Comparison of Glucose Oxidases from *Penicillium Adametzii*, *Penicillium Funiculosum* and *Aspergillus Niger* in the Design of Amperometric Glucose Biosensors

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The properties of amperometric glucose biosensors based on three different glucose oxidases and various redox mediators were evaluated. Glucose oxidases (GOx) from *Penicillium adametzii*, *Penicillium funiculosum* and *Aspergillus niger* and artificial redox mediators, such as ferrocene, ferrocenecarboxaldehyde, α -methylferrocene methanol and ferrocenecarboxylic acid, were used for modifying the graphite rod electrode and amperometrical reagent-less glucose detection. The obtained results were compared using *N*-methylphenazonium methyl sulphate in the solution. Taking into account the experimental kinetic parameters and the stability of the tested enzymatic electrodes, GOx from *Penicillium funiculosum* proved to be more suitable for glucose biosensor design in comparison with other evaluated enzymes.

Keywords Glucose oxidases from *Penicillium adametzii*, *Penicillium funiculosum* and *Aspergillus niger*, redox mediators, electrochemical biosensor

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Introduction

The electron-transfer pathway between biomacromolecules and an electrode surface is vital in the development of biosensors and some other bioelectronic devices. However, the electron-transfer efficiency for the majority of redox enzymes is low due to their active sites deeply embedded inside the protein shells. For this reason, the sensitivity of designed biosensors can be significantly improved by the application of redox mediators.¹⁻³

The properties of amperometric biosensors are mainly governed by the interaction of the redox enzyme and the artificial redox mediator replacing oxygen and facilitating the electron transfer reaction.⁴ The fast electron-transfer rate between the active site of the enzyme and the electrode surface, sufficient chemical stability of the oxidized and the reduced forms of mediator under the experimental conditions, no secondary reactions, a redox potential of the mediator away from the redox potential of interfering species, independence of the potential on the pH of the medium and their non-toxic nature are very important for the efficient action of amperometric

biosensors. Various materials, like synthetic polymers,^{5,6} natural polymers,^{7,8} carbon-based nanostructures, such as graphene, single- or multi-wall carbon nanotubes,⁹⁻¹¹ metal nanoparticles^{12,13} and composite matrixes,^{14,15} have been used in order to improve efficient electron transfer between the enzyme and the electrode surface.

Enzymatic biosensors are valuable devices for chemical and biomedical analyses, biotechnology, and contamination monitoring, as well as food and agro products processing.¹⁶ This promising and growing technology could substitute a great number of analytical methods currently employed at both laboratory and industrial levels, due to its simplicity and low cost. Therefore, emphasis has been focused on the development of fast and reliable methods for glucose detection due to their relevance in such fields as clinical diagnostics, food industry, and biotechnology. Generally, electrochemical techniques have been preferred as analytical signal determination methods owing to their advantages, such as low detection limit, miniaturized size, rapid response, reproducible results, high sensitivity and excellent selectivity, multifunctionality and low sample volume.^{11,12} Moreover, electrochemical methods may be applied for investigating non-transparent samples and they are less sensitive to non-specific adsorption at interfaces, which is very important for the evaluation of real samples.¹⁷ Enzyme-based

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Fig. 1 Generalized scheme of electrochemical biosensor based on glucose oxidase and redox mediators (ferrocene, α -methylferrocene methanol, ferrocenecarboxaldehyde or ferrocenecarboxylic acid). M(ox.). Oxidized form of redox mediator, M(red.). Reduced form of redox mediator.

amperometric biosensors are an attractive choice among these techniques.

