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Katedra analytické chemie

Pokročilé analytické postupy využívající cholinesterasy

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Advanced analytical procedures using cholinesterases

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V Pardubicích dne 21. 6. 2019

Adam Kostelník

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Souhrn

Cholinesterasy jsou enzymy, které jsou pro svou citlivost k určitým látkám používány k jejich průkazu. Jedná se o látky ze skupin pesticidů, bojových plynů či léčiv. Tato disertační práce se zabývá přípravou biosenzorů založených na cholinesterasach. Cílem práce bylo připravit cholinesterasové biosenzory jednoduché konstrukce jako levnější alternativy ke standardně používaným analytickým metodám. V této práci byly zkoumány i interakce některých inhibitorů s cholinesterasami metodou *in silico*. Tato práce je složena z komentářů k jednotlivým publikacím, jejichž detailní popisy jsou shrnuty v přiložených publikacích.

Klíčová slova

biosenzor, acetylcholinesterasa, butyrylcholinesterasa, neurotoxické látky, inhibitor, spektrofotometrie, elektrochemie, kolorimetrie, *in silico*

Summary

Cholinesterases are enzymes, which are sensitive to compounds from pesticide, nerve agents and drug groups and they can be used for their determination. This thesis deals with preparation of biosensors based on cholinesterases with simple construction as cheaper alternatives to standard analytical methods. It was also examined interactions of inhibitors with cholinesterases by *in silico* method. This work is consisted from comments on publications deal with preparation of cholinesterase biosensors, while details are summarized in enclosed publications.

Key words

biosensor, acetylcholinesterase, butyrylcholinesterase, neurotoxic compounds, inhibitor, spectrophotometry, electrochemistry, colorimetry, *in silico*

Obsah

Seznam obrázků a zkratek

Seznam obrázků

Seznam zkratek

AChE	acetylcholinesterasa
BChE	butyrylcholinesterasa
DN	dibukainové číslo (z angl. dubucain number)
SPR	rezonance povrchových plasmonů (z angl. surface plasmon resonance)

1. Úvod

Analýza jedovatých látek je v současné době prováděna zejména chromatografickými technikami často v kombinaci s hmotnostním detektorem, který umožňuje přímou identifikaci neznámé látky. Nicméně toto přístrojové vybavení je finančně nákladné a jeho použití je vázáno na laboratoř, do které musí být vzorky nejprve dopraveny, což může být časově náročný proces. Proto se pro analýzu přímo v terénu používají přenosná zařízení, některé z nich pracují na principu biosenzorů, které sice nemusí přímo rozlišit, o jakou látku se jedná, ale alespoň mohou ukázat na určitou skupinu látek. Časté je použití biosenzorů s enzymovým rozlišovacím prvkem.

První objevy na poli enzymů se uskutečnily v počátcích 19. století a asi o sto let později se toto pole začalo velmi bouřlivě rozvíjet, kdy počty nově objevených enzymů raketově rostly. V té době, tedy zhruba v polovině 20. století, se enzymy dostaly do hledáčku v souvislosti s přípravou biosenzorů, když Clark a Lyons představili svůj biosenzor pro stanovení glukózy, který se stal vůbec prvním biosenzorem v historii. Od té doby urazily biosenzory velký kus cesty a řada z nich našla uplatnění v rutinních analýzách.

Historie cholinesterasových biosenzorů je poměrně mladá, první publikace se objevily asi před třiceti lety, nicméně rychlý rozvoj přinesl na toto téma nespočet publikací. Své uplatnění nacházejí v mnoha oblastech, především pak v analýzách životního prostředí, kontrole kvality potravin, klinické praxi či dokonce na bitevním poli. Je tomu tak díky citlivosti cholinesteras k různorodému spektru látek, které fungují jako jejich inhibitory a které byly v minulosti připraveny uměle, nebo jsou přírodního charakteru.

Současné trendy na poli cholinesterasových biosenzorů se ubírají cestou tvorby funkčních, i když často relativně složitých, konstrukcí. Snahou této disertační práce bylo připravit cholinesterasové biosenzory jednoduché konstrukce snadno adaptovatelné na použití v terénních podmínkách, kde je jednoduchost a rychlost žádanou vlastností. Rovněž byly zkoumány dosud nepopsané interakce některých cholinesterasových inhibitorů s cholinesterasami metodou *in silico*.

2. Cíle práce

- Rešerše problematiky cholinesteras a jejich využití k diagnostice a analýze neurotoxických látek. Porovnání acetylcholinesterasy a butyrylcholinesterasy.
- Vypracování laboratorních postupů pro biochemickou diagnostiku využívající stanovení cholinesterasemie za použití nových či neobvyklých substrátů a reakčních činidel.
- Konstrukce metod s imobilizovanou cholinesterasou ke stanovení neurotoxických látek a optimalizace imobilizačních postupů.
- Konstrukce detekčního systému využívajícího magnetické mikročástice s vázanou cholinesterasou.
- Zavedení laboratorního postupu stanovení aktivity cholinesteras využívající kvantové tečky.
- *In silico* predikce interakce inhibitorů s cholinesterasami.
- Ověření laboratorních postupů a metod za využití reálných vzorků plasmy.

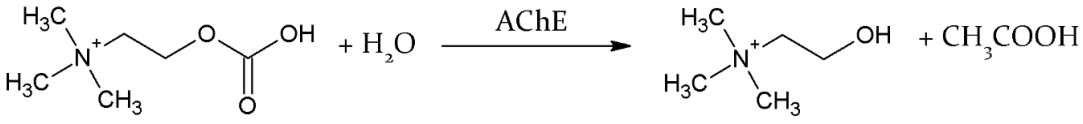
3. Teoretická část

3.1. Cholinesterasy

Cholinesterasy řadíme do skupiny hydrolytických enzymů štěpící estery cholinu. Rozlišujeme dvě cholinesterasy – acetylcholinesterasu (AChE) a butyrylcholinesterasu (BChE), a to jako globulární nebo asymetrickou formu. Globulární formy obsahují jednu (G1), dvě (G2) nebo čtyři (G4) podjednotky a jsou buďto ve volné formě nebo jsou vázány k membráně (např. G2 forma v membráně erytrocytů nebo G4 forma v mozku). Rozpustná forma BChE se vyskytuje z 95 % jako G4 tetramer a G1 či G2 formy jsou její degradační formy. Podobně jako BChE tak i rozpustná forma AChE se vyskytuje jako G4 tetramer, sekretovaný nervovými či svalovými buňkami. Asymetrické formy obsahují jednu (A4), dvě (A8), největší z nich (A12) má pak tetramerové podjednotky tři [1, 2].

3.1.1. Acetylcholinesterasa

AChE (3.1.1.7) je enzym zodpovědný za rychlé ukončení přenosu informací v nervovém systému hydrolýzou neurotransmiteru acetylcholinu na cholin a kyselinu octovou (Obr. 1). Kromě synaptických štěrbin v mozku se vyskytuje rovněž na nervosvalových ploténkách a erytrocytech, kde rozkládá do krve uvolněný acetylcholin.



Obrázek 1. Rovnice hydrolýzy acetylcholinu.

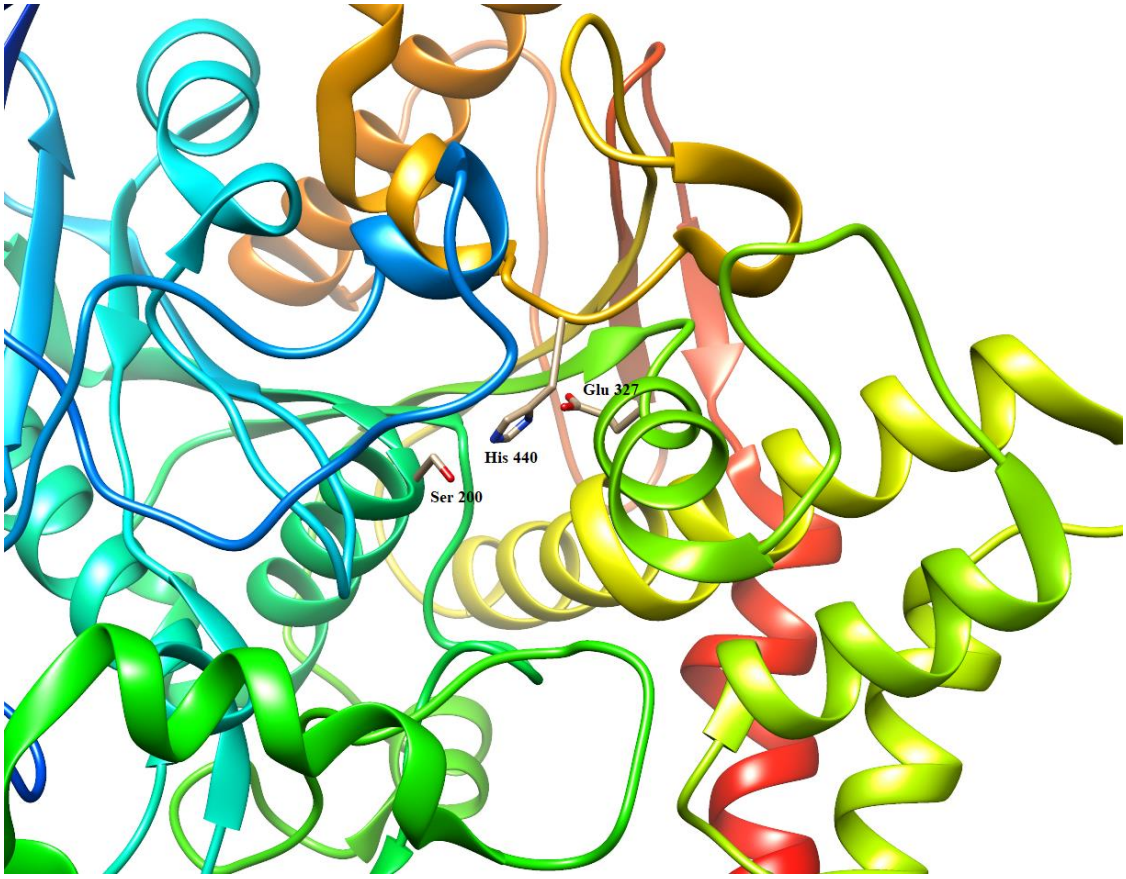
3.1.1.1. Struktura acetylcholinesterasy

Strukturu AChE lze výborně demonstrovat na AChE izolované z elektrického úhoře (*Torpedo californica*), která je dobře dostupná a počátky výzkumu cholinesteras jsou spojeny právě s ní (Obr. 2). Aktivní místo enzymu se sestává z několika částí:

1. Esterové místo – je zodpovědné za samotnou enzymovou aktivitu. Je tvořeno katalytickou triádou aminokyselin Ser 200, Glu 327 a His 440.

2. Anionické místo – je zodpovědné za správnou orientaci acetylcholinu vůči esterovému místu. Tvoří ho aminokyseliny Trp 84, Tyr 121 a Phe 338.

Kromě těchto důležitých částí je vstup do kavity aktivního místa AChE tvořen aromatickými aminokyselinami (tzv. aromatická štěrbina), které mají za úkol zvyšovat selektivitu pro zachycení acetylcholinu nevazebnou interakcí kation-π s kvartérním dusíkovým atomem acetylcholinu a aromatickým systémem aminokyselin. Poblíž vstupu do aktivního centra se nachází periferní anionické místo, které je cílem některých enzymových inhibitorů [3, 4].



Obrázek 2. Struktura AChE izolované z *Torpedo californica*. Ve struktuře jsou zobrazeny aminokyseliny aktivního místa, tzv. katalytické triády Ser 200, Glu 327 a His 440.

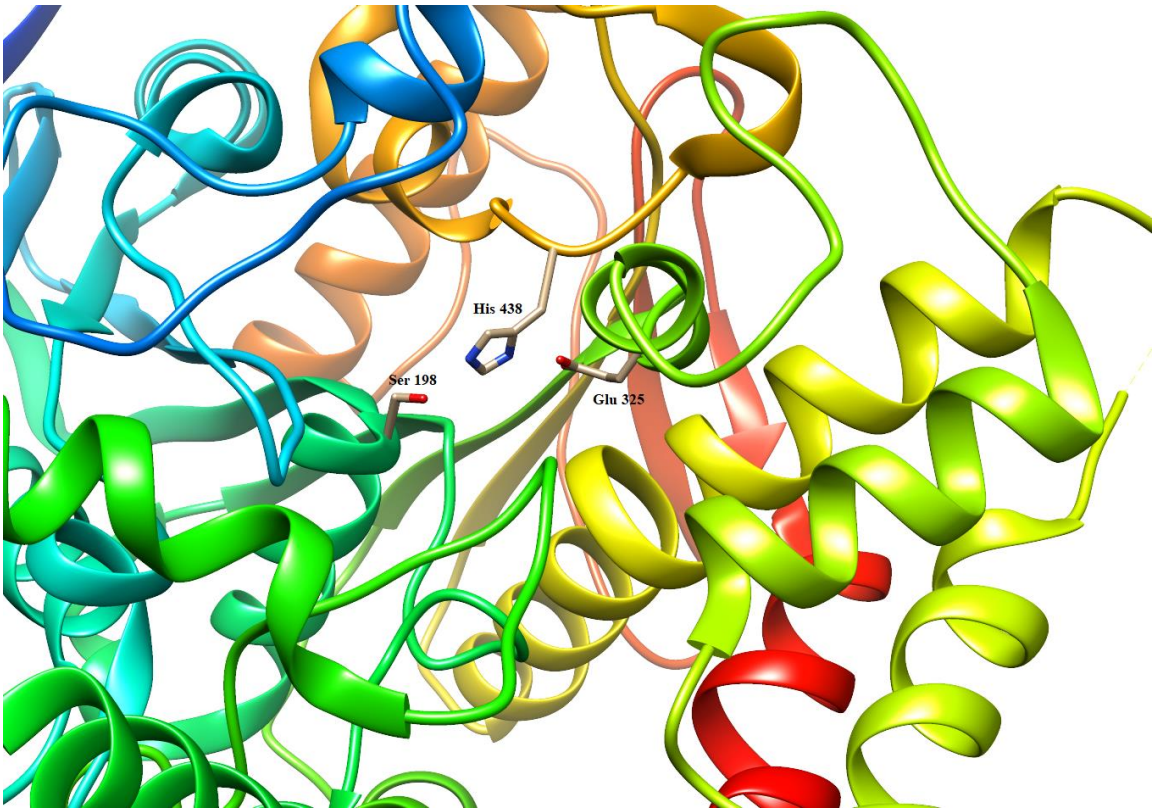
3.1.2. Butyrylcholinesterasa

Na rozdíl od AChE zůstává funkce BChE (3.1.1.8) stále neznámá. Je syntetizována v mnoha tkáních, hlavně v ledvinách, mozku, srdci a v játrech. Díky své afinitě k řadě látek se podílí na detoxifikaci jedovatých sloučenin jako např. kokain, heroin či aspirin, ale i klinicky využívaných preparátů jako např. myorelaxanty (sukcinylcholin nebo mivakurium). V této souvislosti se vyšetřuje typ BChE, kromě normální A varianty existuje i K varianta, která má zhoršené odbourávání myorelaxantů, což může potenciálně ohrozit život pacienta při chirurgickém výkonu. Proto se stanovuje tzv. dibukainové číslo (DN), což je procento inhibice BChE v přítomnosti dibukainu.

Homozygotní A varianta je k dibukainu citlivá, má DN ≥ 75, AK heterozygoti mají DN 40–70, homozygoti s K variantou mají DN < 20.

3.1.2.1. Struktura butyrylcholinesterasy

Aktivní místo BChE je stejně jako u AChE tvořeno katalytickou triádou Ser 198, Glu 325 a His 438 (lidská BChE) (Obr. 3) a anionickým místem, nicméně hlavní rozdíl proti struktuře AChE je ve složení aromatické štěrbiny. Struktura BChE obsahuje celkem 8 aromatických aminokyselin v porovnání se 14 u AChE. Tento podstatný rozdíl je důvodem, proč je BChE schopna štěpit širší spektrum substrátů, než je tomu v případě AChE. Rozdíl je i v periferním anionickém místě, ve struktuře BChE je méně vyvinuté, tudíž látky schopné enzymové inhibice v tomto místě AChE na BChE nepůsobí, nebo jen minimálně [3, 5-8].

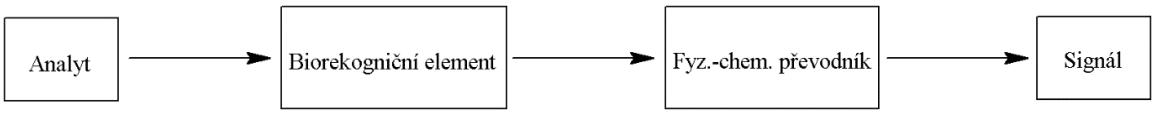


Obrázek 3. Struktura lidské BChE. Ve struktuře jsou zobrazeny aminokyseliny aktivního místa, tzv. katalytické triády Ser 198, Glu 325 a His 438.

Své využití cholinesterasy našly v biosenzorech pro stanovení cholinesterasových inhibitorů, a to především v kontrole životního prostředí a potravin, při výzkumu nových léků ve farmaceutickém průmyslu či ve vojenské oblasti.

3.2. Biosenzory

Historie biosenzorů se začala psát v polovině 20. století, kdy Clark a Lyons představili světu vůbec první biosenzor pro stanovení glukózy v krvi založený na enzymu glukosaoxidasa. Od té doby se pole biosenzorů začalo velmi rychle rozvíjet a rozkvět cholinesterasových biosenzorů nastal asi o tři dekády později, na přelomu 80. a 90. let minulého století, ze kdy lze vypátrat první publikace na toto téma. Biosenzor můžeme definovat jako analytické zařízení, které se sestává z biologické části poskytující specifitu vůči analytu a fyzikálně-chemického převodníku, který převádí biologické interakce na měřitelný signál [9, 10]. Obecné schéma biosenzoru je na obrázku 4.



Obrázek 4. Obecné schéma biosenzoru.

3.2.1. Biorekogniční elementy

Biorekogniční element zodpovídá za samotnou rozlišovací schopnost a specifitu biosenzoru ke stanovovanému analytu. Zde zmiňme nejčastější typy biorekognič-ních elementů:

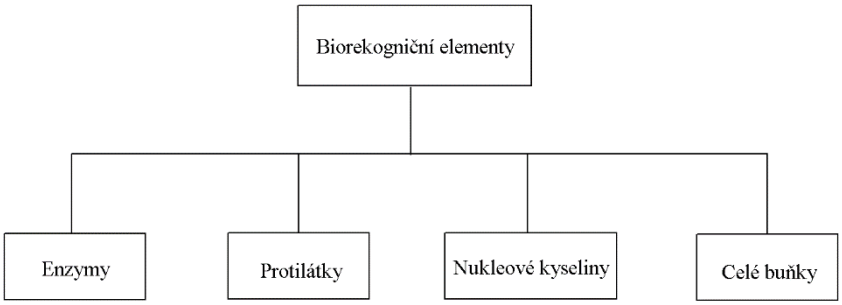
Enzymy – přírodní katalyzátory schopny rozpoznat specifické substráty a katalyzovat jejich přeměnu, čímž se výborně hodí pro konstrukci biosenzorů. Interakce enzym-substrát poskytuje enzymovým biosenzorům vysokou senzitivitu a selektivitu, navíc je rychlá a levná. Na druhou stranu použití enzymů vyžaduje specifické podmínky jako je např. teplota, pH atd. Nejčastěji enzymové biosenzory najdeme v klinické diagnostice (např. rutinní stanovení glukózy) či hodnocení kvality životního prostředí (např. detekce pesticidů).

Protilátky – neboli imunoglobuliny, detekují antigen na základě specifického složení aminokyselin ve vazebném místě antigenu a imunoglobulinu. Vazba imunoglobulinu s antigenem je díky tomu vysoce specifická a selektivní. Využití imunosenzorů je především v klinické diagnostice (např. stanovení protilátek při autoimunitních onemocněních) nebo při hodnocení kvality potravin (např. detekce patogenních bakterií) či životního prostředí.

Nukleové kyseliny – v biosenzorech založených na nukleových kyselinách je jednovláknový řetězec DNA (ssDNA) navázán na povrch převodníku a při interakci s komplementárním řetězcem ze vzorku dojde k hybridizaci na dvouvláknovou DNA

(dsDNA). Aplikace nacházejí tyto biosenzory v detekci patogenů při kontrole potravin či v klinické diagnostice (např. dědičné choroby).

Méně častým, a proti výše uvedeným biorekogničním elementům spíše unikátním typem, jsou biosenzory využívající celých buněk používané především v kontrole potravin, životního prostředí, klinické diagnostice atp. [11-16]. Biorekogniční elementy shrnuje obrázek 5.



Obrázek 5. Biorekogniční elementy.

3.2.2. Fyzikálně-chemické převodníky

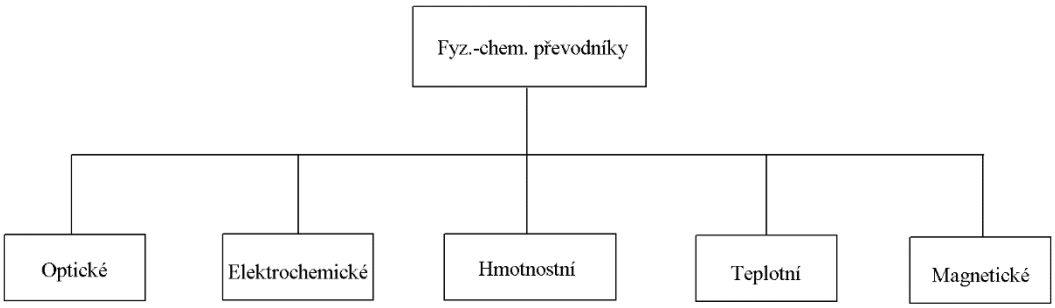
Fyzikálně-chemické převodníky jsou zodpovědné za převod biochemické reakce na měřitelný signál. Rozlišujeme pět typů fyzikálně-chemických převodníků: optické, elektrochemické, hmotnostní, teplotní, magnetické. Teplotní a magnetické převodníky představují spíše raritní typy, a proto se zmiňme především o prvních třech, které jsou ve spojení s cholinesterasami využívány více.

Optické převodníky – do optických převodníků řadíme metody kolorimetrické, flourescenční, luminiscenční či resonanci povrchových plasmonů (SPR). Fluorescenční převodníky využívají fluorescenčních značek, fluoroforů, u kterých při pohlcení světla o tzv. excitační vlnové délce dochází k excitaci elektronu ze základního energetického stavu do stavu o vyšší energii. Při návratu elektronu do základního stavu dojde k vyzáření světla o tzv. emisní vlnové délce. Jako příklady používaných flouroforů uveďme např. fluorescein, fluorescein isothiokyanát atd. Chemiluminiscenční detekce využívá zase značky luminiscenční, hlavně luminol nebo jeho deriváty. SPR generuje na rozmezí dvou fází (např. sklo a kapalina) ozářených polarizovaným světlem v určitém úhlu povrchové plasmony, což vede k redukci intensity odraženého světla v tzv. resonančním úhlu. SPR lze využít k detekci vazby biorekogničního elementu na povrch fyzikálně-chemického převodníku.

Elektrochemické převodníky – v této skupině jsou nejčastěji používány převodníky amperometrické a potenciometrické. Amperometrické převodníky jsou založeny na

měření proudové odezvy (při konstantním potenciálu elektrody), která je přímo úměrná koncentraci měřeného elektroaktivního analytu, tzn. analyt se musí oxidovat nebo redukovat. Potenciometrické převodníky jsou zase založeny na měření změny potenciálu mezi referentní elektrodou se stálým potenciálem a měrnou elektrodou, jejíž potenciál je závislý na koncentraci analytu ve vzorku. Elektrochemické převodníky jsou široce používány např. v medicíně či při monitorování životního prostředí, hlavně díky nízké ceně, vysoké citlivosti a možnosti měření v zakalených vzorcích.

Hmotnostní převodníky – jsou schopny reagovat na změny v hmotnosti látky navázané na jejich povrchu. Typicky se jedná o krystaly, které mění svou oscilační frekvenci na základě vloženého napětí a hmotnosti látky na jejich povrchu. Své využití nachází hmotnostní převodníky např. v detekci patogenů či specifických interakcí protilátek s antigeny [17-19]. Fyzikálně-chemické převodníky jsou shrnuty na obrázku 6.



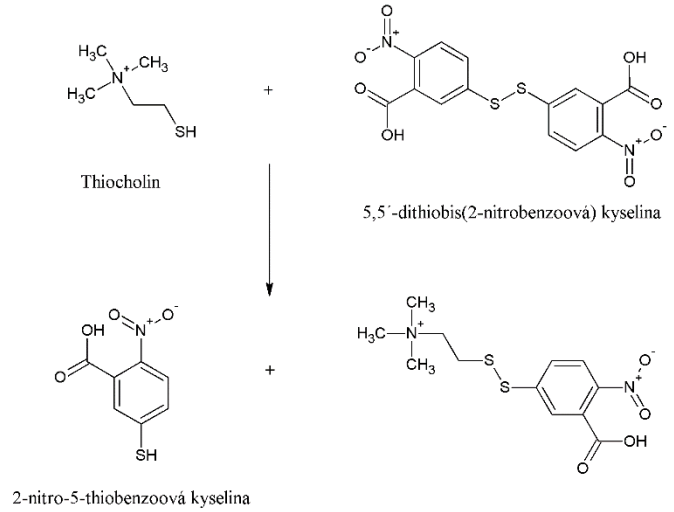
Obrázek 6. Fyzikálně-chemické převodníky.

3.3. Metody stanovení cholinesterasové aktivity

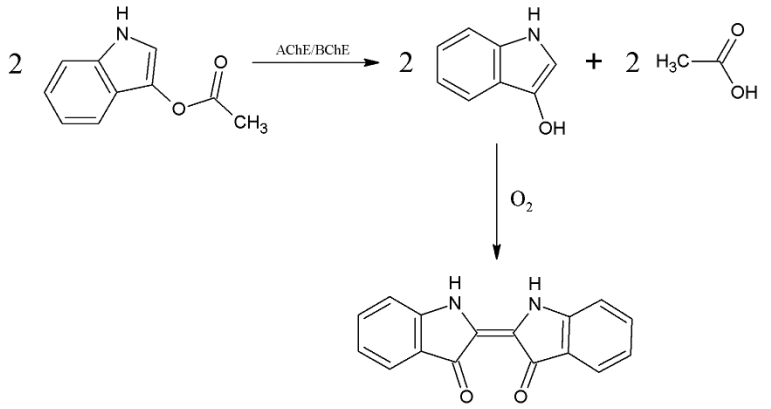
Potřeba stanovovat aktivitu cholinesteras vychází z jejich citlivosti k nejrůznějším inhibitorům. Obecné rozdělení biosenzorů bylo uvedeno výše, v následujícím přehledu jsou uvedeny příklady konkrétních metod, které lze v měření cholinesterasové aktivity použít a na nichž je možné postavit konstrukci cholinesterasových biosenzorů.

3.3.1. Optické metody

Nejvíce používanou metodou, a dá se říci, že v dnešní době již považovanou za „zlatý standard“, je metoda stanovení podle Ellmana. Ten svou práci publikoval již v roce 1961 a zakládá se na reakci Ellmanova činidla, 5,5´-dithiobis(2-nitrobenzoové) kyseliny, s thiolovou skupinou thiocholinu vzniklého enzymovým štěpením substrátu acetylthiocholinu, popř. butyrylthiocholinu. Výsledný barevný produkt 2-nitro-5-thiobenzoová kyselina je spektrofotometricky měřitelná při 412 nm [20]. Princip Ellmanova stanovení je na obrázku 7. Výhodou Ellmanovy metody je rychlost, jednoduchost a do jisté míry i zavedenost v analýze cholinesterasové aktivity. Nevýhodou metody je nestabilita Ellmanova činidla, které je citlivé na světlo [21]. Jednou z alternativních možností spektrofotometrického stanovení je použití indoxyl acetátu, ten je hydrolyzován na modré indigo, které absorbuje ve vlnových délkách kolem 600 nm (Obr. 8). Indoxyl acetát je také schopný fluorescence a možné je použití i dalších látek jako např. resorufinů či derivátu fluoresceinu [22-25], své využití v měření cholinesterasové aktivity našly i kvantové tečky [26-29]. Výhodou použití fluorescence je snížení limitu detekce v porovnání se spektrofotometrickým stanovením a stabilita použitých fluorescenčních činidel.



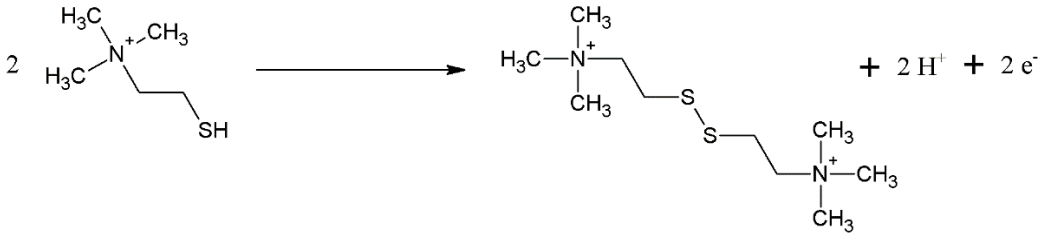
Obrázek 7. Princip stanovení cholinesterasové aktivity Ellmanovou metodou.



Obrázek 8. Princip stanovení cholinesterasové aktivity založený na rozkladu indoxyl acetátu.

3.3.2. Elektrochemické metody

Elektrochemická detekce se zakládá především na voltametrických technikách, square-wave voltametrii, cyklické voltametrii či diferenční pulzní voltametrii. Thiocholin vzniklý enzymovým rozkladem alternativního substrátu acetylthiocholinu/butyrylthiocholinu je oxidován na dithiocholin a při potenciálu asi 0,65 V (vs. Ag/AgCl) jsou registrovány volné elektrony. Velikost proudové odezvy je přitom úměrná koncentraci thiocholinu v roztoku, a tím enzymové aktivitě, při stanovení cholinesterasových inhibitorů je úměrná nepřímo. Princip elektrochemické detekce je na obrázku 9. Oxidační potenciál thiocholinu lze snížit na asi 0,4 V modifikací pracovní elektrody. K tomuto účelu lze použít různých modifikátorů, např. kobalt ftalocyanin, hexakyanoželeznatan železitý či karbonové nanotyčinky [30, 31]. Jinou možností stanovení cholinesterasové aktivity je kombinace cholinesterasy s cholinoxidasou. Toto stanovení zakládá na oxidaci vzniklého cholinu cholinoxidasou na betain a peroxid vodíku, který je elektrochemicky stanoven [32, 33]. Výhodou použití elektrochemických metod je nízký detekční limit či možnost měření v zakalených vzorcích.



Obrázek 9. Princip elekrochemického stanovení cholinesterasové aktivity.

3.3.3. Metody zakládající na měření pH

Metody měření pH byly jedny z prvních, které se používaly pro stanovení cholinesterasové aktivity a přinesly značné ulehčení oproti prvotním metodám, které byly velmi časově i instrumentálně náročné [34, 35]. Během hydrolýzy cholinových esterů dochází k produkci kyselin, čehož je možné využít pro stanovení enzymové aktivity. Při stanovení dochází k přechodu v relativně úzkém pásu pH (asi 6,0–8,0), používají se proto indikátory s barevným přechodem v této oblasti, např. fenolová červeň, neutrální červeň atd. [36, 37]. Detekci barevné změny lze provádět spektrofotometricky, čímž dochází k prolínání s optickými metodami (Obr. 10). Další možností měření pH je použití iontově-senzitivního tranzistoru s efektem pole [38-40] nebo použití skleněné elektrody [41].

Obrázek 10. Barevná změna fenolové červeně před enzymovou reakcí (vpravo) a po enzymové reakci (vlevo).

