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Electrochemical Determination of Activity of Acetylcholinesterase Immobilized on Magnetic Particles

Adam Kostelnik[1], *Alexander Cegan*[1], *Miroslav Pohanka*[2,3*]

[1]Faculty of Chemical Technology, University of Pardubice, Studentska 95, Pardubice, Czech Republic
[2]Faculty of Military Health Sciences, University of Defense, Trebesska 1575, Hradec Kralove, Czech Republic
[3] Department of Geology and Pedology, Mendel University in Brno, Czech Republic
[*]E-mail: miroslav.pohanka@gmail.com

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Acetylcholinesterase (AChE) is an enzyme which terminates action of neurotransmitter acetylcholine in cholinergic system. Activity of enzyme is commonly assayed by Ellman´s method which has significant disadvantages like instability of reagents. Inability to use enzyme from the mixture repeatedly is another disadvantage. Therefore, we developed process using N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) to immobilize AChE on the surface of magnetic particles, which enable to separate enzyme and use it repeatedly. To determinate activity of enzyme, we optimized square wave voltammetry (SWV) method where screen-printed sensor with Prussian blue (PB) modified working electrode was performed. We report limit of detection equal 8.1 μM for a tested inhibitor of AChE tacrine. Comparing to the standard Ellman´s test, the immobilized enzyme has limited sensitivity to interferences caused by organic solvents. The proposed method is readily to practical application.

Keywords: Acetylcholinesterase; tacrine; magnetic particles; screen-printed electrode; square wave voltammetry

1. INTRODUCTION

AChE is an enzyme hydrolyzing neurotransmitter acetylcholine to choline and acetic acid, thereby it terminates excitation of cholinergic system [1]. George L. Ellman introduced new colorimetric method for determination activity of AChE in 1961. This method is based on hydrolysis of acetylthiocholine (ATChCl) by AChE to thiocholine and acetic acid. In the next step, thiocholine reacts with Ellman´s reagent 5,5´-dithiobis(2-nitrobenzoic acid) (DTNB) to produce 5-thio-2-nitro benzoic acid providing yellow coloration to solution and highly absorbing at 412 nm [2]. Because of

daylight instability of DTNB there is an advantage in use of electrochemical methods for AChE activity determination [3]. In this case acetylthiocholine is also hydrolyzed by AChE but due to thio group presented in thiocholine molecule, electrochemical oxidation results in forming of disulfide bond and free electrons are released (Fig. 1).

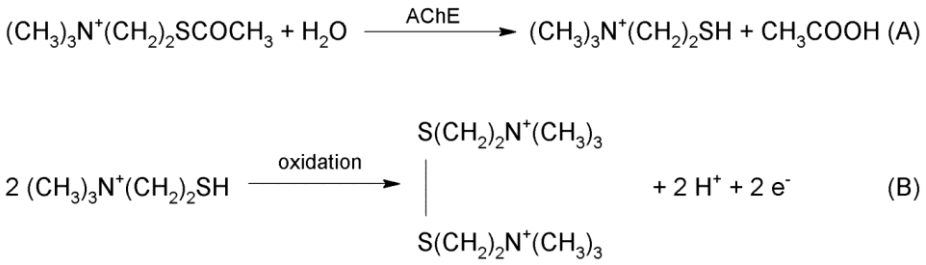

Figure 1. Hydrolysis of acetylthiocholine by AChE (A), thiocholine oxidation (B)

Screen-printed electrodes are a response to demand of miniaturization in last years and because of low cost became ideal for mass production, plus they have good reproducibility and reliability. Silver or carbon ink is typically used to printing process. Carbon ink is widely used because it is relatively cheap, easy to modify and chemically inert. On the other hand, overpotential of graphite electrodes is very close to the much expensive electrode from nobel metals. Number of mediators can be mixed into ink to modify electrode surface [4-6]. Although thiol moieties undergo electrochemical oxidation, relatively high potential is needed (0.7 V). Added mediators like cobalt phthalocyanine, PB, CdS quantum dots or cobalt hexacyanoferrate can reduce this potential [7,8]. PB has been described to cathodic reduction of H_2O_2 but applications for ascorbic acid, hydrazine and thiols such as cysteine, N-acetylcysteine, glutathione or thiocholine determination was presented as well [9,10].

