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Recent Advances in Amperometric Acetylcholinesterase Biosensor

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Abstract: Amperometric acetylcholinesterase (AChE) biosensors have emerged as a highly sensitive and rapid technique for toxicity analysis in environmental monitoring, food safety and quality control. AChE biosensors have superior properties over the existing techniques such as high-performance liquid chromatography or gas chromatography, because they can provide rapid, sensitive, simple and low-cost on-site detection. This review will highlight the research carried out during the last 5 years on AChE biosensor for determination of organophosphorous and carbamate pesticides in a wide range of samples. In this paper, various immobilization protocols and modified electrode methods used for constructing AChE biosensors are also introduced in detail. Future prospects toward the development of selective, sensitive biosensing systems are discussed. *Copyright © 2012 IFSA.*

Keywords: Biosensor, Acetylcholinesterase, Pesticides residue, Immobilization, Modified electrode.

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1. Introduction

Organophosphorous (OP) and carbamate pesticides (see structures in Fig. 1) [1] have been used widely in agriculture to protect crops and seeds before and after harvesting. They also have contributed significant health and economic benefits to society. At the same time, these compounds inhibit acetylcholinasterase (AChE) that hydrolyses the neurotransmitter acetylcholine (ACh), often causing severe impairment of nerve functions of human or even death [2-4].

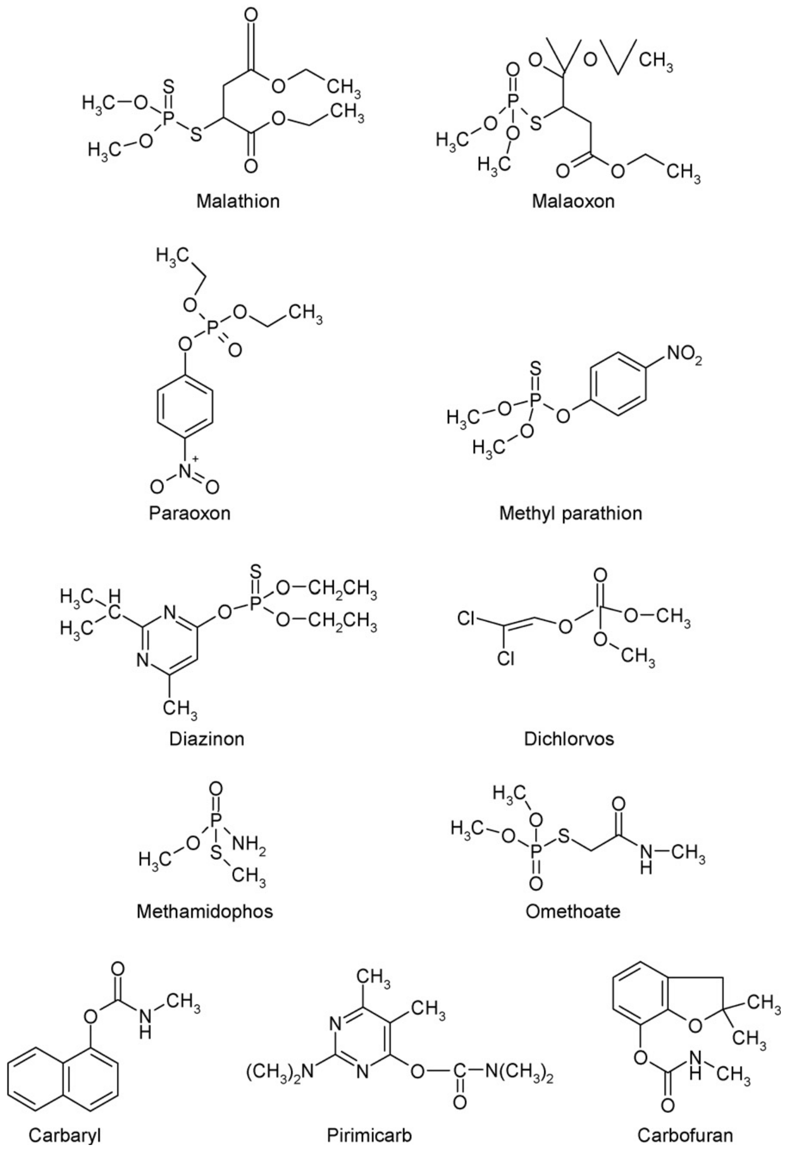


Fig. 1. Structures of the main pesticides used as targets in AChE biosensors.

At the present, the identification and quantification of pesticides are generally based on classical standardized chromatographic analysis techniques, such as gas chromatography (GC), high-pressure liquid chromatography (HPLC), capillary electrophoresis (CE) and mass spectrometry (MS). These analytical techniques have been described and reviewed extensively in the literature. They are very sensitive and reliable. However, they have some disadvantages such as complex, time consuming, require costly, bulky instrumentation and so on. Therefore, the development of rapid determination and reliable quantification detection methods has become increasingly important for human health and environment protection.

