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Application of Electrochemical Sensors for Determination of Anticholinesterase Activity and Immobilization of Enzyme

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Abstract: Cholinesterases are important enzymes, which are responsible for the rapid elimination of acetyclholine after its releasing at cholinergic synapses. High activity of cholinesterases is present in patients with Alzheimer´s disease. Therefore, testing of new inhibitors of cholinesterases are important. For determination of the inhibition efficiency several methods can be used. In our case, the anticholinesterase activity expressed by IC_{50} of five carbamate compounds were tested using screen-printed electrodes and square-wave voltammetry. The next part of our work was focused on testing of two types of immobilization of enzyme on the surface of screen-printed electrodes. Kinetic parameters (K_m, V_m) were determined for immobilized and soluted enzyme. Obtained results were disscused.

Keywords: Screen-printed electrodes; Square-wave voltammetry; Cholinesterases; IC_{50}.

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Introduction

Cholinesterases (CHEs) constitute a group of esterases that hydrolyse choline esters at a higher rate than other esters. CHEs are divided into two groups. First one, acetylcholinesterase (ACHE), also known as real cholinesterase, is a key component of cholinergic brain synapses and neuromuscular junctions and is an enzyme composed of a catalytic triad in the active site consisting of three amino acids, namely serine (Ser), histidine (His), and glutamic acid (Glu). Second one, butyrylcholinesterase (BCHE) is also known as pseudocholinesterase, non-specific cholinesterase or simply cholinesterase. An important feature distinguishing BCHE present in serum and glia from ACHE present in neurons and erythrocytes is its kinetics with regards to concentrations of acetylcholine (ACH). BCHE is less substrate specific for ACH than ACHE [1, 2, 3, 4].

Acetylcholine, as a neurotransmitter, is synthesized in the pre-synaptic neurons by the enzyme choline acetyltransferase from choline and acetyl-CoA, and released by exocytosis into the synaptic cleft (Fig. 1). Reversible binding of ACH to the ACH receptor on the post-synaptic neuron initiates a signal and neurotransmission continues. Unbound ACH is captured by ACHE, hydrolysed and the choline returned to the pre-synaptic neuron for reuse [1, 5].

Alzheimer´s disease (AD) is one of the most common forms of dementia in adults. It is age-dependent neurodegenerative disorder that destroys brain cells, causing problems with memory, thinking and behaviour consequently, severe enough to affect work, lifelong hobbies or social life [6]. The physiopathology of AD is complex. There are several causes of this disease. Cholinergic hypothesis of AD stems from the following findings at post-mortem and biopsy studies: (a) deficits in acetylcholine synthesis and in the activity of choline acetyltransferase and high-affinity choline uptake; (b) reduced acetylcholine release and metabolism; (c) the loss of nicotinic, but not muscarinic cholinergic receptors [7].

Presently five drugs are used for symptomatic treatment of AD. Four, called cholinesterase inhibitors (CHEIs), tacrine (Cognex®), donepezil (Aricept®), rivastigmine (Exelon®), and galantamine (Razadyne®, previously known as Reminyl®), slow the metabolic breakdown of acetylcholine, an important brain compound involved in nerve cell communication. This slows the progression of cognitive impairment and can be effective for some patients with AD. These four medications are all approved for the treatment of mild to moderate symptoms of Alzheimer’s disease. The fifth medication, memantine (Nameda®) acts on the glutamatergic system by blocking NMDA-type glutamate receptors and is approved for the treatment of moderate to severe AD [1, 8].

