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Bioelectrochemical application of some PQQ-dependent enzymes

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Abstract

This paper focuses on the use of PQQ-dependent enzymes (PQQ enzymes) in amperometrical biosensors and gives emphasis on their innovative designs and applications. The study covers some aspects in the evolution of biosensors based on PQQ enzymes. Main attention is focused on the electrochemical properties of PQQ enzymes as very promising materials for the formation of electrochemical biosensors. Immobilization approaches and redox mediators recently used in PQQ enzymes based biosensors are reviewed. The acceptance of polypyrrole as a very promising immobilization matrix for some PQQ enzymes is discussed. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Glucose dehydrogenase; Glycerol dehydrogenase; Alcohol dehydrogenase; Polypyrrole

1. Introduction

The effective combination of biochemistry and electrochemistry in analytical devices could provide the basis for the direct electrical detection of a wide range of analytes with great sensitivity and specificity.

In the development of enzyme-based amperometric biosensors, oxidases have been mostly used as a biological recognition element. The main drawback of the oxidase-based biosensor is its response dependence on oxygen concentration in the sample. This drawback causes trouble in designing biosensors for undiluted blood, microbiological media, and other biological samples with unstable oxygen concentration. A number of attempts have been made to replace oxygen by artificial electron acceptor but the problem remains since an artificial acceptor exhibiting a high enough chemical affinity and capability to compete with oxygen has not been found yet. To overcome this problem NAD-dependent dehydrogenases have been applied [1]. Although these sensors are not dependent on oxygen, the necessary addition of the free-diffusing coenzyme NAD^+ or the use of free-diffusing redox mediator caused severe restrictions for the application of these sensors.

The development of enzyme biosensors also gained from novel enzymes and bioengineered proteins. Significant improvement was achieved when oxidases and NAD-dependent dehydrogenases were replaced by PQQ enzymes, which catalyzes the transfer of electrons from substrate to an electron acceptor other than oxygen [2,3]. Moreover, some of PQQ-dependent enzymes are able to transfer electrons directly to solid surfaces [4] or to conducting polymers [5]. PQQ-dependent dehydrogenases were first reported as potential objects for new biosensors about 15 years ago. Later a gene-engineered apo-PQQ-dependent glucose dehydrogenase (PQQ-GDH) was successfully used for commercial electrochemical glucose test strips. PQQ, as cofactor and redox shuttle, was additionally added in the test strips. However, this apo-PQQ-GDH bonds PQQ cofactor very weakly and test strips in biosensing device can be used only as single-use analytical tools [2].

We have purified some new PQQ-dependent dehydrogenases with more tightly bonded cofactors from new sources [6–8] and designed a number of glucose [9–15], glycerol [16] and ethanol [17,18] biosensors.

2. Materials and methods

PQQ-GDH was purified from *Erwinia* sp. 34-1 [6]. PQQ-dependent glycerol dehydrogenase (GlycDH) was purified from the membrane fraction of *Gluconobacter* sp. 33 [7]. Alcohol dehydrogenase (QH-ADH) was purified from *Gluconobacter* sp. 33 [8].

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The sensors were prepared following previously optimized protocols according to Refs. [4,5,9–18]. Three enzyme immobilization methods were used: cross-linking by glutaraldehyde [4,9–11,13,16,17], entrapment into conducting polymer polypyrrole (Ppy) [5,14,15] and covalent binding using functionalized polymeric matrix [12,18]. The responses of biosensors to addition of analytes were investigated in stirred 0.1 M acetate buffer containing 0.1 M KCl under potentiostatic conditions at +300 mV according protocols described in Refs. [4,5,9–13,16–18], and at +500 mV vs. Ag/AgCl if [osmium-bis-*N*,*N*′ -(2,2′ -bipyridyl)-*N*-(pyridine-4-yl-methyl-(6-pyrrole-lyl-hexyl)-amine)chlorode]chloride (Os-complex) copolymerized with pyrrole was used as redox mediator and immobilization matrix [14,15].

