Author's Accepted Manuscript

www.elsevier.com/locate/talanta

Electrochemical biosensor based on polyelectrolyte complexes for the determination of reversible inhibitors of acetylcholinesterase

Alexey Ivanov, Regina Davletshina, Indira Sharafieva, Gennady Evtugyn

PII: S0039-9140(18)31147-0
DOI: https://doi.org/10.1016/j.talanta.2018.10.100
Reference: TAL19228

To appear in: *Talanta*

Received date: 16 August 2018
Revised date: 26 October 2018
Accepted date: 29 October 2018

Cite this article as: Alexey Ivanov, Regina Davletshina, Indira Sharafieva and Gennady Evtugyn, Electrochemical biosensor based on polyelectrolyte complexes for the determination of reversible inhibitors of acetylcholinesterase, *Talanta,* https://doi.org/10.1016/j.talanta.2018.10.100

Electrochemical biosensor based on polyelectrolyte complexes for the determination of reversible inhibitors of acetylcholinesterase

Alexey Ivanov, Regina Davletshina, Indira Sharafieva, Gennady Evtugyn*

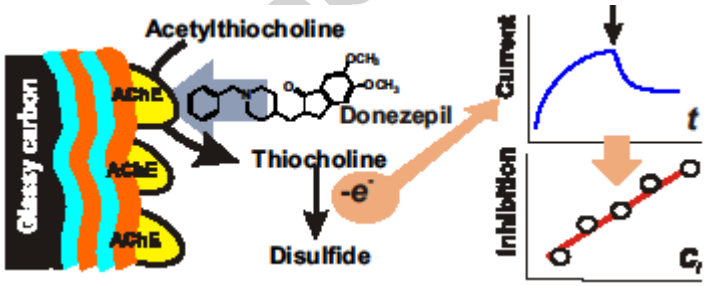
A.M. Butlerov' Chemistry Institute of Kazan Federal University, 18 Kremlevskaya street, Kazan 420008, Russian Federation

*Corresponding author: Gennady.Evtugyn@kpfu.ru

Abstract.

Reversible inhibitors of acetylcholinesterase are increasingly applied in the Alzheimer's disease therapy. Their determination with biosensors requires special attention to the enzyme immobilization due to importance of electrostatic and steric factors affecting accessibility of the active site to the substrate and inhibitor present simultaneously. In this work, polyelectrolyte complexes have been assembled from electrochemically inactive components with implementation of acetylcholinesterase from electric eel. The influence of the complex components and enzyme position on the assembling efficiency was explored using surface plasmon resonance and kinetic parameters of the enzyme with amperometry with Co phtalocyanine mediator. The biosensor developed showed robust response toward donepezil and berberine with limit of detection of 0.46 and 70 nM, respectively, within 1 minute after inhibitor addition. The sensitivity of determination coincides or is better than characteristics of biosensors based on covalently attached AChE described in the literature. The biosensor was tested on the example of drug determination in spiked samples of artificial urine (recovery 103% for 0.6 nM donepezil and 110% for 0.1 μM berberine).

Graphical Abstract:



Keywords: acetylcholinesterase biosensor, reversible inhibition, polyelectrolyte complex, Alzheimer's disease drug

1. Introduction

Acetylcholinesterase (AChE) is one of the enzymes most frequently used in electrochemical biosensors for inhibitor determination [1, 2]. The interest to this enzyme was first initiated by its high sensitivity toward chemical warfare agents exerting neurotoxic effect. Although the international programs of chemical warfare destruction sufficiently decreased risks related to their application, terrorist attacks are continuously reported starting from the Tokyo subway sarin attack (1995) and finishing with recent accidents in Syria civil war [3]. Chemical nerve agents exert irreversible inhibition of the AChE resulted in sever health damage and death of poisoned people. Later on, anticholinesterase organophosphates and carbamates have found a broad application as insecticides in agriculture. From 1950-s, they are on the third place in the world market among other pesticides due to low stability in the environment and high efficiency of action. Analytical aspects of the development and application of the AChE biosensors for irreversible inhibition detection have been the subject of recent comprehensive reviews [4-7].

Recently, the attention to the AChE biosensors has been increased due to another area of their application related to the detection of drugs for neurodegenerative diseases, especially, Alzheimer's disease, a leading cause of age related dementia [8]. The effect of the AChE inhibitors is related to the suppression of acetylcholine hydrolysis. This compensates for lower rate of its natural synthesis catalyzed by choline acetyltransferase [9, 10]. Galantamine, rivastigmine, tacrine and donepezil (DON) are approved at the moment for clinical treatment of neurodegenerative diseases [11]. They show positive influence on cognitive function, living activity, but exert rather high toxicity.

For this reason, further efforts both in searching new anti-dementia drugs and their sensitive detection in biological fluids are demanded. Most of the drugs mentioned showed competitive inhibition of the AChE related to binding of anionic site of the enzyme. The methodology of the determination of reversible inhibition differs from that of irreversible inhibitors dominating in the AChE biosensor reports.

In modern clinical analysis, anticholinesterase drugs are commonly determined with HPLC coupled with extraction protocols [12-14]. Being sensitive, such conventional instrumentation is time and labor consuming and cannot be used outside the laboratory as the point-of-care devices.

Biosensors for inhibition measurements offer new opportunities in clinical analysis and medicine [15]. There are considered as alternative to conventional analytical techniques appropriate for fast screening of biochemical targets and pharmacokinetics study. However, only

few articles have been published for determination of reversible AChE inhibitors that offer specific requirements to the accessibility of the active site to the substrate and inhibitor present in the sample simultaneously.

In this work, electrochemical biosensors based on the AChE physically entrapped in polyelectrolyte complexes assembled on the electrode surface has been elaborated for the determination of two reversible inhibitors, i.e., DON as anti-dementia drug and berberine (BER) as reversible AChE inhibitorapplied in Chinese medicine.

The interaction of DON with AChE was first studied on the enzyme from *Torpedo Californica*, which structure and active site composition were at that time discovered. It was shown that DON is oriented along the active site gorge from anionic subsite at the bottom to peripheral anionic site at the top [16]. Hydrophobic π-staking interactions with aromatic residues also contribute to the inhibition complex formation as was later shown for human AChE [17].

Berberine is an isoquinoline alkaloid present in some medicinal herbs (*Hydrastis Canadensis*, *Cortex phellodendri* and *Rhizoma coptidis*). It exerts reversible inhibition on AChE first characterized in 2002 [18]. Its positive effect on Alzheimer's disease was proved on rat models by intragastric administration [19].

For the first time, the conditions of the AChE immobilization in the polyelectrolyte complexes consisting of electrochemically inactive components were characterized by the surface plasmon resonance (SPR) technique and the analytical characteristics of the drug determination were obtained in model solutions and spiked samples of artificial urine.

2. Experimental

2.1. Reagents

AChE from electric eel (EC 3.1.1.7, 1468 units /mg protein), acetylthiocholine chloride (ATCh), Carbon black (CB, N220) was produced by Cabot Corporation (Ravenna, Italy). Co phtalocyanine, polyallylamine (PAA, mol. mass 65000), poly(diallyldimethylammonium chloride) (PDDA, mol. mass 400 000-500 000), polystyrene sulfonate (PSS, mol. mass 1 000 000), 11-mercaptoundecanoic acid (MUA, 95%), 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Sigma-Aldrich, Germany. Working solutions were prepared using Milli-Q® type 1 water. The AChE, ATCh, DON and BER stock solutions were frozen and stored at -20^{o}C between measurements and diluted before use. Final AChE solution was prepared with 0.05 M phosphate buffer solution containing 0.1 M NaCl (pH = 7.8) or 0.05 M MES (pH = 5.5)

and adjusted to the pH value required. Electrochemical measurements were performed in Britton-Robinson buffer solution. Artificial urine samples contained 10 mM $CaCl_2$, 6 mM $MgCl_2$, 80 mM, 16 mM Na_2SO_4, 2 mM potassium citrtrate, 20 mM KH_2PO_4, 21 mM KCl, 18 mM NH_4Cl, 9 mM creatinine and 416 mM urea. All other reagents were of analytical grade.