Glucose oxidase (GOx, EC 1.1.3.4) has an extensive, over 50-year-long, application record in amperometric glucose biosensor design.^{18,19} However, enzyme catalytic action was most thoroughly investigated only for GOx from *Aspergillus niger*.^{1,20} Glucose oxidases derived from various strains of *A. niger* are also used in the fabrication of the majority of commercial glucose sensors. Given the expanding market demand, the studies in many research centres around the globe have been focused on the search and further selection of novel hyperactive strains-enzyme producers. For instance, screening among fungi of various genera for GOx-generating activity performed by the team from Institute of Microbiology, National Academy of Sciences, Belarus enabled to sort out most promising cultures of *Penicillium adametzii* and *Penicillium funiculosum*. The heterogeneity of the above-mentioned fungi was revealed, and the most stable variants were isolated (*P. adametzii* LF F-2044.1 and *P. funiculosum* BIM F-15.3).²¹ Three-stage induced mutagenesis and adaptation selection to riboflavin resulted in strain *P. funiculosum* 46.1, distinguished by a 3-times higher level of GOx synthesis as compared to initial strain *P. funiculosum* BIM F-15.3. The GOx preparations from *Penicillium* fungi were derived and characterized.^{22,23}

Usually, GOx from *A. niger* and different redox mediators are used for amperometric glucose detection; however, glucose oxidases obtained from other strains of fungi might also improve the performance of biosensors. Thus, the aim of our study was to perform comparative analysis and to evaluate the properties of amperometric glucose biosensors based on commercial GOx from *A. niger* and GOx from new industrially attractive enzyme sources – *P. adametzii* LF F-2044.1 and *P. funiculosum* 46.1 using some ferrocene-based redox mediators (Fig. 1). The presented results can provide valuable information about the interactions between different glucose oxidases and redox mediators, which is important for applications of developed systems for glucose detection.

Experimental

Reagents and chemicals

GOx from *P. adametzii* (GOx_{*P.adametzii*}), from *P. funiculosum* (GOx_{*P.funiculosum*}) and ferrocene (FC) were provided by the Institute of Microbiology (Minsk, Belarus). GOx from *A. niger* (E.C.1.1.3.4.) (GOx_{*A.niger*}), 1,4-benzoquinone and 25% glutaraldehyde were purchased from AppliChem GmbH (Darmstadt, Germany). D-(+)-glucose was obtained from Carl Roth GmbH&Co (Karlsruhe, Germany). *N*-methylphenazonium methyl sulphate (PMS), α -methylferrocene methanol (α -MF), ferrocenecarboxaldehyde (FCAlD) and ferrocenecarboxylic acid (FCA) were dispatched by Sigma-Aldrich (St. Louis, MO) and Fluka (Buchs, Switzerland). Graphite rod (GR) electrodes (3 mm diameter, 99.999%, low density) were supplied by Sigma-Aldrich (St. Louis, MO). Solutions of FC, FCAlD, FCA and α -MF were prepared in acetonitrile. A glucose solution in distilled water was allowed to mutarotate for at least 24 h in order to reach equilibrium of α - and β -forms. All other chemicals used in the present study were of analytical grade or of highest quality. All solutions were based on deionised water purified with Millipore S.A system (Molsheim, France).

Production of GOx preparations

The recoveries of GOx_{*P.adametzii*} and GOx_{*P.funiculosum*} were performed by the ultrafiltration-based concentration of cultural liquid resulting from submerged fermentation of penicillin. Ultrafiltration was conducted using laboratory device based on hollow fiber membrane element MPVE PS-10M-0.2 (MIFIL, Minsk, Belarus) and UFYA-47 cell (Biotest Co., Kirishi, Russia). A hollow fiber-membrane element contained membrane composed of aromatic polysulfone with a protected surface PS-10M (nominal molecular weight retention limit 10 kDa). For the cell ultrafiltration membrane UPM-10 forming a porous polymeric film based on aromatic polysulforamide Sulfon-IT or polypropylene support (retention limit 10 kDa) (Vladipore, Vladimir, Russia) was applied. A solution of GOx_{*A.niger*} was freshly prepared from lyophilized enzyme powder in the 0.05 mol L⁻¹ sodium acetate buffer, pH 6.0.

GOx activity assay

Spectrometric determination of GOx activity is based on the enzymatic reduction of 1,4-benzoquinone to 1,4-hydroquinone and measuring the rate of increase of 1,4-hydroquinone absorbance at 290 nm.²⁴ A unit of enzyme activity is defined as the amount of enzyme sufficient to catalyse the transformation of 1.0 mmol L⁻¹ 1,4-benzoquinone into 1,4-hydroquinone during 1 min at 25°C.

