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Bi-enzyme sensor based on thick-film carbon electrode modified with electropolymerized tyramine

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Abstract

Bi-enzyme sensor based on thick-film epoxy-carbon electrode modified with polytyramine has been developed and examined for the determination of peroxidase substrates and cholinesterase inhibitors. Polytyramine was obtained on the electrode surface by repeated scanning of the potential from +600 to +1800 mV vs. Ag/AgCl in tyramine solution. The enzymes were immobilized in the polytyramine matrix by cross-linking with glutaraldehyde. The biosensor developed provides a reliable and inexpensive way for preliminary testing of common environmental pollutants with a single sensor in accordance with assumed toxic effect by the choice of appropriate substrate and measurement conditions. The bi-enzyme sensor makes it possible to determine substituted phenols and aromatic amines in the micromolar range of their concentrations and anticholinesterase pesticides with detection limits of 0.1 (Coumaphos) and 0.03 μmol l^{-1} (Chloropyrifos-methyl).

Keywords: Biosensor; Enzyme sensor; Electropolymerization; Polytyramine

1. Introduction

The increasingly stringent standards for the discharge of wastewaters call for the development of novel sensitive techniques for the preliminary estimation of quantities and toxic effects of the pollutants released in the environment. Electrochemical enzyme sensors are considered an alternative to the conventional spectrometric and chromatographic techniques for pollutant determination due to their low cost, simplified sample treatment, fast and sensitive response. Biosensors can be easily combined with conventional electrochemical equipment and implemented in the automated systems for the environmental monitoring or wastewater treatment control. Common pollutants present in the environment at ppm–ppb level affect key enzymes of metabolic pathways in living beings. This could be used for quantification of the pesticides, heavy metals, fluorides, cyanides, substituted phenols and thionic substances. The enzyme sensors based on cholinesterase [1–3], tyrosinase [4,5], urease [6,7] and peroxidase [5,8] have been described for this purpose. However, practical application of enzyme sensors is often limited by the problems of interpretation of their response in multicomponent media, e.g. wastewaters. The combination of several enzymes in a sensor array provides in some cases the identification of pollutants. Thus, cholinesterase, alkaline and acid phosphatases were proposed to use in three-electrode system developed for the determination of pesticides and heavy metals in one sample [2]. Similar pollutants were simultaneously determined in the biosensing system involving glassy capillaries covered with physically sorbed urease and cholinesterase [6]. However, the increase in the number of sensors in the array complicates the manufacture and the measurement protocol and hence increases the time necessary for sample testing and data interpretation. The immobilization of several enzymes on the same transducer is an alternative to the series of mono-enzyme sensors. In this work, we proposed to use for this purpose two enzymes, i.e. cholinesterase previously examined in testing industrial

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discharges [9] and peroxidase described both for the detection and removal of easily oxidized phenols which are typical components of municipal sewages [10,11]. Both enzymes were immobilized onto the polytyramine matrix that was successfully used for the development of various enzyme sensors [12–14].

2. Experimental

Voltammetric measurements were carried out in a standard three-electrode electrochemical cell thermostated at 25 ± 1 °C using the Ecotest-VA analyzer (Econix-Expert, Moscow, Russia). Biosensor response was measured in DC mode with Pt auxiliary and Ag/AgCl reference electrodes at the potential scan rate of 150 mV s^{-1}. Thick-film planar electrodes made of an epoxy-graphite composite on PVC plates were provided for the investigations by "IVA", Ekaterinburg, Russia. All the measurements were carried out in phosphate buffer saline (PBS, 0.002 mol l^{-1}, pH 7.0, NaCl 0.1 mol l^{-1}).

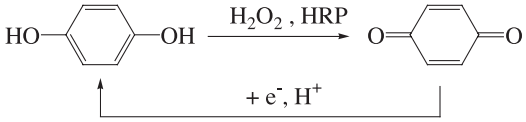
Electropolymerization of tyramine was performed from its 0.01 mol l^{-1} solution in ethanol/PBS mixture (1:3, pH 7.0) by potential cycling in-between +600 and +1800 mV at the scan rate of 50 mV s^{-1} as described elsewhere [12]. After that the electrode was washed and the enzymes were immobilized from PBS. Butyrylcholinesterase from horse serum (ChE, specific activity 500 U mg^{-1} of protein) and horseradish peroxidase (HRP, specific activity 250 U mg^{-1} solid) were immobilized by 5 min treatment with 2% glutarladehyde. The final loading of 1 E of each enzyme per electrode was used in all the experiments. The enzyme sensors were stored in PBS or in dry conditions at 4 °C for at least 3 months.