2.2. Apparatus

Electrochemical measurements were performed in 5 mL non-thermostated cell at ambient temperature with Pt wire auxiliary electrode and double-junction Ag/AgCl (3 M KCl) reference electrode (Metrohm Autolab b.v., the Netherlands). The AChE biosensor was assembled using glassy carbon electrode (20 mm cylindrical rod with the cross-section area 2.83 mm^2 capsulated in polytetrafluoroethylene tube) mechanically polished to a mirror like surface. Voltammograms and chronoamperograms were recorded using potentiostat-galvanostat AUTOLAB PGSTAT 302N (Metrohm Autolab).

SPR measurements were performed on the 25 mm BK-7 glass disk covered with the 50 nm thick Au film with the Autolab ESPRIT (Metrohm) equipped with two-channel cuvette and the 670 nm laser with the p-polarization. An incidence angle shift of the laser beam was recorded as the SPR signal expressed in millidegrees (m^o).

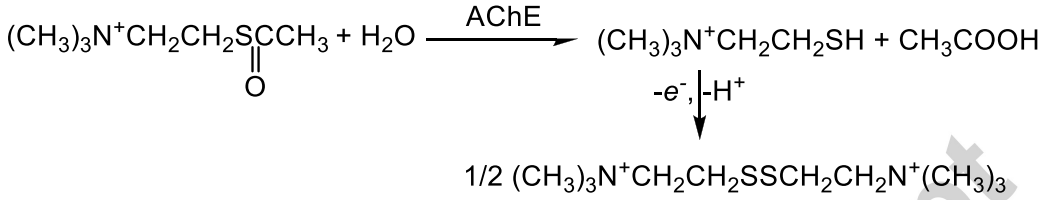
Statistical data treatment was performed using Origin 8.1 Software (OriginLab).

2.3. The acetylcholinesterase biosensor preparation

For electrode modification, two dispersions containing 1 mg/mL of Co phtalocyanine and 1 mg/mL of CB were first prepared by 30 min. sonication in dimethylformamide. Glassy carbon electrode polished and cleaned were covered with 1 μL of the dispersions mixed in 4:1 vol. ratio and dried at 70^{o}C. After that, polyelectrolyte solutions with alternating positive and negative charges were deposited one by one. Each layer was adsorbed from 10 μL of solution for 10 min followed by multiply washing of the electrode with deionized water. Finally, enzyme was immobilized by physical adsorption from phosphate buffer solution. For this purpose, 1.5 μL of solution of the 0.05 U/μL AChE were placed onto the polyelectrolyte layer and allowed drying at ambient temperature. The electrode was washed as described above and dried if not used. In some cases, additional polyelectrolyte layer has been deposited on the enzyme for its protection. The AChE sensors were stored in dry conditions at 4 ^{o}C.

2.4 Biosensor signal measurements

The AChE activity was measured at a constant electrode polarization (chronoamperometric mode) by anodic current related to the mediated oxidation of thiocholine formed in reaction (1).



(1)

For this purpose, the AChE biosensor was immersed in phosphate buffer solution, pH = 7.8, and polarized at 200 mV for 180 s to reach a constant background current. After that, the ATCh solution was added and the shift of the current i_0, which corresponded to the thiocholine oxidation in the absence of an inhibitor, was measured. For the inhibition degree (*I%*) determination, the inhibitor solution was then added to the biosensor contacted with the substrate and appropriate current *i* was recorded in 60 s after addition. Inhibition degree was calculated in accordance with Eq. (2).

$$I\% = \frac{i_0 - i}{i_0} 100\% \quad (2)$$

2.5. The SPR investigation of the polyelectrolyte layer capacity toward acetylcholinesterase

The surface of the golden coating of the SPR chip was first modified with 1 mM MUA solution in ethanol under 30 min. stirring. Then the surface was rinsed with deionized water. Consecutive deposition of the oppositely charge polyelectrolytes was performed as described above for glassy carbon electrode by treatment of the chip with 100 μL of their solutions and 10 min. conditioning. The AChE adsorption was performed by placing 70 μL of the ACHE solution in phosphate buffer for 30 min. The amounts of the AChE adsorbed were estimated by the shift of the SPR angle measured prior to and after the AChE immobilization. Maximal theoretic angle shift (670 m^o) was calculated as described elsewhere [20] assuming the AChE mol mass 260 kDa and spherical shape of the molecule. Immobilization efficiency (*Im*) was calculated from the SPR angle shift ϑ corresponded to the enzyme implementation (3).

$$Im = \frac{\vartheta}{670}100\% \quad (3)$$

3. Results and discussion

3.1. Influence of the polyelectrolyte complex composition on the efficiency of acetylcholinesterase immobilization

The efficiency of immobilization (*Im*) characterizes the amount of the enzyme that can be kept in the surface layer and depends on the number and relative position of the binding sites of polyelectrolyte molecules. The AChE globule can be ascribed as a dipole with the vector directed to the active site cavity [21]. For this reason, the enzyme can be immobilized in the polyelectrolyte complex via electrostatic interactions with positively charged components. Besides, hydrogen bonds and hydrophobic interactions should be considered. They explain possibility to form stable complexes with negatively charged carbon nanotubes in weakly basic media (pH 7.4-8.0) that is preferable for inhibition measurements and causes negative charge of the protein globule (the AChE pI = 5.5) [22]. Driving forces of immobilization pre-determine spatial orientation of the AChE globule and hence can affect kinetics of the substrate hydrolysis and inhibition. The charge, chemical structure and order of deposition highly influence resulting parameters of polyelectrolyte complexes, i.e., total charge density, hydrophobicity and polymer chain rigidity. Besides, the increase of the ionic strength of the solution and of the polyelectrolyte concentration promote globulization of the polyelectrolyte molecules and hence increase external and internal roughness of the layer surface [23, 24]. In turn, higher roughness resulted in some cases in exponential growth of the polyelectrolyte coatings obtained by their stepwise assembling and in a higher effective square of the films [25, 26]. Such consequences can improve enzyme immobilization.

Fig. 1 represents the dependency of the SPR signal on the number of polyelectrolyte layers. Measurements were performed after equalization of the electrode in phosphate buffer solution, pH = 7.8, and correspond to the amounts of adsorbed components stable at conditions commonly used for the AChE inhibition estimation.

PAA deposition results in bigger quantities of the complex attached to the surface against

those in case of the PDDA use. This can be attributed to the increased internal heterogeneity of the complex and external roughness of the coating in case of PAA resulted in a higher effective square and bigger adsorption of the oppositely charged components. Decrease of the PAA concentration from 0.5 to 0.1 mg/mL insignificant decreases the quantity of the complexes formed after the PSS addition.

Immobilization efficiency determined for various assemblies of polyelectrolytes is illustrated by Fig. 2 for different amounts of the enzyme present in the immobilization mixture.

Maximal *Im* value accessible in the experimental conditions was calculated from the experimental data on Fig. 2 by nonlinear data fitting using the function (4) where c_{AChE} is concentration of the enzyme in the aliquot used for immobilization per electrode.

$$Im = Im^{\max} \frac{c_{AChE}^{n}}{k^{n} + c_{AChE}^{n}} \tag{4}$$

Eq.(4) describes Hill equation that is often used for description of sigmoidal dependencies in biochemical investigations [27]. Here *n* is the Hill coefficient describing cooperativity of interactions. For n<1 (see Table 1) binding of the enzyme on the support decreases the approaching of other enzyme molecules, This coincides well with electrostatic mechanism of enzyme immobilization, The *k* value is equal to the concentration of enzyme corresponded to the occupation of 50% binding sites available for enzyme on the support surface. The coefficient of determination R^2 represents fitting quality. It was calculated from the total sum of squares (TSS) and residual sum of squares (RSS) ascribing deviation of the model and experimental data (5).

$$R^2 = \frac{Explained\,variation}{Total\,variation} = \frac{TSS - RSS}{TSS} = 1 - \frac{RSS}{TSS} \tag{5}$$

The results of the $Im^{\max}$ approximation are presented in Table 1.