3. Results and discussion

3.1. Principle designs of bioelectrochemical sensors based on PQQ-dependent enzymes

The principal schemes of action of PQQ enzymes are presented in Fig. 1. PQQ enzymes have two major possibilities to transfer electrons to conducting solid or polymeric surfaces: (i) via redox mediators (Fig. 1A); (ii) via intrinsic redox chains (Fig. 1B). Intrinsic electron transfer is a characteristic feature of some of PQQ enzymes containing number of hemes-*c*. QH-ADH, via intrinsic electron transfer chain, can directly transfer electrons to some conducting surfaces [4,5].

3.2. Application of PQQ-dependent glucose dehydrogenases in glucose biosensors

PMS [9], as water-soluble redox mediator, was applied in some biosensors based on PQQ-GDH. Soluble mediators are useful for the construction of single-use test strips. More promising for design of biosensors devoted for continuous measurements are insoluble mediators like polyquinonic polymers. Reagentless glucose biosensors can be created during immobilization of PQQ-GDH on the carbon electrode modified with: (i) redox polymer obtained by electropolymerization of *p*-ferrocenylphenol [13]; (ii) electrochemically deposited number of heterocyclic compounds [10]; (iii) by irreversibly adsorbed and electropolymerised naphthoquinone or benzodiazepine derivatives [11]; or (iv) PQQ-GDH modified with an enzymatically synthesized polyarbutin, or included into such polymer [12]. The surface of carbon electrode in these cases is covered either with a multi-layer of heterocyclic molecules or by oligomers and polymers. This leads to formation of infrastructure containing semi-oxidized heterocyclic compounds. This network of electrochemically reversible groups facilitates charge transfer from the active center of enzyme to the electrode surface. Reduced PQQ-GDH cannot directly transfer electrons to the surface of metals, carbon or to conducting polymer-polypyrrole. But if polypyrrole matrix is modified with osmium complexes [14,15], such redox polymer excellently accepts electrons from the reduced form of the PQQ-GDH.

The linear parts of calibration curves of biosensors based on PQQ-GDH are in the range of 1–20 mM. This interval is determined by K_m of the native enzyme and immobilisation technique, because, if immobilisation by glutaraldehyde is applied, biosensors acts in semi-diffusion mode. The linear part of the calibration curve can be extended either by application of the additional diffusion layer of PVA [10] or if PQQ-GDH is included into hydrophobic polypyrrole layer [15]. Thermostability of PQQ-GDH was about twice lower in comparison with glucose oxidase. In addition to the thermal inactivation, the process of PQQ elimination can be observed under certain conditions. For example, the fast inactivation of the biosensor was observed in phosphate buffer. When phosphate buffer was replaced with acetate buffer, the activity of the biosensor was restored. This process was faster and more efficient in the presence of 1 mM Ca^{2+} or

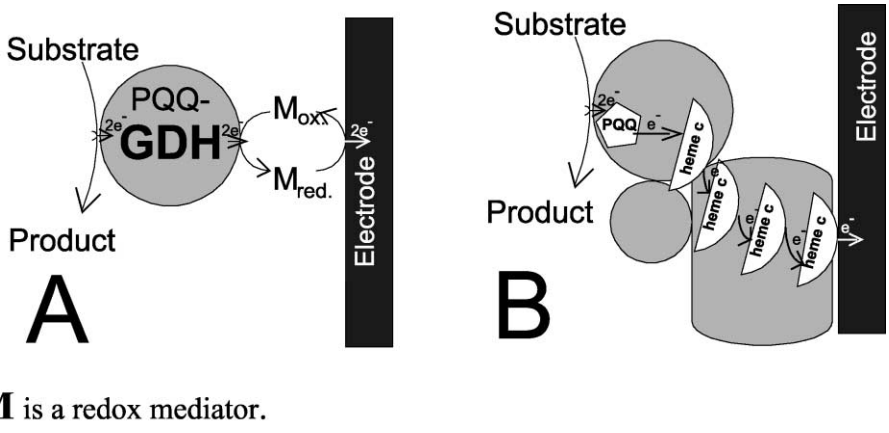

Fig. 1. Principal action schemes of the PQQ enzymes based biosensors. A—single redox center based quinoproteins; B—multi redox center-based quinohemoproteins.

