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REVIEW ARTICLE

Biosensors based on cholinesterase inhibition for insecticides, nerve agents and aflatoxin B_1 detection (review)

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Abstract The present review reports the research carried out during last 9 years on biosensors based on cholinesterase inhibition for nerve agents, organophosphorus and carbammic insecticides, and aflatoxin B_1 detection. Relative applications in environmental and food areas are also reported. Special attention is paid to the optimization of parameters such as enzyme immobilization, substrate concentration, and incubation time in the case of reversible inhibition by aflatoxin B_1 or irreversible inhibition by organophosphorus and carbamic insecticides, and nerve agents in order to optimize and improve the analytical performances of the biosensor. Evaluation of selectivity of the system is also discussed.

Keywords Biosensors · Inhibition · Insecticides · Cholinesterase · Nerve agents · Aflatoxin

Introduction

It is well known that in the measurement of analytes by means of biosensors two different approaches can be carried out: i) if the enzyme metabolises the analyte, the analyte can be determined measuring the enzymatic product; ii) if the analyte inhibits the enzyme, the decrease of the enzymatic product formation can be measured and correlated to the analyte concentration. In the latter case, this type of biosensor is called "biosensor based on enzyme inhibition". The first biosensor based on cholinesterase (ChE) inhibition for detection of nerve agents was developed by G.Guilbaut in 1962 [1] and from this one, a lot of ChE biosensors were developed for several compounds such as heavy metals [2, 3], organophosphorus and carbammic insecticides [4–8], toxins [9, 10], glycoalkaloids [11, 12], drugs [13–15], fluoride [16, 17], cocaine [18, 19] and nicotine [20, 21]. In details, during the last 9 years more than 100 papers were published on the ChE biosensors. As reported in the Fig. 1 the 78% of the papers reports ChE biosensors for insecticide detection (), 3% for drugs (), 3% for nerve agents (), 2% for heavy metals (), 5% for glycoalkaloids (), 4% for toxins such as aflatoxin B_1 (AFB_1) () and the last part for other inhibitors such as fluoride, nicotine and cocaine measurement. This trend is due to several factors:

The *lower* percentage of papers based on ChE biosensor for measurement of:

i) Heavy metals is due to the low sensitivity towards this type of inhibitor (ppm levels)
ii) AFB_1 is owing to the very recent discovery of the AFB_1 power to inhibit the acetylcholinesterase (AChE)
iii) Nerve agents are ascribed to the high level of safety required to measure them
iv) Drugs because the ChE biosensor can be used as screening analysis of them but for quality drugs measurement the U.S. Food and Drug Administration requires high selective methods
v) Glycoalkaloids because are toxic compounds found only in Solanaceae plant family such as potato

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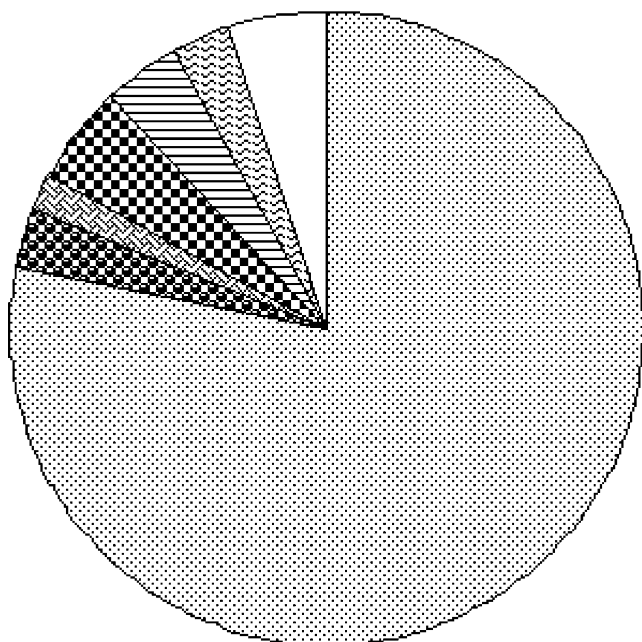


Fig. 1 Inhibitor distributions in enzymatic cholinesterase biosensor investigations. Inhibitor: insecticides (), drugs (), nerve agents (), heavy metals (), glycoalkaloids (), toxins () and last part for other inhibitors such as fluoride, nicotine and cocaine

The *higher* percentage of the papers based on ChE biosensors reports measurements of insecticides owing to the very high sensitivity towards these compounds (ppb levels) because of their simple procedure for safety manipolation than nerve agents, also their wide use and thus their presence in food and environment.

Nowadays, several reviews based on ChE inhibition biosensors have been published. Taking in consideration the papers published from 2000, six reviews appeared focused on ChE biosensors. In 2006 Andreescu and Marty [22] wrote an interesting review published on Biomolecular Engineering Journal based on ChE inhibition, principally related to organophosphorus and carbammic compounds detection. The review reports the research efforts over the last 20 years in AChE biosensors showing also the different configurations and fabrication techniques, particularly those based on low-cost electrochemical sensors. In 2008 Pohanka et al. wrote a short review in Protein and Peptide Letters, focusing it on ChEs immobilization and on the ways of converting ChE activity into an output signal [23]. In the 2009, the same research group has reviewed in Current Medicinal Chemistry Journal AChE and butyrylcholinesterase (BChE) biosensors for the detection of various compounds such as organophosphorus and carbammic insecticides and nerve agents [24]. In the latter case, it was highlighted the possibility of using the ChE biosensor as a tool in medicinal chemistry and toxicological research. The use of ChE based amperometric biosensors for the assay of anticholinergic compounds was also reviewed by the same authors on Interdisciplinary Toxicology Journal [25]. The same year, the use of esterase enzymes for detection of chemical neurotoxic agents was reported in Proteins and Peptide Letters by Manco et al. reviewing the biosensors based on ChE or carboxylesterase [26]. The last review on ChE biosensor was reported in 2009 on Sensor journal by Periasamy et al. [27]. This interesting review reports a recent research work on ChE biosensor using nanomaterials for organophosphorus insecticides detection.

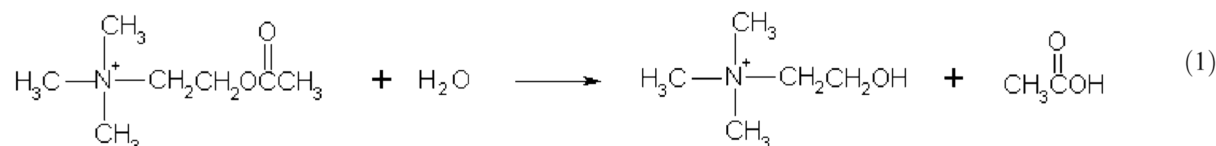
In order to avoid overlappings and repetitions of already existing reviews we attempted to concentrate this paper on the biosensors based on ChE inhibition for organophosphorus and carbammic insecticides, nerve agents and aflatoxin B_1 underlying the different types of inhibition (reversible or irreversible). Our purpose was focused on how to optimise the analytical performance of ChE biosensor such as reaching the lowest detection limit or reducing the interferences by the diagnosis of the inhibition type.

Biosensor based on cholinesterase inhibition

In order to develop a biosensor based on enzyme inhibition, in our view, it is relevant to know the structure of ChE enzyme and the mechanism of inhibition in order to better optimise several parameters which affect the degree of inhibition such as enzyme loading, incubation time, reaction time, concentration of substrate, pH and immobilisation method. In this way, a briefly description of the structure of ChE enzyme and its kinetic will be reported and correlated to the target analytes of this review (organophosphorus and carbammic insecticides, nerve agents and AFB_1).