Výše zmíněné metody mohou využívat různé podoby cholinesteras. Nejčastější, a v souvislosti s cholinesterasami nejvíce používanou, je chemická či fyzikální imobilizace, na druhou stranu prostá fyzikální adsorpce se v dnešní době příliš neuplatňuje.

a) Fyzikální adsorpce – tento přístup, kdy se enzym ponechá při laboratorních podmínkách volně zaschnout na povrchu, je nejjednodušším způsobem imobilizace. V literatuře byly ovšem nalezeny i práce zabývající se adsorpcí AChE na povrch porézních materiálů. Autoři dosáhli slibných výsledků v porovnání s volným enzymem, což činí tento typ materiálu slibným v budoucí konstrukci biosenzorů [42-44], pro stanovení pesticidů byla zase AChE sorbována na povrch elektrochemického senzoru [45].
b) Chemická imobilizace – nabízí opakované využití enzymu na rozdíl od fyzikální adsorpce, při které zpravidla při prvním měření dojde k odmytí enzymu z povrchu. Pro chemickou imobilizaci enzymu se využívá např. karboimidů, sukcinimidů či

glutaraldehydu, které váží enzym kovalentní vazbou. Jako dobrý příklad platformy pro chemickou imobilizaci cholinesteras mohou posloužit magnetické částice, jejichž výhodou je právě opakované použití např. v průtokových analyzátorech, dobrá stabilizace navázaného enzymu a nízká cena analýzy plynoucí z opakovaného použití. Na druhou stranu v přítomnosti inhibitorů vážící se kovalentní vazbou do aktivního místa cholinesterasy dojde k nevratné inhibici, což do jisté míry smývá výhodu imobilizace, ale po případné reaktivaci inhibované cholinesterasy je možné opětovné použití [46-48]. V literatuře je možné dohledat jen několik prací věnující se imobilizaci AChE pouze na magnetické částice pro stanovení organofosfátových pesticidů či insekticidů [49-55].

c) Fyzikální imobilizace – v tomto případě se jedná o zachycení enzymu do membrány. Pro imobilizaci cholinesteras existuje celá řada materiálů pro přípravu membrán, např. agar, chitosan nebo želatina. Výhodou je jednoduchá příprava, při které se enzym smíchá s materiálem membrány a nechá se zatuhnout. Následně lze membránu stabilizovat pomocí síťovacích činidel, např. epichlorhydrin, akrylamidy nebo glutaraldehyd [56, 57]. Další výhodou fyzikální imobilizace je nízká cena a možnost opakovaného použití. Nevýhodou je ztráta katalytických vlastností, protože substrát hůře proniká do aktivního centra skrze materiál membrány. Spojení cholinesteras s kotvením do želatinové či chitosanové membrány pro stanovení organofosfátových i karbamátových pesticidů bylo předmětem řady publikací [58-61].

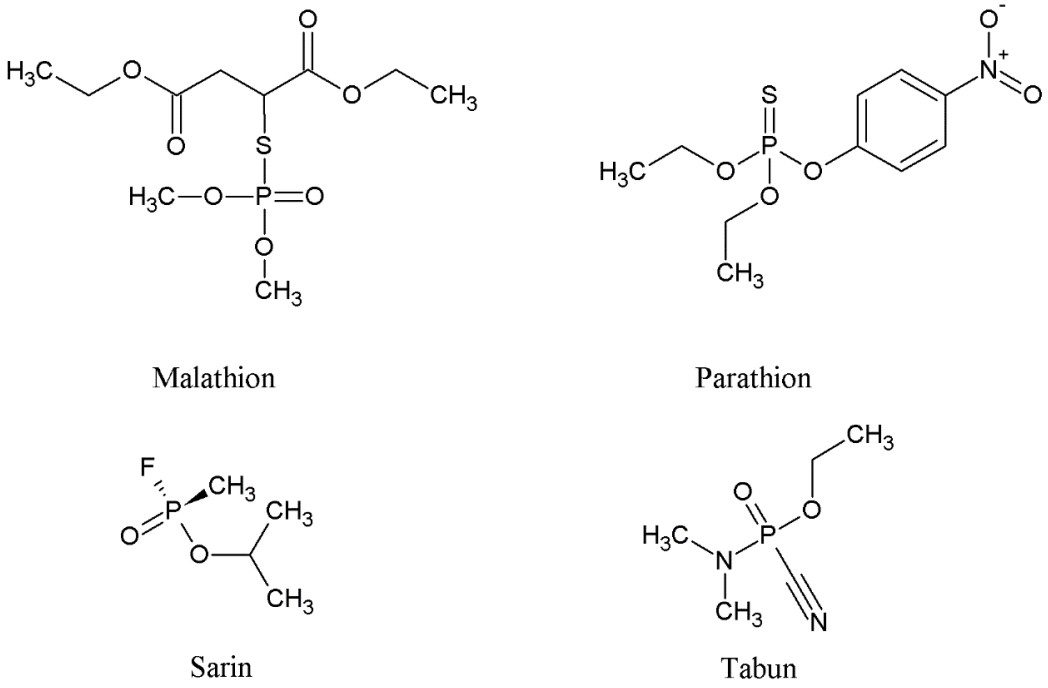
3.4. Inhibitory cholinesteras

Inhibitory cholinesteras tvoří chemicky heterogenní skupinu látek, které je možné stanovit cholinesterasovými biosenzory využívající metody uvedené v předchozí kapitole. Z převážné části jde o inhibitory AChE, která je klinicky významnější než BChE, proto jsou níže uvedeny spíše inhibitory AChE. Co se týče inhibitorů BChE, afinitu k ní má i velká část inhibitorů AChE. Nicméně existuje i selektivní inhibitor BChE, tetraisopropyl pyrofosforamid, který je možné použít pro rozlišení obou cholinesteras v neznámém vzorku [62, 63].

3.4.1. Inhibitory esterového místa

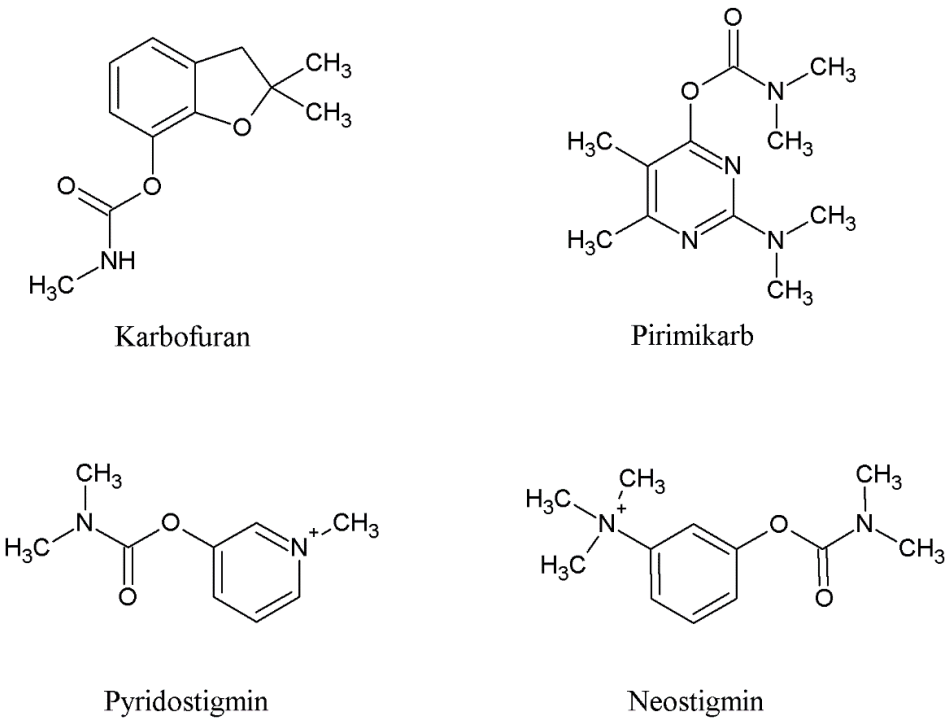
Do inhibitorů esterového místa řadíme organofosfáty a karbamáty, které jsou buďto ireverzibilními, či pseudoireverzibilními inhibitory obou cholinesteras. Mechanismus účinku těchto látek spočívá ve vazbě na serinové residuum v aktivním místě enzymu, čímž je znemožněna přeměna substrátu. Ireverzibilní vazbu vytváří látky ze skupiny organofosfátů, např. malathion či parathion (po předchozí aktivaci na oxo formu), používané jako pesticidy. Po určitém čase dochází u cholinesterasy inhibované organofosfáty k tzv. „stárnutí“ enzymu, kdy dochází k dealkylaci části molekuly organofosfátu, ale jelikož enzym zůstává fosforylován, je nadále neaktivní. Tomuto procesu lze zabránit podáním tzv. reaktivátorů. Chemicky se jedná o oximové sloučeniny – používány jsou pralidoxim, obidoxim nebo látka HI-6 [64-66]. Mechanismus působení oximových reaktivátorů spočívá v nukleofilní atace záporně nabitého kyslíku na atom fosforu v molekule organofosfátu a serinové residuum se tak stává opět aktivní v přeměně substrátu [67].

Speciální skupinou organofosfátových sloučenin jsou látky souhrnně označované jako nervově paralytické látky. Většinou se jedná o těkavé, extrémně toxické látky používané hlavně pro vojenské účely, např. pro soman byla po intramuskulárním podání kryse nalezena LD_{50} 69 μg/kg [68]. První z nich, tabun, byl syntetizován krátce před II. světovou válkou a jejich produkce pokračovala do 60. let 20. století. Řadíme sem např. sarin, soman, tabun či látku VX [69]. Některé organofosfáty jako inhibitory AChE našly využití v humánní medicíně, takovým příkladem je metrifonát, původně používaný jako antihelmetikum, který byl navržen k léčbě Alzheimerovy nemoci. Nicméně při dlouhodobém užívání se projevily jeho závažné vedlejší účinky vedoucí ke stažení z trhu [70]. Struktury některých organofosfátů jsou na obrázku 11.



Obrázek 11. Chemické struktury vybraných organofosfátů.

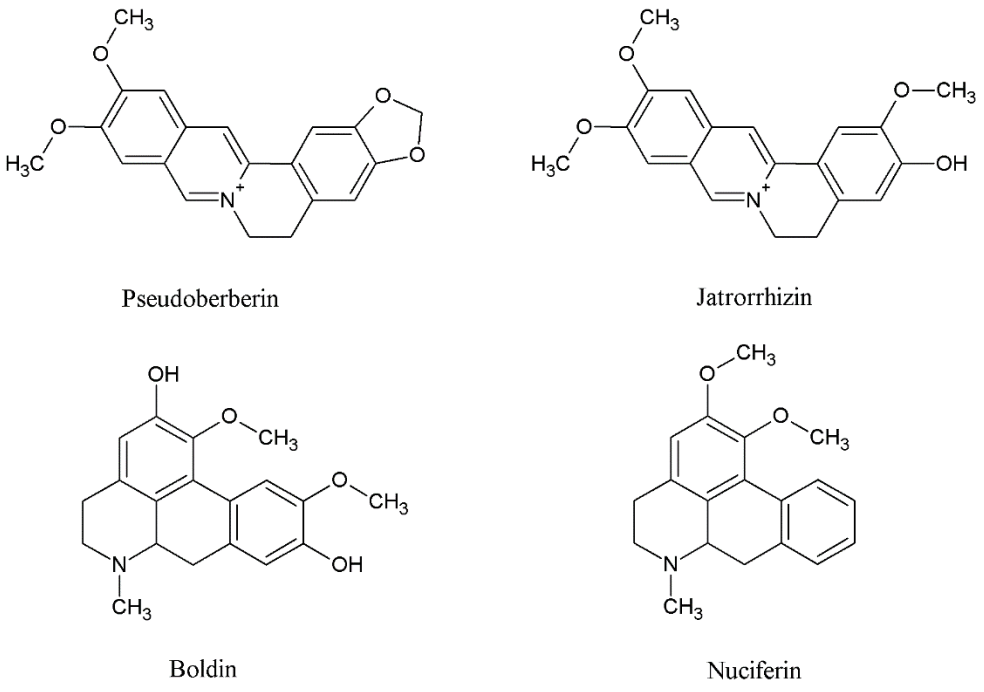
Karbamáty na rozdíl od organofosfátů vytváří se serinovým residuem cholinesterasy pseudoireverzibilní vazbu, která se po čase rozpadá a enzym se stává opět aktivním. Rozpad vazby cholinesterasa-karbamát je ale silně ovlivněn délkou postranního řetězce karbamátu, nicméně může být urychlen přídavkem jiných látek [71, 72]. Patří sem látky jako např. karbofuran či pirimikarb, dříve hojně používané jako pesticidy. Kromě pesticidů jsou karbamáty využívány v klinické praxi jako léčiva, např. neostigmin, pyridostigmin, fyzostigmin či rivastigmin (Obr. 12) [3].



Obrázek 12. Chemické struktury vybraných karbamátů.

3.4.2. Inhibitory anionického místa

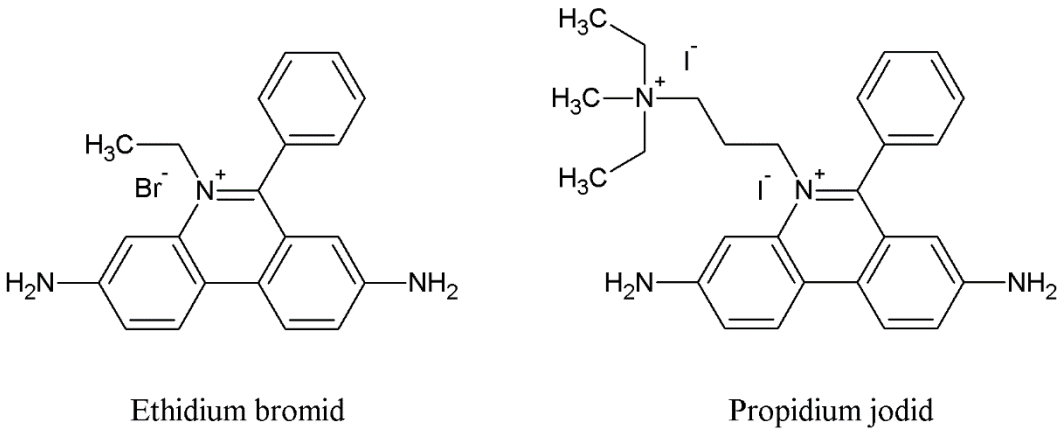
Na rozdíl od inhibitorů esterového místa jsou tyto látky inhibitory reversibilními. Společným znakem je přítomnost aromatických jader, které zajišťují průnik aromatickou štěrbinou AChE, a dusíku, popř. kvarterního dusíku, který zajišťuje interakci s aminokyselinami anionického místa. Řadí se sem např. akridinové či teterahydroakridinové deriváty, jejichž příkladem je takrin, jež je diskutován níže jako látka pro léčbu Alzheimerovy choroby [3]. Velkou skupinou látek vážící se do anionického místa jsou rostlinné alkaloidy, jako např. protoberberinové alkaloidy (pseudoberberin, jatrorrhizin), aporfinové alkaloidy (boldin, nuciferin) atd. (Obr. 13). V literatuře je popsána celá řada látek rostlinného původu, které mají cholinesterasovou aktivitu [73-76]. Patří sem i látky, které našli uplatnění jako léčiva, jedná se např. o galantamin nebo huperzin A, ty jsou diskutovány v následující kapitole o Alzheimerově chorobě.



Obrázek 13. Chemické struktury vybraných rostlinných alkaloidů.

3.4.3. Inhibitory periferního anionického místa

Inhibice periferního místa cholinesteras není příliš častá. Do periferního anionického místa se váží např. fluorescenční barviva používané v analýze DNA, ethidium a propidium (Obr. 14) [77, 78]. Do periferního anionického místa se váží i inhibitory s duálním mechanismem účinku, např. deriváty bis-takrinu či léky pro Alzheimerovu nemoc donepezil a huperzin A. Zdá se, že hliník a další ionty kovů taktéž inhibují AChE v tomto místě, ačkoliv mechanismus této interakce nebyl ještě úplně pochopen [79-82].



Obrázek 14. Chemické struktury fluorescenčních barviv vážících se do periferního anionického místa.

3.5. Stavy spojené se změnami v hladině cholinesteras

Inhibitory cholinesteras jsou významným pomocníkem v léčbě některých stavů, při kterých dochází ke změnám v cholinergním systému. Nejznámějšími příklady takových stavů jsou Alzeimerova choroba a myasthenia gravis, u nichž je indikováno podávání cholinesterasových inhibitorů.

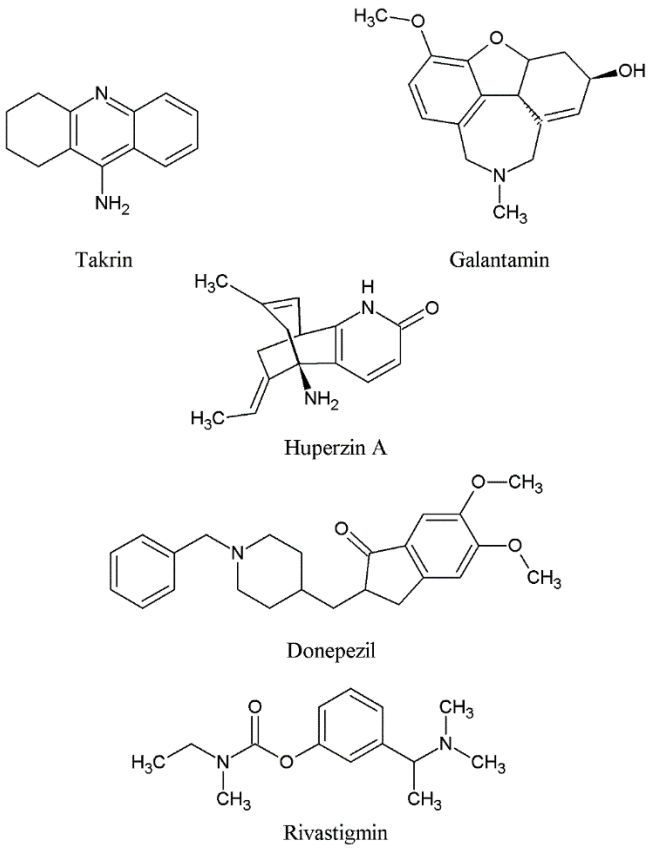
3.5.1. Alzheimerova choroba

Alzheimerova choroba byla poprvé popsána německým lékařem Aloisem Alzheimerem v roce 1906. Je to nejčastější typ demence u lidí starších 65 let. Hlavním příznakem počátku nemoci je neschopnost zapamatovat si nové informace, dalšími příznaky jsou např. poruchy paměti ovlivňující denní život, problémy s řešením běžných úkolů, problémy s řečí či psaním atd. [83, 84]. Jak onemocnění vzniká není jasné, nicméně existuje několik hypotéz, ze kterých je v současnosti nejvíce podporována tzv. cholinergní hypotéza, kdy v důsledku poruchy syntézy acetylcholinu dochází k jeho nedostatku v synaptické štěrbině. Proto se podáním inhibitorů AChE zvyšuje jeho koncentrace a tím dochází ke zlepšení kognitivních funkcí [85, 86]. Používanými inhibitory jsou galantamin, donepezil, rivastigmin a další, které jsou uvedeny níže (Obr. 15). U těžších stádií je podáván blokátor ionotropních receptorů excitačních aminokyselin – memantin. Nefarmakologická léčba spočívá v rehabilitačních programech zaměřených na kognitivní, řečové funkce či reedukační aktivity z běžného života [87].

a. Takrin (Cognex®) – chemicky 1,2,3,4-tetrahydro-9-aminoakridin, byl vůbec první syntetický lék používaný na léčbu Alzheimerovy nemoci. Jedná se o reversibilně působící nekompetitivní inhibitor AChE. Z důvodu vysoké hepatotoxicity se ovšem od jeho užívaní upouští a je nahrazován jinými terapeutiky či méně toxickými analogy [88-91].
b. Galantamin (Reminyl®) – systematickým názvem (4aS,6R,8aS)-5,6,9,10,11,12-hexahydro-3-methoxy-11-methyl-4aH-[1]benzofuro[3a,3,2-ef][2]benzazepin-6-ol, je přírodní alkaloid izolovaný z rostlin čeledi *Amarylidaceae* (např. *Galanthus woronowii*) a hojně používaný v léčbě Alzheimerovy nemoci. Kromě přírodní verze je k dispozici i verze syntetická. Jde o reversibilní kompetitivní inhibitor AChE, který působí dvojím mechanismem, kromě inhibice AChE alostericky moduluje afinitu

nikotinových receptorů k acetylcholinu. Optimální terapeutická dávka se pohybuje v rozmezí 16–24 mg/den [92, 93].

c. Donepezil (Aricept®) – 1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]methylpiperidin, je hojně používaný v léčbě Alzheimerovy nemoci. Jde o reverzibilní inhibitor, který je selektivní pro AChE a je zhruba 10krát účinnější než takrin. Terapeutická dávka se pohybuje mezi 5–10 mg/den [94-96].
d. Rivastigmin (Exelon®) – (S)-N-ethyl-3-((1-dimethyl-amino)ethyl)-N-methylphenylkarbamát, je spolu se dvěma výše zmíněnými látkami široce využívaný inhibitor při léčbě Alzheimerovy nemoci. Na rozdíl od donepezilu či galantaminu není specifickým inhibitorem AChE, ale díky karbamátové vazbě má afinitu i k BChE. Terapeutická dávka se pohybuje v rozmezí 1–4 mg/den nebo 6–12 mg/den, v závislosti na stavu nemocného [97, 98].
e. Huperzin A – neboli (1R,9S,13E)-1-amino-13-ethyliden-11-methyl-6-azatricyklo[7.3.1.02,7]trideca-2(7),3,10-trien-5-on, je přírodní alkaloid izolovaný z čínské rostliny *Huperzia serrata* a v Číně byl schválen k terapii Alzheimerovy nemoci již v roce 1994. Od té doby bylo vypracováno mnoho klinických studií, které prokázaly výrazné zlepšení kognitivních funkcí po jeho podání. Jedná se o vysoce selektivní inhibitor AChE, v čínské praxi podávaný v dávkách 300–500 μg/den [99-101].



Obrázek 15. Chemické struktury klinicky používaných léčiv.

3.5.2. Myasthenia gravis

Myasthenia gravis je autoimunitní onemocnění charakteristické svalovou slabostí až ochablostí. Vyskytuje se buď jako oční forma, kdy jsou postiženy oční víčka, nebo jako generalizovaná forma s přítomností protilátek proti acetylcholinovému nikotinovému receptoru (anti-AChR), díky kterým dochází k destrukci receptoru a následně snížené schopnosti přenášet signály. Proto je nutné zajistit vyšší koncentraci acetylcholinu v synaptické štěrbině, čehož lze docílit podáváním inhibitorů AChE. U některých pacientů se anti-AChR nevyskytují, proto se provádí vyšetření na přítomnost protilátek proti svalově specifické tyrosinkinase (anti-MuSK). V těžších případech dochází k postižení svalů mimických, dýchacích, objevují se problémy s polykáním atd. Velmi nebezpečnou komplikací je tzv. myastenická krize, při které může dojít až k zástavě dechu [102, 103]. Možná léčba spočívá v podávání inhibitorů AChE, hlavně pyridostigminu 30–60 mg, každých 3–6 hod. U progredující nemoci, kdy již léčba inhibitory AChE neúčinkuje, se přistupuje k podávání kortikosteroidů či azathioprinu, cyklosporinu atd. [103-105]. Někdy se při léčbě přistupuje k tymektomii, nicméně se

nezdá, že by operativní odstranění thymu mělo přínos, jelikož i u těchto pacientů může znovu dojít k rozvinutí myastenická krize [106, 107].

3.5.3. Otravy způsobené nervově paralytickými látkami

Acetylcholin působí jako přenašeč vzruchů v centrální a periferní nervové soustavě a jeho hromadění při otravách nervově paralytickými látkami, a tím inhibici AChE, vede k typickým příznakům otravy. Rozlišujeme celkem tři základní příznaky těchto otrav – centrální, nikotinové a muskarinové. Centrální příznaky otrav se projevují nadměrným drážděním neuronů centrální nervové soustavy (např. poruchy vědomí, křeče, třes, bolesti hlavy atd.). Nikotinové příznaky jsou spojeny s inhibicí na autonomních gangliích a kosterních svalech (např. křeče, tachykardie, fascikulace atd.) a muskarinové příznaky souvisí se žlázami a hladkým svalstvem (např. slzení, slinění, kašel, bradykardie atd.). [108] Z důvodu organofosfátového původu nervově paralytických látek a tím spojené tvorby ireverzibilní vazby s cholinesterasami je léčba těchto stavů spojená s reaktivátory cholinesteras, které byly zmíněny výše. V polních podmínkách, kdy lze očekávat nasazení ze strany nepřítele, je možné podat krátkodobě působící inhibitory cholinesteras, které se váží do aktivního místa přednostně a brání tím vazbě nervově paralytické látky. Používá se pyridostigmin, který ale na rozdíl od organofosfátů neprochází hematoencefalickou bariérou, a proto je neúčinný v ochraně centrálního nervového systému. Podává se proto v kombinaci s centrálně působícími anticholinergiky, antikonvulzivy nebo cholinesterasovými reaktivátory [109-111]. Zajímavou možností profylaxe proti otravě organofosfáty do budoucna je podávání BChE, na kterou se organofosfáty váží přednostně, snad kvůli svému nadbytku v plasmě, a díky tomu chrání fyziologicky důležitější AChE. Tento postup má však i svou nevýhodu, protože jedna molekula BChE dokáže navázat pouze jednu molekulu organofosfátu, proto je potřeba podávat velké koncentrace. Celý proces je prozatím ve fázi klinických testů [112].

4. Experimentální část

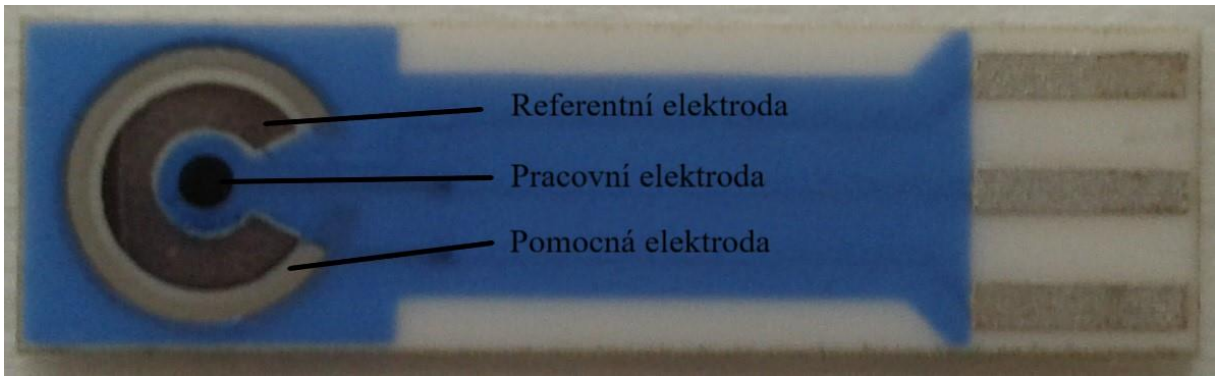
4.1. Přístroje a zařízení

Evolution 201 UV-Visible Spectrofotometer (Thermo Scientific Inc., Waltham, MA, USA). Elektrochemický analyzátor PalmSens řízený softwarem PSTrace 4.8.1 (PalmSens BV, Houten, Nizozemsko). Mobilní telefon Sony Xperia MT27i se zabudovaným 5 Mpx fotoaparátem a LED světlem, používající operační systém Android 2.3.7. (verze zařízení 6.0.B.3.184, se senzorem Sony Exmor R^{TM} CMOS) (Sony Corporation, Tokyo, Japonsko). 3D tiskárna Prusa i3 (Prusa Research, Praha, Česká republika). Třepačka Thermo-Shaker PST-60HL (Biosan, Riga, Lotyšsko). Destilační aparatura pro přípravu destilované vody Aqua Osmotic (Tišnov, Česká republika).

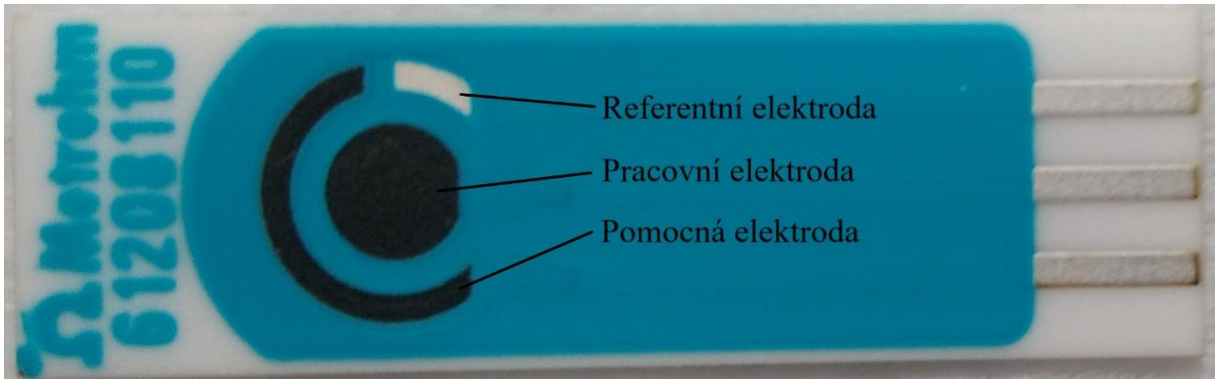
4.2. Chemikálie a materiál

Acetylcholinesterasa z elektrického úhoře (typ V-S, lyofilizovaný prášek, ≥ 1000 U/mg proteinu, molekulová hmotnost 280 kDa), lidská rekombinantní acetylcholinesterasa (exprimována v buňkách HEK 293, lyofilizovaný prášek, ≥ 1000 U/mg proteinu), butyrylcholinesterasa z lidského séra (lyofilizovaný prášek, ≥ 50 U/mg proteinu), acetylcholin chlorid (čistota ≥ 99 %), acetylthiocholin chlorid (čistota ≥ 99 %), butyrylthiocholin jodid (čistota ≥ 98 %), indoxyl acetát (čistota ≥ 95 %), N-(3-dimethylaminopropyl)-N′-ethylkarbodiimid hydrochlorid (čistota ≥ 98 %), 5,5′-dithiobis(2-nitrobenzoová) kyselina (čistota 99 %), 9-amino-1,2,3,4-tetrahydroakridin hydrochlorid hydrát (čistota ≥ 99 %), galantamin hydrobromid (čistota ≥ 94 %, z lilií druhu *Lycoris*), donepezil hydrochlorid monohydrát (čistota ≥ 98 %), karbofuran (čistota 98 %), tetraisopropyl pyrofosforamid, boldin (analytický standard), chitosan z krunýřů krevet (nízká viskozita), magnetické částice na povrchu aktivované karboxylovou skupinou (koncentrace částic 18–22 mg/ml), glutaraldehyd (50% vodný roztok), N-acetyl-L-cystein (čistota ≥ 99 %), hexakyanoželeznatan železitý, acetátový pufr pH 5,0 (100 mM, složení: 0,2 M kyselina octová a 0,2 M octan sodný do 100 ml a upraveno na pH 5,0), fosfátový pufr pH 7,4, (10 mM, tablety, složení: 0,0027 M chlorid draselný a 0,137 M chlorid sodný), dimethylsulfoxid (čistota ≥ 99 %), 2-propanol (čistota ≥ 99,5 %), Tween-20 (dynamická viskozita 250-450 mPa·s) byly dodány firmou Sigma-Aldrich (St. Louis, MO, USA). Želatina (čistota p.a.), absolutní ethanol (čistota p.a.), kyselina octová (čistota p.a.), octan sodný trihydrát (čistota p.a.) poskytla firma PENTA (Praha, Česká republika). Fenolová červeň, sodná sůl (čistá, rozpustná ve vodě) byla zakoupena u firmy Acros

Organics (Thermo Scientific Inc., Waltham, MA, USA). Biperiden (1 ml ampule o koncentraci účinné látky 5 mg/ml) od firmy Knoll AG (Ludwigshafen, Německo). Filtrační papír (velikost pórů 1PS) od firmy Whatman (Maidstone, UK). Indikátorové pH papírky pH Fix 6,6–7,7 od firmy Macherey-Nagel (Düren, Německo). Tištěné elektrody od firmy BVT (Brno, Česká republika) o rozměrech 25,4 × 7,26 × 0,63 mm s uhlíkovou pracovní elektrodou o průměru 1 mm, chloridostříbrnou referentní elektrodou a platinovou pomocnou elektrodou (Obr. 16) a tištěné elektrody od firmy Metrohm (Herisau, Švýcarsko) o rozměrech 34 × 10 × 0,5 mm s uhlíkovou pracovní elektrodou o průměru 4 mm, stříbrnou referentní elektrodou a pomocnou uhlíkovou elektrodou (Obr. 17).



