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Review

Recent approaches to improving selectivity and sensitivity of enzyme-based biosensors for organophosphorus pesticides: A review

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ABSTRACT

Pesticide determination has attracted great attention due to the fact that they exhibit high acute toxicity and can cause long-term damage to the environment and human lives even at trace levels. Although classical analytical methods (including gas chromatography, high performance liquid chromatography, capillary electrophoresis and mass spectrometry) have been effectively used for analysis of pesticides in contaminated samples, they present certain limitations such as time-consuming sample preparation, complexity, and the requirement of expensive instrumentation and highly skilled personnel. For these reasons, there is an expanding need for analytical methods able to provide simple, rapid, sensitive, selective, low cost and reliable detection of pesticides at trace levels. Over the past decades, acetylcholinesterase (AChE) biosensors have emerged as simple, rapid and ultra-sensitive tools for toxicity detection of pesticides in the environment and food. These biosensors have the potential to complement or replace the classical analytical methods by simplifying or eliminating sample preparation and making field-testing easier and faster with significant decrease in cost per analysis. With the recent engineering of more sensitive AChE enzymes, the development of more reliable immobilization matrices and the progress in the area of microelectronics, AChE biosensors could become competitive for multi-analyte screening and soon be used for the development of portable instrumentation for rapid toxicity testing of samples. The enzymes organophosphorus hydrolase (OPH) and organophosphorus acid anhydrolase (OPAA) have also shown considerable potential in OP biosensor applications and they have been used for direct detection of OPs. This review presents the recent advances in the fabrication of enzyme biosensors for organophosphorus pesticides (OPs) and their possible applications for toxicity monitoring of organophosphorus pesticide residues in real samples. The focus will be on the different strategies for the biosensor construction, the analytical performance of the biosensors and the advantages and disadvantages of these biosensor methods. The recent works done to improve the analytical performance, sensitivity and selectivity of these biosensors will also be discussed.

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1. Introduction

Pesticides (e.g. insecticides, herbicides, and fungicides) are widely used in agriculture to eliminate or control a variety of agricultural pests that can damage crops and livestock and reduce farm productivity. Although pesticides are directly applied to plants and soils, only 1% of pesticide sprayed is delivered to the intended target [1]. Accidental release of pesticides due to spills, leaking pipes, underground storage tanks, and waste dumps may lead to their persistence in the environment for a long period of time [1]. As a result, pollution due to the uncontrolled use of these pesticides has become one of the most alarming challenges.

The organophosphorus pesticides (OPs) are synthetic esters, amides, or thiol derivatives of the phosphoric, phosphonic, phosphorothioic, or phosphonothioic acids [2,3] which are used extensively to control agricultural, household and structural pests globally. OPs break down quickly when exposed to light and air, are less persistent in the environment, are not subject to bioaccumulation and biomagnification, and do not release toxic break down products [2]. These features justify their application in the agricultural and veterinary practices of the modern world [2]. Consequently, they are favored over organochlorine (OC) pesticides such as DDT. However, it is not known whether OPs ever degrade fully since they have been detected in soil and drinking water long after application [4].

OPs are among the most acutely toxic pesticides and their residues in the environment can cause long-term damage to human health even at trace levels. They belong to the toxicity class I (highly toxic) or toxicity class II (moderately toxic), according to the EPA classification [5]. The toxicity of OPs is based on the inhibition of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7), which is essential for the functioning of the central nervous system of mammals and insects. This results in the accumulation of acetylcholine (Ach) neurotransmitter, which interferes with muscular responses and causes respiratory and myocardial impairment and even death [6]. Although exposure to a single compound or active ingredient may not exceed the level considered to be without acceptable risk for either humans or environmental species, concurrent exposures to numerous OP compounds could exceed a safe level because of increased AChE inhibition. The toxicity of OPs is reported to vary considerably, depending on the chemical structure of the pesticide [6].

Due to the high acute toxicity of the OPs [4,7], as well as the registered chronic effects [4], the OP residue limits in food, drinking water and environmental samples are subject of regulation and control [2,4]. As a result, their rapid detection and reliable quantification has become increasingly necessary. Numerous analytical methods including gas chromatography (GC), liquid chromatography (LC), high-performance liquid chromatography (HPLC), mass spectrometry (MS), capillary electrophoresis (CE), surface plasmon resonance (SPR), fluorimetry, ultraviolet spectroscopy and GC–MS have been developed for analysis of OPs in contaminated samples [2,5,6]. Although these methods have been used successfully for the detection of OPs, they present several disadvantages due to the fact that they are time-consuming, require sample pre-treatment, expensive instrumentation, highly skilled personnel and they can only be used in laboratories. For these reasons there is an expanding need for field deployable analytical methods able to provide simple, rapid, sensitive, selective, low cost and reliable detection of OPs at low concentrations.

Numerous biosensors have been developed over the last decades in an attempt to satisfy these needs. Among the different types of biosensors, the enzyme-based electrochemical biosensors are especially desirable for field applications since they are easy to manufacture and deploy [8]. Furthermore, it is possible to miniaturize the required instrumentation providing compact and portable analysis devices, and they can potentially be engineered to be highly selective and sensitive [9]. These biosensors are well documented and have emerged in the past few years as the most promising alternative to detect OPs [8]. The applied electrochemical transduction mode of the electrochemical biosensors is commonly potentiometric or amperometric [10]. The potentiometric determinations are based on the measurement of the emf of a galvanic element, constituted by an indicator and a reference electrode [10]. The potential of the indicator electrode depends on the analyte concentration, according to the Nernst equation, while the potential of the reference electrode remains constant. The exponential character of the relationship between the potential of the indicator electrode and the analyte concentration defines the wide dynamic concentration range determinations (3–4 decades), but also defines the low accuracy and precision of the method [10]. The amperometric determinations involve the measurement of the current response of an indicator electrode, as a function of the concentration of the electroactive species present in solution, at a constant potential [10]. The amperometric detection normally presents advantages such as high sensitivity, precision, linearity of the calibration plot and the ability to control the process through the electrode potential [10]. This review therefore focuses more on the amperometric detection method.

The most commonly reported electrochemical OP biosensors are based on indirect detection by AChE enzyme. A large number of documents including several review articles on AChE-based electrochemical biosensors for the detection of OP compounds are available in literature, with limits of detection (LOD) ranging from micro-molar to pico-molar levels depending on the source of AChE enzyme, technique, and transduction mechanism employed [10]. Various amperometric and potentiometric AChE-based biosensors have been reported. The potentiometric AChE biosensors detect the pH shift resulting from the decrease (in the presence of OP pesticides) of the acid released during the enzyme catalyzed hydrolysis of the choline esters [10]. The amperometric AChE biosensors are based on the measurement of the change in concentration of the electroactive product thiocholine, produced as a result of hydrolysis of acetylthiocholine. When OPs are present, AChE is inhibited and, therefore, less thiocholine is produced [6]. The conventional or native AChE-based biosensors have LOD comparable to the classical laboratory techniques listed previously, but they have limitations such as poor selectivity, sensitivity and long analysis time due to the incubation period. In addition to sensitivity and selectivity, another difficulty of conventional AChE biosensors is the poor resolution of pesticide mixtures. They provide information on a family of compounds and not on individual compound in a family. In this sense, the use of recombinant AChE has been reported allowing dramatic enhancement of the sensitivity of these devices [9,11]. Although these biosensors show a high sensitivity, they often lack selectivity due to the fact that they react with any cholinesterase inhibitor. To address the selectivity issue, different AChE variants having different sensitivities and selectivities for various OPs have been used in array based sensors for discrimination between the different OPs [9]. The arrays of

sensors have been used in order to generate multidimensional data, which can be chemometrically processed to obtain detailed information.

The other preferred strategy to overcome the disadvantages of inhibited AChE-based biosensors is to investigate enzymes that are capable of direct and selective recognition, and hydrolysis of OPs. Alternative biosensing routes for the direct detection of OPs involving the use of organophosphorus hydrolase (OPH) enzymes (also known as phosphotriesterases, PTE) and organophosphorus acid anhydrolase (OPAA) enzymes have thus been reported [12–21]. The OPH and OPAA biosensors are based on direct detection of OPs and they have been characterized aiming at decreasing analysis time. Several OPH-based amperometric, potentiometric, or optical biosensors have been reported in literature [18]. Unlike AChE, OPH enzymes catalyze the hydrolysis of OPs with a high turnover rate, can potentially be reused and are, therefore, suitable for continuous monitoring of OPs. OPH exhibits the capability to hydrolyze a large variety of OP compounds (such as paraoxon, parathion, coumaphos) by producing less toxic products, such as *p*-nitrophenol and diethyl phosphate [22]. The amperometric OPH biosensors rely on the detection of the electroactive product of the hydrolysis reaction. However, the use of these biosensors is limited to selected few OPs with electroactive leaving groups and majority of the reported amperometric OPH biosensors are based on anodic detection of the hydrolysis product *p*-nitrophenol at high overpotentials [3,23]. Due to these high operating potentials, interferences constitute a problem as well as surface fouling caused by the phenolic groups [23].