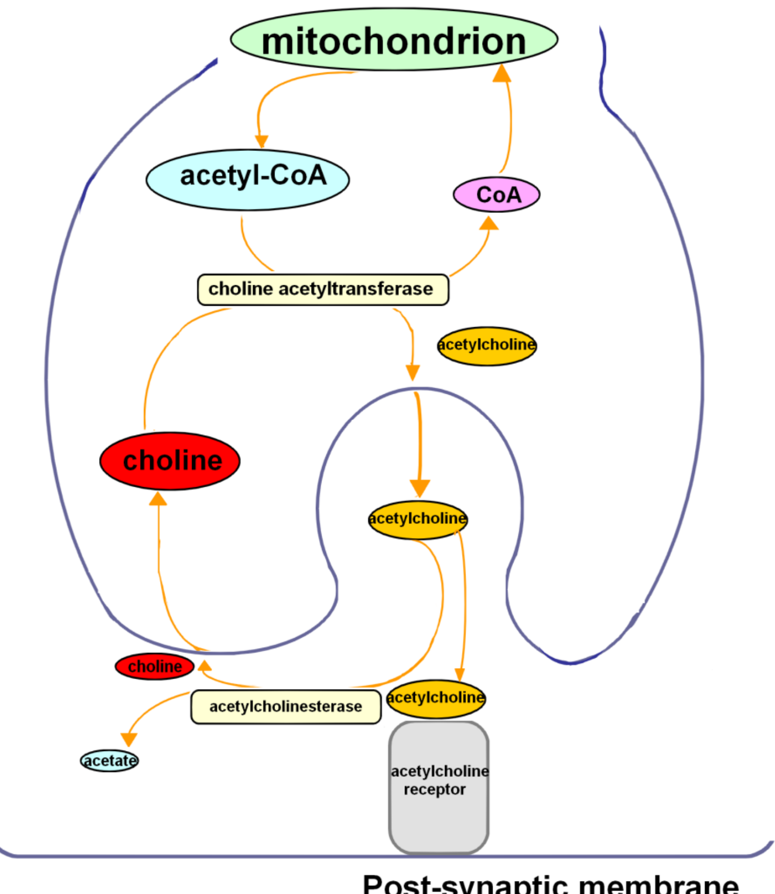

Fig. 1: *Synthesis and hydrolysis of acetylcholine.*

The inhibitors of acetylcholinesterase should increase the efficiency of cholinergic transmission by preventing the hydrolysis of released acetylcholine, thus making more acetylcholine available at the cholinergic synapse. CHEIs can be divided into three groups: tertiary amino compounds, organophosphates and carbamates (pseudo-irreversible inhibitors) [7].

The effectiveness of cholinesterase inhibitors should be judged by the value of IC_{50}. It is the concentration of inhibitor that causes a reduction of rate of inhibited (v_i) reaction to one half compared to uninhibited (v_0) reaction, i.e $v_i = \frac{v_0}{2}$.

Biosensors are analytical devices composed of three parts: bio-recognition element, physico-chemical convertor, and detector [9]. Biosensor utilizes the sensitivity and selectivity of a bio-receptor attached onto the surface of a physical transducer. The transducer is able to respond to and transform a bio-chemical and/or physico-chemical property into a measurable

signal as a result of a bio-recognition event between the bio-receptor and its target analyte (Fig. 2) [10].

The most important step in the development of an enzyme biosensor is the stable attachment of the enzyme onto the surface of the working electrode. This process is governed by various interactions between enzyme and the electrode material and strongly affects the performance of the biosensor in the term of sensitivity, stability, response time and reproducibility [10].

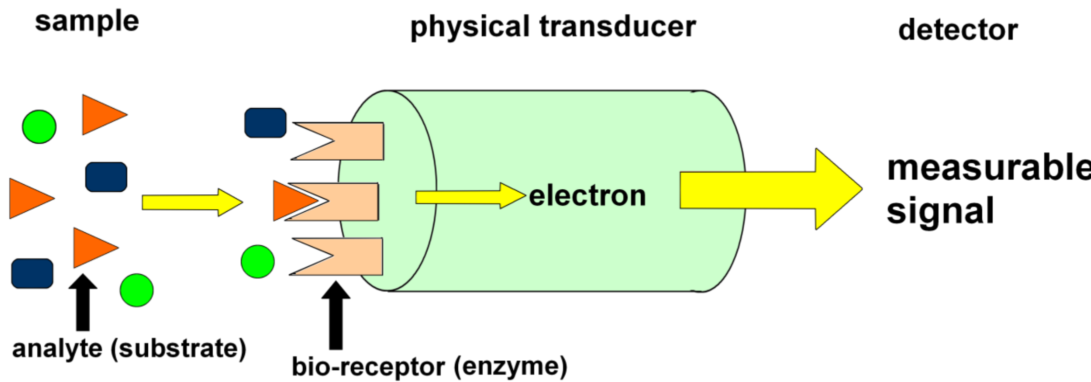

Fig. 2: *Scheme depicting the functional principles of an amperometric biosensor.*

Various immobilization strategies capable of deposition of cholinesterases onto the working electrode surface have been used [10]. There are several types of immobilization, such as physical adsorption, covalent coupling, self-assembled monolayer, physical entrapment etc. [10, 11].