Obrázek 16. Tištěná elektroda od firmy BVT.



Obrázek 17. Tištěná elektroda od firmy Metrohm.

4.3. Metody

Zde uvedené metody jsou pouze obecným shrnutím metod použitých v této disertační práci. Detailní postupy, včetně použitých koncentrací a objemů jednotlivých látek, jsou uvedeny v přiložených publikovaných pracích.

4.3.1. Stanovení cholinesterasové aktivity

4.3.1.1. Ellmanova metoda stanovení aktivity cholinesteras

Do standardní spektrofotometrické kyvety (šířka optické dráhy 1 cm) byla pipetována 5,5´-dithiobis(2-nitrobenzoová) kyselina, roztok cholinesterasy a acetylthiocholin/butyrylthiocholin. Všechny použité roztoky byly ve fosfátovém pufru pH 7,4. Absorbance roztoku byla měřena ihned po přídavku substrátu a následně po dvou minutách reakce. Ze změny absorbancí byla následně vypočtena aktivita cholinesterasy použitím molárního absorpčního koeficientu 14,150 $l \times mol^{-1} \times cm^{-1}$ pro 2-nitro-5-thiobenzoovou kyselinu [113].

4.3.1.2. Elektrochemické stanovení aktivity AChE

Elektrochemické stanovení aktivity AChE bylo použito v kombinaci s magnetickými částicemi. Pro tento účel byla použita metoda square-wave voltametrie a tištěné elektrody od firmy BVT nebo Metrohm. AChE imobilizovaná na magnetických částicích byla po proběhnutí enzymové reakce odseparována od roztoku, který obsahoval thiocholin. Roztok byl následně analyzován a koncentrace thiocholinu, resp. velikost proudové odezvy byla přímo úměrná enzymové aktivitě. Při stanovení cholinesterasových inhibitorů byla jejich koncentrace nepřímo úměrná velikosti proudové odezvy.

4.3.1.3. Hodnocení cholinesterasové aktivity fotografickou detekcí

Fotografie byly pořízeny za použití tmavé komory (Obr. 18) a následně byly vyhodnocovány v programu GIMP 2.8.16 (open source software) použitím funkce Color Picker. Byly analyzovány body červeného, zeleného a modrého kanálu (RGB body), které pro formát obrázku jpg nabývají hodnot od 0 do 255. Rozdíly těchto hodnot před a po reakci potom odpovídaly enzymové aktivitě. Vhodnost použití jednotlivých kanálů byla vždy stanovena experimentálně.

4.3.2. Posuzování vlivu organických rozpouštědel na AChE

Pro každou metodu založenou na AChE je nutno stanovit vliv organických rozpouštědel, které se při testování cholinesterasových inhibitorů mohou do analýzy dostat (např. při extrakci inhibitorů ze vzorků životního prostředí). Proto byl vliv organických rozpouštědel testován v každé publikaci zabývající se imobilizací AChE. Použit byl ethanol, 2-propanol a dimethylsulfoxid.

4.3.3. Imobilizace cholinesteras

V této disertační práci bylo použito celkem pět imobilizačních postupů AChE, které jsou zde pouze stručně shrnuty, detailní postupy jednotlivých imobilizací jsou uvedeny v publikacích v kapitole výsledků.

První imobilizační postup AChE byl proveden s komerčními magnetickými částicemi aktivovanými karboxylovou skupinou, za použití N-(3-dimethylaminopropyl)-N´-ethylkarbodiimid hydrochloridu, který s aminovou skupinou AChE a karboxylovou skupinou na povrchu magnetických částic vytvořil kovalentní vazbu.

Ve spolupráci s výzkumnou skupinou v Brně (doc. RNDr. Pavel Kopel, Ph.D., Mendelova univerzita v Brně, Vysoké učení technické v Brně – CEITEC) byl na nově syntetizované magnetické částice proveden druhý imobilizační postup AChE. Celkem bylo připraveno pět nových typů magnetických částic s různou povrchovou modifikací, ze kterých byla vybrána jedna finální a pomocí glutaraldehydu byla AChE imobilizována na povrch.

Fyzikální zachycení do želatinové membrány bylo třetím typem imobilizačního postupu. Tekutá želatina byla smíchána s AChE a při laboratorní teplotě se nechala zatuhnout na povrchu filtračního papíru.

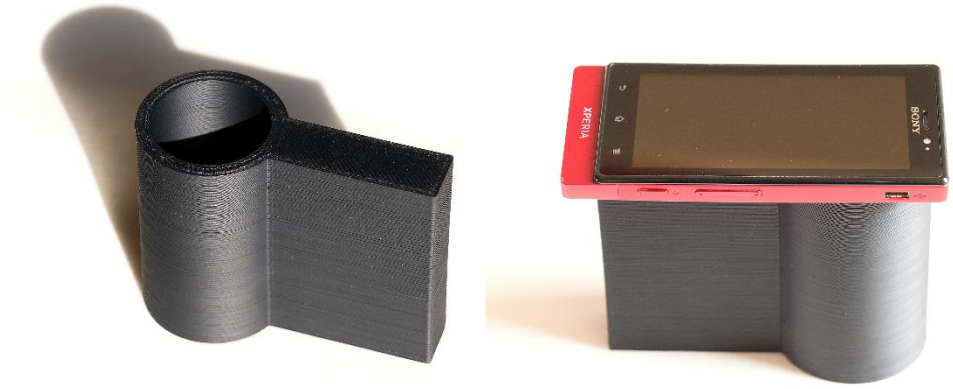
Adsorpce AChE na povrch pH citlivého proužku, a následné překrytí želatinovou membránou mající za úkol stabilizovat adsorbovaný enzym, byla čtvrtým typem imobilizace použitým v této disertační práci.

Pátou imobilizací byla vazba na povrch aktivovaného chitosanu. Po zatuhnutí přes noc na filtračním papíře byl povrch chitosanu aktivován N-(3-dimethylaminopropyl)-N´-ethylkarbodiimid hydrochloridem pro kovalentní navázání AChE.

4.3.4. 3D tisk

Metoda 3D tisku byla použita pro vytvoření tmavé komory pro fotografickou detekci ve tvaru válce s plochou pro podepření mobilního telefonu (Obr. 18). Návrh byl vytvořen v programu Autodesk® 123D® Design (Autodesk, San Rafael, CA, USA). Z tohoto programu byl návrh importován do programu Prusa3D Slic3r 3mm (součást softwaru 3D tiskárny), kde byla provedena finální úprava návrhu a vygenerován souřadnicový kód pro tiskárnu. Velikost vytvořeného objektu byla 80 mm na výšku, 105 mm na délku a vnitřní průměr válce komory 40 mm. Tiskové parametry tiskárny: materiál akrylonitril butadien styren ve filamentu

o průměru 3 mm, teplota trysky 285 °C, teplota podložky 100 °C, tloušťka vrstvy při tisku 0,1 mm.



Obrázek 18. Tmavá komora (vlevo) vytištěná 3D technologií a celkové uspořádání při fotografické detekci (vpravo).

4.3.5. Výpočet LOD, LOQ

Výpočty limitů detekce a kvantifikace byly prováděny v programu Origin (OriginLab, Northampton, MA, USA). Limit detekce byl vypočítán jako trojnásobek směrodatné odchylky vůči pozadí, limit kvantifikace jako desetinásobek směrodatné odchylky vůči pozadí.

5. Výsledky a diskuze

5.1. Výsledek 1

Adam Kostelník, Alexander Čegan, Miroslav Pohanka, „Electrochemical determination of activity of acetylcholinesterase immobilized on magnetic particles“ *International Journal of Electrochemical Science*, 11 (6), 4840–4849, 2016 (IF = 1,369)

Magnetické částice se pohybují ve středu zájmu již řadu let a jsou používány pro imobilizaci nejrůznějších molekul proteinové povahy, včetně enzymů [114-118]. Na druhou stranu existuje jen málo publikací zabývající se imobilizací enzymu AChE na magnetické částice [49-55], přestože použití magnetických částic skýtá mnoho výhod. Hlavní výhodou je znovupoužití enzymu, chemická stabilizace navázaného enzymu a nelze opomenout ani ekonomickou stránku.

Potřeba imobilizovat AChE na povrch magnetických částic vyplývá z faktu, že při standardním spektrofotometrickém stanovení podle Ellmana zůstává enzym nadále aktivní (v případě, že není ireverzibilně inhibován) a spolu s ostatními reagenciemi je znehodnocen v odpadu. To nenahrává ekonomičnosti, jelikož enzym představuje nejdražší položku stanovení.

V této práci byla AChE imobilizována na povrch komerčních magnetických částic aktivovaných karboxylovou skupinou za použití N-(3-dimethylaminopropyl)-N´-ethylkarbodiimid hydrochloridu a enzymová aktivita byla stanovena elektrochemicky metodou square-wave voltametrie. Použité částice prokázaly svou schopnost vázat enzym, nicméně se neukázaly příliš vhodné pro opakované měření. Při promývání mezi jednotlivými měřícími cykly došlo skokově k jejich degradaci, čímž byla odmyta i část vázané AChE a došlo ke ztrátě katalytických vlastností. Kvůli degradaci magnetických částic se ztrácí výhoda imobilizace v porovnání se spektrofotometrickým stanovením, na druhou stranu k degradaci částic docházelo až při šestém měřícím cyklu, což stále představuje značnou úsporu v porovnání se spektrofotometrickým stanovením. Nicméně v citovaných pracích zabývající se imobilizací AChE na magnetické částice dosáhnul např. Istamboulie et al. stability magnetických částic nejméně 10 cyklů, Dzudzevic Cancar et al. zase publikoval stabilitu až 20 cyklů [50, 54].

Lehkou nevýhodou může být časová náročnost stanovení v porovnání se spektrofotometrickým stanovením, jelikož z důvodu přeměny substrátu na thiocholin a následné separace magnetických částic před elektrochemickým měřením se prodlužoval čas potřebný pro stanovení.

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Electrochemical Determination of Activity of Acetylcholinesterase Immobilized on Magnetic Particles

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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).

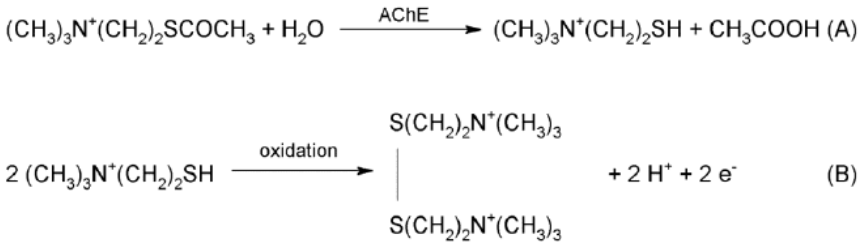

Figure 1. Hydrolysis of acetylthiocholine by AChE (A), thiocholine oxidation (B)

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.

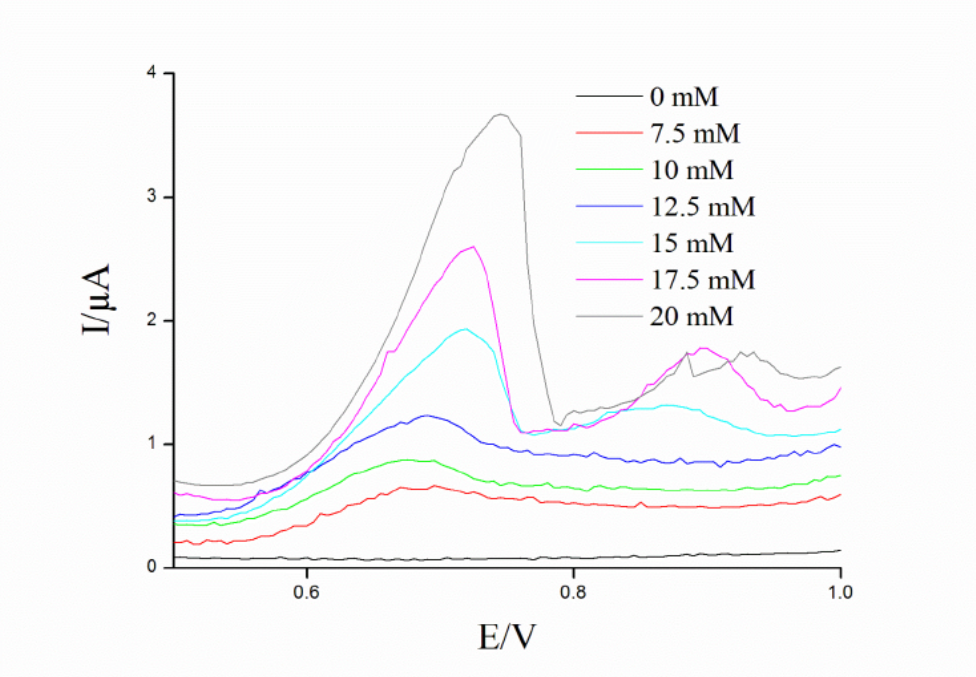

Figure 2. SWV curves of N-acetyl-L-cysteine performed in PBS 7.4; scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl referent electrode and platinum auxiliary electrode

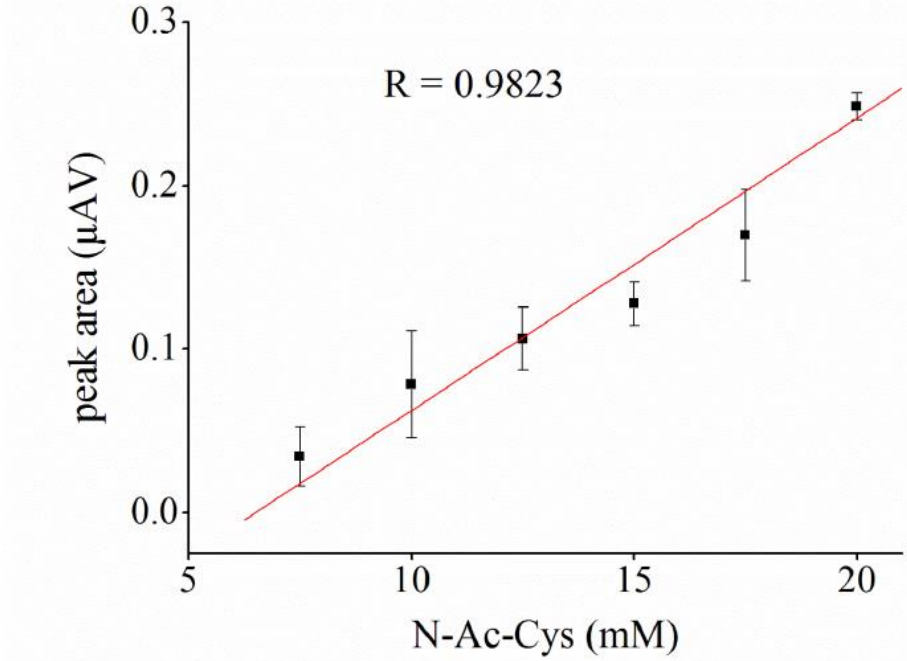

Figure 3. Optimization curve of N-acetyl-L-cysteine performed in PBS 7.4; scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl referent electrode and platinum auxiliary electrode.

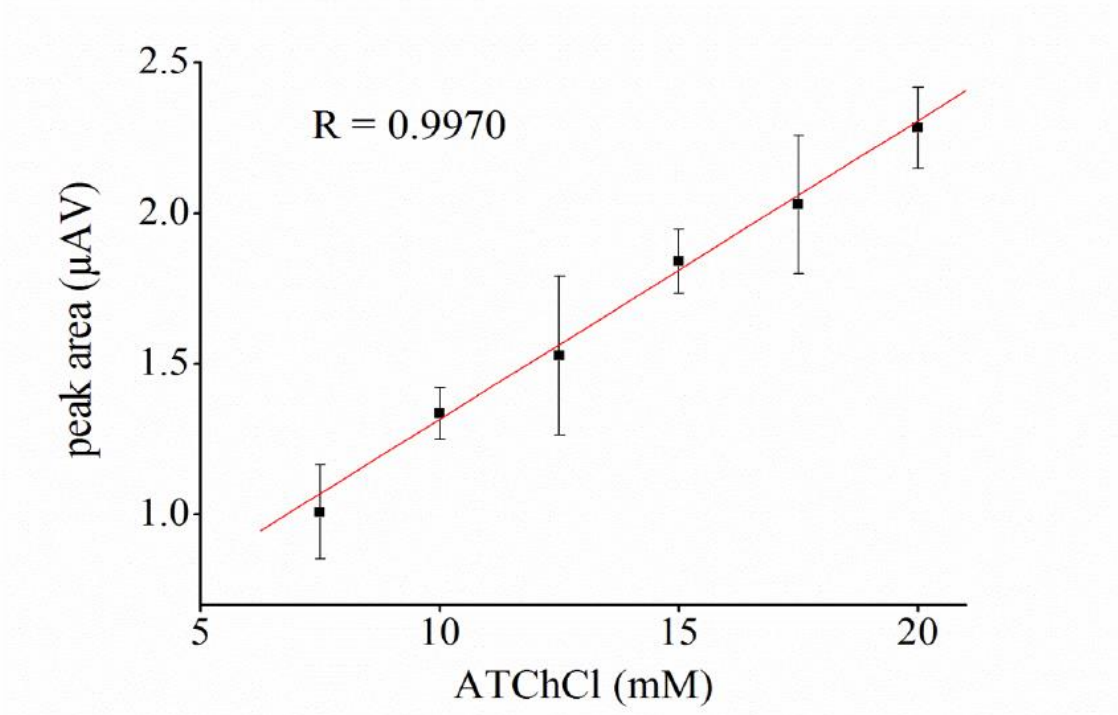

Figure 4. Optimization curve of acetylthiocholine performed in PBS 7.4; scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl referent electrode and platinum auxiliary electrode.

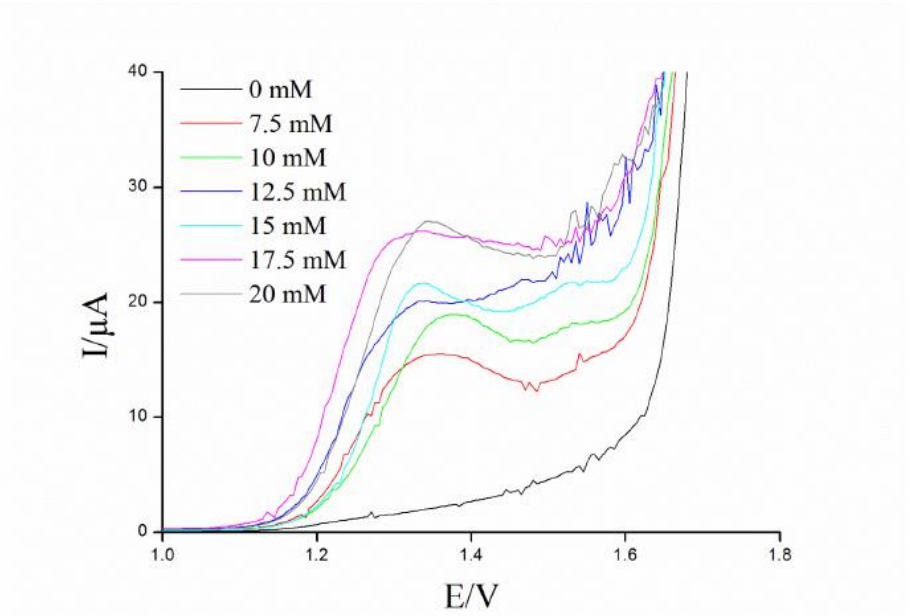

Figure 5. SWV curves of acetylthiocholine performed in PBS 7.4; scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl referent electrode and platinum auxiliary electrode

3.2. Tacrine calibration

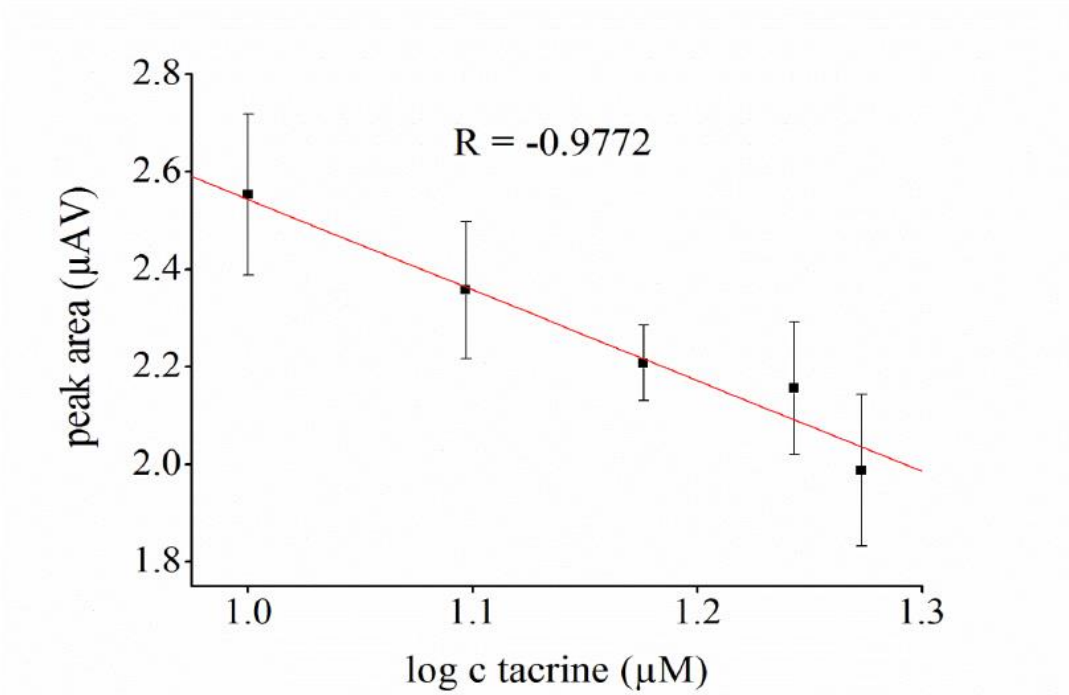

Figure 6. Tacrine calibration curve performed in PBS 7.4; scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl referent electrode and platinum auxiliary electrode.

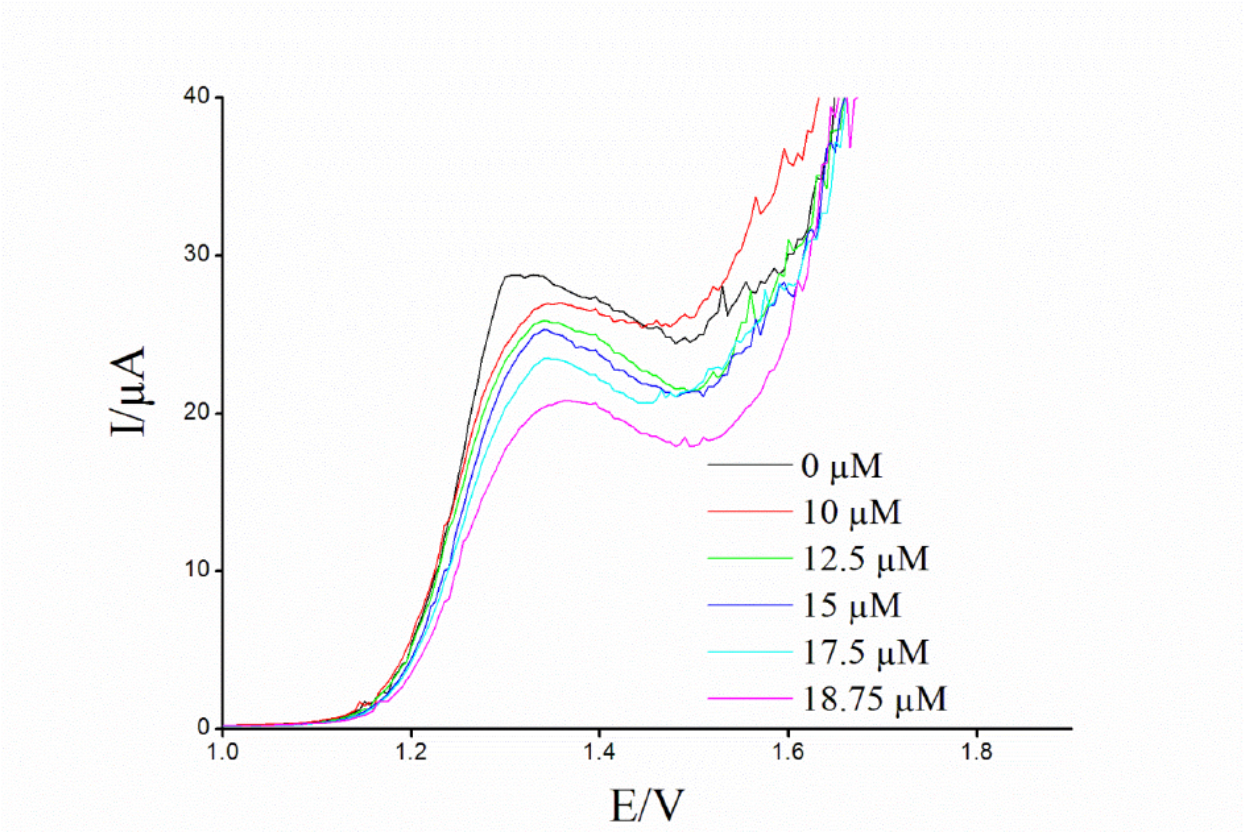

Figure 7. SWV curves of tacrine performed in PBS 7.4; scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl referent electrode and platinum auxiliary electrode

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.

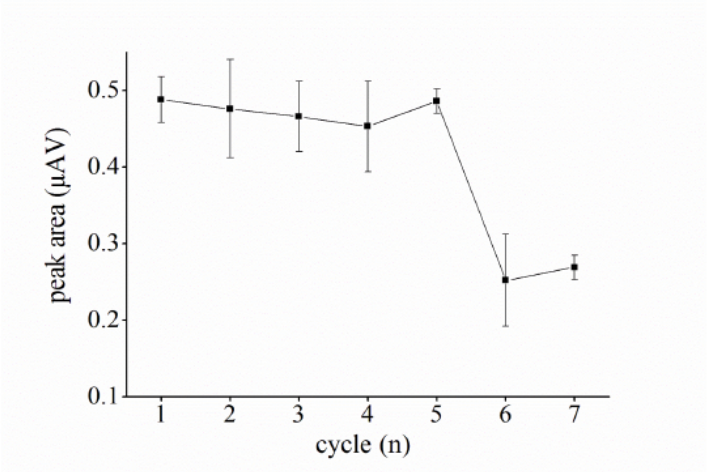

Figure 8. Reproducibility of measurement with MPs-AChE performed in PBS 7.4; scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl referent electrode and platinum auxiliary electrode.

3.4. Method interferences

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

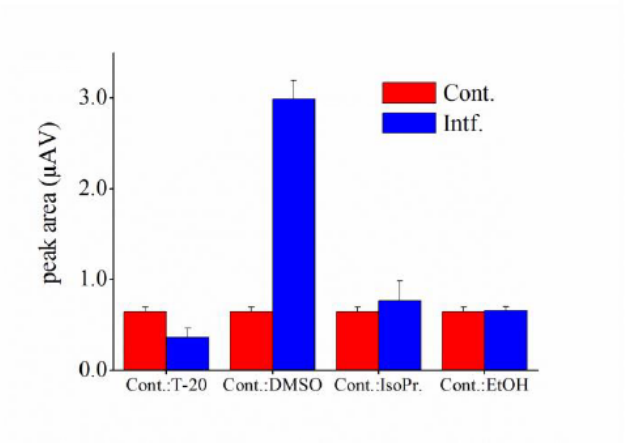

Figure 9. Method interferences (T20 = Tween-20; DMSO = dimethyl sulfoxide; IsoPr = isopropyl alcohol; EtOH = ethanol; Cont. = control; Intf. = interference); scanning range 0 – 1.8 V, potential step 0.005 V, amplitude 0.010 V and frequency 1 Hz; screen-printed electrode carbon working electrode, AgCl 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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5.2. Výsledek 2

Adam Kostelník, Pavel Kopel, Alexander Čegan, Miroslav Pohanka, „Construction of an acetylcholinesterase sensor based on synthesized paramagnetic nanoparticles, a simple tool for neurotoxic compounds assay“, *Sensors*, 17 (4), 676–688, 2017 (IF = 2,475)

Nedostatečná stabilita komerčních částic byla důvodem imobilizace AChE na nově syntetizované magnetické částice připravené výzkumnou skupinou na Mendelově univerzitě v Brně. Částice byly připraveny redukcí borohydridem sodným z dusičnanu železitého s následnou modifikací povrchu, detailní postup přípravy částic a jednotlivých modifikací byl uveřejněn v publikaci. V porovnání s komerčními magnetickými částicemi, které se pohybovaly ve velikostech mikro, byly nově připravené magnetické částice velikosti nano, čímž se lehce prodlužovala doba potřebná k magnetické separaci. Nejedná se však o limitující krok, jelikož použití silnějšího magnetu by tento problém odstranil. Aktivita AChE byla opět stanovována elektrochemicky metodou square-wave voltametrie.

Různé povrchové modifikace nově připravených magnetických částic byly testovány pro schopnost vázat AChE. V případě povrchové modifikace tetraethyl orthosilikátem (TEOS) nabízí povrch volné hydroxylové skupiny, na které se AChE může vázat. Již dříve byla tato modifikace úspěšně použita pro vazbu enzymu celulasy [119]. Kombinace TEOS a 3-aminopropyltriethoxysilanu zase nabízí volné aminové skupiny. I tato modifikace již byla úspěšně zkoumána pro vazbu enzymu [120], proto byl předpoklad úspěšné vazby i v případě AChE. Modifikace magnetických částic N^1-(3-trimethoxysilylpropyl)diethylentriaminem, který dosud nebyl pro imobilizaci enzymů použit, byla nakonec zvolena pro další měření. Nabízí totiž na povrchu celkem dvě volné iminové skupiny a jednu aminovou skupinu, které se ukázaly být ve vazbě AChE nejlepší.

Předností vybraných magnetických částic byla výborná stabilita v porovnání s komerčně dodanými částicemi použitými v předchozí práci. Z publikovaných prací jiných autorů je vidět, že magnetické částice syntetizované v laboratoři vykazují vysokou stabilitu [54, 55, 119, 120], nicméně nelze říci, že všechny komerčně dodávané částice jsou méně stabilní než ty laboratorně připravené, jak dokazují jiné publikované práce [50, 52]. Výhodou vysoké stability připravených částic s navázanou AChE je možnost jejich uplatnění především v průtokových systémech při stanovení cholinesterasových inhibitorů.

V této práci byly také stanovovány cholinesterasové inhibitory, navíc bylo zkoumáno obnovení aktivity AChE po inhibici galantaminem a sledována byla i dekarbamoylace aduktu

AChE-karbofuran. Galantamin, jakožto kompetitivní inhibitor AChE, je možné z aktivního centra inhibovaného enzymu vytlačit nadbytkem substrátu. To je ovšem neekonomické, proto byly zkoumány levnější alternativy, a to vymytí pufrem o jiném pH. Fosfátový pufr o pH 7,4, který byl prostředím pro enzymovou reakci, byl nahrazen pufrem acetátovým o pH 5,0. Ten se ukázal pro tento účel vhodný, jelikož se aktivita AChE vrátila na původní hodnotu, pravděpodobně díky změně náboje aminokyselin v aktivním centru, které mají slabou nekovalentní interakci s galantaminem na starosti.

