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Carbofuran assay using Gelatin based Biosensor with Acetylcholinesterase as a Recognition Element

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Carbofuran is a neurotoxic pesticide acting as an inhibitor of enzyme acetylcholinesterase (AChE). The fact that carbofuran is able to inhibit AChE can be used for its assay using an appropriate sensor. Here, electrochemical biosensor having immobilized AChE and using acetylthiocholine as substrate was performed. The biosensor was consisted from a layer containing electric eel AChE and screen printed sensors. AChE was immobilized into gelatin membrane. The immobilization procedure was optimized considering temperature of drying and concentration of gelatin in mixture. Long term stability and interference of organic solvent was done as well. In the final experiment, carbofuran was assayed using the prepared biosensor. Limit of detection 5.01×10^{-10} mol/l (0.111 ng/ml) was achieved.

Keywords: biosensor; acetylcholinesterase; pesticide; inhibitor; screen printed electrode; voltammetry; amperometry

1. INTRODUCTION

Acetylcholinesterase (AChE; EC 3.1.1.7) is an enzyme being involved in cholinergic nervous system composed from both brain with spinal cord and peripheral nerves [1]. AChE plays a unique role in the system as a part terminating neuro-transmission by a fast hydrolysis of neurotransmitter acetylcholine in nerve junction [2]. Importance of AChE for pharmacology purposes is underlined by number of compounds inhibiting the enzyme. Drugs for Alzheimer disease treatment, galantamine, rivastigmine, donepezil and huperzine, are examples of AChE´s potent inhibitors [3,4]. Beside drugs, AChE is a target of inhibitors such organophosphorus and carbamate insecticides or nerve agents.

Compounds such as carbofuran, sarin, soman, tabun and VX can be exampled as toxins inhibiting AChE [5-10].

Because AChE is inhibited by the neurotoxic compounds and its activity can be simply assayed in vitro, the enzyme has been recognized a suitable biorecognition element applicable for construction of analytical devices for assay of the neurotoxic compounds. Device or method using AChE provides no or lowered signal in presence of the neurotoxic compound. The opportunity to assay structurally different compounds using one device is an advantage of AChE application as a biorecognition element. For the assay purposes, both standard optical and electrochemical tests are known [11]. Owing to the electrochemical tests, oxidation of thiocholine released from acetylthiocholine or oxidation of choline by cholineoxidase are common mechanisms of assay [12].

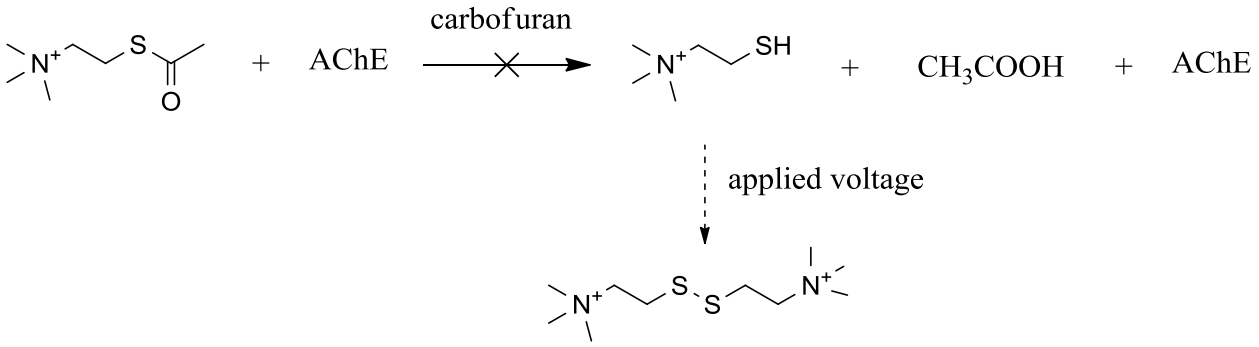

Figure 1. Chemical principle of AChE based assay. Acetylthiocholine is converted by AChE in thiocholine and acetic acid. The thiocholine is oxidized by applied voltage to dithiocholine in presence of applied voltage. Carbofuran or other AChE inhibitor stops the reaction.

