UNIVERSITY OF PARDUBICE

FACULTY OF CHEMICAL TECHNOLOGY

Department of Analytical Chemistry

Adam Kostelník

Advanced analytical procedures using cholinesterases

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Author: **Mgr. Adam Kostelník**

Supervisor: **prof. Ing. Alexander Čegan, CSc.**

Supervisor – specialist: **prof. RNDr. Miroslav Pohanka, Ph.D., DSc.**

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References

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Abstract

This thesis deals with preparation of cholinesterases biosensors for determination of cholinesterase inhibitors. Chemical or physical immobilization procedures were used for preparation of individual biosensors. These procedures involved immobilization of cholinesterase onto surface of commercial or laboratory synthetized magnetic particles and immobilization into or onto surface of membrane. Chemical immobilization was used for assay of cholinesterase inhibitors in combination with magnetic particles. Membrane immobilization was used for colorimetric assay of cholinesterase inhibitors by mobile phone camera. All prepared biosensors proved their function in cholinesterase inhibitors assay. Application of prepared biosensors is expected in environment and food control, pharmaceutical industry, military or biological analysis. This thesis is consisted from comments on individual publications dealing with cholinesterase biosensors preparation, while details are summarized in enclosed publications.

Abstrakt

Předložená disertační práce se zabývá přípravou cholinesterasových biosenzorů pro stanovení cholinesterasových inhibitorů. Pro přípravu jednotlivých cholinesterasových biosenzorů byly použity chemické nebo fyzikální imobilizační postupy. Ty zahrnovaly imobilizaci cholinesteras na povrch komerčních nebo laboratorně připravených magnetických částic a imobilizaci do nebo na povrch membrány. Chemická imobilizace byla použita pro elektrochemické stanovení cholinesterasových inhibitorů v kombinaci s magnetickými částicemi. Membránová imobilizace byla použita pro kolorimetrické stanovení cholinesterasových inhibitorů v kombinaci s fotografickou detekcí mobilním telefonem. Všechny připravené biosenzory prokázaly funkčnost při stanovení cholinesterasových inhibitorů. Použití zde připravených biosenzorů je předpokládáno v oblastech kontroly životního prostředí a potravin, farmaceutickém průmyslu, vojenství nebo analýzách biologických vzorků. Tato práce se skládá z komentářů k jednotlivým publikacím na téma přípravy cholinesterasových biosenzorů, jejich detailní popisy jsou shrnuty v přiložených publikacích.

Keywords

Acetylcholinesterase, butyrylcholinesterase, neurotoxic compounds, inhibitor, biosensor

Klíčová slova

Acetylcholinesterasa, butyrylcholinesterasa, neurotoxické látky, inhibitor, biosenzor

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Introduction

Analysis of poisonous compounds is currently done mainly by chromatographic techniques often in combination with mass detector, which allows direct identification of unknown compound. However, these instruments are expensive and their usage is linked to laboratory, where samples have to be transported, which can be time consuming process. Therefore, use of portable instruments for analysis in field conditions is required. Some of them are working on biosensor principle, which do not have to distinguish compound, but they can point out to certain compound group. Common is use of biosensors with enzyme recognition element.

First findings on field of enzymes were done at the begging of 19. century and about a hundred years later this field started expand very rapidly, when numbers of found enzymes grew quickly. At this time, at half of 20. century, enzymes got into interest in context of biosensors preparation, when Clark and Lyons introduced their biosensor for glucose determination, which became the very first biosensor in history. Since that time, biosensors traveled a long way and many of them found application in routine analysis.

History of cholinesterase biosensors is quite young, first publications were published about 30 years ago, however fast advancement brought countless numbers of publications. Cholinesterase biosensors found their applications in many fields, especially in environment, food control, clinical practice or even on battle field. This is due to cholinesterases, which are enzymes sensitive to compounds, involved in aforementioned applications, which act as a natural or artificial cholinesterases inhibitors.

Current trends in cholinesterase biosensors are focusing on construction of functional but relatively complicated biosensors. Hence, the main effort of this thesis was preparation of biosensors with simple construction, easy adaptable to usage in field conditions, where simplicity and time of analysis is required property. Part of the thesis was research of undescribed interactions of some cholinesterases inhibitors with cholinesterases by *in silico* method.

1. Aims of the thesis

- To create methods for assay of cholinesterase activity using new or unusual cholinesterase substrates.
- To create methods with immobilized cholinesterase for neurotoxic compounds assay and optimization of immobilization procedures.
- To create methods with immobilized cholinesterase on magnetic particles surface.
- To create method for assay of cholinesterase activity using quantum dots.
- To make *in silico* prediction about interaction of cholinesterases with inhibitors.
- To verify functionality of created methods with standard spectrophotometric test and real plasma sample.

2. Theoretical part

2.1. Cholinesterases

We distinguish two cholinesterases, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Role of AChE is known for a long time, it is responsible for terminating neurotransmission in cholinergic nerves; treatment of Alzheimer disease with cholinesterase inhibitors is directed to AChE. On the other hand, role of BChE is still unclear, nevertheless it is known for ability to split compounds like cocaine, heroin, procaine, aspirin or succinylcholine and therefore it is involved in their detoxification. Structure of active site of both cholinesterases involve a few common sings: (i) esteratic site, which contains catalytic triad of Ser, Glu and His and which is responsible for own catalytic activity, (ii) anionic subsite, responsible for right orientation of substrate, (iii) aromatic gorge, responsible for substrate specificity of AChE, BChE has wider aromatic gorge, which gives ability to split greater spectrum of substrates, (iv) peripheral anionic subsite, which is probably target for enzyme modulators, BChE has this site less developed, therefore some modulators, which potentially influence AChE have no or small effect to BChE (Pohanka 2011, Pohanka 2013).

