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Short Review

Electrochemical Biosensors based on Acetylcholinesterase and Butyrylcholinesterase. A Review

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Two cholinesterases are known: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The both enzymes are presented in the body under physiological conditions and the both enzymes have broad use in pharmacology, toxicology and analyses. In the current paper, construction of electrochemical biosensors, a specific phenomenon in analytical chemistry, is reviewed. Development of voltammetric and potentiometric methods using standard electrodes, nanostructured materials, micro and nanoparticles is described here commonly with depicting of reaction principles and selection of substrates for the cholinesterases. Survey of actual literature is also provided within the article and disparate analytes used for the biosensors testing are introduced.

Keywords: Acetylcholinesterase; biosensor; butyrylcholinesterase; conductometry; nerve agent; pesticide; potentiometry; voltammetry

1. INTRODUCTION

While AChE (EC 3.1.1.7) is an enzyme with broad interest from pharmacologist developing new drugs, BChE (EC 3.1.1.8) has lower importance because of not clear biological function [1,2]. BChE is expressed by livers and secerned to blood plasma where can be used as a liver function test [2]. Role of AChE is well known because it is a part of cholinergic nervous system where it terminates excitation by hydrolysis of a neurotransmitter acetylcholine in nerve junctions, neuro-muscular junctions, blood and elsewhere [3-5]. The enzymes are sensitive to a wide number of chemical compounds that are able to inhibit them. The inhibition is relevant in therapy of some neurodegenerative disorders like Alzheimer disease or myasthenia gravis but nerve agents used in military and some agricultural pesticides are also inhibitors [6-8]. Considering physiological impact of

the inhibitors, the compounds can be indicated as neurotoxins interacting with cholinergic nervous system resulting in hyperaccumulation of acetylcholine because of AChE inhibition [9,10]. Cholinesterases are a diagnostic marker and analytical tools for measuring of their activity are constructed beside application in biosensors and similar techniques [11]; however, major focus in biosensors research is given on use of cholinesterases as biorecognition components.

The current review is focused on biosensors having cholinesterase as their crucial part important. Survey of actual literature in the field of cholinesterase based biosensors is given and examples of their application for inhibitors assays are given. The work attempts to provide critique look on the field.

2. CHOLINESTERASES AND THEIR INHIBITORS

Inhibitors of cholinesterases are a diversified group of chemical compounds able to interact with cholinesterases in some of the crucial structures responsible for substrate distribution or hydrolysis. A lot of inhibitors have equal affinity toward AChE and BChE but huge number of them is either selective to one of them or they have unequal affinity. It is caused by small structural dissimilarities between the two enzymes. In a global view, the similarities can be found over the whole family of enzymes esterases – lipases where cholinesterases belong [12,13]. Regarding to quaternary structure, the both AChE and BChE can occur in form of monomer G_1, dimer G_2, trimer G_3 and tetramer G_4 [14]. Molecular weight around 69 kDa for AChE subunit and 85 kDa for BChE subunit is known [15]. Cholinesterase can be further linked to a glycosylphosphatidylinositol anchor enabling fixation to cell membrane [16,17]. Three forms of AChE are currently known: $AChE_T$, $AChE_H$ and $AChE_R$. The forms are typical for mammals but evolutionary lower organisms like nematodes also have it [18]. From the three mentioned, $AChE_T$ is the most common type of AChE presented in the brain. It can be linked with Proline-Rich Membrane Anchor or anchored to collagen giving exact location to the enzyme [18,19]. $AChE_H$ is typical for blood where it is located on erythrocytes and it is associated with glycosylphosphatidylinositol anchor [20,21]. The third form of AChE, $AChE_R$, is not a common type and it is believed that it is expressed under stress and pathologic conditions [22,23].