Construction of reliable and readily to use biosensors with immobilized AChE is aim of the present experiment. Though biosensors based on AChE are known and a lot of promising devices have been constructed, simple and low cost biosensor being reliable enough is not currently available. Here reported experiment is devoted to preparation and characterization of a biosensor prepared by immobilization of AChE using gelatin and standard screen printed electrodes. Environmental, pharmacological and military application is expected of the constructed biosensor. Here, performance of the biosensor on assay of carbofuran was chosen after conisdering of carbofuran practical importance. Assay principle is obvious from figure 1. Carbofuran, or other inhibitors of AChE, stops conversion of acetylthiocholine so electrochemical reaction cannot be initiated.

2. EXPERIMENTAL PART

2.1. Immobilization procedure

AChE from electric eel was used as a biorecognition element. The enzyme was purchased from Sigma-Aldrich (Saint Louis, Missouri, USA) in lyophilized form with specific activity 16.7 μkat/mg protein and dissolved in phosphate buffered saline (PBS). Activity of enzyme was adjusted up 0.25 U/μl (4.17 nkat/μl) for 1 mM acetylthiocholine as substrate. In a separate tube, gelatin (Sigma-Aldrich)

was solved in PBS in concentration range 0.0068 – 1 % w/w. In a total 5 µl of AChE solution was mixed with 5µl of gelatin solution or PBS and applied on ceramic part (site opposite to electrodes) of screen printed sensor (BVT Technologies, Brno, Czech Republic) sized 25.4×7.3×0.6 mm. The screen printed sensor had platinum dot shaped (1 mm diameter) working, silver covered with silver chloride reference and platinum reference electrode. The biosensor was remained to dry at 37°C at thermostatic box. After drying, the biosensor was rinsed by saline solution in order wash out un-reacted parts of membrane. The used screen printed sensor is depicted as figure 2.

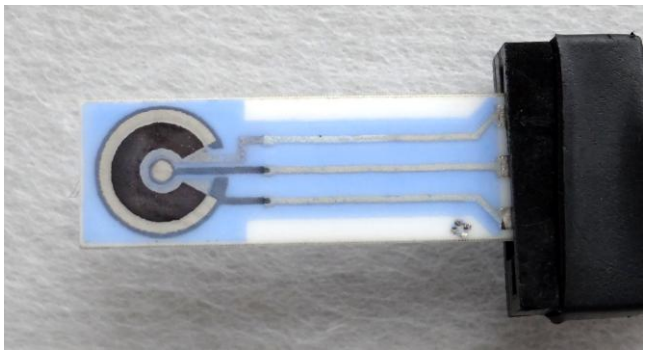

Figure 2. Screen printed sensor used for biosensor construction.

2.2. Biosensor performance

Carbofuran (Sigma-Aldrich) diluted in deionized water with 10 % v/v isopropanol was used as a sample. Calibration scale of carbofuran 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} mol/l was prepared. Biosensor was immersed into plastic reaction cell with volume 2 ml and linked to the PalmSens (PalmSens BV, Houten, Netherlands) device and connected with a computer. The device was controlled by software PSLite 1.8 (PalmSens BV). 800 µl of PBS and 100 µl of sample was injected into the reaction cell and let to incubate for 10 minutes. After that, reaction was started by addition of 100 µl of 10 mmol/l acetylthiocholine chloride. Accumulation of thiocholine was measured using chronoamperometry with previously found potential 640 mV [12]. After the assay, used electrodes were decontaminated by immersion into 30% calcium hypochlorite and wasted.

2.3. Data processing

Experimental data were processed in software Origin 8 (OriginLab Corporation, Northampton, MA, USA). Median inhibitory concentrations (IC_{50}) were calculated using Hill equation with hill coefficient adjusted up n=1. Limit of detection was calculated from confidence interval (95%) in calibration plot. All samples were assayed in tetraplicate.

3. RESULTS AND DISCUSSION

Screen-printed electrodes, which are used as economical electrochemical substrates, have gone through significant improvements over the past few decades with respect to both their format and their printing materials. Because of their advantageous material properties, such as disposability, simplicity, and rapid responses, screen-printed electrodes have been successfully utilised for the rapid in situ analysis of numerous compounds [13]. Due to numerous fields of applications, their design and materials used for their fabrication must be considered, because these properties markedly influence detection limits and linearity [14-17].

3.1. Immobilization

Using of gelatin for immobilization of biological part of a biosensor seems to be effective, which has been shown in detection of creatinine and peroxide [18-20]. Three temperatures and six concentrations of gelatin were chosen for immobilization purposes. The reached experimental data are displayed as figure 3. The temperatures were laboratory one (24 °C), and elevated temperatures (30 °C and 37°C) reached in an incubation box. We proved that increased concentration of gelatin in the mixture with AChE is beneficial for amount of captured AChE activity. Control immobilization where no AChE was applied into the mixture provided biosensors with no registered current when used in the assay therefore the current can be attributed to AChE only.

