

Electrochemical acetylcholinesterase biosensor for detection of cholinesterase inhibitors: study with eserine

Nina Lokar¹, Veno Kononenko², Damjana Drobne², Danilo Vrtačnik¹

¹University of Ljubljana, Faculty of Electrical Engineering, Laboratory of Microsensor Structures and Electronics, Ljubljana, Slovenia

²University of Ljubljana, Biotechnical Faculty, Department of Biology, Ljubljana, Slovenia

Abstract: Cholinesterase inhibitors are widely used as pesticides, as chemical warfare agents and as drugs to treat symptoms of Alzheimer's disease. Therefore, it is a high need to develop methods for their detection which are fast, sensitive, and reliable. This paper reports a preliminary work in the development of an electrochemical biosensor based on acetylcholinesterase (AChE) which is constructed by immobilization layers – cysteamine/glutaraldehyde/AChE on thin layer gold electrode for detection of cholinesterase inhibitors. Eserine (physostigmine) was used as a test inhibitor. The enzyme immobilization efficacy was evaluated by measuring activity of immobilized enzyme via Ellman's method. The enzyme activity of the initial reduction of 33% in five days remained after that stable for at least one week. Chronoamperometric response to substrate acetylthiocholine chloride (ATCl) was assumed to follow Michaelis-Menten kinetics. After exposure biosensor to 25 μM eserine for 10 min, 70% inhibition of enzyme was detected. Reactivation factor of inhibited AChE was determined as 0.016 min^{-1} .

Keywords: Electrochemical biosensor; Acetylcholinesterase; Eserine; Ellman assay; Chronoamperometry; Cyclic voltammetry

Elektrokemijski acetilholinesterazni biosenzor za detekcijo holinesteraznih inhibitorjev: raziskava z eserinom

Izveček: Holinesterazni inhibitorji se pogosto uporabljajo kot pesticidi, kot kemična bojna sredstva ali kot zdravila za zdravljenje simptomov Alzheimerjeve bolezni, zaradi česar je razvoj hitrih, občutljivih in zanesljivih metod za njihovo zaznavanje izrednega pomena. Članek podaja rezultate začetnih raziskav v razvoju elektrokemijskega biosenzorja za detekcijo inhibitorjev acetilholinesteraze (AChE). Biosenzor je zgrajen na steklenem substratu, na katerem je strukturirana tankoplastna zlata elektroda. Na njej so imobilizirani cisteamin/glutaraldehyd/AChE. Kot testni inhibitor je uporabljen eserin (fizostigmin). Učinkovitost imobilizacije encima smo spremljali z merjenjem aktivnosti imobiliziranega encima z Ellmanovo metodo. Po petih dneh od izdelave biosenzorja se je aktivnost encima stabilizirala. Kronoamperometrični odziv na substrat acetiltioholin klorid (ATCl) je predpostavljeno sledil kinetiki Michaelisa in Mentenove. Po 10 minutni izpostavljenosti biosenzorja 25 μM eserinu smo detektirali 70% inhibicijo encima. Reaktivacijski faktor inhibiranega AChE je bil 0.016 min^{-1} .

Ključne besede: elektrokemijski biosenzor; acetilholinesteraza; eserin; Ellmanova metoda; kronoamperometrija; ciklična voltametrij

*Corresponding Author's e-mail: nina.lokar@fe.uni-lj.si

1 Introduction

In 1962, Clark and Lyons proposed the initial concept of glucose enzyme electrodes which led to a powerful analytical instrument for the detection of glucose in samples from patients with diabetes. This resulted in

popularization of biosensors [1, 2]. In comparison with the conventional analytical techniques (such as chromatography and mass spectrometry), biosensors are typically easy to use, suitable for *in situ* measurement, and low cost [3]. Biosensors are remarkably diverse,

comprising a wide range of combinations of biorecognition and transduction elements [4, 5]. Enzymes are the most often used bio-recognition elements, whereas the most widely applied biosensors are based on electrochemical transduction method [6].

Enzyme-based biosensors are implemented in direct or indirect form [5]. One example of indirect form option is cholinesterase (ChE) biosensors which are based on enzyme inhibition [7, 8]. They are useful tools for detection of ChE inhibitors. The most widespread applications of such biosensors are detection of pesticides (organophosphates and carbamates) and warfare nerve agents (sarin) [6]. ChE inhibitors are also recognized as prevailing choice (galantamine, rivastigmine, donepezil) in the treating of Alzheimer's disease symptoms [9]. For these applications, the inhibitors detection and analysis are of great importance. Eserine has been used by many researchers as a reference standard in the evaluation of new ChE inhibitors [10]. Eserine can be found naturally in the Calabar bean. Eserine binds at both the anionic and esteric sites of acetylcholinesterase (AChE), forming a drug-enzyme complex. The mechanism is not completely reversible [11].