2.2. Cholinesterase inhibitors

Inhibitors of cholinesterases are heterogenous group of substances. Mostly, there are AChE inhibitors due to its clinical importance against BChE. In case of BChE, most of AChE inhibitors have affinity to BChE, however selective inhibitor (tetraisopropyl pyrophosphoramide) of BChE exists and could serve to distinguish both enzymes in unknown sample. Inhibitors of cholinesterases can bind into all important parts of enzyme active site. Inhibitors of esteratic site are carbamates and organophosphates, anionic subsite inhibitors involve inhibitors with aromatic cores and nitrogen or quaternary nitrogen and inhibitors of peripheral anionic subsite are inhibitors with dual mechanism of action (Pohanka 2012b). Cholinesterase inhibitors are important in treatment of Alzheimer disease but they are used in many other fields (pharmaceutical industry, agriculture etc.). Therefore, there is need for their determination to control their levels in patients, industry workers, food and so on. For these purposes, cholinesterase biosensors have been developed.

2.3. Cholinesterase biosensors

Biosensor is device which could be define as analytical device consists from biological part, which provides specificity to analyte(s) and physicochemical transducer, transfers biological interaction into measurable signal. Biosensors period started in half of 20. century, when first biosensor was introduced, and grew very quickly. Cholinesterase biosensors are often constructed as optical or electrochemical ones, however e.g. mass detected biosensors exist. Typical optical cholinesterase biosensor is based on Ellman´s assay utilizing thiocholine esters, but numbers of other substrates for cholinesterase optical biosensors are available. Electrochemical biosensors also using thiocholine esters or in combination of cholinesterase and choline oxidase, hydrogen peroxide can

be detected (Pohanka 2015a). Cholinesterase biosensors using bound form of the enzyme rather than free form.

2.4. Cholinesterase immobilization techniques

Biosensors for cholinesterase inhibitors determination using free form of the enzyme are not common nowadays as enzyme is the most expensive part and during first use is debase in waste, therefore immobilization took place in construction of cholinesterase biosensors. Immobilization process significantly increase usability of cholinesterase biosensors in assay of cholinesterase inhibitors. Immobilization of cholinesterases can be carry out in two main ways. Very successful method for cholinesterase bonding is chemical immobilization. Good example of chemical immobilization is attachment of cholinesterase onto magnetic particles (MPs). It provides good stabilization for the enzyme and big benefit is reuse of bound cholinesterase. Despite benefits that chemical immobilization to MPs offers, only a few publications to this topic were published (Istamboulie et al. 2007, Dominguez et al. 2015, Günther and Bilitewski 1995, Gan et al. 2010, Dzudzevic Cancar et al. 2016, Lui et al. 1997, Ben Oujji et al. 2012). Physical immobilization or entrapment in membrane is the second option in cholinesterase immobilization process. Very popular membrane materials for cholinesterases are gelatin and chitosan. Many authors paid attention to construction of membrane immobilized cholinesterase-based biosensors for assay of cholinesterase inhibitors (Timur and Telefoncu 2004, Pohanka et al. 2013b, Pohanka 2012a).

3. Methodology

Here introduce methods create only a brief list of used methods, details of each method are in published papers included in the thesis.

3.1. Assay of cholinesterase activity

3.1.1. Ellman´s assay

Into standard spectrophotometric cuvette was pipetted 5,5´-dithobis(2-nitro benzoic) acid, cholinesterase solution and acetylthiocholine/butyrylthiocholine. All the solutions were in phosphate buffer saline pH 7.4. Absorbance of solution in cuvette was measured immediately after addition of substrate and then after two minutes of reaction. Cholinesterase activity was calculated from absorbance differences using extinction coefficient 14.150 $l \times mol^{-1} \times cm^{-1}$ for 2-nitro-5-thiobenzoic acid (Eyer et al. 2003).

3.1.2. Electrochemical assay

Electrochemical assay was used in combination with MPs. MPs with immobilized cholinesterase was pipetted into cuvette with phosphate buffer saline pH 7.4 and acetylthiocholine. After enzymatic reaction, medium contains thiocholine was analyzed by square-wave voltammetry and thiocholine concentration or current yield respectively, was directly proportion to enzymatic activity. Current yield during cholinesterase inhibitors measurement was indirectly proportional to enzymatic activity.

3.1.3. Photographic assay

Photography were taken with smart phone using a dark chamber prepared by 3D print technology and evaluated in GIMP 2.8.16 (open source software) using Color picker function. There were analyzed points of red, green and blue channel (RGB points) at the photography. For jpg. picture format it takes values between 0 and 255. Differences between these values before and after reaction were equal to enzymatic activity. Suitability of used channel was assessed experimentally during each analysis.

3.2. Cholinesterase immobilization

- First immobilization of AChE was done onto commercial MPs with surface activated by carboxyl group and using N-(3-dimethylaminopropyl)-N´-ethylcarbodiimide hydrochloride (EDC). It forms covalent bond with amino group of AChE and carboxyl group onto MPs surface.
- Second immobilization of AChE was done onto newly synthetized MPs prepared by research group in Brno. In total, five new types of MPs were prepared with various surface modification, then one modification was chosen and AChE was covalently immobilized onto surface using glutaraldehyde.
- Third type was physical immobilization into gelatin membrane. Liquid gelatin was mixed with AChE and let solidify in ambient temperature onto filter paper surface.
- Adsorption of AChE onto surface of pH sensitive strip with stabilization of enzyme in gelatin membrane was the fourth type of immobilization.

- Fifth immobilization of AChE was done onto surface of activated chitosan. After solidify overnight onto filter paper surface, chitosan was activated by EDC reagent for covalent bonding of AChE.

3.3. Influence of organic solvents to cholinesterases

Organic solvents have to be tested in every cholinesterase assay as enzymatic activity can be influenced. Organic solvents are used as pesticides solvents. Tested organic solvents were following: ethanol, isopropyl alcohol and dimethyl sulfoxide.