Rychlost rozpadu karbamátové vazby záleží na délce postranního řetězce molekuly karbamátu [121]. Wetherell a French při pokusech s fyzostigminem zjistili, že dekarbamoylace AChE probíhá rychleji u primátů (makak, kočkodan) než u morčecího modelu, proto je navrhli jako model pro porovnání dekarbamoylačních časů s lidskou variantou [122]. Pro karbofuranem inhibovanou lidskou BChE byl nalezen poločas rozpadu karbamátové vazby asi 2 hodiny [123]. V této práci byl poločas rozpadu vazby karbofuran-AChE stanoven na cca 3,5 hodiny při použití AChE z elektrického úhoře, která je lidské AChE velmi blízká [124], a mohla by proto rovněž posloužit jako model při porovnávání dekarbamoylačních časů, navíc je dostupnější než výše uvedené.

sensors

MDPI

Article

Construction of an Acetylcholinesterase Sensor Based on Synthesized Paramagnetic Nanoparticles, a Simple Tool for Neurotoxic Compounds Assay

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Abstract: Magnetic particles (MPs) have been widely used in biological applications in recent years as a carrier for various molecules. Their big advantage is in repeated use of immobilized molecules including enzymes. Acetylcholinesterase (AChE) is an enzyme playing crucial role in neurotransmission and the enzyme is targeted by various molecules like Alzheimer's drugs, pesticides and warfare agents. In this work, an electrochemical biosensor having AChE immobilized onto MPs and stabilized through glutaraldehyde (GA) molecule was proposed for assay of the neurotoxic compounds. The prepared nanoparticles were modified by pure AChE and they were used for the measurement anti-Alzheimer's drug galantamine and carbamate pesticide carbofuran with limit of detection 1.5 μM and 20 nM, respectively. All measurements were carried out using screen-printed sensor with carbon working, silver reference, and carbon auxiliary electrode. Standard Ellman's assay was used for validation measurement of both inhibitors. Part of this work was the elimination of reversible inhibitors represented by galantamine from the active site of AChE. For this purpose, we used a lower pH to get the original activity of AChE after inhibition by galantamine. We also observed decarbamylation of the AChE-carbofuran adduct. Influence of organic solvents to AChE as well as repeatability of measurement with MPs with AChE was also established.

Keywords: acetylcholinesterase; magnetic particles; electrochemistry; screen-printed sensor; carbofuran; galantamine; nanomaterial; nanoparticles

1. Introduction

AChE plays a significant role in termination of signals in the cholinergic system. The mechanism of the action is based on degradation of neurotransmitter acetylcholine into non active choline and acetic acid [1]. Measuring of AChE activity is important in diagnostics or serves as a tool in analytical chemistry in inhibitor determination. A commonly acknowledged method for activity determination is Ellman's reaction but there is potential for pH or electrochemical detection [2–7]. There is a demand for the determination of AChE inhibitors in a wide spectrum of used compounds

like Alzheimer's disease drugs (donepezil, rivastigmine, huperzine, galantamine), pesticides (carbofuran, malaoxon, malathion), and chemical warfare agents (sarin, soman, tabun, VX) [8]. MPs have been known for many years and can be prepared from many materials but most frequently iron oxides are utilized. They can be applied in a wide range of applications as can be seen from some reviews [9–15]. MPs have been used for many years in protein immobilization as a good platform for the attachment of proteins through free carboxyl, hydroxyl, thio, or amino groups on their surface [16–18]. Many protocols for enzyme immobilization on MPs have already been reported [4,19–21]. Silane-coupling reagents (like 3-aminopropyltrimethoxysilane or 3-aminopropyltriethoxysilane) are easily used for surface modification of synthetized MPs plus provide free amino groups which can be used for enzyme immobilization [22–24]. There are many different approaches proposed for the synthesis of MPs such as coprecipitation reactions, aggregation reactions, sol-gel reactions, etc. [24]. For example, maghemite nanoparticles were prepared by sodium borohydride reduction of iron chloride in ammonia solution [25,26]. The nanoparticles are easily prepared and moreover, there are hydroxo groups on their surface which are very suitable for the further modifications. The nanoparticles were used to cover Dovex for sarcosine separation as a potential prostate cancer marker. Maghemite beads modified by tetraethyl orthosilicate and 3-aminopropyl triethoxysilane can be applied for binding of H7N7 virions [27] whereas the beads covered by polyvinylpyrrolidone and gold were utilized for fluorescence resonance emission transfer (FRET)-based sarcosine detection [28].

AChE-based assays have typical lack in their inability to be used repeatedly. In this work, we focused our effort to the development of a magnetic nanoparticles based biosensor for the determination of neurotoxic compounds. We also hypothesize that the particles can be used repeatedly and lower costs in this way.

2. Materials and Methods

2.1. Materials and Equipment

Acetylcholinesterase from electric eel, lyophilized powder (≥1000 units/mg protein), acetylthiocholine chloride (ATChCl), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), GA solution (50%), galanthamine hydrobromide, carbofuran (98%), phosphate buffer saline (PBS) pH 7.4, isopropyl alcohol (i-PrOH), dimethyl sulfoxide (DMSO), tetraethyl orthosilicate (TEOS), 3-aminopropyltriethoxysilan (APTES), N^1-(3-Trimethoxysilylpropyl)diethylenetriamine (BAATMS), sodium borohydride, ammonia, iron(III) nitrate nonahydrate, sodium triphosphate, and calcium nitrate tetrahydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (EtOH), methanol (MeOH), sodium acetate and acetic acid were obtained from PENTA (Prague, Czech Republic). SWV assay was performed using electrochemical device PalmSens (PalmSens BV, Houten, The Netherlands) connected with computer and operated by software PSTrace 4.8.1 (PalmSens BV, Houten, The Netherlands). Screen-printed sensors (Metrohm, Herisau, Switzerland) were sized 34 × 10 × 0.5 mm with a 4 mm diameter carbon working electrode, silver reference electrode, and carbon auxiliary electrode.

2.2. Solutions Preparation

ATChCl solutions were prepared in concentration range from 1.25 to 20 mM. Galantamine solutions were prepared in concentration range from 25 to 100 μM. Both solutions were prepared in PBS buffer pH 7.4 and final concentration in cuvette was 10-fold less. Carbofuran inhibitor was dissolved in isopropyl alcohol in concentration range from 1.56 to 25 μM with 40-fold less concentration in cuvette. Glutaraldehyde solution (2.5%) was prepared freshly before use. All solutions were prepared into plastic microtube. For Ellman's assay, all solutions were prepared in PBS 7.4. ATChCl solution was prepared in concentration 10 mM and DTNB in 1 mM. Concentration range of galantamine was from 25 to 100 μM and from 1.56 to 25 μM for carbofuran. Final concentrations in the cuvette were 10-fold less for ATChCl and galantamine and 40-fold less for carbofuran. Carbofuran was preincubated with AChE before substrate addition for 10 min.

2.3. Synthesis of Magnetic Particles

2.3.1. Preparation of Maghemite

Sodium borohydride (1 g) dissolved in 3.5% ammonia (50 mL) was poured to stirred solution of iron(III) nitrate nonahydrate (7.48 g) dissolved in water (400 mL) [29]. The mixture was then heated at 100 °C for 2 h. The dark product was separated by magnet and washed several times with water. Obtained magnetic particles were combined with water to total 50 mL and 10 mL of the solution was used for every surface modification.

2.3.2. Preparation of MAN-34

The maghemite was poured to i-PrOH (150 mL), 28% ammonia solution (20 mL) and TEOS (3.33 mL) were added. This mixture was stirred and heated at 40 °C overnight. The product was separated by magnet and washed several times with water and finally dried at room temperature.

2.3.3. Preparation of MAN-37-NH_2

The maghemite, i-PrOH (150 mL), 28% NH_3 (20 mL), and TEOS (3.33 mL) were mixed, stirred and heated at 40 °C for 2 h and APTES (3.33 mL) was added and the mixture was heated for next 1 h. The product was stirred overnight at room temperature, separated by magnet and washed several times with diluted EtOH (75%). Finally, the product was left in 20 mL of EtOH (75%).

2.3.4. Preparation of MAN-38-1-NH_2

It was prepared similarly to MAN-37 but without TEOS and the product was dried after washing.

2.3.5. Preparation of MAN-161-NH_2

The maghemite suspension was diluted with MeOH (100 mL), BAATMS (0.2 mL) and 28% NH_3 (1 mL) were mixed and stirred for 2 h. Water (50 mL) was added and stirred overnight. The product was separated by magnet and washed several times with water. Finally, the product was left in 50 mL of water.

2.3.6. Preparation of MAN-164

Sodium triphosphate (0.368 g) in 50 mL of water was added to the maghemite suspension and stirred for 2 h. Then, 1 M $Ca(NO_3)_2 \cdot 4H_2O$ (6 mL) was poured into the solution, continued by stirring overnight. The modified maghemite was separated by magnet, washed several times with water, and left in water (50 mL).

2.4. Preparation and Comparison of Particles with Bound AChE (MPs-AChE)

MAN 34 and MAN 38-1-NH_2 MPs were obtained as solid dust and were prepared in a concentration of 20 mg/mL for analysis. MAN 37-NH_2, MAN 161-NH_2, and MAN 164 MPs were suspended in solution. Before use, all MPs were well homogenized and 400 µL of each were pipetted into microtube and washed three times with 1 mL of PBS 7.4. In the next step, 400 µL of GA solution (2.5%) was added and shaken for one hour (600 rpm). After washing three times with 1 mL of PBS 7.4, 300 µL of AChE (activity for acetylthiocholine 26 U) solution was added and shaken for two hours (600 rpm). Finally, MPs-AChE were washed three times with 1 mL PBS 7.4 to remove unbound enzyme and resuspended in another 400 µL of PBS 7.4. The principle of immobilization is depicted in Figure 1. Comparison was performed using Ellman's assay as following: 400 µL of DTNB, 450 µL of PBS 7.4, 50 µL of MPs-AChE and 100 µL of 10 mM ATChCl were mixed in standard 1.5 mL cuvette. After incubation lasting 25 min, yellow medium was separated from MPs-AChE into a clean cuvette followed by measurement of absorbance in 412 nm.

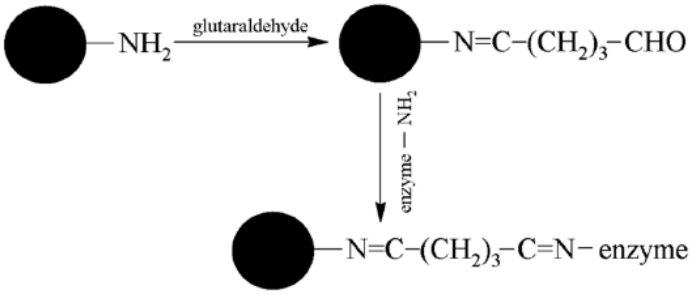

Figure 1. Immobilization principle of enzyme using glutaraldehyde.

2.5. Electrochemical Assay

850 µL PBS 7.4, 50 µL of MPs-AChE (0.39 U per electrode), and 100 µL of 10 mM ATChCl were placed into a microtube. In the case of inhibition measurement, inhibitor was added into microtube in the appropriate concentration. For competitive inhibitors, concentration of substrate was in a concentration which did not influence competition for enzyme. After the incubation step, lasting 25 min, the reaction medium containing thiocholine was separated from MPs-AChE into clean cuvette and SWV assay was performed as described above. Setting for SWV was following: 0–1.1 V scanning range, 0.005 V potential step, 0.010 V amplitude, and frequency 1 Hz. These conditions were successfully used for thiocholine measurement in our previous work [4].

2.6. Validation Measurement

In a cuvette, 400 µL of Ellman's reagent, 450 µL PBS 7.4, 50 µL of AChE, and 100 µL of 10 mM ATChCl were mixed together and an absorbance in 412 nm was measured after incubation lasting 2 min. Activity of AChE was then calculated using extinction coefficient ε = 14,150 L·mol^{-1}·cm^{-1} [30].

2.7. Galantamine Inhibited MPs Activity Restore

Activation of MPs inhibited by galantamine was done using acetate buffer (AC) pH 5.0. MPs were incubated for 5 min and then washed three times with AC pH 5.0 followed by washing by PBS 7.4 three times.

3. Results and Discussion

3.1. Synthesis and Comparison of Magnetic Particles

We have prepared five modifications of superparamagnetic particles to compare their ability to bind AChE. The surface was modified by silane (MAN-34), a combination of silane and APTES (MAN-37-NH_2), and APTES only (MAN-38-1-NH_2). Magnetic particles MAN-161-NH_2 were modified with triamine, whereas MAN-164 were modified with calcium triphosphate only. Synthetized MPs were tested for ability to bound AChE with addition of bridging GA molecule and the enzyme activity was measured by Ellman's assay. MAN 38-1-NH_2 particles were rejected from testing because their size does not allow to form homogenous solution which is necessary for analysis. It was found that surface modification of MAN-164 particles did not allow groups for enzyme binding and simple physical adsorption do not fulfill requirement on repeated using of enzyme on their surface, therefore they were rejected for this purpose as well. Only MAN 34, MAN 37-NH_2, and MAN 161-NH_2 particles allow to AChE binding with/without GA molecules through either absorption in the case of MAN-34 or amino groups present on their surface. The ability of MPs to bind AChE was characterized by enzyme activity related to weight of particles took to analysis in 50 µL (Figure 2). The best results exerted MAN 161-NH_2 particles which were able to bind the highest amount of enzyme in the smallest quantity of MPs, probably due to the high density of amino groups in BAATMS molecule per square of particle. In the case of BAATMS, the secondary amines can non-covalently interact with enzymes. This can lead to a higher affinity. Although, MAN

161-NH_2 particles prepared without GA molecule showed bigger enzyme activity (probably due to the presence of triamine), MAN 161-NH_2 particles prepared with GA were chosen because of higher enzyme activity in a second measuring cycle. It was probably caused by leaching of AChE from MPs prepared without GA molecule. MAN 37-NH_2 and MAN 34 particles were able to bind AChE but did not prove to be appropriate for further measurement for smaller yields of linked enzymes. However, TEOS with APTES molecule (MAN-37-NH_2) seems to exert weaker binding, while only the TEOS molecule (MAN-34) provides a better signal only due to non-specific interaction.

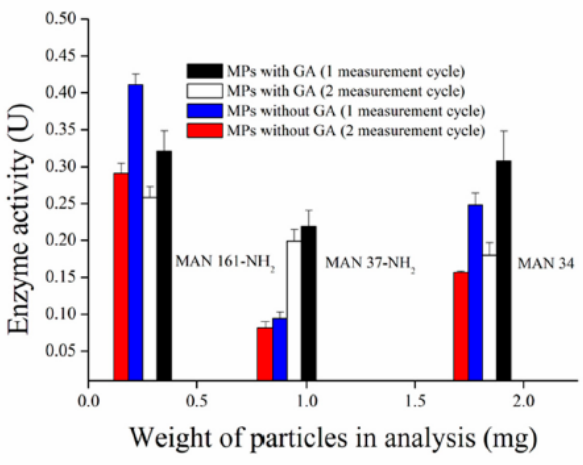

Figure 2. Comparision of binding process of AChE to different magnetic particles measured by Ellman's assay. MPs with GA = magnetic particles prepared with glutaraldehyde, MPs without GA magnetic particles prepared without glutaraldehyde. Error bars indicate standard error of the mean for $n = 3$.

3.2. Substrate Measurement

Saturation curve for AChE and ATChCl as a substrate was performed in concentration range from 0.125 to 4.0 mM with K_M value calculated to 4.56 mM compare to native enzyme, where K_M value was calculated to 99.57 μM (Figures 3 and 4). K_M value seems to be strongly affected by immobilization process as was also reported by Gabrovska et al. where K_M value of immobilized AChE was approximately 3 mM compare to 0.9 mM of free AChE [31] or it could be influenced by external processes like irradiation as was described by Barteri et al. who calculated K_M value to 1.37 mM [32]. A high value of K_M was also provided by human mutant AChE [33,34]. Peaks forming at the potential 427 ± 22 mV were revealed during this assay.

3.3. Inhibitors Measurement

Inhibitors of AChE contain wide range of substances capable reduce its activity, especially drugs used in Alzheimer's disease treatment or pesticides in agriculture can be mentioned as the broadly available [35]. As a model molecules for our measurement, galantamine was chosen as being representative of competitive inhibitor and carbofuran was chosen as the noncompetitive inhibitor. Carbofuran as a carbamate inhibitor needs time to bind to AChE. This time was investigated while five minutes was found to be sufficient for creating this bind. Concentration of substrate for these measurements was chosen to be 1 mM of ATChCl, due to economic reasons, when thiocholine peaks were still big enough for detection. We performed calibration curves in a concentration range from 2.5 to 10 μM of galantamine (Figures 5 and 6) and from 39 to 625 nM of carbofuran (Figures 7 and 8) and method was validated to standard Ellman's assay (Figures 9 and 10). There are no interferences in plasma as potential real sample towards thiocholine measurement. Potential after binding of carbofuran was found to be shifted in 565 ± 20 mV. SWV assay performed only with carbofuran did not reveal any peak in the record, thus this shift is caused by interaction of carbofuran with AChE. Analysis of inhibitors is nowadays performed mainly by chromatography techniques, however,

determination of AChE is a usable tool. We calculated the limit of detection for galantamine to 1.5 μM and 20 nM for carbofuran. Nevertheless, there is a drawback of this method in a relatively narrow linear range for galantamine, resulting in poor detection limit against already published methods [36,37]. On the other hand it is well established that sensitivity is decreased once the enzyme is immobilized and it is also supported by recently-achieved results [5]. Carbofuran detection limit in ppb, using AChE as recognition tool, is considered to be a good result as was previously described in literature [38–40].

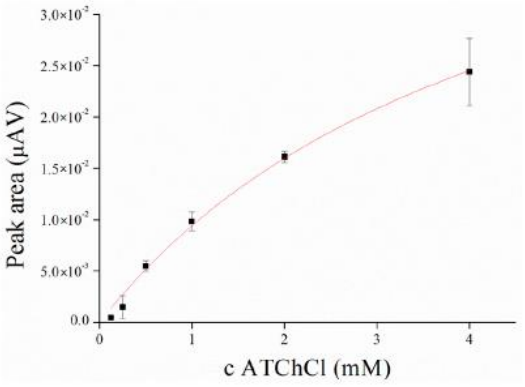

Figure 3. Saturation curve of AChE and acetylthiocholine as a substrate performed in PBS 7.4. Hill funciton with a coefficient of cooperativity n = 1 was used for fitting. Error bars indicate standard deviation for n = 3.

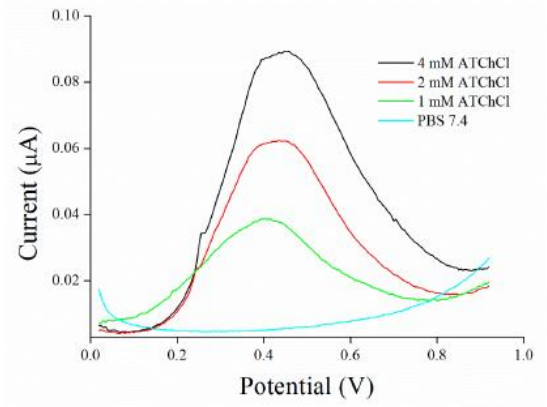

Figure 4. SWV curves of acetylthiocholine performed in PBS 7.4.

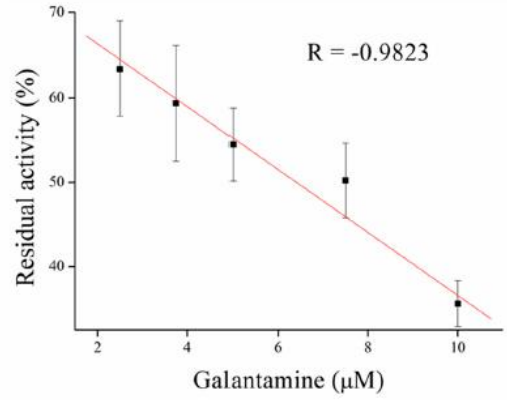

Figure 5. Galantamine calibration curve performed in PBS 7.4. Error bars indicate standard deviation for n = 3.

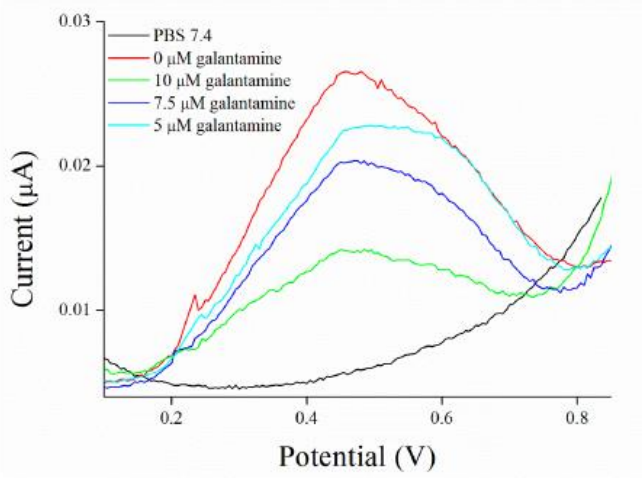

Figure 6. SWV curves for galantamine performed in PBS 7.4.

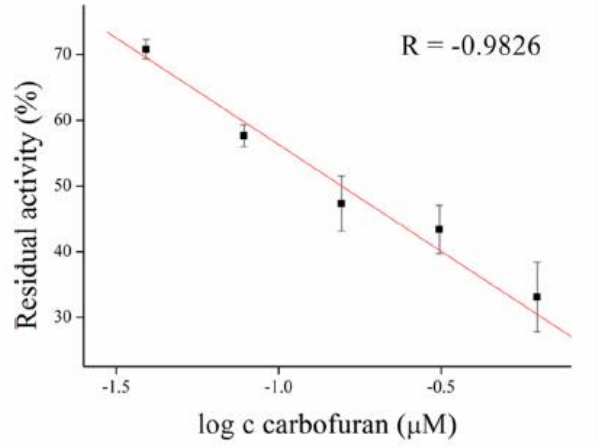

Figure 7. Carbofuran calibration performed in PBS 7.4. Error bars indicate standard deviation for $n = 3$.

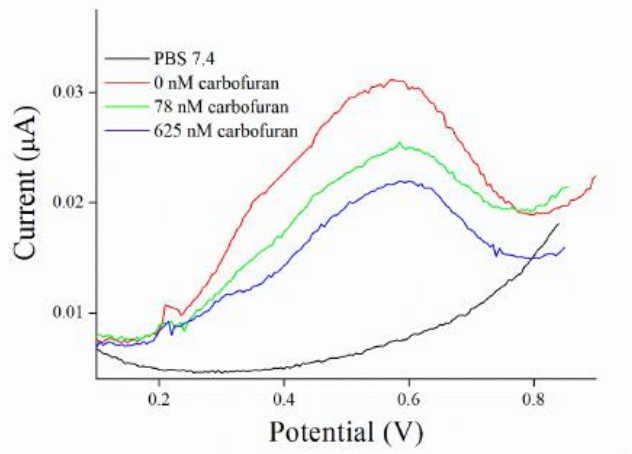

Figure 8. SWV curves of carbofuran performed in PBS 7.4.

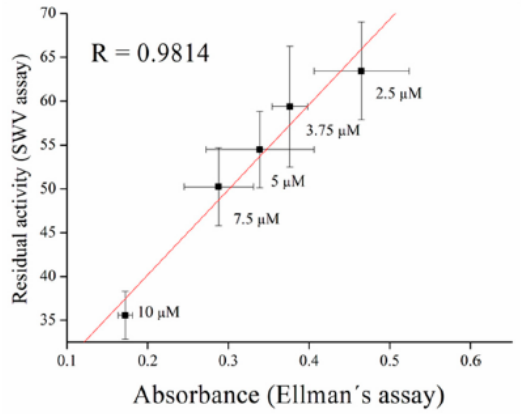

Figure 9. Validation of galantamine measurement compared to standard Ellman's assay. Error bars indicate standard deviation for $n = 3$.

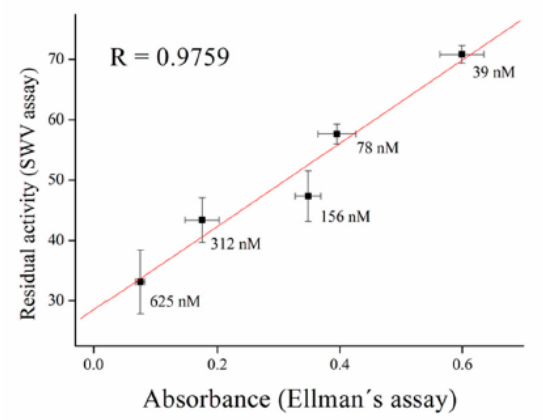

Figure 10. Validation of carbofuran measurement compared to standard Ellman's assay. Error bars indicate standard deviation for $n = 3$.

Galantamine can inhibit enzymes as a competitive inhibitor until it is eliminated from the active site. Therefore, we washed out galantamine from AChE to get original enzyme activity. For this purpose, we tested PBS pH 7.4 and AC pH 5.0. Phosphate buffer appeared to be inapplicable for galantamine elimination when we did not wash out galantamine from the active site, while AC proved to be efficient and the enzyme appeared to be active again. The probable mechanism is in changes of charge of amino acids in the active site of the enzyme in pH 5.0 and disintegration of interaction between AChE and galantamine (Figure 11).

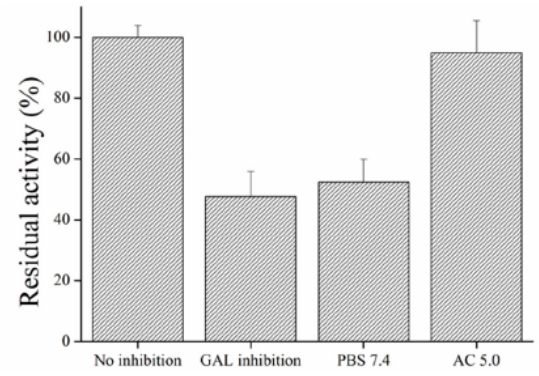

Figure 11. Wash out of galanthamine from galanthamine-inhibited magnetic particles. Error bars indicate standard deviation for $n = 3$.

The mechanism of action of carbamate inhibitors to AChE is caused via blocking Ser residuum in the active site [41]. However, this bond undergoes spontaneous degradation over time or can be potentiated by exogenous substances [42]. Moreover, time of decarbamylation depends on length of side chain of carbamate [43]. Carbofuran adduct with BChE showed half-life about 2 h [44], while we found the half-life of the carbofuran adduct with AChE to be about 3.5 h (Figure 12).

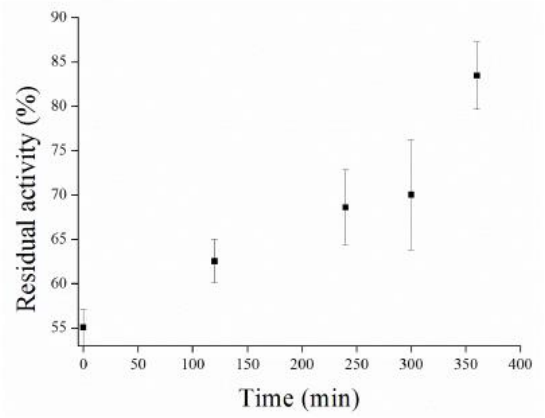

Figure 12. Spontaneous decarbamylation performed in PBS 7.4. Error bars indicate standard deviation for $n = 3$.

3.4. Influence of Organic Solvents

Organic solvents have to be considered as interference in every AChE measurement. Denaturation of enzyme molecules could have critical influence on analysis and thus it is important to keep percentage of solvents on minimum [5]. I-PrOH, EtOH, and DMSO were tested for their inhibition properties on AChE, all in 2.5% concentration. In the quoted work, DMSO was identified as a solvent capable to decrease AChE activity even in very small concentrations [45]. In tested concentrations, we found strong inhibition of AChE. Both alcohols appeared to be potent inhibitors of AChE as described earlier [46] (Figure 13).

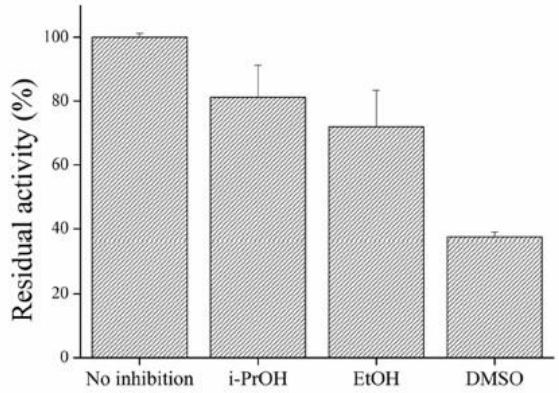

Figure 13. Organic solvents performed in PBS 7.4.

3.5. Repeated Measurement with MPs-AChE

The idea of immobilization of enzymes onto MPs is in the possibility to use it repeatedly. The immobilization process via GA molecules was used before [20]. We discovered this process to be more efficient considering repeatability of measurements compared to our previous results, where immobilization to commercial MPs activated with carboxyl group and EDC reagent was used [4]. There were another immobilization protocols proposed earlier, however they require sophisticated

procedures [19,47] (Figure 14). We claim that thus-modified MPs could create a good platform for automatic analyzers, microfluidics, or other uses where long stability of particles is required.

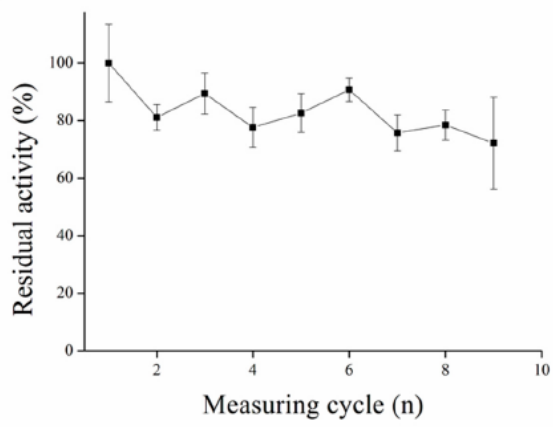

Figure 14. Repeatability of magnetic particles with AChE performed in PBS 7.4. Error bars indicate standard deviation for $n = 3$.

4. Conclusions

Synthetized MPs proved their ability to attach enzymes onto their surface via amino groups and GA molecule interaction. Compared to the immobilization process using commercial MPs and EDC reagents, prepared MPs and the use of GA appeared to be more useful when speaking about repeated measurements. We conclude that thus-prepared MPs could provide a good platform for analysis where long reagent lifetimes are needed—e.g., flow systems and so on. Inhibitors of AChE galantamine and carbofuran were measured with detection limits of 1.5 μM and 20 nM, respectively. Validation of this method was successfully performed by Ellman's assay. Lower pH buffers were used to restore enzyme activity of inhibited particles by galantamine and spontaneous decarboxylation of the AChE-carbofuran adduct was also observed. Every analysis using AChE is influenced by organic solvents and we proved their inhibition potential in this application as well.

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Author Contributions: Adam Kostelnik performed the experiments and wrote the paper; Pavel Kopel synthetized magnetic particles; Miroslav Pohanka participated in manuscript preparation and provided the experimental idea; Alexander Cegan participated in study design and manuscript preparation.

Conflicts of Interest: The authors declare no conflict of interest.

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5.3. Výsledek 3

Adam Kostelník, Alexander Čegan, Miroslav Pohanka, „Color change of phenol red by integrated smart phone camera as a tool for the determination of neurotoxic compounds“, *Sensors*, 16 (9), 1212–1222, 2016 (IF = 2,475)

Imobilizace enzymů do membrán jsou častým řešením a v porovnání s chemickou imobilizací jednodušší a levnější. V minulosti bylo uveřejněno na toto téma nespočet publikací, používají se membrány např. z želatiny, chitosanu, alginátu nebo sol-gel membrány [125-128]. Rozmach technologií udělal z mobilního telefonu každodenního společníka pro většinu světové populace. Ani analytická chemie, biochemie, imunochemie či mikrobiologie nezůstala v jeho aplikaci pozadu. V posledních letech vzniklo mnoho publikací zabývajících se spojením mobilního telefonu a stanovením vybraných analytů [129-135]. Důvodem je jeho snadná obsluha, kterou zvládne i neškolený personál, takže ho lze použít např. v ordinacích lékařů nebo v malých laboratořích, které nedisponují drahým laboratorním vybavením, a jeho velkou předností je přenosnost, což činí tento typ analýzy ideální pro domácí péči.