Polyelectrolyte complex involving PAA show maximal capacity toward the enzyme among all the assemblies tested. However, if the PAA was deposited directly onto the MUA monolayer, the adsorption was minimal. Addition of the PSS-PAA polyelectrolyte pairs remarkably increases the adsorption of the enzyme (see Fig.2, curves 2-4). Maximal capacity toward the AChE was achieved with the PAA-PSS-PAA complex. Moreover, decrease of the PAA concentration from 0.5 to 0.1 mg/mL did not significantly affect the enzyme

immobilization. The following deposition of the fourth PSS layer expectedly decreased the capacity due to electrostatic repulsion of the charge outer surface of the complex and the enzyme globules. Replacement of the PAA by PDDA in combination with the PSS decreases the adsorption (Fig.2, curve 5). The consecutive addition of the PSS-PDDA pairs influences the adsorption in a similar manner.

It should be mentioned that maximal *Im* value achieved remains much lower than that corresponded to the monolayer deposited on the electrode. This might be due to two reasons, i.e., rather high roughness of the surface accessible for the enzyme and due to rather crude description of the monolayer in the framework of the model used. However, maximal concentration of the enzyme immobilized is not a primary goal for inhibition measurement. Vice versa, lower enzyme activity often provides lower inhibitor concentrations detected because of higher part of the enzyme inactivated in target interactions. Affinity of the substrate toward the enzyme active site is more important. For this reason, kinetic parameters of enzymatic reaction have been explored for various assemblies of the polyelectrolyte complexes.

3.2. Kinetic parameters of the acetylthiocholine hydrolysis

Amperometric signal of the AChE catalyzed hydrolysis of ATCh was recorded with the glassy carbon electrode modified with CB and Co phtalocyanine, which previously were examined for mediated thiocholine oxidation and showed excellent efficiency and low working potential [28, 29]. Co phtalocyanine does not form separate peak on voltammogram. However, the current recorded at 150-250 mV regularly increases with the thiocholine concentration. CB stabilizes Co phtalocyanine on the electrode interface and increases the surface area and efficiency of electron transduction. In case of further polyelectrolyte assembling, negative charge of carboxylate groups on the surface of CB particles acts as anchor for binding positively charged components of the complex. The signal was measured at 200 mV offering most reproducible and stable current in the whole range of the ATCh concentrations used.

The influence of the current on the substrate concentration was monitored for various polyelectrolyte complexes. Concentration curves obtained were used for estimation of efficiency of the AChE immobilization and calculation of the Michaelis constant (K_M). For this purpose, electrochemical form of the Michaelis-Menten equation (6) describing the concentration dependence of the current was used. Here i is the thiocholine oxidation current, i_{max} is its maximal level corresponding to the saturation of the immobilized enzyme with the substrate and

c_S is the bulk ATCh concentration.

$$i = \frac{i_{\max} \cdot c_S}{c_S + K_M} \tag{6}$$

Eq. (5) was applied as approximation model for the calculation of the apparent $i_{\max}$ and K_M values (Table 2). The ATCh concentration was varied between 1.0 μM and 10.0 mM and the current (i) values were measured in triplicate for each of 14 different concentrations with at least three individual biosensors. In Table 2, average ± standard deviation values are presented.

The comparison of the results showed remarkable difference in the parameters of the layers including PAA and PDDA. In case of PAA, $i_{\max}$ is mostly affected by the nature of the component contacting the enzyme. The PSS outer layer provides higher currents against those with PAA due to electrostatic attraction of cationic ATCh molecules. In case of PDDA, such an influence is much milder. Recorded currents regularly decrease with the total number of polyelectrolyte layers. Similar decay was also observed for increasing number of PSS-PAA pairs. The SPR data indicated increase of the amounts of immobilized enzyme with the number of polyelectrolyte layers, so that current decrease might be due to progressive limitation of the thiocholine transfer from the enzyme location through the polyelectrolyte layer to the electrode. Bigger current decay in the complexes with the PAA outer layer and PSS as counter ion is probably related to a high density of negative charge and hydrophobicity of the PSS core, which do not promote the enzyme adsorption.

The apparent K_M value regularly decreases with the number of polyelectrolyte layers in the complexes PDDA-PSS while the PAA-PSS complexes rather slowly affect its value. Even more, the K_M was slightly bigger if PAA was an outer layer. With the PSS outer layer, the K_M obtained with the PAA containing complexes was twice less and became minimal among all the polyelectrolyte complexes considered.

Variation of the apparent K_M values depends on the electrostatic interactions of the enzyme microenvironment with the charged substrate molecule. the In PAA case, positive surface charge hinders diffusion of the positively charge substrate to the enzyme active site. Considering dipole character of the enzyme globule, the AChE active site is located in negatively charged part of the molecule that is deeper immersed in the polyelectrolyte matrix than oppositely charged free part of the protein. Electrostatic limitations of the substrate diffusion are confirmed by the results obtained with PSS deposited onto the enzyme adsorbed (PAA-PSS-

PAA-AChE-PSS). The K_M decreased threefold against that obtained with the same complex with outer PSS (PAA-PSS-PAA-AChE). For the following determination of reversible inhibitors that are also cationic substances, the complex (PAA-PSS-PAA-AChE-PSS) was chosen. In addition to the lowest K_M value corresponded to high affinity of cationic substrate to the enzyme active site, this complex stabilized the enzyme structure and prevented enzyme desorption during the biosensor operation.

3.3. Conditions for inhibition measurement

Inhibition caused by DON and BER was evaluated by consecutive addition of the substrate and inhibitor to the buffer solution followed by the measurement of appropriate current shift. All the measurements were performed at a constant polarization of the AChE biosensor at 200 mV which was found sufficient for mediated oxidation of thiocholine and generation of reliable and reproducible signal.

First, pH dependence of the inhibition effect was considered (Fig. 3). In weakly basic media, a broaden maximum of the inhibition degree at pH = 7.8 was obtained corresponded to the pH dependence of free AChE. Other experiments were performed at this pH value.

As could be seen, BER exerts much lower inhibition effect which is about constant in rather broad range of the pH. This might be due to difference in the DON and BER structure (Figure 4).

The reversible AChE inhibition is controlled by interaction between the cationic center of an inhibitor molecule and anionic site of the enzyme. In case of DON, salt form of the drug was used with protonated amino group active in such an interaction. The reaction is also promoted by hydrophobic interactions with aromatic residues near the active site gorge of the AChE molecule [16, 17]. Meanwhile, quaternary ammonium group in BER molecule is sterically hindered so that inhibitory activity of BER is lower than that of DON.

Fig. 5 displays the effect of the ratio of the substrate/inhibitor concentration ratio on the inhibition degree. DON is more active inhibitor was used in these experiments. For comparison, concentration dependence of the response obtained prior to inhibitor addition is presented.

Relative decay of the current recorded prior and after addition of 10 nM DON regularly decreases with increased ATCh concentration in accordance with kinetics of competitive inhibition [30]. Meanwhile the deviation of the signal also increases with the anodic current recorded. Thus, the following experiments have been performed using 0.2 mM ATCh concentration as a compromise between the biosensor signal and accuracy of its recording. It should be mentioned that contrary to other works on the AChE biosensors, implementation of the AChE in the polyelectrolyte layer does not allow considering different enzyme quantities because the activity of immobilized enzyme is established by intrinsic properties of polyelectrolytes involved in the complex by self-assembling. Independency of the AChE signal on the concentration of the AChE used on the stage of complex assembling was confirmed in independent experiments. Thus, variation of the AChE concentration in aliquot within 0.03-0.20 U/μL resulted in 3% deviation of maximal signal. This is comparable with the sensor-to-sensor repeatability of the above parameter reached for each enzyme concentration.