The ChE activity was quantified amperometrically with the peak current of anodic oxidation of thiocholine formed in reaction (1) at +150 mV.

$$\begin{aligned}&(CH_3)_3N^+CH_2CH_2SC(O)CH_3 + H_2O\\ &\quad \overset{ChE}{\rightarrow} (CH_3)_3N^+CH_2CH_2SH + CH_3COOH\\ &2(CH_3)_3N^+CH_2CH_2SH - 2e^-\\ &\quad \rightarrow (CH_3)_3N^+CH_2CH_2SSCH_2CH_2N^+(CH_3)_3 \end{aligned} \quad (1)$$

Acetylthiocholine chloride was used as the ChE substrate. The inhibition was estimated as a relative decay of the signal toward acetylthiocholine measured after 10 min incubation of the bi-enzyme sensor in the pesticide solution. Coumaphos and Chloropyrifos-methyl (Riedel-de-Haen, Seelze, Germany) were pre-oxidized to their phosphoryl analogs with bromine for 10 min to increase their inhibitory effect.

HRP reaction was monitored by the peak current referred to the cathodic reduction of the product formed in the enzymatic oxidation of a substrate as shown in Eq. (2) for hydroquinone as an example.

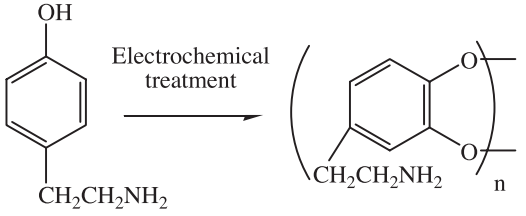


(2)

Substituted anilines and phenols were tested as HRP substrates. Their peak potentials varied from −250 to −150 mV. The response was measured in the presence of 1.0 mmol l^{-1} of hydrogen peroxide 10 min after the substrate injection.

3. Results and discussion

Repeated cycling of the electrode potential in 0.1 mol l^{-1} tyramine solution resulted in the formation of the low-conductive film growing on the electrode surface and the progressive decrease of appropriate currents in the range of potential scanning (Eq. (3)) [12–15].



(3)

The polytyramine film obtained flaked away from the bare electrode left to stay in PBS in several days. The deposition of Nafion suspension on the electrode either before or after the tyramine polymerization increased the stability of polytyramine coating up to several weeks for the electrodes stored in PBS at room temperature. In optimal conditions, 2 μl of the 5% Nafion suspension were spread on the 4 mm^2 electrode surface. For performing the following experiments, five cycles of potential scanning were chosen as a compromise between the response value and its reproducibility.

The tyramine polymerization was then applied for a simple and effective immobilization of the enzymes. The ChE and HRP solutions in PBS were first spread and dried on the electrode modified with polytyramine and then treated with glutaraldehyde. The latter formed covalent bonds with the amino groups of the proteins and of the polymer side chains. The stability of the signal of the bi-enzyme sensor obtained was found to be even higher than the characteristics of polytyramine films on the bare electrode. The biosensor retained 80% of the initial HRP activity for at least 3 months and 50% for 6 months when stored in PBS at 4 °C. The ChE activity increased by 10–30% during the first several weeks and then stabilized for 3–4 months (Fig. 1). The signal measurements were performed with the same sensor for each enzyme separately by selecting the

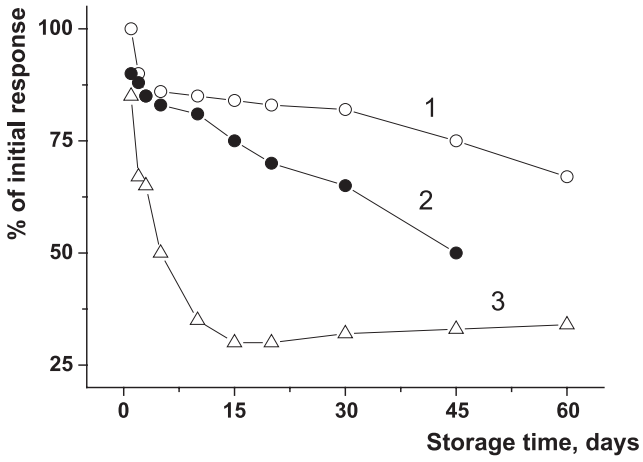


Fig. 1. The stability of the HRP activity during the storage of enzymatic sensors at 4 °C in PBS. (1) Bi-enzyme sensor with the ChE and HRP implemented in polytyramine matrix; (2) HRP implemented in polytyramine matrix alone; (3) the HRP implemented in gelatin layer. The response toward hydroquinone was measured 1.0 mmol l^{-1} of hydrogen peroxide in 10 min after the substrate addition.

appropriate working potential and substrate. The activity of immobilized enzymes did not affect each other. No memory effects were observed when the ChE and HRP activities were measured in random order during the whole time of biosensor storage. The stability of the HRP immobilized alone was found to be lower than that in the presence of ChE in the same surface layer. The entrapment of HRP in the gelatin layer also showed a remarkable decay of enzyme activity during the storage period (Fig. 1).