Electrode pre-conditioning and modification by redox mediators and GOx

In order to avoid any electrode contamination by the oxidation products and to obtain a clean electrode surface, the surface of the GR electrode was hand-polished with fine emery paper, washed with ethanol and distilled water, and dried at room temperature before modification procedure. During GR electrode modification process 3.0 μ L of redox mediator solution in acetonitrile (0.044 mol L⁻¹) was dropped on the electrode surface and evaporated; this step was repeated three times. After the 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 enzymatic activity (U). During modification of the electrode surface, each following drop of a 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°C in a closed vessel. Prior to all electrochemical measurements, working electrodes were thoroughly washed with deionised water in order to remove any non-cross-linked enzyme. Then electrodes were sealed into a silicone tube to prevent contact of the electrode side surface with the solution.

Electrochemical measurements

All electrochemical measurements were performed in a three-electrode electrochemical cell system using computerized potentiostat PGSTAT 302N/Autolab (EcoChemie, Netherlands) with GPES 4.9 software. A conventional three-electrode system comprising a prepared working GR electrode (modified as was described above), 2 cm² platinum as an auxiliary electrode and Ag/AgCl/3 mol L⁻¹ KCl Metrohm (Herisau, Switzerland) as a reference electrode were employed for all electrochemical experiments. All electrochemical measurements were performed at room temperature in stirred 0.05 mol L⁻¹ sodium acetate and sodium phosphate buffer with 0.1 mol L⁻¹ KCl (A-PBS), pH 6.0. The dependence of the amperometric signal on the substrate concentration was studied at +600 mV vs. Ag/AgCl/3 mol L⁻¹ KCl. In order to compare the results obtained using a reagentless biosensor, control experiments with a GR electrode modified only with GOx and in the presence of 2 mmol L⁻¹ governed PMS in the solution were performed.

Stability test of GOx from different fungal species

GR electrodes modified with various GOx at the same level of active enzyme (measured in U) were prepared as previously described. Prepared electrodes were stored in a closed vessel over buffer solution at +4°C prior to use in experiments. The electrochemical measurements were performed at room temperature in stirred A-PBS, pH 6.0, in the presence of 2 mmol L⁻¹ PMS.

Calculations

The amperometric signal of all tested electrodes showed a hyperbolic dependence on the substrate (glucose) concentration, and it is in agreement with Michaelis-Menten kinetics. The kinetic parameters: maximal current under saturated substrate conditions ($I_{\max}$) (corresponds to the maximal rate of enzymatic reaction $V_{\max}$, according to Michaelis-Menten kinetics) and apparent Michaelis constant (K_M^{app}) are the corresponding a and b parameters of hyperbolic function $y = ax/(b + x)$, which was used for an approximation of results. Parameter b of hyperbolic function corresponds to the substrate concentration at half of $I_{\max}$. The nonlinear regression method is very simple, quick and convenient for our results approximation.

Results and Discussion

The glucose biosensors have been widely used in the diagnosis of diabetes mellitus, in the food quality control and research.¹⁹ Recently, many efforts have been focused on the development of reliable glucose biosensors based on electrochemical,²⁵ chemiluminescent²⁶ or other methods.²⁷ So far the GOx is one of the most popular objects in designing and modelling of enzymatic biosensors due to its commercial availability, high purity, good stability, high specificity for commercially important analytes, rapid turnover rate and relatively low cost.¹⁸ The main strategies for amperometric glucose biosensor design according to the position of the enzyme can be divided into two

Table 1 Characteristics of the tested glucose oxidases

Enzyme	GOx _{P.adametzii}	GOx _{P.funiculosum}	GOx _{A.niger}
Initial activity, U/mL	1017.9	1660.1	1055.5
Specific activity, U/mg of protein	75.8	65.6	164.6
The amount of enzyme per sensor			
U	9.5	9.5	9.5
μL	9.3	5.7	9.0
mg of protein	0.12	0.14	0.06
mol of enzyme	$3.75 \cdot 10^{-7}$	$4.61 \cdot 10^{-7}$	$4.75 \cdot 10^{-7}$

main groups: (i) a soluble enzyme is in the solution; (ii) enzyme is immobilized on the electrode surface. In our case several different GOx and redox mediators were immobilized on the electrode, except PMS used for control experiments. In this study, the efficiency of GOx from *Penicillium* and *Aspergillus* species in the design of amperometric glucose biosensors based on various redox mediators was investigated and compared.

