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Application of PQQ-GDH Based Polymeric Layers in Design of Biosensors for Detection of Heavy Metals

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A novel feature of biologically active polymer created by copolymerisation of glutaraldehyde (GA) with enzyme pyroloquinoline quinone dependent glucose dehydrogenase from *Gluconobacter sp. 34* (PQQ-GDH) was its sensitivity to heavy metals. Examination of the influence of Cd^{2+} and Pb^{2+} ions on biologically active membranes was investigated. Both metal ions showed reversible inhibition of PQQ-GDH/GA copolymer. Inhibition effects of enzymatic activity of PQQ-dependent glucose dehydrogenase were applied in biosensors devoted for detection of Cd^{2+} and Pb^{2+} ions. Basic characteristics of biosensors were determined. Here presented biosensor can be applied for detection of Cd^{2+} and Pb^{2+} ions in concentration range of $15 \cdot 10^{-5}$ mol/l.
Keywords: heavy metals, cadmium, lead, biosensors, PQQ enzymes, glucose dehydrogenase, inhibition.

INTRODUCTION

Number of methods has been used to immobilize biological molecules in sensor's membrane and to preserve high level of their activity. One of the most popular immobilization of enzymes is cross-linking by glutaraldehyde [1]. Such enzyme-based layers are often used in design of biosensors. Manufacturing and application of biosensors cause some problems, the main of which are: optimization of enzyme immobilization process to preserve biomolecular enzyme structures and maximal value of enzyme activity and development of approaches to accomplish a repeated analysis of substances which are reversible or irreversible inhibitors of the above mentioned enzymes. Very often during application of biosensors activity of immobilized enzymes dramatically decreases. One of the factors decreasing its activity is the influence of heavy metals. On the other hand decrease of enzymatic activity can by successfully exploited in biosensors devoted for detection of heavy metals. The content of heavy metals in body fluids, foods, and environmental samples is of wide concern and its determination is of great interest since the pollution of water by heavy metals is dramatically increased during the 20th century.

Effects induced by metals are various from irritant and acute or chronic systemic toxic effects to teratogenic, mutagenic and carcinogenic effects [2]. Each metal has its own spectrum of affinity constants for organic binding: elevated values are observed for molecules rich in –SH groups, towards which metals such as Pb, Cd, As and Hg show particular reactivity. The reactivity for a wide range of biological ligands lies at the basis of the damaging action of the metal ion at the molecular level and determinates toxicity of the adsorbed metal. Once adsorbed, heavy metals have few opportunities to produce toxic effects as a result of the presence of homeostatic mechanisms, such as intestinal control of absorption and presence of specific transport proteins. The competition between essential and toxic elements for protein binding sites lies at the basis of the toxicity of some of them. The toxic effects of elements are partly due to direct inhibition of the enzymatic systems, partially due to the indirect alteration of the essential metal-ions equilibrium and the consequent inhibition of their biological availability [2]. Metals form a vast variety of coordination compounds, whose stability is determined by specific equilibrium constants. The marked tendency to form compounds ensures that metals in vivo are invariably complexed with particular biological groups, such as sulfhydrilic (-SH), amine ($-NH_2$), oxhydrilic (-OH), disulfuric (-SS-), and carboxylic (-COOH). These groups also pertain to important protein molecules with catalytic, structural or transport functions.

In earlier it works was shown that activity of PQQ-dependent glucose dehydrogenase depends on concentrations of some metals [3]. This enzyme found wide application in design of biosensors devoted for detection of glucose concentration in the various samples , especially in assays where oxygen may have an influence on the results [5 – 11]. Their coenzyme pyrroloquinoline quinone (PQQ) is located at the active site of the enzyme tightly coordinated to a Ca^{2+} ion [11, 12]. The Ca^{2+} ion plays a key role in the enzyme mechanism [13] and all of PQQ-containing dehydrogenases whose structures have been determined also contain a Ca^{2+} ion bonded to the PQQ at the active site and are likely to have a similar mechanism of action. [14]. The competition experiments with *E. coli* PQQ-GDH suggest that enzyme has two sites for binding divalent metal ions; these are the active site in which the ion is bound to amino acids and to PQQ, and a

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second inhibition site [14]. Some of metal ions lead to inactive enzyme.