This paper reports construction of an AChE biosensor based on immobilization layers of cysteamine, glutaraldehyde, and AChE, which are chemically bound to the thin gold electrode layer. Cyclic voltammetry (CV) and chronoamperometry (CA) were applied to demonstrate the feasibility of fabricated biosensor structure for the detection of one well-known and important neurotransmitter inhibitor eserine.

2 Materials and methods

2.1 Chemicals

Following compounds were purchased from Merck (Germany): 95-98% H_2SO_4 , 30% H_2O_2 , 100 mM cysteamine, glutaraldehyde 5% (v/v), AChE from electric eel (50 U/mL), 100 mM K-P buffer solution with pH = 8.0 (47 mL 1 M K_2HPO_4 + 3 mL 1 M KH_2PO_4 + 450 mL milliQ water), 5 mM (if not specified differently) substrate acetylthiocholine chloride solution (ATCl), and 10 mM eserine. 0.1 M KCl containing 2 mM ferri/ferro-cyanide were prepared and purchased from Merck and Fluka (Belgium), respectively.

2.2 Instruments

Spectrophotometry was carried out by microplate reader Cytation 3 from BioTek (Germany).

CV and CA measurements were carried out using potentiostat EmStat3+ Blue equipped with PSTrace 5.3 software from PalmSens BV (The Netherlands). Miniature Ag/AgCl, 3 M KCl reference electrode, model ET073 was from eDAQ Pty Ltd (Australia).

2.3 Biosensor structure

Electrodes. Replaceable chip, containing an array of 6 thin layer gold working electrodes (Figure 1a) was manufactured. Gold electrodes were applied on glass substrate using microfabrication processes – sputtering of Cr/Au with thicknesses 30 nm/120 nm, photolithography patterning and wet etching. The diameter of the working electrodes was 0.3 cm, resulting in an apparent geometric area of 0.07 cm². As counter (or auxiliary) electrode, platinum wire was used, and it was wrapped around the external reference electrode (Figure 1b). All three electrodes were installed in an originally designed electrochemical cell with reservoir volume of 5 mL (Figure 1c). Chip with working electrodes in electrochemical cell was connected to potentiostat (Figure 1d) with spring tips.

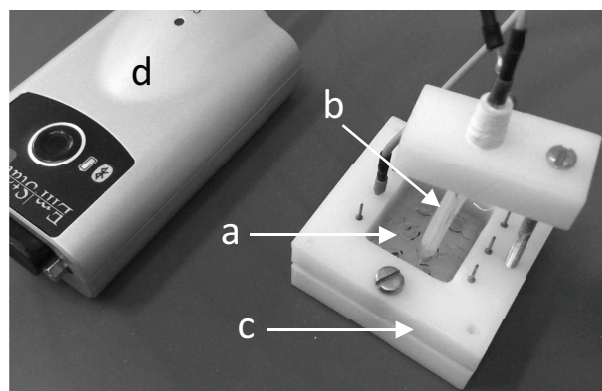


Figure 1: Experimental setup of biosensor.

AChE immobilization. Gold electrode chip was first cleaned with piranha solution (H_2SO_4 : H_2O_2 in volume ratio 3:1), for 10 minutes. Thereafter, the electrode chip was well rinsed with ultrapure water and imme-

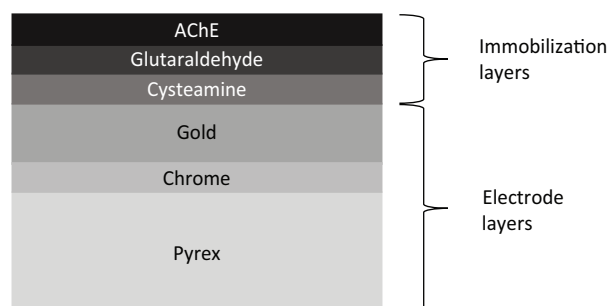


Figure 2: AChE immobilized gold electrode.

diately immersed in cysteamine, glutaraldehyde aqueous solution and in AChE solution as described in [12]. Schematic description of resulting sample structure is shown in Figure 2.