The properties of the examined glucose oxidases are described in the Table 1. It is evident that commercial product GOx_{A.niger} is distinguished by superior specific activity (164.6 U/mg protein) as compared to enzyme preparations derived from fungi of genus *Penicillium* (65.6 and 75.7 U/mg protein). 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. The initial activity of GOx_{P.adametzii} preparation was 1017.9 U mL⁻¹, and GOx_{P.funiculosum} – 1660.1 U mL⁻¹ and commercially available GOx_{A.niger} – 1055.5 U mL⁻¹. Thus, after evaluations we found that to obtain 9.5 U of GOx immobilized on the GR electrode, 9.3 μL of GOx_{P.adametzii}, 5.7 μL of GOx_{P.funiculosum} and 9.0 μL of GOx_{A.niger} have to be deposited on each electrode. It corresponded to molar GOx concentrations of $3.75 \cdot 10^{-7}$, $4.61 \cdot 10^{-7}$ and $4.75 \cdot 10^{-7}$ mol L⁻¹, respectively.

Another very important parameter for the biocatalytic activity of enzyme and for the analytical signal of GOx-modified electrode is the optimal pH value for each enzyme. Investigations were conducted in the A-PBS (pH 5.0 – 9.0). Enzyme catalytic activity was assessed by the estimation of maximal rate of 1,4-benzoquinone reduction to 1,4-hydroquinone, Michaelis constant (K_M^{app}), which is an important indicator of substrate affinity, and the efficiency of glucose oxidation reaction ($k_{\text{cat}}/K_M^{\text{app}}$). It was found that for GOx_{P.funiculosum} and GOx_{A.niger} the optimal K_M^{app} values were observed at pH 6.0 – 8.0, for GOx_{P.adametzii} – at pH 6.0 – 7.0. The higher affinity of enzymes to glucose was observed using GOx from *Penicillium* fungi (3.4 – 7.7 times higher). The most efficient substrate binding determined from the $k_{\text{cat}}/K_M^{\text{app}}$ values were observed at pH 7.0 for GOx_{P.adametzii}, pH 5.0 – 7.0 – for GOx_{P.funiculosum} and pH 6.0 – 8.0 – GOx_{A.niger}. The optimal pH for GOx_{P.funiculosum} and GOx_{P.adametzii} was considerably lower than for GOx_{A.niger}. It is necessary to note that the efficient substrate binding of glucose oxidases from *Penicillium* fungi was equal at pH 6.0. At this pH value GOx_{A.niger} showed good results. Thus, the further electrochemical studies were performed in the A-PBS, pH 6.0.

Firstly, the performance of electrodes modified with different GOx was evaluated using PMS (2.0 mmol L⁻¹) dissolved in the A-PBS, pH 6.0 (Fig. 2). GOx is a homodimer composed of two identical subunits. It contains two molecules of flavin adenine dinucleotide (FAD). FAD is cofactor, which acts as an initial electron acceptor and it is required for oxidation of glucose.

Fig. 2 Calibration plots of graphite rod electrodes based on different glucose oxidases in the presence of PMS. 1. Glucose oxidase from *Penicillium funiculosum*, 2. Glucose oxidase from *Penicillium adametzii*, 3. Glucose oxidase from *Aspergillus niger*. Changes in the anodic current are presented as a function of the glucose concentrations in A-PBS pH 6.0 containing 2.0 mmol L^{-1} PMS at $+0.6 \text{ V}$ vs. Ag/AgCl/ 3 mol L^{-1} KCl.

