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ORIGINAL ARTICLE

Optimization of acetylcholinesterase immobilization onto screen printed platinum electrode

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Summary
This article is focused on an optimization of acetylcholinesterase immobilization on screen printed platinum electrodes. An acetylcholinesterase layer cross linked by glutaraldehyde and another non cross linked were compared according to several parameters including background current, current before and current after inhibition by paraoxon. The percentage of inhibition was also calculated. The results obtained confirmed the importance of glutaraldehyde cross linking in the design of the acetylcholinesterase based biosensor.

Keywords: acetylcholinesterase – immobilization – cross linking – biosensor – electrochemical

INTRODUCTION

A number of instrumental analytical methods for the detection of organophosphorous compounds have been developed and their efficacy has been recently reviewed (Margariti et al. 2007). Biosensors are one of the most approachable methods; miniaturization and minimization of the consumption of reagents especially with preservation of good analytical parameters are the main advantages (Schulze et al. 2003). Several biorecognition components can be theoretically considered for biosensor construction. However, those including acetylcholinesterase (AChE; EC 3.1.1.7) or butyrylcholinesterase (BChE; EC 3.1.1.8) seem to be the most wide-spread (Skládal 1996). Cholinesterases are sensitive to noncompetitive inhibition by organophosphates and carbamates (Patočka et al. 2004) and even these inhibitors are industrially designed to the highest toxic efficacy for either military or agricultural purposes. The structural properties of AChE as well as its developmental characteristics have recently been the objects of intensive investigation (Wiesner et al. 2007). Although inhibition of cholinesterases is commonly considered as irreversible, some strategies for reactivation have been proposed. Oxime compounds were considered the best reactivators (Kuča et al. 2003, Musílek et al. 2007).

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The toxicity effect on viable organisms such as *Daphnia magna* can be suitable for the prescreening of organophosphates present in the environment (Vesela et al. 2006).

Biosensors with harboring cholinesterase have been prepared on diverse electrodes. Biosensors with either platinum or carbon matrix electrodes are frequently mentioned in the literature. Sotiropoulou and Chaniotakis (2005) combined AChE with a nanoporous carbon matrix resulting in a very low limit of detection (10^{-12} M) for dichlorvos assay. In another application, graphite electrodes with a prussian blue modifier, and AChE were used for the quantitative analysis of dichlorvos, fenthion and diazinon in organic solvents (Ciucu and Ciucu 2002). Immobilization of cholinesterase on a graphite-based electrode can be established by mechanical capturing onto cobalt phthalocyanine as a modifier and a acetylcellulose binder (Skládal et al. 1995, 1997). Platinum is another promising material for electrode construction in the manner described by Skládal and Krejčí (1996). A similarly based biosensor was employed for the pharmacological study of the reactivator HI-6 (Pohanka et al. 2007). The optimization of AChE immobilization onto platinum electrodes is the subject of this work. Cross-linking is considered as a stabilization step and a comparison will be made of a non cross-linked with a cross-linked layer.

MATERIAL AND METHODS

Chemicals

Acetylcholinesterase as lyophilized powder (2,000 U/mg of protein) and acetylthiocholine chloride (ATChCl) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Paraoxon-ethyl was purchased from Labor Dr. Ehrenstorfer-Schafers (Augsburg, Germany) and diluted with deionized water up to a final concentration of 100 ppm. Rgw Millipore system was used throughout for deionized water production. Glutaraldehyde and salts for phosphate buffered saline (PBS) preparation were purchased from Lachema (Brno, Czech Republic).

Immobilization procedure

Two types of immobilization procedures were considered for biosensor construction. 1µl of AChE solution (0.1 U/µl) was injected onto screen printed electrode (Pt, obtained from BVT Brno, Czech Republic) surface, the drop was let to dry and the injection was repeated five times. AChE immobilization on the first electrode was realized in a wet chamber; the second electrode was placed in a wet chamber with a glutaraldehyde saturated atmosphere. The biosensor was washed by PBS and let dry after the immobilization procedure.