Three sites of a cholinesterase are responsible for its enzymatic activity: active site, aromatic gorge and β anionic site (elsewhere known as peripheral anionic site). If we will try to imagine way of a substrate or an inhibitor, the compound meets cholinesterase on its β anionic site which is located on molecule surface. While AChE has β anionic site highly developed by presence of aromatic amino acids, BChE is not well equipped in this point of view. In example, Tyr 70 (Y70), Asp 72 (D72), Tyr 121 (Y121), Trp 279 (W279) and Tyr 334 (Y334) are the crucial amino acids in AChE (numbering valid for AChE from *Torpedo californica*) [24]. The huge number of aromatic amino acids has crucial significance for interaction with the both substrate acetylcholine and inhibitors by chemical interaction cation-π or π-π. The second part of path toward active site is called aromatic gorge. As the name proposes, it is composed from aromatic amino acids giving to the aromatic gorge properties close to peripheral anionic site: ability to bound compounds by cation-π and π-π interactions. While AChE has the gorge widely covered by aromatic amino acids, BChE had significantly lower number when

considered 14 aromatic residues in AChE against 8 in BChE [25]. When a compound passes the β anionic site and aromatic gorge, it will get to active site where, in case of AChE, natural substrate acetylcholine is split to acetic acid and choline. The active site has two subsites: the esteratic (esteric or ester in other sources) one and the α anionic subsite. While the α anionic subsite is responsible for proper orientation of acetylcholine by fixing it via interaction cation-π the esteratic subsite has the ability to split ester bound by a nucleophilic substitution mechanism. Amino acids Ser 200 (S200; numbering valid for *Torpedo californica* type of AChE), Glu 327 (E327) and His 440 (H440) are involved in the mechanism [26]. Just the Ser 200 is the terminal amino acid interacting with ester bound by its free hydroxyl moiety.

The both cholinesterases are inhibited by many chemical compounds as can be perceived from table 1. Two groups of inhibitors make covalent modification of the both cholinesterases resulting in their incapability to hydrolyze substrates: organophosphates and carbamates. These inhibitors are able to make stable ester with serine in esteratic subsite of active site. While organophosphates provide stable esters and mechanism of inhibition is standardly irreversible, carbamates makes instable esters with cholinesterases and carbamate moiety leaves the cholinesterase by spontaneous hydrolysis resulting in a full restoration of enzyme activity [27,28]. The mechanism of cholinesterase inhibition by a carbamate is called as pseudoirreversible. Nerve agents like sarin, soman, tabun, cyclosarin or VX can be introduced as notable organophosphorus inhibitors serving in military warfare or can be misused for terrorist activities [10,29,30]. These compounds causes irreversible inhibition of the both AChE and BChE, they are easily distributed throughout the organism and are able to cross blood brain barrier. Many former and currently used pesticides like organophosphates malaoxon and paraoxon or carbamate carbofuran are inhibitors of cholinesterases [31]. Some other pesticides like organophosphates malathion, parathion and chlorpyrifos are metabolically transferred to their analogous oxo-forms malaoxon, paraoxon and chlorpyrifos-oxon being responsible for the final inhibition of cholinesterases [32-34]. The former drug for Alzheimer disease and an anthelmintic metrifonate (trichlorfon) is also organophosphorus compound inhibiting cholinesterases [35,36]. Carbamate inhibitors of cholinesterases with pseudoirreversible mechanism of inhibition have broad use in pharmacology because of their use in therapy of e.g. Alzheimer disease or myasthenia gravis. Drug for Alzheimer disease rivastigmine is a blood brain barrier crossing inhibitor of cholinesterases while neostigmine and pyridostigmine serving in the therapy of myasthenia gravis and for anesthesia purposes have no or limited ability to reach brain hence they typically inhibit cholinesterases in the blood and peripheral nerves [5,37].