Oxidized form of PMS is reoxidizing the active site of GOx and then reduced form of PMS is transferring electrons to the GR electrode. The hyperbolic dependence of amperometric signal on glucose concentration in the range of $0.1 - 200 \text{ mmol L}^{-1}$ was observed for electrodes modified by all tested glucose oxidases (Fig. 2).

The highest I_{max} recorded for GR electrode modified with $\text{GOx}_{P.funiculosum}$ was $51.76 \mu\text{A}$. For glucose oxidases from $\text{GOx}_{P.adametzii}$ and $\text{GOx}_{A.niger}$ I_{max} values were 25.39 and $12.29 \mu\text{A}$, respectively. Thus, for electrodes modified with $\text{GOx}_{P.funiculosum}$ and $\text{GOx}_{P.adametzii}$ mediated with PMS, 4.2 and 2.1 times higher amperometric signals were registered when compared with commercial $\text{GOx}_{A.niger}$. K_M^{app} for $\text{GOx}_{P.funiculosum}$ and $\text{GOx}_{A.niger}$ were very similar – 7.6 and 7.8 mmol L^{-1} , while for $\text{GOx}_{P.adametzii}$ it was 6.2 mmol L^{-1} . This effect is related to lower diffusional limitations, because slightly smaller amount of $\text{GOx}_{P.funiculosum}$ was added and hence lower protein content deposited on the electrode formed thinner layer.

The stability of different glucose oxidases was evaluated using PMS in the solution. It is obvious that glucose oxidases derived from different species have different stability (Fig. 3). It was determined that $\text{GOx}_{P.adametzii}$ and $\text{GOx}_{P.funiculosum}$ exhibited higher stability than commercial $\text{GOx}_{A.niger}$. After 7 days the amperometric signal of electrode modified with $\text{GOx}_{A.niger}$ decreased by 40.0%, while signal of electrodes modified with $\text{GOx}_{P.adametzii}$ and $\text{GOx}_{P.funiculosum}$ decreased by 4.1 and 9.5%. Significantly higher stability of $\text{GOx}_{P.adametzii}$ and $\text{GOx}_{P.funiculosum}$ allows efficient operation of prepared electrodes for a longer period. After 30 days of continuous operation the I_{max} of commercial enzyme decreased by 80%, while for $\text{GOx}_{P.adametzii}$ and $\text{GOx}_{P.funiculosum}$ it decreased by 21 and 42%.

The performance of a biosensor depends not only on the nature and specific properties (e.g. stability) of biocatalyst, but also on the mediators that act as electron carriers between the active site of the enzyme and the electrode surface. The use of soluble redox mediator complicates perspectives of biosensor application to provide wellness of diabetic patients. In order to simplify the performance of biosensors and improve the practical

Fig. 3 Stability of electrodes modified with GOx from different species of fungi: electrochemical signal vs. time. 1. Glucose oxidase from *Penicillium funiculosum*, 2. Glucose oxidase from *Penicillium adametzii*, 3. Glucose oxidase from *Aspergillus niger*. Experiments were performed in A-PBS pH 6.0 containing 2 mmol L^{-1} PMS at $+0.6 \text{ V}$ vs. Ag/AgCl/ 3 mol L^{-1} KCl.

potential of created systems, water insoluble redox mediators were immobilized on the surface of graphite electrode. The different glucose oxidases were engaged in the design of working electrode (Fig. 1). GR electrode was modified with the same amount of each redox mediator (FC, FCAld, α -MF or FCA) and appropriate amount of corresponding GOx (special attention was paid to assessment of $\text{GOx}_{P.adametzii}$ and $\text{GOx}_{P.funiculosum}$ to reach the equivalent amount of active enzyme on the surface of GR electrode (Table 1)). A fast biosensor preparation procedure exploiting the volatility of glutaraldehyde was used in our study. The effects of selected mediators and corresponding GOx on the kinetic parameters of immobilized enzyme and analytical signal of biosensors were evaluated and compared.