The aim of the present study was to show that PQQ-dependent glucose dehydrogenase employed in design of enzymatic biosensors can be used as an alternative for detection of heavy metals.

EXPERIMENTAL

Chemicals. PQQ-dependent glucose dehydrogenase from *Gluconobacter sp. 34*. was purified according method published earlier [15]. The enzyme had an activity of 61 Uml^{-1} and a concentration of 20 mg protein ml^{-1}. Carbon electrodes (ULTRA "F" PURITY ultra ® carbon) 3 mm in diameter were obtained from Ultra Carbon Division of Carbon USA, RAVEN-M. Phenazine methosulfate (PMS), 2,6-dichloroindophenol (DCIP) and glucose were received from Sigma (St. Louis, USA).

Electrode preparation. Enzyme modified graphite electrodes were prepared as follows: first, rods of spectroscopic graphite were cut, and polished on fine emery paper (Tufback, Durite P1200, Allar, Sterling Heights, MI, USA), followed by rinsing the electrode surface with water and drying at room temperature before coating with enzyme. Next, 6 µl of enzyme solution were added on the electrode surface and the coated electrodes were kept for 7 minutes over the vapuor of 25 % solution of glutaraldehyde at room temperature.

Electrochemical measurements. Electrochemical measurements were performed by polarographic analyser PA2 (Czech Republic) with XY recorder Endim 622.01 (USA) at three electrode mode. Working electrode potential was 250 mV *vs* Ag/AgCl. All experiments were done in 0.05 mol/l acetate buffer (pH 6.0) containing 0.1 mol/l KCl and 1 mmol/l PMS.

Spectrophotometrical measurements were carried out by spectrophotometer Specord UV VIS (Germany) at 600 nm. The GDH-PQQ activity was assayed in a dye-linked system containing phenazine methosulfate (PMS) and 2,6-dichloroindophenol (DCIP). The 1-ml assay mixture contained 20 mmol/l acetate (pH 6.0), 20 mmol/l D-glucose, 10 µl PQQ-GDH, 100 µmol/l DCIP and 60 µmol/l PMS. One unit of enzyme activity was defined as the amount of enzyme that catalyses the reduction of 1 µmol of DCIP per min.

RESULTS AND DISCUSSION

The polymer with biological activity was made from enzyme glucose dehydrogenase copolymerized with glutaraldehyde. Enzyme solution was deposited on the tip of carbon electrode. Then copolymerization was carried out at room temperature and near neutral pH by crosslinking protein moiety of enzymes with glutaraldehyde according following reaction:

2 E —NH_2 + **CHO—CH_2—CH_2—CH_2—CHO** ⟶
PQQ-GDH glutaraldehyde (GA)

⟶ **E—NH—CO—CH_2—CH_2—CH_2—CO—NH—E** .
copolymer PQQ-GDH/GA

Under such conditions crosslinking of enzyme molecules adsorbed on the surface of electrode occurs, polymer loses of solubility but its biological activity remains.

Such biologically active polymers were employed in design of electrochemical biosensors for detection of glucose concentration. For this carbon electrode was covered by approximately 15 µm thick layer of PQQ-GDH/GA copolymer. Electrochemical signals were generated by redox mediator based system, where phenasine methosulphate was employed as redox shuttle between active site of PQQ-GDH and conducting electrode surface (Fig. 1).

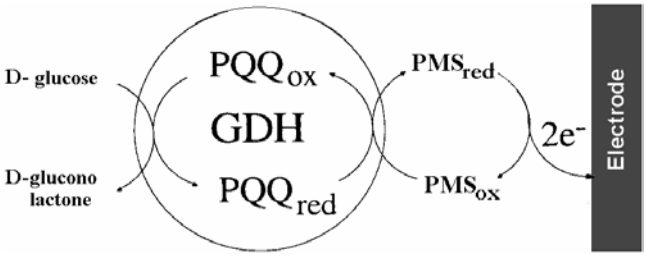


Fig. 1. Principal scheme of mediated bioelectrocatalysis

Bioelectrochemical system allowed us to register electrocatalytical currents depending on glucose concentrations in presence of constant PMS concentration (Fig. 2).