Another advantage of the polytyramine matrix is a significant decrease of the potential necessary for thiocholine oxidation. This product formed in the ChE reaction is oxidized on bare electrode at +450...+680 mV [1,2]. This process is complicated with the sorption of sulfur containing by-products. As a result, the recurrent electrochemical cleaning of the electrode is usually recommended. The signal of bi-enzyme sensor developed toward acetylcholine did not show any tendency to decay in at least 10 consecutive measurements performed during a working day. Probably that was due to the low working potential (about +150 mV). This value is typical rather for mediated ChE sensors [1] and does not lead to the necessity of electrochemical removal of by-products from the electrode surface. The decrease in the working potential for acetylcholine oxidation in comparison with that observed on non-modified electrode could result from the favorable coordination of thiocholine cations provided by free amino groups of the side chains of the polymer. The ability of polytyramine to form complexes with metal ions and to affect their electrochemical behavior is well known [15].

The results of the determination of HRP substrates and ChE inhibitors are summarized in Table 1. The detection limit refers to the analyte concentration resulting in the shift of the signal which is equal to three standard deviations of the background current, i.e. 0.1 μA for the substrate determination or about 8% inhibition for the pesticide determination. The concentration range corresponds to the linear part of appropriate calibration curves in the plots of the current, μA, against [substrate, mol l^{-1}] for the HRP substrates and in the plots of inhibition, %, against log[pesticide, mol l^{-1}] for ChE inhibitors. The sensitivity was calculated from the slope of the linear part of calibration curves. As shown the characteristics of determination of HRP substrates are similar. Aniline has probably been polymerized in the measurement conditions. This made the signal irreproducible at lower concentrations of the analyte. The formation of oligomeric by-products is also confirmed by a significant increase of the background current after the measurement of aniline signal. The lowest detection limits were obtained for aminophenols and *p*-chloroaniline. This agrees with their high reactivity in the non-enzymatic oxidation observed on bare electrode. A high activity of *p*-chloroaniline is promoted by easy removal of chloride on the cathodic part of the substrate cycle (Eq. (2)). The *p*-aminophenol is very sensitive to both enzymatic and non-enzymatic oxidation.

The sensitivity of pesticide detection was found to be similar to that obtained for other ChE-sensors. Thus, the detection limits of 0.04 μmol l^{-1} for Coumaphos and 1 μmol l^{-1} for Chloropyrifos-methyl were obtained with the ChE sensor based on the screen-printed carbon electrode modified with Nafion [16]. The initial ChE activity can be restored by treatment of the sensor with the 0.1% 2-pyridine aldoxime methiodide (2-PAM) solution for 10 min. Up to 10 consecutive measurements of the pesticide inhibition can be performed with the same sensor a day.

Table 1
Analytical characteristics of bi-enzyme sensors based on thick-film epoxy-carbon electrodes modified with electropolymerized tyramine

Peroxidase substrates			
Analyte	Detection limit (mmol l^{-1})	Concentration range (mmol l^{-1})	Sensitivity (mA (mol l^{-1})$^{-1}$ cm^{-2})
Aniline	0.3	0.7–10	1.3
o-Nitroaniline	0.010	0.015–0.25	25
m-Nitroaniline	0.025	0.05–0.21	11
p-Nitroaniline	0.015	0.25–0.50	42
p-Metoxyaniline	0.01	0.03–1.0	31
p-Chloroaniline	0.004	0.006–0.06	347
m-Toluidine	0.015	0.03–0.45	100
3,4-Dichlorophenol	0.09	0.12–0.22	125
2-Amino-4-nitrophenol	0.003	0.005–0.23	1700
Hydroquinone	0.01	0.02–0.078	1126
Cholinesterase inhibitors			
Pesticide	Detection limit (μmol l^{-1})	Concentration range (μmol l^{-1})	Sensitivity (% of inhibition per decade)
Coumaphos	0.1	0.2–0.8	45
Chloropyrifos-methyl	0.03	0.07–3	31

4. Conclusion

The co-immobilization of cholinesterase and peroxidase onto the electrode modified with polytyramine provides an effective and easy way to develop an enzyme sensor for preliminary monitoring of environmental pollutants due to simple manufacture and measurement procedure, fast and reliable response and variety of the analytes to be detected. Due to the electropolymerization procedure there is no serious limitations in size or shape of the transducer used. The manufacture protocol can be standardized and adapted to other transducers available, e.g. screen-printed electrodes on plastic supports.

The enzymes implemented into the sensor assembly do not interfere with each other. This means that the appropriate enzyme activities related to the specific substrates and inhibitors present in the sample can be characterized at once with a singe biosensor by making choice of appropriate measurement conditions in accordance with the assumed sample content and/or its predominant toxic effect. In comparison with gelatine and other matrices described [16] polytyramine stabilizes the HRP activity during the storage and decreases the working potential of thiocholine oxidation. This diminishes the interfering influence of electroactive species present in the sample. The bi-enzyme sensor developed makes it possible to determine organophosphate pesticides, aromatic amines and phenols in micromolar range of their concentrations.

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