Physical adsorption is the easiest and the least denaturing method of immobilization. The procedure consists of simple deposition of enzyme onto the electrode material and attachment of the enzyme through weak bonds such as Van der Waals and electrostatic interactions. This technique does not involve any functionalization of the electrode materials or covalent links and is generally non-destructive for enzyme activity. However, the biosensors with adsorbed enzyme suffer from a poor operational and storage stability [10].

Covalent coupling is the most widely used procedure for immobilization of enzyme. Typically, the procedure involves modification of the transducer with a bifunctional cross-linker such as glutaraldehyde that will bind on the support side the activated amino, carboxyl or hydroxyl groups and on the other end with the biomolecules [10, 12]. The mixture of glutaraldehyde and protein, in dependence of concentrations of components, creates

spontaneously the reticulum on the surface of sensor. Reaction of aldehyde group of glutaraldehyde with amino group of protein leads to creation of Schiff base (Fig. 3).

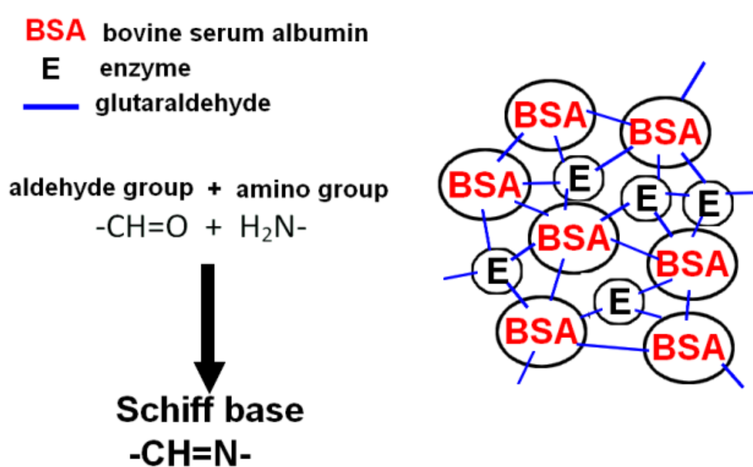

Fig. 3: *Covalent coupling of aldehyde group of glutaraldehyde with amino group of protein (E - enzyme)*.

Experimental

Chemicals, Reagents, Stock and Standard Solutions

Acetylcholinesterase (E.C. 3.1.1.7) lyophilizate from electric eel., acetylthiocholine (ATCH) iodide, BSA (bovine serum albumin), DTNB (5,5´-dithiobis-2-nitrobenzoic acid, Ellman´s reagent), and glutaraldehyde were all from *Sigma-Aldrich Praha* (Czech Republic). Dioxan p.a. was bought from *Lach-Ner* (Czech Republic). 0.1 M phosphate buffer saline (PBS) pH 7.4 was prepared from NaCl, KH_2PO_4, $Na_2HPO_4.12H_2O$ and KCl.

Substitued carbamates (Table I) were synthesized in the Institute of Organic Chemistry and Technology, University of Pardubice. 0.01 M analytical solutions of all carbamates were prepared by dissolving in dioxane and then this solutions were diluted by demineralized water as necessary.

To prepare the solution of ACHE, about 10 mg of enzyme preparation was dissolved in 20 ml of PBS and kept at –6 °C in plastic tube in the freezer. The actual activity of ACHE was verified every day using Ellman´s method.

Reaction mixture with glutaraldehyde was prepared by mixing of 1 mg of BSA (final concentration in reaction mixture was 1 mg·ml^{-1}), 1 ml of ACHE and 0.03 ml of glutaraldehyde (concentration of glutaraldehyde in reaction mixture was 30 %).

Table I: *Carbamates inhibitors.*

Inhibitor	Structure
1	C_6H_5–NH–C(=O)–O–CH_2–C_6H_4–O–C(=O)–CH_3
2	C_6H_5–NH–C(=O)–O–CH_2–C_6H_4–Cl
3	C_6H_5–NH–C(=O)–O–CH_2–C_6H_4–NO_2
4	C_6H_5–NH–C(=O)–O–CH_2–C_6H_4–O–CH_3
5	C_6H_5–NH–C(=O)–O–CH_2–C_6H_4–O–C_6H_5

Instrumentation and Electrodes

For determination of enzyme activity and 50% inhibitory concentration of tested carbamates, Ellman´s method (ELM) was used. This method is based on hydrolysis of substrate (acetylthiocholine for ACHE) to thiocholine (TCH) and acetic acid (HA). TCH reacts with 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) and produces yellow product 5-merkapto-2-nitrobenzoate, which is detected by diode-array spectrophotometer Hewlett-Packard 8453 at the wavelength 412 nm [13].