Magnetic particles have shown ability in many applications such in enzyme immobilization, immunoassay, DNA or RNA purification and so on. This is achievable by groups on particles surface (-COOH, -OH etc.) which interact with $–NH_2$ or –SH protein groups [11-13]. Only a few protocols dealing with AChE immobilization to magnetic particle surface are described [14-17]. However many protocols using AChE biosensors was presented [18,19].

This work is focused on immobilization of AChE on the magnetic particles surface using EDC which was described to form amide bonds in aqueous medium [20], optimize square wave voltammetry method using PB-modified screen-printed electrode to observe AChE activity with and without presence of tacrine and also establish method´s interferences. It is expected that the assay will bring significant improvement to the current assays.

2. EXPERIMENTAL PART

2.1. Materials

Acetylcholinesterase from electric eel as lyophilized powder (≥ 1000 units/mg protein), ATChCl, carboxy functionalized magnetic particles, EDC, 9-amino-1,2,3,4-tetrahydroacridine

hydrochloride hydrate (tacrine), N-acetyl-L-cysteine, Prussian blue soluble, phosphate buffer saline (PBS) pH 7.4, dimethyl sulfoxide (DMSO), Tween-20 and isopropyl alcohol were purchased from Sigma-Aldrich (St. Louis, MO, USA), denatured ethanol was obtained from PENTA (Prague, Czech Republic).

2.2. Instruments and measurement conditions

Screen printed sensors (BVT, Brno, Czech Republic) were sized 25.4 × 7.26 × 0.63 mm and contained dot shaped carbon working electrode with diameter 1 mm, circle shaped platinum auxiliary electrode and circle shaped argent chloride referent electrode. The assay was performed using electrochemical device PalmSens (PalmSens BV, Houten, Netherlands) connected with computer and processed by PSLite 1.8 (PalmSens BV, Houten, Netherlands) software. Setting for SWV was following: scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz. Prussian blue modified electrode process was following: 3 mg of Prussian blue was dispersed in 1 ml of PBS 7.4 and shaken then 30 µl of dissolved portion was pipetted onto electrode surface and air-dried in laboratory temperature.

2.3. Solutions preparation

Solutions of ATChCl and N-acetyl-L-cysteine in PBS 7.4 were in concentrations 75, 100, 125, 150, 175, 200 mM. Calibration solutions for tacrine in PBS 7.4 were in concentrations 750, 700, 600, 500, 400 µM and prepared from stock solution (2 mM). Each solution was prepared into disposable Eppendorf tube and final volume was set to 1 ml. Resulting concentration in cuvette was 10-fold less in case of ATChCl and N-acetyl-L-cysteine and 40-fold less in case of tacrine.

2.4. Preparation of magnetic particles with immobilized AChE (MPs-AChE)

Carboxy functionalized magnetic particles (400 µl) were pipetted into 5 ml tube and two times washed by 1 ml of PBS pH 7.4, washing buffer was always fully removed then 200 µl of EDC (3 mg in 200 µl of PBS pH 7.4) was added. Whole mixture was shaken 15 minutes (600 rpm), followed by two times washing by 1 ml of PBS pH 7.4 to eliminate unbound enzyme. Buffer was fully removed and 300 µl PBS pH 7.4 and 300 µl of AChE was added and shaken for 2 hours (600 rpm). Finally were magnetic particles three times washed by PBS 7.4, resuspended into 200 µl of PBS 7.4 and stored in fridge.

2.5. Voltammetry assay

400 µl of PBS 7.4, 25 µl of MPs-AChE and 25 µl of tacrine solution was added into cuvette and incubated 10 minutes in laboratory temperature, then 450 µl PBS 7.4 and 100 µl of 175 mM acetylthiocholine solution was added. Final volume was 1 ml and whole process was

performed in magnetic holder. After one hour incubation was solution separated from magnetic particles and pipetted into clean cuvette. Measuring with Prussian blue modified electrode was performed and voltammogram was recorded.