The developments of AChE, OPH and OPAA biosensors require immobilization of the enzyme onto the working electrode surface and thus, the immobilization is the key to obtaining a highly sensitive biosensor. Immobilization process is governed by various interactions between the enzyme and the electrode material and strongly affects the performance of the biosensor in terms of stability, response time, sensitivity and reproducibility [6]. Recently, with the rapid development of nanotechnology, various nanomaterials have been applied as biosensor materials, in an attempt to improve sensitivity, and several techniques for immobilization of the enzyme onto the electrode surface such as physical entrapment, physical adsorption, covalent coupling, self-assembled monolayer, oriented immobilization and cross-linking have been reported in literature [3,7]. Carbon nanotubes are the most widely used nanomaterials in electrochemical biosensors. This is because of their chemical stability, mechanical strength, stiffness and improved electron transfer properties attributed to the changes in the energy bands close to the Fermi level [25]. Both single walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) have been used for enzyme immobilization even though the drawbacks of the SWCNTs such as high cost and low dispersibility favor the MWCNTs application, in spite of the high surface area and low electrical percolation thresholds of the SWCNTs [3].

In this review, we provide an overview of enzyme-based electrochemical biosensors that have been used for the detection of OPs in various sample matrices up to date. Discussions on the different biosensor techniques for OPs are highlighted with emphasis on different enzymes that have been employed in the construction of the biosensors and the efforts that have been made to improve the sensitivity and selectivity of these biosensors. The recent advances in the development of these biosensors, including the transducer modification and the genetic engineering of the biological recognition element are reviewed. The application of the developed biosensors for analysis of OPs in real samples is also presented.

2. Biosensors

Biosensors are defined as analytical devices incorporating a biological recognition element (enzyme, antibody, whole-cell, DNA, receptor or microorganisms) which is integrated within a physicochemical signal transducer or transducing micro system [26]. The signal transducing element (electrode, optical detector, piezo crystal and others) converts the biochemical response into electric and optic signals which are amplified, measured and decoded by an appropriate electronic unit [26]. Most electrochemical detectors, such as amperometric and potentiometric detectors, are surface detectors. They respond to substances that are either oxidizable or reducible and the electrical output results from an electron flow caused by the chemical reaction that takes place at the surface of the electrodes. The choice of a biological element for a biosensor assembly depends on the biological targeting of toxic species and specificity of detection of their specific interactions. In this regard, various prototypes of biosensors have been proposed and investigated for OP detection utilizing different biomaterials, e.g. enzymes, antibodies, and whole cells [27]. A biosensor can produce either individual or successive digital electronic signals that are equivalent to the concentration of a single analyte or a group of analytes being monitored [27].

In the last two decades, research in the field of biosensors has increased significantly owing to the fact that there is need for cheap, fast, simple, sensitive, and field-deployable analytical tools that are able to provide real-time quantitative and qualitative information about the sample composition with minimum sample preparation. The main trends in modern electroanalysis include development of biosensors based on the progress in biochemical methods of molecular recognition and development of measuring devices of a large integration scale, including sensor arrays. In comparison to other instrumental methods of analysis, electroanalytical instrumentation is relatively easy to miniaturize.

The enzymatic biosensors are considered to be amongst the most popular analytical tools that have emerged during the last few decades as they provide a viable, handy, and potentially portable alternative. In these enzymatic biosensors, the enzyme (recognition element) reacts selectively with its substrate (target analyte). Enzymatic biosensors can measure the catalysis or the inhibition of enzymes by the target analyte, i.e.:

a) the enzyme can metabolize the analyte, and such, the concentration of the analyte is determined through measuring the catalytic transformation of the analyte by the enzyme; or,
b) the enzyme can be inhibited by the analyte, so the concentration of the analyte is associated with a decrease in formation of the enzymatic product [27].

Enzymatic biosensors can, therefore, detect the produced or the consumed species, respectively. Commonly, only one enzyme is used in enzymatic biosensors but the progress in this field involves combining enzymes to obtain multi-enzyme array systems where several enzymes are incorporated into the same platform [27]. Despite the popularity of enzymes as analytical tools, optimization of their specific features such as selectivity, sensitivity, stability and accuracy is still required. In order to improve these experimental parameters, research efforts focus on the production of new biomolecules with tailor-designed properties and protein engineering is emerging as a possible strategy to improving the performance of enzymes [9]. This is accompanied by the use of new materials, including nanomaterials and nanostructures. The two main strategies in protein engineering are the rational design, which combines site-directed mutagenesis with the detailed knowledge of enzyme structures and functions or computational models, and the directed evolution, which does not require

scientific knowledge since it is based on the random synthesis of a pool of mutated enzymes and the subsequent selection by an iterative process [9]. In the case of biosensors for OPs, enzymes such as AChE, OPH and OPAA have been recognized as potential recognition elements and are, therefore, discussed in this review.

3. Biosensors based on cholinesterase enzymes

Cholinesterases (ChEs) are important enzymes present in vertebrates and insects, which hydrolyze the neurotransmitter acetylcholine in the nervous system In the body, ChE is responsible for transmission of nerve impulses to the cholinergic synapses and is connected with human memory and Alzheimer's disease [28]. AChE and butyrylcholinesterase (BChE) are the two types of cholinesterase enzymes and they have been widely used as bioreceptors for the fabrication of biosensors. AChE in nerve synapses terminates nerve impulse transmission by hydrolyzing the neurotransmitter acetylcholine. BChE acts as a backup for AChE, and as a scavenger for poisons that might inhibit AChE activity. BChE was of little interest to research scientists until the US Department of Defense allocated millions of dollars for the mass production of pure human BChE to use for protection against the toxicity of nerve agents [29]. A prerequisite for the commercialization of enzymes is the availability in large amounts of extremely pure, functional and stable enzymes at low costs. In this sense, several types of AChE enzymes have been purified from different sources such as *Horse serum (hs)*, *Human erythrocites (he)*, *Human blood (hb), Bovine erythrocites (be)* or from the electric organ of the *Electric eel* (*ee*) and are commercially available and have been used for biosensor fabrication.

ChEs are inhibited by a number of compounds including organophosphate and carbamate pesticides, nerve agents, natural toxins, heavy metals, and some drugs [6,28] although organophosphate pesticides are the progressive irreversible inhibitors of ChEs. The toxic effect of OPs is mainly related to their ability to irreversibly bind to the esteratic active site of the enzyme and hence prevent the transmission of nerve impulses. This may lead to a severe impairment of nerve functions or even death [6]. Depending on the extraction source, ChEs can have different substrate specificity and susceptibility to inhibitors [28].

Biosensors based on AChE as well as BChE were first reported during the 1980s. In the course of the years, there has been a continuous improvement of ChE-based biosensors due to the gradual improvement of transducer devices and the availability of pure enzymes [6]. Several generations of ChE biosensors have, therefore, been developed and recently, there have been numerous reports on the development of ChE biosensor methods for the detection of OPs. The operating principle of these biosensors is based on the fact that the activity of ChEs is inhibited by the OPs and the degree of inhibition is dependent on the concentration of the inhibitor in contact with the enzyme [30,31]. Various electrochemical methods for monitoring AChE and BChE activity have been published [1,6,9,16,28–31]. The most important step in biosensor development is the stable immobilization of the enzyme onto the electrode surface. This process is governed by the interactions between the enzyme and the electrode material and strongly affects the stability, sensitivity, response time and reproducibility of the biosensor. Several techniques for immobilization of the enzymes on transducers have been reported [24,28]. For increased stability and high activity of the immobilized enzyme, covalent coupling has been the most widely used and preferred procedure for fabrication of biosensors [24,28].

It is known that the determination of OPs by ChE inhibition biosensors is very sensitive, but indirect, requiring incubation for a period of time. The LOD is known to vary with the electrode configuration, the immobilization technique used to attach the enzyme and the incubation time of the pesticides with the enzyme [28]. The inhibition measurement results in the decrease in the biosensor response so that the life time of a biosensor is reduced to 10–15 measurements irrespective of the stability of the immobilized enzyme. Drawbacks of these methods are also the lack of selectivity and the need, in some cases, of enzyme reactivation/regeneration [5]. The ChE enzyme can be reactivated with oxime type-reactivation agents such as pyridine-2-aldoxime methochloride (2-PAM). The necessity of permanent reloading or reactivation of an enzymatic layer normally complicates the operation of a biosensor, especially under field conditions, and diminishes the reproducibility of the results obtained. To overcome the irreversibility of the ChE inhibition by OPs, several assay systems have been developed including the use of replaceable enzymatic membranes based on commercial supports and the manufacture of cheap disposable biosensors with the enzyme directly immobilized on the surface. In the first case, ChE is immobilized on the commercial membranes (nylon, cellulose nitrate and others). The membranes obtained can be stored singly and are used in a biosensor assembly when necessary [32]. The replacement of the enzyme membrane becomes very simple and can be done in field conditions. In the second case, screen-printed electrodes (SPE) or field-effect transistors are used with an ultrathin film of the enzyme directly bonded to the active surface of a sensor. The application of micro-fabrication techniques and small amounts of an enzyme used for immobilization make the measurement cheap enough for a single use of the biosensor with no reactivation of the inhibited cholinesterase. All the described biosensors give a sum parameter expressed as total *anti*-AChE activity using paraoxon as reference [32]. These assays are, therefore, unsuitable for compound-specific multi-analyte detection, and as OPs and carbamates inhibit the enzyme to a different extent, the relevance of such a parameter is debatable [32].