Cholinesterase enzymes

The principal biological role of AChE is the termination of the nervous impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine.



$$H_3C-N^+(CH_3)_2-CH_2CH_2OC(=O)CH_3 + H_2O \longrightarrow H_3C-N^+(CH_3)_2-CH_2CH_2OH + CH_3C(=O)OH \quad (1)$$

Early kinetic studies indicated that the active site of AChE contains two sub-sites, the esteratic and anionic sub-sites, corresponding respectively, to the catalytic site and choline-binding pocket [28]. The esteratic site contains a serine residue which reacts with the substrate and, also, with the organophosphates (insecticides and nerve agents) and carbamates (insecticides). This site is similar in the multiple forms of AChE (*Electrophorus, Torpedo*, rat and chicken) and it is also located in the BChE enzyme. For this reason, it is possible to use several species of AChE and BChE enzymes to develop a ChE biosensor for insecticides and nerve agent detection. The AChE enzyme is also peculiarly characterized by a deep and narrow gorge that penetrates halfway into the enzyme and widens out close to its base where there is the active site [29]. The substrate can arrive to the active site penetrating into the gorge. Dougherty et al. [30] presented theoretical considerations as well as the experimental data on the aromatic character of the gorge which plays a key role for the detection of AFB_1. As reported recently by Hansmann et al. [31] the AFB_1 inhibits AChE by binding at the peripheral site, located at the entrance of the active site (at the tryptophane residue). This behaviour is peculiar of AChE enzyme; for this reason, as reported below, the AChE is more sensitive than BChE for AFB_1 detection [31, 32]. The knowledge of the structure of ChE enzyme can be an instrument to understand which type of ChE should be used in order to develop a biosensor with increasing selectivity; in fact, if it is required to measure insecticides in a food sample in which can be present both AFB_1 and insecticides, the insecticides could be measured using BChE, in order to reduce the interference of AFB_1.

Enzyme inhibition

Biosensors based on enzyme inhibition have found wide application for detection of toxic analytes that inhibit the normal enzyme function. The detection of the analyte is simply based on the determination of the difference in enzyme activity in the presence and absence of inhibitor, according to the following the Eq. 2:

$$I\% = [(A_0 - A_i)/A_0] \times 100 \qquad (2)$$

where A_0 is the activity in absence of inhibitor, and A_i in presence of inhibitor. Important parameters are defined as: "incubation time", the time of contact between enzyme and inhibitor, "reaction time", the time of the reaction between substrate and enzyme. The linear range is usually comprised between 20% and 80% of inhibition and the detection limit is usually defined as the amount of inhibitor which gives the decrease 20% of inhibition [33].

The formula reported above is used by both reversible and irreversible inhibition biosensors, but there is a substantial difference between these two kind of systems. Irreversible inhibition (i.e. nerve agents) is characterised by covalent bonding between the ChE enzyme and the inhibitor, and thus requires either a new biosensor after the inhibitor measurement or a reactivation of the biosensor in use. Reversible inhibition, on the other hand, is characterised by non-covalent interaction between inhibitor (AFB_1) and AChE enzyme with the consequent restoration of the initial activity after the inhibitor measurement. We can summarise the inhibitor of ChE investigated in this review as:

- Irreversible inhibitors (organophosphorus insecticides and nerve agents)
- Pseudo-irreversible inhibitor (carbammic insecticides)
- Reversible inhibitors (AFB_1)

However in the case of carbamate, the acylated intermediate is slowly hydrolysed to reactivate the enzyme, usually their half life is in order of hours [34, 35] and, because in the biosensor field the times of analysis have to be short (less than 1 h), we can consider also carbamates as irreversible inhibitors.

Cholinesterase biosensors for insecticides and nerve agents detection (irreversible inhibitors)

Organophosphorus, carbammic insecticides and nerve agents

The detection of pesticide residues in food, water and soil is one of the major issues for the analytical chemistry. Pesticides are, in fact, among the most important environmental pollutants because of their increasing use in agriculture. For this reason, most countries have established maximum residue levels (MRL) in food products [36]. Among the pesticides, organophosphorus and carbammic insecticide species are the most used, due to their insecticidal activity and relatively low persistence in environment.

The fact that nerve agents belong to organophosphorus compounds is due to the accidentally discover of these compounds in 1936 by Dr. Gerhard Schrader, working for IG Farben in order to develop new types of insecticide. Schrader experimented numerous fluorine-containing compounds that lead to the preparation of Tabun. After, Sarin, Soman and Cyclosarin were also synthesised (nerve agents G series). The G-series are named labelling Tabun as GA (German Agent A), Sarin as GB, Soman as GD, and Cyclosarin as GF. The V-series is the second family of nerve agents: VE, VG, VM, VR and VX.

To detect the organophosphorus and cabammic compounds chromatographic methods such as High Performance

Liquid Chromatography (HPLC) or Gas Chromatography (GC) usually coupled to mass spectrometry (MS) [37–40] are used as reference methods, but they present strong drawbacks such as complex and time-consuming treatments of the samples, i.e. extraction of pesticides, extract cleaning, solvent substitution etc. [41–43]. Moreover, the analysis usually has to be performed in a specialised laboratory by skilled personnel and it is not suitable for "in situ application". ChE-based biosensors are considered as one of the best alternatives for the detection of these compounds [44, 45].

Measurement protocol of insecticides solution

The inhibitory effect of insecticides on ChE was evaluated by determining the enzymatic activity after and before the exposure of the biosensor to the inhibitor. To do this, the measurement can be carried out using three different protocols.

In the first one, that in this context we called protocol A, the measurement can be performed by means a *single step*. In details, the biosensor was immersed in a buffer solution, the substrate was then added and the signal registered, after, the inhibitor was added in the same solution and a decrease of current was observed (Fig. 2, protocol A). The concentration of insecticide is then calculated measuring the enzymatic activity before and after the addition of inhibitor in solution [46]. This method is not so often adopted because the absence of incubation time allows to reach a higher detection limit.

In the second protocol (called protocol B) the measurement is carried out using *two steps*: the biosensor is

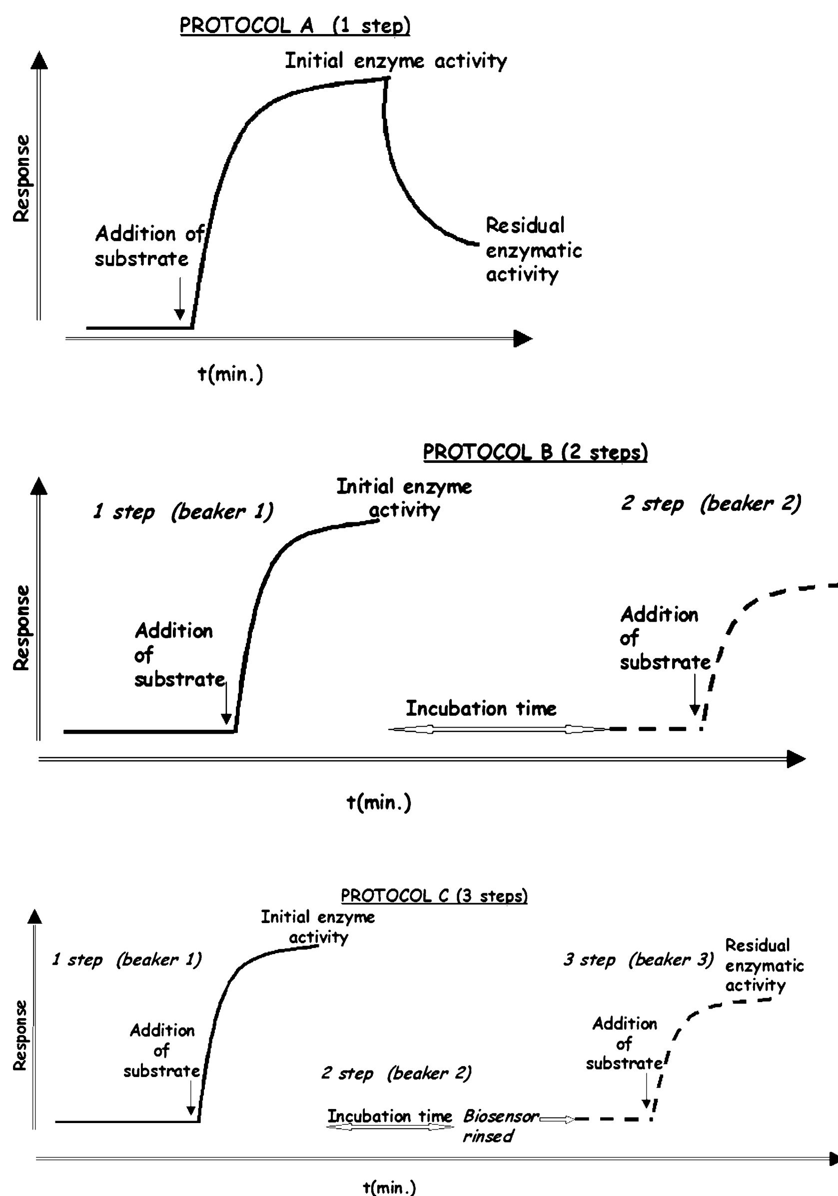


Fig. 2 Description of measurement protocols and response using a single step (protocol A), two steps (protocol B) and three steps (protocol C) mode for insecticides detection

immersed in a buffer solution, the substrate is then added and the signal registered (step 1). The ChE biosensor is then immersed in the insecticide solution for a certain period (incubation time) and after, in the same insecticide solution, the substrate is added and the residual activity measured registering the signal (step 2). The concentration of insecticide is then calculated applying the Eq. 2 (Fig. 2, protocol B) [47]. In this protocol is possible also to add in the working solution both insecticide and substrate (in the step 2) but as reported by Nikolelis et al., this system is less sensitive then the previous reported (protocol B) [48].