3.3. DON and BER determination

The calibration curves of DON and BER obtained with a constant ATCh concentration of 0.2 mM are non-linear (Fig. 5) and can be fitted by logistic four-parameter function (7).

$$I,\% = \frac{A_1 - A_2}{1 + (c_I / b)^p} + A_2 \qquad (7)$$

Here c_I is an inhibitor concentration, A_1 and A_2 are upper and lower limits of the curve, ideally near 0 and 100%, respectively, and b characterized half inhibition value of an inhibitor concentration and p reflects sensitivity of the enzyme toward inhibitor (slope of the middle part of the curve). Similar dependencies are frequently used for the description of the immunochemical assay results [31]. Both cases describe similar models with reversible formation of 1:1 complex inactive from the point of view of detection system used. Appropriate calibration curves are presented in Fig. 6 and corresponding fitting parameters summarized in Table 3.

Limit of detection (LOD) was estimated on the level of 15% inhibition which corresponds to 5% deviation of the current recorded (S/N = 3 criterion). Similar cut-off value is often used in the works describing irreversible inhibition evaluated with the AChE biosensors [1, 2, 28, 32]. Sensitivity of an inhibitor determination was expressed by its concentration IC_{50} corresponded to 50% inhibition. In case of DON, covalent AChE immobilization resulted in increase of the IC_{50} value against free enzyme and the parameter obtained in this research. Hence, implementation of the enzyme in the polyelectrolyte complex retains native enzyme structure and its accessibility toward reversible inhibitor.

The characteristics of DON and BER determination obtained with the AChE biosensor based on polyelectrolyte complex are comparable or better than those reported for other biosensors (Table 4).

It should be mentioned that enzymes from various source were applied in biosensor assembly. This complicates direct comparison of the biosensor performance. The only method offered lower LOD value, utilized electropolymerized redox active layer with two enzymes, i.e., choline oxidase and AChE attached to its surface. Higher sensitivity can be probably referred to synergic effect of the analyte on all the components of the layer. Besides, linear range of concentrations near this LOD value is very narrow and for this reason can hardly be used for real sample assay.

Regarding BER, its interaction with the AChE was first characterized as competitive-non-competitive in 2002 [18]. Since that time, a number of reviews considered various aspects of inhibition by BER and its derivatives as anti-dementia drugs [39, 40]. However, only few reports have been devoted to analytical applications of BER-AChE interaction. This approach was applied in spectrophotometric detector in liquid chromatography [38] and in a similar amperometric device coupled with the FIA system [37]. Lower sensitivity of the BER determination vs. DON can be explained, as in the case of pH sensitivity of the signal, by higher steric limitation of the enzyme-inhibitor interaction.

As BER inhibition is less investigated and detectable concentrations are higher than those of DON, the parameters of calibration curves were also determined with different substrate concentration. Thus, decrease of the ATCh concentration from 0.2 to 0.1 mM decreased the IC_{15} value to 60 nM whereas its increase to 0.5 and 2.0 mM obviously increased this parameter to 107 and 187 nM. The IC_{50} concentrations varied as follows: 0.54 (0.1 mM ATCh), 0.93 (0.5 mM

ATCh) and 1.24 μM (2.0 mM ATCh). The upper quantification level estimated by reaching 85% inhibition varied for the same substrate concentration range from 5.6 to 16.9 μM. Thus, the behavior of the AChE biosensor coincides well with kinetic of reversible inhibition assuming competitive or mixed mechanism of inhibition [18].

3.4. Biosensor precision, lifetime and real sample analysis

Although the polyelectrolyte complexes are rather sensitive to physical contacts, the use of CB - Co phtalocyanine underlying layer provides stability of the biosensor assembly sufficient for multiple use and rather long storage. All the results were obtained with the biosensor with enzyme layer additionally covered with the PSS layer. The sensor-to-sensor repeatability determined for ten biosensors prepared from the same set of reagents was found to be 4.5% for 0.2 mM ATCh and 5.5% for 1.0 mM ATCh. The storage of the biosensors in dry conditions at 4 ^{o}C did not affect the response toward the substrate and DON for at least six weeks. After that, the activity of the AChE began regularly decreasing by 10% per weak. Nevertheless, inhibition degree remained quite stable until reaching 50% decay of the response. Reversible character of inhibition assumes possibility of biosensor recovery by washing out the inhibitors after measurements. Indeed, it can be performed by simple washing with working buffer solution. Unfortunately, this procedure significantly increases deviation of the response toward the inhibitor on second (third etc.) cycle of measurement – washing. For robust reliable drug determination single use of the AChE biosensor is recommended. This will also avoid possible mistakes related to the impurities of the samples with biological materials of other patients.

Applicability of the AChE biosensor developed for the real samples analysis was proved by testing artificial urine samples spiked with the drugs investigated. Samples contained main urine components including inorganic salts, urea and creatinine. Prior to measurements, the samples were diluted with phosphate buffer solution in 1:1 (DON) or 1:3 (BER) vol. ratio and adjusted to pH = 7.8. It was preliminary shown that the inhibitory effect of the samples prior to inhibitor addition was negligible. The addition of DON to its final concentration 0.6 nM in diluted artificial urine resulted in the detection of 0.62±0.15 nM, n =5, or 103±24%, based on non-linear calibration graph plotted using standard analyte. Rather high deviation of the results is because of the rather flat course of the curve (compare Fig. 5) in this concentration region. Increase of the DON concentration to 10 nM decreased error to 6% , which is comparable with

the deviation of the signal in standard solution. For 0.1 μM BER the measurement result (0.11±0.01 μM, n = 5) corresponded to the 110±10% recovery.

It should be mentioned that DON is predominantly released from the human being via urine (57% of daily dose) [41] so that the biosensor can be used to monitor this drug. BER is metabolized to some products with even higher anti-cholinesterase activity (berberrubine and thalifendine) that are excreted together with parent drug so that the biosensor can be used only for semi-quantitative BER assessment [42]. In both cases, no effect of sample matrix on inhibition measured with the AChE biosensor developed was found.

4. Conclusion

In this work, polyelectrolyte complexes based on electrochemically inactive polycations and polyanions have been explored as matrix for electrostatic AChE immobilization. The polyelectrolyte complex obtained by rather simple protocol of drop casting of oppositely charged agents onto the electrode surface was characterized using PCR method to establish the role and possible mechanism of influence of the polyelectrolyte nature and content. Based on this consideration, it was supposed that the self-assembling protocol provides site-oriented immobilization of the dipole AChE molecule in the layer so that active site remains fully accessible for interaction with the substrate and inhibitor. Appropriate interactions were monitored by amperometric system consisted of carbon black – Co phtalocyanine mediator catalyzed oxidation of thiocholine, a product of enzymatic hydrolysis of ATCh, an artificial substrate. Kinetic parameters of enzymatic reaction showed preferable position of the enzyme in outer layer of the polyelectrolyte complex and electrostatic control of the substrate access affecting the current recorded.

The AChE biosensor developed showed robust response toward two model drugs one of which is approved (DON) and the second one considered as promising in the Alzheimer's disease therapy (BER). They competitively decreased the response with decreased substrate and increased inhibitor concentration. The concentration dependence of the signal was fitted with the four-parameter logistic curve. The sensitivity of determination coincides or is better than characteristics of biosensors based on covalently attached AChE described in the literature. Potential application of the biosensor described was shown by determination of the drugs in spiked samples of artificial urine with satisfactory recovery.

Acknowledgements

Financial support of Russian Foundation for Basic Research (RFBR), grant No 17-03-00381 is gratefully acknowledged.

Declarations of interest: none

Conflict of interests

No conflict of interests is declared.