Reversible inhibitors of AChE and BChE are a wide group of compounds where structural similarities are hard to find. Plant alkaloids, aromatic compounds, metal containing coordination complexes or even metals themselves can be introduced as the inhibitors. Typical reversible inhibitors of cholinesterases acts as non-competitive one and they are either selective for AChE or they have at least significantly higher affinity to AChE than to BChE because of more frequent aromatic amino acids in AChE. These inhibitors make their inhibition by interactions with the aromatic amino acids in the α anionic subsite of active site, β anionic site and aromatic gorge. In this regard, galantamine (naturally occurring as an alkaloid in Caucasian snowdrop) is an exception because it acts as a competitive inhibitor with significantly higher affinity toward AChE than BChE [8,38,39]. Huperzine

and donepezil, important drugs used for Alzheimer disease therapy, can be introduced as typical non-competitive inhibitors of AChE. While huperzine is a group of structurally close alkaloids from *Huperzia* genus of Lycophytes where huperzine A is the most effective type [40-42], donepezil is a piperidyl derivative selective to AChE [43,44]. It was developed by Pfizer and Eisai and sold under trade name Aricept. Tacrine, a former drug for Alzheimer disease, has the very same mechanism AChE inhibition like huperzine and donepezil [45-48]. Some metallic ions were unveiled as weak non-competitive inhibitors of AChE with decreased affinity in a row Cu^{2+}> Al^{3+}> Ca^{2+}> Fe^{3+} [49]. Caffeine is another inhibitor of cholinesterases with significantly higher affinity toward AChE than BChE [50-54]. Aflatoxins, secondary metabolites from *Aspergillus* molds, have high affinity toward AChE while it is no inhibitor of BChE [55-58]. Ethidium and propidium are other non-competitive inhibitors of AChE [1,59-61].

Table 1. Basic facts about compounds inhibiting AChE and BChE

Inhibitor	Importance	Mechanism	Enzyme	References
Nerve agents (organophosphates) – sarin, soman, tabun, cyclosarin, VX	Poisoning compounds known from chemical warfare	Irreversible inhibition by covalent bound on serine in active site	AChE and BChE – equal affinity	[10,29,30]
Organophosphates chlorpyrifos-oxon, paraoxon, malaoxon	Former pesticides and metabolic products of less toxic pesticides chlorpyrifos, parathion, malathion	Irreversible inhibition by covalent bound on serine in the active site	AChE and BChE – equal affinity	[32-34]
Carbofuran (a carbamate compound)	Pesticide	Pseudo-irreversible inhibitor of cholinesterases, it makes covalent bound to serine in the active site but the bound is spontaneously hydrolyzed	AChE and BChE – equal affinity	[31]
Rivastigmine, neostigmine, pyridostigmine (carbamate compounds)	Drugs	Pseudo-irreversible inhibitor of cholinesterases, it makes covalent bound to serine in the active site but the bound is spontaneously	AChE and BChE – equal affinity	[5,37]

		hydrolyzed		
Galantamine	Drug for Alzheimer disease and secondary metabolite from a Caucasian snowdrop	Competitive inhibitor	AChE >>BChE	[8,38,39]
Huperzine	Drug for Alzheimer disease and secondary metabolite from *Huperzia* genus of Lycophytes	Non-competitive inhibitor	AChE >>BChE	[40-42]
Donepezil and tacrine	Current (donepezil) and former (tacrine) drugs for Alzheimer disease	Non-competitive inhibitor	AChE	[43-48]
Caffeine	Secondary metabolite from multiple plants, drug	Non-competitive inhibitor	AChE >>BChE	[50-53]
Cu^{2+}, Al^{3+}, Ca^{2+}, Fe^{3+}	Water soluble metallic ions	Non-competitive inhibitor	AChE	[49]
Aflatoxins	Natural toxins from *Aspergillus* genus	Non-competitive inhibitor	AChE	[55-58]

3. TOWARD BIOSENSORS

In a common sense, biosensors are analytical devices where a sensor part is in a tight junction to a part of biological origin. While the sensor part provides measureable signal, the part of biological origin provides selectivity to the assayed analyte [62]. The history of biosensors has started since early 1960s when Clark and Lyons made their pioneer work on biosensors for a fast assay of glucose using enzyme glucose oxidase and an electrode sensor [63,64]. There is currently known many types of biosensors using optical, voltammetric, piezoelectric and other sensor parts. Enzymes, sequences of DNA, antibodies, receptors, whole cells or tissues can be introduced as the parts of biological origin suitable for a biosensor construction. Biosensors having an electrochemical sensor and either AChE or BChE as the part of biological origin are discussed in this review.