The third one (protocol C called also "medium exchange method") is performed in *three steps*: The biosensor is immersed in a buffer solution, the substrate is then added and the signal registered (step 1). After, the ChE biosensor is immersed in the insecticide solution for a certain period (incubation time) (step 2). After that, the biosensor is rinsed several times with distillate water. The biosensor is then immersed in a new solution of buffer and the substrate added, thus residual activity was measured (step 3) (Fig. 2, protocol C). The concentration of insecticide is then calculated applying the Eq. 2 [49, 50]. Using the "medium exchange method" is possible to avoid both i) electrochemical and ii) enzymatic interferences. The electrochemical interferences, which can be present in the real sample tested, were eliminated because the residual enzymatic activity was measured in a new substrate phosphate buffer solution in absence of real sample. The enzymatic interferences such as reversible inhibitors [51, 52] as well as detergents [53, 54] are avoided because after the incubation step the biosensor is washed with distilled water and, in this way, only the inhibitor covalently linked to the enzyme (organophosphorus and carbammic compounds) is measured. The need for adopting a medium exchange method in the protocol for insecticide measurements has been demonstrated in literature [50]. In details, using the medium exchange method in presence of 200 ppb of sodium dodecyl sulfate (SDS), the limit value for waste waters, no inhibition was observed while in the case of measurement of the enzymatic activity following the protocol B an inhibition of 88% was observed. With this procedure, the enzyme acts as a high affinity capture agent for the insecticide, and, because of the irreversibility of the inhibition, the successive enzymatic reaction can be carried out in a fresh buffer solution, thereby circumventing the effect of reversible inhibitors such as also the AFB_1 present in real samples.

Measurement protocol of nerve agent gases

For nerve agents measurement in gas phase using BChE biosensor the following procedure was reported using a portable system [55]: the drop of buffer containing butyrylthiocholine was placed onto the BChE biosensor, the potential applied and the signal recorded. After the incubation time, the surface of the working electrode was wet with phosphate buffer, and then the biosensor was exposed to Sarin gas. After, the residual activity was measured (step 3). The concentration of nerve agents is then calculated applying the Eq. 2. Using this procedure it was possible to detect the Sarin at the concentration of 0.1 mg·m^{-3} as reported in the Fig. 3 [55].

Transducers for cholinesterase biosensor development

ChE biosensors used for irreversible inhibitors such as nerve agents or organophosphorus and carbammic insecticides can be classified regarding the type of transducer adopted. As reported in the Fig. 4, the type of transducer most used is the electrochemical one for several reasons and among them: cost-effective (especially in the case of screen printed electrodes), fast response, miniaturisable and used also in the case of coloured solutions. In literature it is also reported the use of piezoelectric, fiber optic and surface plasmon resonance (SPR) transducer. A briefly description of these biosensors is described below in order to have an overview of ChE biosensors developed in function of the transducer utilised.

Electrochemical biosensors

The electrochemical biosensors can be classified as bi-enzymatic in which the ChE is coupled to choline oxidase (ChOx) enzyme and mono-enzymatic system in which only ChE is used as biocomponent.

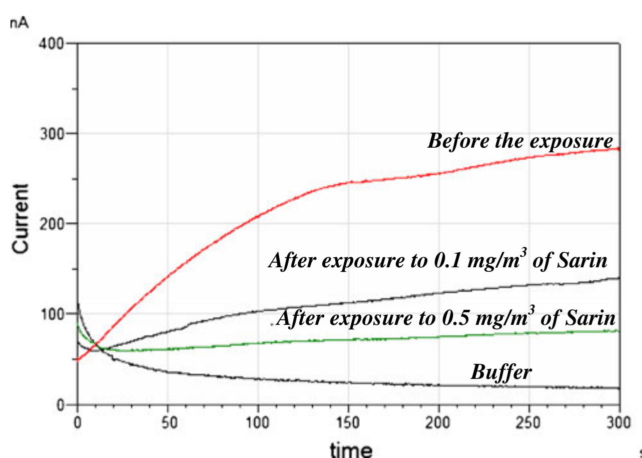


Fig. 3 Original recording obtained using BChE biosensor. Signal recorded in phosphate buffer (**a**) and in a solution of butyrylthiocholine (5 mM) before the exposure of the biosensor to Sarin gas (**b**) and after 1 min exposure to 0.1 mg/·m^{-3} (**c**) and to 0.5 mg·m^{-3} (**d**) of Sarin gas (reproduced with permission of Arduini et al. [55])

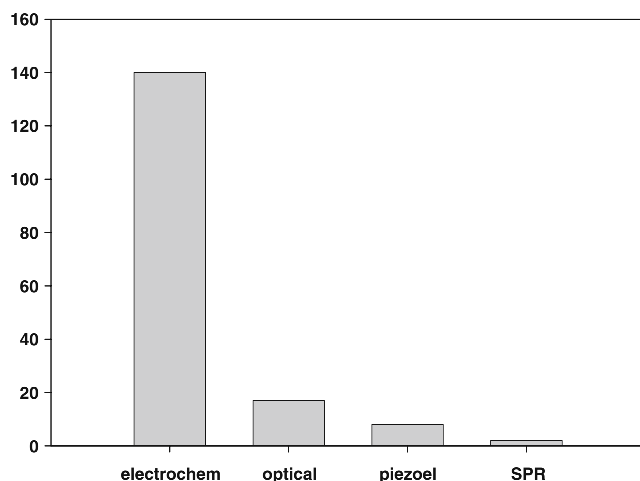


Fig. 4 Distribution of transducers used for the design of ChE biosensors for the detection of insecticides, nerve agents and aflatoxin B_1 (electrochem = electrochemical, piezoel = piezoelectric, SPR = surface plasmon resonance)

Bi-enzymatic systems

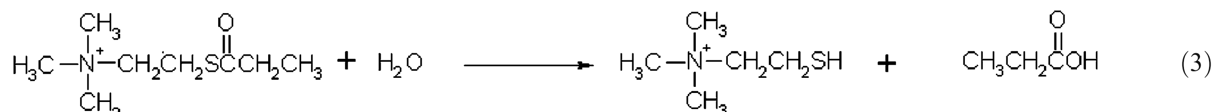
The bi-enzymatic ChE biosensor is constructed using: AChE, that hydrolyses the substrate acetylcholine to choline and acetic acid (or butyrylcholine in the case of BChE) and ChOx that oxidises the choline to betaine with the production of H_2O_2. The use of ChOx is necessary in the case of amperometric biosensors because the enzymatic products of the reaction (Eq. 1, choline and acetic acid) are not electroactive.

The enzymatic activity can be detected by means the O_2 decrease measurement using Clark's electrode [56] or the increase of H_2O_2. In the latter case, the enzymatic product H_2O_2 is measured amperometrically at around +600 mV vs Ag/AgCl using a platinum electrode [57] or in order to reduce the applied potential, by means of the use of redox mediators such as ferophthalocyanine [58], Prussian Blue [59] or ii) by adopting novel materials such as carbon nanotubes [60].