References

1. Y. Miao, N. He, J.-J. Zhu, History and new developments of assays for cholinesterase activity and inhibition, Chem. Rev. 110 (2010) 5216-5234. doi: 10.1021/cr900214c.
2. M.Pohanka, Electrochemical biosensors based on acetylcholinesterase and butyrylcholinesterase. A review, Int. J. Electrochem. Sci. 11 (2016) 7440-7452. doi: 10.20964/2016.09.16.
3. C. Jefferson, Origins of the norm against chemical weapons, Intern. Affairs 90 (2014) 647-661. https://doi.org/10.1111/1468-2346.12131
4. F. Arduini, A. Amine, D. Moscone, G. Palleschi, Biosensors based on cholinesterase inhibition for insecticides, nerve agents and aflatoxin B1 detection (review), Microchim. Acta 170 (2010) 193-214. doi: 10.1007/s00604-010-0317-1
5. M.Pohanka, Cholinesterases in biorecognition and biosensors construction: A review, Anal. Letters 46 (2013) 1849-1868. https://doi.org/10.1080/00032719.2013.780240.
6. E.A. Songa, J.O. Okonkwo, Recent approaches to improving selectivity and sensitivity of enzyme-based biosensors for organophosphorus pesticides: A review, Talanta 155 (2016) 289-304. https://doi.org/10.1016/j.talanta.2016.04.046.
7. B. Rajangam, D.K. Daniel, A.I. Krastanov, Progress in enzyme inhibition based detection of pesticides, Eng. Life Sci. 18 (2018) 4-19. https://doi.org/10.1002/elsc.201700028.
8. J.M. Wilkins, E. Trushina, Application of metabolomics in Alzheimer's disease, Frontiers Neurol. 8 (2018) 719. doi: 10.3389/fneur.2017.00719.
9. D. Munoz-Torrero, Acetylcholinesterase inhibitors as disease-modifying therapies for Alzheimers disease, Curr. Med. Chem. 15 (2008) 2433-2455. https://doi.org/10.2174/092986708785909067
10. A.K. Sahoo, J. Dandapat, U.C. Dash, S. Kanhar, Features and outcomes of drugs for combination therapy as multi-targets strategy to combat Alzheimer's disease, J. Ethnopharm. 215 (2018) 42-73. https://doi.org/10.1016/j.jep.2017.12.015.
11. N. Bassil, G.T. Grossberg, Novel regimens and delivery systems in the pharmacological treatment of Alzheimer's disease, CNS Drugs 23 (2009) 293-307. https://doi.org/10.2165/00023210-200923040-00003.
12. L. Liu, Z.Chen, Analysis of four alkaloids of *Coptis chinensis* in rat plasma by high performance liquid chromatography with electrochemical detection, Anal. Chim.Acta 737 (2012) 99-104. https://doi.org/10.1016/j.aca.2012.05.054
13. L.A. Marques, M. Giera, H. Lingeman, W.M.A. Niessen, Analysis of acetylcholinesterase inhibitors: bioanalysis, degradation and metabolism, Biomed. Cromatogr. 25 (2011) 278-299. https://doi.org/10.1002/bmc.1573
14. S. Yıldırım, N. U. Çolak, A. Yaşar, Application of dispersive liquid-liquid microextraction for the determination of donepezil in urine by HPLC using a core-shell column, J. Liquid Chromat. Rel.Techn. 41 (2018) 66-72. https://doi.org/10.1080/10826076.2017.1373669.
15. L. El Harrad, I. Bourais, H. Mohammadi, A. Amine, Recent advances in electrochemical biosensors based on enzyme inhibition for clinical and pharmaceutical applications, Sensors 18 (2018) 164. doi:10.3390/s18010164.
16. G. Kryger, I. Silman, J. L. Sussman, Structure of acetylcholinesterase complexed with E2020 (Aricept®): implications for the design of new anti-Alzheimer drugs, Structure, 7 (1999) 297-307. https://doi.org/10.1016/S0969-2126(99)80040-9.
17. J. Cheung, M.J. Rudolph, F. Burshteyn, M.S. Cassidy, E.N. Gary, J. Love, M.C. Franklin, J.J. Height. Structures of human acetylcholinesterase in complex with pharmacologically important ligands. J. Med. Chem. 55 (2012) 10282-10286. doi: 10.1021/jm300871x.

18. L.P. Kuznetsova, E.B. Nikol'skaya, E.E. Sochilina, M.D. Fadeeva, E. Inhibition of human blood acetylcholinesterase and butyrylcholinesterase by some alkaloids, J. Evol. Biochem. Physiol. 38 (2002) 35-39. https://doi.org/10.1023/A:1015561103682.
19. F. Zhu, C. Qian, Berberine chloride can ameliorate the spatial memory impairment and increase the expression of interleukin-1beta and inducible nitric oxide synthase in the rat model of Alzheimer's disease, BMC Neurosci. 7 (2006) 78. doi:10.1186/1471-2202-7-78
20. J. Lahiri, L. Isaacs, J. Tien, G. M. Whitesides, A strategy for the generation of surfaces presenting ligands for studies of binding based on an active ester as a common reactive intermediate: A surface plasmon resonance study, Anal. Chem. 71 (1999) 777-790. doi: 10.1021/ac980959t.
21. H. Dvir, I. Silman, M. Harel, T.L. Rosenberry, J.L. Sussman, Acetylcholinesterase: from 3D structure to function, Chem. Biol. Interact. 187 (2010) 10-22. doi: 10.1016/j.cbi.2010.01.042.
22. Y. Zhang, M.A. Arugula, J.S. Kirsch, X. Yang, E. Olsen, A.L. Simonian, Layer-by-layer assembled carbon nanotube-acetylcholinesterase/biopolymer renewable interfaces: SPR and electrochemical characterization, Langmuir 31 (2015) 1462-1468. doi: 10.1021/la503474w.
23. M. Losche, J. Schmitt, G. Decher, W.G. Bouwman, K. Kjaer, Detailed structure of molecularly thin polyelectrolyte multilayer films on solid substrates as revealed by neutron reflectometry, Macromolecules 31 (1998) 8893-8906. doi: 10.1021/ma980910p.
24. P. Nazaran, V. Bosio, W. Jaeger, D.F. Anghel, R.v. Klitzing, Lateral mobility of polyelectrolyte chains in multilayers, J. Phys. Chem. B. 111 (2007) 8572-8581. doi: 10.1021/jp068768e.
25. J.E. Wong, F. Rehfeldt, P. Hanni, M. Tanaka, R.v. Klitzing, Swelling behavior of polyelectrolyte multilayers in saturated water vapor, Macromolecules 37 (2004) 7285-7289. doi: 10.1021/ma0351930.
26. M. Elzbieciak, M. Kolasinska, S. Zapotoczny, R. Krastev, M. Nowakowska, P. Warszynski, Nonlinear growth of multilayer films formed from weak polyelectrolytes, Colloid. Surf. A. 343 (2009) 89-95. https://doi.org/10.1016/j.colsurfa.2009.01.034
27. S. Goutelle, M. Maurin, F. Rougier, X. Barbaut, L. Bourguignon, M. Ducher, P. Maire, The Hill equation: a review of its capabilities in pharmacological modelling, Fundam. Clin. Pharm. 22 (2008) 633-648. doi: 10.1111/j.1472-8206.2008.00633.x.
28. A.N. Ivanov, R.R. Younusov, G.A. Evtugyn, F. Arduini, D. Moscone, G. Palleschi, Acetylcholinesterase biosensor based on single-walled carbon nanotubes - Co phtalocyanine for organophosphorus pesticides detection, Talanta 85 (2011) 216-221. https://doi.org/10.1016/j.talanta.2011.03.045.
29. N.B. Oujji, I. Bakas, G. Istamboulié, I. Ait-Ichou, E. Ait-Addi, R. Rouillon, T. Noguer, Sol-gel immobilization of acetylcholinesterase for the determination of organophosphate pesticides in olive oil with biosensors, Food Control 30 (2013) 657-661. https://doi.org/10.1016/j.foodcont.2012.09.005
30. I.H. Segel, Enzyme Kinetics, Wiley J. & Sons, New York, 1975. ISBN-13: 978-0471303091.
31. M.A.O 'Connell, B.A. Belanger, P.D. Haaland, Calibration and assay development using the four-parameter logistic model, Chemometrics Intel. Lab. Syst. 20 (1993) 97-114. https://doi.org/10.1016/0169-7439(93)80008-6
32. G. Evtugyn, Biosensors for detection of anticholinesterase agents, In: Biosensors for Security and Bioterrorism Applications. Advanced Sciences and Technologies for Security Applications. (Nikolelis D.P., Nikolei G.-P. eds.) Springer, Cham, 2016, pp. 349-384. https://doi.org/10.1007/978-3-319-28926-7_17.
33. A.J. Veloso, P.M. Nagy, B. Zhang, D. Dhar, A. Liang, T. Ibrahim, S. Mikhaylichenko, I. Aubert, K. Kerman, Miniaturized electrochemical system for cholinesterase inhibitor detection, Anal. Chim. Acta 774 (2013) 73-78. http://dx.doi.org/10.1016/j.aca.2013.02.033