Eectrochemical biosensors have emerged the past few years as the most promising alternative to detect pesticides due to the high sensitivity inherent to the electrochemical detection and the possibility of portability and miniaturization [5-7]. Among them, amperometric AChE biosensors as a combination of enzymatic reactions with the electrochemical methods have shown satisfactory results for pesticides analysis, where the enzyme activity was employed as indicator of quantitative measurement of insecticides [8]. When AChE is immobilized on the working electrode surface, its interactions with the substrate of acetylthiocholine (ATCl) produce the electroactive product of thiocholine [3].The reaction equation is shown as follows [9-10].

$$\text{acetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{thiocholine} + \text{acetic acid} \qquad (1)$$

$$\text{2thiocholine} \longrightarrow \text{dithio-bis-choline}+2H^{+}+2e^{-} \qquad (2)$$

Furthermore, the anodic oxidation current is inversely proportional to the concentration of organophosphorus pesticides (OPs) in samples, and the exposed time as well. The procedure of the preparation of AChE biosensor and pesticides detection is shown in Fig. 2 [11-12].

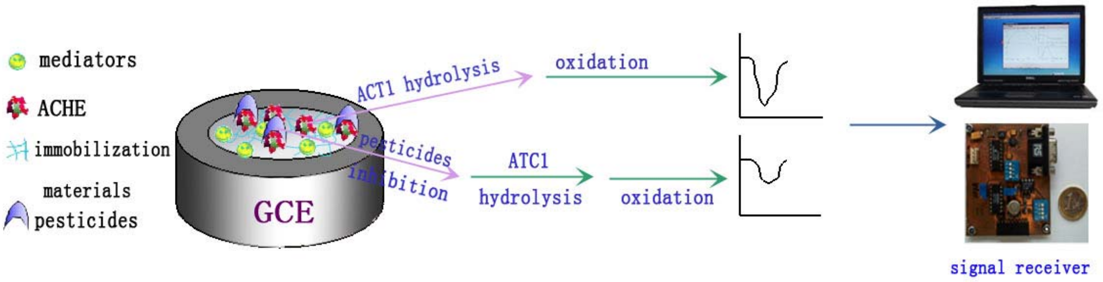


Fig. 2. The procedure of the preparation and detection of amperometric AChE biosensor.

Amperometric AChE biosensors are considered as sensitive and accurate methods in the pesticides determination due to their liner relationship between the current and concentration of the pesticides at relatively low substrate concentration. Many groups have reported these satisfied results about amperometric AChE biosensors for pesticides detection [2, 5, 8, 13-23]. Moreover, these biosensors had been the subject of several reviews [24-25] before five years ago. However, there is a time gap between current status in the field and the most recent reviews. Thus, in this review, we specifically provide an overview of the research carried out during the last 5 years relative to AChE inhibition-based biosensors for food and environment safety. We will review the key steps to construct an AChE biosensor including enzyme resource, the immobilization protocols used for formation of a bio-recognition interface, the electrode modification. We also will discuss the trends and challenges associated with designing a reliable AChE sensor for practical applications in detail.

2. AChE Source

It is well known that Drosophila melanogaster and the Electric Eel are commercially available and are the most widely used for biosensor fabrication. AChE has different substrate specificity and susceptibility to inhibitors from various extraction sources. Some research groups have reported the influences of different AChE sources on the enzyme activity and specificity to OP and carbamate pesticides [26-27]. Hence, in order to improve the performance characteristics of the AChE biosensors, the design and production of the appropriate enzymes is crucial. It is very difficult to find a natural enzyme with all the features required to construct the ideal biosensor. Nevertheless, protein engineering is helping researchers to manipulate enzymes in order to produce tailor-designed bio-recognition molecules for their integration into biosensing platforms.

Initial biochemical studies revealed that Drosophila melanogaster acetylcholinesterase (Dm. AChE) is 8-fold more sensitive than Electric Eel enzyme toward Ops, but the sensitivity has been increased to 12-fold by introducing a mutation [28]. Latter some research groups also have verified that the use of genetically modified AChE in biosensors has significantly increased their sensitivity to inhibition by OP pesticides [20-21, 29-30]. The design of mutation enzyme is to increase the affinity for the target analyte favouring the accessibility of the active site. Since the active site of Drosophila melanogaster (Dm) AChE, the most active wild form, is buried 20Å inside the protein and the entrance to the active site is very narrow, mutations have been mainly addressed to alter some residues of that region [31]. The replacement of glutamic acid 69 (Glu69), located at the rim of the active site gorge, by amino acids with bulky side chains, such as tryptophan (Trp) or tyrosine (Tyr), has been demonstrated to increase significantly the inhibition constant, ki, for dichlorvos [32]. This effect has been suggested to be due to interactions between those chains and the insecticide, which favour its way towards the buried active site. Double mutations, e.g. replacing Tyr71 by aspartic acid (Asp) additionally to the Glu69 substitution, have led to even more sensitive enzymes.