The bioelectrocatalytical reaction can be written as,

$$\begin{aligned} &S + E \leftrightarrow SE_{ox} \rightarrow P + E_{red} \\ &M_{ox} + E_{red} \leftrightarrow ME_{red} \rightarrow M_{red} + E_{ox}\,, \\ &M_{red} \rightarrow M_{ox} \end{aligned} \qquad (1)$$

where S is the substrate (glucose), P is the product, E is the enzyme, M_{ox}, M_{red} are the oxidized and reduced forms of a redox molecule (PMS).

The ping-pong mechanism is assumed for the enzyme reaction, whose rate v_S, in the steady state is given by

$$v_S = \frac{k_{cat} E}{1 + K_S / S + K_M / M_{ox}}\,, \qquad (2)$$

here k_{cat} is the catalytic constant, K_S and K_M are the Michaelis constant for S and M_{ox}, respectively; and E, S and M_{ox} are the respective concentrations. The current I, measured at the electrode reflects v_S, as expressed by the following approximate equation [16]:

$$I_{app} = FAM \cdot \sqrt{\frac{2 n_S n_M D_M k_{cat} E}{2K_M + M}}\,, \qquad (3)$$

here F, A, M, and D_M are the Faraday's constant, electrode surface area, bulk concentration of mediator M, and its diffusion coefficient, respectively; n_S and n_M are the number of electrons involved in Eq. 1, respectively. I_{app} expresses I within the error of ±5 % and allows us to determine kinetic parameters from the bioelectrocatalytic current. Under the condition indicated in Fig. 2, where current increases with increasing concentration of glucose, I_{max} = 49.5 mA, K_S = 6.8 mmol/l. This K_S value is higher in comparison with such constant for PQQ-GDH monomer (K_S = 0.47 mmol/l) measured spectrophotometrically; this denotes, that PQQ-GDH/GA copolymer acts in the diffusion mode. The current of biosensor was linear in the range 0.1 to 3 mmol/l of glucose with sensitivity 4.5 µA/mM.

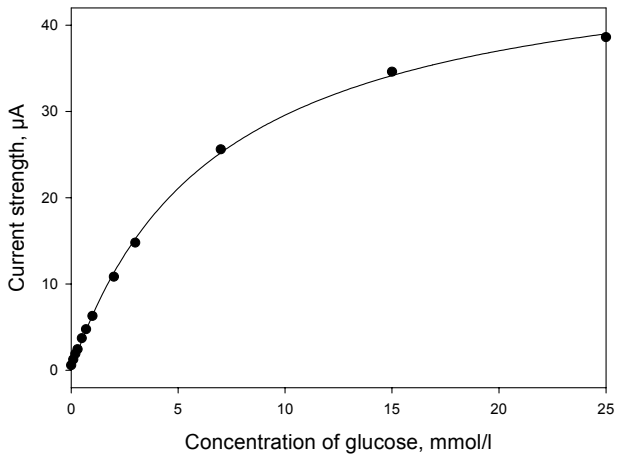


Fig. 2. PQQ-GDH/GA based biosensor response to glucose. Measurements were held amperometrically in 0.05 mol/l Na acetate buffer pH 6.0 with 0.1 mol/l KCl and 1 mmol/l PMS

The kinetic measurements executed with soluble PQQ-GDH monomer with presence of cadmium ions give us the evidence, that there is a non-competitive inhibition case (Fig. 3).

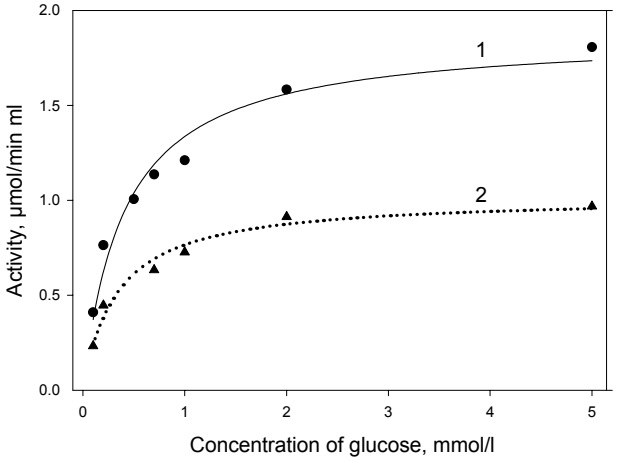


Fig. 3. Effect of cadmium addition on activity of the biologically active monomer PQQ-GDH. (1 – increase of activity without Cd^{2+}, 2 – when 20 mmol/l cadmium ion are added).