3.4. Three-dimension print

Method of three-dimension print was used for creation of dark chamber for photographic assay in shape of tube with flat area as a support for smart phone. Chamber was designed in Autodesk® 123D® Design (Autodesk, San Rafael, CA, USA). Final adjustment of designed chamber was done in Prusa3D Slic3r 3mm (part of printer software) and coordinates for printer were generated. Final size of created chamber was 80 mm in height, 105 mm in length and inner diameter of tube was 40 mm. Settings of the printer: acrylonitrile butadiene styrene as material in 3 mm filament, temperature of nozzle 285 °C, temperature of the bottom plate 100 °C, thickness of single layer was 0.1 mm. Photo of prepared dark chamber can be seen of figure 1.

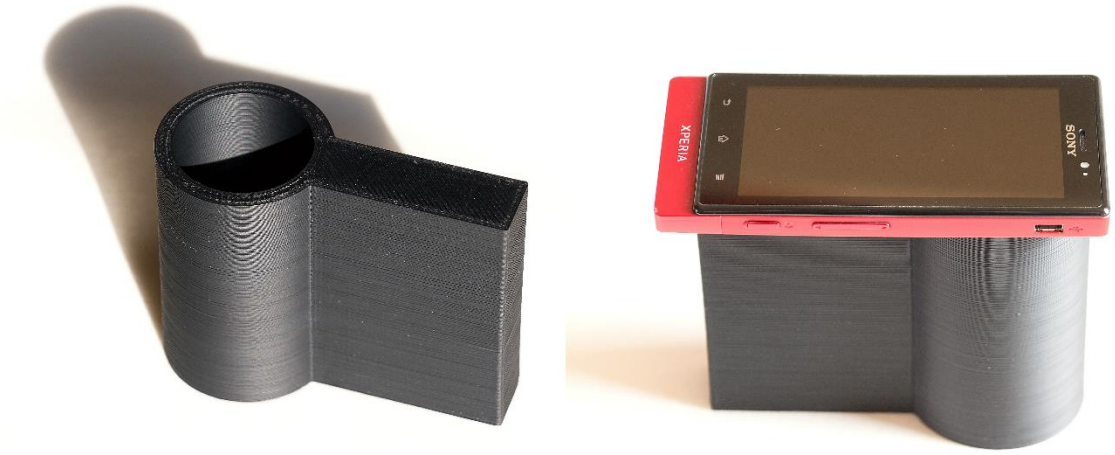

Figure 1. Dark chamber for photographic assay (left) and setting with smart phone (right).

4. Results and Discussion

4.1. Electrochemical determination of activity of acetylcholinesterase immobilized on magnetic particles

MPs are in center of interest for many years and they are used for immobilization of various molecules of protein origin including enzymes (Roque et al. 2009, Koneracká et al. 1999, Rossi et al. 2004, Martinkova et al. 2016, Otari et al. 2019). On the other hand, only a few publications dealing with immobilization of AChE onto MPs (Dzudzevic Cancar et al. 2016, Istamboulie et al. 2007, Lui et al. 1997, Günther and Bilitewski 1995, Dominguez et al. 2015, Gan et al. 2010, Ben Oujji et al. 2012), though use of MPs offers many benefits. Main advantage is reuse, chemical stabilization of immobilized enzyme and also economical view cannot be omitted.

Demand for AChE immobilization onto MPs surface leads from fact, that after Ellman´s standard spectrophotometric assay, AChE is still active (if it is not irreversibly inhibited) and it is debase in waste together with other chemicals. This approach is not very economical as enzyme is the most expensive part of the assay.

In this work, AChE was immobilized onto commercially available MPs surface using EDC reagent and enzyme activity was assayed by square-wave voltammetry method. Used MPs showed their ability to bound the enzyme, however they did not show suitable for repeated measurement. During washing step between measuring cycle sudden degradation of MPs occurred and part of immobilized AChE was washed out which result in loss of catalytical properties. Due to this degradation, benefit of immobilization is lost compare to spectrophotometric assay, nevertheless this degradation occurred in sixth measuring cycle, which still represents considerable saving compare to spectrophotometric assay. Compare to used particles, in cited publications dealing with AChE immobilization onto MPs, for example Istamboulie et al. achieved at least ten cycles and Dzudzevic Cancar published stability of even twenty cycles (Dzudzevic Cancar et al. 2016, Istamboulie et al. 2007).

Slight drawback of the method can be seen in time consuming process compare to spectrophotometric assay due to transformation of substrate into thiocholine and following separation step of MPs before electrochemical assay.

4.2. Construction of an acetylcholinesterase sensor based on synthetized paramagnetic nanoparticles, a simple tool for neurotoxic compounds assay

Insufficient stability of commercial particles was the reason for AChE immobilization onto newly synthetized MPs prepared by research group at Mendel university in Brno. MPs were prepared by reduction of sodium borohydride from iron (III) nitrate followed by surface modification. Compare to commercial particles, which were micro size, newly prepared particles were nano size, which slightly increase time needed for magnetic separation. However, this is not a limitation step because use of stronger magnet would solve this problem. Activity of AChE was again assayed by square-wave voltammetry method.

Various surface modifications of newly synthetized MPs were tested for ability to bind AChE. In case of surface modification by tetraethyl orthosilicate, there are free hydroxyl groups on the surface, which can bind AChE. This modification was successfully used for immobilization of enzyme cellulase earlier (Roth et al. 2016). Combination of tetraethyl orthosilicate and 3-aminopropylthiethoxysilane offers free amino groups and also this modification was successfully used in enzyme bonding (Ibrahim et al. 2014), therefore there was assumption of successful immobilization of AChE. Surface modification of MPs by N^1-(3-trimethoxysilylpropyl)diethylenetriamine, which was not used for immobilization of enzymes yet, was chosen for further measurement. It offers two imino groups and one amino group, which showed to be the best option in AChE immobilization.