For determination of IC_{50}, screen-printed three-electrode sensors (AC1.W2.RS) from *BVT Technologies* were used too (Fig. 4). These sensors were employed for determination of kinetic parameters (Michaelis constant – K_M, maximum rate – V_m) of enzyme reaction with immobilized and soluble enzyme as well. Using acetylthiocholine as a substrate for ACHE thiocholine is produced during enzymatic reaction. Thiol-containing compounds are known to be oxidizable at the electrode surface, and the anodic oxidation current of thiocholine is measured using square-wave voltammetry method. Hydrolysis of ATCH and oxidation of originated TCH is shown in following reactions.

$$\text{acetylthiocholine} + H_2O + \text{ACHE} \longrightarrow \text{thiocholine} + \text{acetic acid} \quad (1)$$

$$2\ \text{thiocholine (red)} \longrightarrow \text{thiocholine (ox, dimeric)} + 2\,H^+ + 2\,e^- \quad (2)$$

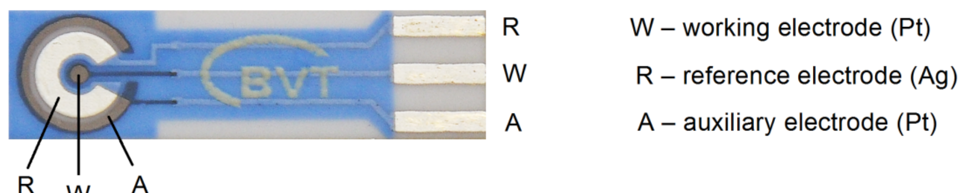

Fig. 4: *Screen-printed electrode from BVT Technologies Brno [14].*

Determination of IC_{50}

Ellman´s Method. Total volume of reaction mixture was 2 ml. Reaction mixture with no inhibition was prepared by mixing of 0.4 ml DTNB, 0.08 ml 0.001 M ATCH, X ml ACHE and Y ml PBS and reaction mixture of inhibited reaction by mixing 0.4 ml DTNB, 0.08 ml 0.001 M ATCH, X ml ACHE, Y ml of inhibitor and Z ml PBS. Volume of enzyme was chosen so that the enzyme activity in final reaction mixture was 0.2 $U \cdot ml^{-1}$.

The reaction was started by addition of ATCH. The dependence of absorbance (A, 412 nm) *vs*. time (t) was registered. The reaction rate ($v = \Delta A/\Delta t$) was calculated for all reactions (uninhibited and inhibited). The dependence of v_o/v_i *vs.* concentration of inhibitor was constructed (v_o – rate of uninhibited reaction, v_i – rate of inhibited reaction). The regression curve was calculated with corresponding equation ($y = k*x + q$). The IC_{50} value (x in regression equation) was calculated for $y = 2$ (coming from the definition of IC_{50}). Each measurement was performed in two replicates.

Measurements with Screen-Printed Sensors. Total volume of reaction mixure was 0.55 ml. Reaction mixture with no inhibition was prepared by mixing of X ml PBS, Y ml ACHE, and 0.0055 ml 0.1 M ATCH. Reaction mixture with inhibition contained X ml PBS, Y ml ACHE, Z ml inhibitor, and 0.0055 ml ATCH. Volume of enzyme was chosen so that the enzyme activity in final reaction mixture was 0.5 $U \cdot ml^{-1}$.

After 5 min incubation the dependence of current (I) *vs*. potential was registered. For each reaction, unhibited and inhibited, current corresponds to the reaction rate. The peak of oxidised thiocholine was visible at potential about 700 mV. The dependence I_o/I_i *vs.* concentration of inhibitor was constructed (I_o – current height of thiocholine peak for uninhibited reaction, I_i – current height of thiocholine peak for inhibited reaction). The regression curve was constructed and the corresponding equation was defined ($y = k*x + q$). The IC_{50} value (x in regression equation) was again calculated for $y = 2$. Each measurement was performed in two replicates.