Measuring setup

The measuring principle could be presented according to the following equation (Fig. 1).

Acetylthiocholine is hydrolyzed by AChE into acetic acid and thiocholine. Applied voltage +410 mV causes oxidation of thiocholine.

The biosensor was placed into 1 mM ATChCl in PBS and the steady state current was measured (i_0). After that, 10 µl of 100 ppm paraoxon was applied and allowed to incubate for 20 minutes. The output current was measured after washing the biosensor surface with PBS (i_i). The background current (i_b) was obtained when ATChCl was displaced by PBS. The percentage of inhibition (I) was calculated using the following equation:

$$I = \left(\frac{i_i - i_b}{i_0 - i_b}\right) \times 100$$

RESULTS AND DISCUSSION

Both described biosensors: the first with non-cross linked AChE and the second with an AChE layer stabilized by glutaraldehyde cross-linking were used for measuring following the background signal by measurement of the equilibrium current in 1 mM ATChCl before and after paraoxon inhibition. The values obtained served for percent of inhibition computing and are summarized in the following table 1.

The biosensor with cross-linked AChE and non-cross linked AChE provided a low background current that did not exceed 10 nA. Another current increased in the presence of ATChCl. The biosensor with non cross-linked AChE provided a current nearly 40 nA lower in comparison with the glutaraldehyde cross linked AChE biosensor. This phenomenon is probably caused by elution of non cross-linked AChE during the immobilization procedure. At the beginning of the experiments, we expected partial denaturation of AChE in the presence of glutaraldehyde. However, this phenomenon was not shown. If denaturation occurred during the immobilization procedure, its contribution was strikingly lower that that caused by the washing of the non cross-linked AChE.

The immobilization procedure presented correlated quite well with that used by Skládal and Krejčí (1996); however, they did not consider optimization of the immobilization procedure. Some other protocols were found suitable for AChE immobilization. We can mention enzymes immobilization on the screen printed sensor through a histidine tail (Andreescu et al. 2001) or an immobilization protocol based on AChE capturing into poly(vinyl alcohol) bearing styrylpyridinium groups (Devic et al. 2002). Although these immobilization protocols seem to be applicable, the immobilization procedures are quite complicated

and the costs per one analysis could increase, so that immobilization through simple polymeration by glutaraldehyde is more approachable. We could consider employing borhydride for the stabilization of Shiff bonds. This type of stabilization is suitable for immuno-analysis and is able to prolong the life of the biorecognition layer especially during the regeneration procedure. We consider the approximate lifetime of an AChE based biosensor to be one positive measuring cycle and no drastic regeneration procedures are demanded. On the other hand, in the future a more stable biorecognition layer could be needed, especially if the reactivators of AChE are improved.

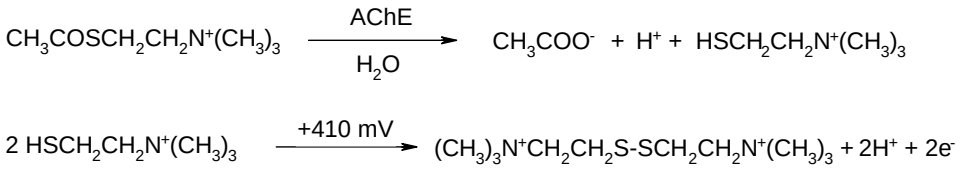

Fig. 1. **Chemical principle of the AChE based biosensor.**

We consider AChE cross-linking by glutaraldehyde should be a part of the immobilization procedure. Our experiments confirmed the importance of stabilization by glutaraldehyde cross linking and we expect the preparation of biosensors based only on cross-linking as a possibility for future experiments and the practical performance of the biosensor described for the detection of organophosphates.