Thus, the decrease of steady state current I_{app}, which is proportional to enzyme reaction rate, in the presence of inhibitor metal ions will be conditioned by inhibitor concentration.

PQQ-GDH/GA polymer showed significant sensitivity to heavy metals. Its sensitivity to Cd^{2+} and Pb^{2+} ions was investigated and it was found that metal concentrations within range of 0.3 to 3 mmol/l significantly reduce bio-catalytical current (Figs. 4 and 5).

Linear regression graphs in the double-reciprocal plots for cadmium and lead, regarding to results of Figs. 4 – 5, gives the crossed linear lines on the axis (Fig. 6).

Measurements were held spectrophotometrically in 0.05 mol/l Na acetate buffer pH 6.0 with 0.1 mol/l KCl, 60 µmol/l PMS and 100 µmol/l DCIP.

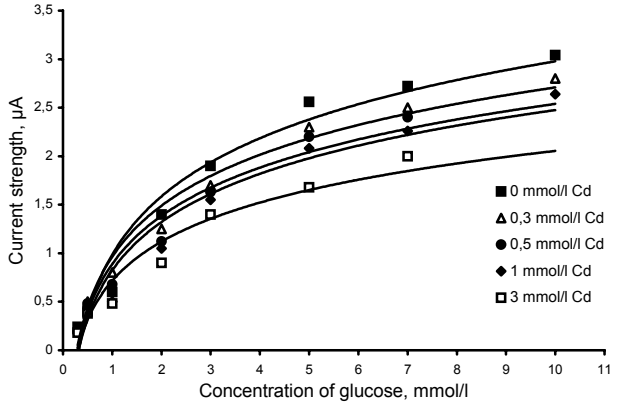


Fig. 4. Effect of different cadmium concentrations on activity of the biologically active copolymer PQQ-GDH/GA

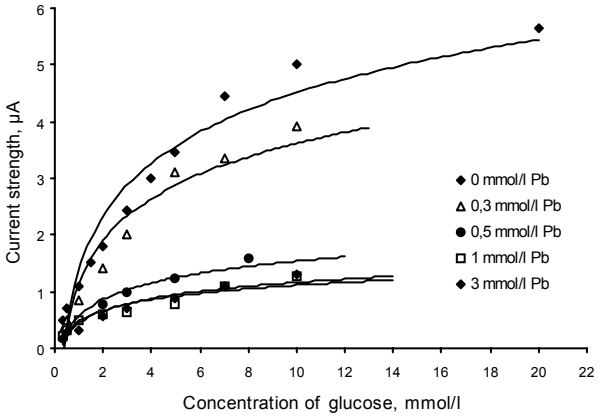


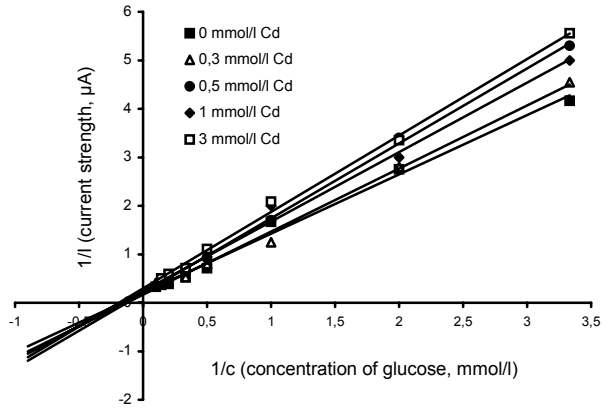
Fig. 5. Effect of different lead concentrations on activity of the biologically active copolymer PQQ-GDH / GA. Measurements were held amperometrically in 0.05 mol/l Na acetate buffer pH 6.0 with 0.1 mol/l KCl and 1 mmol/l PMS

Measurements carried out amperometrically in 0.05 mol/l Na acetate buffer pH 6.0 with 0.1 mol/l KCl and 1 mmol/l PMS.