34. E. Fabini, A. Tramarin, M. Bartolini, Combination of human acetylcholinesterase and serum albumin sensing surfaces as highly informative analytical tool for inhibitor screening, J. Pharm. Biomed. Anal. 155 (2018) 177-184. https://doi.org/10.1016/j.jpba.2018.03.060.
35. J. Turan, M. Kesik, S. Soylemez, S. Goker, M. Kolb, M. Bahadir, L. Toppare, Development of an amperometric biosensor based on a novel conducting copolymer for detection of anti-dementia drugs, J. Electroanal. Chem. 735 (2014) 43-50. https://doi.org/10.1016/j.jelechem.2014.10.007
36. M.-S. Chae, Y.K. Yoo, J. Kimc T.G. Kim, K.S. Hwang, Graphene-based enzyme-modified field-eff ect transistor biosensor for monitoring drug eff ects in Alzheimer's disease treatment, Sens. Actuators B 272 (2018) 448-458.
37. M. Vandeput, C. Parsajoo, J. Vanheuverzwijn, S. Patris, Y. Yardim, A. le Jeune, A. Sarakbi, D. Mertens, J.-M. Kauffmann, Flow-through enzyme immobilized amperometric detector for the rapid screening of acetylcholinesterase inhibitors by flow injection analysis, J. Pharm. Biomed. Anal. 102 (2015) 267-275. http://dx.doi.org/10.1016/j.jpba.2014.09.012.
38. L. Wang, Y. Zhao, Y. Zhang, T. Zhang, J. Kool, G.W. Somsen, Q. Wang, Z. Jiang, Online screening of acetylcholinesterase inhibitors in natural products using monolith-based immobilized capillary enzyme reactors combined with liquid chromatography-mass spectrometry, J. Chromatogr. A 1563 (2018) 135-143.
39. H.-F. Ji, L. Shen, Berberine: a potential multipotent natural product to combat Alzheimer's disease, Molecules 16 (2011) 6732-6740; doi:10.3390/molecules16086732.
40. Y. Jiang, H. Gao, G. Turdu, Traditional Chinese medicinal herbs as potential AChE inhibitors for anti-Alzheimer's disease: A review, Bioorg. Chem. 75 (2017) 50-61. https://doi.org/10.1016/j.bioorg.2017.09.004
41. P.J. Tiseo, C.A. Perdomo, L.T. Friedhoff, Metabolism and elimination of ^{14}C-donepezil in healthy volunteers: A single dose study. Br. J. Clin. Pharmacol, 46 (1998) 19-24. doi: 10.1046/j.1365-2125.1998.0460s1019.x.
42. J.- Y. Ma, R. Feng, X.- S. Tan, C. Ma, J.- W. Shoum J. Fu, M. Huang, C.- Y. He, S.- N. Chen, Z.- X. Zhao, W.- Y. He, Y. Wang, J.- D. Jiang, Excretion of berberine and its metabolites in oral administration in rats, J. Pharm. Sci. 102 (2013) 4181-4192.

Fig. 1. The dependence of the SPR signal on the number of polyelectrolyte layers consecutively deposited on the Au surface of the SPR chip modified with MUA

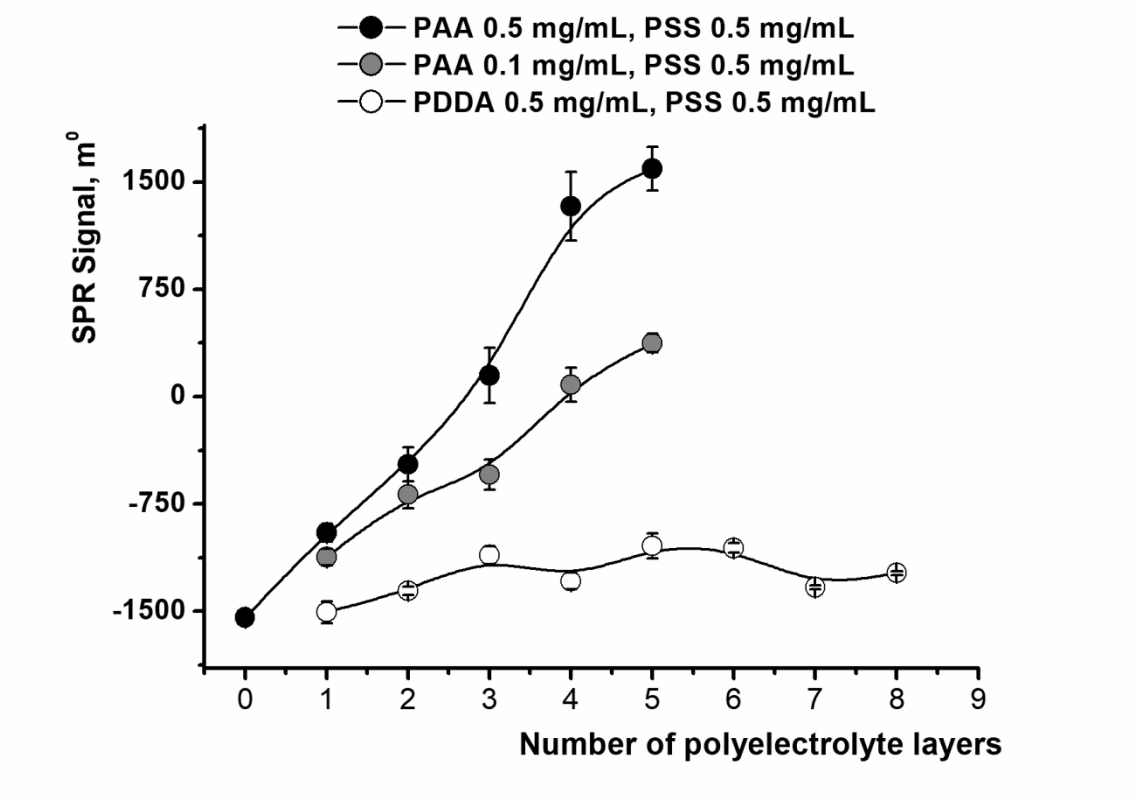
Fig. 2. Efficiency of the AChE immobilization at various assemblies of the polyelectrolyte complexes and enzyme concentration taken for immobilization. 1 – PAA 0.5 mg/mL, one layer; 2 – PAA 0.5 mg/mL + PSS 0.5 mg/mL, three layers; 3 - PAA 0.1 mg/mL + PSS 0.5 mg/mL, three layers; 4 - PAA 0.1 mg/mL + PSS 0.5 mg/mL, four layers; 5 - PDDA 0.5 mg/mL + PSS 0.5 mg/mL, three layers; 6 - PDDA 0.5 mg/mL + PSS 0.5 mg/mL, five layers; 7 - PDDA 0.5 mg/mL + PSS 0.5 mg/mL, six layers. Average ± standard deviation calculated from three repetitions.