3.1. Acetylcholinesterase biosensors

The active site of AChE is characterized by a catalytic coordinated triad of three essential amino acids: histidine, serine, and aspartic acid [3]. The enzyme catalysis occurs when the triad's anionic binding site attracts the positively charged quaternary ammonium group of AChE. The serine hydroxyl group attacks and cleaves the ester after its deprotonation by a neighboring histidine group in the triad. In the presence of an inhibitor such as OP, the nucleophilic serine hydroxyl group located at the active site is covalently bound to the phosphorus atom of the OP [6].

The most commonly used enzyme for the development of OP biosensors is the enzyme AChE and researchers worldwide have devoted their efforts to developing AChE-based inhibitor biosensors based on various immobilization and detection technologies [6,10,28,30,31]. These biosensors have emerged as simple, rapid and highly sensitive tools for pesticide analysis and have been in development for over 20 years. More recently, AChE has been incorporated in very sensitive and selective OP biosensor systems. As previously mentioned, researchers have paid attention to two types of AChE inhibition-based biosensors according to their transduction mode (potentiometric and amperometric). Generally, the amperometric inhibition biosensors are more sensitive than the potentiometric biosensors. The potentiometric AChE biosensors detect OPs in a single step, using a range of pH-sensitive transducers, varying from the traditional pH glass electrodes to the ion selective field effect transistors [10]. The equipment is simple and involves the use of commercially available devices. However, the drawbacks of this method include the non-linearity of the curve obtained and the long response time which varies from 2 to 10 min depending on the time required to reach

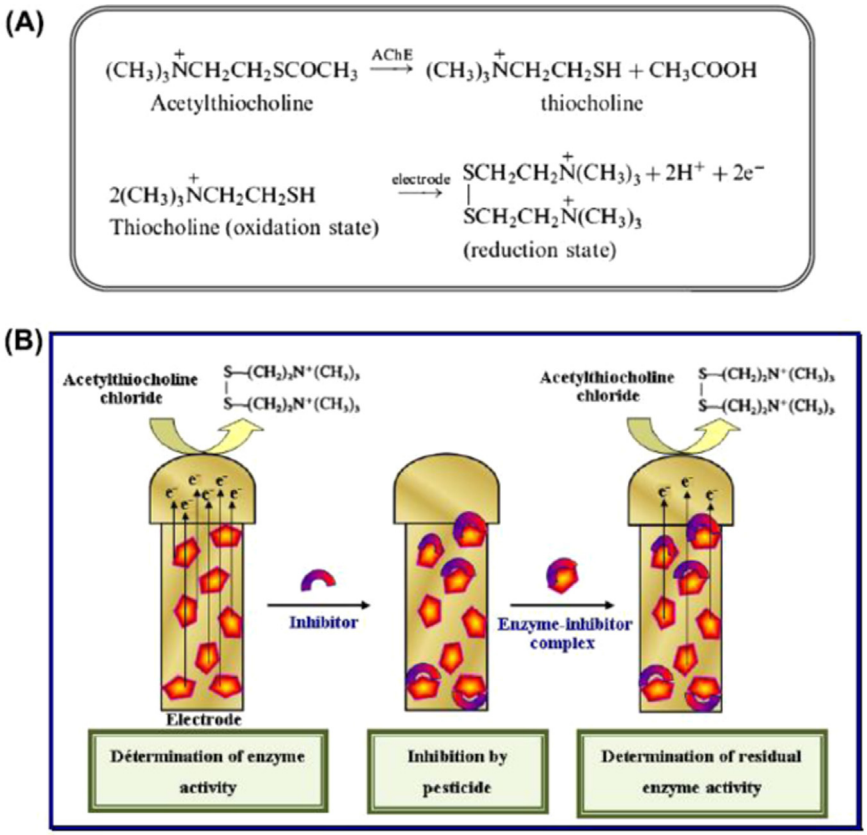


Fig. 1. (A) Electrochemical reactions involved in response measurements of AChE biosensors. (B) Strategy for the measurement of immobilized AChE inhibition in pesticide solution. Reproduced from Ref. [6], with permission from Elsevier.

equilibrium at the interface between the biosensor and the solution, since the measurements are performed under no current flow conditions [10].

The amperometric biosensors based on inhibition of AChE activity have been extensively studied to detect the presence of OPs in various sample matrices. With these biosensors, the target inhibitor irreversibly inhibits the activity of the immobilized AChE enzyme resulting in a decrease in signal that is proportional to the amount of target analyte present in the test solution. The detection mechanism for OPs is as follows: the immobilized AChE can catalyze the hydrolysis of acetylthiocholine, and the enzymatic reaction product is the electroactive thiocholine, which can produce an irreversible oxidation peak [33]. When the analyte is not present in the solution, the substrate acetylthiocholine is converted into thiocholine and acetic acid. In the presence of an inhibitor such as OP, the hydrolysis of acetylthiocholine is decreased or even null [6] resulting in reduced oxidation of thiocholine. The oxidation peak current of thiocholine is inversely proportional to the concentration of OPs. By monitoring the oxidation peak current of thiocholine before and after inhibition, the OP concentration can be determined. The general reaction mechanism of an electrochemical biosensor based on AChE is shown in Fig. 1A, while the procedure for the preparation of the AChE biosensor and its application for pesticide detection is illustrated in Fig. 1B.

The main advantage of the electrochemical biosensor methods that are based on the use of a native form of AChE is that they are selective towards pesticides and heavy metals and principally all these compounds can be detected. However, the major drawbacks of these methods arise from the facts that: (1) it is only possible to measure a sum parameter of AChE inhibition and no qualitative or quantitative information about an individual inhibitor in a complex solution can be obtained, i.e., the different AChE-inhibiting compounds in solution cannot be measured selectively. (2) These protocols involve multiple steps requiring measurement of the uninhibited activity of AChE, followed by incubation of the sensor with the analyte sample for 10–15 min (and even longer) and the measurement of the AChE again to determine the degree of inhibition. (3) A final step of reactivation/regeneration of the enzyme is necessary, which in many cases is partial and in some cases not possible due to irreversible inhibition. Most AChE-inhibition based biosensors described so far are based on the mechanism described above and they provide a sum parameter as signal, which is expressed as the total anti-AChE activity. Although the AChE biosensor method cannot selectively detect and quantify different pesticides, it can nevertheless be used for rapid pre-screening of samples to discriminate between samples that do or do not contain AChE inhibitors. Therefore, should AChE inhibitors be targeted, the biosensor test would help to increase the number of tested samples.

To date, the reliability issue that originates from poor selectivity and stability of these biosensors, as well as insufficient detection limits for direct trace level analysis is being investigated and addressed by various researchers worldwide. Despite the efforts to develop AChE biosensors, their analytical characteristics still require improvement in order to meet the required specifications for real sample applications. It is reported that the enzyme used in a biosensor design should be sensitive enough to decrease the threshold detection as low as possible and should also be selective towards the target analyte or class of analytes for which it is designed for [28]. Most of these biosensors employ commercially available *ee*AChE. However, it has been suggested that this enzyme might not be the optimal choice in terms of selectivity, sensitivity and stability. For instance, a study on a biosensor employing *ee* and human AChE showed that the sensitivity of the

biosensor was not sufficient for all organophosphates and carbamates in the range of the maximum residue limit of infant food although the paraoxon detection limit of the *ee*AChE biosensor was 1 μg/L [34].

3.1.1. Recent advances in the development of acetylcholinesterase biosensors for OP detection

The main strategies to improving the performances of the AChE biosensors for OPs have recently emerged and they include the genetic engineering of the biological recognition element as well as the use of nanomaterials for transducer modification.

The nanotechnological approach in electrochemical biosensor development takes advantage of the electroanalytical properties of the nanostructures, their action as electron transfer mediators or electrical wires, large surface to volume ratio, structural robustness, and biocompatibility [10]. This results in electrode potential lowering, enhancement of the electron transfer rate with no electrode surface fouling, sensitivity increase, stability improvement, and interface functionalization [10]. Various nanomaterials have been used as AChE immobilization matrices during the development of biosensors for OPs [24, 25, 35–38]. The results of these studies demonstrate that modification of the transducer with nanomaterials enables detection of OPs in the nano-mole to pico-mole range and enhances stability of the biosensors [10].