For all the three temperatures, efficacy of immobilization increased up to gelatin concentration 0.25% w/w. No further improvement of immobilization was found when concentration of gelatin was increased above 0.25% w/w. Applied temperature during drying had significant role like the concentration of gelatin. Increased temperature had a positive effect to amount of immobilized AChE. The optimal concentration of gelatin was same for all three temperatures. Owing to the reached data, use of immobilization mixture containing 0.25 % gelatin and drying at 37 °C has been chosen as optimal for biosensor construction.

Immobilization is a crucial step in biosensor preparation. Immobilized AChE is more resistant to external influences [21]. AChE can be bound to the electrode surface by a covalent immobilization using chemically modified particles [22]. In one experiment, Zhang and co-workers covalently immobilized AChE on a chitosan-coated microplate [23]. The covalent immobilization is more elaborative than capturing into membrane and it has lower reproducibility. Capturing of AChE into membrane is considered as an approachable method as it can stabilize the enzyme in environment where it is quickly degraded [24].

AChE is the most expensive element in the constructed biosensor. We expect that AChE is more than half of material costs needed for biosensor construction. Effort to immobilize AChE with a good efficacy is necessary for pertinent commercialization of the device.

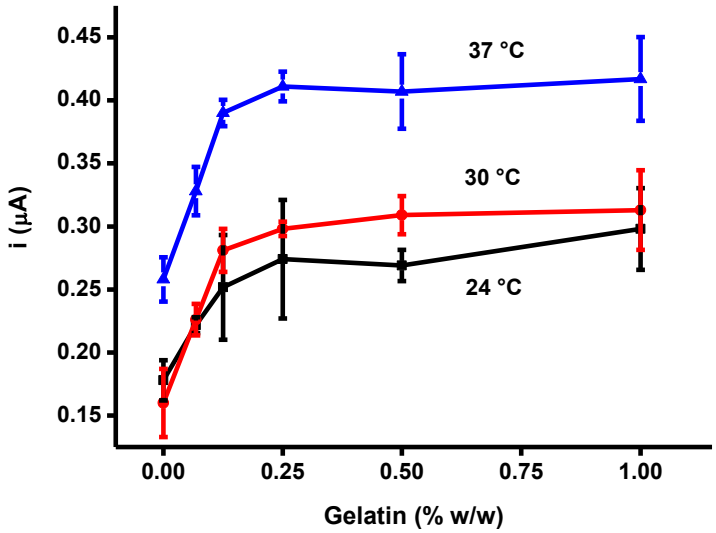

Figure 3. Optimization of AChE immobilization procedure. Error bars indicate standard deviation for n=4.

3.2. Interference

AChE is an enzyme sensitive to organic solvents presence. Both polar and non-polar solvents can inhibit AChE with quite low median inhibitory concentration [25-27]. As the solvents are inhibitors of AChE, they can interfere in an AChE based assay. Moreover, the solvents are needed for neurotoxins samples collection and extraction. Alcohol solvents are applicable for extraction of both chemical inhibitors and natural toxins with impact on AChE [28]. For the experiment, methanol, ethanol and 2-propanol were chosen as polar solvents miscible with organophoshporus and carbamate inhibitors of AChE. Inhibitory effect of the organic solvents is obvious from curves represented in figure 4.

IC_{50} values were 12.2% w/w for methanol, 13.8% w/w for ethanol, and 15.8% for 2-propanol. We can infer that the IC_{50} increases with length of carbonyl scaffold in solvent molecule. The lowest inhibitory effect (i.e. the highest IC_{50} value) had 2-propanol. However, all the solvents are quite potent inhibitors once presented in higher level. For the assay purposes, concentration of solvents should not exceed 10 %. For the more inhibiting solvents preferred maximal level 5 % should be obeyed. Higher content of solvents should be carefully considered and avoided in order to not initiate alteration in interaction of specific inhibitor such as orgonophoshorus toxin with AChE active site [29]. Samples prepared by extraction to organic solvent should be diluted by water or saline solution. The experimental data are not surprising. Similar conclusions were done by Scheerle and co-workers [30]. They proved quite strong inhibition of AChE caused by methanol and isopropanol. Some other compounds such as acetone and acetonitrile had inhibitory potency higher than the hydroxyl contained solvents. Owing to the findings, alcohol solvents should be preferred for samples manipulation. It should be emphasized that the assay of unknown sample described here is based on application of 100

µl of sample and 900 µl of other reagents (acetylthiocholine and buffer). Extract containing inhibitor in a pure alcohol have maximal level of alcohol just 10 % so an extensive interference will not take place.