Advantage of chosen MPs was fine stability compare to commercial MPs used in previous work. From another published works can be seen that MPs synthetized in laboratory show high stability (Ibrahim et al. 2014, Dzudzevic Cancar et al. 2016, Roth et al. 2016, Ben Oujji et al. 2012), however it cannot be said, that all commercial particles are less stable than the synthetized one, how prove some published papers (Lui et al. 1997, Istamboulie et al. 2007). Benefit of high stability of prepared particles is application especially in flow systems for assay of cholinesterase inhibitors.

In this work, cholinesterase inhibitors were also assayed and moreover there was research of AChE activity restore after inhibition by galantamine and also decarbamylation of AChE-carbofuran adduct. Galantamine as competitive inhibitor of AChE is possible to displace from enzyme active center by excess of substrate. It is not very economical, therefore we investigated cheaper variant, wash out by buffer with different pH. Phosphate buffer pH 7.4, which created environment for enzymatic reaction was replaced by acetate buffer pH 5.0. It showed to be effective as activity of AChE returned back to origin values, probably due to change of charge of amino acids in active center of AChE, which weakly interact with galantamine.

Speed of decarbamylation depends on length of side chain of carbamate molecule (Reiner 1971). Wetherell and French during their experiments with physostigmine found out that decarbamylation of AChE is faster in primates (macaque, marmoset) than in guinea pig model, therefore they proposed it to be a model for comparison of decarbamylation times with human variant (Wetherell and French 1991). Half-life for BChE inhibited by carbofuran was published to be about two hours (Li et al. 2009). In this work, half-life of carbofuran-AChE was determined to be about three and half hour, when using electric ell AChE, which is very close to human one (Dvir et al. 2010), therefore it could also serve as model for comparison of decarbamylation times, moreover it is more available than aforementioned ones. Scheme of measuring process is on figure 2 and it is equal to both methods using MPs.

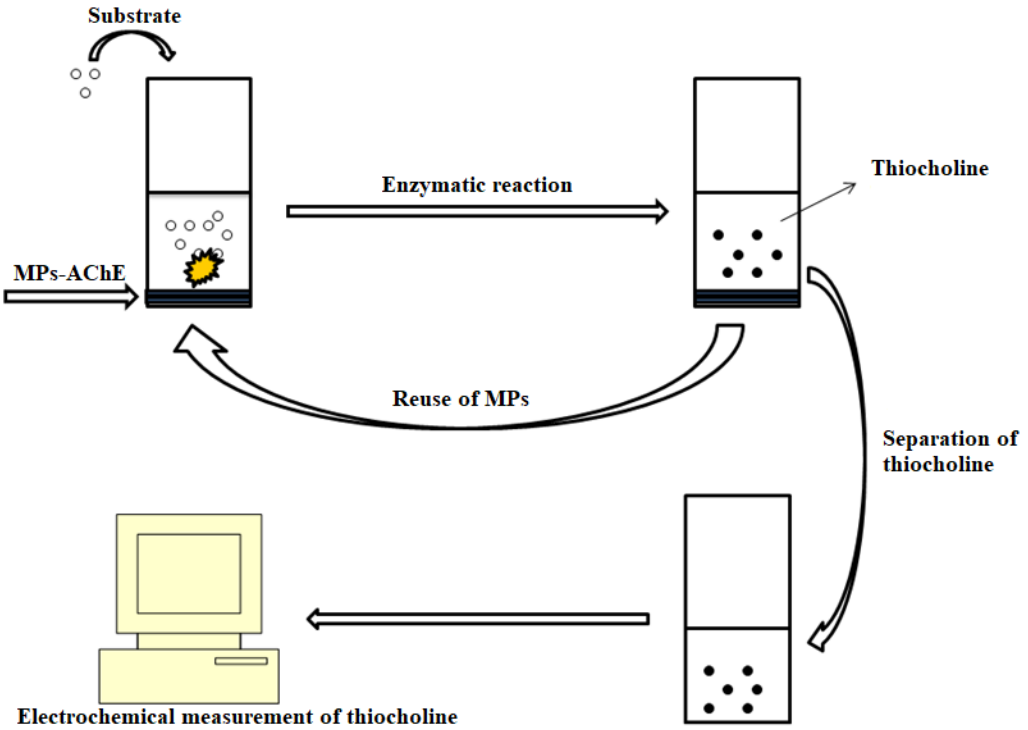

Figure 2. Scheme of electrochemical determination of AChE activity.

4.3. Color change of phenol red by integrated smart phone camera as a tool for the determination of neurotoxic compounds

Immobilization of enzymes into membranes are often solution and compare to chemical immobilization more simple and cheaper. In the past, there were published many papers dealing with this topic and used materials for membranes are e.g. gelatin, chitosan, alginate or sol-gel membranes (Warner and Andreescu 2016, Pohanka et al. 2013a, Lonshakova-Mukina et al. 2018). Expansion of technology made from mobile phone everyday partner for major part of humanity. Neither analytical chemistry, biochemistry, immunochemistry or microbiology did not stay behind with his application. In recent years, there were published many papers dealing with connection of mobile phone and assay of certain analytes (Rateni et al. 2017, Pohanka 2015b, Cai et al. 2012, Martinez et al. 2008, Wang et al. 2017, Mahato and Chandra 2019). Reason why is easy manipulation, which can be handled by untrained personal, so it can be used in doctor´s offices or small laboratories, which do not have expensive laboratory equipment and big advantage is portability thus this kind of analysis is ideal for home care.