The fact that cholinesterases are inhibited by the neurotoxic compounds can be easily utilized in their assay. Scale of inhibition is then proportional to concentration of inhibitor and the inhibitor is assayed thereof. Simplified principle of a biosensor based either on AChE or BChE is depicted in figure 1. The idea of a cholinesterase based biosensor has a big advantage in huge amplification of signal because cholinesterases have big turnover rate [1]. It results in a phenomenon that one molecule of an inhibitor prevent from splitting of thousands molecules of the used substrate. The assay has also

a significant disadvantage: the biosensor cannot be easily used repeatedly in case of positive prove of inhibitor.

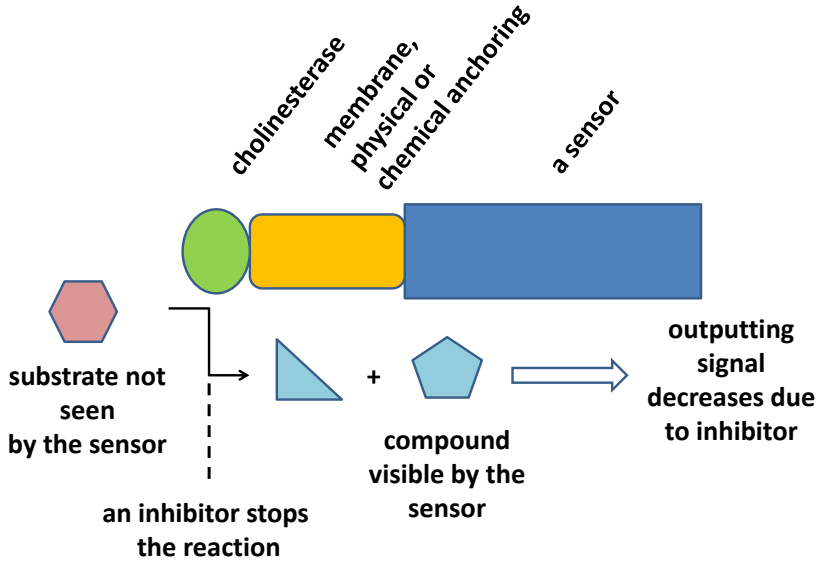

Figure 1. Schema of a biosensor containing either AChE or BChE

4. BIOSENSORS BASED ON VOLTAMMETRY

Voltammetry can be performed in several ways when cholinesterase selected as a recognition part. It is readily to be used when acetylthiocholine chosen as a substrate for AChE respectively butyrylthiocholine as a substrate for BChE. The substrates are converted to thiocholine and acetic respective butyric acid. Liberated thiol group is electrochemically active moiety that can be oxidized by an applied voltage and current is proportional to concentration of the thiols. Chemical principle of the assay is depicted in figure 2.