3. RESULTS AND DISCUSSION

3.1. Optimization of thiocholine electrochemistry

SWV method was optimized using N-acetyl-L-cysteine from 7.5 to 20 mM (Fig. 2, Fig. 3) and we obtained peak of N-acetyl-L-cysteine at average 0.9 V with Prussian blue non-modified electrode but when Prussian blue modified electrodes were used peak in 0.745±0.005 V was discovered. Peak of thio group in similar potential 0.764±0.041 V was obtained by Pohanka et al. during measurement of glutathione and Foroughi et al. at potential 0.6 V when N-acetyl-L-cysteine measure [21,22]. Another oxidation of N-acetyl-L-cysteine can also occur in higher potential which results in formation of second peak [23]. As was described earlier, Prussian blue is able to decrease potential needed to thio moiety oxidation and we confirmed it during N-acetyl-L-cysteine measurement [24]. For identification of thiocholine peak we made optimization curve from 7.5 to 20 mM of ATChCl (Fig. 4, Fig. 5). Though ATChCl and N-acetyl-L-cysteine have structural similarity in our conditions we found out thiocholine peak in potential 1.320±0.020 V which unfortunately makes analysis longer.

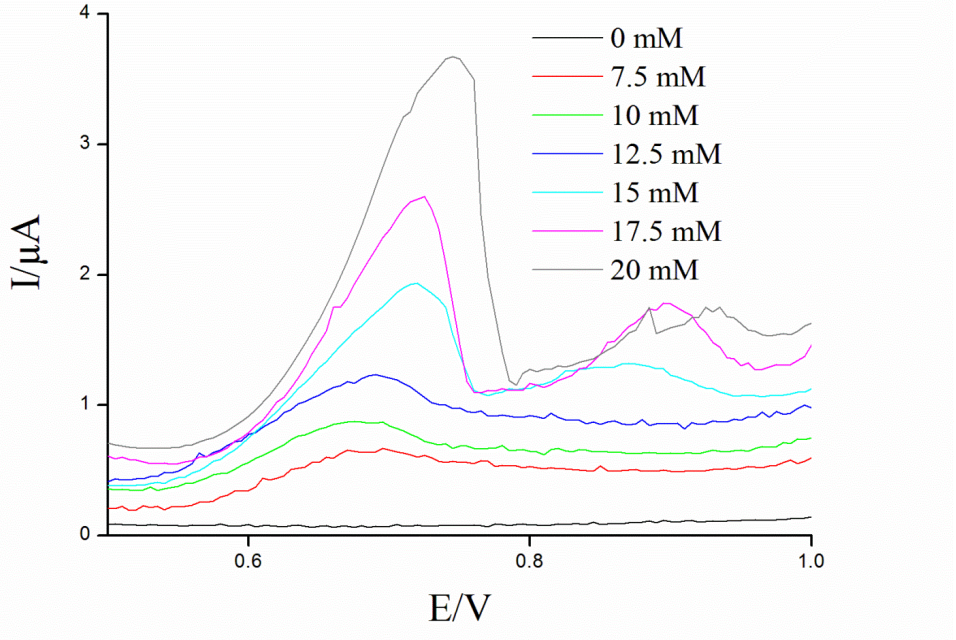

Figure 2. SWV curves of N-acetyl-L-cysteine performed in PBS 7.4; scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl referent electrode and platinum auxiliary electrode