In this work, mobile phone was used for detection of color change of phenol red as indicator of AChE activity for cholinesterase inhibitors determination. For purpose of taking photography we prepared dark chamber by three-dimensional print technology, which also served as underlay of mobile phone, so it was during whole analysis at the same position. Evaluation of obtained photography can be carry out by several approaches (e.g. shades of grey or CMYK system (Grudpan et al. 2015)), nevertheless we used RGB (red-green-blue) system, which is common in photography evaluation. General problem of enzyme immobilization is deterioration of catalytic properties because of structural change, in membrane immobilization there is additional problem in substrate diffusion throughout membrane material. In our work, diffusion of substrate

to AChE was fast enough to do not make analysis time longer, which is key factor in biosensors application. Probably it was caused by structure of membrane, because there was not used of crosslink reagent, which would increase density of membrane and slow down substrate diffusion.

Due to immobilization of AChE into gelatin membrane onto surface of filter paper matrix in combination with photographic detection prepared biosensor is fast, cheap and portable alternative for cholinesterase inhibitors determination in field conditions.

4.4. Acetylcholinesterase inhibitors assay using colorimetric pH sensitive strips and image analysis by a smartphone

Second work published on topic of indicator color change in combination with mobile phone used commercial pH sensitive strips (type of indicator is not specified by supplier, however from color change and measuring range we could conclude about phenol red). Measuring of pH could be considered as preliminary exam and most of commercially available pH strips suffer to indicator leaching, which is undesirable, mainly if composition of sample has to be preserved. This problem can be solved by using of pH strips with chemically immobilized indicator, moreover immobilized indicator can be regenerated and used again (Zaggout 2006, Liu et al. 2005).

In this work, AChE was adsorbed onto surface of pH strips with immobilized indicator and covered in gelatin membrane for better stabilization, thus prepared pH strips were used for assay of cholinesterase inhibitors. As well as in previous work, time for color change of indicator due to substrate diffusion throughout membrane was needed. Compare to previous work, this time was slightly higher as enzyme was not in the membrane but below it. On the other hand, this arrangement has advantage in compactness because enzyme and indicator are immobilized in one place, thereby we can avoid preparation and pipetting of indicator, which is often problematic in field conditions. Moreover, improvement of arrangement brought reduction of problems with pipetting of indicator to membrane in shape of bubble used in previous work (tearing and molding of pipetted drops)

Considerable difference in prepared biosensors from previous work and here is in sensitivity to organic solvents. Organic solvents could contact cholinesterases in analysis of pesticides (as used pesticides solvents) and they have potential to influence each assay, therefore their influence to cholinesterases should be tested. Whereas in this work AChE is influenced by organic solvents, the same solvents used in previous work did not influenced AChE activity. It could be explained by different order, membrane in previous work provided better stabilization to AChE while it was right inside membrane compare to this work, where solvents could diffuse to AChE throughout cellulose material of pH strip. Nevertheless, influence of organic solvent can be removed by control measurement, when only solvent without inhibitor is used. Principle of prepared biosensor is depicted on figure 3 and it is equal to previous method using phenol red.

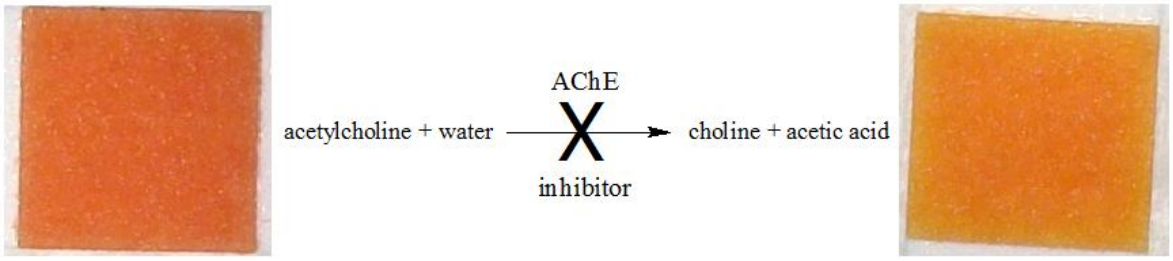

Figure 3. Principle of color change of pH sensitive strip based on AChE. Red color of the strip (left) was changed into orange (right) due to pH change.

4.5. Superficially bound acetylcholinesterase based on a chitosan matrix for neurotoxic compound assay by a photographic technique

Despite popularity of AChE immobilization into membranes of diverse materials, immobilization onto surface of membrane is another option, although published papers were dealing more with immobilization onto composites than membrane itself (Du et al. 2007, Jeyapragasam and Saraswathi 2014, Norouzi et al. 2010, Rodrigues et al. 2018). Very popular material for surface immobilization is derivative of natural polymer chitin – chitosan, which offers amino groups on his surface, which are capable to bind enzyme with or without activation. Moreover, due to biocompatibility it creates ideal environment for enzyme immobilization (Malmiri et al. 2012, Cao et al. 2015).

In this work, AChE was immobilized onto surface of chitosan activated by EDC reagent for assay of cholinesterases inhibitors together with photographic technique. Advantage of immobilization of AChE onto chitosan surface is in access of substrate into enzyme active center. Whereas in case of immobilization into membrane substrate has to diffuse throughout membrane material, in case of surface immobilization enzyme is accessible to substrate thus assay time is not influenced by substrate diffusion. Here we used indoxyl acetate (IA) as a substrate, which is cleaving by AChE into indoxyl, which is spontaneously oxidized into blue indigo. Use of IA has advantage in better color change against phenol red used in previous works. Color change of phenol red is from red to yellow, however color change of IA from white to blue is better controlled even by naked eye, therefore it can be used for semiquantitative analysis, when mobile phone is not used. Small affinity of AChE to IA is disadvantage of the method, therefore we had to use high concentration of IA and prolong time of analysis. Principle of reaction is on figure 4.

As in previous works, sensitivity of AChE to organic solvents was tested. As expected, activity of AChE was decreased by used organic solvents as it was immobilized onto membrane surface. Notwithstanding, influence of solvent was not significantly higher compare to previous works, which well demonstrates stabilization effect of immobilization, when enzyme is in direct contact with solvent.