Since AChE can be inhibited by several compounds (not only organophosphate and carbamate insecticides, but also some toxins). Bucur et al. [33] developed biosensors for carbaryl, carbofuran and pirimicarb. Although they identified the best mutant for each pesticide, the high sensitivity provided by all biosensors towards all analytes did not allow the discrimination between them. However, to this day, the combination of engineered AChEs with different sensitivity levels for different analytes can not obtain satisfied selective biosensors.

3. AChE Immobilization

The immobilization of AChE is a key step in optimizing the analytical performance of an AChE biosensor in terms of response, sensitivity, stability, and reusability. The immobilization strategies most generally employed are physical or chemical methods. In general, they mostly fall into following methodologies:

(1) Physical adsorption: Physical adsorption generally consists of simple deposition of AChE onto the surface of working electrode and attachment of AChE through weak bonds such as Van der Waals forces and electrostatic interactions between the AChE and the transducer. It is the easiest and the least denaturing method, and it is generally non-destructive for enzyme activity because this technique does not involve any functionalization of the electrode materials or covalent links. However, this mode of immobilization are random orientation and weak attachment, therefore, the biosensors with adsorbed enzyme suffer from a poor operational and storage stability. Sotiropoulou's group immobilized AChE into the nano-structured conductive carbon with this technique. Using this biosensor, the monitoring of the organophosphorus pesticide dichlorvos at 1×10^{-12} M [34].

(2) Covalent coupling: Covalent coupling method immobilization AChE is the most widely used procedure. AChE can be covalently linked to the surfaces of a transducer through formation of a stable covalent bond between functional groups of AChE and the transducer. By comparing the performances of covalent with non-covalent immobilization method for the immobilization of AChE onto screen-printed carbon electrodes, it was concluded that when AChE was immobilized with non-covalent, the sensors detection limit was found to be about 10^{-10} M for the organophosphate pesticide dichlorvos, lower than covalent immobilization method [7]. The procedure provides increased stability of the AChE but decreases the activity of AChE and is generally poorly reproducible. Recently, this approach has also been utilized to immobilize acetylcholinesterase with glutaraldehyde [8, 10, 35, 36].

(3) Physical entrapment: Physical immobilization methods such as entrapment in sol-gel matrices and photo-polymerized monomers have also been used for AChE biosensors. The sol-gel process is a synthethic inorganic procedure, well known in material chemistry. Some research groups have reported the protocols using sol-gel to immobilize AChE. Shi et al. reported an AChE biosensor by entrapping AChE in Al_2O_3 sol-gel matrix in a screen-printed 3-electrode plastic chip. The biosensor

can be found the highest current signal at an operating potential of 0.25 V, and the detection limit for dichlorvos is achieved at 10 nM in the simulated seawater for 15 min inhibiting time [37]. Zejli et al. developed an AChE biosensor by entrapping AChE in Al_2O_3 sol-gel matrix on the sonogel-carbon electrode. The biosensor showed optimal activity at an operating potential of 210 mV, and the detection limit achieved for chlorpyriphos-ethyl-oxon was 2.5×10^{-10} M at a 10 min incubation time [38]. Yin et al. reported that using gold nanoparticles (AuNPs) and silk fibroin (SF) colloid immobilized AChE, and the detection limits were found to be 2×10^{-11} M for methyl paraoxon [39]. Zinc oxide sol-gel has also been used as a matrix for immobilization of AChE. The immobilized enzyme retained its enzymatic activity up to three months when stored in phosphate buffered saline (pH 7.4) at 4^{o}C [40]. Some research groups also obtained satisfied results that using the silica sol-gel incorporating gold nanoparticles (AuNPs) composite immobilized AChE onto GCE surface [11, 13].

Another physical entrapment method that could be applied for AChE immobilization involves encapsulation of enzymes during the electrodeposition of a conducting polymer such as polypyrrole (PPy) or polyaniline (PANI) in the presence of enzyme [41]. The method enables control of polymer/enzyme layer thickness, is extremely simple and rapid but requires a significant amount of enzyme. Polypyrrole and polyaniline copolymer doped with multi-walled carbon nanotubes was used to immobilize AChE. The synthesized PANI-PPy-MWCNTs copolymer presented a porous and homogeneous morphology which provided and ideal size to entrap enzyme molecules [5]. AChE was immobilized onto a three-dimensional porous network based on Au nanoparticles-polypyrrole anaowires, it exhibited a strong synergetic effect on improving the sensing properties of OP pesticides [3]. Valdés-Ramírez et al. reported that AChE was immobilized by entrapment in a photocrosslinkable PVA-SbQ polymer on a screen printed graphite electrode [30]. The principle of entrapment of enzymes in the photo-sensitive polymer was shown in Fig. 3 [19].