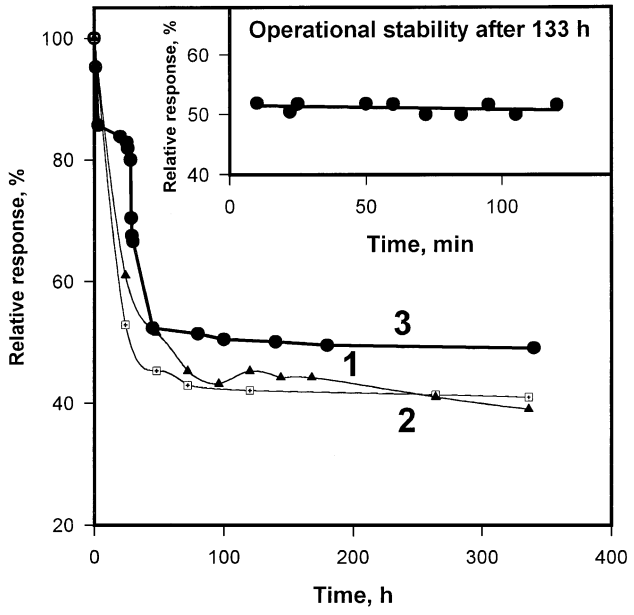

Fig. 2. Storage stability of glucose (1), glycerol (2) and ethanol (3) biosensors at +4 °C. Inset: Operational stability of ethanol biosensor at +25 °C in 0.05 M Na-acetate buffer, pH 6.0, at +300 mV vs. Ag/AgCl. Relative responses were calculated from relation of actual response with biosensor response immediately after preparation (100%).

Mg^{2+} ions. Immobilization of PQQ-GDH into carbon paste increased the stability of the enzyme more than three times [11]. The operational stability of PQQ-GDH immobilized by glutaraldehyde is presented in Fig. 2, curve 1.

These glucose biosensors were used for the determination of glucose in wines and soft drinks. The data were compared with standard refractometric method. Good correlation ($r = 0.9778$) was obtained [11]. It was also shown that response of the biosensor does not depend on oxygen concentration in the samples.

3.3. Glycerol dehydrogenase (GlycDH) in glycerol biosensors

Graphite electrode was selected as the most suitable surface for GlycDH immobilization. The reduced form of PQQ in this enzyme does not transfer electrons to modified carbon electrode and a soluble mediator like PMS should be used. The calibration graph of biosensor was linear to 8 mM of glycerol [16]. Approximation of experimental data by hyperbola model leads to parameters $K_m^{app} = 10.4$ mM and $I_{max} = 5.6$ μA. These data, together with the extended linear region of the steady-state currents vs. concentration of glycerol (about 12 times longer than can be expected from the K_m value for glycerol (the calculated K_m value of GlycDH for glycerol was 0.83 mM) reported for the purified glycerol dehydrogenase, indicated that the biosensor acts in a diffusion-limited mode.

Comparison of biosensor signals to 1–10 mM glycerol was obtained in oxygen-free and oxygen-containing solutions. It was shown that signals of biosensor in oxygen-free sample were higher only on about 4%. This side-reaction has negligible impact because electrochemical oxidation of reduced PMS on the electrode surface is much faster. The selectivity of the obtained biosensor to various mono- and polyhydroxylic compounds was also investigated [16]. At pH 6.0, significant interference of glucose, sorbitol and mannitol was observed but at pH 8.0, responses to glycerol were significantly higher compared to aforementioned substances. No biosensor responses to ethanol and methanol were observed at all. Interfering response generated by fructose, methanol and dulcitol can be neglected because the concentrations of those compounds normally are lower than that of glycerol in tested beverages.