Monoenzymatic systems

In a monoenzymatic system the reaction monitored is the one reported in Eq. 1. In this case the enzymatic activity can be measured by means of different electrochemical transducers:

i) potentiometric: the reaction can be monitored by the measurement of the pH variation using a pH electrode [61], ISFETs [62, 63], and electrodes modified with polymers [64–66]. A BChE based light addressable potentiometric sensor was developed by Mourzina et al. [67]. Recently current driven ion fluxes of a polymeric membrane ion-selective electrode for BChE potentiometric biosensing was published on Journal of American Chemical Society [68].
ii) conductimetric: the reaction can be monitored by measurement of conductivity variation [69, 70]
iii) amperometric: for the monoenzymatic amperometric biosensors, a synthetic substrate must be used; in fact, acetylthiocholine was adopted instead of acetylcholine. The enzymatic reaction hydrolyses the acetylthiocholine to acetic acid and thiocholine (Eq. 3) and the thiocholine, being electrochemically active, can be measured.



(3)

The monoenzymatic biosensor was developed using a platinum working electrode on which the AChE was immobilised and measuring the thiocholine at +450 mV vs Ag/AgCl [71]. In order to reduce the applied potential and the electrochemical interferences, two approaches can be followed: I) the use of redox mediators such as cobalt phthalocyanine (CoPc) [72], Prussian Blue [73], Tetracyanoquinodimethane (TCNQ) [74], Cobalthexacyanoferrate [75], Potassium ferrycyanide [76] or ii) the use of novel materials [77, 78] such as carbon nanotubes.

Optical biosensor

An optical transducer was also utilised to detect insecticides [79, 80]. A fiber-optic photometer based on the use of solid-state opto-electronic components was developed by the researcher group of prof. Wolfbeis [81]. A sol-gel based fiber optic biosensors were developed using pH sensitive fluorescent indicators [82, 83]. The ChE activity was also measured by spectrophotometric detection [84] or via chemiluminescent reaction [85] in a flow injection system.

Piezoelectric biosensor

The insecticides can be measured also by means of piezoelectric biosensors [86, 87]. The paraoxon was immobilised on the sensing surface pre-incubating with BChE. In the presence of diisopropylfluorophosphate (DFP), the binding of BChE to the surface-bound paraoxon decreased proportionally to the DFP present in the sample [88]. The detection of organophosphate and carbamate was also carried out measuring the precipitation of an enzymatic reaction product over quartz crystal microbalance (QCM) [89, 90].

Surface plasmon resonance (SPR) biosensor

Recently, ChE biosensors using a SPR were reported in literature. The AChE was immobilised on SPR biosensor chip surface and in presence of insecticides a changing of intensity SPR angles was observed [91, 92].

Immobilisation

After the choice of transducer, the enzyme immobilisation is an important step in the biosensor design. Several types of immobilisation were investigated in order to obtain sensitive and stable ChE biosensors.

The physical immobilisation such as adsorption is one of the simple procedure to immobilise the biocomponent onto the transducer [93, 94]. AChE was immobilised by adsorption on screen printed electrodes modified with multiwall carbon nanotubes (MWCNTs). In this way, some μL of AChE solution were dropped on the MWCNT modified electrode surface and allowed to dry at room temperature under a current of air. The electrode was then rinsed twice with buffer to remove the loosely adsorbed enzyme molecules on MWCNTs [77]. This was an important step to avoid the leakage of the enzyme during the measurement. AChE was also physically adsorbed on polyvinylpyrrolidone K 30 [95] or calcium carbonate-chitosan composite [96]. One of the most sensitive biosensors was obtained immobilising the AChE by physical adsorption in nanostructured carbon matrix as reported by Sotiropoulou and Chaniotakis [97]. This system allows obtaining a very stable biosensor under continuous operation conditions (L_{50}>60 days) and very low detection limit for dichlorvos at picomolar levels. This promising result can be ascribed, as suggested by the authors, at i) the properties of the activated carbon to preconcentrate the insecticides and ii) the hyperactivity of enzyme within the nanopores. In fact, it is possible to reach lower detection limit using enzyme immobilised than enzyme in solution if the matrix in which the enzyme is immobilised is able to preconcentrate insecticides [98].

Another type of immobilisation is the enzyme entrapment in matrix [99–103]. Andreescu et al. have reported the immobilisation of ChE by encapsulation in sol-gel prepared by TMSO (Tetramethoxysilane) and MTMSOS (Methyltrimethoxysilane) or by entrapment in poly(vinylalcohol) bearing styrylpyridinium (PVA-SbQ) showing in both cases a storage stability of several months [104]. Anitha et al. have immobilised ChE in a thin sol-gel derived from TEOS (tetraethoxysilane) [105]. Du et al. have developed a sol-gel derived silicate network assembling gold nanoparticles that provided a biocompatible microenvironment around the enzyme allowing the storage stability of 3 weeks at 4°C [106].

A novel recent approach to immobilise AChE consists in the layer by layer electrostatic self assembly of AChE on MWCNTs modified glassy carbon electrode [107]. The CNT was initially NaOH treated in order to assume a negative charge and then was dipped into a solution of cationic poly(diallyldimethylammonium chloride) (PDDA) which leads to the adsorption of positively charged polycation layer (CNT-PDDA). After the negatively charged AChE was adsorbed on CNT-PDDA to obtain CNT-PDDA-AChE. Finally, in order to avoid the leakage of AChE from the electrode surface, another PDDA layer was absorbed resulting in sandwich structure of PDDA/AChE/PDDA. This system allows a low detection limit of paraoxon equal to 0.4×10^{-12} M. Carbon nanotubes were also used to synthesise with gold a nanocomposite [108] or combined with chitosan [109] for AChE biosensor construction.

One of the most used types of enzyme immobilisation is the chemical immobilisation by means of cross-linking with glutaraldeyde. This method confers to the biosensor high working stability even if there is usually a decrease of the enzymatic affinity towards its substrate. This behaviour owes to the distortion of the enzyme structure with consequent $K_{Mapparent}$ higher than K_M obtained for ChE in solution [50, 55, 110]. An example of chemical immobilisation is based on a non conducting polymer electrosynthetised onto the electrode and after the enzyme was immobilised by crosslinking with glutaraldehyde [111, 112]. Several immobilisations were carried out successfully using ChE immobilised by cross-linking method with glutaraldehyde vapour [113–117] or making an enzymatic membrane onto the electrode with ChE, Nafion® and glutaraldehyde [118, 119]. In the last case it was demonstrated that the use of albumin bovine serum at 3% increases the enzyme stability [50, 120].

Another interesting approach is the immobilisation of ChE carried out by affinity methods using concanavalin A [121, 122] or Ni-His affinity binding [123–125] which allows to an oriented disposition of the enzyme on the transducer. Andreescu et al., for example reported the

possibility to immobilise AChE enzyme by a metal chelate. The immobilisation has taken placed using a electrode modified with nickel complex able to bind a histidine present in an engineered AChE [123]. Recently Instamboulie et al. have developed a highly sensitive detection of organophosphorus insecticides using magnetic microbeads and Ni-His affinity binding. This method, owing to the absence of diffusion barriers, showed a lower detection limit and a fast response time than a biosensor based on enzyme immobilised by entrapment on azide. This system, however, is characterised by not so high reproducibility [126].

Another type of immobilisation consists of the enzyme immobilised close to the electrode surface with a high degree of control over the molecular architecture of the recognition interface by means of self assembled monolayer (SAM) [127]. The affinity of thiols for some metal surfaces, particularly gold, makes alkanethiols ideal for the preparation of modified electrodes. Somerset et al. have developed a gold electrode modified with mercaptobenzothiazole and either poly(o-methoxyaniline) or poly(2,5-dimethoxyaniline) [128, 129]. An AChE based amperometric biosensor was developed by immobilisation of the enzyme onto a self assembled modified gold electrode using as 3-mercaptopropionic, glutaraldehyde and (N'-cyclohexy-N'-(2-morpholinoethyl) carbodiimide methyl-p-toluenesulfonate by Pedrosa et al. [130, 131]. The acetylcholinesterase biosensor was also constructed by means of gold nanoparticles and cysteamine assembled on glassy carbon paste [132] or by single walled carbon nanotubes wrapped by thiol terminated single strand oligonucleotide (ssDNA) on gold [133].