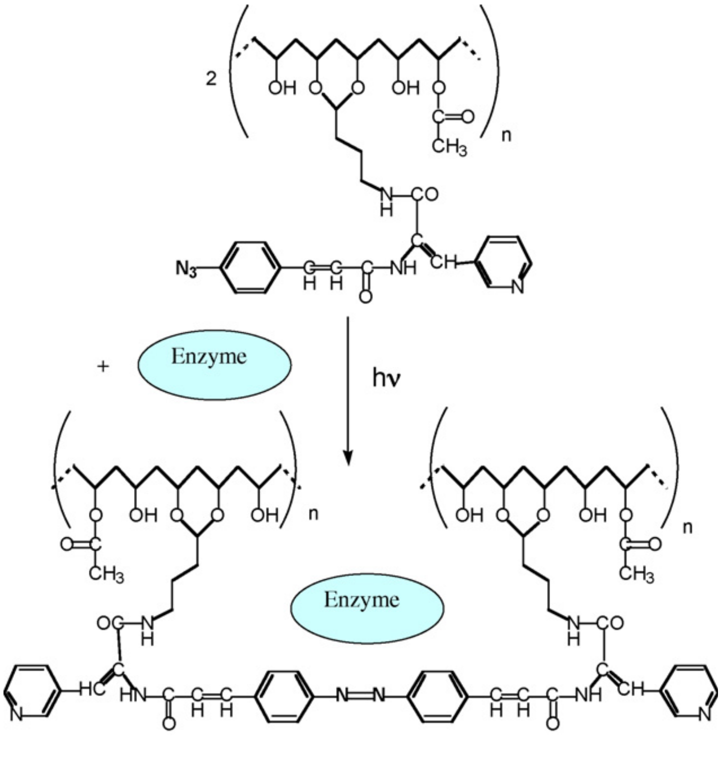


Fig. 3. The entrapment of enzymes in photo-sensitive polymer.

However, these methods suffer from several limitations such as leaking of biocomponent and possible diffusion barriers which restrict the performance of biosensor.

(4) Self-assembled monolayer (SAM): SAM may be generated by the spontaneous chemisorption of molecules onto a gold surface, thus, SAM as a platform can link biomolecules either using direct chemical linkages or by encapsulation with the help of polymeric supports. It is built of long-chained n-alkylthiols with derivatized organic functional groups, which are easily linked to the gold film via the thiol groups [42]. This technique was used to immobilize analyte derivatives onto the surface of gold-coated sensors. For example, Viswanathan et al. reported the method of the self-assembled monolayers (SAMs) of single walled carbon nanotubes (SWCNT) wrapped by thiol terminated single strand oligonucleotide (ssDNA) on gold to prepare nano-size polyaniline matrix for acetylcholinesterase (AChE) enzyme immobilization. The pesticides were determined through inhibition of enzyme reaction. The biosensor has been applied for the determination of methyl parathion and chlorpyrifos in spiked river water samples with the detection limit of 1×10^{-12} M [43]. Du et al. reported an immobilization method of AChE on cysteamine self assembled monolayers modified gold electrode for carbaryl detection. The detection linear ranges were from 1 to 50 ng/mL and from 50 to 500 ng/mL, with a detection limit of 0.6 ng/mL [15]. Another kind of AChE/dendrimers polyamidoamine (PAMAM)-Au/Carbon nanotubes (CNTs) layer- by-layer self-assembled technique is employed in the detection of carbofuran in samples with the range of 4.8×10^{-9} M to 0.9×10^{-7} M and a detection limit of 4.0×10^{-9} M [44]. Similar strategies are also reported for the preparation of AChE biosensor [17, 45-47]. In summary, this method possesses easy formations of ordered, pinhole free and stable monolayers, ensures the orientation and spatial control of the enzymes in the immobilization process and the absence of diffusion barriers, and provides suitable microenvironment for biomolecule immobilization, but the reproducible layering of biomolecules remains a major limitation.

The one-step protocol, based on the incubation of the gold electrode into an enzyme solution, not only simplified the construction of the biosensor but also provided more reproducible results. Du et al. reported one-step electrochemically deposited interface of chitosan-gold nanoparticles and one-step synthesis of multiwalled carbon nanotubes-gold nanocomposites for fabricating amperometric AChE biosensor, respectively. Gong et al also reported one-step fabrication of three-dimensional porous calcium carbonate-chitosan composite film as the immobilization matrix of acetylcholinesterase. These one-step protocols all obtained satisfying results [6, 18].

(5) Oriented immobilization: The aforementioned immobilized methods such as adsorption, covalent binding, entrapment within a polymeric matrix and crossing-linking may involve multiple-point attachment, loss of biomolecule activity , biomlecule leaking, random biomolecule orientataion and large diffusion barriers, which render biosensors poorly sensitive, unstable and with long response times. New trends focus on the development of protocols for the oriented immobilization of enzymes through specific functional groups located at their surface. In this way, active sites may be faced towards the target analytes present in the sample, and substrates and products may freely diffuse in the biological layer [1]. The procedure of oriented immobilization enzymes for pesticides detection was shown in Fig. 4.

