



Biosensor for Pesticides Based on Valerolacton Copolymer

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Summary: A construction of amperometric biosensor based on immobilized acetylcholinesterase and cholin oxidase is described and its application in the detection of organophosphate pesticides through enzyme inhibition measurements is discussed. The bioactive component of the sensor consists of acetylcholinesterase or cholin oxidase covalently immobilized on two types new polymeric synthetic membranes. Two types of the copolymers were used for the synthesis of membranes – the copolymer of polyacrylamide and acrylonitrile and the new copolymer of poly-(hexanlactam)-co-block-poly-(δ -valerolactone) with aliphatic polyester. It is investigated the technical characteristics of biosensor like, response time, linear range and operating stability. The factors affecting the inhibition and reactivation processes were investigated too.

Keywords: Biosensor, Pesticides, Enzyme, Acetylcholinesterase, Cholin Oxidase, Paraoxon.

1. INTRUDUCTION

Two main structures of biosensors were used for detection of pesticides – immunosensors and enzyme electrodes. The measurement of enzyme inhibition in pesticide biosensors has been performed using a variety of transducers and enzyme substrate systems. The most commonly used transducers have been pH-sensitive devices, such as glass pH electrodes, ion-selective field-effect transistors and metal-metal oxide electrodes. Amperometric devices have also become popular transducers for pesticides biosensors. Enzyme inhibition in these systems is normally assessed using either acetyl- or butyrylthiocholin as the substrate reagents, both of which hydrolyse to produce the electrochemically detectable species, thiocholine [1-4]. Amperometric enzyme-based sensing of pesticides is more commonly performed, however, using a bi-enzymatic reaction (between acetyl-or butyrylcholinesterase and choline oxidase) and hydrogen peroxide detection. Optical-based pesticides biosensors have also been described in recent years.



Wolfbeis and Koeller [5] reported the first fiber-optic device based on immobilized acetylcholinesterase that has been applied for sensing pesticides, although this was not a biosensor in a “true” sense. It consists of acetylcholinesterase immobilized on nylon beads and packed in a reactor column. A buffered proprietary substrate solution is passed through the column, where it is converted from a red solution into a blue dye-thymol blue and fluorescent dye [6, 7] upon action by the enzyme.

For extremely high price of enzymes, are used only immobilized on different carriers.

The purpose of the presented study is the construction of amperometric enzyme sensor for determination of pesticides based on covalently immobilized acetylcholinesterase and cholin oxidase on new synthetic membranes.

2. MATERIALS AND METHODS

Two types of the copolymers were used for the synthesis of membranes - the copolymer of polyacrylamide and acrylonitrile and the new copolymer of poly-(hexanlactam)-co-block-poly-(δ -valerolactone) with aliphatic polyester. The central B-block was synthesized. Powdered copolymer of polyacrylamide and polyacrylonitrile /200 mg/ dissolves in 5 ml dimethylformamide. By spin coating method the obtained mixture was poured out in thin membranes with thickness 0.05 mm. The membranes were heat treated in 50°C until the solvent evaporating. The membranes were activated by phosphate buffer with pH 7.5 and 12.5 ml formaldehyde for 4 hours in 45°C, according to [9]. Subsequently the activated membranes were washed up with distilled water to entire evaporating of the formaldehyde.

For obtaining the other new copolymer, was used hexanlaktam (HL), which was dried in eksikator in 60°C preliminarily with P₂O₅. Bi-functional polyester was synthesized via bulk anionic polymerization with δ -valerolactone in concentration of 3200 g/mol. By spin-coating method was obtained membranes with thickness 0.05 mm and they were activated by the above mentioned method.



The more appropriate copolymer for construction of biosensors was the copolymer of poly-(hexanlactam)-co-block-poly-(δ -valerolactone) because it can't be dissolved in solvents except in HCOOH and chloroform in the fixed correlation.

Cholin oxidase /EC 1.1.3.17/ isolated from *Alcalygenes sp.* and acetylcholinesterase /EC 3.1.1.7/ from *Electrophorus electricus* /FLUKA/ were with specific activity 12 U/mg and 850 U/mg respectively according [10] and [11]. The procedure of covalently immobilization of two enzymes (1% - solutions) was done separately and simultaneously in 0.1 M phosphate buffer, pH 5.5 for 8 hours.

After this the membranes were washed up with distilled water and 0.1 M NaCl until no absorbance was observed at 280 nm in the rinsing water.

3. RESULTS AND DISCUSSION

3.1. Preparation of the enzyme membranes

The immobilized enzymes were characterized regarding their pH, relatively activity (Table 1).

Table 1. Catalytic properties of the immobilized enzymes

Enzyme	Amount of bound protein [mg.g ⁻¹] dry carrier	Specific Activity U/mg	Relatively activity %	pH opt
acetylcholinesterase	3.2	120	94%	8.0
Cholin oxidase	20.8	9.36	75%	8.0
Simultaneously Acetylcholinesterase and cholin oxidase	18.4	80.40 6.15	-	8.0

For immobilized enzymes, separately and simultaneously was determined the temperature and pH optimum, quantity bound protein, specific and relatively activity. It can be seen from the table,



the relatively activity for both enzymes is high and pH optimums are similar for the free one.