V této práci byl mobilní telefon použit pro detekci barevné změny fenolové červeně jako indikátoru aktivity AChE při stanovení cholinesterasových inhibitorů. Pro potřeby pořizování fotografií byla metodou 3D tisku připravena tmavá komůrka, která zároveň sloužila pro podepření mobilního telefonu, jenž tak byl během celé analýzy ve stejné poloze. Vyhodnocení získaných fotografií lze provádět několika způsoby (např. odstíny šedé nebo doplňkové barvy v systému CMYK [136]), nicméně zde byl použit systém RGB, který je využíván nejvíce. Obecným problémem imobilizací enzymů je zhoršení katalytických vlastností z důvodu změny struktury, u imobilizace do membrány se přidává problém prostupu substrátu skrze materiál membrány. V této práci ale probíhala difúze substrátu membránou k AChE dostatečně rychle na to, aby se čas analýzy zbytečně neprodlužoval, což je pro biosenzory rozhodujícím faktorem. Pravděpodobně to bylo zajištěno strukturou membrány, protože nebylo použito síťovací činidlo, které by zvětšilo hustotu sítě materiálu membrány, čímž by se prostup substrátu k enzymu zpomalil.

Díky imobilizaci AChE do želatinové membrány na povrchu papírové matrix v kombinaci s fotografickou detekcí je připravený biosenzor rychlou, levnou a přenosnou alternativou pro stanovení cholinesterasových inhibitorů v polních podmínkách.

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Color Change of Phenol Red by Integrated Smart Phone Camera as a Tool for the Determination of Neurotoxic Compounds

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Abstract: The use of a cell phone as a detection system is easy, simple and does not require trained personnel, which is in contrast to standard laboratory instruments. This paper deals with immobilization of acetylcholinesterase (AChE) in a gelatin matrix, and phenol red, as an indicator of AChE activity, is used in order to establish a method that is easily compatible with a camera device. AChE splits acetylcholine into choline and acetic acid, which changes the pH of a medium, resulting in a phenol red color change. The coloration changed in presence of an AChE inhibitor. Measurements were performed on 3D-printed, tube-shaped holder, and digital photography, with subsequent analysis of red-green-blue (RGB), served for assay purposes. Calibration of AChE inhibitors, tacrine and galantamine, was performed, with limit of detection equal to 1.1 nM and 1.28 μM, respectively. Interferences were also measured, resulting in a proof-of-method stability. The method was further successfully validated for the standard Ellman's assay, and verified on murine plasma samples spiked with inhibitors.

Keywords: acetylcholinesterase; phenol red; smart phone; drop assay; colorimetry; inhibitor; biosensor

1. Introduction

In the body, the enzyme acetylcholinesterase (AChE) terminates stimulation in a cholinergic system by hydrolysis of acetylcholine into choline and acetic acid [1]. The activity is typically measured in diagnoses or in analytical chemistry for the determination of inhibitors [2–5]. The most common AChE activity assay is typically based on acetylthiocholine and 5,5′-dithiobis(2-nitro benzoic acid) as a chromogenic reagent, but it can be assayed electrochemically as well [6–8]. The acetic acid produced by the aforementioned reaction can also be employed in an AChE activity assay, and acidification of the medium is detected using an acid-base indicator. Several indicators can be used, for example, brilliant yellow, neutral red, phenol red (PR), and so on [9]. Enzyme immobilization is advantageous for its lower costs and the reuse of enzymes [10]. Different matrixes can be used for enzyme entrapment, such as chitosan, alginate, agarose, agar, or gelatin [11,12]. Gelatin is a natural product made of collagen, which undergoes irreversible gelatinization in temperatures under 40 °C [13]. This material has broad biocompatibility and no reported toxicity [14]. Smart phones are widely-spread in the population, and most of them have integrated cameras with high-resolutions, autofocus, and digital zooms [15]. Several applications of smart phones in analytical chemistry, e.g., immunosensors or blood analysis sensors, have been introduced thus far [16]. Digital photography can be evaluated by disparate models, but the RGB channel intensity model is the most common method [17–19]. In this

work, a biosensor, based on immobilized AChE with colorimetric determination of activity using a camera and phenol red reagent, is proposed for the assay of neurotoxic compounds and is compared to the standard measuring protocol.

2. Experimental Section

2.1. Materials and Instruments

AChE from electric eel, as a lyophilized powder (⩾1000 units/mg protein), acetylcholine chloride (AChCl), acetylthiocholine chloride (ATChCl), 9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate (tacrine), galanthamine hydrobromide, tetraisopropyl pyrophosphoramide (iso-OMPA), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), 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 and gelatin were obtained from PENTA (Prague, Czech Republic). Phenol red (PR) and sodium chloride salt were supplied by ACROS ORGANICS (Thermo Scientific Inc., Waltham, MA, USA). Filter paper (1PS) was obtained from Whatman (Maidstone, UK). Color change was detected using a Sony Xperia MT27i with a 5 Mpx camera and an LED light, using the Android 2.3.7. operating system (device version number 6.0.B.3.184) (Tokyo, Japan). Murine plasma samples were obtained from 20 female BALB/c mice, which were purchased from Velaz (Unetice, Czech Republic). The mice were kept under standard ambient temperature and humidity 50% $\pm$ 10%. Light and dark periods lasted 12 h. The mice were sacrificed at the age of 8 weeks, by cutting of the carotid under carbon dioxide narcosis. Blood was taken into lithium heparin treated tubes (Dialab, Prague, Czech Republic) and centrifuged at 1000$\times$ *g* for 5 min. Fresh plasma was kept at -80 °C until use in the assay. The whole experiment was both permitted and supervised by the ethical committee of the Faculty of Military Health Sciences (Hradec Kralove, Czech Republic).

2.2. Preparation of Gelatin with Immobilized AChE

Gelatin was prepared according the protocol determined by Lourenço et al. [20]. Four-hundred milligrams of powdered gelatin were dissolved in 1 mL of distilled water (60 °C) by stirring for 20 min; then, 2 mL of PBS 7.4 (60 °C) was added and mixture was stirred for another 30 min. After cooling down, the aforementioned mixture was poured into 300 µL of AChE (activity for acetylthiocholine 5.65 $\times$ 10^{-9} mol/s/µL) and 300 µL of water and stirred. Then, 50 µL of the final mixture was spread over Whatman filter paper and dried at laboratory temperature for 2 h. Filter paper was stored in a dark box with a saturated humidity of PBS 7.4 at 4 °C overnight.

2.3. Solutions Preparation

AChCl solutions were prepared in concentration ranges from 10 mM to 0.16 mM in disposable tubes, and the final volume was set to 1 mL. Tacrine solutions were prepared in concentration ranges from 62.5 to 3.91 nM. Each solution was prepared in PBS 7.4, and the final concentration in drops was 5 times less for AChCl, and 4-fold less in the case of tacrine. Concentration of PR in water was set to 5 $\times$ 10^{-4} M. All solutions for the Ellman's assay were prepared in PBS pH 7.4. DTNB solution was prepared in a concentration of 1 mM and ATChCl, 10 mM. Concentration ranges of tacrine were from 39.1 to 625 nM. Final concentrations in cuvette were 10-fold less concentration of ATChCl and 40-fold less in the case of tacrine. Iso-OMPA was prepared in PBS 7.4 at a concentration of 1 mM; final concentration in plasma samples was 0.1 mM.

2.4. Measuring Process

Thirty-five microliters of PBS, 25 µL of PBS 7.4 or tacrine solution, 20 µL of PR, and 20 µL of 5 mM AChCl were consequently added to the surface of paper with immobilized AChE. After 5 min of incubation, the surface of the paper was photographed using a smart phone camera placed on a 3D-printed, tube-shaped holder. The holder was printed using a Prusa i3 (Prusa Research, Prague,

Czech Republic) using acrylonitrile butadiene styrene shaped into 3-mm filaments. The nozzle temperature was set at 285 °C and the bottom plate temperature was 100 °C during the printing procedure. The individual layers deposited on the final object were 0.1-mm thick. The size of the holder was 80 mm in height, 105 mm in length, and the inner diameter of the tube was 40 mm. Organization of the whole device can be seen in Figure 1.

(**a**) (**b**)

Figure 1. Tube-shaped holder (**a**) prepared using 3D-printing technology, and the final settings for photography (**b**).

2.5. Ellman's Assay

Four hundred microliters of DTNB, 25 µL AChE, 25 µL tacrine solution, 450 µL PBS 7.4, and 100 µL of 10 mM ATChCl were added to a standard cuvette. Absorbance was measured at 412 nm immediately and after 5 min of incubation.

2.6. Data Processing

Photography was processed in GIMP 2.8.16 (open source software) using the Color Picker function, and RGB colors were obtained. K_M value for AChE and ACh as substrates were calculated in Origin software (OriginLab, Northampton, MA, USA) using non-linear curve fitting using the Hill function with the coefficient of cooperativity set to number one. In the Origin software, K_M was calculated as the concentration responding to half of the maximal velocity, which was calculated as the upper limit of the curve. Limit of detection for AChE inhibitor tacrine was calculated by linear regression in Origin as a signal to noise ratio equal to three.

3. Results and Discussion

Mobile phones have proven their ability to serve as detection systems in recent years [16,21,22], and represent a cheaper variant for detection [23]. Colorimetric detection for various molecules [24,25], and also measurement of pH, were introduced [26]. A paper platform offers the possibility of use in the construction of paper-based devices for colorimetric detection [27]. Here, we used paper as a platform for a gelatin matrix and phenol red was used for colorimetric detection. First, we selected the color channel that is suitable for the performance method. Color change in RGB was observed in green and blue channels, the red channel was without change (Figure 2). While the both blue and green channels were suitable for the assay, we decided work with the blue channel further because of the greater difference between maximal and minimal color intensity values. On the other hand, the green channel can be chosen, for example, for reference purposes or the function method whenever necessary.

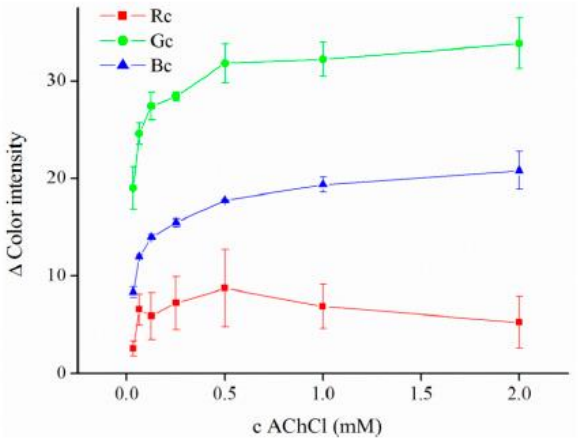

Figure 2. Δ Color intensity for different concentrations of AChCl in all RGB channels. Rc = red channel; Gc = green channel; and Bc = blue channel. Error bars indicate standard error of the mean for $n = 5$.

In neutral pH of PBS 7.4, PR gives a red color. After addition of AChCl, immediately forming acetic acid acidifying medium and the PR turns yellow (Figure 3). We performed a saturation curve for AChE and ACh as a substrate in concentration ranges from 0.031 to 2.00 mM, and the calculated K_M value was equal to 2.9×10^{-4} mM (Figure 4).

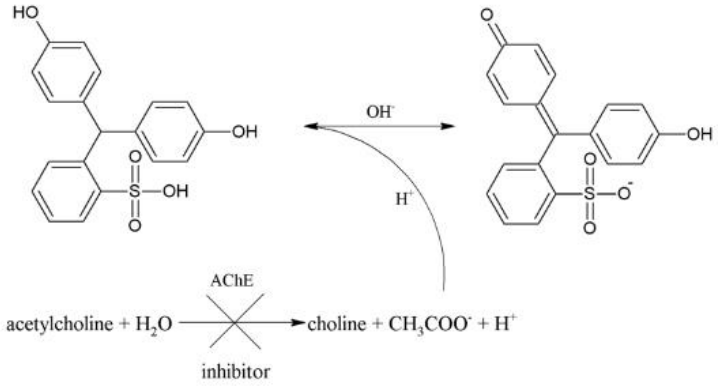

Figure 3. Principle of reaction based on color change of phenol red.

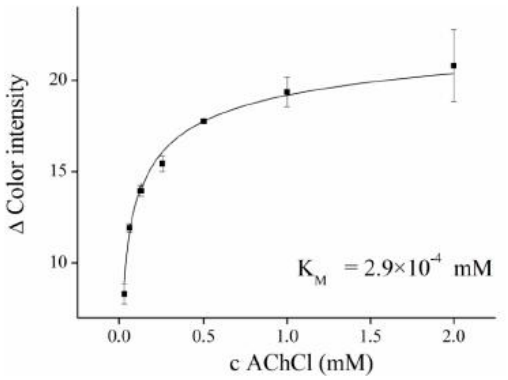

Figure 4. Satuation curve for AChE and acetylcholine as a substrate. The Hill function was used for fitting. Δ Color intensity was observed in the blue channel. Error bars indicate standard error of the mean for $n = 5$.

Gelatin was previously used as a matrix for AChE immobilization in the detection of organophosphates [28] and carbamates [29]. It appears to be a suitable material, which is well penetrable for substrates and inhibitors, but does not affect enzyme activity [30–32]. In our work, we used AChE inhibitor tacrine as a model molecule with a high affinity toward the enzyme. We performed a calibration curve in the concentration ranges from 0.98 to 15.63 nM of tacrine, and limit of detection for entrapped AChE was calculated to be equal to 1.1 nM (Figure 5). Comparable results of 10 nM of tacrine, were achieved by Pohanka and Vlcek [33], who measured color intensity using indoxyl acetate.

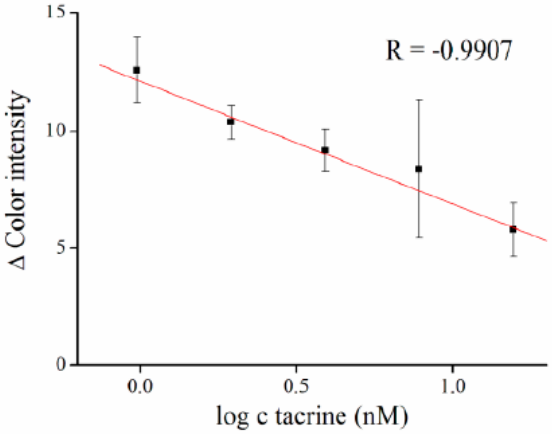

Figure 5. Tacrine calibration curve. Concentration of tacrine is given in logaritmus. Error bars indicate standard error of the mean for $n = 5$.

The method for tacrine determination was validated, compared to Ellman's assay and a correlation coefficient of R = 0.9463 was achieved. These results show that analysis using a smart-phone-integrated camera is viable for assay of AChE inhibitors (Figure 6). The experimental data can be extrapolated using a linear model with a good coefficient of determination. Though both methods were equally suitable for the assay, the camera-based assay had several advantages over the standard spectrophotometry test, such as stability of the enzyme and portability of the assay.

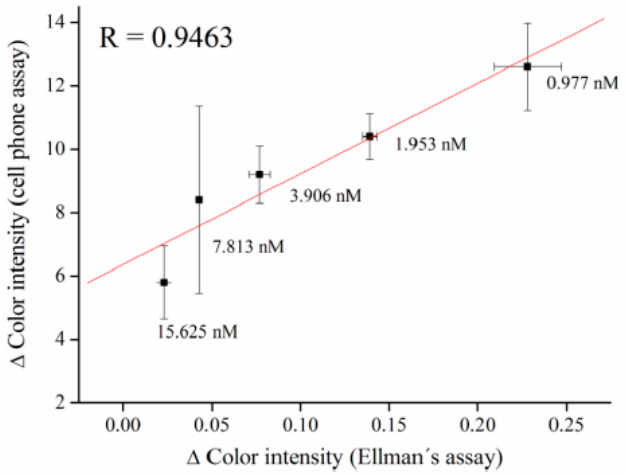

Figure 6. Method validation compared to standard Ellman's assay. The vertical axis represents Δ Color intensity of the cell phone assay and the horizontal axis represents Δ Color intensity of Ellman's assay. Error bars for the cell phone assay indicate standard error of the mean and, for Ellman's assay, a standard deviation for $n = 5$.

For verifying the assay, tacrine was spiked into murine plasma with the same concentrations that were used in the calibration measurement. Butyrylcholinesterase (BChE) naturally occurs in plasma, and because of its affinity towards acetylcholine, inhibition of measurement of AChE activity is required. For this purpose, iso-OMPA, which acts as selective inhibitor of BChE, is used [34]. Results showed feasibility for assay of tacrine with a correlation coefficient of R = 0.9247 (Figure 7).

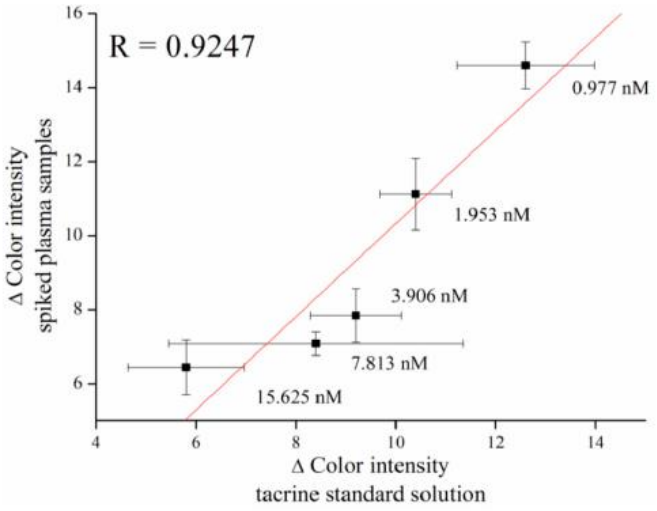

Figure 7. Verifying assay of tacrine in plasma samples compared to a standard tacrine solution. Error bars indicate standard error of the mean for $n = 5$.

Calibration for galantamine inhibitor, an anti-Alzheimer drug, was performed as well. Galantamine was measured in concentration ranges from 6.25 to 200 µM, with a limit of detection equal to 1.28 µM (Figure 8).

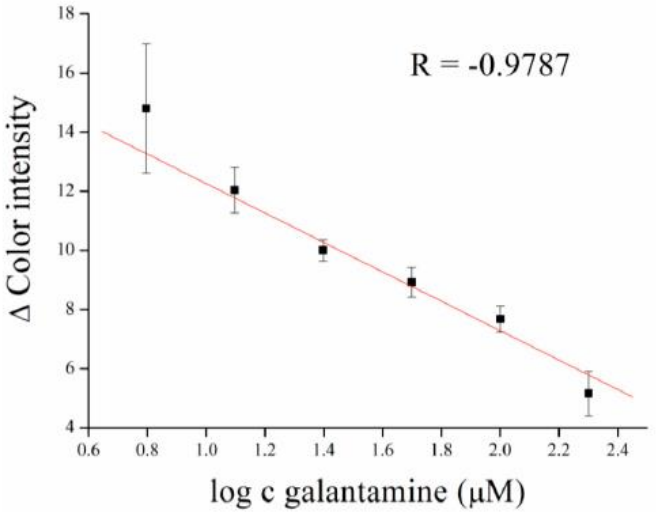

Figure 8. Galantamine calibration curve. Concentration of galantamine is given in logaritmus. Error bars indicate standard error of the mean for $n = 5$.

Verifying of the galantamine assay was performed in murine plasma, as well as tacrine. Galantamine was spiked into plasma samples in appropriate concentrations and a correlation coefficient of R = 0.9941 was achieved (Figure 9).

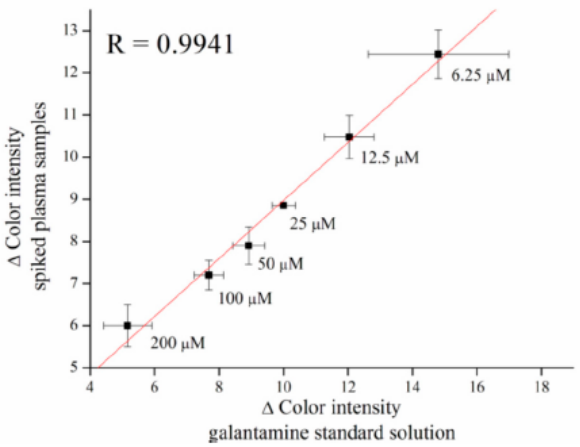

Figure 9. Verifying assay of galantamine in plasma samples compare to standard galantamine solution. Error bars indicate standard error of the mean for $n = 5$.

As was described earlier, the activity of AChE can be reduced using organic solvents, such as methanol, ethanol, and isopropyl alcohol, in small concentration [29]; hence, the organic solvents can be noted as interferents of the AChE-based assay. For the purpose of interference testing, we tested organic solvents DMSO, Tween-20, isopropyl alcohol, and ethanol. All tested solvents were in 5% concentration, except for Tween-20, which was set at 0.25% because of its limited solubility. In the literature, DMSO is known as an inhibitor of AChE, even if present in small concentrations [35], nevertheless it has no effect to the immobilized enzyme. The same conclusion can be made for Tween-20, which also did not inhibit AChE, and this is in compliance with published results [36]. Alcohols are able to inhibit AChE, however, this inhibition occurs in quite high concentrations [29]. We found no interferences toward to AChE activity in the tested concentrations of alcohols. However, isopropyl alcohol caused a decrease of surface tension, which results in a spill of reaction medium (drop) on the paper platform. Ethanol showed the same behavior, but to a lesser extent. This could be used for a tentative resolution of solvent, which was used for the dissolution of AChE inhibitor (Figure 10). We assume that the resistance to organic solvents is caused by the stabilization of the enzyme in the membrane, which protects from denaturation. On the other hand, the phenomenon was not primary aim for our study, and deeper insight should be sought prior to coming to a clear conclusion on this issue.

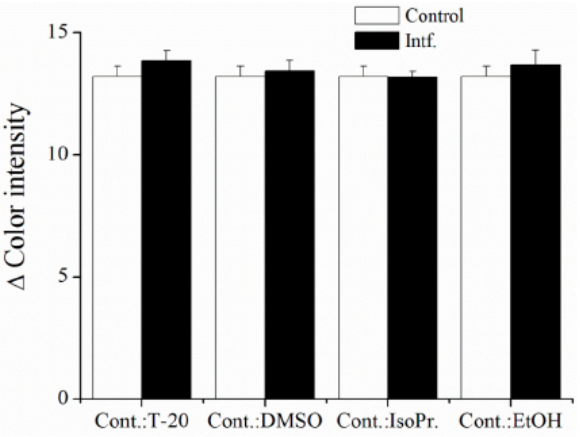

Figure 10. Interferences of organic solvents. T-20 = Tween-20; DMSO = dimethyl sulfoxide; IsoPr. = isopropyl alcohol; EtOH = ethanol; Cont. = control; Intf. = tested organic solvent. Error bars indicate standard error of the mean for $n = 5$.

The presented method is suitable for measurement of AChE inhibitors, and the analysis is quick, has a low cost, and is portable; however, the limit of detection is worse than that of the standard method due to the immobilization of AChE in a gelatin matrix. The fabrication time of the gelatin entrapped enzyme takes around 3 h, but subsequent measurements are quick. A comparison of the presented method with the standard Ellman's assay and literature are summarized in Table 1.

Table 1. Method comparison with standard Ellman's assay and literature.

	LOD Achieved	Fabrication Time	Assay Time	Necessary Equipment	Possibility to Check the Assay by a Naked Eye	Determination of Analyte Exact Concentration
Presented camera based assay	Tacrine: 1.1 nM Galantamine: 1.28 µM	3 h	10 min	None—only smartphone	Yes	Yes
Standard Ellman's assay like here presented	Tacrine: 1.2 pM Galantamine: 18.3 nM	NA	10 min	Spectrophotometer	Yes	Yes
Dipstick assay [37]	Neostigmine, paraoxon: both approx 10^{-7}	Aprox. 1 h	45 min	None	Yes	No
Colorimetric assay [33]	Tacrine: 10 nM	Aprox. 1 h	45 min	None	Yes	No
Flow fluorimetric assay [38]	Galantamine: 0.5 µM	NA	10 min	Fluorimeter, pumps, reaction coil	No	Yes

4. Conclusions

Using a smart phone for detection enables a quick and low-cost analysis without the need for sophisticated laboratory methods and trained personnel. We successfully used this method for the determination of AChE activity entrapped in a gelatin matrix. AChE inhibitors tacrine and galantamine were assayed with limits of detection equal to 1.1 nM and 1.28 µM, respectively. Organic solvents were tested for methods interference, and no interference toward AChE activity was observed, while alcohols caused a spill of drops during analysis. The method was correlated with the Ellman's assay, and results showed that it is usable for the determination of AChE inhibitors. Verifying the assay was performed in murine plasma, and the results showed that the presented method is suitable for measurements in real samples.

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Author Contributions: Adam Kostelnik performed experiments and wrote paper; Miroslav Pohanka participated in paper preparation and the experiment's idea.

Conflicts of Interest: The authors declare no conflict of interest.

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5.4. Výsledek 4

Adam Kostelník, Alexander Čegan, Miroslav Pohanka, „Acetylcholinsterase inhibitors assay using colorimetric pH sensitive strips and image analysis by a smartphone“, *International Journal of Analytical Chemistry,* vol. 2017, 2017 (IF = 1,479)

Druhá práce publikovaná na téma barevné změny indikátoru snímané mobilním telefonem využívala komerční pH citlivé proužky (o jaký indikátor se jedná výrobce neuvádí, ale z rozsahu měřitelného pH a barevného přechodu lze usuzovat na fenolovou červeň). Měření pH je možné považovat za předběžnou zkoušku a většina komerčně dostupných pH papírků trpí na uvolňování indikátoru do roztoku, což není žádoucí, hlavně pokud je potřeba zachovat složení vzorku před samotnou analýzou. Existují však i papírky s chemicky kotveným indikátorem, které tento problém řeší. Navíc lze kotvený indikátor regenerovat a použít ho znovu [137, 138].

V této práci byla AChE adsorbována na povrch pH proužku s kotveným indikátorem a pro větší stabilitu překryta želatinovou membránou, takto připravené proužky byly použity ke stanovení cholinesterasových inhibitorů. Stejně jako v předchozí práci byl z důvodu difuze substrátu membránou potřeba čas k dosažení barevné změny indikátoru. V porovnání s předchozí prací se tento čas lehce zvýšil, protože enzym nebyl přítomen v membráně, ale až pod ní. Na druhou stranu má toto uspořádání výhodu v kompaktnosti, jelikož enzym i indikátor jsou zakotveny na jednom místě, čímž odpadá příprava roztoku indikátoru a jeho pipetování, které je v polních podmínkách často obtížné. Navíc vylepšení uspořádání přineslo redukci problémů s pipetováním roztoků k membráně ve tvaru bubliny použité v předchozí práci (odtrhávání a slévání kapek).

Znatelný rozdíl v připravených biosenzorech z předchozí práce a zde je v citlivosti k organickým rozpouštědlům. Ty mohou s cholinesterasami přijít do kontaktu při analýze pesticidů (jako použitá rozpouštědla) a mají potenciál ovlivnit každé stanovení, proto je potřeba jejich vliv testovat. Zatímco v této práci je AChE organickými rozpouštědly ovlivněna, ta samá rozpouštědla použitá v předchozí práci na AChE vliv nemají. To se dá vysvětlit různým způsobem uspořádání – membrána v předchozí práci poskytovala enzymu, který byl pevně zakotven přímo v ní, větší ochranu než membrána v této práci, kde se organické rozpouštědlo mohlo k enzymu dostat skrze celulosový materiál pH proužku. Vliv organického rozpouštědla lze ovšem odstranit kontrolním měřením, kdy se použije příslušné organické rozpouštědlo bez inhibitoru.

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Research Article

Acetylcholinesterase Inhibitors Assay Using Colorimetric pH Sensitive Strips and Image Analysis by a Smartphone

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Smartphones are widely spread and their usage does not require any trained personnel. Recently, smartphones were successfully used in analytical chemistry as a simple detection tool in some applications. This paper focuses on immobilization of acetylcholinesterase (AChE) onto commercially available pH strips with stabilization in the gelatin membrane. AChE degrades acetylcholine into choline and acetic acid which causes color change of acid-base indicator. Smartphone served as a tool for measurement of indicator color change from red to orange while inhibitors blocked this process. AChE inhibitors were measured with limits of detection, 149 nM and 22.3 nM for galanthamine and donepezil, respectively. Organic solvents were measured for method interferences. Measurement procedure was performed on 3D printed holder and digital photography was evaluated using red-green-blue (RGB) channels. The invented assay was validated to the standard Ellman's test and verified on murine plasma samples spiked with inhibitors. We consider that the assay is fully suitable for practical performance.

1. Introduction

AChE is an enzyme splitting the neurotransmitter acetylcholine in cholinergic synapsis into choline and acetic acid [1]. Sensitivity of AChE to neurotoxic compounds (anti-Alzheimer's drugs, pesticides, and nerve agents) can be use in their measurement [2]. Commonly used method is based on reaction of thiocholine, formed from acetylthiocholine during enzymatic hydrolysis, with Ellman's reagent producing yellow 5-thio-2-nitrobenzoate measurable by spectrophotometry in 412 nm [3]. Despite broad use, this method has some drawbacks like instability of Ellman's reagent and hemoglobin interference [2, 4, 5]. Furthermore, there is possibility of measuring enzyme activity electrochemically [6, 7]. Colorimetric detection can be also based on pH measurement. Many acid-base indicators are known for this purpose, when phenol red was used in our work previously [8, 9]. Different techniques for enzyme immobilization and matrix like gelatin were described as well [10–13]. Simple physical immobilization of enzyme onto cellulose and stabilization into gelatin matrix was successfully used for preparation of biosensor in an application where AChE was embedded into gelatin membrane on a paper matrix and performed for the assay of neurotoxic compounds [14, 15]. In the cited papers, there was no, however, made evaluation of enzyme activity by a camera because a naked eye assay and another type of substrate were preferred. Gelatin provides good properties for enzyme immobilization combined with biocompatibility and zero toxicity [16]. Modern mobile phones dispose high resolution cameras which gives them ability to serve as a tool for diagnostics [17, 18]. Previously mobile phones have been used for some applications in analytical chemistry [19, 20]. This paper deals with preparation of biosensor based on commercial pH strips with immobilized AChE and stabilized in gelatin membrane. Performance of the biosensor was verified on neurotoxic compounds. This approach offers easy way for AChE inhibitors determination, especially if we considered well-established spectrophotometric assay; for the presented method here, no special equipment or trained personnel are required. There is also innovation in

evaluation of color based reaction compared to previously described methods. The major advantage of our assay is based on the opportunity to link it to a smartphone which is considered as the detector device providing wide availability to less equipped laboratories and for field tests without any expensive gear.

2. Material and Methods

2.1. Materials and Devices. Acetylcholinesterase from electric eel (≥1000 units/mg protein), acetylcholine chloride (AChCl), acetylthiocholine chloride (ATChCl), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), donepezil hydrochloride monohydrate, galanthamine hydrobromide, tetraisopropyl pyrophosphoramide (iso-OMPA), phosphate buffer saline (PBS) pH 7.4, dimethyl sulfoxide (DMSO), and isopropyl alcohol were purchased from Sigma-Aldrich (St. Louis, MO, USA); denatured ethanol and gelatin were supplied by PENTA (Prague, Czech Republic). Indicator strips pH-Fix 6.0–7.7 were obtained from Macherey-Nagel (Düren, Germany). Color change was detected by Sony Xperia MT27i with 5 Mpx camera and LED light using operation system Android 2.3.7., device version number 6.0.B.3.184 (Tokyo, Japan). For 3D print, 3D printer Prusa i3 from Prusa Research (Prague, Czech Republic) was used. Murine plasma samples were obtained from 20 female BALB/c mice which were purchased from Velaz (Unetice, Czech Republic). The mice were kept under standard ambient temperature and humidity 50 ± 10%. Light and dark periods lasted equally for 12 hours each. The mice were sacrificed in the age of 8 weeks by cutting of carotid under carbon dioxide narcosis and the blood was taken into tubes pretreated with lithium heparin (Dialab, Prague, Czech Republic) and centrifuged at 1,000 ×g for 5 minutes. Fresh plasma was kept at −80°C until use in the assay. The whole experiment was both permitted and supervised by ethical committee Faculty of Military Health Sciences (Hradec Kralove, Czech Republic).