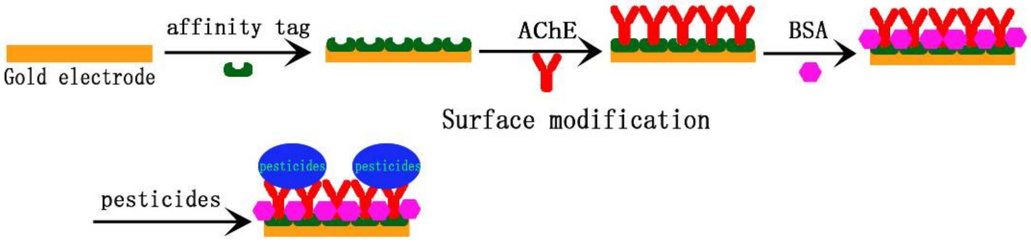


Fig. 4. Schematic representation of oriented immobilization enzymes.

Istamboulie et al. oriented immobilization AChE by metal chelate-functionalised magnetic microbeads. This method is based on the ability of some metal ion (Ni^{2+}, Cu^{2+}, Zn^{2+}) to bind strongly but reversibly to peptides and proteins containing histidine (His) or cysteine (Cys) residues. In affinity chromatography, chelating agent nitrilotriacetic acid (NTA) and iminodiacetic acid (IDA) are generally used to bind these metal ions on solid supports to perform His-taggedenzyme purification. In this work, pre-activated magnetic beads carrying Ni-IDA complexes have been used to immobilize a genetically modified AChE (B394) having engineered a hexa (histidine) tail. This method provides a basis for controlled and oriented immobilization of the enzyme on different supports, opening the way for new approaches to enzyme immobilization. The general principle of this procedure is presented in Fig. 5 [19].

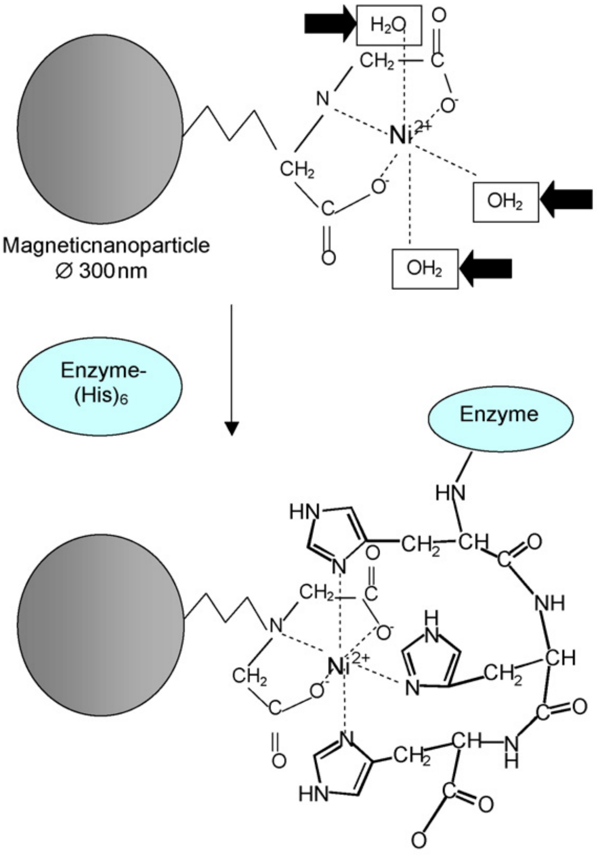


Fig. 5. Schematic representation of enzyme immobilization on magnetic microbeads via Ni–His affinity. Black arrows show histidine-exchangeable water molecules.

(6) Immobilization on membrane materials: Enzyme was usually immobilized on electrode surfaces directly, which has many disadvantages, such as weak protection of the immobilized enzyme, elaborate pretreatment of the electrode surface before the actual immobilization, etc. Therefore, in the recent years, the membrane support materials become important alternative immobilization AChE strategies. Because it can prolong the enzyme storage life, possess the convenience of storing only the membranes, not whole electrodes and it can be easily replaced when enzyme's activity is lost, and have not the necessity of constantly cleaning the electrode surface after each immobilization procedure. Moreover, there are multiple options for analyte detection (one electrode-multiple membranes-multiple enzymes). Recently, many research groups reported that enzyme was immobilized on different membrane materials, indicating the higher activity of AChE, higher sensitivity of sensor and simplifier operation [10, 48-52].

4. Electrode Modification

It is well known that the principle of amperometric AChE biosensor is to detect the concentration of the pesticides by measuring the change of the oxidation current of thiocholine. However, thiocholine oxidation occurs at relatively high potential that cause high background current and interference from other electroactive compounds [53]. Thus, appropriate selection of the electrode modified material and immobilization chemistry is essential for a reliable biosensor [24]. However, in most of the cases, native materials do not meet these requirements and therefore additional modifications are necessary in order to allow further attachment of the enzyme. To overcome this problem, mediators, such as tetracyanoquinodimethane (TCNQ) [33], Prussian blue [54,10], cobalt-phthalocyanine (CoPC) [55, 30] and some nanoparticles including carbon nanotubes (CNT) [13, 18, 52], gold nanoparticles [2, 11], CdTe quantum dots [15] have been successfully used to decrease the applied potential versus Ag/AgCl or saturated calomel electrode (SCE), hindering the oxidation of other compounds thus reducing the interferences. These electronic mediators are generally modified on the surface of the working electrode.