The stability of the obtained membranes was investigated for bi-enzymatic system and for 20 consecutive measurements the specific activity was a constant.

3.2. Amperometric biosensor

For the construction of the biosensor, an amperometric oxygen sensor was used, as produced at HANA instruments HI 9142. The oxygen sensor of Ag /AgCl was placed in an electrolytic cell covered with a teflon membrane, permeable to oxygen and fed with a polarizing d.c. voltage of 0.8 V. The membranes with enzymes – acetylcholinesterase and cholin oxidase immobilized on synthetic membrane were fixed to the surface of the oxygen electrode by means of a dialysis membrane and an O-ring. The biosensor was dipped for each measurement into the measuring cell, containing 7.5 mM of Tris buffer, pH 8.0 at 25°C. The initial acetylcholinesterase activity was then measured by injecting replicate samples of the substrate solutions (100mM acetylcholine prepared in the Tris buffer) through valve. After that the solution of pesticide-diethyl-4-nitro phenyl phosphate /the technical 90% paraoxon/, provides of Aldrich chemical UK was injected in the measuring cell. The inhibited enzyme activity was measured by decreasing of consumption rate of the oxygen. The electrode was washed up by distilled water and in the experimental cell was injected regenerative solution immediately. The whole measurement cycle was repeated for sampling other pesticide solutions. The percentage inhibition (or reactivation) at each measurement step was evaluated using the expression (1):

$$\text{Inhibition (\%)} = [(E_0 - E)/E_0].100 \quad (1)$$

where E_0 is the initial inhibited sensor response time and E is the inhibited (or reactivated) sensor response time.

As a first step was explored the sensitivity of the biosensor toward acetylcholin. For substrat was used acetylcholin chloride /FLUKA/.

From the curves at different concentrations of acetylcholin it is seen that the response time of biosensor is 1-2 minutes in contacting a



static state's evidence (Fig. 1a). Range working of the substrate is 2.5 - 25 mM. The results of the 20-th measurements are shown on Fig. 1a. Subsequently was investigated the possibilities of the biosensor for detection of pesticides. The organophosphate compound – paraoxon was picked out. It is mentioned above. The inhibition of acetylcholinesterase is normally attributed to the acylation of the serine – OH in the active site of the enzyme by these compounds.

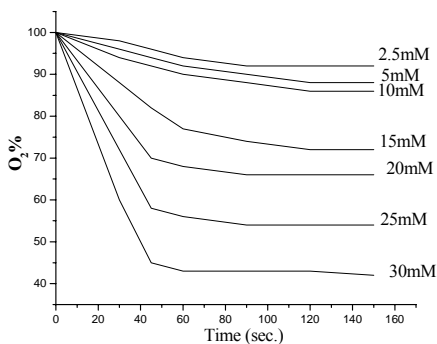


Fig. 1a. Kinetics curves of biosensor's response time for substrate acetylcholin

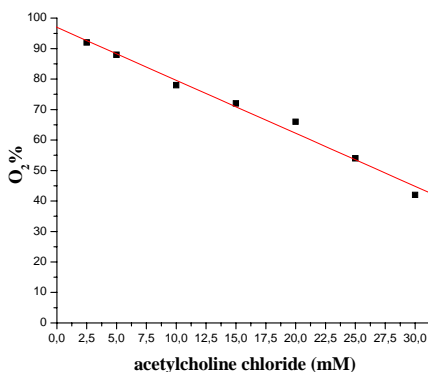


Fig. 1b. Calibration curve for acetylcholin obtained by biosensor



The reaction with acetylcholinesterase is analogous to the enzyme-substrate reaction, whereby a Michaelis enzyme-pesticide complex is first formed, followed by the transfer of the pesticide acyl groups to the serine – OH of the enzyme, and the concomitant release of the side product HB. However, unlike the acylated ester formed from the choline ester, the rate of hydrolysis of the phosphorylated and carbamylated acetylcholinesterase was very slow [7].

The factors affecting the inhibition and reactivation processes were investigated.