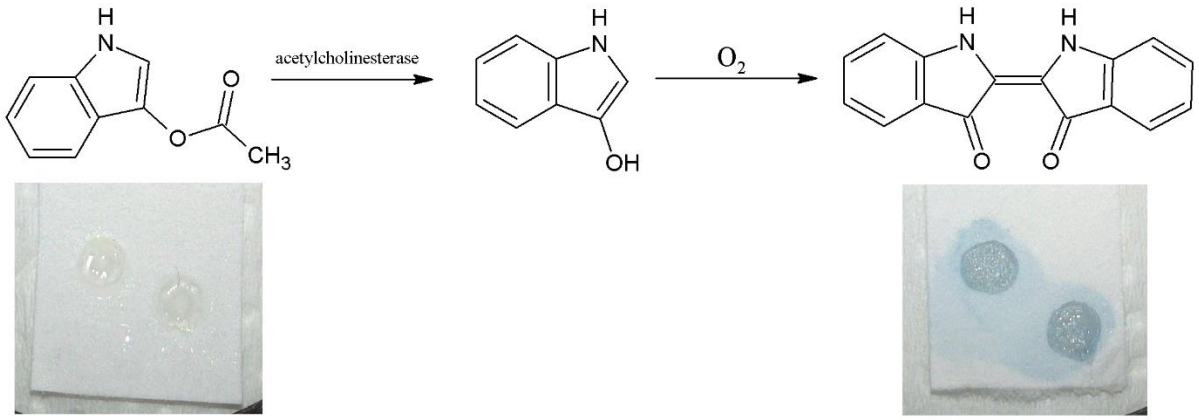

Figure 4. Scheme of reaction based on indoxyl acetate and AChE. At the beggining, indoxyl acetate is colourless (left) and is changed into indoxyl, which is then oxidized into indigo blue (right).

4.6. Anti-Parkinson drug biperiden inhibits enzyme acetylcholinesterase

Computer modeling or *in silico* is important tool used for prediction of potential interactions of target structure with certain type of ligand. It is used abundantly e.g. in protein engineering or pharmaceutical industry in preparation of new drugs. For *in silico* purposes, it is possible to use protein databases like UniProtKB, Protein Data Bank (PDB), Protein Structure Classification Database (CATH) and so on, which eliminate demanding experimental part needed to obtain protein structures (Gupta et al. 2014). For purpose of this work, protein structure of AChE was taken from PDB database, structures in this database are mostly created by X-ray crystallography, nuclear magnetic resonance or electron microscopy (Berman et al. 2000), which are the most used methods in this field.

Though, biperiden (Akineton®) is used in treatment of parkinsonism (Brocks 1999) and in therapy of convulsions at poisoning of cholinesterase inhibitors (Kassa and Fusek 2000, Capacio and Tsung-Ming 1991), there is no information about interaction with AChE. Therefore, interaction of AChE with biperiden was closely investigated. For better understanding of interaction and getting more data we performed experimental study to find out which inhibition mechanism is applied in AChE-biperiden interaction. We revealed uncompetitive mechanism, which in context of cholinesterase inhibitors and inhibitors in general, is very unique, because cholinesterase inhibitors act mostly with noncompetitive mechanism (Pohanka 2015a).

According our results interaction of biperiden with AChE is happening in peripheral anionic subsite, see figure 5. It is important to say that this is *in silico* prediction and more effort should be done this way, especially crystallographic structure should be obtained. Even though, biperiden proved to be weak inhibitor of AChE, his structure could serve as leading structure in future cholinesterase inhibitors research and thus to treatment of Alzheimer disease and other neurodegenerative disorders.

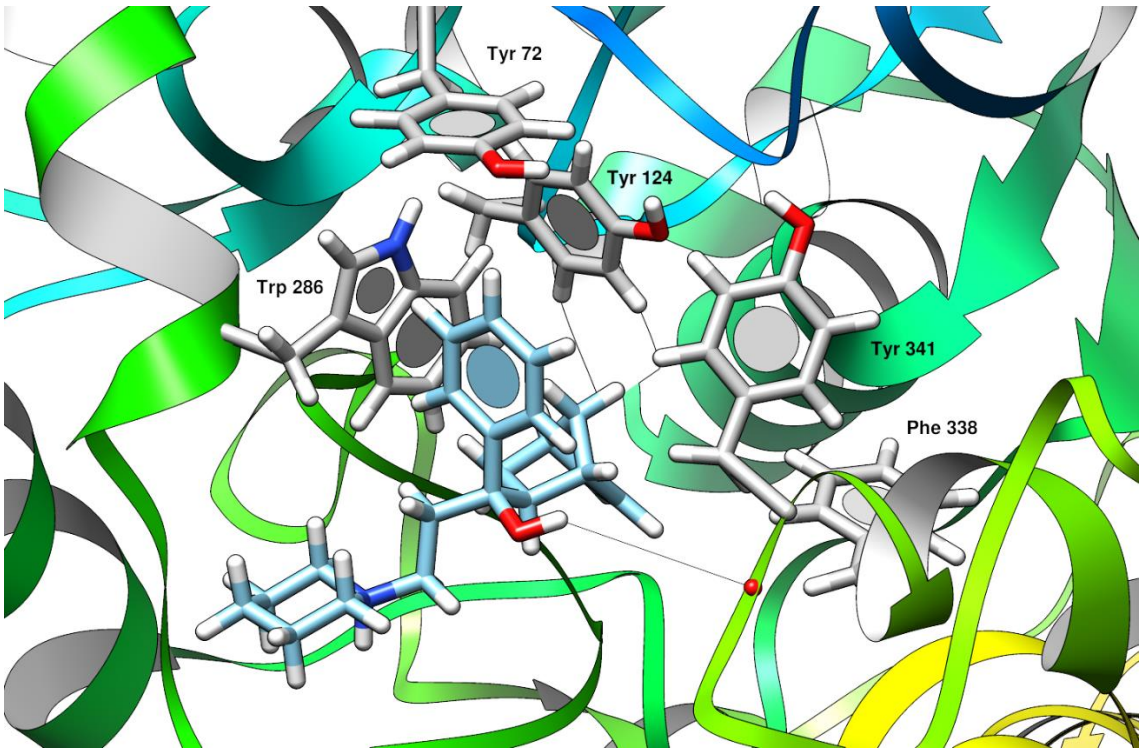

Figure 5. Predicted position of biperiden in AChE peripheral anionic subsite. C = grey (in enzyme) and light blue (in biperiden), H = white, N = blue, O = red. Black line represents hydrogen bond.

