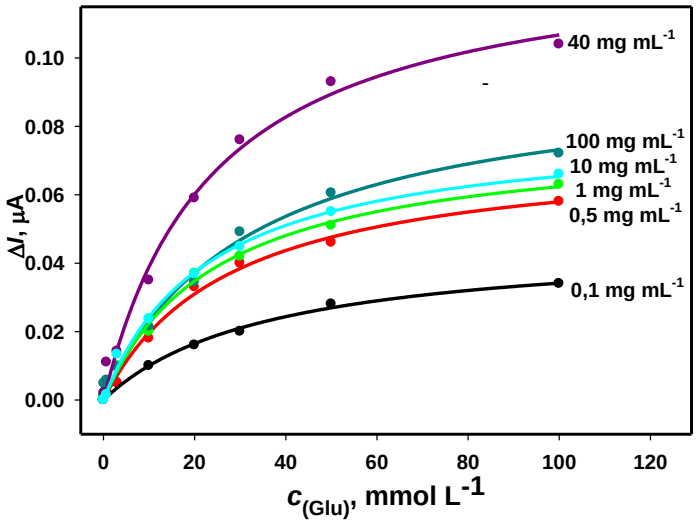

Fig 13 Calibration plots of Pt electrodes based on polypyrrole layer after the incubation in solutions of different GOx concentrations. Changes of anodic current are presented as a function of glucose concentrations in 0.05 mol L^{-1} sodium acetate buffer, pH 7.0, containing 2 mmol L^{-1} PMS at + 0.3 V *vs.* Ag/AgCl/KCl$_{3M}$.

Firstly, the dependence of analytical signal on the concentration of GOx in the used solutions was performed. The GOx solutions of 0.1, 0.5, 1, 10, 40 and 100 mg mL^{-1} concentrations and 10 min time of Pt/PPy electrode incubation at different concentrations of the enzyme in solution were chosen. The calibration plots

of Pt/PPy electrodes after the incubation in different concentrations of GOx solutions are presented in figure 13.

As it is illustrated, the increase of analytical signal was observed after Pt/PPy electrode incubation in GOx solutions from 0.1 to 40 mg mL^{-1}. When the concentration of GOx in the solution is 100 mg mL^{-1} the analytical signal is 1.4 times lower if compared with that in GOx solution of 40 mg mL^{-1}. In the next step the influence of incubation period was investigated. The results are presented in figure 14.

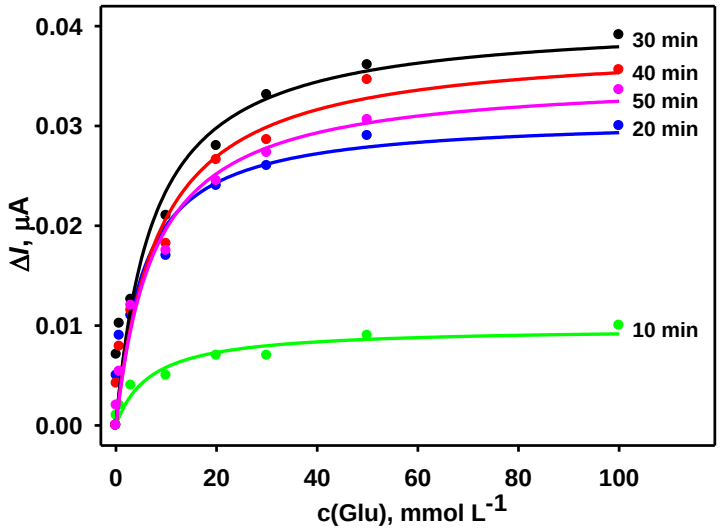

Fig 14 Calibration plots of Pt electrodes based on polypyrrole layer after the incubation in 40 mg mL^{-1} concentration of GOx for different period of time. Changes in anodic current are presented as a function of glucose concentrations in 0.05 mol L^{-1} sodium acetate buffer, pH 7.0, containing 2 mmol L^{-1} PMS at + 0.3 V *vs.* $Ag/AgCl/KCl_{3M}$.

As it is illustrated in figure 14, the increase of analytical signal was observed up to 30 min of Pt/PPy electrode incubation in GOx solution of 40 mg mL^{-1} concentration. After 40 min of incubation the analytical signal decreases, and it is more visible, when the incubation time is 50 min. Consequently, system with electrochemically synthesized polypyrrole layer on Pt electrode could be applied for the determination of glucose oxidase or glucose.