Recently, some new trends about electrode modification focus on the new modification materials and composite modification materials applications. Istamboulie et al. demonstrated poly (3,4-ethylenedioxythiophene) (PEDOT), a new conducting polymers materials for AChE biosensor applications. The new material as mediator is suitable for thiocholine oxidation, allowing the measurement of AChE activity at 100 mV vs. Ag/AgCl [22]. In addition, the nanocomposites are also gradually used for constructing AChE biosensor, for example, it is well known that carbon nanotubes (CNT) are an important class of materials with unique and interesting properties, like high mechanical strength, high aspect ratio, high electrical and thermal conductivity, metallic and structural characteristics. At the same time, Au nanoparticles have also been reported to provide immobilization sites for the enzymes retaining their biocatalytic activity. They also permit direct and fast electron transfer between redox species and the electrode. These properties make Au nanoparticles an attractive material for sensing biological reactions. At present, some research groups reported the synthetical effect of Au nanoparticles and CNTs for OP pesticides sensing [4, 18]. Others composite modification materials were also reported such as CdTe quantum dots/gold nanaoparticles by Du et al. [16] and multi-walled carbon nanotube/cobalt phthalocyanine by Moraes et al. [56], respectively.

Various immobilization protocols and electrode modification materials (mediators) used for constructing AChE biosensors were show in Table 1 in detail.

5. New Trends and Challenges

5.1. Miniaturization

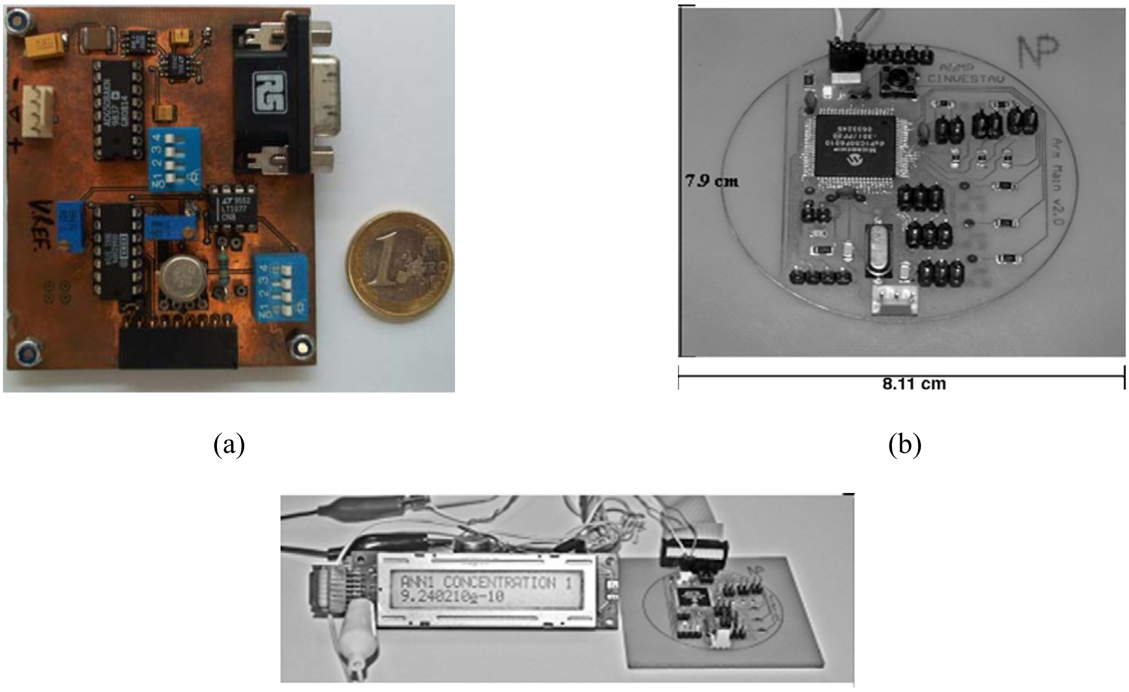
New analytical approaches are oriented to the development of portable systems with high accuracy, low-cost, short-time response, and that can provide qualitative information about the composition of a sample with minimum preparation. With this aim, Steinberg and Lowe [58] described the development of a potentiostat with low-power consuming and high accuracy. A portable electro-chemical instrument which can be used in neurotoxic agents in water have been designed by Nagatani et al. [59] and Hildebrandt et al. [12, 60], respectively. Fig. 6A was the picture of the miniaturized electronic plate that functions as a potentiostat designed by Hildebrandt group.

Table 1. Amperometric AChE biosensors for pesticides detection.