The alternative strategy that has been explored by researchers worldwide, aiming at improving sensitivity and selectivity of the AChE-based inhibitor biosensors, involves the incorporation of the biorecognition elements with tailor made properties into the biosensing platform. Recently, advances in biotechnology and molecular biology have made possible the genetic engineering and production of numerous recombinant AChE enzymes that are highly efficient, have specific and high inhibition constants for the desired analytes, and are more sensitive and selective thus paving way for interesting perspectives in many practical applications. The most commonly used strategy to achieve more sensitive and selective enzymes and consequently biosensors is to increase the affinity for the target analyte favoring the accessibility of the active site [9]. Thus, site-directed mutagenesis on specific amino acids has consequently been used to fine-tune the AChE's characteristics, in order to further increase its stability, selectivity and sensitivity, and produce optimized enzymes that would in turn be suitable for the development of novel biosensors [11]. It has been reported that the combination of engineered AChEs with different sensitivity levels for different analytes enables the construction of selective biosensors and that enzymes extracted from different sources have different sensitivities and selectivity toward pesticides [9]. For instance, the AChE extracted from the *Drosophila melanogaster (dm)* can be up to 8-fold more sensitive than the AChE from *ee*. Thus, several works aiming at large-scale production of mutant AChE enzymes from wild *dm* have been published [39]. For example, Andreescu et al. [40] immobilized recombinant *dm*AChE on nickel-modified thick-film electrodes in order to improve the detection limits. This work reported detection limits for paraoxon, dichlorvos and chlorpyrifos ethyl-oxon to be 10^{-9} mol L^{-1}. Nunes et al. [41] also illustrated the use of *dm*AChE variants, which displayed great sensitivity to methamidophos with one of the seven tested mutants AChE(Dros)-B03 mutant decreasing the detectable methamidophos concentration to a value as low as 1.4 ppb (μg/L) compared to 4.8 ppb which was achieved with the wild type *dm*AChE and 53 ppb which was achieved with *ee*AChE.

De Oliveira Marques et al. [39] investigated the performances of genetically modified AChE from *dm* and from commercial sources, *ee*, *be* and *he* as biological receptors for the detection of methamidophos pesticide based on inhibition studies and the results indicated that the most engineered variant of AChE from *dm* showed enhanced sensitivity toward methamidophos. Among the 24 *dm*AChE variants that were tested, 12 presented a sensitivity comparable to the commercially available *ee*AChE, but higher than AChEs from *be* and *he*. The sensor constructed with genetically modified enzymes showed better characteristics with respect to detection limit and sensitivity compared with those using commercial *ee*AChE. The lower detection limit of 1 ppb was obtained with D375G, Y370F, Y374A, F376L mutant of *dm*AChE, compared to 90 ppb for the commercial *ee*AChE.

Sotiropoulou et al. [11] demonstrated the use of a highly sensitive double mutant (E69YY71D) of the *dm*AChE for the detection of dichlorvos and this mutant decreased the detection limit of the biosensor down to 10^{-17} mol L^{-1}, which was five orders of magnitude lower compared to the *ee*AChE-based biosensor and eight orders of magnitude lower than other biosensors. Bucur et al. [42] also presented an automatic method able to identify the presence of neurotoxic insecticides using omethoate as a model compound. In this work, two sensitive AChEs from the wild type *dm* (wt-*dm*) and the E69W mutant were used. The enzymes were immobilized on SPE by entrapment in a photocrosslinkable PVA-SbQ polymer used with an FIA system with three channels, each one of them simultaneously analyzing the same sample using biosensors based on different AChEs. The biosensor based on the wt-*dm* had a LOD of 2×10^{-6} mol L^{-1} omethoate, while the one based on the E69W mutant presented a LOD of 1×10^{-7} mol L^{-1}. The commercial *ee*AChE was also used and was found to be resistant to omethoate i.e. *ee*AChE was not inhibited by omethoate. These results clearly indicate that by properly choosing the enzyme, the determination of OPs can be largely improved in terms of number of detectable compounds, sensitivity and selectivity. Although this approach allowed the specific detection of analytes, selectivity, which is a less mentioned but still a difficult task for the inhibition-based biosensors that are plagued by multiple interferences was not achieved.

In another study, Istamboulie et al. [43] presented a biosensor for OPs based on immobilization of a highly sensitive genetically engineered AChE (B394) by affinity interactions on metal chelate-functionalized magnetic beads. The results illustrated that the use of the B394 enzyme allowed lowering both the IC50 and LOD by a factor of 100 when compared to *ee*AChE. The oriented and site-specific immobilization combined with the high specificity of the B394 mutant allowed more sensitive detection of chlorpyriphos-oxon and chlorfenvinphos pesticides in concentrations as low as 1.3×10^{-11} mol L^{-1} (IC10).

Valdés-Ramírez et al. [44] also described the construction of an amperometric biosensor for the highly sensitive detection of dichlorvos, based on the inhibition of AChE. The sensitivity of *ee*AChE, genetically engineered AChE (B394) and the wild type AChE (B1) from *dm* were tested and compared. The enzymes were immobilized by entrapment in a photocrosslinkable PVA-SbQ polymer on a screen printed graphite electrode. The enzyme activity was estimated amperometrically by measuring the thiocholine produced by the enzymatic hydrolysis of acetylthiocholine. The pesticide was measured in the presence of 5% acetonitrile without loss of enzyme activity. The best sensitivity was achieved with the *dm* mutant B394 with a detection limit of 7×10^{-11} mol L^{-1} as compared to 1×10^{-8} mol L^{-1} with the B1 *dm* and 6×10^{-7} mol L^{-1} with the *ee*. The results of this work also demonstrated that the use of genetically modified AChE decreased considerably the detection limit (with four orders of magnitude) as compared to biosensors based on wild type *dm* and *ee*AChEs.

The development of an automated flow-based biosensor that employs genetically modified AChE enzymes B394, B4 and wild type B131 was reported by Mishra et al. [45]. The biosensor was based on SPE that was integrated into a flow cell. The enzymes were immobilized on cobalt (II) phthalocyanine (CoPC) modified

electrodes by entrapment in a photocrosslinkable polymer (PVA-AWP). The automated flow-based biosensor was successfully used to quantify chlorpyriphos-oxon (CPO), ethyl paraoxon (EPOx) and malaoxon (MOx). The total analysis time for the assay was less than 15 min. The best LODs were obtained with B394.

The use of several biosensors with different inhibition degrees also envisages the possibility to manufacturing sensitive and selective pesticide arrays [9]. In this aspect, Schulze et al. [34] reported that the sensitivity of AChE biosensors to insecticides could be increased substantially by engineering AChE B of *Nippostrongylus brasiliensis (nb)*. The introduction of 10 single and 4 double mutations into the AChE peptide chain led to an increase in sensitivity of 10 of the 11 insecticides tested. The combination of three mutants with the wild-type enzyme in a multienzyme biosensor array enabled the detection of 11 out of the 14 most important organophosphates and carbamates at concentrations below 10 μg/kg, the maximum residue limit of infant food. The detection limit for pirimiphos methyl was reduced from 10 μg/L to a value as low as 1 ng/L (3.5×10^{-12} mol/L). The newly created biosensors exhibited extraordinary high storage stability. There was no loss of sensitivity of *nb*AChE B, immobilized on SPE, even after 17-month storage at room temperature. All these studies reveal that the use of genetically engineered AChEs can improve the analytical characteristics of the corresponding biosensor systems.

Indeed, all the studies done with genetically engineered AChEs demonstrated improved sensitivities with a confirmation that the AChEs extracted from different sources portray different sensitivities. However, only the use of genetically engineered AChEs to construct biosensors does not address the selectivity issue and it still remains a challenge when it comes to real application of these biosensors. This calls for investigations of ways of further modification of AChE enzymes in order to improve the selectivity of these biosensors. Table 1 is a compilation of the results obtained by different researchers for the detection of selected OPs. The results demonstrate that the use of genetically modified AChE decreased considerably the detection limit as compared to biosensors based on *ee*AChE. Usually genetic engineered enzymes show better sensitivity than enzymes from natural sources, varying from two to five orders of magnitude [11,46]. It can be observed that some authors reported exceptional results with LODs as low as 10^{-17} mol L^{-1}. This high performance could be due to the association of a good transducer and the genetically engineered enzyme used. The incubation time reported by the researchers is 10 min which is reasonable enough for analysis.