4.7. Inhibition of acetylcholinesterase and butyrylcholinesterase by a plant secondary metabolite boldine

Cholinesterases are known for their sensitivity to various inhibitors. One of current research trend in cholinesterase inhibitors is focusing to inhibitors of natural origin. Predominantly it is focusing on AChE inhibitors due to new inhibitors for Alzheimer disease treatment. Many of such natural structures were identified in the past, often with very promising results including nowadays used inhibitors galantamine and huperzine A (Mukherjee et al. 2007, Murray et al. 2013).

Differences in active site of AChE and BChE predict their sensitivity to varied types of inhibitors. Narrow gorge consisted from aromatic amino acids in AChE provides greater resistance to high molecular inhibitors, and in opposite way in case of BChE. It also depends on place where inhibition is happening, in context of natural substances (if we exclude natural carbamates) inhibition takes place in anionic or peripheral anionic subsite of cholinesterase. In this work, we investigated influence of plant aporphine alkaloid boldine to both cholinesterases. Boldine was mentioned in earlier work as AChE inhibitor with promising inhibition constant (Mollataghi et al. 2012), however influence on BChE and inhibition mechanism was not investigated. We did not succeed in verifying of published values of inhibition constants in our conditions and obtained results were several times greater.

From obtained inhibition constants, we could conclude to place where inhibition is happening. Inasmuch as inhibition constants are practically the same, inhibition probably occurs in anionic subsite, whereas peripheral anionic subsite in BChE is less

developed. Noncompetitive inhibition mechanism created another clue toward inhibition in anionic subsite as noncompetitive inhibitors act there (Pohanka 2015a). Interaction of boldine with both cholinesterases was predicted by *in silico* according published papers of similar aporphine derivatives (Yang et al. 2014) and inhibition in anionic subsite was confirmed, as can be learned from figure 6 and figure 7.

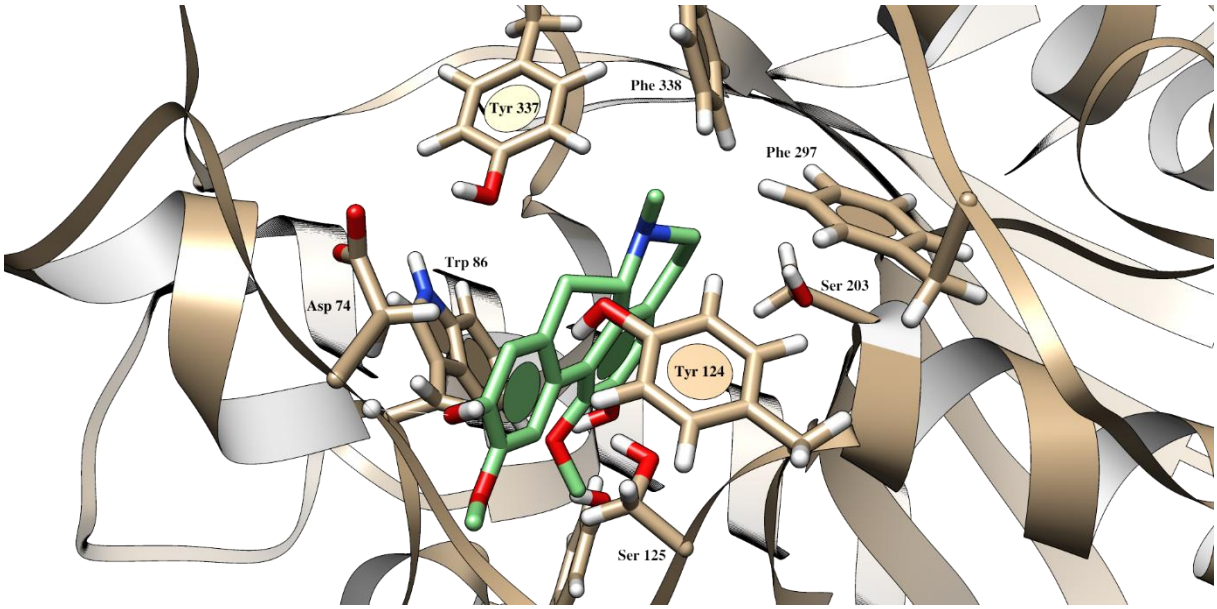

Figure 6. Predicted position of boldine in AChE active center. C = grey (in enzyme) and green (in boldine), H = white, N = blue, O = red. White line represents hydrogen bond.