Calibration plots of GR electrode modified with redox mediator and $\text{GOx}_{P.adametzii}$ show that the highest I_{max} was attained with FC – $27.19 \pm 0.90 \mu\text{A}$, accordingly the K_M^{app} was $19.67 \pm 2.08 \text{ mmol L}^{-1}$ (Table 2). For α -MF the I_{max} was $15.29 \pm 0.48 \mu\text{A}$ and the K_M^{app} – $26.07 \pm 2.36 \text{ mmol L}^{-1}$. In case of FCAld and FCA the I_{max} was very low – 7.96 ± 0.51 and $5.44 \pm 0.16 \mu\text{A}$, respectively (Fig. 4(2)). The affinity of enzyme to each mediator can be estimated using the $I_{\text{max}}/K_M^{\text{app}}$ ratio.²⁸ In this context, FC also appears to be the best among all studied mediators with the highest $I_{\text{max}}/K_M^{\text{app}}$ ratio equal to $1.38 \mu\text{A}/\text{mmol L}^{-1}$. The R for hyperbolic correlations was in the range $0.9905 - 0.9986$ indicating that bioelectrochemical catalysis process could be well described by Michaelis-Menten kinetics. It can be concluded that for $\text{GOx}_{P.adametzii}$ FC is the most suitable redox mediator because relatively high analytical signal and the affinity to enzyme were observed.

Another set of calibration plots was obtained using GR electrodes based on the same redox mediators and $\text{GOx}_{P.funiculosum}$ (Fig. 4(1)). In this case, well-defined hyperbolic relationships of amperometric signals vs. increasing glucose concentrations were registered (R was in the range of $0.9938 - 0.9990$). Very close maximal currents $31.37 \pm 1.48 \mu\text{A}$ and $32.22 \pm 1.72 \mu\text{A}$ were observed using α -MF and FCA (Table 2). However, the difference between K_M^{app} values was very high – 18.84 ± 2.68 and $45.19 \pm 1.83 \text{ mmol L}^{-1}$, respectively. Thus, the highest

Table 2 Estimated kinetic parameters for different glucose oxidases and various redox mediators

Electrode	$K_M^{\text{app}}/\text{mmol/L}$	$I_{\text{max}}/\mu\text{A}$	$I_{\text{max}}/K_M^{\text{app}}, \mu\text{A}/\text{mmol L}^{-1}$	R
GR/FCA/GOx _{P.funiculosum}	45.19 ± 1.83	32.22 ± 1.72	0.71	0.9976
GR/FCAld/GOx _{P.funiculosum}	18.10 ± 1.69	18.64 ± 1.30	1.03	0.9973
GR/ α -MF/GOx _{P.funiculosum}	18.84 ± 2.68	31.37 ± 1.48	1.67	0.9938
GR/FC/GOx _{P.funiculosum}	17.91 ± 1.27	29.01 ± 0.62	1.62	0.9990
GR/FCA/GOx _{P.adametzii}	9.07 ± 0.57	5.44 ± 0.16	0.60	0.9905
GR/FCAld/GOx _{P.adametzii}	8.79 ± 1.01	7.96 ± 0.51	0.91	0.9948
GR/ α -MF/GOx _{P.adametzii}	26.07 ± 2.36	15.29 ± 0.48	0.59	0.9986
GR/FC/GOx _{P.adametzii}	19.67 ± 2.08	27.19 ± 0.90	1.38	0.9980
GR/FCA/GOx _{A.niger}	49.11 ± 0.63	11.68 ± 0.31	0.24	0.9985
GR/FCAld/GOx _{A.niger}	9.37 ± 2.22	11.00 ± 0.88	1.17	0.9988
GR/ α -MF/GOx _{A.niger}	15.29 ± 0.15	11.95 ± 0.45	0.78	0.9865
GR/FC/GOx _{A.niger}	18.79 ± 1.60	28.41 ± 0.74	1.51	0.9989

GR. Graphite rod electrode, FCA. Ferrocenecarboxylic acid, FCAld. Ferrocenecarboxaldehyde, α -MF. α -methylferrocene methanol, FC. Ferrocene.