Comparison of different glucose oxidases and some electron transfer mediators in the design of amperometric glucose biosensors

The aim of this study was to perform comparative analysis and to evaluate properties of amperometric reagent-less glucose biosensors based on commercial GOx from *Aspergillus niger* and GOx from new industrially attractive enzyme sources – *Penicillium adametzii* LF F-2044.1 and *Penicillium funiculosum* 46.1, and artificial redox mediators, such as ferrocene (FC), ferrocenecarboxaldehyde (FCAld), α-methylferrocenemethanol (α-MF) and ferrocenecarboxylic acid (FCA). In order to prepare electrodes modified with the same quantity of active enzyme units (U), the activity of GOx obtained from different species of fungi was estimated spectrophotometrically.

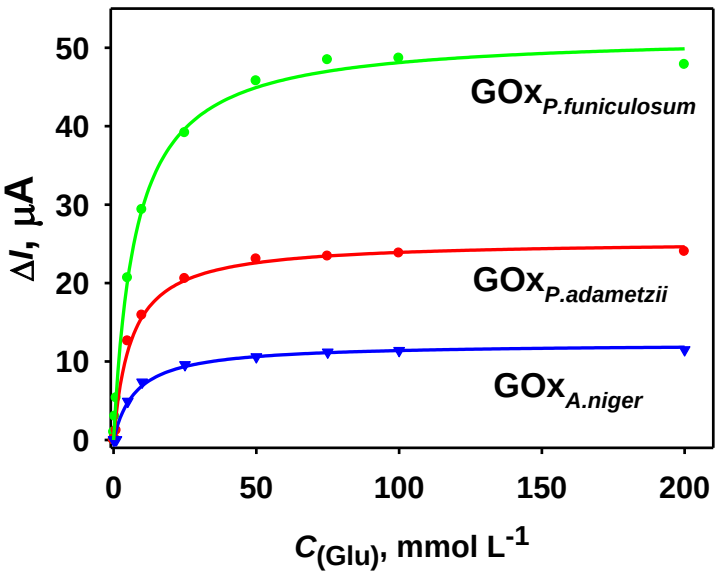

Fig. 15 Calibration plots of graphite electrodes based on different glucose oxidases in the presence of PMS. Changes in anodic current are presented as a function of glucose concentrations in acetate-phosphate buffer, pH 6.0, containing 2.0 mmol L^{-1} PMS at +0.6 V *vs.* $Ag/AgCl/KCl_{3M}$.

Firstly, the performance of electrodes modified with different GOx was evaluated using PMS (2.0 mmol L^{-1}) dissolved in the phosphate buffer pH 6.0 (Fig. 15). The highest I_{max} was observed for GR electrode modified with GOx from *Penicillium funiculosum* ($GOx_{P.funiculosum}$) and it was 51.76 μA. For glucose oxidases from *Penicillium adametzii* ($GOx_{P.adametzii}$) and *Aspergillus niger* ($GOx_{A.niger}$) I_{max} values were

25.39 and 12.29 μA, respectively. Thus, for electrodes modified with $GOx_{P.funiculosum}$ and $GOx_{P.adametzii}$ mediated with PMS, 4.2 and 2.1 times higher amperometric signals were registered when compared with commercial enzyme $GOx_{A.niger}$. $K_{M(app.)}$ for $GOx_{P.funiculosum}$ and $GOx_{A.niger}$ were very similar – 7.6 and 7.8 mmol L^{-1}, respectively; while for $GOx_{P.funiculosum}$ it was 6.2 mmol L^{-1}. This effect is related to lower diffusional limitations, because smaller amount of $GOx_{P.funiculosum}$ was added and hence lower protein content deposited on the electrode formed thinner layer.