2.2. Solutions Preparation. AChCl solutions were prepared in concentration range from 0.31 to 10 mM and placed in microtubes. Galanthamine solutions were prepared in concentration range from 1.6 to 25.00 μM. Donepezil solutions were prepared in concentration range from 0.31 to 5.00 μM. Each solution was prepared in PBS 7.4 and final concentration in microtube was 10 times lower. Gelatin was prepared in 1% concentration by stirring of 10 mg of gelatin in 1 ml of water for 20 min. All solutions for Ellman's assay were prepared in PBS 7.4. DTNB solution was prepared in concentration 1 mM and ATChCl in 10 mM. Concentration range of galanthamine was from 62.5 to 100 μM and 13 to 20 μM in case of donepezil. Final concentrations in cuvette were 10-fold less concentration of ATChCl and 40-fold less in case of galanthamine or donepezil. Iso-OMPA was prepared in PBS 7.4 in concentration 1 mM; final concentration in plasma samples was 0.1 mM.

2.3. Preparation of pH Strips with AChE. To pH strips 10 μl of AChE (activity for acetylthiocholine 1.73×10^{-8} mol/s/μl) was added and let to dry in laboratory temperature. Then pH strips were covered by 10 μl of 1% gelatin. After drying in laboratory temperature pH strips were stored in 4°C until used in the assay.

2.4. Preparation of 3D Printed Holder. Holder was created in Autodesk 123D Design (open source software). 3D printer setup was as follows: acrylonitrile butadiene styrene shaped in 3 mm filaments was used as material, nozzle temperature was at 285°C and bottom temperature at 100°C, and individual deposited layers were 0.1 mm thick. Size of holder was 80 mm in height and 105 mm in length and inner diameter of tube was set to 40 mm (Figure 1).

2.5. Smartphone Assay. The smartphone assay was made in the following way: 450 μl of PBS pH 7.4 and 50 μl of 10 mM AChCl solution were added to microtubes and strip was put into it. After incubation of 15 min, excess of reaction medium was drained and picture of the still wet pH strip was taken. During photographing, the camera was placed on the 3D printed holder and the strip inside; hence, no outer light influenced the photography and integrated LED light was the only one source. Distance between the strip and camera was also constant just due to the holder.

2.6. Ellman's Assay. To standard cuvette 400 μl of DNTB solution, 25 μl of AChE, 475 μl of PBS, and 100 μl of ATChCl were added. Absorbance was measured in 412 nm immediately and after incubation of 2 min.

2.7. Data Processing. RGB color values were obtained by processing of photography in GIMP 2.8.16 (open source software) using Color Picker function. ΔColor intensity was obtained as follows: intensity of strip before reaction − intensity of strip after the reaction. This difference corresponds to AChE activity in different concentration of used substrate or inhibitor. K_M value for AChE and AChCl substrate was calculated using nonlinear curve fitting by Hill function with coefficient of cooperativity $n = 1$. Limit of detection was calculated as signal to noise ratio equal to three. For these purposes, Origin software 8 PRO (OriginLab, Northampton, MA, USA) was used.

3. Results and Discussion

AChE splitting acetylcholine into choline and acetic acid resulted in decreasing pH of medium. Our method is based on color change of indicator in pH sensitive zone of pH strip from red to orange (Figure 2). During photography processing color change was observed in green channel while red and blue ones were without change.

3.1. Gelatin Optimization. Amount of gelatin was tested in 0%, 0.001%, 0.01%, 0.1%, 1%, and 10% concentration. The strips were covered with 10 μl of gelatin in tested concentration and dipped into PBS 7.4 and color change of strip into red was observed after 5 min. Gelatin in 10% concentration did not allow us to enter buffer to pH sensitive zone and color did not change into red one while it stayed orange which was represented by decreasing of color intensity (Figure 3). Color

(a) (b)

FIGURE 1: Tube shaped holder printed by 3D print technology (a) and the holder with a smartphone adjusted on the hole to provide photographs by an integrated camera.

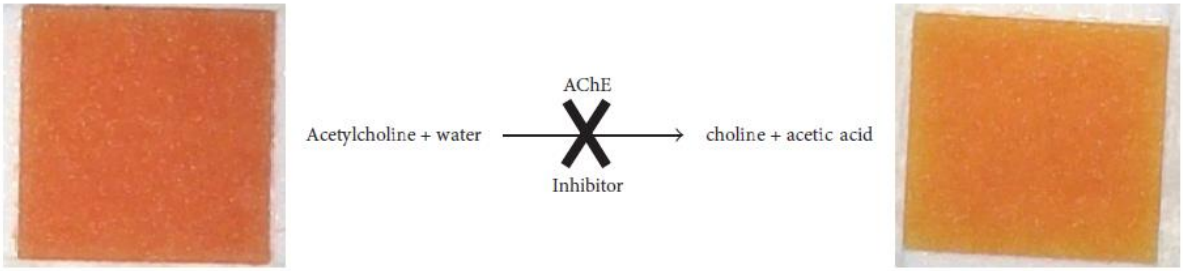

FIGURE 2: pH sensitive method using AChE principle.

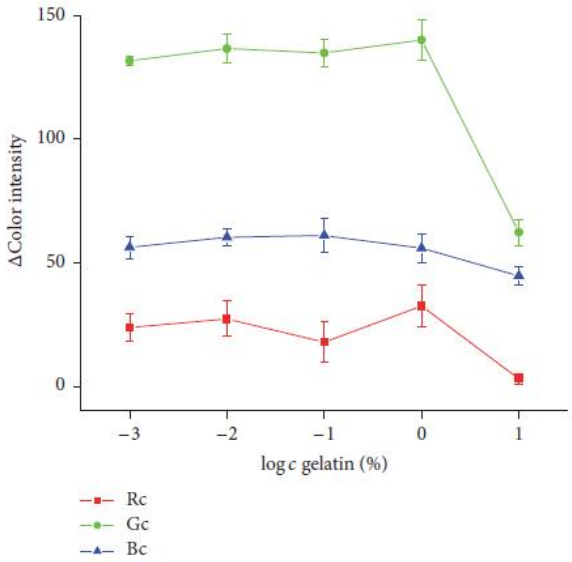

FIGURE 3: Gelatin optimization. Drop in ΔColor intensity indicates that pH strip did not change into red color. Error bars represent standard error of the mean for $n = 3$. Rc = red channel, Gc = green channel, and Bc = blue channel.

change into red was held while using gelatin in 1% and less concentration, but for further measurement 1% gelatin was chosen just for the better stabilization effect for AChE.

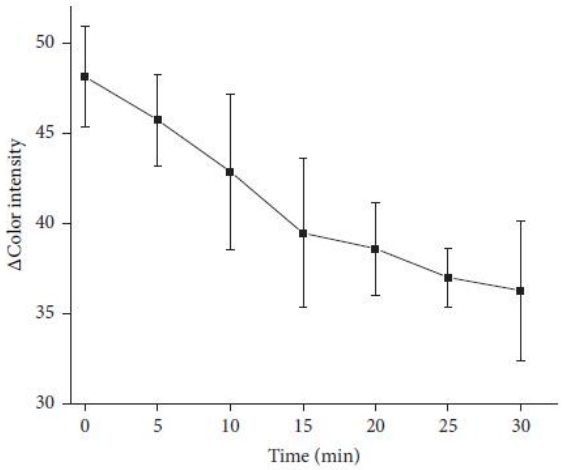

FIGURE 4: Optimization of pH strip incubation time with ATChCl as a substrate. Error bars represent standard error of the mean for $n = 3$.

3.2. Time Optimization. Strip with AChE and 1% gelatin was incubated with 1 mM AChCl. Incubation time with AChCl was observed in 5 min intervals from 0 to 30 min. The biggest color change was held up to 15 min. Over this time color change was not significant for longer incubation time (Figure 4).

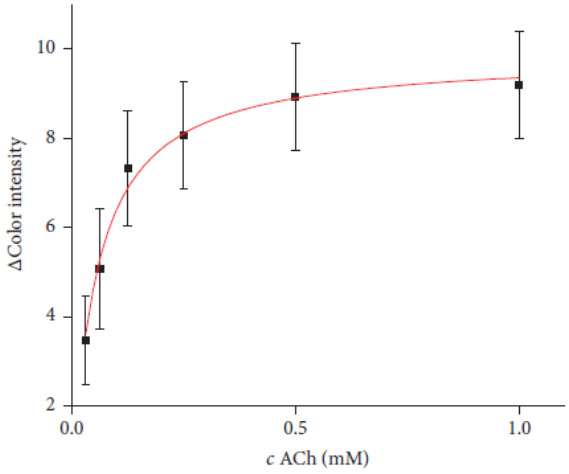

FIGURE 5: Saturation curve for AChE and AChCl as a substrate. Error bars represent standard error of the mean for $n = 3$.

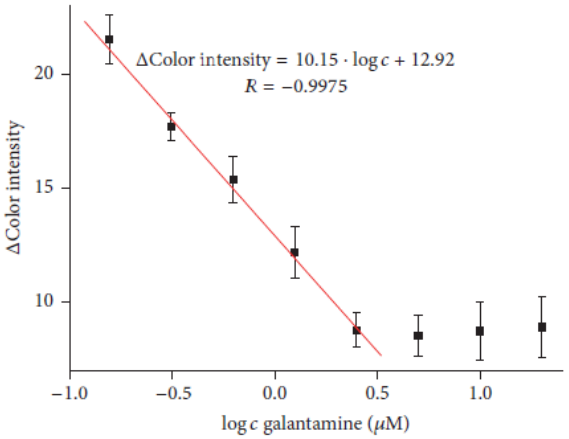

FIGURE 6: Calibration curve of galanthamine; concentration is given in logarithm. Error bars represent standard error of the mean for $n = 3$.

3.3. Substrate Measurement. Saturation curve for AChE and AChCl as a substrate was measured in concentration range from 0.031 to 1.0 mM and K_M value was calculated as described above to 54.26 μM (Figure 5) while 73.9 μM was reported by Xu and coworkers [24]. K_M value however depends on type of used buffer as proved by Wille et al. using human AChE isolated from erythrocytes and achieved K_M equal to 71.4 μM in MOPS buffer, 98.2 μM in PBS buffer, 0.1 mM in Tyrode buffer, and 0.122 mM in Tris buffer when pH was set to 7.4 [25]. There are also big differences between organisms as shown by Shaonan et al. who worked with AChE isolated from fish species and found out values above 0.1 mM [26] and by Jiang et al. who reported K_M to be 63.85 μM when measured with mosquito AChE [27]. Recombinant enzymes exhibit slightly higher K_M values compared to wild types as showed in experiments with mice AChE carried out by Boyd et al. who found out K_M to be 46 μM in wild type and 58 μM for recombinant AChE [28].

3.4. Inhibitors Measurement. AChE is sensitive to neurotoxic compounds like drugs, nerve agents, or pesticides. Some of these compounds are widely used in treatment of Alzheimer disease [29]. Galantamine and donepezil can be examples for the currently available drugs [30]. We performed calibration curve of galanthamine in concentration range from 0.156 to 2.5 μM with limit of detection calculated to 149 nM and quantification limit of 0.5 μM was achieved (Figure 6). Linearity of the assay is limited to 2.5 μM when higher concentrations appeared to be indistinguishable. Comparing to the standard Ellman's assay, it is only 10 times higher detection limit, when 18.3 nM of galanthamine was achieved in our experiment. From previously published methods for galanthamine measurement based on AChE inhibition we can conclude that our method is competitive [21, 22]. Although detection limits are similar, there is advantage in fabrication time of pH strip with immobilized AChE which is not time consuming.

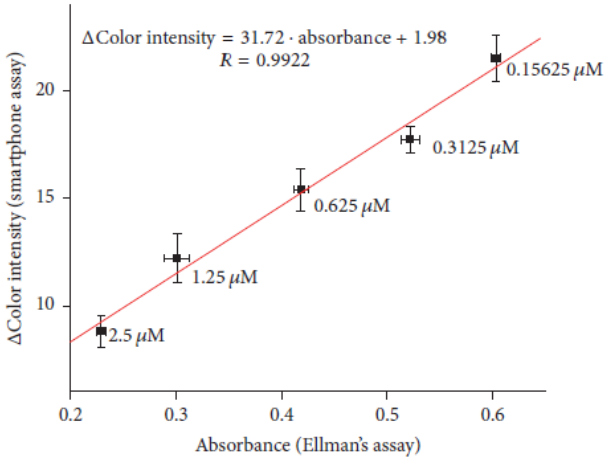

FIGURE 7: Validation of galanthamine assay compared to standard Ellman's assay. Error bars for smartphone assay represent standard error of the mean and for Ellman's assay standard deviation for $n = 3$.

Validation of method for galanthamine measurement was done using standard Ellman's assay with correlation coefficient of 0.9922 (Figure 7).

Method was verified using murine plasma. Plasma samples were pretreated by iso-OMPA, selective inhibitor of butyrylcholinesterase [31], which naturally occurs in plasma and has ability to split acetylcholine. Then galanthamine was spiked into plasma samples in appropriate concentrations and smartphone assay has been performed (Figure 8). For measurement with plasma PBS buffer had to be replaced by water, because plasma strongly buffered itself; then PBS buffer was not required.

In literature, there are plenty of references about determination of donepezil by chromatography techniques [32, 33]. Likewise, there are electrochemical methods for donepezil measurement with similar detection limits as our method [23,

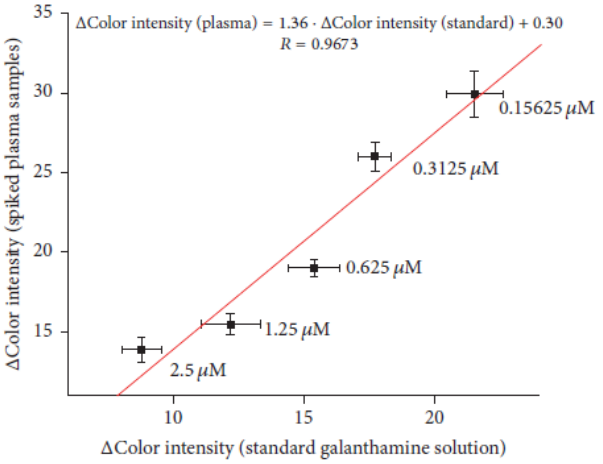

FIGURE 8: Verifying of galanthamine assay in plasma samples compared to standard galanthamine solution. Error bars represent standard error of the mean for $n = 3$.

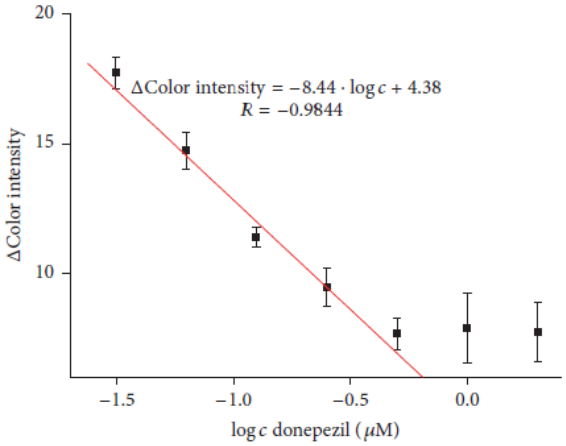

FIGURE 9: Calibration curve of donepezil; concentration is given in logarithm. Error bars represent standard error of the mean for $n = 3$.

34] and some spectrophotometric methods for quantification of donepezil in pharmaceuticals or human plasma [35, 36]. However, no evidence about determination of donepezil via inhibition of AChE was found. Donepezil calibration curve was done in concentration range from 0.031 to 0.50 μM and limit of detection equal to 22.3 nM was achieved while limit of quantification was found to be 0.2 μM (Figure 9). Also, above calibration range there is limited linearity. For comparison, detection limit achieved by Ellman's assay in this experiment was equal to 3.82 nM.

Validation of method for donepezil measurement was performed using standard Ellman's assay with correlation coefficient equal to 0.9895 (Figure 10).

Verifying of donepezil in plasma samples was done as well. Donepezil was spiked in appropriate concentrations into pretreated plasma samples and smartphone assay was

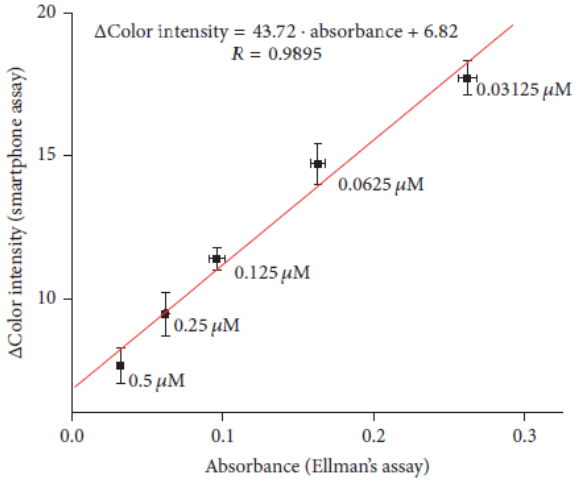

FIGURE 10: Validation of donepezil assay compared to standard Ellman's assay. Error bars for smartphone assay represent standard error of the mean and for Ellman's assay standard deviation for $n = 3$.

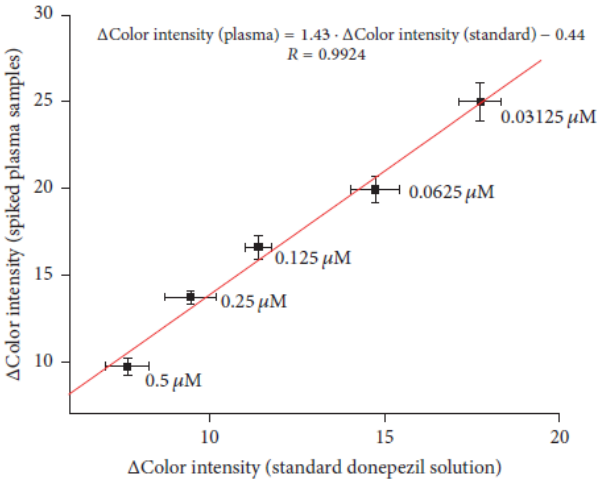

FIGURE 11: Verifying of donepezil assay in plasma samples compared to standard donepezil solution. Error bars represent standard error of the mean for $n = 3$.

performed (Figure 11). PBS buffer was also replaced by water for reason mentioned in the principle based on pH change where a strong buffering could interfere. It appears that both measurements are in a good correlation.

3.5. Organic Solvents. Because AChE activity can be reduced by organic solvents in small concentration [37], we decided to consider them as possible interferents in the AChE based assay. For this purpose ethanol, isopropyl alcohol, and dimethyl sulfoxide were tested, all in 5% concentration. Influence of DMSO to AChE activity has been investigated before and results showed that DMSO slightly decreases enzyme activity which was confirmed in our work [38].

Table 1: Comparison of presented assay with Ellman's assay and literature.

	Detection limit	Fabrication time	Assay time	Necessary equipment
Presented smartphone assay	Galanthamine: 149.1 nM Donepezil: 22.3 nM	Aprox. 1 hour	15 min	Smartphone
Standard Ellman's assay	Galanthamine: 18.3 nM Donepezil: 3.82 nM	NA	10 min	Spectrophotometer
Spectrophotometric assay [21]	Galanthamine: 0.05 nM	Aprox. 3.5 hours	Aprox. 20 mn	Spectrophotometer
Potentiometric assay [22]	Galanthamine: 5.4 nM	Aprox. 10 hours	Aprox. 10 min	Electrodes, potentiometer
Square-wave voltammetry [23]	Donepezil: 151 nM	NA	Aprox. 20 min	Electrodes, electrochemical analyser

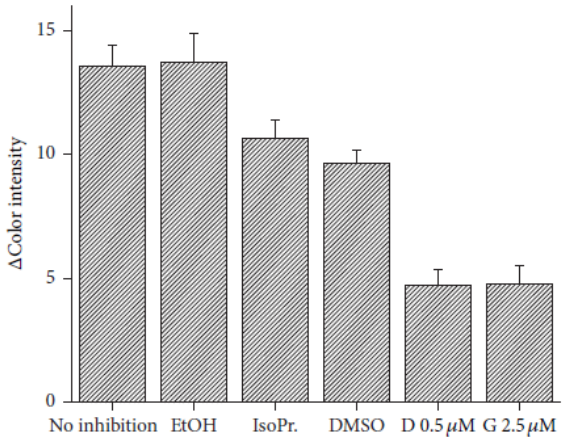

Figure 12: Method interferences by organic solvents. EtOH = ethanol, IsoPr. = isopropyl alcohol, DMSO = dimethyl sulfoxide, D 0.5 μM = donepezil in 0.5 μM concentration, and G 2.5 μM = galanthamine in 2.5 μM concentration. Error bars represent standard error of the mean for $n = 3$.

Ethanol has been reported to decrease AChE activity [37, 39]; however, there is evidence that at low concentration it can enhance enzyme activity [38, 40]. No activity change against uninhibited enzyme was observed in our work. Isopropyl alcohol as well as ethanol can drop AChE activity [41] which is in agreement with our results. To compare used inhibitors, donepezil and galanthamine are included in graph (Figure 12).

4. Conclusion

Here, the presented method proved its ability for determination of neurotoxic compounds with promising limits of detection for galanthamine and donepezil, 149 nM and 22.3 nM, respectively. Assay was successfully validated to the standard Ellman's spectrophotometric test and showed feasibility of measurement in plasma samples. Additionally, no specialized equipment and trained personnel are required; combined with low cost, portability, easy preparation, and miniaturization are considered as big advantages of the here invented method. Comparison of presented method with standard Ellman's assay and literature is given in Table 1.

Competing Interests

The authors declare that there is no conflict of interests with regard to the publication of this paper.

Acknowledgments

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5.5. Výsledek 5

Adam Kostelník, Miroslav Pohanka, „Superficially bound acetylcholinesterase based on a chitosan matrix for neurotoxic compound assay by a photographic technique“, *Analytical Letters*, 51 (10), 1622–1632, 2018 (IF = 1,206)

Navzdory popularitě imobilizací AChE do membrán různých materiálů je imobilizace na povrch membrány další možností imobilizace, ačkoliv se publikované práce zabývají spíše imobilizací na komposity než na samotnou membránu [139-142]. Velmi populárním materiálem pro povrchovou imobilizaci je derivát přírodního polymeru chitinu – chitosan, který nabízí na svém povrchu aminové skupiny, jež jsou schopny vazby enzymu bez nebo po předchozí aktivaci. Navíc díky své biokompatibilitě tvoří ideální prostředí pro enzymovou imobilizaci [143, 144].

V této práci byla AChE imobilizována na povrch chitosanu aktivovaného N-(3-dimethylaminopropyl)-N´-ethylkarbodiimid hydrochloridem pro stanovení cholinesterasových inhibitorů fotografickou detekcí. Výhodou imobilizace AChE na povrch chitosanové membrány je v přístupu substrátu k aktivnímu centru enzymu. Zatímco v případě imobilizace do membrány musí substrát projít materiálem membrány, v případě povrchové imobilizace je enzym pro substrát volně přístupný a čas analýzy tak není ovlivněn rychlostí difuze substrátu membránou. V této práci byl jako substrát použit IA, který je AChE rozkládán na indoxyl, ten se následně spontánně oxiduje na modré indigo. Výhodou použití IA proti fenolové červeni použité v předchozích pracích je v barevném přechodu. Zatímco fenolová červeň v analýze přechází z červené do žluté, barevná změna z bílé do modré je lépe kontrolovatelná i pouhým okem a hodí se tak pro semikvantitativní vyhodnocení v případech, kdy není použito mobilního telefonu. Nevýhodu použití IA jako substrátu je malá afinita AChE k němu. Z toho důvodu musela být použita vysoká koncentrace IA a prodloužen čas analýzy, aby byla zajištěna odečitatelnost výsledků.

Stejně jako v předchozích pracích byla testována citlivost imobilizované AChE k organickým rozpouštědlům. Dle očekávání byla aktivita AChE použitými rozpouštědly snížena, jelikož byla imobilizována vně membrány, nicméně ne výrazněji než v předchozích případech, což dobře demonstruje stabilizační vliv imobilizace v případě, kdy je enzym přímo v kontaktu s rozpouštědlem.

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Superficially Bound Acetylcholinesterase Based on a Chitosan Matrix for Neurotoxic Compound Assay by a Photographic Technique

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ABSTRACT

Smartphones have become popular in the last decade and they have been used in analytical chemistry as easy detection systems with comparable parameters to standard laboratory equipment. This work focuses on the attachment of enzyme acetylcholinesterase on the previously activated chitosan. Acetylcholine splits the neurotransmitter acetylcholine, as a natural substrate, into choline and acetic acid. However, this compound can cleave alternative substrates, e.g., indoxyl acetate, which was used in this work. The conversion of the substrate is blocked or slowed down by inhibitors from which galantamine and tacrine were tested as model inhibitors with detection limits of 1.1 and 0.18 µM, respectively. The measurement procedure was performed on a three-dimensional printed holder and red–green–blue channels were used for digital photography evaluation. The method was validated using a standard Ellman's spectrophotometric assay. We successfully attached acetylcholinesterase on the chitosan surface that was used for the inhibitor assay. The long-term stability of the immobilized enzyme as well as the sensitivity to organic solvents were also tested. The proposed method appeared to be suitable for the rapid and inexpensive assay of neurotoxic compounds.

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Acetylcholinesterase; chitosan; colorimetry; immobilization; indoxylacetate; inhibitor

Introduction

Neurotransmission in cholinergic system is terminated by enzyme acetylcholinesterase that cleaves neurotransmitter acetylcholine into choline and acetic acid which prevents the stimulation of acetylcholine receptors (Soreq and Seidman 2001). It is well documented that the enzyme is sensitive to broad spectrum of molecules like organophosphorus and carbamate pesticides (e.g., carbofuran, parathion, malathion), warfare agents (e.g., sarin, soman, VX), anti-Alzheimer drugs (e.g., donepezil, galantamine, rivastigmine), and toxins (Pohanka 2011; Pohanka 2012b, 2013b). The activity of acetylcholine is assayed mostly by Ellman's test using acetylthiocholine as a substrate (Ellman et al. 1961), but there have been reported methods using alternative substrates as well (Guilbault and Kramer 1965; Rhee et al. 2003).

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The most common method for enzyme immobilization is based on entrapment into membrane from various matrixes, e.g., gelatin, alginate, and so on. These membranes are constructed by the sol–gel technique and create a physical barrier for substrate to the enzyme. Numerous assays based on this procedure have been published so far (Mogharabi et al. 2012; Kostelnik, Cegan, and Pohanka 2016a; Martinkova and Pohanka 2016). On the other hand, there is the possibility to use groups on the surface of the rigid membrane. This require activation by glutaraldehyde or imides like *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, and the enzyme is chemically attached to the membrane. Thus, the assay is not limited by the diffusion of molecules through the membrane barrier. Chitosan is one of these compounds, as it has hydroxy as well as amino groups on its surface, and thus it is a good candidate for covalent immobilization of a protein on the surface (Tanriseven and Ölçer 2008; Singh et al. 2011; Cao et al. 2015).

Smartphones became popular and they are a huge commercial success in the population in recent years. They have been utilized in many applications in analytical chemistry, where they are appreciated as low cost and easy-to-use devices without special training requirements (Shen, Hagen, and Papautsky 2012; Pohanka 2015; Kostelnik, Cegan, and Pohanka 2017).

In this work, we modified a paper platform by chitosan and attached acetylcholinesterase on its surface with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride. The enzyme activity was assayed using indoxyl acetate as an alternative acetylcholinesterase substrate and a smartphone as detection system. We infer that the proposed immobilization procedure in combination with the photographic detection provides a reliable tool for the determination of neurotoxic compounds and would be introduced as a widely available and cheap approach with properties comparable to more elaborative methods.

Materials and methods

Materials and devices

Acetylcholinesterase (electric ell, activity ≥1000 U/mg of protein), chitosan from shrimp shells, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, indoxyl acetate, acetylthiocholine chloride, 5,5′-dithiobis(2-nitrobenzoic) acid, phosphate buffered saline pH 7.4, galantamine, tacrine, 2-propanol and dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol was supplied from Penta (Prague, Czech Republic). Filter papers type 1PS were purchased from Whatman (Maidstone, UK). The color change was observed using Sony Xperia MT27i (device version number 6.0. B.3.184; Tokyo, Japan) with 5 Mpx camera and a light-emitting diode light. They both operated under Android system 2.3.7. A three-dimensional printer Prusa i3 (Prusa Research, Prague, Czech Republic) was also used.

Preparation of three-dimensional printed holder

The holder was in silico proposed in Autodesk 123D Design (open software). The size of the holder was 80 mm in height, 105 mm in length and 40 mm inner diameter of the tube. The printing was made using the three-dimensional printer Prusa i3. The setup for the three-dimensional printer was: acrylonitrile–butadiene–styrene as the material, nozzle

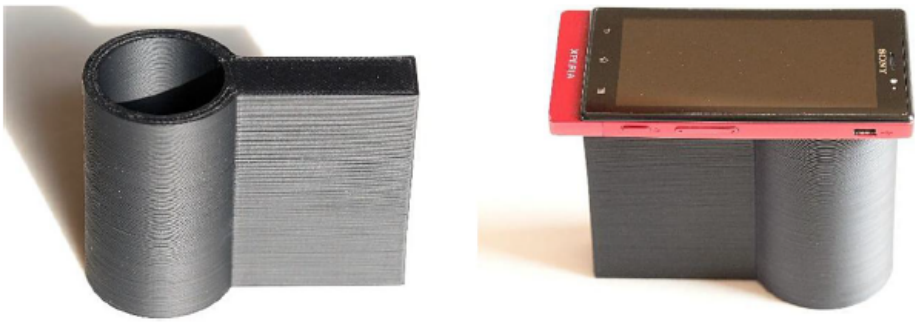

Figure 1. Three-dimensional printed holder (left) and final setting for photography (right).

temperature of 285°C, bottom temperature of 100°C and individual deposit layer of 0.1 mm (Figure 1).

Preparation of filter paper with acetylcholinesterase immobilized on the chitosan surface

Ten milligrams of chitosan were dissolved in 1 ml of acetic acid (0.2 M) with stirring for 30 min. Filter paper was cut into $10 \times 30\ mm^2$ strips. In total, 10 µl of chitosan solution were pipetted on one end and dried under standard ambient laboratory temperature and pressure and artificial light. Another 10 µl were added on the same place and dried overnight. On the dried chitosan spot, 10 µl of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (5 g/ L) in phosphate buffered saline were added and washed with 3 ml of phosphate buffered saline after 1 h of incubation. The material was dried and 10 µl of acetylcholinesterase (activity for acetylthiocholine was set to 28 U) was added for another 1 h and again washed with a mild stream of phosphate-buffered saline with a total volume of 3 ml. The prepared filter paper was used immediately in the assay or stored at 4°C.