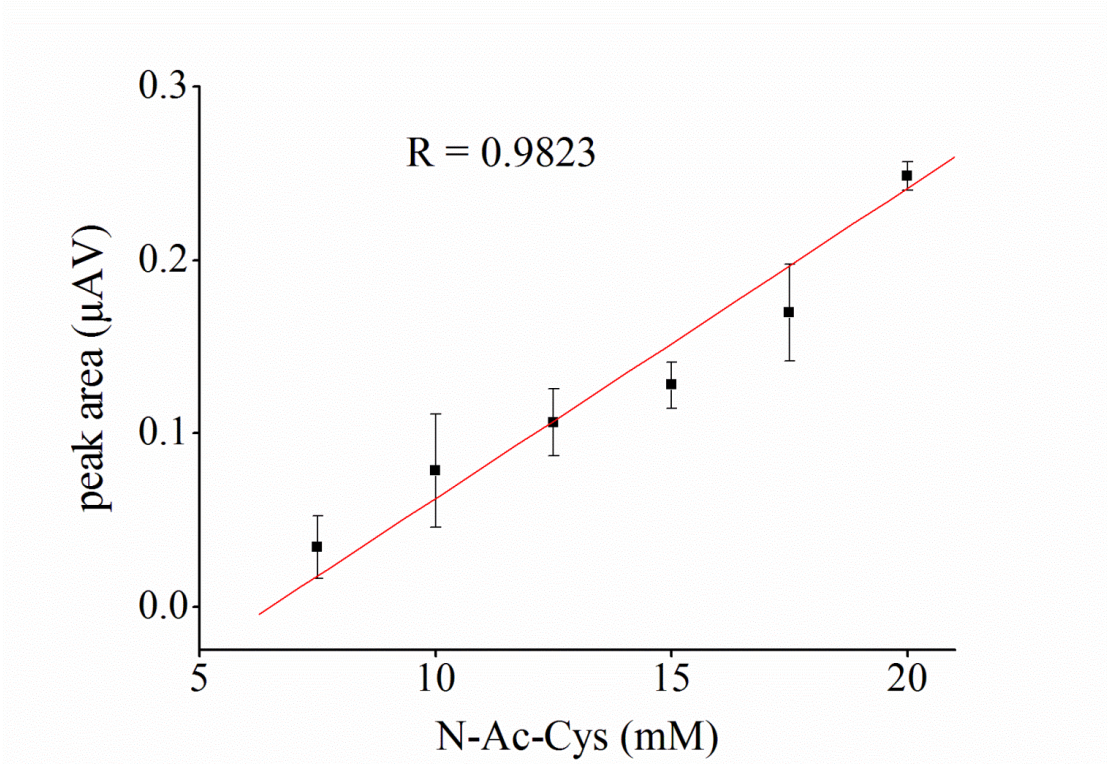

Figure 3. Optimization curve of N-acetyl-L-cysteine performed in PBS 7.4; scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl referent electrode and platinum auxiliary electrode.