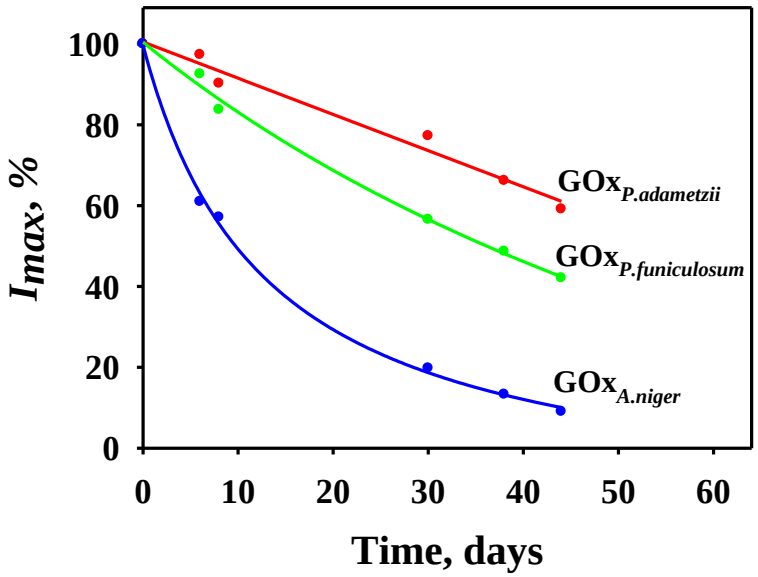

Fig. 16 Stability of electrodes modified with GOx from different species of fungi: electrochemical signal *vs* time. Experiments were performed in acetate-phosphate buffer, pH 6.0, containing 2 mmol L^{-1} PMS at +0.6 V *vs* $Ag/AgCl/KCl_{3M}$.

The stability of different glucose oxidases was evaluated using PMS in the solution. It was determined that $GOx_{P.adametzii}$ and $GOx_{P.funiculosum}$ exhibited higher stability than commercial $GOx_{A.niger}$ (Fig. 16). After 7 days the amperometric signal of electrode modified with $GOx_{A.niger}$ decreased by 40.0 %, while signal of electrodes modified with $GOx_{P.adametzii}$ and $GOx_{P.funiculosum}$ decreased by 4.1 % and 9.5 %. Significantly higher stability of GOx obtained from *P. adametzii* and *P. funiculosum* allows efficient operation of prepared electrodes for a longer period of time. After 30 days of continuous operation the I_{max} of commercial enzyme decreased by 80 %, while for enzymes purified from *P. adametzii* and *P. funiculosum* it decreased by 21 % and 42 % for $GOx_{P.adametzii}$ and $GOx_{P.funiculosum}$, respectively.

The performance of biosensor depends not only on the nature and specific properties (e.g. stability) of biocatalyst, but also on the mediators used for electron transfer between the active site of the enzyme and the electrode surface. In order to evaluate potential of different GOx (special attention was paid to assessment of $GOx_{P.adametzii}$ and $GOx_{P.funiculosum}$) in biosensor composition, various redox mediators were engaged in the design of working electrode. GR electrode was modified with the same amount of each redox mediator (FC, FCAld, α-MF or FCA) and appropriate amount of corresponding GOx to reach the equivalent amount of active enzyme on the surface of GR electrode.

Calibration plots of GR electrode modified with redox mediator and $GOx_{P.adametzii}$ show that the highest I_{max} was attained with FC – 20.27 μA, accordingly the $K_{M(app.)}$ was 17.74 mmol L^{-1}. For α-MF the I_{max} was 15.29 μA and the $K_{M(app.)}$ – 26.07 mmol L^{-1} (Fig. 17). It can be concluded that for $GOx_{P.adametzii}$ FC is more suitable as redox mediator because relatively high analytical signal and low diffusion limitations were observed. In case of FCAld and FCA the I_{max} was 7.76 and 5.44 μA**,** respectively.

Very close analytical signals 31.37 μA and 32.22 μA were observed using $GOx_{P.funiculosum}$ with α-MF and FCA. However $K_{M(app.)}$ values were different: 18.84 and 45.19 mmol L^{-1}, respectively. It may be presumed that for $GOx_{P.funiculosum}$ α-MF is the most suitable redox mediator due to relatively high analytical signal and low limitations for the α-MF to diffuse within immobilized enzyme based layer. The same tendency in the registered analytical signal was observed in the system where $GOx_{P.funiculosum}$ and mediators were analysed spectrophotometrically and spectrofuorometrically in the solution in order to evaluate activity of enzyme. Using $GOx_{P.funiculosum}$ and other redox mediators, registered analytical signals were lower, e.g.: the I_{max} for FC based electrode was 23.92 μA and for FCAld –18.64 μA.