In order to increase the storage stability in a dry state, which is a key point to commercialise the ChE biosensor, the immobilisation should maintain the enzyme activity also in a dry state for several days. For this purpose, several stabilizer mixtures were employed for an additional stabilization of enzyme as well as reported by Gibson's group [134–137]. For example, Vakurov et al. have investigated different types of immobilisation using *Drosophila* AChE. The enzyme non-covalently immobilized onto polyethyleneimine modified screen-printed carbon electrodes showed an improvement of stability when compared to non-immobilized AChE, AChE covalently immobilized onto dialdehyde and polyethyleneimine modified electrodes. Several stabilizer mixtures were also employed for an additional stabilization of AChE, demonstrating higher storage stability in the dry state with dextran-sulphate/sucrose or polygalacturonic acid/sucrose mixtures [138].

Reactivation

In the case of irreversible inhibition the inhibitor binds covalently the active site. In order to have repeated measurements, it was necessary to i) reactivate the ChE biosensor or ii) employ microfrabrication technique which allows the use of a small amount of the enzyme making the measurement cost effective for a single use of the biosensor with no reactivation of the inhibited ChE.

In the case of reactivation, this step is usually performed with oximes by a nucleophilic attack at the phosphorylated enzyme, enabling the release of insecticides from the catalytic site of ChE [139]. AChE inhibited by organophosphorus insecticides can be reactivated by means of pralidoxime chloride [109, 140] or 2-pyridinealdoxime methochloride [141]. Reactivation of the inhibited AChE was investigated using both 2-PAM (Pyridine-2-aldoxime methyliodide) [107] and TMB-4 (4-formylpyridinium bromide dioxime). TMB-4 was found to be a more efficient reactivator under repeated use, retaining more than 60% of initial activity after 11 reuses, whereas in the case of 2-PAM, the activity retention dropped to less than 50% after only six reuses [142]. In another case of AChE inhibition, the reactivation was carried out with repetitive injections of substrate, if the concentration of insecticides is lower than 1 ppm [48] or adding 0.4 mM sodium fluoride [143]. Reactivation of inhibited AChE is dependent on both amount of reactivator and the time of phophorylated enzyme state; in fact if the enzyme is phoshorylated (inhibited) and left for a period of time without exposing it to the reactivator a phenomenon called "ageing" occurs. In the ageing there is a molecular rearrangement of the alkylphosphate groups attacked to the residue serine which renders the inhibited enzyme more resistant to reactivation becoming permanently inhibited [144]. However the ageing changes in function of the inhibitor tested: Du et al. have in fact observed that after 5 h of paraoxon exposing at 25 nM the activity was restored at 90% [145] while the ageing of Soman is very fast [146].

Effect of substrate, incubation time, enzyme loading and pH

In the case of irreversible inhibition, the high substrate concentration can be chosen in order to have a higher output signal. For AChE biosensor a concentration of 1 acetylthiocholine mM was adopted [50]. Usually in the case of AChE biosensor, the acetycholine or acetylthiocholine was chosen and for BChE butyrylcholine or butyrulthiocholine. However the investigation of the effect of acetylcholine substrate using BChE biosensor or butyrylcholine in the case of AChE biosensor was investigated. The results obtained showed that neither AChE nor BChE biosensor is entirely specific to its basic substrate; AChE catalysed 100% of its substrate and 15% of butyrylcholine, while BChE 100% its natural substrate and 35% acetylcholine [114]. In order to obtain higher sensitivity in the case of

biosensor format for insecticides and nerve agents, acetylcholine or acetylthiocholine for AChE biosensor and butyrylcholine or butyrylthiocholine for BChE biosensor is highly suggested.

The incubation time is the reaction time of the enzyme with the inhibitor. For irreversible inhibition it is possible to achieve lower detection limits using longer incubation times; in fact, usually the degree of the enzyme inhibition increases with the incubation time [147] until reaching a plateau [148]. The incubation time is usually chosen as compromise between a sensitive measurement and a measurement carried out in a reasonable time [149]. In literature is possible found incubation times comprised between few minutes (5 min [46], 10 min [120], 15 min [119], 30 min [50], 40 min [111]) until 2 days [110]. In our opinion, the incubation time should be not longer than 1 h because one of the biosensor advantage than i.e. HPLC should be the short time of analysis. In addition, the detection of carbamates using a long incubation time can allow to the reactivation of the enzyme inhibited. In order to increase the sensitivity of the biosensor, in our view it is better varying the enzyme loading than to use the incubation time longer than 1 h. In fact, for irreversible inhibition the degree of inhibition depends of the enzyme concentration. In details, the enzyme concentration should be chosen taking in consideration that: i) the amount of enzyme immobilised should give a measurable signal and that ii) the lowest amount of enzyme is necessary to achieve the lowest detection limit. In this context is very useful to have a highly sensitive enzymatic product detector and an enzyme immobilisation that does not decrease the enzymatic activity. An example can be the biosensor reported by Sofiropoulou et al. in which they have used a very low concentration of highly sensitive double mutant of the *Drosophila melanogaster* immobilised in porous carbon that allows the detection of dichlorvos down to 10^{-17} M [150] or the sonochemically fabricated AChE microelectrode arrays that allows dichlorvos, paraoxon, parathion and azinphos detection down the concentration of $\sim 1\times 10^{-17}$ M, 1×10^{-17} M, $\sim 1\times 10^{-16}$ M and $\sim 1\times 10^{-16}$ M, respectively [151]. The sensitivity can be also increased by using the cholinesterase enzyme from different sources [152–156].

For the selection of the pH, it should be considered that certain enzymes have ionic groups on their active site and these groups must be in a suitable form such as the serine group in the catalytic site of ChE enzymes. As reported in literature, the optimum pH for the free enzyme is pH=8 while the pH can be shifted when the enzyme is immobilised to pH=7 as in the case of AChE immobilised onto Ca-alginate gel beads [157]. However the acid pH should be avoided, in fact in the case of insecticide detection during wine fermentation [118] or in orange juice [158] it is necessary to adjust the pH towards neutral value.

Measurement of insecticides and nerve agents in presence of organic solvents

In general, the extraction of pesticides is carried out using organic solvents as reported in the official methods for pesticides detection (EPA) [43], but is important also the choice of an appropriate organic solvent to reduce the enzyme inactivation.

To understand the possibility to use the organic solvents for insecticide detection with biosensor, their effect on ChE activity was investigated. This effect has been shown to be quite variable and dependent on the immobilisation used and on the polarity of the organic solvent. The influence of acetonitrile, methanol and ethanol on ChE immobilised into polyvinyl alcohol functionalised with methyl pyridinium methyl sulfate (PVA-SbQ) has been reported showing an increase of the output current in 5% of acetonitrile and 10% in ethanol [159–161]. The influence of ethanol was investigated also by Wilkins et al. using ChE immobilised by polyethylenimine and glutaraldehyde [162]. The biosensor constructed by SAM immobilisation was employed to diazinon and fenthion in acetone-saline phosphate buffer solution or ethanol-saline phosphate buffer solution with satisfactory results [163]. Generally the amount of organic solvent should be not higher than 5%, as also reported by Pohanka et al., suggesting that convenient solvents were propan-1,2-diol and isopropanol [71]. An interesting approach was reported by Arduini et al. [164] and Schulze et al. [165]. They demonstrated that organic solvents, which are completely insoluble in aqueous phase, such as hexane or octanol in the first case, or octanol in the second case, caused only a marginal reduction of enzyme activity (less than 5%) and can be used for the extraction and measurement of insecticides without effect on enzyme activity. However it has been also demonstrated that concentrations of hydrophilic solvents higher than 10% can influence the enzymatic activity [164] or the enzymatic kinetics [159].

A different method was chosen by Campanella et al., demonstrating the suitability of a bi-enzymatic biosensor (BChE + ChOx) to work in the organic phase (chloroform-*n*-hexane 1:1 mixture) [166].