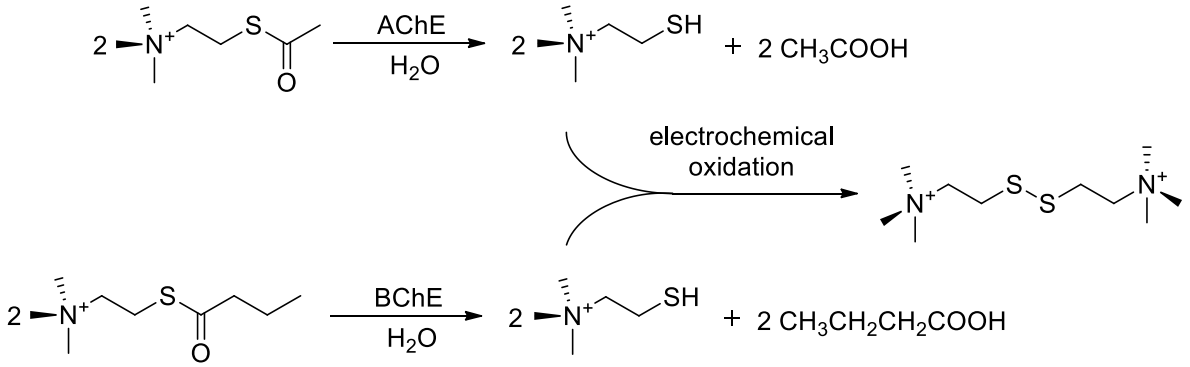

Figure 2. Voltammetric determination of cholinesterases activity using acetylthiocholine (top reaction) and AChE or butyrylthiocholine and BChE (bottom reaction).

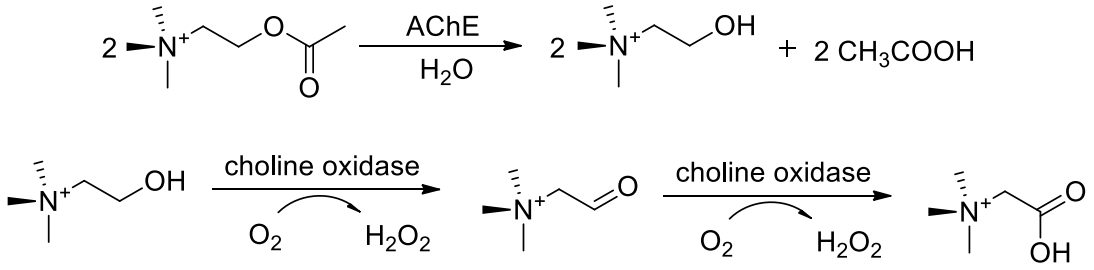

Figure 3. Production of choline by AChE and following oxidation of choline up to betaine by choline oxidase.

Combination of a cholinesterase with choline oxidase is another was how to make the reaction visible by voltammetry. Principle can be learned from figure 3. The assay uses combination of two enzymes either AChE or BChE and choline oxidase. In the first step, cholinesterase get to arise choline from either acetylcholine or butyrylcholine and the arising choline is oxidized in the second step by choline oxidase [65,66]. Betaine and hydrogen peroxide are the final products of chemical reaction.

The voltammetry was frequently chosen for development of cholinesterase based biosensors which can be learned from following examples. Nesakumar and coworkers elaborated a proposal of AChE based biosensors based on standard platinum working electrode further modified with ZnO nano particles [67]. The authors successfully adopted electrochemistry of reaction based on AChE catalyzed splitting of acetylthiocholine and following redox reaction thiocholine by cyclic voltammetry. The biosensor was applied for carbosulfan assay in rice samples resulting in limit of detection equal to 0.24 nmol/l. Santos and coworkers adopted cyclic voltammetry and impedance spectroscopy for the determination of pesticide carbaryl [68]. In the biosensor, AChE was immobilized on a layer composed from poly(amidoamine) with cysteamine core and the final device was suitable for the determination of carbaryl with limit of detection 0.032 µmol/l and linear range of the assay was from 1 to 9 µmol/l of carbaryl. Chen and coworkers constructed a nanocomposite from AChE, nafion, carbon nanotubes, tin oxide nanoparticles and chitosan [69]. The reaction of thiocholine was improved by application of ferrocyanide/ferricianide redox pair and the constructed biosensor was suitable for the determination of chlorpyrifos with limit of detection 0.05 µg/l using differential pulse voltammetry. Voltammetry was also performed with BChE based biosensors. The work by Turan and coworkers can be mentioned [70]. The authors immobilized BChE on graphite electrode chemically improved by poly(5,6-bis(octyloxy)-4,7-di(thieno[3][3,2-b]thiophen-2yl)benzo[c][1,2,5]oxoadiazole) which allowed embedding of the enzyme in the hydrophobic milieu. The constructed biosensor used butyrylthiocholine iodide as an enzymatic substrate and it was performed for the determination of paraoxon with limit of detection 0.212 µmol/l. Co-immobilization of two enzymes is more elaborative process that construction of a biosensors having only one enzyme. On the other hand, such biosensor can provide some improvement like use of a native substrate acetylcholine comparing to the artificial acetylthiocholine. In work by Hatefi-Mehrjardi, AChE and cholineoxidase were co-immobilized on surface of the mercaptopropinoic acid self-assembled monolayer on a gold electrode [71]. The biosensor was used for the determination of pesticide carbaryl with limit of detection 5.96 nmol/l and linear range 10 nmol/l – 1 mmol/l. The aforementioned applications are depicted in table 2.