Fig. 4 Calibration plots of graphite rod electrodes based on different glucose oxidases and various redox mediators. 1. Glucose oxidase from *Penicillium funiculosum*, 2. Glucose oxidase from *Penicillium adametzii*, 3. Glucose oxidase from *Aspergillus niger*, A. Ferrocene, B. α -methylferrocene methanol, C. Ferrocenecarboxaldehyde, D. Ferrocenecarboxylic acid. Changes in anodic current are presented as a function of glucose concentration in A-PBS pH 6.0 containing 2 mmol L⁻¹ PMS at +0.6 V vs. Ag/AgCl/3 mol L⁻¹ KCl.

$I_{\text{max}}/K_M^{\text{app}}$ ratio equal to 1.67 $\mu\text{A}/\text{mmol L}^{-1}$ was observed for α -MF. Another redox mediator FC also showed good results – $I_{\text{max}}/K_M^{\text{app}}$ ratio was 1.62 $\mu\text{A}/\text{mmol L}^{-1}$. In this case the maximal current was $29.01 \pm 0.62 \mu\text{A}$ and K_M^{app} was $17.91 \pm 1.27 \text{ mmol L}^{-1}$. The registered analytical signals were lower using other redox mediators. It may be presumed that for GOx_{P.funiculosum} α -MF is the most suitable redox mediator, while FC also shows good affinity to GOx_{P.funiculosum} and high analytical signal. The spectrophotometric and spectrofluorometric studies of GOx_{P.funiculosum} activity in the solution using α -MF and FC showed the same tendency in the registered analytical signal.²⁹

Electrodes based on commercial GOx_{A.niger} showed very similar patterns of the calibration plots to electrodes based on GOx_{P.adametzii} when different redox mediators were used (Fig. 4(3)). The highest I_{max} was achieved with FC – $28.41 \pm 0.74 \mu\text{A}$, accordingly the K_M^{app} was $18.79 \pm 1.60 \text{ mmol L}^{-1}$. The I_{max} for α -MF, FCA and FCAld-based electrodes are in close proximity – 11.95 ± 0.45 , 11.68 ± 0.31 and $11.00 \pm 0.88 \mu\text{A}$, respectively (Table 2). However, K_M^{app} values of α -MF, FCA and FCAld-based electrodes were very different – 15.29 ± 0.15 , 49.11 ± 0.63 and $9.37 \pm 2.22 \text{ mmol L}^{-1}$, respectively. I_{max} for the FC-based electrodes was 2.4 – 2.6-times higher in comparison with that of α -MF, FCA and FCAld based electrodes. The highest $I_{\text{max}}/K_M^{\text{app}}$ ratio, which was equal to 1.51 $\mu\text{A}/\text{mmol L}^{-1}$, was observed for FC. Calculated R for hyperbolic correlations was in the range of 0.9965 – 0.9989. As

a result, FC was selected as the best redox mediator among the others in GOx_{A.niger} tests. The electrode based on this mediator demonstrated relatively high I_{max} and low K_M^{app} .

Registered kinetic parameters of GOx-based electrodes depend not only on the fungal species as enzyme sources, but also on the applied redox mediators. Figure 4 and Table 2 illustrate that the highest I_{max} was attained using GOx_{P.funiculosum} with all tested redox mediators. The differences in K_M^{app} among the three enzymes and the same mediators could be explained by differences in the rates of the mediator reaction. It should be noted that both GOx_{P.adametzii} and GOx_{A.niger} act in a similar way with all studied redox mediators, but GOx_{P.funiculosum} behaves in a different manner.

Typical hyperbolic dependence of current vs. glucose concentration, which is well described by Michaelis-Menten kinetics, is observed in all examined variants (Figs. 2 and 4). The K_M^{app} values for *Aspergillus* GOx mainly varies from 16.95 to 33.0 mmol L^{-1} ,^{30–32} whereas for *Penicillium* GOx, it is in the range of 3.3 – 19.0 mmol L^{-1} .^{29,33,34} For biosensors based on immobilized and differently mediated GOx_{A.niger} various K_M^{app} were reported: 22 mmol L^{-1} (GOx was immobilized in the matrix of sol-gel organic-inorganic hybrid material),³⁵ 10.8 mmol L^{-1} (GOx was immobilized in a Pt nanoparticles/mesoporous carbon matrix and cross-linked with GA),³⁶ 14.9 mmol L^{-1} (enzyme was immobilized via layer-by-layer self-assembly of carbon nanotubes, gold nanoparticles and