The only approach so far that aims at solving the AChE biosensor selectivity problem involves the application of multi-sensor arrays that are combined with the data processing of artificial neural networks (ANNs) [9,28,32,47]. In these cases, ANNs are particularly suited for various tasks in data processing, such as memorizing, associating, recognizing patterns, or modeling nonlineal multivariate data. ANNs are non-parametric calibration methods specially created to process non-linear information: these tools have an ability to learn and extract X–Y relationships from a set of training samples [46]. The fundamental processing element of an ANNs is the neuron. Neurons are arranged in layers that make up the global network architecture. ANNs have been applied to liquid chromatography (LC), MS and voltammetric methods to quantify and resolve pesticide mixtures from different families, most of the time a combination of OPs and carbamates or triazines [46]. Thus, various researchers have adopted and successfully used ANNs for data processing in order to resolve pesticide mixtures from the response of an inhibition biosensor array.

Bachmann and Schmid [49] pioneered the development and use of a sensitive screen-printed amperometric multielectrode biosensor based on the principle of AChE inhibition and chemometric data analysis using ANNs for rapid discrimination of paraoxon and carbofuran in mixtures. In this work, four types of native or recombinant AChEs (from *ee, be*, *dm* and *rat brain*) were immobilized by screen printing on four-electrode thick-film sensors in sets containing each AChE. The multisensors were used for quantitative inhibition analysis of binary reagent mixtures combining the AChE-multisensor with feed-forward ANNs. The sensors registered a detection range of 0.2–20 mg/L for both analytes with an overall assay time of less than 60 min The individual inhibition pattern of each AChE–analyte combination enabled the discrimination of both analytes by the use of neural network data processing. Thus, paraoxon and carbofuran in mixtures displaying a concentration range of 0–20 mg/L for each analyte could be analyzed. Bachmann et al. [32] then attempted to develop improved biosensor systems based on recombinant *dm*AChE. He presented the use of engineered variants of *dm*AChE as biological receptors of AChE-multi-sensors for the simultaneous detection and discrimination of OPs and carbamates in binary mixtures of insecticides. These systems were based on a combination of amperometric multielectrode biosensors with chemometric data analysis of sensor outputs using ANNs. The multisensors were used for inhibition analysis of binary paraoxon and carbofuran mixtures in a concentration range of 0–5 μg/L followed by data analysis using feed-forward ANN. Malaoxon and paraoxon in composite solutions of 0–5 μg/L were also discriminated using these multisensors. Since then, a number of biosensor systems employing the use of multisensor arrays combined with ANNs have been reported. Most of the works reported aimed at resolving a mixture of pesticides from different families (i.e. between OP and carbamate pesticides) and only a few works have resolved a pesticide mixture from the same family (i.e. both OP pesticides), where the inhibition responses may be quite similar making its resolution more difficult.

In this respect, Valdés-Ramírez et al. [44] developed an amperometric biosensor array to resolve pesticide mixtures of dichlorvos and methyl paraoxon. The target of this work was to develop a very sensitive, simple, relatively inexpensive and trustworthy method of analysis to resolve dichlorvos and methyl

Table 1
Comparison of limits of detection for AChEs (from different sources) when applied to OP analytes.

Enzyme Source	Electrode material	Detection technique	Limit of Detection (mol L^{-1})	Analyte	Incubation time	Reference
*dm*AChE (E69Y Y71D)	AChE/Carbon pellet	Amperometry-FIA	$\sim 1 \times 10^{-17}$	Dichlorvos	10 min	[11]
*ee*AChE	AChE/Carbon pellet	Amperometry-FIA	1×10^{-8}	Dichlorvos	10 min	[11]
*dm*AChE (E69Y Y71D)	AChE/Carbon pellet	Amperometry-FIA	1×10^{-12}	Paraoxon	10 min	[11]
*dm*AChE	AChE/CoPC/SPE	Electrochemical-FIA	$\sim 1 \times 10^{-17}$	Dichlorvos	NR	[48]
*dm*AChE	AChE/CoPC/SPE	Electrochemical-FIA	$\sim 1 \times 10^{-16}$	Parathion	NR	[48]
*dm*AChE	AChE/CoPC/SPE	Electrochemical-FIA	$\sim 1 \times 10^{-16}$	Azinphos	NR	[48]
*dm*AChE	AChE/PVA-SbQ/SPE	Amperometry	7×10^{-11}	Dichlorvos	10 min	[44]
*ee*AChE	AChE/PVA-SbQ/SPE	Amperometry	6×10^{-7}	Dichlorvos	10 min	[44]

Note: NR, not reported.

paraoxon pesticide mixtures, a case where both pesticides are OPs. The biosensor array was used in a flow injection system, in order to operate the inhibition procedure automatically and faster. The sensors used were three screen-printed amperometric biosensors that incorporated three different AChE enzymes: the wild type *ee* and two different genetically modified enzymes, B1 and B394 mutants, from *dm*. The inhibition response triplet was modeled using an ANN which was trained with mixture solutions that contain dichlorvos from 10^{-4} to 0.1 μM and methyl paraoxon from 0.001 to 2.5 μM. This system was considered an inhibition electronic tongue. In comparison with the previous works by Bachmann and Schmid [49] and Bachmann et al. [32], the proposed alternative was fast, portable, automated, and was able to resolve a mixture of two OPs, a challenging case.

In an attempt to address the selectivity of the previously described sensor [43], Istamboulie et al. [18] developed amperometric AChE biosensors for resolving mixtures of chlorpyrifos oxon (CPO) and chlorfenvinfos (CFV) pesticides. Three different biosensors were built using the wild type *ee*, genetically modified *dm*AChE B394 and B394 co-immobilized with a PTE. ANNs were used to model the combined response of the biosensors to the two pesticides. Specifically two different ANNs were constructed. The first one was used to model the combined response of B394+ PTE and EE biosensors and was applied when the concentration of CPO was high and the other, modeling the combined response of B394+ PTE and B394 biosensors, was applied with low concentrations of CPO. The results showed that it is possible to selectively quantify mixtures of the pesticides CPO and CFV.

In another study, Crew et al. [50] described a biosensor array based on six AChE enzymes for use in a novel automated instrument incorporating a neural network program. Electrochemical analysis was carried out using chronoamperometry and the measurement was taken 10 s after applying a potential of 0 V vs. Ag/AgCl. The total analysis time for the complete assay was less than 6 min. The array was used to produce calibration data with six OPs in the concentration range of 10^{-5} mol L^{-1} to 10^{-9} mol L^{-1} to train a neural network. It was envisaged that this analytical system could provide a rapid detection system for the early warning of contamination in water and food.

Alonso et al. [51] also presented a one-step inhibition biosensor method that can be used to determine mixtures of three OPs. The array of biosensors was designed using two AChEs from *dm* (wild-type and genetically modified) and ANN was used to solve mixtures of CPO, CFV and Azinphos methyl-oxon (AZMO) insecticides. The three insecticides were determined with low error due to the direct measurement step. In comparison with previous work, this method was faster, simpler and resolved three compounds using two enzymes. This was an important step toward building a more selective, more efficient array with a pre-trained network suited for in situ applications.

Although selectivity has been achieved by combining multisensor arrays with ANNs, the implementation of ANNs in dedicated systems such as Digital Signal Processors (DSPs)-based implementations, Field-Programmable Gate Arrays (FPGAs)-based implementations and microcontrollers (RISC)-based implementation has some challenges, such as accuracy, compatibility with other peripherals, speed and high cost [52]. Alonso et al. [52], therefore, proposed a low-cost hardware implementation of ANNs using a dsPIC® microcontroller to resolve mixtures of pesticides measured by amperometric AChE biosensors. The ANNs had the same accuracy as that of specialized ANN software such as MATLAB®. The response of three biosensors to different concentrations of CPO and CFV was modeled by two ANNs, which were implemented on the dsPIC®. The performances of the ANNs were good and this implementation was proposed for developing low-cost analytical chemical specialized tools.

3.1.2. Practical applications of acetylcholinesterase biosensors

The AChE biosensors are designed to detect AChE inhibitors such as OPs, carbamate pesticides, heavy metals, nerve agents, natural toxins, and some drugs [6,28] although pesticides are the progressive irreversible inhibitors of AChEs and they have mostly been used to detect pesticides in water, food and environmental matrices. Most of the AChE biosensors designed for practical applications employ immobilized enzymes and the detection limit is said to vary with the electrode configuration and the immobilization technique used to attach the enzyme [28]. However, the practical application of immobilized AChE has a significant limitation in that the inhibition of the immobilized enzyme normally results in a decrease of AChE activity so that repetitive use of the same biosensor becomes limited without enzyme reloading or reactivation. Moreover, the addition of a particular reagent can regenerate the enzyme activity but extends its lifetime for only a few cycles, since the original activity of the enzyme can never be restored to 100%. Another factor that limits the application of the AChE biosensors that are based on irreversible enzyme inhibition is the long analysis time due to the necessary time of incubation (10–20 min). These factors limit the applications of these biosensors especially if they were intended for in situ measurements [28]. Therefore, a single experiment approach, like the use of disposable SPE is justified and highly desired in the case of irreversible inhibition [30]. These electrodes are usually prepared by screen-printing technology which allows mass production with significant reduction in the price per electrode [28]. Amine et al. [30] suggests the use of biosensors based on reversible enzyme inhibition combined with flow injection analysis when high frequency of analyses is required.