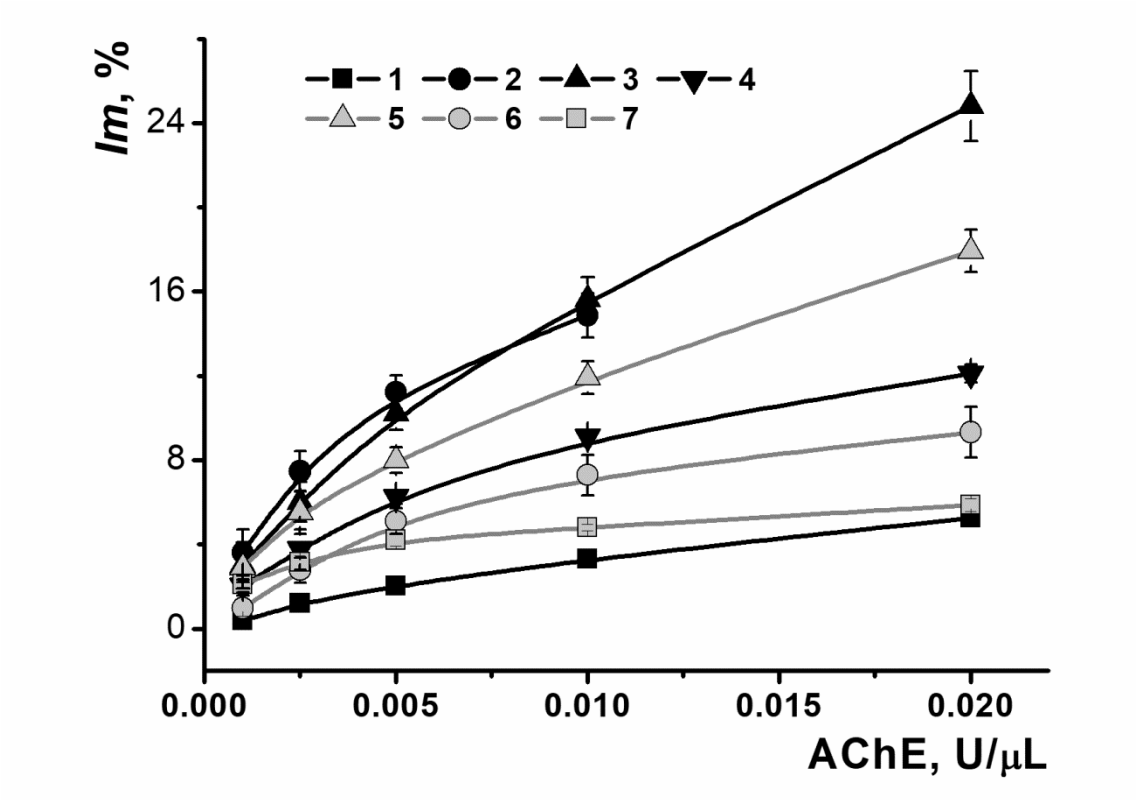
Fig. 3. pH Dependence of the DON and BER inhibition measured with the AChE biosensor based on PAA-PSS-PAA-AChE-PSS polyelectrolyte complex. ATCh 0.2 mM, average of three replications

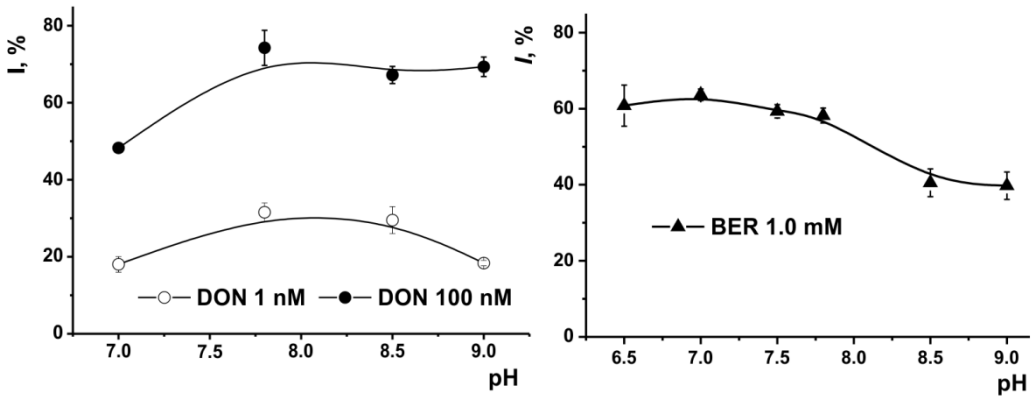
Fig. 4. Chemical structures of reversible AChE inhibitors studied. DON – donepezil, BER - berberine

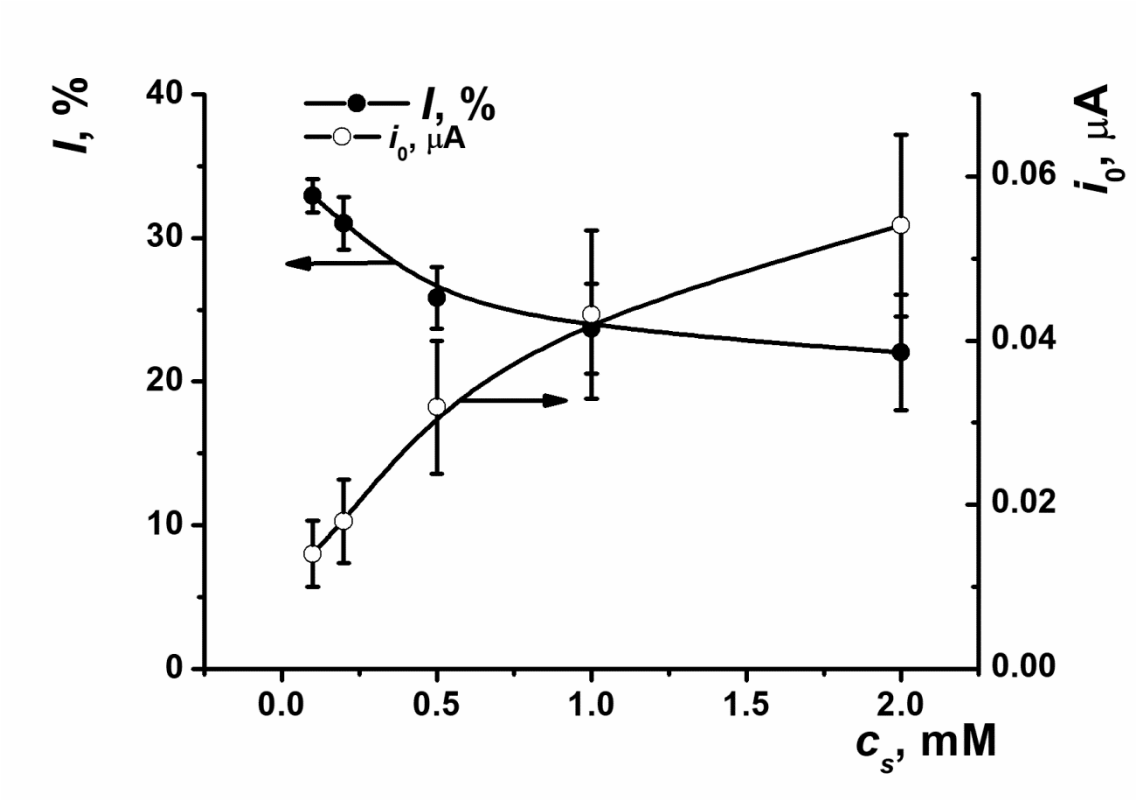
Fig. 5. The dependence of the inhibition degree (I, %) and anodic current of thiocholine oxidation (i_0, mA) on the ATCh concentration. The AChE biosensor based on PAA-PSS-PAA-AChE-PSS polyelectrolyte complex. DON 10 nM, Average ± standard deviation calculated from three repetitions

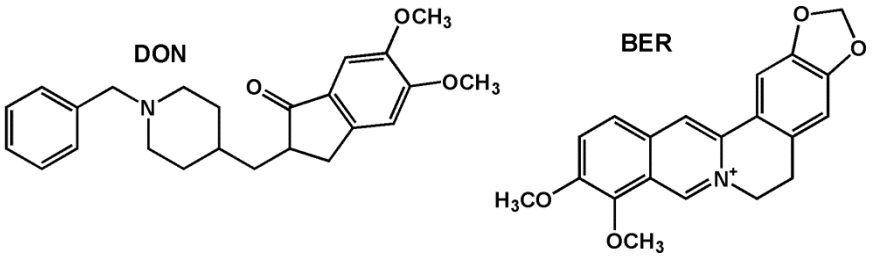
Fig. 6. Determination of DON and BER with the AChE biosensor based on PAA-PSS-PAA-AChE-PSS polyelectrolyte complex. ATCh 0.2 mM, Average ± standard deviation calculated from six repetitions. Lines represent fitting results based on four-parameter logistic function