Table 2. Voltammetric biosensors based on cholinesterases

Cholinesterase	Assayed compound	Principle of assay	Reported limit of detection	References
AChE	carbosulfan	acetylthiocholine hydrolysis to thiocholine and followed by cyclic voltammetry	0.24 nmol/l	[67]
AChE	carbaryl	cyclic respective linear sweep voltammetry of created thiocholine	0.032 µmol/l	[68]
AChE	chlorpyrifos	differential pulse voltammetry of thiocholine	0.05 µg/l	[69]
BChE	paraoxon	voltammetry of thiocholine from butyrylthiocholine iodide	0.212 µmol/l	[70]
AChE, cholineoxidase	carbaryl	chronoamperometry respective cyclic voltammetry of products from choline oxidation	5.96 nmol/l	[71]

Cholinesterase based voltammetric biosensors can be easily performed in a routine assay because of simplicity and typically good analytical parameters. On the other hand, there are significant disadvantages. In case of thiocholine oxidation, the biosensors are sensitive to interference by low molecular weight compounds that can be simply oxidized. Low molecular weight antioxidants like ascorbic acid, glutathione, uric acid and flavonoids can be mentioned. Thiol containing proteins are another potential intereferents. Price of primary commodities necessary for a biosensor construction is another drawback of voltammetric biosensors. Noble metals or at least outputting connectors covered with conductive layer are common in these biosensors. High prices of the materials restrain from simple introducing of final devices to mass production. Considering the aforementioned drawbacks, further miniaturization saving costs in combination with stable membranes protecting from interferences can be helpful for practical application of the biosensors.

5. BIOSENSORS BASED ON POTENTIOMETRY

AChE or BChE based biosensors working on potentiometric principle are suitable for the determination of enzyme activity using acetylcholine respective butyrylcholine. While acetic acid or butyric acid released from acetylthiocholine or butyrylthiocholine in voltammetric assay have no role in the measurement, potentiometric biosensors use them. The typical principle of a cholinesterase

based potentiometric biosensor is based on the acidification of solution by the aforementioned organic acids. Principle of such assay is depicted in figure 4.

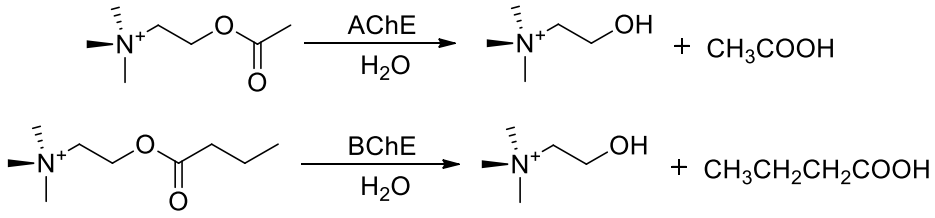

Figure 4. Hydrolysis of acetylcholine (upper reaction) by AChE and butyrylcholine (bottom reaction) by BChE providing monovalent organic acids: acetic acid and butyric acid.