Immobilization of ACHE on Electrochemical Sensor

All procedures of immobilization were realized using three-electrode sensors from *BVT Technologies* (Fig. 4).

Physical Adsorption. This type of immobilization was based on overspread of X ml of enzyme onto the surface of working electrode (final activity of enzyme in reaction mixture was 0.5 $U \cdot ml^{-1}$). Sensor was dried, placed into the Petri dish, and kept in the fridge.

Cross-Linking. At the beginning, the reaction mixture was prepared as described above. From this mixture, X ml was overspread onto the surface of working electrode (according to the actuall activity of enzyme) and sensor was dried, placed into the Petri dish, and kept in the fridge.

Sensors with immobilized ACHE were used for determination of K_M and V_m , stability in time, and comparison of two types of immobilizations.

Stability in Time. After drying of sensor with immobilized enzyme the dependence of current (I) *vs*. potential was registered. Reaction mixture was prepared by mixing of 0.5445 ml PBS and 0.0055 ml 0.1 M ATCH. This process was repeated after 24, 48 and 72 hours. The stability of immobilized enzyme was verified for both techniques, i.e. physical adsorption and cross-linking with glutaraldehyde. The dependence of current (I) *vs*. time was constructed.

Results and Discussion

Screen-printed sensors can provide many advantages such as measurements in low total volume of reaction mixture, good reproducibility, possibility to use one sensor for a number of inhibitors without adsorption onto surface, and short time of measurement when an appropriate electrochemical technique is used.

Inhibition Efficiency

Two methods were used for determination of inhibition efficiency of tested substituted carbamates. Values of IC_{50} obtained using Ellman´s method (ELM) and voltammetry (SWV) are shown in Table II. Two current peaks can be seen on the square-wave voltammogram during oxidation of thiocholine. The first one with maximum at +0.7 V vs. Ag/AgCl was attributed to oxidation of thiocholine and it was used further for peak height evaluation. The second peak probably corresponds to some other subsequent electrochemical process, where

oxidation form of thiocholine is involved, because it is observed only in the presence of thiocholine in solution. The solutions of buffer and inhibitors did not give any oxidation signals in monitored potential window.

It is obvious that the values of IC_{50} of tested inhibitors obtained by ELM and SWV methods differ. This is probably caused by different reaction conditions for these two methods. In ELM, thiocholine reacts with DTNB and absorbance of the yellow product (TNB) is detected by spectrophotometer while in voltammetry the thiocholine is directly oxidized by applied potential and current peak is recorded.

Table II: *Values of IC_{50} for tested inhibitors.*

Inhibitor	**IC_{50} (mol·ml^{-1})**	
	ELM	SWV
1	$8.17 \cdot 10^{-6}$	$4.80 \cdot 10^{-4}$
2	$5.75 \cdot 10^{-6}$	$5.17 \cdot 10^{-4}$
3	$2.71 \cdot 10^{-4}$	$3.48 \cdot 10^{-4}$
4	$3.85 \cdot 10^{-5}$	$5.47 \cdot 10^{-4}$
5	$2.14 \cdot 10^{-4}$	$3.06 \cdot 10^{-4}$