2.4 Measurement procedure

AChE activity measurement. AChE hydrolyses ATCl to acetate and thiocholine chloride (TChCl) which can be further measured spectrophotometrically [13]. Standard spectrophotometric Ellman colorimetric assay [14] was used to check the efficacy of the enzyme immobilization procedure on the gold surface. Test gold plated glass with 0.2 cm² had AChE immobilized as described previously. The absorbance of yellow product 5-thio-2-nitrobenzoate (TNB) was measured after reaction time of 10 minutes and the product quantity was evaluated using the Lambert-Beer law as Eq. 1 [15]:

$$\text{Product quantity} = \frac{AV}{\epsilon l} \quad (1)$$

where A is absorbance at 405 nm, V is volume of the sample (products TChCl, Ellman's reagent, K-P buffer), ϵ known molar extinction coefficient of TNB ($\epsilon = 14,150 \text{ M}^{-1}\text{cm}^{-1}$ [16] and l is the beam length in the sample, respectively. In our case parameters were: $V = 250 \mu\text{L}$, $l = 0.75 \text{ cm}$.

CV and CA techniques [17] were applied to measure enzyme activity on immobilized gold electrode chip. In the case of measurement by CV, the input voltage

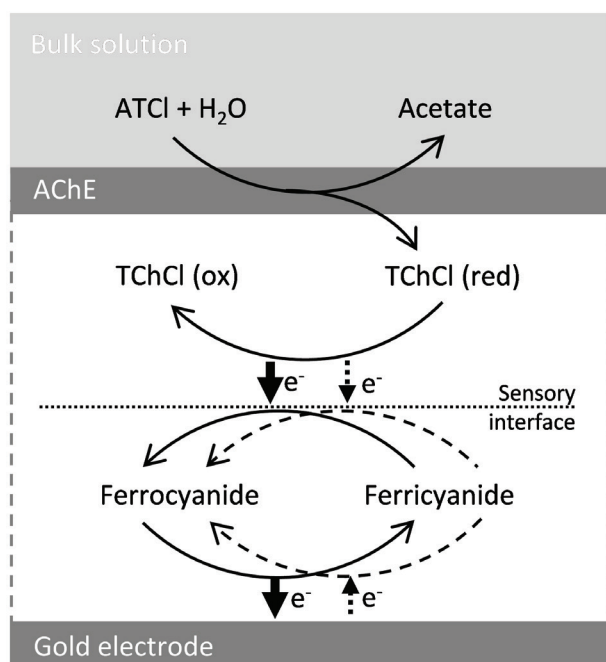


Figure 3: Sequence of reactions occurring at AChE biosensor.

was scanned from -0.1 V to +0.6 V, with potential step of 0.01 V and scan rate of 0.06 V/s. CA measurement was carried out at DC potential of 0.4 V. Measurements, if not specified differently, were performed 10 minutes after exposure substrate ATCl to AChE enzyme. Substrate was dissolved in K-P buffer and solution of ferricyanide with KCl. Sequence of reactions occurring at an AChE biosensor is shown schematically in Figure 3. The result of the ATCl hydrolysis is the electrochemically active TChCl, which exchanges an electron with an electrochemical mediator ferricyanide when passing from reduction to oxidation state. Mediator transfers the electron to the electrode via redox reactions. Oxidation and reduction process on cyclic voltammogram are referred to solid and dashed lines in Figure 3, respectively [18, 19]. Electrons on the electrode are detected by external electrical circuit (potentiostat).

AChE inhibition measurement. AChE biosensor is based on enzymatic inhibition mechanism. Eserine as analyte inhibits the normal enzyme function. The enzyme inhibition is therefore determined by the difference in measured electric currents in the absence (i_0) and presence (i) of eserine. The inhibition is calculated as Eq. 2 [20]:

$$\text{Inhibition} = (i_0 - i) / i_0 \quad (2)$$

Measuring protocol of AChE inhibition was performed in three steps. The biosensor was immersed in the substrate ATCl solution and solution of ferricyanide with KCl, for 10 min and the signal i_0 was measured (step 1). Then, the AChE biosensor was rinsed three-times with the K-P buffer solution (step 2) and immersed in a new substrate and ferricyanide solution with eserine addition for reaction and incubation time of 10 min, then residual activity i_i was measured (step 3).

3 Results and discussion

Activity of the immobilized enzyme was evaluated spectrophotometrically by measuring the quantity of produced thiocholine chloride product. The activity was monitored in time period of 12 days (Figure 4).

From Figure 4 it can be seen that after 33% initial decrease of enzyme activity in five days, the activity remained steady for at least one week. The reason for large initial loss of activity can be attributed to stabilization of biostructure or incomplete covalent binding of the enzyme, surface defects and impurity of enzyme.