Table 1. **The most important parameters of biosensor feasibility testing.** The meaning of the symbols is as follows: i_b background current, i_0 and i_i is current (all in nA) before and after inhibition by paraoxon. I is the percentage of inhibition. Two types of biosensors are included: with immoblized AChE without cross linking and the second with AChE cross linked by glutaraldehyde.

	glutaraldehyde cross-linked	non cross-linked
i_b	8±2	6±4
i_0	167±8	125±7
i_i	110±11	93±9
I	35.8	26.1

CONCLUSIONS

Two AChE immobilization procedures were tested. Both of them were based on adsorption on screen printed platinum electrodes. Cross-linking by glutaraldehyde was recommended as an important part of biosensor preparation and this method for designing biosensors is considered optimal for future experiments.

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REFERENCES

Andreescu S, Magearu V, Lougarre A, Fournier D, Marty JL: Immobilization of enzymes on screen-printed sensors via an histidine tail. Application to the detection of pesticides using modified cholinesterase. Anal. Lett. 34:429–540, 2001.

Ciucu A, Ciucu C: Organic phase amperometric biosensor for detection of pesticides. Roum. Biotechnol. Lett. 7:667–676, 2002.

Devic E, Li D, Dauta A, Henriksen P, Codd GA, Marty JL, Fournier D: Detection of anatoxin-a(s) in environmental samples of cyanobacteria by using a biosensor with engineered acetylcholinesterase. Appl. Environ. Microbiol. 68:4102–4106, 2002.

Kuča K, Patočka J, Cabal J: Reactivation of organophosphate inhibited acetylcholinesterase activity by α,ω-bis-(4-hydroxyiminomethyl-pyridinium)alkanes *in vitro*. J. Appl. Biomed. 1:207–211, 2003.

Margariti MG, Tsakalof AK, Tsatsakis AM: Analytical methods of biological monitoring for exposure to pesticides: recent update. Ther. Drug Monit. 29:150–163, 2007.

Musílek K, Kuča K, Jun D, Doležal M: *In vitro* reactivation potency of bispyridinium (*E*)-but-2-ene linked acetylchlinesterase reactivators against tabun-inhibited acetylcholinesterase. J. Appl. Biomed. 5:25–30, 2007.

Patočka J, Kuča K, Jun D: Acetylcholinesterase and butyrylcholinesterase – important enzymes of human body. Acta Medica (Hradec Kralove) 47:215–228, 2004.

Pohanka M, Jun D, Kuca K: Amperometric biosensor for evaluation of competitive cholinesterase inhibition by the reactivator HI-6. Anal. Lett. 40:2351–2359, 2007.

Schulze H, Vorlová S, Villatte F, Bachmann TT, Schmid RD: Design of acetylcholinesterases for biosensor applications. Biosens. Bioelectron. 18:201–209, 2003.

Skládal P: Compensation of temperature variations disturbing performance of an amperometric biosensor for continuous monitoring. Sens. Actuat. B Chem. 28:59–62, 1995.

Skládal P: Biosensors based on cholinesterase for detection of pesticides. Food Technol. Biotechnol. 34:43–49, 1996.

Skládal P, Krejčí J: Performance of the amperometric biosensor with immobilized butyrylcholinesterase in organic solvents. Collect. Czech. Chem. Commun. 61:985–991, 1996.

Skládal P, Nunes GS, Yamanaka H, Ribeiro ML: Detection of carbamate pesticides in vegetable samples using cholinesterase-based biosensors. Electroanalysis 9:1083–1087, 1997.

Sotiropoulou S, Chaniotakis NA: Lowering the detection limit of the acetylcholinesterase biosensor using a nanoporous carbon matrix. Anal. Chim. Acta 530:199–204, 2005.

Vesela S, Ondruska V, Kuca K, Patocka J: Test with *Daphnia magna*: A new approach to prescreen toxicity of newly synthesized acetylcholinesterase reactivators. J. Enzyme Inhib. Med. Chem. 21:427–432, 2006.

Wiesner J, Kříž Z, Kuča K, Jun D, Koča J: Acetylcholinesterases – the structural similarities and differences. J. Enzyme Inhib. Med. Chem. 22:417–424, 2007