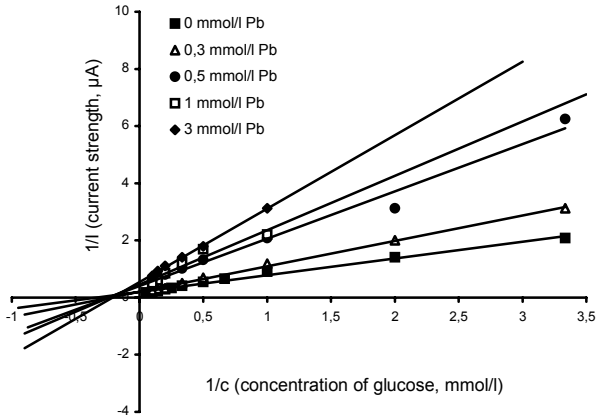
After almost all lines have crosses on the abscisse axis, noncompetitive inhibition has been assumed. It means that the heavy metals deforms the active centre of the enzyme but don't combine at the same place where the substrate does. So, the inhibitor can combine to the enzyme and (or) to the *ES* complex, forming an inactive compound. Elimination of the Cd^{2+} ions from the solution, the primary activity of PQQ-GDH/GA resets, what shows that the compound is unstable and Cd^{2+} spells from the enzyme and of the enzyme-substrate complex. Pb^{2+} ions formed a considerably more stable combination with the enzyme.

During the next step the same PQQ-GDH/GA biopolymer was employed in design of biosensor devoted for detection of heavy metals. The model of action of here proposed biosensor was based on the inhibition of PQQ-GDH by heavy metal ions (Cd^{2+} or Pb^{2+}). To obtain the analytical signal by PQQ-GDH/GA based biosensor, addition of constant glucose and constant electron transfer mediator phenazine methosulfate was obligatory. Therefore important part of this work was to found optimal concentrations of both compounds. It was shown that by variation of concentrations of PMS and glucose is possible

to change the detection diapason of Cd^{2+}. The most extended Cd^{2+} detection diapason was determined if concentration of glucose was no less than 45 mmol/l and concentration of PMS was 1 mmol/l. The sensitivity of PQQ-GDH to both Cd^{2+} and Pb^{2+} ions depends on incubation time (Fig. 7).



a



b

Fig. 6. Linear regression, drawn on Lineweaver-Burk coordinates, according to the graph of inhibition with Cd^{2+} (a) and Pb^{2+} (b) of the PQQ-GDH/GA polymer

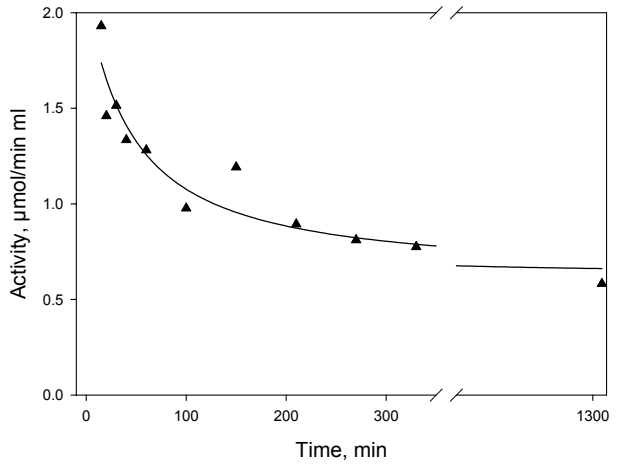


Fig. 7. Influence of preincubation time of biologically active monomer PQQ-GDH with cadmium ions on its activity. Measurements were done spectrophotometrically with 5 mmol/l glucose and 1 mmol/l Cd^{2+}

The inhibition of sensor by Cd^{2+} ions same as Pb^{2+} ions depends on the concentration of the ions in the solution (Fig. 8).