After confirming immobilized enzyme activity by Ellman's method, AChE biosensor performance was investigated by CV and CA measurements on biosensor

electrode structure. Figure 5 shows CV response obtained in absence and in presence of ATCI.

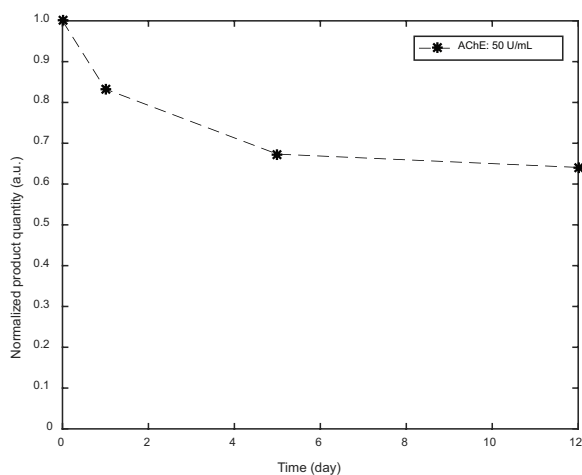


Figure 4: Spectrophotometrical measurement of time dependent AChE activity.

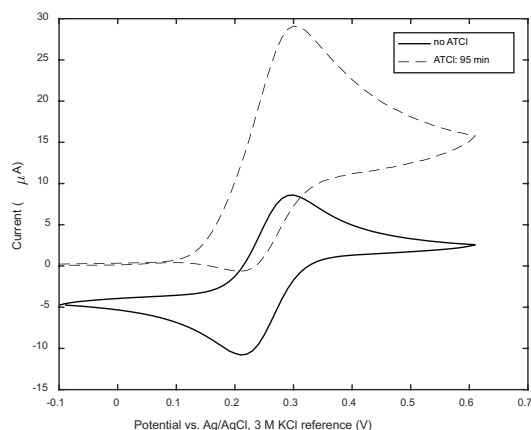


Figure 5: Cyclic voltammogram obtained in absence (solid line) and in presence of substrate ATCI (discontinuous line) in the presence of redox couple ferri/ferro-cyanide. The reaction with substrate refer to ATCI hydrolysis, catalysed by the enzyme AChE.

Addition of ATCI causes an increase in concentration of TChCl and consequently of the ferrocyanide, resulting in an increase of the anodic peak current. On the contrary, the cathodic peak current is proportional to the amount of ferricyanide that decreases after the addition of ATCI. This can be seen in Figure 5, where anodic peak current I_{pa} increases and cathodic peak current I_{pc} decreases with time of TChCl present. Therefore, we can determine a ratio I_{pa}/I_{pc} . The ratio of the peak currents is equal to one for reversible system of ferro/ferri-cyanide, shown by solid line in Figure 5. Larger peak current difference is obtained for reaction with ATCI, shown by dashed line in Figure 5, for the reaction time of 95 min. The corresponding ratio of the peak currents was therefore determined as 3.1. This confirms higher rate of oxidation and consequently concentration of produced TChCl.

Figure 6 shows measured current by CA technique and fitted result (solid line) versus ATCI concentration.

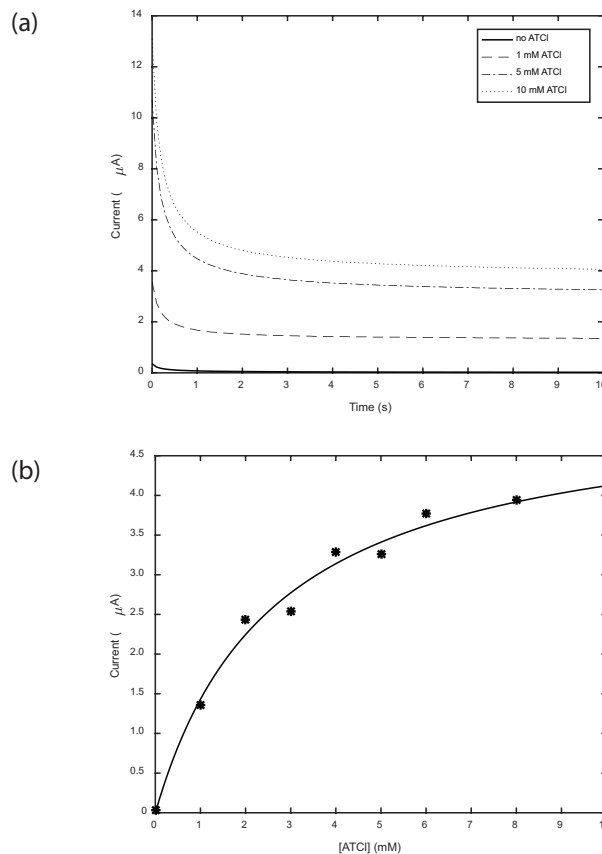


Figure 6: Selected chronoamperometric curves (a) from which (at 10 s) calibration plot of ATCI using the AChE biosensor is constructed (b).