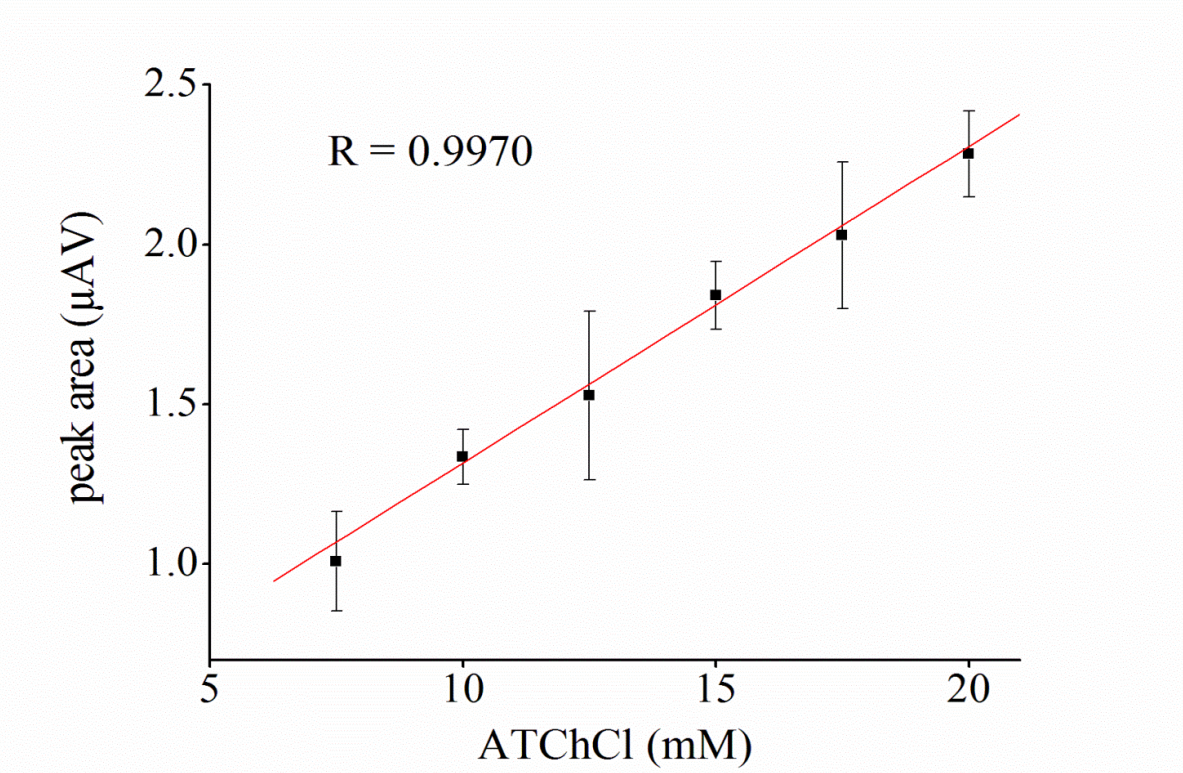

Figure 4. Optimization curve of acetylthiocholine performed in PBS 7.4; scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl referent electrode and platinum auxiliary electrode.

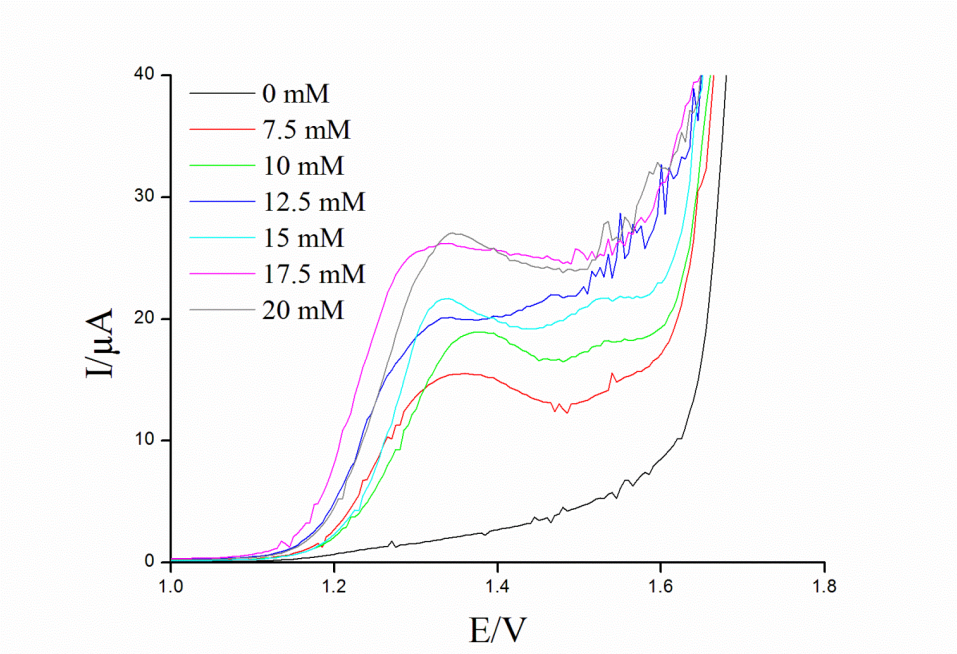

Figure 5. SWV curves of acetylthiocholine performed in PBS 7.4; scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl referent electrode and platinum auxiliary electrode