Another major factor that limits the applicability of the AChE biosensor methodology has been the poor selectivity of these biosensors (i.e. the capacity to detect selectively only one analyte). AChE is known to be inhibited by several OPs and carbamate pesticides so in a mixture of these compounds, only an anti-AChE activity index can be calculated [30]. Despite the significant amount of scientific research dedicated to AChE biosensors in general, little success has been realized through their real practical applications and the transfer of these devices from pristine research laboratory conditions to real life and commercial applications still presents a major challenge. To date, the existing native AChE biosensors and those employing genetically engineered AChEs still suffer from inability to correctly differentiate OPs and identify particular analytes, so the selectivity for these biosensors is still very poor. Due to this limitation, the majority of AChE sensors reported in literature have been tested on standard solutions and not on real samples. AChE biosensors have mainly been attractive for measuring the total toxicity of the samples, rather than specific OPs in solution. Amine et al. [30] suggests that in this case, the inhibition assay can be considered as a “Family Doctor”; further analyses can be performed by a “Specialist Doctor”, such as a HPLC method, able to identify and quantify each inhibitor. The non-contaminated samples normally do not show any inhibition when measured with AChE biosensors, so they do not require further analysis with HPLC, saving time and money. Therefore these biosensors can be considered as excellent, fast and cost-effective devices for toxicity screening of OPs. However, the critical parameters of the biosensors (employing native and genetically engineered AChEs) such as enzyme stability, reliability and selectivity still need improvement [28].

The most studied pesticides are paraoxon, dichlorvos, diazinon, aldicarb and carbofuran. Paraoxon is commonly used as a model for AChE inhibition. Some pesticides have nearly no or little inhibitory effect on AChE in their pure form and detection is only possible by oxidizing them to oxon forms, which are much more toxic [28]. This review focuses on the application of AChE

biosensors for the detection of OPs. The strategies used for testing OPs in real samples often involve spiking the samples with a known amount of pesticide in question and evaluating the inhibition degree in a real matrix [28].

An example of practical application of a highly sensitive and rapid food-screening test based on disposable screen-printed AChE (from *ee* and *hu*) biosensors for the direct detection of insecticide residues in infant food was described by Schulze et al. [53]. The biosensor test was used for the detection of pesticides in 26 fruit and vegetable samples as well as 23 samples of processed infant foods and the test was compared with two traditional pesticide multi-residue analysis methods (GC–MS and LC-MS). Each AChE biosensor was incubated in the food sample for 30 min before carrying out inhibition measurements. The proper performance of the test was verified by analyzing several food samples to which different amounts of paraoxon in the concentration range between 5 and 20 μg/kg were added. The inhibition of the *ee*AChE, caused by the incubation in the spiked food, was compared with the inhibition that was observed when equivalent paraoxon concentrations were present in the buffer solution. This biosensor method could detect levels lower than 5 μg/kg and thus fulfilled the demands of the EU. To substantiate the measurements, recovery rates were determined and amounted to an average of 104% in food. With respect to pesticides, the developed biosensor test method presented several advantages over the classical analytical methods and previously described biosensor tests. Using this test, samples could be analyzed within approximately 90 min and it did not involve the complicated extraction, cleanup and measurement steps which often require well-trained personnel and sophisticated equipment. The test method also avoided the use of any organic solvent and no pre-concentration step was needed. However, it was only possible to measure a sum parameter of AChE inhibition and qualitative or quantitative information about the single compound could not be obtained. The authors, therefore, recommended the use of this biosensor test in combination with traditional multi-residue methods as a prescreening test to discriminate between samples that do or do not contain AChE inhibitors.

In another study, practical application of an amperometric biosensor constructed with genetically engineered AChE (B394) has been described by Valdés-Ramírez et al. [44]. The B394 biosensor was used to quantify dichlorvos in a sample of skin apple after extraction with acetonitrile and spiking with known concentration of dichlorvos standard solution. For inhibition measurements, the biosensor was incubated for 10 min in a solution containing the dichlorvos extract in acetonitrile. This solution was prepared in 5% (v/v) of organic sample extract and PBS solution. The results obtained with the apple sample showed a 63% inhibition which was in good agreement with the 65% inhibition percentage obtained with the reference sample (without apple). This work demonstrated the possibility to quantify dichlorvos in solid matrices using biosensors based on *dm*AChE. In addition, the biosensor was able to work in the presence of 5% acetonitrile, which was necessary for the extraction of pesticide from the sample. The authors recommended the use of disposable biosensors which would offer some additional advantages such as mass production, possibility for miniaturization and low cost. However, this study did not report on the selectivity of the developed biosensor and the possibility of its application to complex real samples is not known.

The development of an automated flow-based biosensor (employing genetically modified AChE enzymes B394, B4 and the wild type B131) for the detection of the OPs in milk was described by Mishra et al. [45]. The biosensor was based on SPE that was integrated into a flow cell. The OPs used in this study were chlorpyriphos-oxon (CPO), ethyl paraoxon (EPOx) and malaoxon (MOx). The biosensor B394 successfully determined EPOx, CPO and MOx down to 5×10^{-9} mol L^{-1}, 5×10^{-12} mol L^{-1} and 5×10^{-10} mol L^{-1}, respectively, in milk samples. It was reported that though direct incubation in the milk samples would be advantageous, matrix effects would impede sample analysis and that this biosensor test was more suited to low fat milk samples than those with high fat content. The analyses performed on the calibrants and spiked samples demonstrated that the system could successfully determine OPs in real samples. The tests were completed in less than 15 min with good reproducibility and the biosensor met the requirements set by EU regulations with respect to detection limits for tested milk samples as well as recoveries. The described biosensor food screening test was said to be fast compared to chromatographic methods. The authors, therefore, recommended the proposed system for online monitoring of OPs in milk processing units and collection centers. This method was reported to be inexpensive, sensitive, portable, non-invasive and provided real-time results though it was not selective.

As discussed earlier, the selectivity of AChE biosensors has been reported to be improved by combining multi-sensor arrays with ANNs and this kind of biosensors have been employed successfully for real practical applications to resolve OP mixtures in real samples. For example, Valdés-Ramírez et al. [46] developed an amperometric biosensor array to resolve pesticide mixtures of dichlorvos and methyl paraoxon in real samples of river water and bottled mineral water. The sensors used were three screen-printed amperometric biosensors that incorporated three AChEs: the wild type *eeAChE* and two genetically modified enzymes, B1 and B394 mutants, from *dm*. The biosensor array was used in a flow injection analysis (FIA) system, in order to operate the inhibition procedure automatically. Each sample was divided into two, with the first portions analyzed directly with the FIA system, and the second portions spiked with different amounts of standard dichlorvos and methyl paraoxon. The results obtained according to the ANN response model indicated that bottle water contained traces of dichlorvos (0.008 μ mol L^{-1}) while methyl paraoxon was below the detection limit. On the other hand, when the samples were spiked with the two considered pesticides, the bottled water sample analysis correctly found evidence for the presence of both pesticides, dichlorvos and methyl paraoxon, in this case the amount of pesticide found were 0.0033 μmol L^{-1} of dichlorvos and 1.40 μ mol L^{-1} of methyl paraoxon. The results for the river water sample were also consistent with the spiked concentrations. Average recoveries were 104% for dichlorvos and 118% for methyl paraoxon. These results suggested that the proposed method was able to resolve the considered pesticide mixtures in the real samples tested. With the automated FIA system presented here, certain advantages such as reduced operation cost, short analysis time and improved reproducibility were envisaged in comparison with the standard HPLC procedures. When applied to real samples, the two OPs were determined with low errors using an extremely simple procedure that can be the principle to develop an on-site screening methodology. In comparison with previous works [32,49], the proposed alternative was fast, portable and automated, and resolved a mixture of two OPs, a challenging case.

Istamboulie et al. [18] developed amperometric AChE biosensors to resolve mixtures of chlorpyrifos oxon (CPO) and chlorfenvinfos (CFV) pesticides in real water samples. Three different amperometric biosensors were built using AChEs from wild type *ee*, the genetically modified dmAChE B394 and B394 co-immobilized with a PTE and ANNs were used to model the combined response of the two pesticides. The proposed method was then employed in complex real samples to demonstrate its viability. Seven water samples taken from a lake were analyzed directly and after spiking with CFV and CPO. All samples without added pesticide revealed no inhibitory effect, whereas the spiked ones showed some inhibition, which could be estimated with the

previously trained ANN2, since the amount of spiked pesticide was low. The results of this study indicated that the proposed method was able to resolve the considered pesticide mixtures in the tested real samples. It was, therefore, possible to selectively quantify mixtures of the pesticides CPO and CFV and both pesticides were quantified with low errors from a direct measurement step.