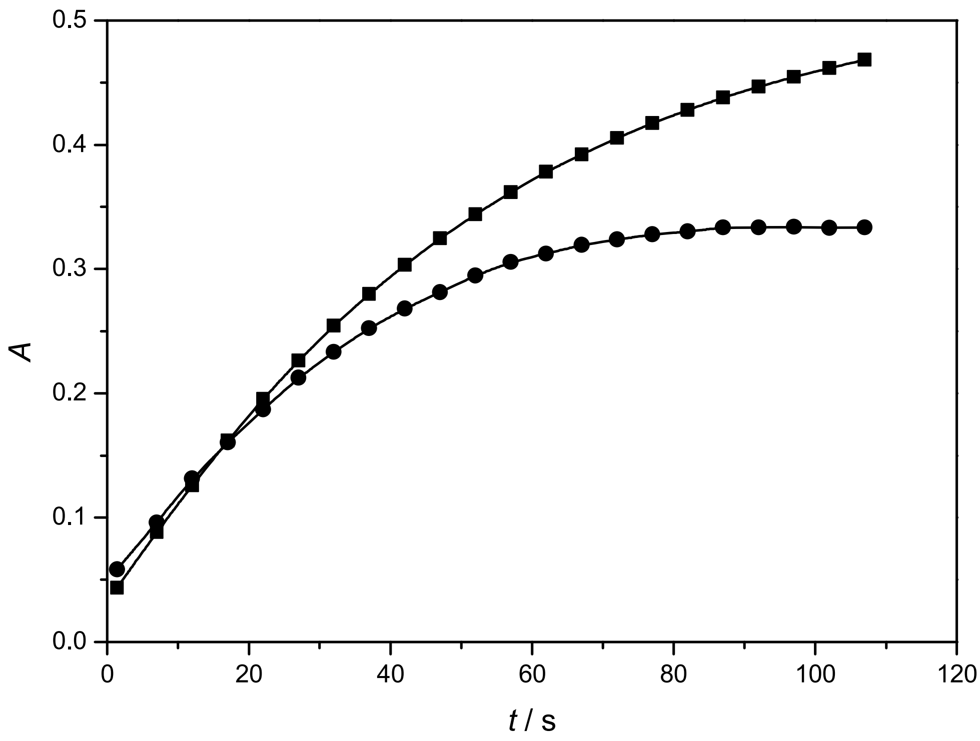

Fig. 5: *Dependence of absorbance* vs. *time*. Uninhibited (■) and inhibited reaction (●). Ellman´s method, c(ATCH) = $4 \cdot 10^{-5}$ M, ACHE activity 0.2 U·ml^{-1}, 0.1 M PBS, c(inhibitor **4**) = $2 \cdot 10^{-5}$ M.

On the basis of results, summarized in Table II, it is possible to conclude that the lowest values of IC_{50} were estimated for inhibitor **2** (4-chlorobenzyl phenylcarbamate) using ELM and inhibitor **5** (3-phenoxybenzyl phenylcarbamate) using SWV. Examples of uninhibited and inhibited reactions for spectrophotometric and voltammetric methods are shown in Fig. 5 and Fig. 6, respectively.

The presence of inhibitor decreases the reaction rate. Absorbance is lower due to less amount of formed yellow reaction product produced from enzymatically generated thiocholine. In voltammetry, the oxidation current of thiocholine is lowered comparing to unihibited reaction as its production is diminished by the inhibitor. The example of determination of IC_{50} is then presented in Fig. 7.