3.2. Tacrine calibration

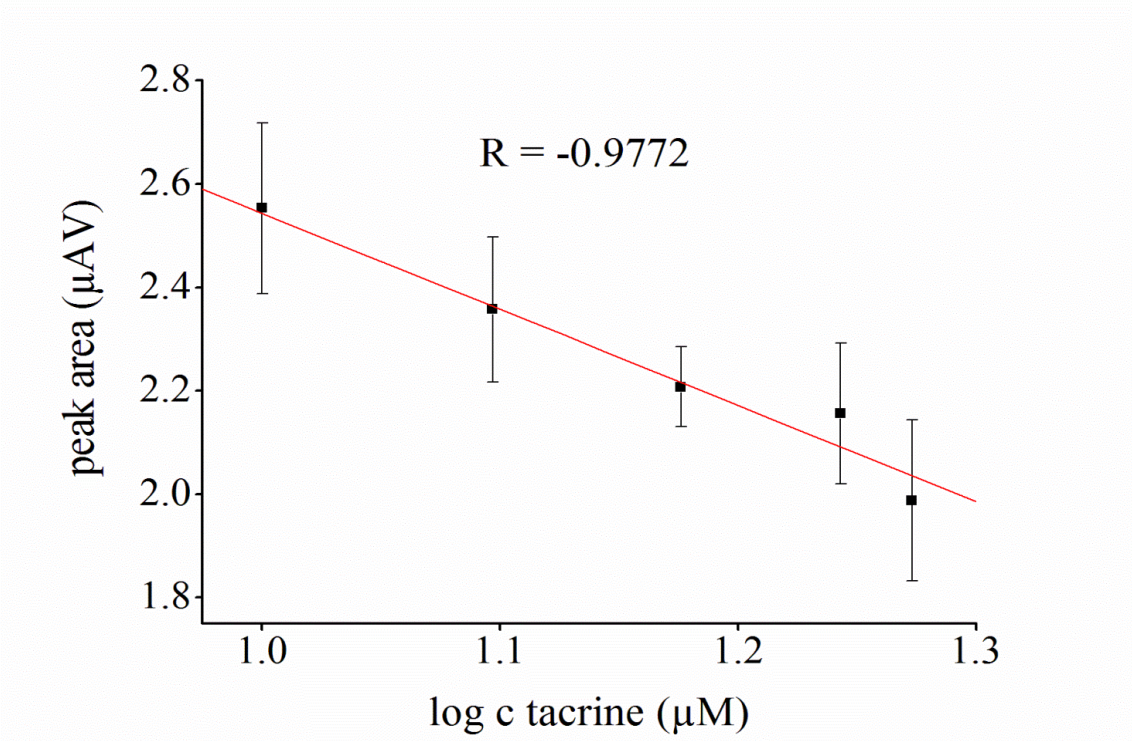

Figure 6. Tacrine calibration curve performed in PBS 7.4; scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl referent electrode and platinum auxiliary electrode.