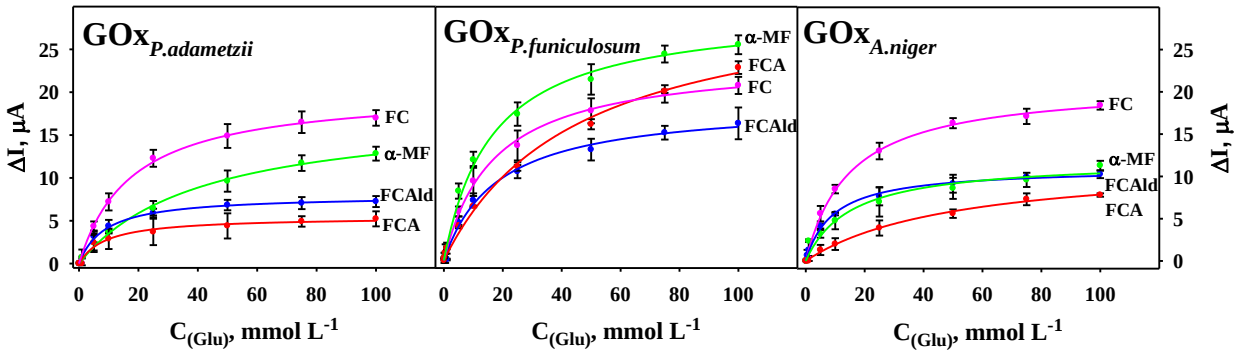

Fig. 17 Calibration plots of graphite rod electrodes based on different redox mediators and different glucose oxidases. FC – ferrocene, α-MF – α-methylferrocene methanol, FCAld – ferrocenecarboxaldehyde, FCA – ferrocenecarboxylic acid. Changes in anodic current are presented as a function of glucose concentration in, acetate-phosphate buffer, pH 6.0, at +0.6 V *vs* Ag/AgCl/KCl_{3M}.

Electrodes based on commercial $GOx_{A.niger}$ shows very similar pattern of calibration plots to electrodes based on $GOx_{P.adametzii}$ when different redox mediators are used with these electrodes (Fig. 17). The highest I_{max} was achieved with FC – 20.96 μA, accordingly the $K_{M(app.)}$ was 15.02 mmol L^{-1}. The I_{max} for α-MF, FCA and FCAld-based electrodes are in close proximity – 11.95, 11.68 and 11.00 μA, respectively. However, $K_{M(app.)}$ values of α-MF, FCA and FCAld-based electrodes were very different – 15.29, 49.11 and 9.37 mmol L^{-1}, respectively. The I_{max} for FC-based electrodes was 1.8 – 1.9 times higher in comparison with that of α-MF, FCA and FCAld based electrodes. As a result, FC was selected as the best redox mediator among the others in this research tested for the $GOx_{A.niger}$. The electrode based on this mediator demonstrated relatively high I_{max} and low $K_{M(app.)}$.

Taking into account evaluated kinetic parameters and the stability of tested enzymatic electrodes, glucose oxidase obtained from *Penicillium funiculosum* is the most suitable enzyme for the development of reagent-less glucose biosensor.

CONCLUSIONS

- ✓ Gold nanoparticles immobilized on graphite electrode with soluble electron transfer mediator provide more effective electron transfer from glucose oxidase to electrode. Biosensor based on electrode modified with 13.0 nm gold nanoparticles and glucose oxidase shows 1.42 times higher analytical signal compared with that of electrode modified only with glucose oxidase.
- ✓ Biosensors based on electrodes modified with 3.5, 6.0 and 13.0 nm gold nanoparticles show 2.14, 2.07 and 1.84 times higher analytical signal if compared with biosensor without gold nanoparticles. The linear detection range of developed biosensor is up to 10 mmol L^{-1}, and limit of detection is 0.08 mmol L^{-1}.
- ✓ Electrode modified with 13.0 nm gold nanoparticles and glucose oxidase is less stable compared with electrode without gold nanoparticles. The $\tau_{1/2}$ for GR/GOx electrode was 49.3 days and for GR/AuNPs/GOx electrode – 19.5 days.
- ✓ Gold nanoparticles present in solution together with soluble redox mediator provide more effective electron transfer from enzyme active center to electrode when concentration of gold nanoparticles is from 0.01 to 0.06 nmol L^{-1}. For biosensor based on 13.0 nm gold nanoparticles in the solution the limit of detection is 0.05 mmol L^{-1} and the linear detection range is from 0.1 to 10 mmol L^{-1} of substrate concentration. The higher concentration of AuNPs in the solution (up to 0.60 nmol L^{-1}) makes the electron transfer in the same system less efficient if compared with that effect of lower concentrations of AuNPs.
- ✓ The polypyrrole layer on the AuNPs and enzyme modified electrodes has positive effect on the linear detection range of glucose. After 3 h of polymerisation the linear detection range for graphite electrodes with GOx, AuNPs and PPy electrodes was extended up to 20 mmol L^{-1} and the limit of detection was determined to be 0.07 mmol L^{-1}. Biosensors based on small AuNPs (3.5 nm) exhibited higher amperometric responses at the same concentration of glucose before and after polypyrrole layer formation.
- ✓ Glucose oxidases obtained from *Penicillium adametzii* and *Penicillium funiculosum* and immobilized on the electrode exhibited higher stability than GOx obtained from *Aspergillus niger*. Using electrode modified with GOx from *P. funiculosum* and redox mediators – α-methylferrocenemethanol and ferrocenecarboxylic acid, the highest analytical signals were registered.