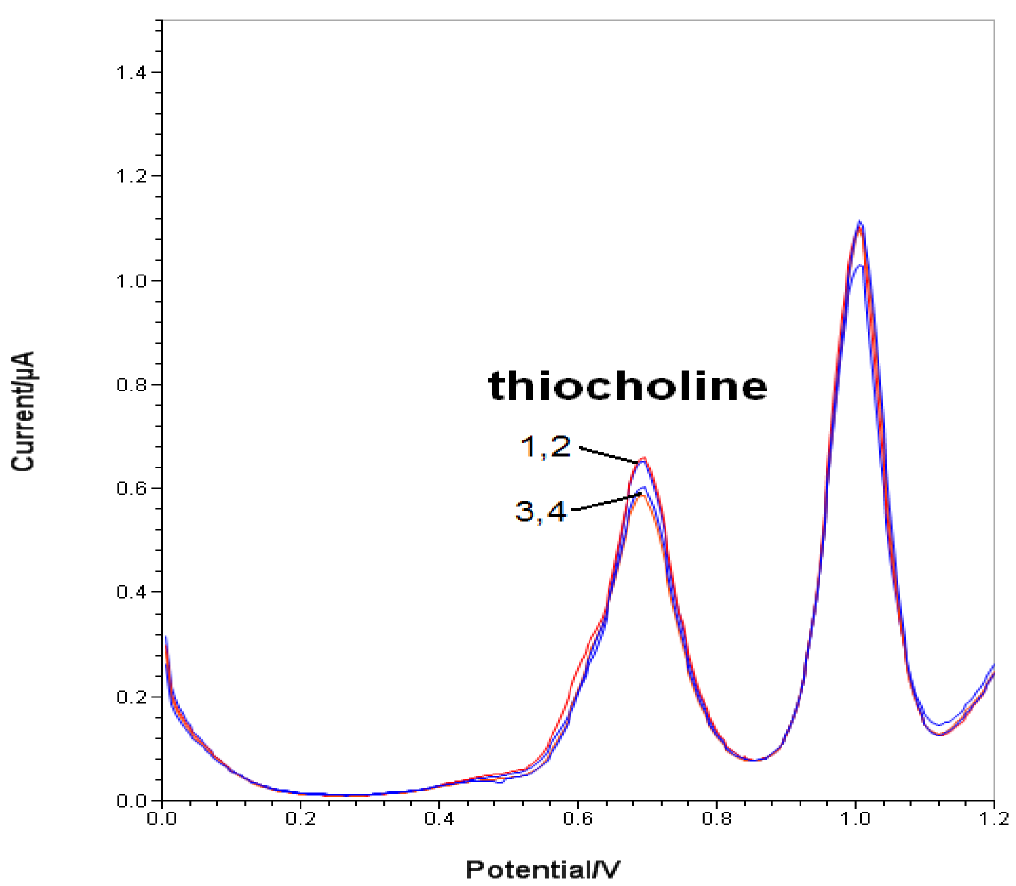

Fig. 6: *Dependence of current* vs. *potential*. Voltammograms for uninhibited (1, 2) and inhibited reaction (3, 4). SWV, c(ATCH) = $1{\cdot}10^{-3}$ M, activity of ACHE 0.5 $U{\cdot}ml^{-1}$, 0.1 M PBS, c(inhibitor **4**) = $1{\cdot}10^{-3}$ M.

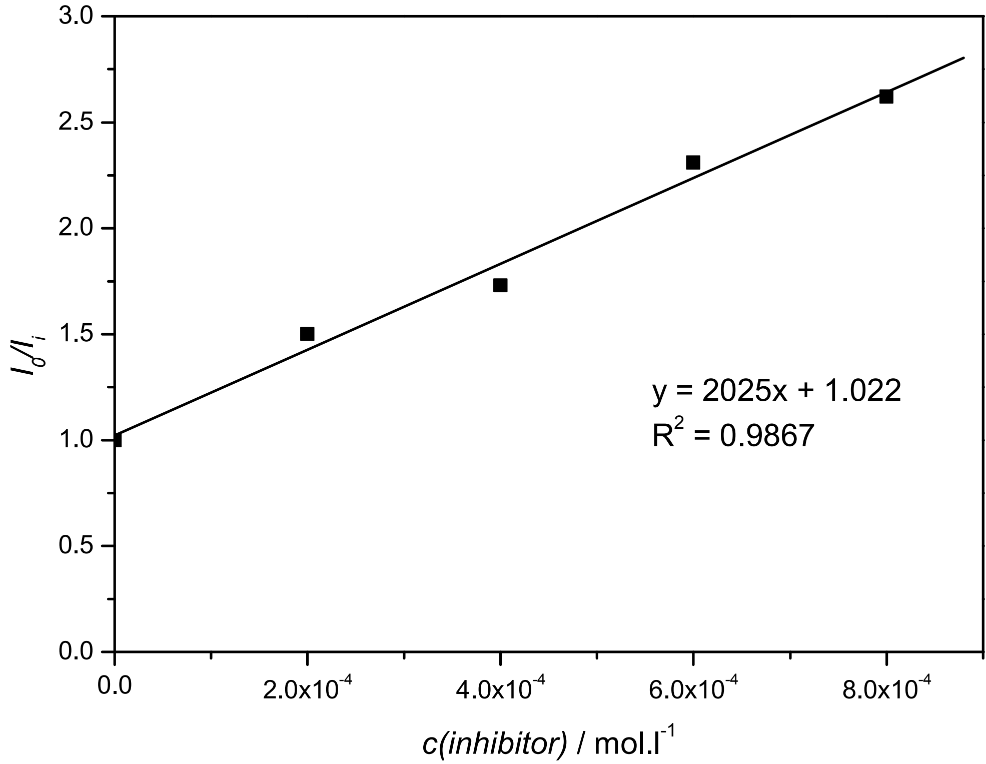

Fig. 7: *Dependence of I_o/I_i vs. concentration of inhibitor **4***. Evalutation of peak heights, SWV, c(ATCH) = $1{\cdot}10^{-3}$ M, activity of ACHE 0.5 U·ml^{-1}, 0.1 M PBS, c(inhibitor **4**) = $1{\cdot}10^{-3}$ M.

Kinetic Parameters

Important kinetic parameters (K_M, V_m) were determined using three-electrode sensors. Firstly, these constant were calculated for reaction mixture with soluble enzyme. Secondly, two types of immobilization (physical adsorption and cross-linking with glutaraldehyde) of ACHE onto surface of sensor were used. Obtained results are presented in Table III.