Crew et al. [50] also employed a biosensor array based on six AChE enzymes (wt (B131), B02, B04, B65, B394 and B421) and a novel automated instrument incorporating a neural network program to detect the OPs (dichlorvos, malaoxon, chlorpyrifos-oxon, chlorpyrifos- methyl-oxon, chlorfenvinphos and pirimiphos-methyl-oxon) in water and food samples. Solutions containing the OPs were prepared at concentrations between 10^{-5} and 10^{-9} mol L^{-1} in 0.05 mol L^{-1} phosphate buffer pH 8.0. The array was used to produce calibration data with six OPs in the concentration range of 10^{-5} mol L^{-1} to 10^{-9} mol L^{-1} to train a neural network. The output of the neural network was subsequently evaluated using different sample matrices. There was no detrimental matrix effects observed from water, phosphate buffer, food or vegetable extracts. Furthermore, the sensor system was not detrimentally affected by the contents of water samples taken from each stage of the water treatment process. The biosensor system successfully identified and quantified all samples where an OP was present in water, food and vegetable extracts containing different OPs. There were no false positives or false negatives observed during the evaluation of the analytical system. The biosensor arrays and automated instrument were evaluated in situ in field experiments where the instrument was successfully applied for analysis of a range of environmental samples. Prior to any field testing, the effectiveness of the neural network in identifying the presence of an OP in a solution was examined using deionized water spiked with each OP at final concentrations between 10^{-5} mol L^{-1} and 10^{-7} mol L^{-1}; the neural network identified the presence of an OP in each of the spiked solutions. Following analysis in the field location, it was evident that the output from the biosensor array was not significantly different from the blank solutions. Consequently, the interpretation of the data by the neural network indicated that no OPs were present in any of the samples. In order to study the effect of food extracts on the biosensor array response, a broad range of raw foods were selected. Extracts of wheat, cabbage, apple, orange and cherry were extracted using a non-toxic solvent based extraction and reconstituted in methanolic 0.05 mol L^{-1} phosphate buffer pH 8 as described by Crew et al. [54]. The chronoamperometric responses obtained in the presence of any of the food extracts were not significantly different from those obtained with buffer solution indicating that no interfering compounds or matrix effects were present. To validate the biosensor array for the analysis of food sample extracts, a selection of different food types were spiked with known quantities of six different OPs then extracted and interrogated using the biosensor system, followed by interpretation by the neural network. The neural network identified the presence of the inhibiting OP in every spiked extract, and conversely indicated the absence of any OP in every sample where no OP was present. Consequently the system did not produce any false positives or false negatives.

The electrochemical analytical system and biosensor array described by Crew et al. [50] clearly demonstrates robustness, precision and portability and its capacity to be taken and operated at a field site. The portability of the system was clearly demonstrated in the field trials and the equipment completed all of the analyses and interpretation of the environmental samples without any malfunction or need for repetition. This study demonstrated that both qualitative and quantitative analysis, using AChE-based electrochemical biosensor arrays, with neural network recognition were readily feasible for the in situ analysis of environmental samples and food extracts.

Another interesting development is described by Mishra et al. [55]. In this work, an automatic flow based biosensor was applied for the detection of binary CPO and malaoxon (MO) mixtures in milk, based on ANN. Genetically modified AChE B394 and B4 were used as a biological recognition elements for sensor development. The enzymes were coupled on SPEs and inserted in a flow cell connected to the potentiostat and syringe pump. The main aim of the ANN setup was to obtain the best performance with the training data. The training data was taken from the inhibitions of enzyme activities which was performed individually using flow based biosensor. The principle of the training model was based on the evaluation of functions to obtain desired outputs and find the lowest error. The network was constructed based on a set of data which was divided in training set and tests set. In order to validate the training model, the network was fed with new set of data and a new test called external test was made as shown in Fig. 2A. All possible combinations of 9 training algorithms and 14 different structures with different number of neurons, hidden layers and different transfer functions were tested. The training algorithms of CPO and MO are shown in Fig. 2B. The concentrations were always chosen at low level. Fig. 2C shows inhibition curve obtained in PB with 7 different concentrations of CPO and MO mixtures in triplicates. In order to model the combined response of CPO and MO, a total set of 19 mixtures were prepared using ANN. The modeling was validated with an external test of 6 milk samples spiked with CPO and MO mixtures. The spiked concentrations of CPO and MO ranged from 5×10^{-10} to 5×10^{-12} mol L^{-1} and 1.01×10^{-10} to 9.17×10^{-11} mol L^{-1}, respectively. The developed automated flow based biosensor system was found to be very rigid, practical and easy to use without human intervention for a long time. The results obtained by the system were highly reproducible and could be reused.

4. Organophosphorus hydrolase biosensors

The organophosphorus hydrolases are a growing class of bacterial enzymes that have recently been recognized and characterized as alternative recognition enzymes for direct detection of OPs. In most journal articles, organophosphorus hydrolases have been referred to by names such as phosphotriesterase, organophosphorus hydrolase, organophosphate-degrading enzymes, or parathion hydrolases. In this article, we will focus on the well-characterized organophosphorus hydrolase (OPH, E. C. 3.1.8.1), which was first isolated from the soil bacteria *Pseudomonas diminuta* MG and *Flavobacterium* sp. This OPH prefers the substrates paraoxon and P-esters, which have a P-O bond. The OPH catalyzed hydrolysis of OP compounds generate hydrogen ions as a result of the cleavage of P-O, P-F, P-CN or P-S bonds [56,57]. Unlike AChE, OPH catalyzes the hydrolysis of OPs with a high turnover rate; therefore, OPH has shown considerable potential in OP biosensor applications. OPH-based biosensor assays are reported as reversible detection systems since they respond by taking the OP compounds as the enzyme substrates, rather than inhibitors. Since the enzymatically catalyzed OP substrates hydrolysis involves pH changes and generates electroactive products, the detection could be performed in a single step, using potentiometric (pH sensitive) or amperometric transducers. The hydrolysis products are usually more polar than the parent OPs and the resulting metabolites possess lower phosphorylating power than the initial OP, and are, therefore, less toxic. Due to these reasons the hydrolysis by OPH is considered a detoxifying reaction [58].

Direct OP analysis can be achieved by the use of OPH electrochemical sensors and, therefore, the direct detection using OPH has been utilized for a broad class of OPs [59]. Since OPH enzymes have a rather wide spectrum of substrate selectivity, they hydrolyze many kinds of organophosphorus compounds including the OPs

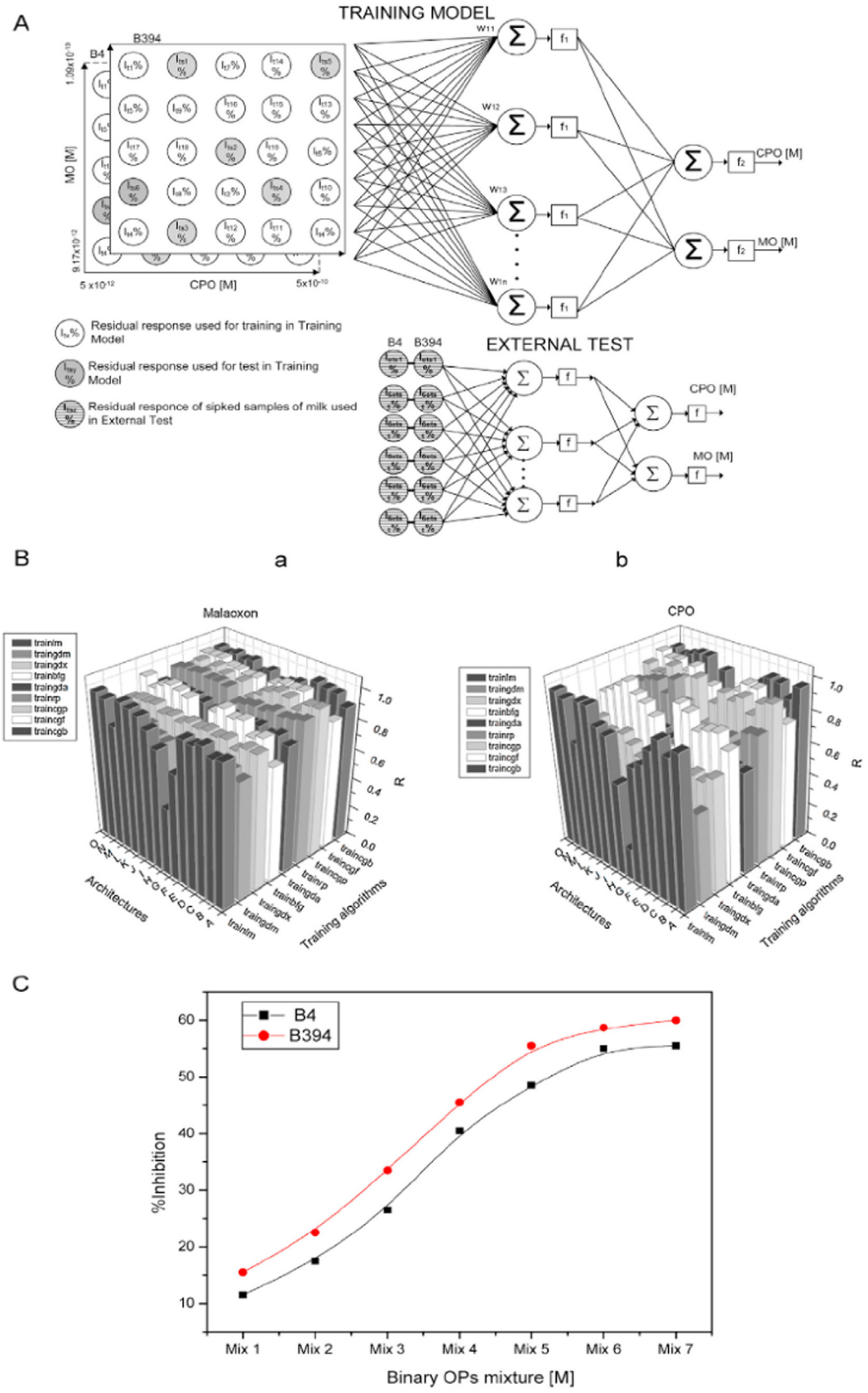
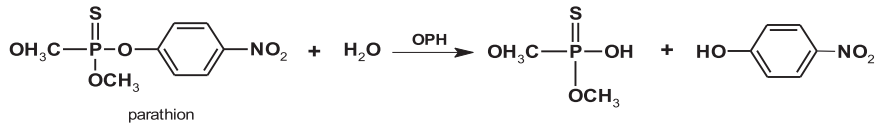