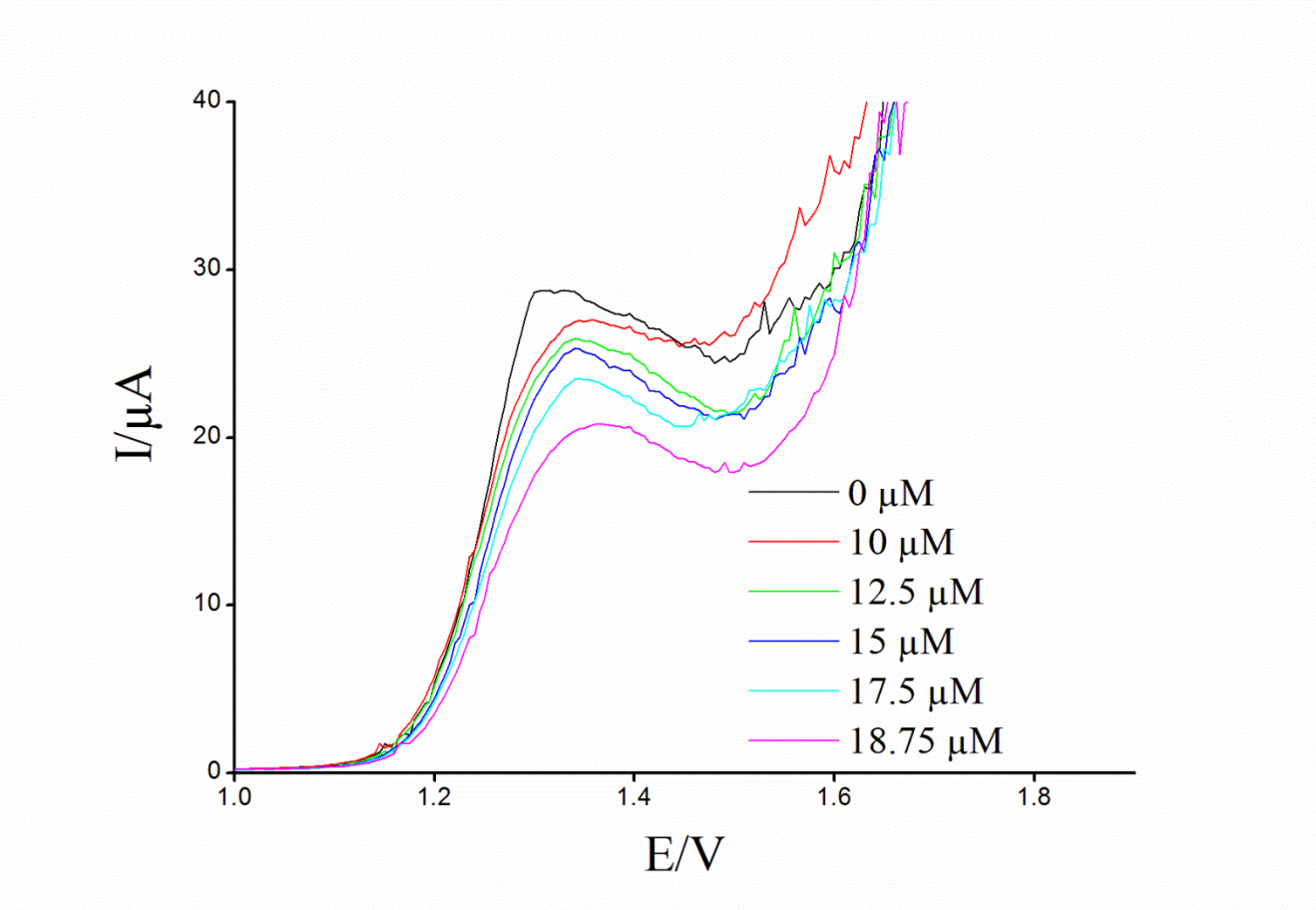

Figure 7. SWV curves of tacrine performed in PBS 7.4; scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl referent electrode and platinum auxiliary electrode

AChE is inhibited by tacrine, reversible inhibitor of AChE [25]. We successfully performed tacrine calibration in final concentrations from 10.00 to 18.75 μM (Fig. 6, Fig. 7). However concentrations over 20 μM return intensity of thiocholine peak into higher potentials and no further increase of inhibition is observed. It is caused by saturation of the enzyme by inhibitor. We calculated LOD for this method to 8.1 μM of tacrine which is comparable to Bollo et al. who achieved LOD 4.8 μM of tacrine [26].

3.3. Reproducibility of measurement with MPs-AChE

We evaluated durability of MPs-AChE thus how long they can be use without decrease of enzyme activity. MPs-AChE was washed three times by 1 ml of PBS 7.4 between each cycle. This is caused by degradation of magnetic particles throughout washing between each cycle (Fig. 8). Products of degradation were presented as reddish brown mist of solution.