Smartphone assay

Before the assay, 40 µl of indoxyl acetate solution in ethanol (100 mM) were added on the second end of the cut of paper with dried chitosan and acetylcholinesterase and ethanol were allowed to evaporate. The paper with indoxyl acetate should be added freshly before smartphone assay, otherwise it would transform into a pink degradation product and the assay was inefficiently performed. The assay was started by the addition of 40 µl of phosphate buffered saline or an inhibitor solution and both ends were folded and pressed together for 1 h. The liquid between both ends was not squeezed out, otherwise it would dry and both ends would stick together which would debase the whole assay. After the reaction, the filter paper was unfolded, excess medium was gently drained out, and a picture of paper was taken by the smartphone camera located on the three-dimensional printed holder. The holder purposely excluded external light and the internal light was on. This setting improved the reproducibility of the assay and also limited the impact of white balance in the camera because half of the photography was created by the black holder and the other half by the filter paper. The assay is based on the conversion of indoxyl acetate by acetylcholinesterase into acetic acid and indoxyl, which then undergoes

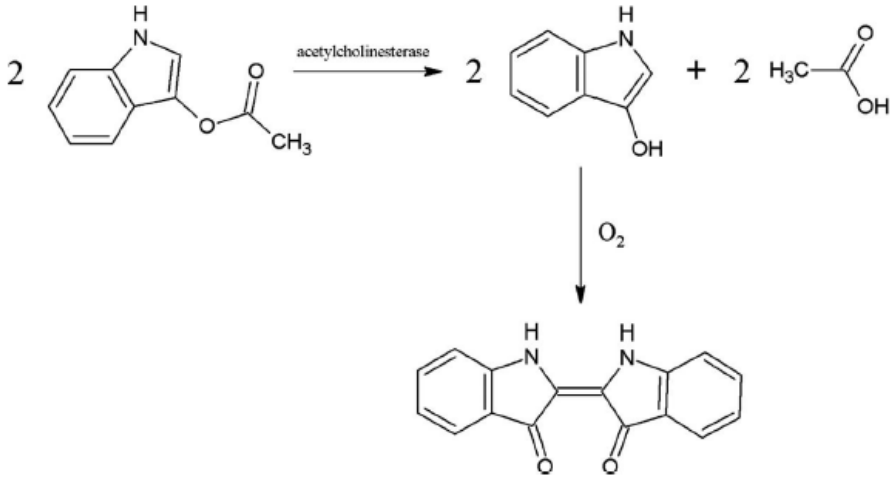

Figure 2. Principle of colorimetric reaction based on indoxyl acetate.

spontaneous oxidation into indigo blue, which provides blue coloration of the solution (Figure 2). This color change was observed in all red–green–blue channels; however, the most suitable was the red.

Ellman's assay

A standard Ellman's spectrophotometric assay was used for validation and was performed as follows: 400 µl of 5,5′-dithiobis(2-nitrobenzoic) acid (1 mM), 25 µl acetylcholinesterase, 475 µl phosphate buffered saline, and 100 µl of acetylthiocholine chloride (10 mM). The absorbance was measured immediately after addition of substrate and then after 2 min of reaction.

Data processing

The color values were obtained by photography processing in GIMP 2.8.16 (open source software) using color picker function and the red–green–blue channels were analyzed. Because the data were collected in standard jpg format which has eight bits, values of color depth from 0 to 256 can be expected. The Δ color depth was then calculated as the difference between color values before and after reaction. The limit-of-detection was calculated as the signal-to-noise ratio equal to three. Calibration curves and correlations were prepared in the Origin 9 Software (OriginLab, Northampton, MA, USA). The Michaelis constant was calculated in Origin using the non-linear Hill function with coefficient of cooperativity $n = 1$.

Results and discussion

Chitosan layers and percentage optimization

For assay purposes, chitosan was tested at 0.5, 1, and 2% concentrations and in different layers (Figure 3). We found out a decrease in activity of acetylcholinesterase with increasing percentage and layers of chitosan. Although the best results were provided by 0.5% chitosan, we decided to use two layers of 1% chitosan for the further assays because more

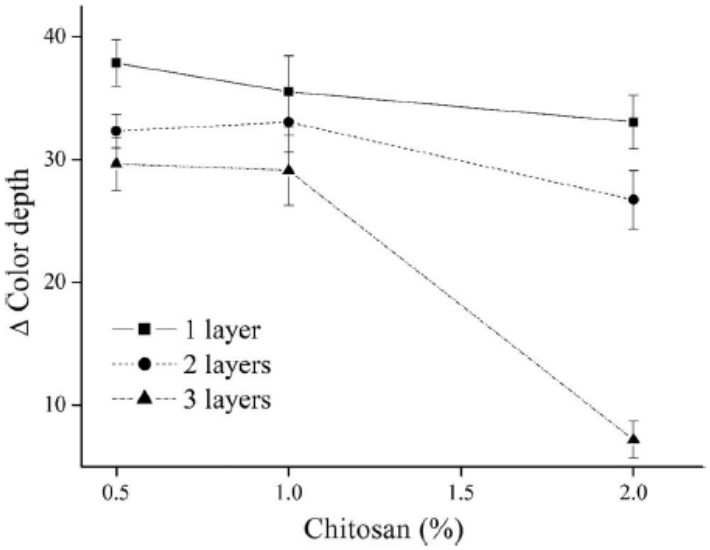

Figure 3. Chitosan layers and percentage optimization. Error bars are for $n = 3$ with ±standard error of the mean.

binding sites on the surface were offered. Alternatively, it is possible to use other layers and concentrations of chitosan, apart three layers of 2% chitosan, where a big drop in the ability to bind enzyme was obtained. It is perhaps steric problem when the medium is too dense that groups are limp and unavailable for bonding.

Immobilization of acetylcholinesterase on the chitosan surface

Acetylcholinesterase was immobilized on the chitosan surface with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride. There are proposed two possible mechanisms how the bond between chitosan and acetylcholinesterase is created: (1) amino groups in chitosan and carboxy groups in enzyme or (2) hydroxy groups in chitosan and amino groups in enzyme. There is sorption of acetylcholinesterase on the chitosan surface as seen from the results. However, using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride provided approximately 25% better results, likely due to the bond between chitosan and acetylcholinesterase. It is difficult to quantify the Michaelis constant value as excess of substrate is needed for assay function, and therefore substrate curve cannot be construct. There is a decrease in the Michaelis constant, when enzyme is physically/chemically immobilized into/on the membrane, which is common and in agreement with already published papers (Gabrovska et al. 2008; Kostelnik et al. 2017). The decrease in Michaelis constant is approximately 29% compared to the native enzyme, which was left to dry on the bare surface of the paper platform (Figure 4). We also tested albumin as a potential matrix for acetylcholinesterase immobilization but this material appeared to be inapplicable because it was immediately dissolved in used buffer in the *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride activation step.

Inhibitor measurements

The sensitivity of acetylcholinesterase to neurotoxic compounds (pesticides, drugs, warfare agents) can be used in construction of acetylcholinesterase-based biosensors

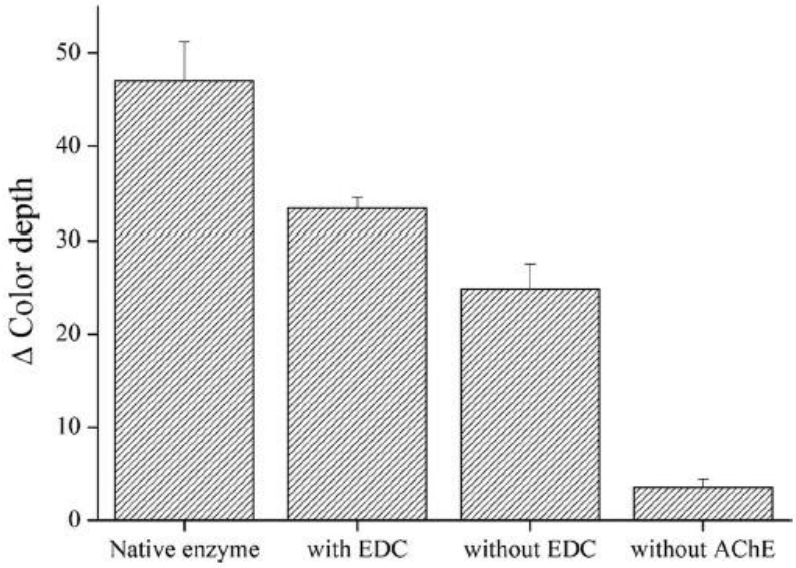

Figure 4. Immobilization of acetylcholinesterase. Error bars are for $n = 3$ with ±standard error of the mean. With/without *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) = immobilization with/without using EDC, without acetylcholinesterase (AChE) = negative control (no AChE used for immobilization).

(Pohanka 2013a). Galantamine and tacrine are drugs for Alzheimer's disease treatment (Čolović et al. 2013), although tacrine was banned from use due to a broad spectrum of side effects. However, this compound is still useful as a model molecule in acetylcholinesterase-based biosensors construction.

We decided to use tacrine for the comparability with the other studies. We obtained a galantamine calibration curve for immobilized acetylcholinesterase in concentration range from 0.97 to 15.6 µM (Figure 5). Validation of the assay was done using the standard

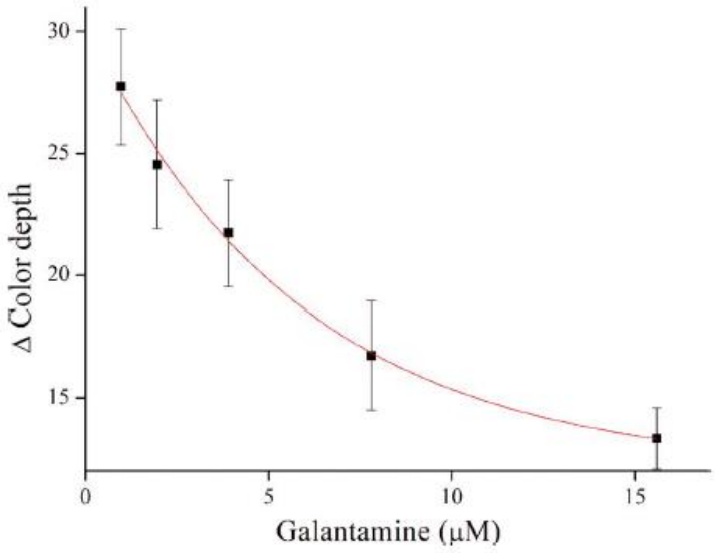

Figure 5. Galantamine calibration curve performed with immobilized acetylcholinesterase on the chitosan surface. Correlation coefficient is 0.9979. Error bars are for $n = 3$ with ±standard error of the mean.

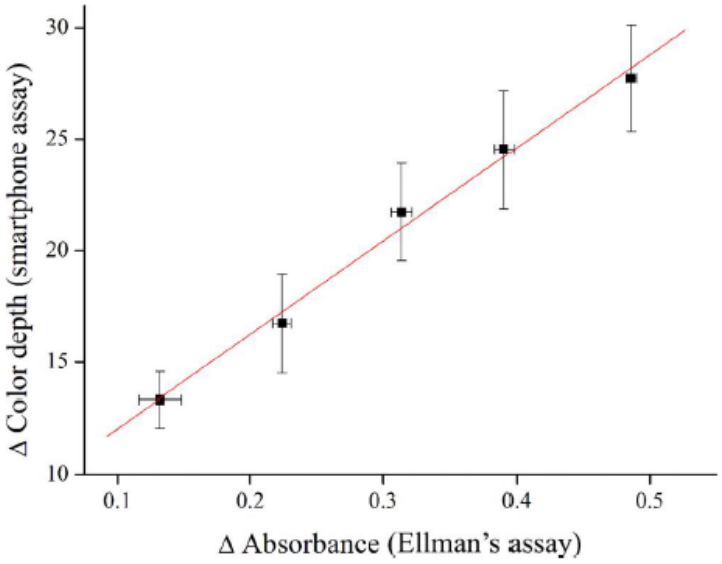

Figure 6. Galantamine assay validation. Correlation coefficient is 0.9959. Error bars for the smartphone assay are for $n = 3$ with ±standard error of the mean and for Ellman's assay for $n = 3$ with ±standard deviation.

Ellman's test (Figure 6). A calibration curve in range from 0.25 to 1.5 μM was obtained for tacrine (Figure 7) and for validation we used Ellman's test (Figure 8). Detection limits were 1.1 μM for galantamine and 0.18 μM for tacrine.

There are more sensitive methods for galantamine determination (White, Andrew Legako, and James Harmon 2003; Cuartero et al. 2013), but similar results were achieved as well (Rhee et al. 2003). Tacrine was also assayed with more sensitive methods (Pohanka and Vlcek 2014; Kostelnik, Cegan, and Pohanka 2016a), but comparable results was obtained (Bollo et al. 2000). However, the present method has some benefits compared

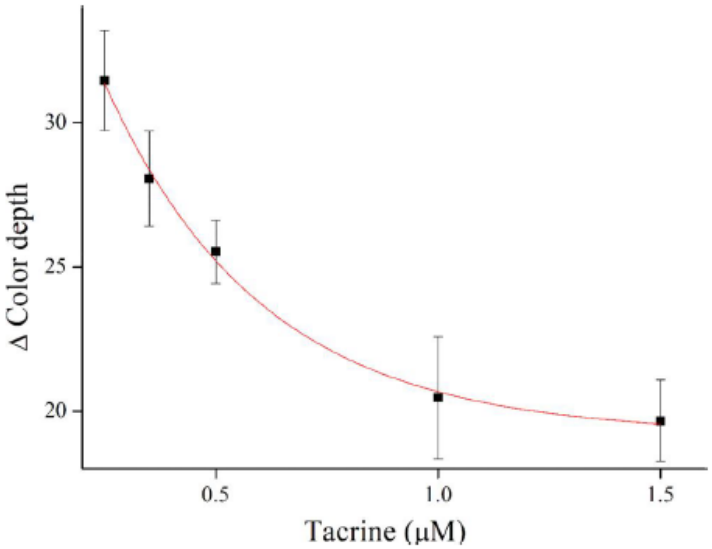

Figure 7. Tacrine calibration curve performed with immobilized acetylcholinesterase on the chitosan surface. Correlation coefficient is 0.9974. Error bars are for $n = 3$ with ±standard error of the mean.

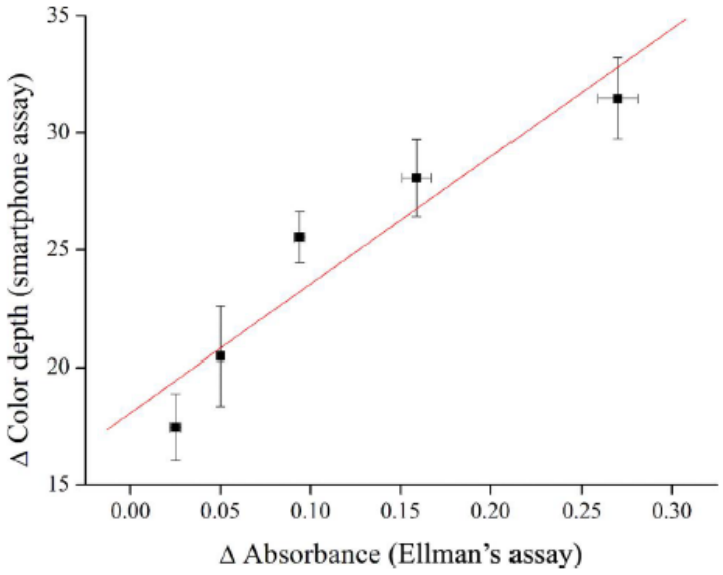

Figure 8. Tacrine assay validation. Correlation coefficient is 0.9481. Error bars for the smartphone assay are for $n = 3$ with ±standard error of the mean and for Ellman's assay for $n = 3$ with ±standard deviation.

to the aforementioned methods that are time consuming and require expensive laboratory equipment, furthermore portability, and long stability (see below). The ability to use the method in field conditions by an unskilled worker equipped only with a smartphone is a major advantage.

Organic solvents

Organic solvents are known for their ability to influence assays based on acetylcholinesterase; thus it is important to keep their percentage in reaction medium as low as possible (Pohanka et al. 2013). We tested three organic solvents, ethanol, 2-propanol and dimethyl sulfoxide for assay interference. All solvents were present at the 5% concentration (Figure 9). Both alcohols appeared to inhibit acetylcholinesterase as reported many times before (Andreescu et al. 2002; Pohanka, Adam, and Kizek 2013; Kostelnik, Cegan, and Pohanka 2016b). Dimethyl sulfoxide was reported for acetylcholinesterase inhibition (Solná et al. 2005), as we confirmed in this work, and it has larger effects than the alcohols.

Long-term stability

As seen from the results, acetylcholinesterase immobilized on the chitosan membrane is stable for 1 month. The activity of acetylcholinesterase varied during the observation period. The maximum activity was reached after 4 days, likely due to maturation of the chitosan membrane. Aging of chitosan was studied before and results indicated structural changes (Bodek and Bąk 1999), which probably occurred here as well. Therefore we suggest that acetylcholinesterase on the chitosan surface became more accessible for the substrate after membrane aging (Figure 10). There are reports where authors achieved long stability after enzyme immobilization into the membrane (Ricci et al. 2003; Pohanka 2012a), though

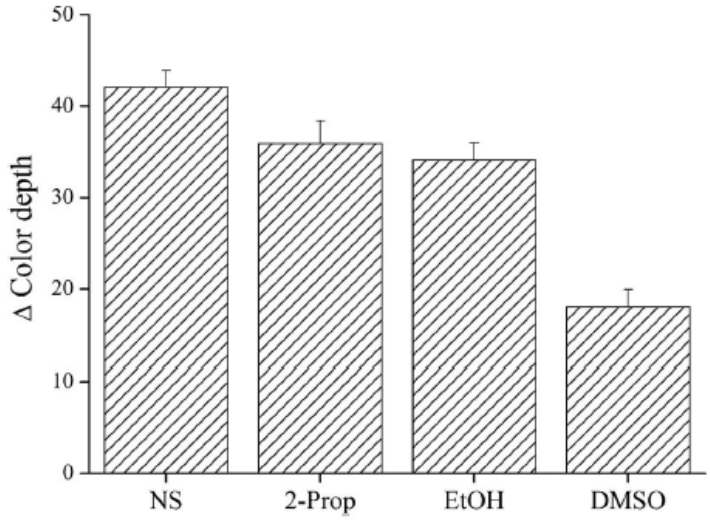

Figure 9. Influence of organic solvents to acetylcholinesterase immobilized on the chitosan surface. Error bars are for $n = 3$ with ±standard error of the mean. NS = no solvent (phosphate buffered saline used only), 2-prop = 2-propanol, EtOH = ethanol, DMSO = dimethyl sulfoxide.

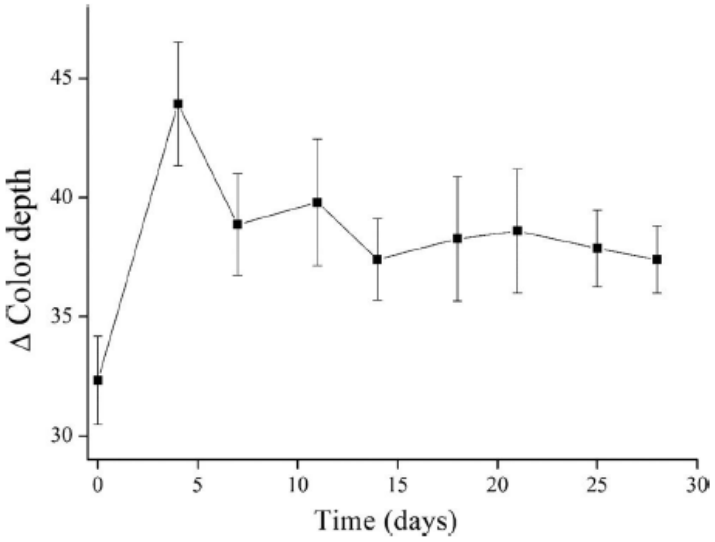

Figure 10. Long-term stability of acetylcholinesterase immobilized on the chitosan surface. Error bars are for $n = 3$ with ±standard error of the mean.

the stability of a superficial immobilized enzyme was investigated and long stability was reported as well (Cao et al. 2015).

Conclusion

We successfully attached acetylcholinesterase on chitosan using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride and performed calibrations for two model inhibitors galantamine and tacrine, with detection limits of 1.1 and 0.18 µM, respectively. The method was successfully validated to the standard Ellman's spectrophotometric protocol. Long stability, low cost, easy preparation, portability and easy detection are considered

to be the main advantages of the present method. Additionally, when a smartphone is absent, the assay can be still easily controlled by naked eye, when color change from white to blue is highly visible, compared to color changes from white to yellow.

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5.6. Výsledek 6

Adam Kostelník, Alexander Čegan, Miroslav Pohanka, „Anti-Parkinson drug biperiden inhibits enzyme acetylcholinesterase“, *BioMed Research International*, vol. 2017, 2017 (IF = 2,583)

Počítačové modelování neboli *in silico* je důležitý nástroj používaný pro zjištění potenciálních interakcí cílové struktury s určitým typem ligandu. Hojně se používá např. v proteinovém inženýrství nebo ve farmaceutickém průmyslu v přípravě nových léčivých preparátů. Pro účely *in silico* je v dnešní době možné sáhnout do proteinových databází jako např. UniProtKB, Protein Data Bank (PDB), Protein Structure Classification Database (CATH) atd., čímž se eliminuje náročná experimentální část, která je k získání proteinových struktur potřebná [145]. Pro potřeby této práce byla proteinová struktura AChE převzata z databáze PDB, struktury, které v této databázi najdeme, jsou z převážné většiny vytvořeny rentgenovou krystalografií, nukleární magnetickou rezonancí nebo elektronovou mikroskopií [146], což jsou zároveň nejčastěji používané metody v této oblasti.

Ačkoliv je biperiden (Akineton®) používán při léčbě parkinsonismu [147] a uplatňuje se i v terapii křečí při otravách cholinesterasovými inhibitory [148, 149], o jeho interakci se samotnou AChE nejsou k dispozici žádné informace. Proto byla interakce AChE-biperiden v této práci zkoumána blíže. Pro lepší pochopení interakce a získání více dat byla nejprve provedena experimentální studie, která měla za úkol zjistit, jaký inhibiční mechanismus se při interakci AChE-biperiden uplatňuje. Odhalen byl akompetitivní inhibiční mechanismus, který je v kontextu cholinesterasových inhibitorů a inhibitorů obecně velmi unikátním typem, jelikož cholinesterasové inhibitory působí většinou nekompetitivním mechanismem [150].

Dle našich výsledků se interakce biperidenu s AChE odehrává v jejím periferním anionickém místě. Je nutno dodat, že se jedná o *in silico* predikci, a bude proto potřeba tuto interakci v budoucnu ověřit sestavením krystalografické struktury. I když se biperiden ukázal jako slabý inhibitor AChE, jeho struktura by v budoucnu mohla posloužit v syntéze účinnějších derivátů, a tím k léčbě Alzheimerovy nemoci či dalších neurodegenerativních poruch.

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Research Article

Anti-Parkinson Drug Biperiden Inhibits Enzyme Acetylcholinesterase

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Biperiden is a drug used in Parkinson disease treatment and it serves also as an antiseizures compound in organophosphates poisoning. It acts as antagonist of muscarinic receptor activated by acetylcholine while the enzyme acetylcholinesterase (AChE) cleaves acetylcholine in synaptic junction into choline and acetic acid. This enzyme is inhibited by various compounds; however there has not been proposed evidence about interaction with biperiden molecule. We investigated this interaction using standard Ellman's assay and experimental findings were critically completed with an in silico prediction by SwissDock docking software. Uncompetitive mechanism of action was revealed from Dixon plot and inhibition constant (K_i) was calculated to be 1.11 mmol/l. The lowest predicted binding energy was −7.84 kcal/mol corresponding to H-bond between biperiden molecule and Tyr 341 residuum in protein structure of AChE. This interaction seems to be further stabilized by π-π interaction with Tyr 72, Trp 286, and Tyr 341. In conclusion, biperiden appears as a very weak inhibitor but it can serve as a lead structure in a pharmacological research.

1. Introduction

Termination of neurotransmission in cholinergic nerves or neuromuscular junctions is done by an enzyme AChE. Mechanism of action is in cleaving of acetylcholine, neurotransmitter interacting with acetylcholine muscarinic receptor (mAChR) and nicotinic receptor (nAChR) [1, 2]. AChE is a target for many toxic compounds like organophosphorus and carbamate pesticides (e.g., parathion, malathion, and carbofuran), warfare agents (e.g., sarin, soman, and VX), or some toxins like aflatoxin B1 [3–7]. Big and important group of AChE inhibitors is created by anti-Alzheimer drugs as donepezil, galantamine, rivastigmine, or huperzine A [8–10].

Biperiden also known under tradename Akineton®, a compound with proper chemical name alpha-bicyclo[2.2.1]hept-5-en-2-yl-alpha-phenyl-1-piperidinepropanol (Figure 1), is an anticholinergic drug used in treatment of Parkinson disease and neuroleptic-induced extrapyramidal motor side effects [11]. It acts as a muscarinic receptor antagonist with high affinity for the M1 muscarinic receptor [12]. Furthermore, it can be used as antiseizures compound in poisoning by organophosphates [13, 14].

Despite the fact that biperiden is used in therapy of parkinsonism, there is no evidence about possible interaction with AChE itself. Some structural motives in the biperiden resemble another AChE inhibitor, compound known as huperzine A. The fact leads us to the idea that biperiden can act as inhibitor and we hypothesize a possible interaction with AChE.

2. Material and Methods

2.1. Chemicals. Acetylcholinesterase as lyophilized powder (electric eel, activity ≥ 1000 units/mg of protein), acetylthiocholine chloride, 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), and phosphate buffer saline (PBS) 7.4 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Biperiden lactate (5 mg/ml) in one-milliliter ampules was obtained from Knoll AG (Ludwigshafen, Germany). Deionized water was prepared by Aqua Osmotic device (Tisnov, Czech Republic).

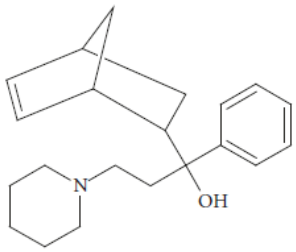

Figure 1: Chemical structure of biperiden molecule.

2.2. Enzymatic Assay with Biperiden. Ellman's method was chosen for the enzyme activity assay and it was performed as follows: 400 μl of DTNB, 25 μl of AChE, 375 μl of PBS 7.4, 100 μl of PBS 7.4 or biperiden, and 100 μl of acetylcholine chloride were pipetted into standard spectrophotometric cuvette. Absorbance was measured immediately after addition of substrate and then after 2 min of reaction. Enzyme activity was then calculated using extinction coefficient for 5-thio-2-nitrobenzoic acid $\varepsilon = 14{,}150\,\mathrm{l} \times \mathrm{mol}^{-1} \times \mathrm{cm}^{-1}$ [15]. Concentration of biperiden was calculated to whole volume of reaction medium in cuvette.

2.3. Data Processing. Dixon plot was created in Origin software (OriginLab, Northampton, MA, USA). K_i for uncompetitive inhibition was calculated from Dixon plot as follows: Slope $= 1/V_{max} \times K_i$. V_{max} for AChE was obtained experimentally (as described in previous section) and calculated in Origin software (OriginLab, Northampton, MA, USA) using nonlinear Hill fitting with coefficient of cooperativity $n = 1$.

2.4. Docking of Biperiden to AChE. SwissDock server (Swiss Institute of Bioinformatics, University of Lausanne, Switzerland) was used for in silico prediction of the lowest free binding energy. The calculation was running online (accessible from http://www.swissdock.ch/) in the Internet browser. Crystal structure of AChE (1C2B) [16] was taken in PDB format and biperiden ligand in ZINC format as required for calculation [17]. UCSF Chimera 1.11.2 software was used for visualization of the results and creating 3D images.

3. Results and Discussion

Biperiden proved to be inhibitor of AChE as seen from the results. From Dixon plot, uncompetitive mechanism of AChE inhibition was revealed (Figure 3). This type of inhibition is very rare and it is more probable for multifold substrate reactions. More typical mechanism for AChE is noncompetitive or competitive inhibition [18]. K_i for biperiden and AChE was calculated to be 1.11 ± 0.20 mmol/l, which equals IC_{50} in this type of inhibition [19]. Figure 2 is displaying saturation curve from which V_{max} for K_i calculation was obtained as described above. Data obtained from experiment are summarized in Table 1.

Interaction of biperiden with AChE was studied using SwissDock server. The lowest binding energy ΔG was equal to −7.84 kcal/mol and corresponds to interaction between biperiden and peripheral anionic subsite. In the lowest energy, there is predicted H-bond between hydroxyl group in biperiden molecule and O atom in Tyr 341 (2.24 Å). This seems to be stabilized by π-π interaction of benzene ring in biperiden with aromatic amino acids of peripheral anionic subsite Tyr 72 (3.43 Å), Trp 286 (3.18 Å), and Tyr 341 (3.05 Å) (Figure 4). As seen from the quoted papers [20, 21], T-shape geometry (or face to age) interaction is the most common between two aromatic systems and it was found to be most abundant interaction in present work. On the other hand, face to face interaction is very rare

Table 1: Data from inhibition assay.

Substrate (mM)	Slope (s × l/mol^2)	Interception (s/mol)	Correlation coefficient
1.0	1.30×10^9	1.60×10^6	0.9647
0.50	1.55×10^9	1.75×10^6	0.9978
0.25	1.87×10^9	2.05×10^6	0.9965

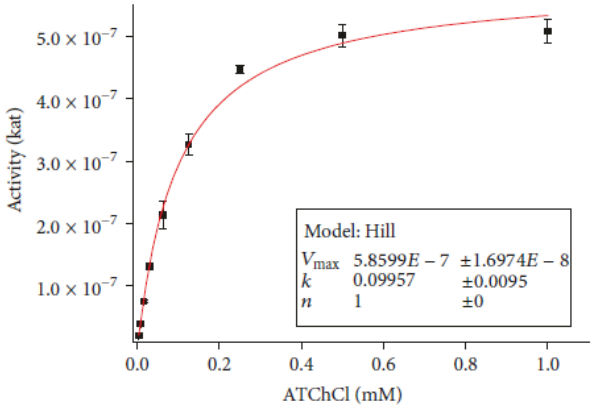

Figure 2: Saturation curve for AChE and acetylthiocholine as a substrate. Error bars indicate standard deviation for $n = 3$.

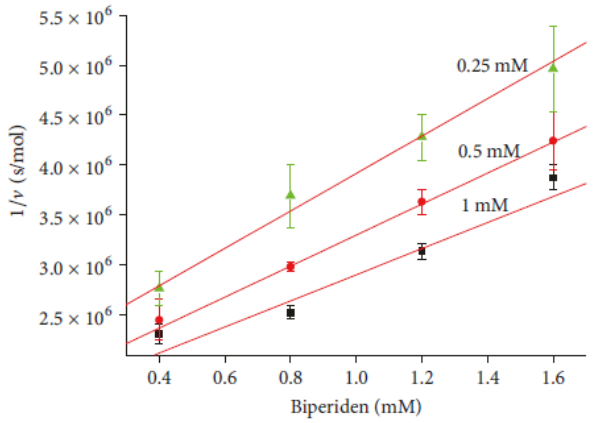

Figure 3: Dixon plot for AChE with different concentrations of substrate (indicated above each line). Error bars indicate standard deviation for $n = 3$.

Table 2: Found results about biperiden interaction with AChE.

Parameter	Findings
Mechanism of inhibition	Uncompetitive
Inhibition constant (equal to IC_{50} in this kind of inhibition)	1.11 ± 0.20 mmol/l
Predicted binding energy ΔG	−7.84 kcal/mol
Predicted interactions	H-bond: Tyr 341 π-π interaction: Tyr 72, Trp 286, Tyr 341

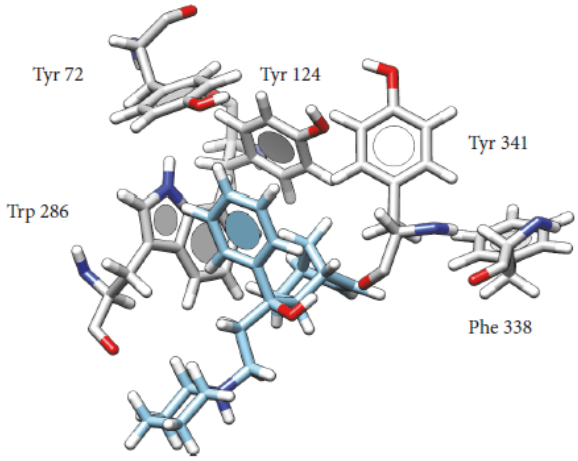

Figure 4: Possible interaction of biperiden with AChE. Atom color: white = H, grey (in AChE) or light blue (in biperiden) = C, blue = N, and red = O.