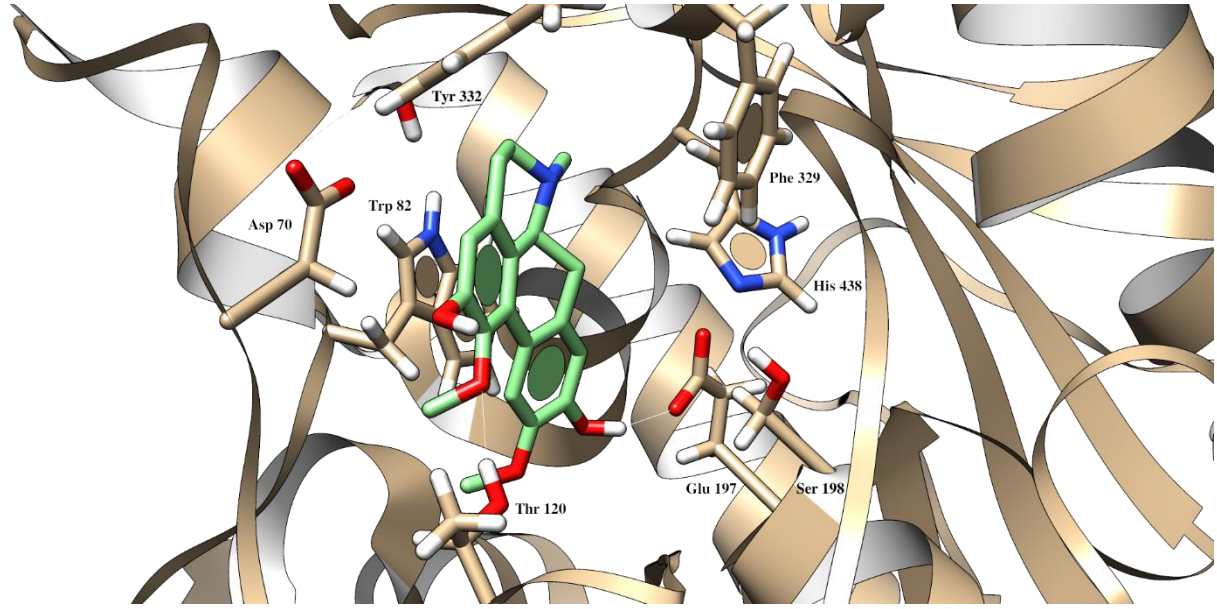

Figure 7. Predicted position of boldine in BChE active center. C = grey (in enzyme) and green (in boldine), H = white, N = blue, O = red. White lines represent hydrogen bonds.

4.8. Assay of cholinesterase activity using quantum dots

Quantum dots are semiconductor crystals of nanometer scale, typical diameter ranges between 2 and 10 nm. Crystals are consisted from groups II to VI or III to V elements, despite cytotoxicity the best success were scored by CdSe and CdTe quantum dots.

Unique are properties of quantum dots – wide absorption spectrum and narrow emission spectrum, which depends on quantum dot size. Due to these properties quantum dots were applied in many parts of research (Chandan et al. 2018, Ghosal and Ghosh 2019).

Photographic detection of indicator color change is possible to apply to detection of fluorescence. In this context we investigated influence of pH to quantum dots fluorescence. Assumption of the experiment was changing fluorescence of quantum dots with increasing or decreasing value of pH. Therefore, we performed experiment, where equal concentration of CdTe quantum dots (1 mg/ml) was added into solutions with different pH and fluorescence intensity was observed (λ_{ex} = 366 nm). From the results can be seen, that application of this procedure is not possible, because quenching of fluorescence is observed in very small rate in pH 5.5 and clearly visible is in pH 3.0 as can be seen from figure 8. If could be theoretically possible to decrease pH enough by enzymatic reaction to make this change visible, denaturation of the enzyme would occur and reaction would stop. Practically it cannot be achieved as pH optimum for cholinesterase is approximately between pH 6.0 and 8.0, moreover acid produced during enzymatic degradation of acetylcholine can decrease pH in tenths, which cannot harm the enzyme. Another reason for abandon of quantum dots application was absence of sufficiently sensitive method for fluorescence detection on department, where doctoral study was realized.

Figure 8. Quetching of quantum dots fluoresnce in different pH. From the left – hydrogen peroxide (possitive control – quetching fluorescence), solution pH 3.0, solution pH 5.5, solution pH 7.4, solution pH 9.0. Upper solution contains AChE after enzymatic reaction.

5. Conclusion

This dissertation thesis is summarizing biosensors based on cholinesterases for detection of neurotoxic compounds, which were prepared during doctoral study. There were prepared biosensors with chemically immobilized AChE onto MPs surface, thereby this research field was extended as not many publications were deal with immobilization of AChE only onto MPs surface. Chemically of physically, AChE was immobilized into/onto membrane from gelatin and chitosan, moreover in combination with photographic technique, where is assumption of following upgrowth in next years as importance of technology will grow in all aspects of human life. Each prepared biosensor proved its function in measurement of cholinesterase inhibitors. Also, there were investigated interactions of cholinesterases with compounds acting as their inhibitors, using method of computer modeling. Studied interactions were not described yet.

Prepared biosensors are simple alternative to analytical methods (especially chromatographic techniques) for neurotoxic compounds assay. Their usage can be intended in many applications. Mainly it is analysis of environment, food control, pharmaceutical industry or analysis of biological material. Thanks to their simplicity, small dimensions, low cost and portability is possible to use them in field conditions out of laboratory. This is especially offered in combination with photographic detection, because mobile phone does not require any special skills or training, therefore it can be used in conditions of small laboratories, doctor´s offices or even in homecare. Modeling of inhibitors interaction with cholinesterases is considered as important part of the thesis. Especially utilization of interaction of biperiden with AChE is expected. This interaction was unknown and it could start exciting direction in synthesis of new cholinesterase inhibitors and leads to potential development of new drugs for treatment of Alzheimer disease or other neurodegenerative disorders.

List of abbreviations

AChE	acetylcholinesterase
BChE	butyrylcholinesterase
EDC	N-(3-dimethylaminopropyl)-N´-ethylcarbodiimide hydrochloride
IA	indoxyl acetate
MPs	magnetic particles

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List of Students´ Published Works

The results of this doctoral thesis are contained in 7 publications with impact factor:

- KOSTELNÍK, Adam, ČEGAN, Alexander, POHANKA, Miroslav. Electrochemical determination of activity of acetylcholinesterase immobilized on magnetic particles. *International Journal of Electrochemical Science*, 2016, vol. 11, no. 6, 4840–4849
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