Inhibitors	AChE source	Immobilization matrix	Mediator/ electrode	LOD	Samples	Incubation time (min)	Reference
Paraoxon,	Electric eel	Al_2O_3 sol-gel	Sonogel-	7.5×10^{-9}M	-	10	[38]
dichlorvos,			carbon	5×10^{-10}M			
chlorpyriphos-			electrode	2.5×10^{-10}M			
ethyl-oxon							
dichlorvos	Electric eel	Al_2O_3 sol-gel		10nM	river	15	[37]
			-/SPE		water		
				1×10^{-12}M			
methyl parathion	Electric eel	Polyaniline matrix		1×10^{-12}M	river	15	[43]
Chlorpyrifos			SWCNT/Au		water		
				2×10^{-11}M			
methyl paraoxon	Electric eel	AuNPs-SF		1×10^{-10}M	-	15	[39]
Carbofuran			AuNPs/pt	2×10^{-19}M			
Phoxim							
				2nM			
dimethoate	Electric eel	MWCNTs-β			garlic	8	[17]
		cyclodextrins	MWCNTs/GC	0.6ng/ml			
malathion	Electric eel	MWCNTs-Au-	E		garlic	8	[18]
		CHIT		0.6ng/ml			
monocrotophos	Electric eel		MWCNTs-	1.0ng/ml	-	10	[2]
malathion	Electric eel	AuNPs-SiSG sol-	Au/GCE		-	15	[5]
		gel	AuNPs/GCE	0.035ppm			
paraoxon	Electric eel	PANI-PPy-	MWCNTs/GC	24ppb	-	10-30	[40]
Aldicarb	Electric eel	MWCNTs	E	25ppb	water	30	[54]
Carbaryl		copolymer		1.3×10^{-11}M			
Chlorpyriphoso-	Drosophila	ZnO sol-gel	ZnO		-	-	[19]
oxon	B394	-	PB/SPE	8ng/ml			
Coumaphos	Electric eel	Magnetic	Magnetic	4×10^{-9}M	honey	20	[57]
		microbeads	microbeads/				
Chlorpyrifosoxon	Electric eel		SPE	150nM	-	10	[22]
		PB	PB/SPE	40nM			

Paraoxon ethyl	Electric eel			40µM	-	25	[36]
Sarin		Entrapment in a	PEDOT:PSS/				
Aldicarb		AWP	SPE	0.6ng/ml		30	[4]
		Cross-linking with	Au-Pt				
Carbaryl	Electric eel	glutaraldehyde	NPs/GCE	4×10^{-9}M	garlic	10	[14]
		AChE droped on	Au-MWNTs/				
Carbofuran	Electric eel	modified electrode	GCE		onion,	6	[44]
		CdTe-Cys SAMs	CdTe-Cys	2.5ng/l	lettuce,		
Dichlorvos			SAMs/Au	15ng/l	cabbage		
Omethoate	Dm	PAMAM-Au/	PAMAM-Au/	5ng/l	-	10	[10]
Trichlorfon		MWNTs	MWNTs/GCE	10ng/l			
Phoxim				7×10^{-11}M			
		CHIT membrane	PB/GCE				
Dichlorvos	Dm mutant				apple	10	[30]
	B394						
		Entrapment in a					
		PVA-SbQ polymer	CoPC/SPE				

LOD: limit of detection, SWNT:single wall carbon nanotube, gold nanoparticles: AuNPs, SF: silk fibroin, MWNTs: multiwalled carbon nanotubes, chitosan: CHIT, silica sol-gel:SiSG, polyaniline: PANI, polypyrrole:PPy, SPE: screen printed electrode, PB: Prussian-blue, PEDOT: poly(3,4-ethylenedioxythiophene), PSS: poly(styrenesulfonate), AWP:polyvinylalcohol-based photopolymer, GCE: glassy carbon electrode, NPs: nanoparticles, SAMs:self-assembled monolayers, Cys:cysteamine, PAMAM:dendrimers polyamidoamine, Dm:Drosophila melanogaster, CoPC:Cobalt phthalocyanine.



(a) (b)

(c)

Fig. 6. Picture of the miniaturized electronic plate that functions as a potentiostat.

5.2. High Throughput and Quantification Detection

AChE-based biosensors have a major drawback: they give a sum parameter of AChE-inhibition without any qualitative or quantitative information about the individual analytes. One approach to solve this problem involves the application of multi-sensor arrays that are combined with the data processing of artificial neural networks. Using micro or nano-sensor arrays will likely become a new trend. Thus, the ability to construct arrays of enzymes will likely allow current multianalyte detection of several compounds to be expanded to accommodate the analysis of perhaps hundreds or thousands of separate compounds [61]. One of the challenges that must be met for this type of system would be the development of parallel computational methods to convert electronic responses for each analyte into meaningful concentration data. In this respect progress has been reported for artificial neural network implementation in single low-cost chip for the detection of insecticides by modeling of enzymatic sensors response [20, 62]. The design of single chip to selectively quantify mixtures of the pesticides chlorpyrifos oxon and chlorfenvinfos by an artificial neural network implementation was shown in Fig. 6B, and the dedicated system was shown in Fig. 6C [62].