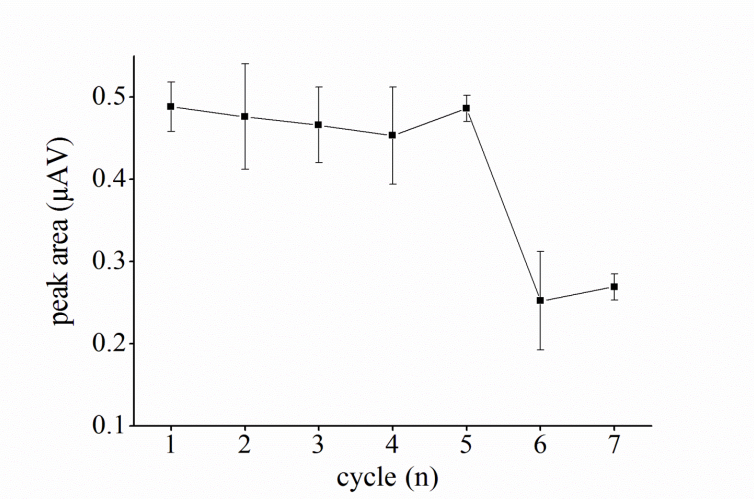

Figure 8. Reproducibility of measurement with MPs-AChE performed in PBS 7.4; scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl referent electrode and platinum auxiliary electrode.

3.4. Method interferences

We established method interferences using Tween-20 (0.025%), DMSO (2.5%), isopropyl alcohol (2.5%) and ethanol (5%).

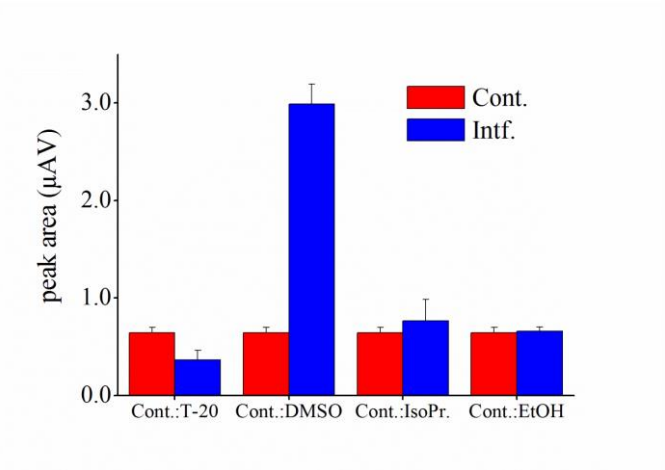

Figure 9. Method interferences (T20 = Tween-20; DMSO = dimethyl sulfoxide; IsoPr = isopropyl alcohol; EtOH = ethanol; Cont. = control; Intf. = interference); scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl referent electrode and platinum auxiliary electrode.

We found out ability of Tween-20 to decrease AChE activity which is surprising because it was used to extraction of AChE from tissue and was not reported any inhibition effect to AChE [27,28]. On the other hand DMSO was able to increased peak intensity to 5-fold more against control although decrease of AChE activity was reported [29,30]. It is probably caused by DMSO oxidation around potential needed to thiocholine oxidation. Ethanol and isopropyl alcohol was presented to decrease

activity of AChE [31,32]. Isopropyl alcohol and ethanol did not exhibited any interference in presented concentrations nevertheless both alcohols have shown to be interfere in higher concentrations than 5 % when caused noise and shift in voltammogram. In higher concentrations, we noticed that isopropyl alcohol probably interact with magnetic particles caused their degradation, ethanol did not show this phenomenon (Fig 9).

4. CONCLUSION

We successfully used EDC for AChE immobilization to magnetic particles and optimized it for use with square wave voltammetry. Compared to standard Ellman´s method there are no instable reagents and the particles can be used repeatedly due to AChE stable bound. Presented method is suitable for electrochemical determination of tacrine to 20 μM and up to presented concentration no inhibition by tacrine is observed because of saturation of the enzyme by inhibitor. Limit of detection for tacrine was calculated to be 8.1 μM. Isopropyl alcohol and ethanol did not inhibit AChE in presented concentrations while Tween-20 was found to inhibit enzyme and DMSO interfere in voltammogram due it is oxidation.

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