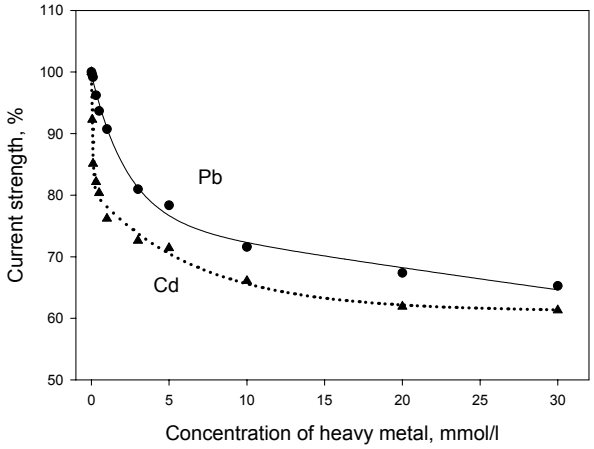


Fig. 8. Dependence of the residual current of the PQQ-GDH/GA based sensor on concentration of heavy metals. Acetate 0.05 mol/l (pH = 6.0) with 0.1 mol/l KCl, 1 mmol/l PMS and 45 mmol/l glucose

The curves of dependence are linearized in semi-logarithmic coordinates. Slopes of the lines reflects the sensitivity of the sensors to heavy metals. Sensitivities are 1.397 %/mM and 1.05 %/mM to Pb and Cd ions, respectively (Fig. 9).

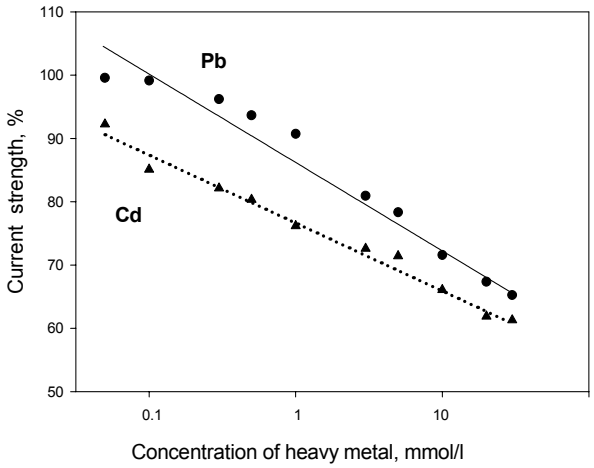


Fig. 9. Decrease of current of biosensor based on PQQ-GDH/GA copolymer in presence of Pb^{2+} and Cd^{2+} ions

After measurements of heavy metals and rinsing of the sensor the bioelectrochemical signal regained to the same value which was registered before addition of heavy metals.

Formed PQQ-GDH/GA copolymer was biologically active within 2 weeks at room temperature if stored in 0.5 mol/l sodium acetate solution at 4 °C.

During investigations of stability it was shown that such bioelectrochemical system based on PQQ-GDH/GA copolymer can operate within 1 – 2 weeks and over 50 heavy metal addition cycles can be performed during this period. However recalibration of this analytical system every 4 hours is required to obtain more objective results.

The range for accurate detection of heavy metals concentrations was in the range of 0.15 – 1.5 mmol/l for the Cd^{2+} and 0.15 – 3.5 mmol/l for the Pb^{2+}. However there in no discrimination between Cd^{2+} and Pb^{2+} ions and results of biosensor are imprecise if the sample contains ions of both metals. This problem can be solved by chemical precipitation one of them from the sample.

CONCLUSIONS AND FUTURE DEVELOPMENTS

It was shown that PQQ-GDH/GA as biologically active copolymer can be employed in design of electrochemical biosensors sensitive to concentration of heavy metals. Investigation of influence of Cd^{2+} and Pb^{2+} showed that inhibition of PQQ-GDH and reduction of registered amperometrical signal has reversible character and depends only on concentration of heavy metals in the sample. Using proposed analytical system total concentration of both heavy metals in the sample can be determined. For exact determination of each metal ion concentration separation/precipitation step is required.

The bioanalytical systems based on PQQ-GDH/GA copolymer are under intensive investigations and development. Kinetic investigations of action mechanism and solution of some here highlighted analytical problems will be presented elsewhere.

Acknowledgment

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