5.3. Integration of Detection System

To improve sample throughput or allow online monitoring of the inhibition processes, flow-injection analysis (FIA) systems have proved to be a popular approach and capable of performing continuous analysis. Recently, the use of micro-fluidic devices as minute FIA systems has attracted increasing attention due to their significant reduction of reagent consumption and low operating costs as well as high throughput capability. He et al. described the development of a silica based, immobilized enzyme micro-reactor, with an integrated micro-fluidic electrochemical detector for the rapid evaluation of enzyme kinetics [63]. The micro-fluidic device was shown in Fig. 7.

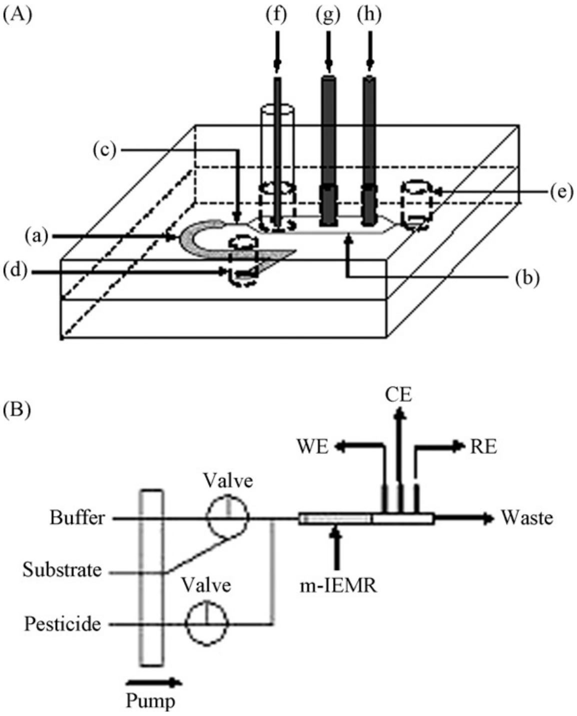


Fig. 7. Schematic of the micro-fluidic device: (a) monolith channel, 600 μm wide, 50 μm deep and 20 mm long; (b) electrochemical detection channel, 1.5 mm wide, 50 μm deep and 20 mm long; (c) link channel, 100 μm wide, 50 μm deep and 5 mm long; (d) inlet, 1.5 mm diameter, to which syringe a pump was linked through a plastic tube (0.5 mm diameter); (e) outlet, 1.5 mm diameter; (f) working electrode (WE, pt disc, 0.5 mm diameter), (g) Pt wire (1 mm diameter) counter electrode (CE) and (h) Ag/AgCl (1mm diameter) reference electrode (RE).

5.4. Real Samples Detections

The majority of AChE biosensors reported to date have been designed for detection of pesticides in water samples. Application to other matrices such as food samples (fruits and vegetates) has been restricted due to the problems related to the use of these devices in the presence of organic solvents extracts. Several works reported that enzyme biosensors can function in a mixed aqueous-organic phase in low amounts of organic solvents [44]. However, these devices suffered from a low sensitivity and have not been used for the quantification of pesticides in solid sample.

Simple sample pretreatment methods for AChE biosensor need further research, because it hinders the real application of biosensor.

5.5. Combined Sensor for OP and Carbamates Detection

Further new trends will likely focus on combined sensor for OP and carbamates detection. For example, multifunctional biosensor will be applied for detecting different analytes simultaneously at food samples. Teller et al. developed a dual piezoelectric/amperometric sensor for the detection of two unrelated analytes in one experiment, which can simultaneously completed the detection of cocaine with a dynamic range from 10^{-9} to 10^{-7} M and the organophosphate chlorpyrifos-oxon range from 10^{-6} to 10^{-8}M [64]. In addition, AChE-choline oxidase based bienzymatic or multienzyme sensor will be paid more attention for OP and carbamates detection due to high sensitivity and reliable [29, 36, 57]. Moreover, Wei et al. developed photoelectron-synergistic catalysis for detecting organophosphorus pesticides exhibits another trends of AChE biosensor. Using composite nanostructured $PbO_2/TiO_2/Ti$

modification material decrease applied potential. This strategy was found to catalyze the oxidative reaction of thiocholine effectively, make the AChE/PbO_2/TiO_2/Ti biosensor detect the substrate at 0.30 V, hundreds mill-volts lower than others reported [65].

6. Conclusions

In summary, amperometric acetylcholinesterase biosensors are strong candidates for screening pesticide residues and they become more and more relevant in environmental and food analysis. Compared to traditional chromatography and other methods, the strengths of AChE biosensor can be described as follows:

- They are very selective and sensitive.
- They can be carried out for use in the field.
- They can work with complete automation and give the results after a short period of time.

However, these AChE biosensors still face many challenges hindering their real applications:

- The data are analyzed on the electrochemical analysis instrument, and they are not miniaturization and portable.
- They only give a sum parameter of AChE-inhibition without quantitative information and high throughput detection.
- They do not combine with FIA system to build integration instrument.
- They have not specific and suitable real samples pretreatment.

Acknowledgments

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