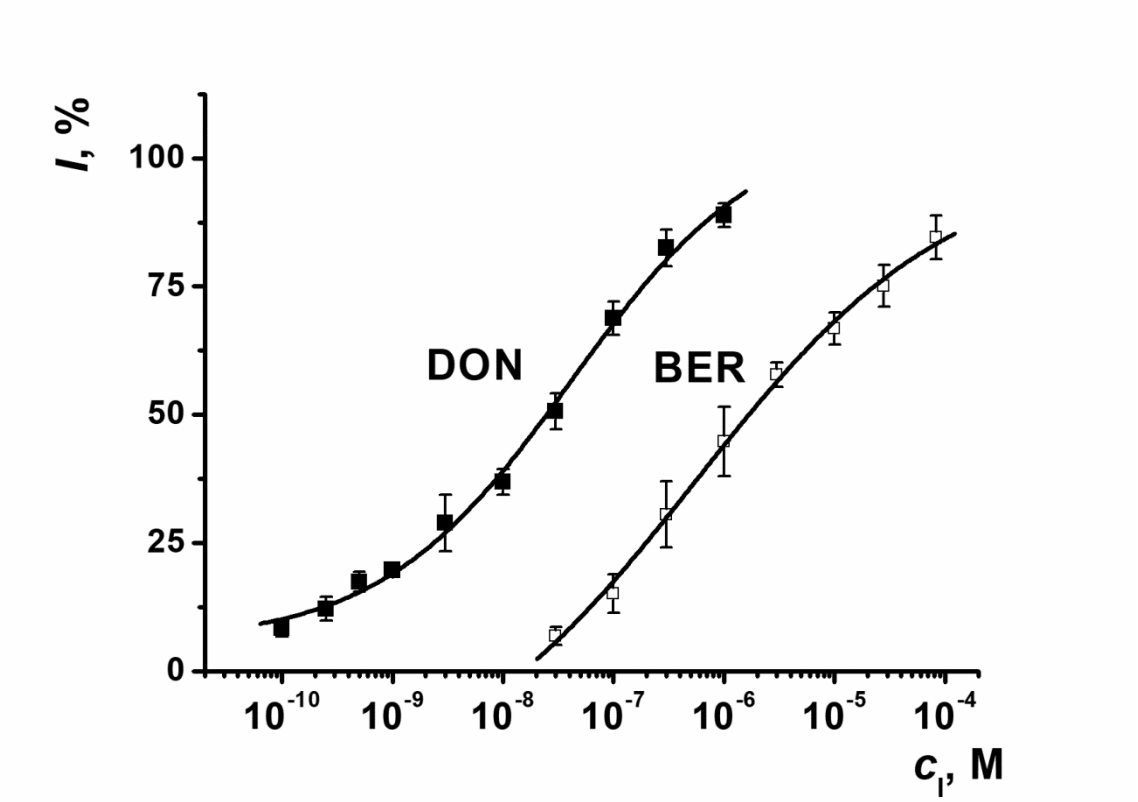


Table 1. Maximal efficiency of the AChE immobilization for various assemblies of polyelectrolyte complexes. Average ± standard error calculated from three repetitions. The line numbers correspond to the complex content denoted in legend to Fig. 2; n, k and R^2 are the Hill model parameters (Eq.4)

No	Im^{max}, %	k, U/μL	n	R^2
1	12.6±1.8	0.023±0.013	1.01±0.13	0.9992
2	23.5±0.2	0.0045±0.0001	1.06±0.01	0.9999
3	137±16	0.13±0.02	0.75±0.08	0.9987
4	22.5±0.9	0.013±0.001	0.87±0.02	0.9999
5	214±25	0.78±0.14	0.62±0.05	0.9993
6	12.1±0.3	0.0056±0.0005	1.33±0.07	0.9992
7	11.0±1.4	0.011±0.002	0.52±0.09	0.9878

Table 2. Kinetic parameters (i_{max} and apparent K_M) of ATCh hydrolysis measured by glassy carbon electrode modified with CB and Co phtalocyanine and covered with polyelectrolyte complexes with physically adsorbed AChE. Concentration of the polyelectrolytes used 0.5 mg/mL. Average ± standard deviation calculated from results obtained with 14 substrate concentrations measured in triplicate

Polyelectrolyte complex content	i_{max} , μA	K_M , mM	R^2
AChE	0.68±0.11	0.7±0.3	0.8879
PAA-AChE	0.65±0.18	1.1±0.1	0.9929
PAA-PSS-AChE	0.11±0.01	0.48±0.04	0.9968
PAA-PSS-PAA-AChE	0.38±0.06	1.9±0.2	0.9967
$(PAA-PSS)_2$-AChE	0.099±0.009	0.59±0.03	0.9993
$(PAA-PSS)_2$-PAA-AChE	0.34±0.03	1.15±0.03	0.9976
$(PAA-PSS)_3$-AChE	0.015±0.005	0.59±0.04	0.9987
PAA-PSS-PAA-AChE-PSS	0.047±0.003	0.51±0.12	0.9430
PDDA-AChE	0.72±0.04	1.63±0.06	0.9998
PDDA-PSS-AChE	0.77±0.05	2.16±0.02	0.9983
PDDA-PSS-PDDA-AChE	0.42±0.04	0.94±0.05	0.9992
$(PDDA-PSS)_2$-AChE	0.36±0.06	0.62±0.06	0.9951
$(PDDA-PSS)_2$-PDDA-ACHE	0.36±0.05	0.65±0.05	0.9958
$(PDDA-PSS)_3$-AChE	0.28±0.03	0.48±0.03	0.9973

Table 3. Approximation parameters based on logistic model for inhibitor determination with the AChE biosensor based on polyelectrolyte complex, six repetitions, average ± standard error

Inhibitor	DON	BER
A_1, %	5.5±3.6	-25±22
A_2, %	107±9	100±5
b, M	$(4.1\pm1.4)\times10^{-8}$	$(5.5\pm1.5)\times10^{-7}$
p	0.51±0.08	0.37±0.05
R^2	0.9964	0.9972
IC_{15}, nM	0.46	70
IC_{50}, nM	24.7	590

Table 4. Comparison of analytical characteristics of the AChE based DON and BER determination with biosensing devices

Method	Enzyme immobilization	LOD, nM	IC_{50} , nM	Ref.
	DON			
Differential pulse voltammetry	Free AChE from human erythrocytes, Au screen-printed electrode	-	28±7	[33]
SPR	Human recombinant AChE bonded to human serum albumin deposited on the Au film		20±1	[34]
Cyclic voltammetry	Covalent binding together with choline oxidase to electropolymerized thiophene derivative	0.07	-	[35]
pH sensitive field effect transistor	Affine immobilization onto graphene via pyrene butanoic acid and carbodiimide binding		47	[36]
Amperometry in FIA mode	Covalent attachment to Au via cysteamine-glutaraldehyde binding		500	[37]
Amperometry	Implementation in polyelectrolyte complexes	0.46	24.7	This work
	BER			
Amperometry in FIA mode	Covalent attachment to Au via cysteamine-glutaraldehyde binding		6450	[37]
Photometric	Capillary enzyme reactor with the AChE immobilized onto the polymethacrylate monolith and coupled with liquid chromatography		3.1±0.6	[38]
Amperometry	Implementation in polyelectrolyte complexes	70	590	This work

Highlights:

1. Acetylcholinesterase was immobilized by entrapment in polyelectrolyte complexes.
2. Enzyme capacity and complex properties were evaluated with surface plasmon resonance.
3. Kinetics of enzymatic reaction depends on electrostatic and steric factors.
4. Anti-dementia drugs (donepezil and berberine) were determined with high sensitivity.