Various compounds have been analyzed using the potentiometric principle and a cholinesterase. Stepurska and coworkers constructed a pH biosensor with immobilized AChE [72]. The biosensor used field-effect transistor (FET) and was suitable for the determination of aflatoxin B1 which was verified even for real samples like sesame, pea and walnuts. Another potentiometric biosensor was constructed by Arruda and coworkers [73]. The biosensor contained AChE immobilized on a self-assembled layer consisting from poly(allylamine) hydrochloride and silicon dioxide nanoparticles and it was presented as a device for assay of the both neurotransmitter acetylcholine in biological samples and for pesticide methamidophos. FET sensor served for studying of AChE activity for acetylcholine and following interaction with an inhibitor carbamylcholine [74].

Table 3. Potentiometric biosensors based on cholinesterases

Cholinesterase	Assayed compound	Principle of assay	Reported limit of detection	References
None immobilized	AChE, acetylcholine	selective membrane for acetylcholine	150 nmol/l for acetylcholine; 2×10^{-5} U/ml for AChE	[75]
AChE	phorate, parathion, chlorpyrifos, methamidophos, and dimethoate	selective membrane based on methylcellulose	10^{-7} mol/l	[76]
AChE	carbofuran, carbaryl	flow-injection pH biosensor with AChE immobilized on silica gel	0.02 – 8 ppm for carbofuran and 0.3 – 10 ppm for carbaryl	[77]

In another work, a sensor covered with a membrane fabricated from a plasticized polymer was made and proved to be suitable for assay of the both AChE and acetylcholine by potentiometric principle [75]. The potentiometric sensor exerted limit of detection 150 nmol/l for acetylcholine and

activity of AChE in a range 2×10^{-5} – 3.8×10^{-1} U/ml was also measured. Zhang and coworkers prepared a potentiometric biosensor having AChE immobilized in a membrane composed from methylcellulose, N,N-dimethylformamide, and bovine serum albumin [76]. The biosensor was used for assay of pesticides phorate, parathion, chlorpyrifos, methamidophos, and dimethoate with corresponding limit of detection equal for the all chosen pesticides 10^{-7} mol/l. In another work, pH selective electrodes were placed into a flow-injection system with AChE immobilized to silica gel [77]. The biosensor was tried on carbamate pesticides carbofuran and carbaryl in real water samples. The authors reported limits of detection 0.02 – 8 ppm for the carbofuran and 0.3 – 10 ppm for the carbaryl. A potentiometric biosensor was also constructed by Timur and Telefoncu [78]. The biosensor was based on AChE immobilized onto chitosan membrane over a pH electrode. The assay was found suitable for the determination of malathion, parathion-methyl, and methamidophos and the biosensor was used repeatedly by application of a oxime reactivator pyridine-2-aldoximethiodide. Examples of potentiometric sensors are shown in figure 3.

Potentiometric cholinesterase biosensors represent a less frequent type of such analytical device when compared to the voltammetric one. This type of biosensors has a big advantage. They can work with the native substrate acetylcholine and use of another reagent is not necessary. The overall simplicity is; however, outweighing by a disadvantage: problems connected with shift of pH. The cholinesterases, like every enzyme, have their pH optimum and they are processed in a buffered solution. For pH biosensors, the buffering capacity shall not be strong enough because releasing of butyric or acetic acid has to cause change of medium pH. In conditions of no or weak buffer is the enzyme unprotected and its activity can strongly variate. False positive finding of an inhibitor in acidic solutions and false negative in basic one can be expected as well.

6. CONCLUSION

Electrochemical biosensors are an extensively searched type of analytical devices with a broad use in analysis of various neurotoxic compounds like drugs, nerve agents and pesticides. They can be constructed by the both AChE and BChE; however, the first enzyme is the more common. Considering sensitivity of the known electrochemical biosensors toward inhibitor, they are able to distinguish very low amount of inhibitors and their limits of detection are close to more expensive and elaborative techniques. On the other hand, these biosensors have another disadvantage as well. Because many of the inhibitors causes irreversible inhibition, such biosensor has to be replaced by a new one immediately after positive prove. The limited use is caused by fact that the cholinesterase biosensors are disposable devices.

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