Table III: *Kinetics parameters for different immobilizations of ACHE.*

Parameter	ACHE in solution	Adsorption	Cross-linking with glutaraldehyde
K_M	0.128 M	0.024 M	0.018 M
V_m	91.07 μA	12.76 μA	7.421 μA

Immobilization of enzyme affects kinetic parameters. Immobilization of enzyme onto the surface of sensor decreases Michaelis constant and maximum rate as well. Significant

decrease both of kinetic parameters was observed in case when the enzyme was cross-linked with glutaraldehyde.

The time stability of biosensor was verified. For both of types of immobilization, the response of biosensor was measured 24, 48 and 72 hours after immobilization of enzyme.

Physical adsorption showed better results than cross-linking with glutaraldehyde. Biosensor exhibited good stability in time; activity of enzyme had fallen to 65 % after 72 hours. The dependence of current vs. time is shown in Fig. 8. The activity of enzyme was undetectable after 24 hours when cross-linked with glutaraldehyde. Therefore, physical adsorption is preferred for experiments.

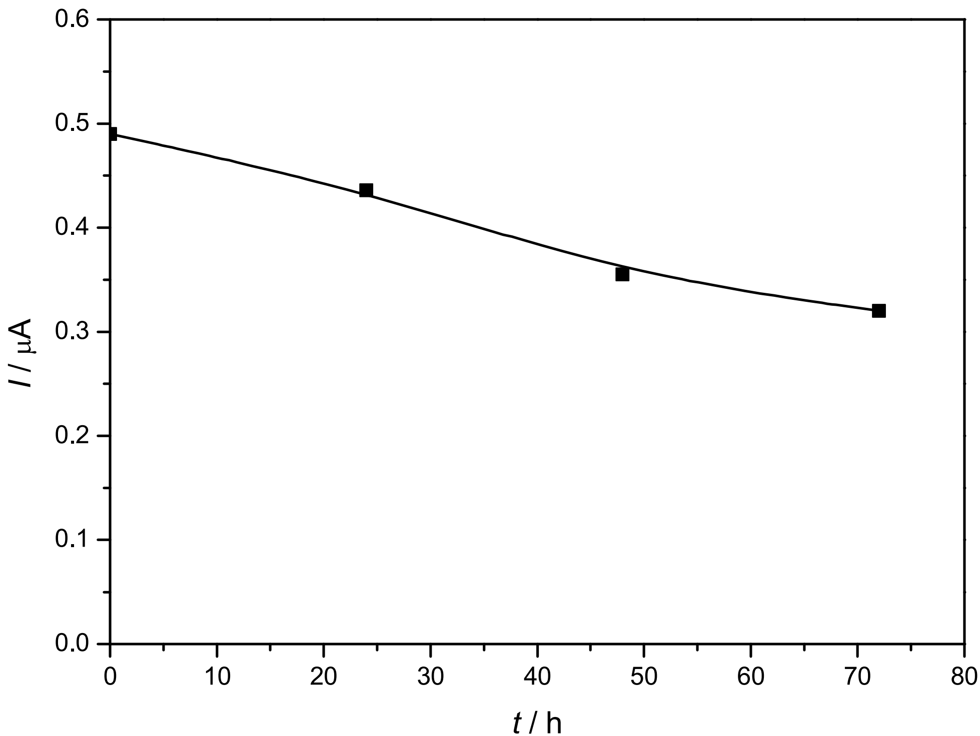

Fig. 8: *Dependence of peak heights* vs. *time for physical adsorption of ACHE*. SWV, c(ATCH) = $1 \cdot 10^{-3}$ M, activity of immobilized ACHE 0.5 $U \cdot ml^{-1}$, 0.1 M PBS.

Conclusions

In this article, two types of immobilization of acetylcholinesterase onto surface of screen-printed electrodes and testing of new inhibitors of cholinesterases have been described. Inhibition efficiency and kinetic parameters obtained by two different methods (based on spectrophotometry and voltammetry) were calculated.

It is possible to conclude that screen-printed sensors with platinum working electrode are suitable for determination of inhibition effects of tested carbamates and are applicable for immobilization of enzyme onto surface of sensor as well, whereas physical adsorption shows better stability of immobilized enzyme in time than cross-linking with glutaraldehyde.

Acknowledgements

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