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AUKSO NANODALELIŲ IR π-π KONJUGUOTO POLIMERO POLIPIROLO TAIKYMAS GLIUKOZĖS BIOLOGINIUOSE JUTIKLIUOSE

SANTRAUKA

Šios daktaro disertacijoje apibendrintų mokslinių tyrimų tikslas – pritaikyti aukso nanodaleles, skirtingas gliukozės oksidazes bei elektrai laidų polimerą polipirolą elektrodo modifikavimui bei gliukozės amperometriniam nustatymui biologiniais jutikliais.

Aukso nanodalelės (AuND) imobilizuotos grafito elektrodo paviršiuje kartu su tirpiu elektronų pernašos tarpininku užtikrina efektyvesnę elektronų pernašą nuo gliukozės oksidazės (GOx) aktyvaus centro elektrodui fermentinės gliukozės oksidacijos metu. Naudojant AuND modifikuotus elektrodus amperometriniais biologiniais jutikliais registruojami apie 2 kartus didesni maksimalūs analiziniai signalai lyginant su elektrodu be AuND. Biologinių jutiklių tiesinės priklausomybės nuo substrato koncentracijos intervalas yra iki 10 mmol L^{-1} gliukozės ir aptikimo riba 0,08 mmol L^{-1} analitės. Po 66 dienų biologiniu jutikliu naudojančiu elektrodą modifikuotą GOx užregistruotas analizinis signalas sudarė 43 % pradinės signalo reikšmės, tuo tarpu naudojant 13,0 nm AuND ir GOx jutiklio analizinis signalas sudarė tik 22 % pradinės reikšmės.

AuND esančios tiriamajame tirpale užtikrina efektyvesnę elektronų pernašą nuo gliukozės oksidazės aktyviojo centro elektrodui, kai jų koncentracija yra nuo 0,01 iki 0,60 nmol L^{-1} ir tirpale yra N-metilfenazino metosulfato. Naudojant 13,0 nm skersmens AuND, biologinio jutiklio aptikimo riba yra 0,05 mmol L^{-1} gliukozės, o tiesiškumo intervalas nuo 0,1 iki 10 mmol L^{-1} substrato. Taip pat nustatyta, kad kuo didesnė AuND koncentracija tirpale (virš 0,60 nmol L^{-1}), tuo mažiau efektyvi yra elektronų pernaša tarp GOx aktyviojo centro ir elektrodo paviršiaus.

Polipirolo sintezė ant elektrodo įtakoja gliukozės biologinio jutiklio su ir be AuND analizinio signalo tiesinės priklausomybės nuo gliukozės koncentracijos intervalą – po 3 val. polimerizacijos visiems elektrodams tiesinės priklausomybės intervalas padidėjo iki 20 mmol L^{-1}, o analitės aptikimo riba yra 0,07 mmol L^{-1}. Naudojant mažiausias 3,5 nm AuND yra registruojamas didžiausias analizinis signalas prieš ir po polipirolo sintezės.

Nustatyta, kad labiausiai biologinių jutiklių elektrodų modifikavimui bei elektrocheminiam gliukozės nustatymui tinka fermentas išskirtas iš *Penicillium funiculosum* bei elektronų pernašos tarpininkai - ferocenkarboksilinė rūgštis ir α-metilferocenmetanolis. Naudodami elektrodą modifikuotą šiuo fermentu registruojame didžiausią analizinį signalą, bei galime modifikuotą elektrodą naudoti ilgesnį laiko tarpą.

CURRICULUM VITAE

Name, surname Jaroslav Voronovič

Bith date and place March 1, 1984; Vilnius, Lihuania

Education:

2009 – 2013 Vilnius University, Faculty of Chemistry, PhD studies.

2007 – 2009 Vilnius University, Faculty of Chemistry, Master‘s degree.

2003 – 2007 Vilnius University, Faculty of Chemistry, Bachelor‘s degree.

2003 Graduation of Vilniaus Juzefo Ingacijaus Kraševskio high school.

Work experience:

2007 – 2010 Vilnius University, Institute of Immunology, Laboratory of Imunoanalysis and Nanotechnology, chemist-engineer.

2010 – 2012 State Research Institute Centre for Innovative Medicine, Division of Immunotechnology, chemist-engineer.

2012 – State Research Institute Centre for Innovative Medicine, Division of Immunology, chemist-engineer

Languages: English (fluent), Russian (fluent), Polish (fluent).

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