Cholinesterase biosensor applications for nerve agents measurement

Despite of the appearance of some papers reporting biosensors for nerve agents, only few papers (summarised in Table 1) have effectively tested nerve agent compounds such as Sarin, Soman, Tabun and VX [55, 71, 167–171]; in

Table 1 ChE biosensors for nerve agents detection

Transducer	Enzyme	Nerve agents detected in solution and relative detection limit	Applications	Ref
Amperometric, screen printed electrode modified with Prussian Blue	BChE	Sarin 12 ppb, VX 14 ppb	Sarin in vapour (0.1 mg/m^3)	[55]
Potentiometric	AChE (biotinilated)	Soman 0.018 and Sarin 0.084 ppb	Sarin in soil	[168]
Amperometric, screen printed electrode	AChE	Tabun 1.48×10^{-8} M, Sarin 5.88×10^{-10} M, Soman 1.07×10^{-8} M, Cyclosarin 9.12×10^{-9} M	–	[71]
Amperometric, glassy carbon electrode modified with gold-platinumNPs	AChE-ChOx	Sarin 4×10^{-8} M		[169]
Planar wave guide absorbance spectroscopy	AChE	Sarin 0.1 ppb	Sarin in vapour (0.014 mg/m^3)	[167]

many papers, in fact, model compounds such as paraoxon or the diisopropylfluorophosphate (DFP) were analysed [107, 172–175].

White et al. [167] demonstrated the application of AChE based detection of Sarin using planar wave guide absorbance spectroscopy. Detection of levels of Sarin as low as 0.1 ppb in solution and 0.014 mg·m^{-3} (2.6 ppb) in vapour are reported. Lee et al. have described an assay system based on biotin-labeled ChE with streptavidin for nerve agent detection in liquid samples. LODs for Soman and Sarin were 0.018 ppb and 0.084 ppb, respectively, for 10 min assay. In addition the system was also employed to detect spiked soil demonstrating that the method provided a quick and reliable way to test the toxicity of contaminated soil or surface [168]. Arduini et al. have developed a BChE biosensor immobilising the enzyme on screen printed electrodes modified with Prussian Blue. The system was challenged towards two different concentrations of Sarin gas (0.1 mg·m^{-3} and 0.5 mg·m^{-3}) at different incubation times (from 30 sec up to 10 min) demonstrating that it is possible to detect the Sarin at the concentration of 0.1 mg·m^{-3} with only 30 sec of incubation time [55]. The AChE biosensor was also developed by Pohanka et al., measuring Tabun, Sarin, Soman, Cyclosarin, and VX in solution with detection limits equal to 5.88×10^{-10} M and 8.51×10^{-10} M for Sarin and VX, respectively [71]. The bi-enzymatic biosensor AChE-ChOx was employed to detect Sarin at nM levels [169]. All these results confirm the possibility to use the ChE biosensor as a prompt system of alarm for people security.

Cholinesterase biosensor for insecticides detection and application in environmental and food safety

As reported recently by Amine at al. [33], despite the elevated number of publications on biosensors based enzyme inhibition, the majority of these systems are not applied to real samples. Some papers which report the applications of ChE biosensors in real samples are showed in Table 2.

A portable fiber optic biosensor was employed to detect real water samples (drinking water, bottle mineral water and ground water) without sample preparation step. The samples were spiked with carbaryl and dichlorvos obtaining insecticide recovery ranges between 85% and 110% [176]. The bi-enzymatic AChE-ChOx based on Prussian Blue screen printed electrodes was used to detect down to 2×10^{-8} M chloropyrifos-methyl, 5×10^{-8} M coumaphos, and 8×10^{-9} M carbofuran in aqueous solution and grape juice. The optimal conditions for grape juice pre-treatment were determined to diminish interference from the sample matrix [119]. The monoenzymatic AChE or BChE based on Prussian blue screen printed electrode was employed by Suprun et al. for monitoring the degradation of insecticides in wine fermentation [118] and by Arduini et al. to detect insecticides in waste water and river water samples. In the former case, in order to decrease the matrix interferences the electrolysis of grape juice and evaporation of ethanol were carried out [50], in the second one the samples did not require any treatment obtaining recovery range values between 79% and 125%. In these cases the organothiophoshate insecticides were electrochemically oxidised before the analysis because, as largely reported in literature, the oxidised form is able to inhibit the enzyme more strongly [177]. This means that organothiophoshate insecticides itself are weak inhibitors of ChE: in fact, only their oxo form is toxic. However in literature (Table 2), sometimes low detection limits using organothiophosphate is shown, and this could be ascribed to i) a possible mistake using parathion spontaneously oxidated to paraoxon ii) the use of organothiophosphate coupled to chemical or electrochemical oxidation in order to have organophophate–oxo iii) the use of a very sensitive biosensor [50, 177].

The omethoate residue was detected in cotton rose hibiscus leaves by means of an amperometric biosensor with a recovery comprised between 98.6% and 107.7% [178]. Several real water samples obtained from Beilun Seaport and the branch river of YangtzeJiang River (Zhejiang province of China) were analysed by means of a mediator free ChE amperometric biosensor in flow

Table 2 ChE biosensors for organophosphorus and carbammic pesticides detection in real samples

Transducer	Enzyme	Pesticide analysed and relative detection limit	Applications in real samples	Ref
Monoenzymatic system				
Amperometric, screen printed electrode modified with Prussian Blue	AChE	Aldicarb 38 ppb, Paraoxon 14 ppb, Parathion methyl 7 ppb	Monitoring wine fermentation	[118]
Amperometric, screen printed electrode modified with Prussian Blue	AChE or BChE	Aldicarb 24 ppb, Carbaryl 25 ppb, Paraoxon 2 ppb, Chlorpyriphos methyl oxon 0.5 ppb	Waste and river water samples	[50]
Amperometric mediator free (Al_2O_3-AChE), flow injection analysis	AChE	Dichlorvos 1×10^{-8} M	Water samples obtained from Beilun Seaport and the branch river of YangtzeJiang River	[179]
Amperometric based on PDMS-PDDA-$AuNP_S$ composite	AChE	Paroxon 0.5 ppt, Parathion 1 ppt, Omethoate 0.1 ppt,	Cottomrose hibiscus leaves	[178]
Amperometric, screen printed electrode modified with TCNQ	AChE	Chlorpyrifos, Carbaryl, Chlorfenvinfos lower than 2 ppb	Wastewater, groundwater and bottled water	[180]
Amperometric, screen printed electrode modified with TCNQ	AChE	Carbaryl 1×10^{-8} M, Pirimicarb 2×10^{-8} M, Carbofuran 8×10^{-10} M	Potable water samples	[184]
Amperometric, screen printed electrode modified with TCNQ	AChE	Paraoxon less than 5 ppb	26 samples of fruit and vegetable; 23 samples of infant food	[158]
Amperometric, screen printed electrode modified with CoPC	AChE, BChE	Paraoxon (K_i=5.40×10^5AChE, 9.30×10^5 BChE), Diazinon (K_i=1.03×10^4AChE, 4.59×10^4 BChE), Chlorfenvinphos (K_i=3.55×10^4 AChE, 1.00×10^7 BChE)	Sheep wool	[183]
Amperometric, screen printed electrode modified with TCNQ	AChE	Chlorpyrifos-oxon 2 ppb	Bottles, river, tap, sea and groundwater, tea, orange juice and milk	[181]
Amperometric, flow injection analysis	AChE	Carbofuran 1.0×10^{-9} M	50 different samples of fruits, vegetables and dairy fruit	[48]
Chronoamperometric, carbon paste electrode modified with CoPc	AChE	Carbaryl 2.0×10^{-6} M	Tomato	[196]
SWV,gold electrode modified with ssDNA/SWCNT	AChE	Methyl parathion and Chlorpyrifos 1×10^{-12} M	River water samples	[133]
Amperometric, gold electrode modified with alkanethiol/MWCNTs	AChE	Carbaryl 0.6 ppb	Garlic samples	[192]
Amperometric, screen printed electrode modified with TCNQ	AChE	Paraoxon 1 ppb, Carbayl 20 ppb	Milk	[185]
Amperometric, screen printed electrode modified with TCNQ	AChE	Paraoxon 1 ppb	Orange juice, baby food	[165]
Amperometric, screen printed electrode modified with CoPc	AChE	Dichlorvos $7x10^{-11}$ M	Apple skin	[191]
Fiber optic	AChE	Carbaryl 108 ppb, Dichlorvos 5.21 ppb	Drinking, bottle and ground water	[176]
Fiber optic	AChE	Propoxur 0.4 ng, Carbaryl 25 ng	Onion, lettuce	[194]
Reflactometer, dipstick	AChE	Parathion 1 ppb, Chlorpyrifos 1 ppb, Malathion 1 ppb, Carbofuran 1 ppb, Carbaryl 1 ppb and Bendiocarb 1 ppb	Rice, lettuce	[193]
Photothermal	AChE, BChE	Paraoxon 20 ppb	Onion, iceberg lattuge, salad	[195]
Piezoelectric, gold sensor	AChE	Paraoxon 0.1 ppt, Chlorpyriphos 0.1 ppb, Chlorfenvinphos 0.14 ppb, Diisopropylfluorophosphate 0.1 ppt	River water samples	[182]
Bienzymatic system				