Storage stability was examined by keeping the biosensor in the dry state at 4 °C. Periodic measurements of the response to glycerol at room temperature were carried out. The slope of biosensor during the first 2 days after preparation decreased considerably, probably due to leakage of weakly immobilized enzyme from surface (half-life period ($\tau_{1/2}$) was 2 days). After this storage period, the remaining sensitivity was about 50% and later decreased very slowly (Fig. 2, curve 2).

3.4. Quinohemoprotein alcohol dehydrogenase—a perspective tool for ethanol biosensors

QH-ADH consists of three subunits, having molecular masses of 83, 52.1 and 16.6 kDa [8]. QH-ADH contains PQQ moiety and at least four heme-*c* moieties. This enzyme can transfer electrons to acceptors in two ways: (i) directly from PQQ, like PQQ-GDH; (ii) via hemes chain. The

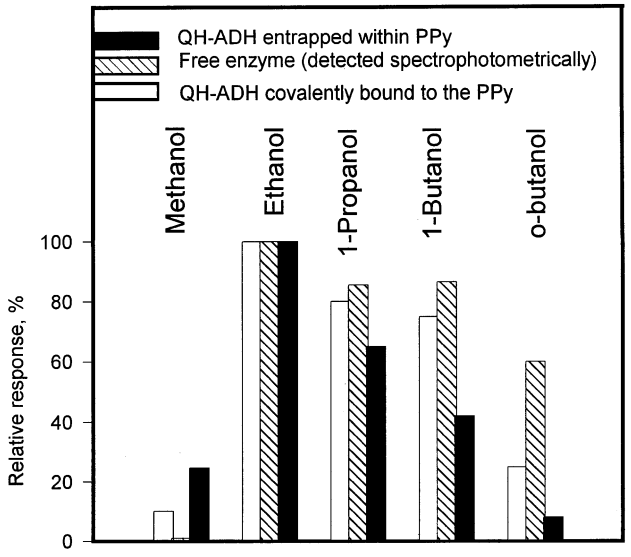

Fig. 3. Comparison of selectivity of ethanol biosensor and native QH-ADH in 0.05 M Na acetate buffer, pH 6.0, at +300 mV vs. Ag/AgCl. Biosensor contained enzyme immobilized: A—into polypyrrole matrix, B—on the surface of electrode coated by electrochemically synthesized polypyrrole.

principal schemes of such systems are shown in Fig. 1. This presumption was confirmed experimentally. QH-ADH transferred electrons not only to the standard mediators [18] or carbon surface modified with ferrocene derivatives [13] but also to the non-modified polypyrrole matrix [5] or even to the carbon surface [4].

The linear part of the calibration curve of the ethanol biosensor depends on the method of QH-ADH immobilisation.

The specificity of QH-ADH- and QH-ADH-based biosensors was investigated using various alcohols (Fig. 3). The selectivity of QH-ADH depends on the immobilization method and differs from native enzyme. This phenomenon can be explained by the diffusion restrictions of higher alcohols in semi-permeable matrix of polypyrrole coating. The results show that the native enzyme prefers three or four carbon atoms containing linear primary alcohols. Methanol is a very bad substrate and it is the advantage of QH-ADH based biosensors. This phenomenon opens good possibilities in the application of such biosensors in food industry, particularly in wine industry. QH-ADH can also oxidize longer alcohols, such as *n*-octanol or *n*-decanol [8], but these substrates are not soluble in water and kinetic characteristics cannot be obtained directly. Secondary and branched alcohols are unfavorable substrates for this enzyme.

The stability behaviour of ethanol biosensors was similar to glucose and glycerol biosensors (Fig. 2). Operational stability during the 2 h period at room temperature was good enough and is shown in Fig. 2 (inset).

4. Conclusions

In this work, the presented and discussed data show that PQQ enzymes are very promising tools when applied to the various designs of amperometrical biosensors. However, problems relating to the instability of biosensors need to be solved.

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