GOx),³⁷ 15.19 mmol L⁻¹ (GOx was immobilized on boron-doped carbon nanotubes by entrapping into poly(*o*-aminophenol) film),³⁸ 8.5 mmol L⁻¹ (GOx was encapsulated in the Nafion-single-walled carbon nanohorns composite film and FCA was used as redox mediator).³⁹ It should be noted that various immobilization techniques and different redox mediators were applied in experiments, and these factors to a great extent influenced the variations of K_M^{app} . In our study calculated K_M^{app} values indicate that in the most cases glucose oxidases immobilized on GR electrodes modified with different redox mediators show sufficient electron transfer efficiency, the substrate easily diffuse, and therefore these electrodes have electrocatalytic activity, which is comparable with other GOx-based electrodes that are mentioned in this paragraph.³⁵⁻³⁹

Among all of the evaluated redox mediators, including those immobilized on GR electrode and dissolved in the buffer, the highest analytical signal for GOx_{*P. adametzii*} was registered with FC immobilized on GR-based electrodes and with PMS dissolved in A-PBS, the I_{max} was equal to 27.19 and 25.39 μ A, respectively. For the GOx_{*P. funiculosus*} very close maximal currents 31.37, 32.22 and 29.01 μ A were observed using α -MF, FCA and FC, while the highest I_{max}/K_M^{app} ratio was using α -MF and FC. I_{max} of 51.76 μ A was achieved with PMS in the solution, and it was 1.65 times higher if compared with that in the presence of α -MF. It should be noted that PMS was used in soluble form therefore its application in practical biosensor design is complicated. For commercial GOx_{*A. niger*} the highest I_{max} of 28.41 μ A was registered using FC, which was immobilized on the GR electrode. Ferrocene as a redox mediator, which is promoting fast electron-transfer reactions, interacts with a wide range of oxidoreductases, including the GOx.⁴⁰ However, reaction rates of glucose oxidases with ferrocene or ferrocene derivatives are different, therefore registered analytical signals of different redox mediators based sensors are significantly influenced by selected ferrocene derivative. The similar results were obtained using GOx_{*A. niger*} (Type II) immobilized on graphite electrode and different electron transfer mediators in the solution (*p*-benzoquinone, toluquinone, duroquinone, thimoquinone, 2,6-dichlorophenolindophenol, 2,6-dibromoinosalicilic acid and others). The highest I_{max} values were registered using *p*-benzoquinone and toluquinone.¹ During the enzymatic oxidation of glucose by the GOx immobilized on the electrode generated reduced form of electron-transfer mediator is oxidised at the electrode surface and in this way the anodic current is generated. The low I_{max} values indicate that the rate of oxidised mediator reduction reaction by the reduced enzyme is low, and this value varied with the mediator. Taking into account calculated kinetic parameters, the stability of tested electrodes and application, GOx_{*P. funiculosus*} was recognized as the most appropriate enzyme for the construction of reagent-less biosensors suitable for glucose determination.

Conclusions

This work was a comparative study, which demonstrated the performance of glucose oxidases from different fungi with several immobilized redox mediators. In this research GOx from *P. adametzii*, *P. funiculosus* and *A. niger* and some ferrocene-based redox mediators were analysed in terms of their suitability for the design of glucose biosensors. Enzymes isolated from *P. adametzii* and *A. niger* showed a similar performance with applied redox mediators, while GOx isolated from *P. funiculosus* behaved in a different way. Significantly higher stability of GOx derived from *P. adametzii* and *P.*

funiculosus in comparison with commercial GOx from *A. niger* was observed. Advanced stability provides the possibility to design enzymatic electrodes with prolonged effective operation periods. Taking into account the evaluated kinetic parameters and the stability of tested enzymatic electrodes, glucose oxidase obtained from *P. funiculosus* was chosen as the most suitable enzyme for the development of a reagent-less glucose biosensor.

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