Table 2 (continued)

Transducer	Enzyme	Pesticide analysed and relative detection limit	Applications in real samples	Ref
Amperometric, screen printed electrode modified with Prussian Blue	AChE-ChOx	Chlorpyrifos-methyl 3×10^{-8} M, Carbofuran 8×10^{-9} M	Grape juice	[119]
Differential pulse voltammetry, screen printed electrode modified with TCNQ	ChOx biosensor; AChE in solution	Chlorpyrifos-methyl 22 ppb	Grape and vine leaf sample	[186]
Amperometric, screen printed electrode modified with Prussian Blue	ChOx biosensor; AChE in solution	Dichlovos 50 ppb	Wheat	[188]
Amperometric, Clark type electrode	BChE-ChOx	Paraoxon 1.5×10^{-8} M, Malathion 1.5×10^{-8} M, Parathion-ethyl 11.5×10^{-9} M, Aldicarb 2.0×10^{-8} M, Carbaryl 2.5×10^{-9} M	Tap, lake and sea water	[47]

injection analysis. The results are in agreement with the results obtained by GC-MS [179]. A portable biosensor was developed by Hildebrandt et al. for screening neurotoxic agents in water samples without sample preparation and processing [180]. The same authors have also applied the portable system in seawater, ground and river water, tea, orange juice and milk [181]. River water sample was also tested by means of piezoelectric biosensor with a recovery between 60.8% and 91% [182]. A highly sensitive and rapid food screening test based on a disposable screen printed AChE biosensor was developed by Schulze et al. The analytical system was successfully validated and applied to 26 fruit and vegetable samples and 23 samples of processed infant food [158]. The organophosphorus and carbammic insecticides were also measured in extract of sheep wool [183], potable, river and lake water samples [47, 133, 184], milk [185], grape and vine leaf samples [186, 187], wheat and durum wheat [188–190], apple skin [191], orange juice [165], garlic [192], lettuce, rice, onion [193, 194], in samples of fruit, vegetable and dairy product [48, 195] and directly in tomato [196]. In the last case, the insecticides were measured without any previous manipulation of the sample, in fact the biosensor was immersed directly in the tomato pulp obtaining a recovery of 83.4% and showing a very low interference of the matrix components. The results reported have showed the real possibility to detect organophosphorous and carbammic insecticides in real sample. However, in our view it is very important to stress the fact that the ChE biosensor can be adopted as screening method to detect organophosphorous and carbammic insecticides in real samples. In fact with a portable instrument [176, 180, 181] and with a simple [165] or even without any treatment of the real sample [196], a measurement of these insecticides can be carried out. However, as reported below, the resolution of mixture of insecticides requires supplementary approachs. In our view, the biosensor can be really useful in routine analysis to detect the presence/absence of these insecticides. Then, only the samples resulted positive will be submitted to further analyses by sophisticated techniques such as HPLC or GC-MS, in order to investigated in details which insecticide is present. This approach can be really advantageous in terms of cost and time of analysis.

Cholinesterase biosensor and bioassay for aflatoxin B detection (reversible inhibitors)

ChE enzyme biosensors can be used also to detect several ChE reversible inhibitors such as glycoalkaloids, heavy metals (Cu, Fe, Mn), nicotine, cocaine, fluoride, and drugs (eserine, amitriptyline, bis(7)-THA, drofenine, 4-aminoquinaldine, neostigmine, tacrine) as reported in our recent work [197]. In this review we have focussed the

work on the reversible inhibition of ChE by AFB_1, because recently we have demonstrated the possibility to use the ChE enzyme for AFB_1 detection. In detail, we will describe how to optimise the biosensor for AFB_1 detection and how to reduce the interferences owing to the possible presence in the sample of organophosphorus and carbamic insecticides. In addition, in this part will be mentioned biosensors together with bioassays (in which the enzyme is in solution), in order to have a wide and useful description of this recent system.

Aflatoxins

The mycotoxin aflatoxins can be produced by several species of the mould *Aspergillus* (*Aspergillus flavus*, *Aspergillus parasitucus* and the rare *Aspergillus nomius*). Their toxicity is due to the capacity of aflatoxins to covalent binding DNA and proteins[198]. The aflatoxin B_1 (AFB_1) is the most acutely and chronically toxic member of the aflatoxin family. The legal limits set for AFB_1 or for total aflatoxins vary significantly from country to country (e.g. for total aflatoxins from 0 to 50 $ng \cdot g^{-1}$) [199]. Their documented impact on both human and animal health and on economic aspects of international trade involving food and animal feeds is reported [200] and for these reasons useful analytical methods are necessary for its detection.

The current reference methods are primarily chromatographic, relying on methods such as high performance liquid chromatography (HPLC) [201] or enzyme linked-immunoassay (ELISA) as an alternative approach [202]. Recently the possibility to detect AFB_1 by means of ChE biosensor was reported with the advantage i) to be a cost effective, miniaturized, easy to use analytical system in respect of the chromatographic technique and ii) to avoid the use specific antibodies and, indirectly, the use of animals in order to produce these “receptors” respect of ELISA system. In this part of the review will be described the parameters that should be investigated in order to obtain a ChE bioassay or biosensor for AFB_1 detection.

Effect of enzyme sources, incubation time and enzyme loading

The first work that has reported the ability to inhibit AChE enzyme was published on Toxicology by Cometa et al. [203]. In this work the inhibition of AChE extracted from mouse brain by AFB_1, was studied obtaining IC_{50} equal 10 ppm. In order to obtain an analytical system with higher sensitivity an investigation of ChEs from various sources such as AChE from *electric eel*, BChE from *equine serum*, AChE from *drosophila melanogaster* wild type and mutants [32], Human recombinant AChE [204], Human BChE, AChE from T*orpedo Californica* [31] was reported in literature demonstrating in all cases the best sensitivity of the AChE from *electric eel* for AFB_1 detection.

When an enzymatic system should be constructed, either in the case of bioassay or biosensor, in our view it is very important to know the type of inhibition. In the case of AChE inhibition by AFB_1 it is known that is a reversible inhibition. A reversible inhibition allows that the degree of inhibition is *independent* of the *incubation time* and of the *enzyme loading*, which means that the time of analysis can be made very shortly because no extended incubation time is required. Furthermore, the amount of enzyme present can be increased in order to obtain a high signal in a short reaction time. The short response time represents an important advantage for AFB_1 detection if compared with insecticides. In details, in fact the investigation of the degree of inhibition at fixed concentration of AFB_1 (60 ppb) using various concentrations of AChE (70 mU mL^{-1}, 40 mU mL^{-1} and 7 mU mL^{-1}) allows to obtain the same degree of inhibition around 50%; and a similar result was obtained in the study of the effect of incubation time (the time of reaction between the AFB_1inhibitor and AChE) on the degree of inhibition [32]. Absence of incubation time is usually chosen [32, 205].

In the case of reversible inhibition is also important to know if the inhibition is competitive or not in nature. In the case of AChE inhibition by AFB_1, as reported in literature [32], the degree of inhibition does not change with substrate concentration indicating that the inhibition is not competitive in nature; this means that the study of substrate is not required to optimise the analytical system thus it is possible to choose the concentration of substrate sufficiently high to give a detectable signal in a short time.

In conclusion, in the case of bioassay or biosensor for AFB_1 detection can be used high concentration of enzyme, no incubation time, sufficient high concentration of substrate because is a reversible inhibition. This means that knowing the type of inhibition is possible to optimise the ChE biosensor for AFB_1 without the investigation of the effect of each parameter (incubation time, enzyme loading and substrate concentration) on the degree of inhibition.