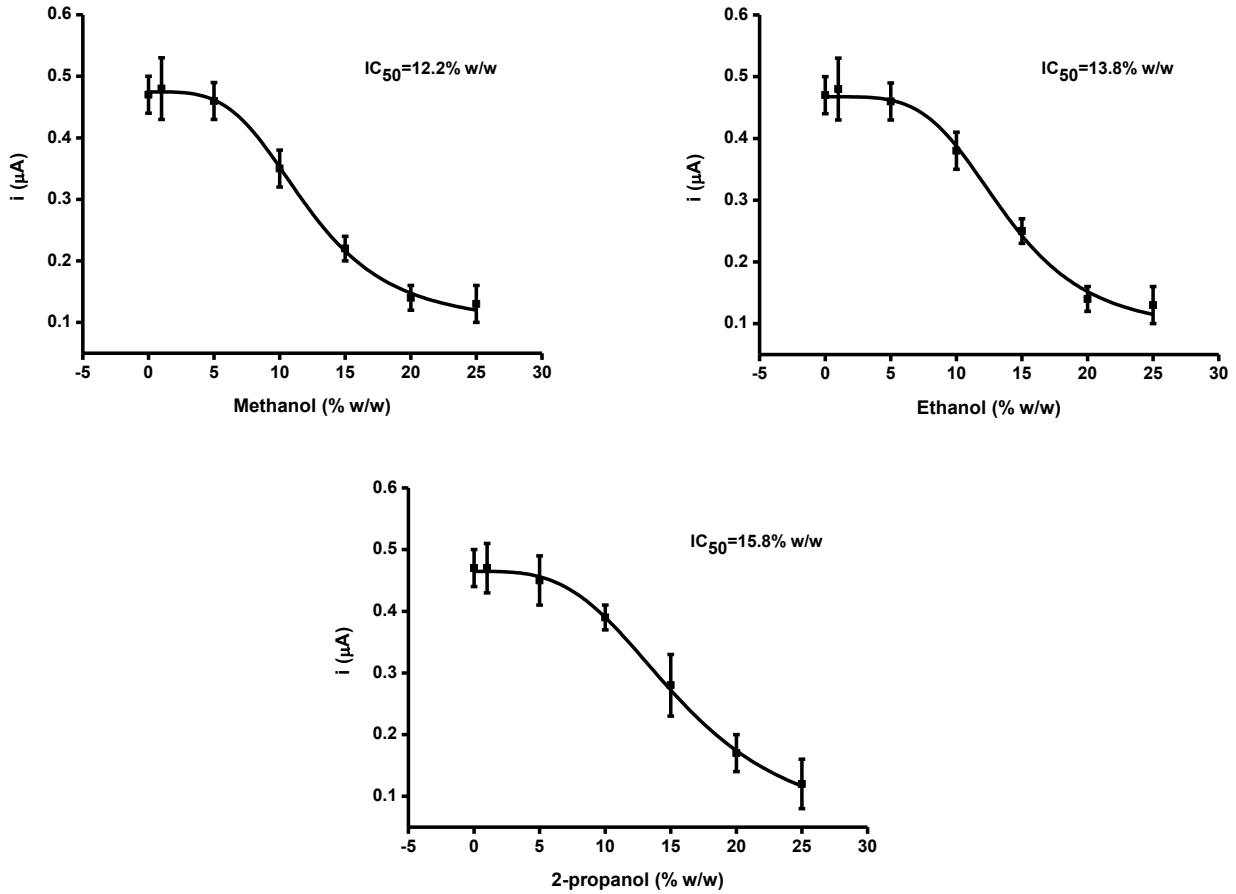

Figure 4. Inhibitory effect of organic solvents. Median inhibitory concentration (IC_{50}) is depicted for every plot. Error bars indicate standard deviation for n=4.