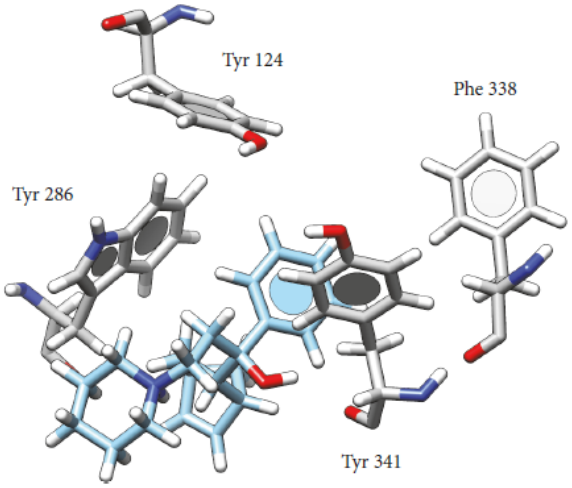

Figure 5: Possible interactions of biperiden with β-anionic site of AChE. Atom color: white = H, grey (in AChE) or light blue (in biperiden) = C, blue = N, and red = O.

due to electrostatic repulsion and it was not observed in this study. As bicycloheptenyl contains double bond, it does not seem to provide any further stabilizing effect. There are conformational changes in protein structure after binding of substrate, leading to decrease of the distance between Phe 338 and Tyr 124 as proved works with 4-oxo-N,N,N-trimethylpentanaminium (acetylcholine analogue) [22]; thus these residues could become active in interaction with this double bond in bicycloheptenyl structure. Conformational changes are fundamental and would explain uncompetitive mechanism of inhibition. Nevertheless, this interaction is alleged and more investigation should be done prior to this conclusion. Results from experiment are summarized in Table 2.

There is another possible interaction followed with release of free binding energy $\Delta G = -7.58$ kcal/mol, where H-bond (2.24 Å) is also predicted between biperiden hydroxyl and O atom in Tyr 341. Additionally, further stabilization seems to be provided by π-π interaction of benzene ring in biperiden with Trp 286 (3.18 Å), Tyr 341 (3.96 Å), and Phe 338 (4.01 Å) in peripheral anionic subsite and cation-π interaction with Tyr 124 (5.84 Å) of AChE (Figure 5).

Biperiden was shown to be weak inhibitor of AChE. For example, most common used cholinesterase's inhibitors like tacrine or donepezil are, respectively, almost 18,500 times and 300,000 times stronger inhibitors of AChE than biperiden [23]. Limited data about biperiden in humans are available. Presumed plasma concentration of biperiden of 5 ng/ml (16 nmol/l) is reached after 1.5 hours when two 2 mg tablets are taken [24]. Typical daily dose is ranging between 2 and 8 mg [25, 26]. However, for causing significant decrease of AChE activity, 342 μg/ml (1.1 mmol/l) is needed as we found out in present work; this means approx. 14 μmol/l for average weight person (80 kg). For humans LD_{50} is not available; for animal models, for example, for rats it is 750 mg/kg (orally); it is approx. 3.0 μmol/l in molar scale. As seen above, for inhibition effect of AChE almost 69,000 times higher dose compared to therapeutic one is required; moreover toxic effect is observed at the same level as needed for causing AChE inhibition. Nevertheless, these results are only hypothetical. Penetration of biperiden to tissues and through blood-brain barrier is based on high lipophilicity and furthermore it is not substrate for efflux transporters like P-glycoprotein which eliminate drugs back into blood flow [27, 28]. At peak concentration of biperiden in plasma, there is approx. 26% occupancy of mAChR in human brain [26]; however no evidence about molar levels in cerebrospinal fluid was found and hence more investigation could be done this way. On the other hand, cerebrospinal fluid levels of, for example, donepezil, were observed [29] and recently changes in concentration between doses were evaluated [30].

Although biperiden is weak inhibitor of AChE and seems to be toxic in high doses, future research based on similar structure derivatives could open interesting direction of AChE inhibitors synthesis and thus in Alzheimer or another neurodegenerative disease treatment.

4. Conclusion

Biperiden was revealed to be inhibitor of AChE in our experiment. However, in comparison with standardly used inhibitors, biperiden is weak inhibitor of AChE; K_i was calculated to be 1.11 mmol/l. In real conditions concentration is too low to cause significant inhibition effect; however in high doses it could become toxic. Future perspectives are seen in further investigation of similar derivatives which could create direction in AChE inhibitors research.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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5.7. Výsledek 7

Adam Kostelník, Miroslav Pohanka, „Inhibition of acetylcholinesterase and butyrylcholinesterase by a plant secondary metabolite boldine“, *BioMed Research International*, vol. 2018, 2018 (IF = 2,583)

Cholinesterasy jsou známy pro svou citlivost k různým inhibitorům. Jeden z trendů výzkumu nových cholinesterasových inhibitorů se ubírá směrem k inhibitorům přírodního původu. Převážně se jedná o inhibitory AChE z důvodu hledání nových inhibitorů použitelných v léčbě Alzheimerovy nemoci. Takových přírodních struktur byla v minulých letech identifikována celá řada, často s velmi slibnými výsledky, včetně dnes používaných inhibitorů jako např. galantamin či huperzin A [151, 152].

Rozdíly v aktivním místě AChE a BChE předurčují jejich citlivosti k různým typům inhibitorů. Úzké hrdlo složené s aromatických aminokyselin při vstupu do aktivního centra AChE jí poskytuje větší odolnost vůči inhibitorům o vyšší molekulové hmotnosti, u BChE je tomu naopak. Také záleží na místu, ve kterém se inhibice uplatňuje, v kontextu přírodních látek (vyloučíme-li přírodní karbamáty) se to u cholinesteras děje buďto v anionickém nebo periferním anionickém místě. V této práci byl zkoumán vliv rostlinného aporfinového alkaloidu boldinu na obě cholinesterasy. Ten byl v dřívější práci označen za inhibitor AChE a inhibiční konstanta naznačovala slibný inhibiční potenciál [153], nicméně nebyl zkoumán jeho vliv na BChE a inhibiční mechanismus. V našich podmínkách se ale publikované hodnoty inhibiční konstanty ověřit nepodařilo a získané výsledky byly několikanásobně vyšší, možné důvody jsou diskutovány v publikaci.

Již z experimentálně získaných inhibičních konstant lze usuzovat na místo působení inhibitoru. Vzhledem k tomu, že inhibiční konstanty nabývají prakticky stejných hodnot, inhibice pravděpodobně probíhá v anionickém místě, jelikož periferní anionické místo v BChE je méně vyvinuté. Nekompetitivní mechanismus inhibice tvořil další vodítko směrem k inhibici v tomto místě, jelikož se nekompetitivní inhibitory váží právě zde [150]. Struktura boldinu byla modelována metodou *in silico* na základě již publikovaných výsledků podobných aporfinových derivátů [154] a inhibice v anionickém místě byla podle očekávání potvrzena.

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Research Article

Inhibition of Acetylcholinesterase and Butyrylcholinesterase by a Plant Secondary Metabolite Boldine

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Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are two enzymes sensitive to various chemical compounds having ability to bind to crucial parts of these enzymes. Boldine is a natural alkaloid and it was mentioned in some older works that it can inhibit some kinds of AChE. We reinvestigated this effect on AChE and also on BChE using acetyl (butyryl) thiocholine and Ellman's reagents as standard substances for spectrophotometric assay. We found out IC_{50} of AChE equal to 372 μmol/l and a similar level to BChE, 321 μmol/l. We conclude our experiment by a finding that boldine is cholinesterase inhibitor; however we report significantly weaker inhibition than that suggested in literature. Likewise, we tried to investigate the mechanism of inhibition and completed it with in silico study. Potential toxic effect on cholinesterases in real conditions is also discussed.

1. Introduction

Cholinesterases are enzymes splitting esters of choline. We distinguish two enzymes belonging to the group of cholinesterases, AChE which is presented in nervous system and terminates neurotransmission and BChE occurring in serum but the particular function of the enzyme remains undiscovered [1, 2]. They are sensitive to broad spectrum of molecules; many of them have artificial origin (e.g., organophosphate and carbamate pesticides or nerve agents) [3]. Research on natural inhibitors which brought substances like physostigmine or galantamine was started long time ago, lately their synthetic versions were also introduced [4, 5].

Boldine, in chemical terminology (S)-2,9-dihydroxy-1,10-dimethoxy-aporphine (Figure 1), is a natural alkaloid with reported antioxidant activity. It was isolated from dozens of plant species from *Monimiaceae and Magnoliaceae* [6, 7] and recently it was reported to inhibit AChE with IC_{50} equal to 8.5 μmol/l [8]; thus it could be potential cholinesterase inhibitor. In this work, we reinvestigated its effect on AChE and also BChE and calculated IC_{50} for both of them. We tried to propose mechanism of interaction of boldine with both cholinesterases as no evidence in literature about this interaction was found. Discussion of potential toxic effect in real conditions is also given. We hypothesize that boldine would serve as a structure for pharmacology research and, furthermore, boldine would act as a multitarget toxin. Unfortunately, data about boldine impact on targeted receptor site are scattered. We decided to investigate interaction between boldine and AChE in higher details than currently available.

2. Material and Methods

2.1. Chemicals. Acetylcholinesterase human, recombinant (expressed in HEK 293 cells, lyophilized powder, ≥1000 U/mg protein; activity for acetylthiocholine chloride was set to 64 U), butyrylcholinesterase from human serum (lyophilized powder, ≥50 U/mg protein; activity for butyrylthiocholine was set to 14 U), boldine (CAS 476-70-0), acetylthiocholine chloride (ATChCl), butyrylthiocholine iodide (BTChI) 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), and phosphate buffer saline (PBS) pH 7.4 were supplied by Sigma-Aldrich (St. Louise, MO, USA); ethanol was purchased from Penta (Prague, Czech Republic).

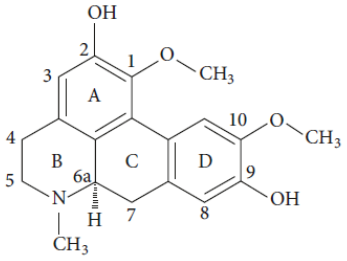

FIGURE 1: Structure of boldine.

2.2. Enzyme Activity Assay. AChE activity was assayed, as triplicate, in standard spectrophotometric cuvette in following way: 400 μl DTNB (1 mmol/l), 25 μl AChE, 450 μl PBS, and 25 μl boldine ethanolic solution, and reaction was started by 100 μl of ATChCl (10 mmol/l). Absorbance was measured immediately and then after 2 min of reaction. BChE activity assay was performed as follows: 400 μl DTNB, 25 μl BChE, 450 μl PBS, and 25 μl boldine ethanolic solution, and reaction was initiated by 100 μl of BTChI (10 mmol/l). Difference in absorbance was measured after 2 minutes.

2.3. Data Processing. Enzyme activity was calculated from absorbance using extinction coefficient for 5-thio-2-nitrobenzoic acid $\varepsilon = 14{,}150\,\mathrm{l} \times \mathrm{mol}^{-1} \times \mathrm{cm}^{-1}$ [9]. Dixon plot was constructed in Origin software (Origin, Northampton, MA, USA) and K_i was then calculated form Michaelis Menten equations.

2.4. Docking Study. UCSF Chimera (version 1.11.2; developed by RBVI with support of NIH, University of California, San Francisco [10]) was used for creating images and molecular modeling. Human AChE (PDB code: 3LII [11]) and BChE (PDB code: 1P0I [12]) were fetched and prepared for docking using Dock Prep tool (added hydrogens, assigned charges). For docking, AutoDock Vina tool was then used, and calculation was run online on Opal server.

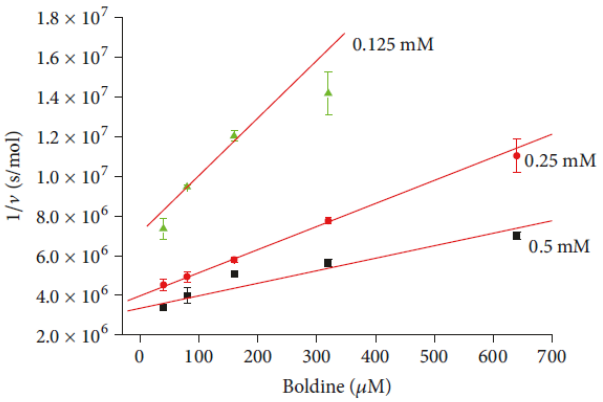

FIGURE 2: Dixon plot for AChE and different concentration of substrate (indicated beside each line). Error bars are for $n = 3 \pm$ standard deviation.

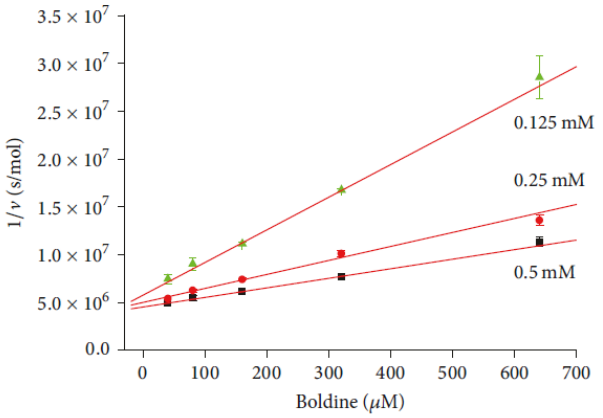

FIGURE 3: Dixon plot for BChE and different concentration of substrate (indicated beside each line). Error bars are for $n = 3 \pm$ standard deviation.

3. Results and Discussion

We investigated effect of boldine on both cholinesterases and found out that it is able to inhibit them. Dixon plot revealed noncompetitive mechanism of inhibition which is the most common inhibition mechanism for cholinesterases [13]. The inhibition of AChE by boldine was already investigated and authors reported IC_{50} 8.5 μmol/l [8]. We found out K_i for boldine and human AChE equal to 372.30 μmol/l and K_i for boldine and human BChE equal to 321.85 μmol/l. K_i obtained from Dixon plot is directly proportional to IC_{50} (Figures 2 and 3). As seen in figures, our findings are not in full agreement with previously achieved result because we found out approximately 40-fold higher IC_{50} values for AChE. This difference might be seen in used enzymes, as we used pure form of human AChE, while in the quoted paper AChE is not specified, and authors refer to original paper published by Ellman, who used bovine erythrocyte cholinesterase [14]. Another difference could be in used boldine isomer where hydrogen atom in position 6a gives two optical forms. We used biologically active (S) isomer while the cited paper does not contain specification of the used isomer. Despite the fact that two isomers exist, our docking results suggest that mentioned hydrogen atom is not crucial for molecule interaction with the enzyme. From the aforementioned facts, we claim that boldine is very weak inhibitor with limited impact for pharmacological research. From the achieved results, we can also learn that IC_{50} for both cholinesterases is practically the same. This is suggesting that inhibition mechanism of boldine towards cholinesterases lies in inhibition of anionic subsite, as peripheral anionic subsite in BChE is missing [1]; thus inhibition via this site seems to be improbable. Data from experiment with both cholinesterases are summarized in Tables 1 and 2.

Docking of boldine to cholinesterases was not found in the literature; thus we tried to investigate mechanism of this interaction. Grid box for boldine docking into AChE was set around Trp 86 and in BChE structure around Trp 82.

Table 1: Data from AChE inhibition assay.

Substrate (mM)	Slope (s × l/mol^2)	Interception (s/mol)	Correlation coefficient
0.5	3.34×10^6	6.31×10^3	0.9532
0.25	3.97×10^6	11.64×10^3	0.9985
0.125	7.13×10^6	28.95×10^3	0.9662

Table 2: Data from BChE inhibition assay.

Substrate (mM)	Slope (s × l/mol^2)	Interception (s/mol)	Correlation coefficient
0.5	4.54×10^6	9.99×10^3	0.9983
0.25	5.01×10^6	14.67×10^3	0.9924
0.125	5.79×10^6	34.14×10^3	0.9988

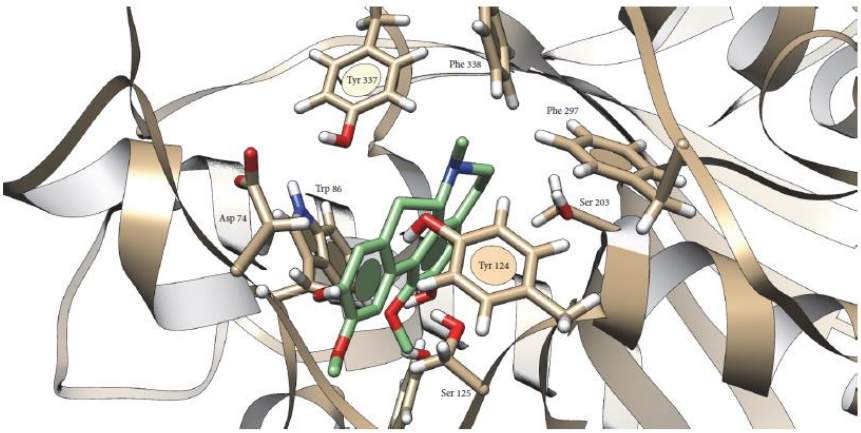

Figure 4: Presumed position of boldine in AChE cavity. Atom color: white = H, red = O, blue = N, grey (in AChE) and green (in boldine) = C. White line represents hydrogen bond.

Docking showed interaction of boldine with AChE in anionic subsite, where H-bond (2.050 Å) with Asp 74 and OH group (position 9) was predicted and stabilized via π-π interaction of Trp 86 and A ring (Figure 4). Similar situation occurred in BChE, where H-bonds between OH group (position 9) and Glu 197 (1.958 Å) and between OCH_3 group (position 1) and Thr 120 (2.387 Å) were predicted. Stabilization is provided by π-π interaction of Trp 82 and D ring (Figure 5). Our results and also previously published docking study of another aporphine derivates [15] support the aforementioned idea about inhibition via anionic subsite of the enzymes. Another clue lies in mechanism, as noncompetitive inhibitors bind to anionic subsite of the enzyme which can be seen from examples of other cholinesterases inhibitors like tacrine and donepezil [13, 16, 17]. Some aporphine derivatives were reported to exert dual activity towards AChE, when, beside anionic subsite, interacting also with peripheral anionic subsite [18, 19]. Therefore, we checked this possible interaction and found out H-bond (2.127 Å) between OH group (position 2) and Ser 293, stabilized by π-π interaction with Trp 286 and ring A (Figure 6). Facts about boldine interaction with cholinesterases are summarized in Table 3.

Boldine is contained in various plants, widely used in Latin America for decades countries and also exported into Europe (e.g., *Peumus boldus*) [20] and it is known as a low toxic secondary metabolite. This is supported by LD_{50} values, as 500–1000 mg/kg (p.o., mice and guinea pigs, resp.) is necessary to cause half probability of fatal poisoning [6]. Considering the fact that plasma concentration after administration of 10 mg/kg (p.o., rats) reaches approximately 1 μmol/l of boldine in its peak [21], the aforementioned lethal dose 500 mg/kg would hypothetically reach 50 μmol/l concentration in the blood or blood plasma. However, higher concentration is needed for significant decay of AChE activity. Thus, even lethal dose is not enough to cause cholinesterase inhibition.

4. Conclusion

Effect of boldine on AChE was reinvestigated, and IC_{50} equal to 372.30 μmol/l was found out compared to previously published 8.5 μmol/l. Thus, we claim that boldine is a cholinesterase inhibitor but it has very low affinity with the enzyme and its inhibitory effect reaches very low scale. We also investigated the effect on BChE (IC_{50} 321.85 μmol/l) and it was found to be at the same level as that on AChE. We investigated inhibition mechanism of boldine to cholinesterases

Table 3: Found facts about boldine and cholinesterases.

Enzyme	Inhibition mechanism	IC_{50} (μmol/l) by spectrophotometry	ΔG (kcal/mol) in silico	Anionic subsite interaction in silico	Peripheral anionic subsite interaction in silico
AChE	Noncompetitive	372.30	−7.6 (a. site) −6.2 (p. site)	H-bond: Asp 74 π-π: Trp 86	H-bond: Ser 293 π-π: Trp 286
BChE		321.85	−9.7	H-bond: Thr 120, Glu 197 π-π: Trp 82	—

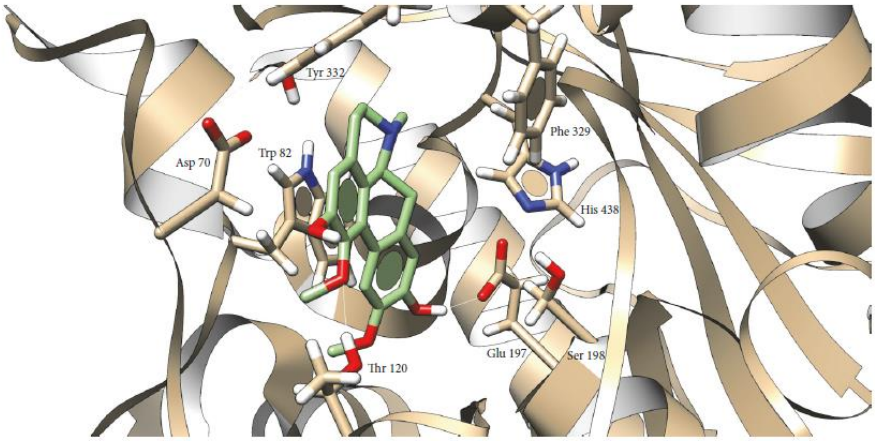

Figure 5: Presumed position of boldine in BChE cavity. Atom color: white = H, red = O, blue = N, grey (in AChE) and green (in boldine) = C. White lines represent hydrogen bonds.

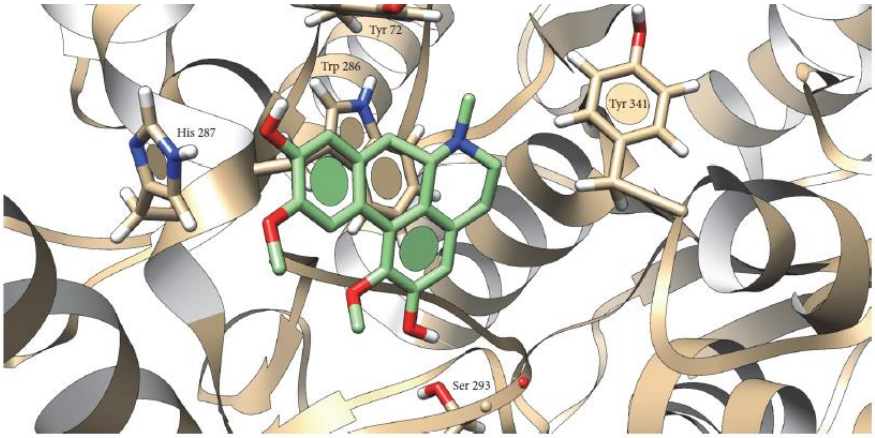

Figure 6: Presumed position of boldine in peripheral anionic subsite of AChE. Atom color: white = H, red = O, blue = N, grey (in AChE) and green (in boldine) = C. White line represents hydrogen bond.

and proposed inhibition mechanism of boldine to AChE by docking. Results are supported by previously published findings and our experimental data and indicate inhibition via anionic subsite. We also discussed theoretical effect of boldine on AChE in real conditions. Considering the conclusions, boldine appears weak inhibitor of cholinesterases, not well suited for pharmacological research. On the other hand, its ability to inhibit cholinesterases is attributed to its biological effect though the only low manifestation is expected.

Conflicts of Interest

The authors declare no conflicts of interest.

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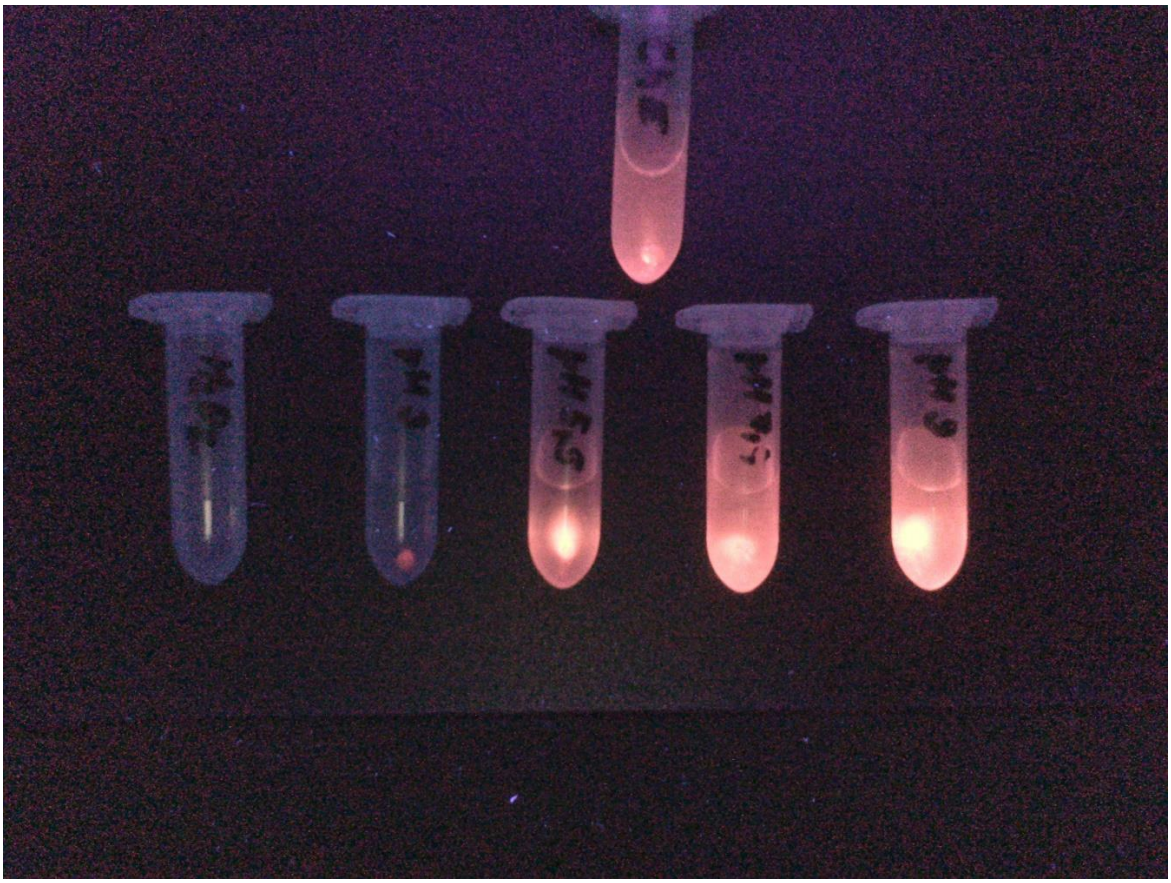
5.8. Výsledek 8 – Stanovení aktivity cholinesteras pomocí kvantových teček

Kvantové tečky jsou polovodičové krystaly nanometrové velikosti, typický průměr se pohybuje mezi 2 a 10 nm. Krystaly jsou složeny s II až VI nebo III až V skupiny prvků periodické tabulky, navzdory své cytotoxicitě zaznamenaly největší úspěch CdSe a CdTe kvantové tečky. Unikátní jsou vlastnosti kvantových teček – široké absorpční spektrum a úzké emisní spektrum, které závisí na jejich velikosti. Díky tomu byly kvantové tečky aplikovány v mnoha oblastech výzkumu [155-157].

Fotografickou detekci barevné změny indikátoru použité v předchozích pracích je možné adaptovat i na detekci fluorescence. V této souvislosti byl zkoumán vliv pH na fluorescenci kvantových teček. Předpokladem experimentu bylo, že se fluorescence kvantových teček mění s klesající nebo stoupající hodnotou pH. Proto byl proveden experiment, kde byla do roztoků o různém pH přidána stejná koncentrace CdTe kvantových teček (1 mg/ml) a sledována intenzita fluorescence (λ_{ex} = 360 nm). Z výsledků je patrné, že aplikace tohoto postupu pro stanovení cholinesterasové aktivity není možná. Důvodem je fakt, že zhášení fluorescence změnou pH se projevuje ve velmi omezené míře zhruba při pH 5,5 a jasný rozdíl je vidět až při pH 3,0 (Obr. 19). Pokud by bylo teoreticky možné snížit pH enzymovou reakcí natolik, že by tato změna byla patrná, docházelo by k denaturaci samotného enzymu, který by se tak vlastně sám degradoval a enzymová reakce by se zastavila. Prakticky tohoto dosáhnout nelze, optimální pH pro funkci cholinesterasy se totiž pohybuje v rozmezí zhruba 6,0–8,0, navíc produkovaná kyselina při rozkladu acetylcholinu je schopna snížit pH maximálně v desetinách, což samotnému enzymu neublíží. Dalším důvodem od upuštění aplikace kvantových teček pro stanovení cholinesterasové aktivity v této disertační práci byla nepřítomnost dostatečně citlivé metody umožňující měření fluorescence na pracovišti, kde bylo studium uskutečněno.



Obrázek 19. Zhášení fluorescence kvantových teček při různém pH. Zleva: peroxid vodíku (pozitivní kontrola – zháší fluorescenci), roztok o pH 3,0, roztok o pH 5,5, roztok o pH 7,4 a roztok o pH 9,0. Nahoře roztok obsahující AChE po proběhlé enzymové reakci.

6. Závěr

Tato disertační práce shrnuje biosenzory založené na cholinesterasach pro detekci neurotoxických látek, které byly zhotoveny během doktorského studia. Připraveny byly biosenzory s chemicky imobilizovanou AChE na magnetických částicích, čímž bylo přispěno k rozšíření této problematiky, jelikož dosud se imobilizací AChE na povrch pouze magnetických částic mnoho prací nezabývalo. Chemicky nebo fyzikálně byla AChE imobilizována v/na membráně z želatiny a chitosanu, navíc v kombinaci s fotografickou detekcí, u které se dá předpokládat, že v dalších letech nabyde na významu, jak poroste důležitost technologií ve všech oblastech života. Všechny připravené biosenzory prokázaly svou funkčnost při stanovení cholinesterasových inhibitorů. Rovněž byly v této práci zkoumány doposud nepopsané interakce cholinesteras s látkami figurujícími jako jejich inhibitory, a to metodou počítačového modelování.

Připravené biosenzory jsou jednoduchou alternativou k analytickým metodám (především chromatografické techniky) detekující neurotoxické látky. Jejich použití lze zamýšlet v celé řadě aplikací. Jedná se zejména o analýzy životního prostředí, kontrolu potravin, farmaceutický průmysl či analýzu biologického materiálu. Díky své jednoduchosti, malým rozměrům a s tím spojené přenosnosti je možné použití i v polních podmínkách mimo dosah laboratoře. Toto nabízí především kombinace s fotografickou detekcí, jelikož mobilní telefon neklade na obsluhu žádné speciální nároky, a proto ho lze využít i v podmínkách malých laboratoří, ordinací lékařů nebo v domácích podmínkách přímo u pacientova lůžka. Počítačové modelování neznámých interakcí inhibitorů s cholinesterasami tvoří důležitou část této práce. Zvláště lze předpokládat využití interakce biperidenu s AChE. Tato interakce nebyla známa a mohla by znamenat start ve výzkumu nových cholinesterasových inhibitorů a vést tak k potenciálnímu objevu nových látek, které najdou své uplatnění v léčbě Alzheimerovi nemoci nebo jiných neurodegenerativních poruch.

7. Publikační činnost

V průběhu studia jsem uveřejnil celkem sedm prací s IF obsahující výsledky disertační práce:

- 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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Podílel jsem se na přípravě dvou publikací s IF:

- MARTINKOVÁ, Pavla, KOSTELNÍK, Adam, VÁLEK, Tomáš, POHANKA, Miroslav. Main streams in the construction of biosensors and their applications. *International Journal of Electrochemical Science*, 2017, vol. 12, no. 8, 7386–7403
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Rovněž jsem se podílel na dvou publikacích bez IF:

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Některé výsledky jsem prezentoval na šesti mezinárodních konferencích formou posteru:

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Na domácích konferencích jsem přednesl tři příspěvky:

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