Immobilisation

On the contrary of ChE biosensor for insecticides detection in which a lot of immobilisations were investigated, in the case of ChE biosensor only four immobilisations were reported in literature. Hansmman et al. have developed a AChE biosensor depositing 3 μL of 1:1 mixture of polyvinylalcohol and the enzyme on cobalt-phthalocyanine modified screen printed electrode and polymerised under neon light at 44°C for 3 h. This sensor allows to detect a

minimum concentration of 3 μM of AFB_1 corresponding to 1 ppm [31]. The physical immobilisation of AChE to detect AFB_1 at ppb levels was investigated by Arduini et al. in order develop an amperometric biosensor using AChE immobilised on Prussian Blue-modified screen-printed electrodes [206]. The AChE immobilised in a gelatine layer allows obtaining a LOD of 100 ppb. Pohanka et al. have developed a biosensor with gelatine layer using human recombinant AChE and obtaining IC_{50}=100 ppb [204]. n our opinion the investigation of different types of immobilisation should be carried out to reach a lower detection limit comparable with the detection limit obtained with the enzyme in solution. The presence of a sensitive biosensor for AFB_1 detection is more advantageous than the ChE biosensor for insecticides, because a reversible inhibition usually is characterised by the total recovery of the enzyme activity after inhibitor measurement by means of a simple washing of the biosensor with an advantage in terms of time of analysis and cost-effective of the system.

Effect of organic solvents

The AFB_1 is normally extracted from many contaminated agricultural samples using mixtures of organic solvents such as methanol, acetonitrile, chloroform or acetone. In the case of bioassays, the effect of methanol on the AChE activity was evaluated. Arduini et al. [32] have investigated first, the effect of methanol on enzymatic activity observing that at 50% methanol, the AChE activity decreased by 30% while the same percentage of methanol does not affect the degree of inhibition This interesting result has demonstrated that it is possible to determine AFB_1 using a percentage of methanol as high as 50%, that is diluting the AFB_1 extracted from the sample only two fold.

The effect of methanol was also evaluated using an electrochemical system in which AChE was present in solution coupled with an amperometric ChOx biosensor. In this case a biosensor response decrease of 15% was observed with only 5% of methanol (v/v) [207]. This high effect was ascribed by the authors to methanol i) onto the AChE enzymatic activity ii) on stability of enzymatic ChOx membrane. The serious effect of methanol for assay based on AChE was also highlighted by Pohanka et al. [205]. These results showed the need to check every time the effect of organic solvents used to extract the AFB_1 on AChE activity in order to avoid a wrong overestimation of the AFB_1.

Cholinesterase bioassay for AFB_1 detection for food safety

The applications of the analytical system based on AChE inhibition for AFB_1 detection in food up to now are limited to the bioassay systems (Table 3). A bioassay with the spectrophotometric detection was applied to detect the AFB_1 in barley samples with a recovery values comprised between 98% and 101% [32]. The bioassay using a ChOx biosensor was applied to detect AFB_1 in olive oil obtaining recovery values higher than 75% [207]. These results seem to confirm the applicability of this system to real samples.

How to improve the selectivity of cholinesterase biosensor

In order to give a complete overview of ChE biosensors is important to stress that in the case of real samples the ChE biosensor is not a selective system because organophosphorus and carbamic insecticides and some other compounds have an inhibition effect on ChE. In fact often is reported the total cholinesterase inhibitors [208] and for this reason Luque de Castro and Herrera in their review mentioned that the inhibition biosensor as questionable

Table 3 AChE biosensors and bioassays for AFB_1 detection

Transducer	Enzyme/immobilisation	Detection limit or IC_{50}	Applications in real samples	Ref
Biosensor				
Amperometric, screen printed electrode modified with Prussian Blue	AChE/entrapment in gelatine layer	100 ppb		[206]
Amperometric, screen printed electrode	AChE/entrapment in gelatine layer	IC_{50}=100 ppb		[204]
Amperometric, screen printed electrode modified with CoPc	AChE entrapment by PVA	1 ppm		[31]
Bioassay				
Optical (Ellman's method)	AChE in solution	10 ppb	Barley	[32]
Eelectrochemical, creen printed electrode modified with Prussian Blue	AChE in solution /ChOx biosensor	10 ppb	Olive oil	[207]
Electrochemical, screen printed electrode	AChE in solution	4.8 ppb		[205]

device [209]. This behaviour can be a disadvantage because other techniques are required in order to evaluate which inhibitor is present. However this aspect can be also an advantage taking in consideration that this system is a screening method. In this way, a relevant example was reported by Dzyadevych et al. [210]. The authors have investigated the photodegradation of methyl parathion and the toxicity assessment of the resulting mixture including the main degradation photoproducts. The monitoring of photodegradation by means of HPLC and ChE biosensor has showed that the inhibition effect with biosensor increases dramatically as soon as the photodegradation begins. In addition the toxicity curve does not exactly follow the curve of appearance of methyl paraoxon which is more toxic than the initial insecticides methyl parathion [210]. These results suggested that some intermediate products can be more toxic than the insecticide itself and the toxicity can be revealed with biosensor but not i.e. with GC-MS or HPLC. This means that biosensors can be very useful tool to understand the presence of possible toxic compounds able to inhibit the ChE, and only the samples in which the inhibition is observed will be measured by the reference method with a relevant saving in terms of time and cost of analysis.

However to improve the selectivity of the system an interesting approach is reported in literature using ChE enzymes sensitive and selective towards a specific insecticide coupled with chemometric calculations [211–213]. In fact, as reported in an interesting review [214], recombinant AChEs have been undertaken to increase the sensitivity of AChE to specific organophosphates and carbamates using site-directed mutagenesis and employing the enzyme in different assay formats. For example, an amperometric biosensor array has been developed to measure insecticides mixture of dichlorvos and methylparaoxon. This system is composed by three screen printed electrodes that incorporate three different AChE enzymes: AChE from *electric eel* and two different genetically modified *Drosophila melanogaster* enzymes. The triplet inhibition responses were then modelled using Artificial Neural Network as processing tool, allowing the resolve of the insecticides mixture [213].

The selectivity was investigated by Korpan et al. by adding the ethylenediamine tetraacetate in the working solution in order to decrease the interferences of heavy metals and also to co-immobilise phosphotriesterase to render the biosensor insensitive to organophosphorus insecticides [215].

The selectivity between reversible (AFB_1) and irreversible inhibitors (insecticides) can be also improved using a kinetic approach [207]. Taking in consideration that for the irreversible inhibition (insecticides) a certain incubation time and a low concentration of the enzyme are necessary, and on the contrary, for the AFB_1 (reversible inhibitors) the degree of inhibition is independent of the enzyme loading and of the incubation time, it is possible suppose that the high concentration of the enzyme adopted for example in the AFB_1 bioassay together with no incubation time allow avoiding the interferences due to the insecticides eventually present in real samples.

This hypothesis was then confirmed in the case of AFB_1 determination in olive oil samples [207]. In this case, the authors in order to evaluate the effect of the insecticide interferences have tested some insecticides at 50 pbb level. Keeping in mind the different types of inhibition in the case of insecticides and AFB_1 i) no incubation time was taken ii) the reaction time was decreased to 1 min iii) the enzyme concentration was increased up to 40 $mU \cdot ml^{-1}$ and optimising the protocol for the AFB_1 extraction from olive oil, no inhibition by insecticides was observed. The results obtained demonstrated that the selection of experimental conditions for sample treatment and measurement should be taken into consideration to avoid interferences from the presence of insecticides in samples during the AFB_1 measurement.

Conclusion

This review highlighted the analytical parameters that should be investigated in order to increase the assay sensitivity using inhibition biosensors. In fact in the case of ChE biosensor for nerve agent and insecticide detection high incubation time and low enzyme loading allows to detect these inhibitors at a very low concentration. Different approaches should be applied in the case of ChE biosensor for AFB_1 detection where no incubation time is required and the degree of inhibition is almost independent of the enzyme loading with a consequent fast analysis time. The knowledge of the type of inhibition allows thus to optimize in a fast way the biosensor in order to increase the performance of the system and also to reduce the interferences, however current efforts in ChE biosensor are directed towards the development of more reliable systems with increase selectivity.

The review reports also a survey of many examples of ChE biosensors for organophosphorus and carbammic insecticides, nerve agents and AFB_1 underlying the application of these biosensors in real samples. Even if in this paper the applications in real samples are not so much reported the results obtained have showed good recovery values allowing also some applications in the field as screening procedures. So the ChE biosensors in our opinion can be considered a valid screening system able to detect a toxic compounds behaving as a “family doctor”.

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