3.3. Biosensors long term stability

Long term stability of biosensors was estimated in a separate experiment. In a total 60 biosensors were prepared and four of them were used for blank assay every week. The biosensors were stored in a dark and dry place in laboratory temperature (SATP conditions). No protective package or wrapping was used. Experimental data can be found in figure 5. Detected current was decreased proportionally to the time of storage. The current decreased up to half of the initial value within two month. The measured current was suitable for assay performance even in the end of the experiment (98 days). However, sensitivity to substrate was decreased on a large scale in the end of the experiment.

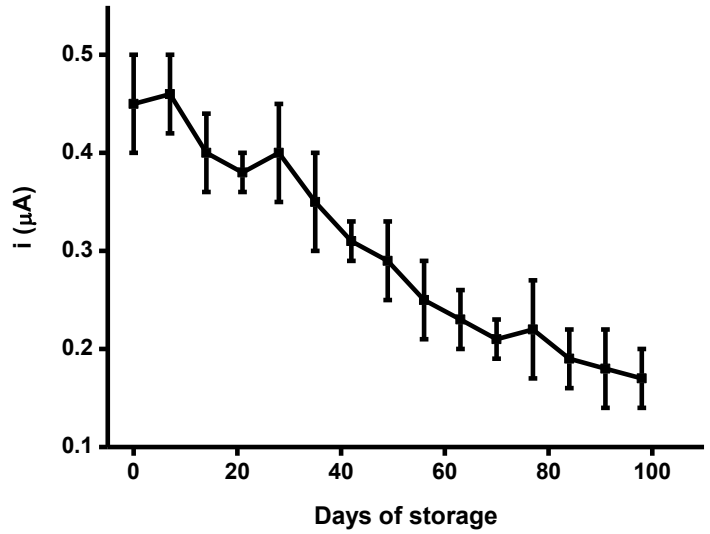

Figure 5. Long term stability of AChE based biosensors. Error bars indicate standard deviation for n=4.

3.4. Carbofuran assay

Carbofuran was chosen as a representative toxins being used in agriculture and exploitable for homicide as well as terrorist purposes. It has quite low median lethal (LD_{50}) dose, which is close to military applicable nerve agents. LD_{50} for intraperitoneal administration into rats is as low as 2 mg/kg [31]. In a comparison, sarin have LD_{50} value close to carbofuran. LD_{50} for sarin per orally administered into rats is 0.8 mg/kg. The other nerve agents had similar LD_{50} values as well [32]. Moreover, use of carbofuran is an ecological problem [33].

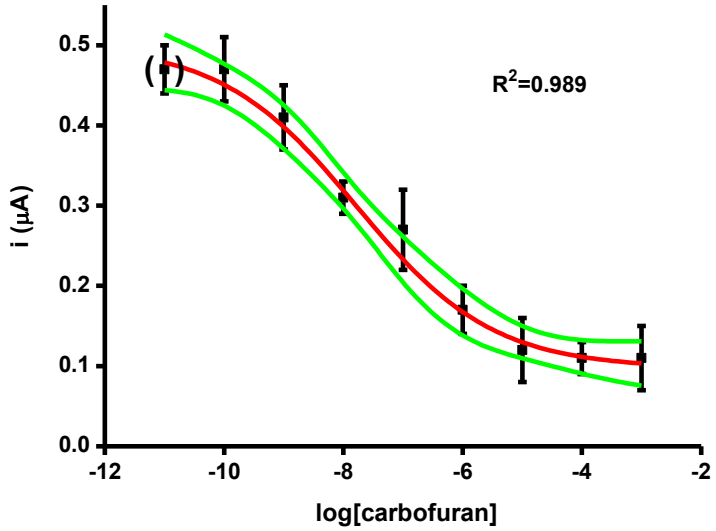

Figure 5. Long term stability of AChE based biosensors. Error bars indicate standard deviation for n=4 and green lines indicate confidence interval 95%.

When processed the calibration plot, coefficient of determination was calculated to be 0.989 and limit of detection was 5.01×10^{-10} mol/l (0.111 ng/ml). When considered sample sized 100 µl, an absolute amount of detected carbofuran was 11.1 pg. The detection limit was slightly better when compared to the quoted papers where limit of detection 0.192 ng/ml was achieved using gold nanocrystal immunosensor [34], 0.33 ng/ml for sol gel immunosensor [34], and 4.0×10^{-9} mol/l for an carbon nanotubes based biosensor [35]. On the other hand, sample consumption is higher in the method described here when compared to the quoted papers.

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