Relation between electrical current and concentration of substrate ATCI was assumed to follow Michaelis-Menten kinetics (Eq. 3) [21], being aware that many other factors can affect the results [22]:

$$V_0 = \frac{V_{max} [S]}{K_M + [S]} \quad (3)$$

where V_0 is initial velocity, V_{max} is maximum velocity, $[S]$ is substrate concentration, and K_M is Michaelis constant. According to the curve fit of measured points in Figure 6 (solid line), the calculated apparent Michaelis constant $K_M^{app} = 2.6 \pm 0.9$ mM was determined.

Finally, after determining production of TChCl by AChE biosensor, AChE inhibition was chronoamperometrically detected by using well-established commercially available AChE inhibitor eserine. The inhibition of AChE was determined by measuring the decrease of the CA signal, which is consequence of eserine addition to the ATCI substrate solution. Result of inhibition for 25 μ M of concentration of eserine is presented in Figure 7.

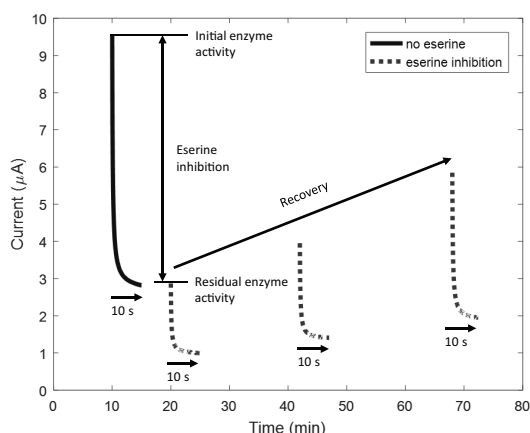


Figure 7: Study of AChE inhibition by eserine.

Both, initial enzyme inhibition in value of 70% and recovery of AChE activity are presented in Figure 7. After 10 minutes of inhibition by eserine, samples were repeatedly rinsed and re-measured, noting the significant recovery of the signal. Note that each measurement, presented in Figure 7, lasted only 10 s, while time on x-axis shows the total time of experiment duration. It can be seen that recovery of AChE activity increases almost linearly with time (20 to 68 min) when system was not exposed to eserine, recovering to 59% and 39% of inhibition at 42 and 68 min, respectively.

An important kinetic parameter of inhibited AChE is the reactivation factor K_{react} . By applying Perola's equation (Eq. 4), the reactivation factor K_{react} was calculated from the inclination of the linear plot obtained by monitoring the inhibition as a function of time [23]:

$$\ln\left(\frac{Inhibition_t}{Inhibition_0}\right) = -K_{react}t \quad (4)$$

where $Inhibition_0$ is the first measured current after inhibitor addition, which corresponded to $t = 0$ of the decarbamylation phase, and $Inhibition_t$ is the inhibition determined as a function of time during the enzyme activity recovery phase. K_{react} of 0.016 min^{-1} was determined, and it is in good agreement with the literature data [24].

4 Conclusions

The AChE electrochemical biosensor is presented. It is successfully applied for detection of eserine as an example of choline inhibitor in water.

Spectrophotometric measurements have shown that five days after AChE immobilisation on gold surface

activity of an enzyme decreased for 33% and then remained stable an extended period of time.

Biosensor was evaluated via CV and CA measurements. CV measurement of enzyme activity resulted in asymmetric cyclic voltammogram, i.e. increase of anodic peak current and decrease of cathodic peak current (ratio 3:1). This confirms higher rate of oxidation and consequently concentration of produced TChCl. Moreover, relation between electrical current and concentration of substrate ATCl up to 10 mM were assumed to follow Michaelis Menten kinetics. Apparent Michaelis constant was determined as $2.6 \pm 0.9 \text{ mM}$.

Our results have shown that $25 \mu\text{M}$ eserine inhibits the activity of AChE for 70%. Reactivation factor of 0.016 min^{-1} was determined, showing reversibility of AChE inhibition by eserine.

Presented work is to be further extended to determine biosensor sensitivity, reliability and concentration dependent inhibition of eserine and other AChE inhibitors.

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