The regeneration of paraoxon-inhibited sensor was possible using 2-pyrimidine aldoxime methiodide (2-PAM), while repetitive substrate injection was necessary to reactivate the biosensor according to previous investigations. The good reactivating property of 2-PAM is due to the cationic center of the aldoxime [12]. This part can attach to anionic site of the inhibited enzyme and become arranged an easier and more proximate interact between the nucleophilic – OH and the phosphorylated esteratic site. As reactive agent of immobilized acetylcholinesterase and for working of biosensor was used solution of 0.1 mM - 2-PAM in 7.5 mM Tris buffer with pH 8.0. Solutions of organophosphate-paraoxon with various concentrations of this reagent were measured by biosensor. Initial solution with concentration 1.5 mM was prepared by dissolving the pesticide in acetone and the standard solutions were prepared by proper spacing in 7.5 mM Tris buffer, pH 8.0. It was obtained curves of biosensor response time by measurement cycle included determination of bound enzyme activity to membrane of biosensor – acetylcholinesterase. Subsequently is done the measurement of enzyme catalytic reaction rate in the probe with injecting of determinately quantity of inhibitor. After that it was done reactivating to 100% initial activity in solution of 2-PAM like the conditions described above and was measured again the enzyme activity. The response time of biosensor was determined by quantity of consumed oxygen. The corresponding calibration curve for paraoxon is illustrated in Fig. 2. The plot appears sigmoidal and exhibits linear response in the concentration ranges $5 \cdot 10^{-7} - 5 \cdot 10^{-6}$ M for paraoxon in the presence of the excess of substrate – 100 mM. The plot also shows the corresponding efficiency of the reactivation steps during the measurement cycle of the changing pesticide



concentration and. On the Fig. 2 is shown the results of 20 measurements.

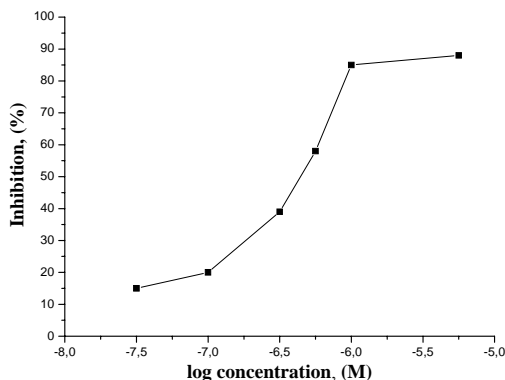


Fig. 2. Calibration plot obtained by biosensor for paraoxon

The obtained results were used for simulating calculations for optimizing the biosensor action.

The biosensor used two-steps enzyme catalytic reaction and electrochemical transformation of the substrate, in this case – oxygen. In this investigation is accepting the symmetric geometry of electrode and homogeny distribution of immobilized enzyme on the membrane. It is used the most simple one-substrate Michaelis's equation, including diffusion coefficients which depend of the membrane thickness, according to, S -acetylcholin, P_1 cholin and P_2 oxygen, which electrochemical is transformed in:

($t > 0$, $0 < x < d$):

$$\frac{\partial S}{\partial t} = D_s \frac{\partial^2 S}{\partial x^2} - \frac{V_1 S}{K_1 + S}$$

$$\frac{\partial P_1}{\partial t} = D_{P_1} \frac{\partial^2 P_1}{\partial x^2} + \frac{V_1 S}{K_1 + S} + \frac{V_2 P_2}{K_2 + P_2}$$

$$\frac{\partial P_2}{\partial t} = D_{P_2} \frac{\partial^2 P_2}{\partial x^2} - \frac{V_2 P_2}{K_2 + P_2}$$



The calculations were performed with the help of software – Matlab. With the similar software were performed the kinetic parameters and technical characteristics of the biosensor with immobilized enzyme. The calculations were obtained by number solving and statistical processing data uses the functions polyfit and polyval.

One of the most important parameter for the determination of properties of the sensor is the optimizing the response time. Distinguish between the experimental fixed values and calculated until reached of the established regime is 6%. It was investigated the response time dependence of the different parameters.

3.3. Dependence of the response time from the diffusion coefficient of the membrane D

It was leading the series of the simulations of the process with changing the values of D to the interval in 10^{-8} - 10^{-5} cm^2s^{-1} . It was determined that with increasing of the value of D the response time decreases. Model equation showed that the response time was the inversely proportional to the diffusion coefficient.

3.4. Dependence of the response time from the thickness of the membrane

According to theoretical investigations response time was the proportional to the thickness of the membrane raised on a square. This is in crucial meaning from a technology viewpoint. This was method for obtaining membranes with twin thickness in the different serieses of the elaboration. The dependence is highly sensitive, because it is in nonlinear quality and the membranes with different thickness influence maximum the output signal.

4. CONCLUSIONS

The biosensor for detection of pesticides with covalently simultaneously immobilized enzymes – acetylcholinesterase and cholin oxidase was constructed. For construction of biosensor the more appropriate membrane is the new copolymer based on the valerolacton, by the reason of its limited solubility in organic solvents. It is very important for detection of pesticides. The



characteristics of the immobilized enzymes on the new copolymer demonstrated light relatively activity and no change in pH optimums. The technical characteristics of the constructed biosensor based on the immobilized enzymes were investigated. The linear range was from 2.5 - 30 mM, response time was 1-2 minutes and the operating stability was 20 days. It was established that the linear range of the biosensor in the same experimental conditions and the used equipment was the absolutely compatible with other researcher's investigations [7, 13, 14].

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