Fig. 2. (A) The ANN constructed based on a set of data which is divided into training set and tests set (external test). (B) Schematics of different structures tested for each output of ANN during setup of training algorithms for CPO and MO. (C) Inhibition curves obtained with different concentrations of binary OPs (CPO and MO) mixtures using biosensor B4 and B394. Reproduced from Ref. [55], with permission from Elsevier.



Scheme 1. OPH-catalyzed hydrolysis of parathion.

paraoxon, parathion, methyl parathion, and others., and nerve agents such as sarin, soman, tabun and VX [23,59]. Of its substrates, OPH can hydrolyze paraoxon the fastest with a rate of 3,170 s^{-1}, while it shows less activity against OP nerve agents [60]. Some OPs can produce chromophoric products, such as *p*-nitrophenol [16], from the hydrolysis of paraoxon, parathion, and methyl parathion, or chlorferon from coumaphos. It is obvious from the reactions shown in Scheme 1 that the reaction product such as 4-nitrophenol can be electrochemically oxidized on the electrode to generate electric current as output signal which can then be detected using potentiometric or amperometric transducers. The amperometric detection has relied on the anodic detection of the *p*-nitrophenol reaction product of OP pesticides such as paraoxon, parathion, methyl parathion, or fenitrothion. Thus, OPH-modified electrodes directly detect OP compounds in a single step, as compared to two steps required for the biosensors constructed using AChE enzymes. In this case, the detection signal is directly proportional to the concentration of the OPs. The advantages of the amperometric OPH detections over the potentiometric detections include the high sensitivity and precision of analysis, the linearity of the calibration plot and the fast sensor response.

The use of OPH enzyme for degradation of OPs as a decontamination protocol has been discussed extensively [61]. Some reviews summarize the performances of the enzymes electrochemical sensors for OP pesticides determination and the principles of their operation [56,62]. Several types of OPH-based amperometric, potentiometric, or optical biosensors have been described [12–14,16]. Because of the high binding specificity of OPH for OPs, the enzyme has been used for detection of OPs both in microbial systems [63] and as a purified enzyme [13] using techniques such as optical, acoustic, potentiometric, and amperometric. The biosensors based on a catalytic reaction are said to be superior to the inhibition mode since they can be potentially reused and are also suitable for continuous monitoring [16]. It has been reported that OPH-based systems allow selective determination of the family of OP compounds that produce nitrophenol upon hydrolysis, in contrast to the AChE enzyme inhibition-based biosensors that respond to any compound that inhibits AChE [15]. OPH biosensors have thus been developed for analysis of individual OPs and more recent developments include the use of mutant forms of OPH [56]. Although these biosensors showed better operational characteristics, their use is limited to the selected few OPs with electroactive leaving groups. An important drawback also represents the complex, long-lasting, and expensive procedure for OPH extraction and purification, performed in specialized microbiological laboratories (to note that these enzymes are not commercially available) [64]. Since most of the amperometric OPH biosensors reported in literature are based on anodic detection of the hydrolysis product *p*-nitrophenol at high anodic potential ($E \geq 0.80$ V/Ag, AgCl) [3], the interferences constitute a problem as well as surface fouling caused by the phenolic groups [23]. The described effects alter the precision of the OPs analysis. A simple approach to overcome this drawback is presented by Stoytcheva et al. [3] and it is based on the application of pulsed amperometric detection. In this sense, the *p*-nitrophenol produced upon the OPH-catalyzed hydrolysis of OPs is detected at a potential of +1.1 V/Ag, AgCl, while the in situ electrode cleaning and reactivation are ensured by applying a cleaning potential of +1.4 V/Ag, AgCl. The other disadvantages of this group of biosensors are the instability of the response (due to enzyme leaking or deactivation), the passivation of the electrode surface and the short life-time at ambient temperature [64]. Potentiometric OPH biosensors on the other hand, rely on detecting the change in local pH as a result of the hydrolysis reaction. These sensors can be used for all OPs that can be hydrolyzed by OPH, but they do not have low LODs [23]. The analytical performances of some selected OPH-based biosensors for the detection OPs are shown in Table 2. Because AChE-inhibition biosensors are not selective to OP pesticides since AChE is also the target of carbamate pesticide, the above OPH enzyme reaction (Scheme 1) has been combined with a variety of transduction schemes to construct simple OPH biosensors for the direct quantification of OP pesticides [15,17,19,21,65].

Table 2
Comparison of limits of detection for OPH-based biosensors when applied to OPs.

Enzyme	Analyte	LOD (μmol L^{-1})	Reference
OPH	Paraoxon	2.0	[12]
OPH	Methyl parathion	2.0	[12]
OPH	Diazinon	2.0	[12]
OPH	Paraoxon	0.1	[15]
OPH	Paraoxon	0.15	[17]
OPH	Methyl parathion	0.8	[17]
OPH/AChE	Paraoxon	0.5	[65]

The recent strategy that has been applied for the improvement of the analytical performances of the OPH-based amperometric sensors relies on biosensors' interface functionalization using nanomaterials [3]. It is reported that due to the electrocatalytic properties of the nanostructures, the electrode potential lowering is achieved, and electrode fouling is avoided, while the large surface to volume ratio, structural robustness, and biocompatibility of the nanomaterials favor biosensors' sensitivity and stability [3]. Carbon nanotubes have widely been used in electrochemical biosensors because of their fascinating electronic, mechanical and optical properties. The SWCNTs normally display higher surface area and low electrical percolation thresholds in comparison to the MWCNTs [25]. However, their higher cost and poorer dispersibility limit their application. Nevertheless, data reported in literature [25] demonstrated that OPH covalently immobilized on SWCNTs conserves much higher activity than OPH conjugated to MWCNTs. In this sense, Pedrosa et al. [21] demonstrated the use of OPH functionalized-SWCNTs and -MWCNTs conjugates for direct amperometric detection of paraoxon, a model OP. The catalytic hydrolysis of paraoxon produced *p*-nitrophenol and the oxidation was monitored amperometrically in real time under FIA mode. OPH covalently immobilized on SWNTs demonstrated much higher activity than OPH conjugated to MWNTs. The dynamic concentration range for SWNT-OPH was 0.5–8.5 μmol L^{-1} with a detection limit of 0.01 μmol L^{-1} (S/N = 3). In addition to this high sensitivity, the immobilized OPH retained a significant degree of enzymatic activity, and displayed remarkable stability with only 25% signal loss over 7 months. These results suggested that covalent immobilization of OPH on CNTs could be used for specific immobilization with advantages of long term stability, high sensitivity, good reproducibility and simplicity.

In another study, Deo et al. [17] developed an amperometric biosensor for OPs based on a carbon nanotubes (CNTs)-modified

transducer and an OPH biocatalyst. The CNT layer led to a greatly improved anodic detection of the enzymatically generated *p*-nitrophenol product, including higher sensitivity and stability. Under the optimal conditions, the biosensor was used to measure as low as 0.15 μmol L^{-1} paraoxon and 0.8 μmol L^{-1} methyl parathion. Such coupling of OPH-based biorecognition and amperometric transduction on CNT transducers was said to be advantageous over AChE-based CNT biosensors that lack specificity towards OP compounds and require addition of the substrate and an incubation period.

The integration of OPH biosensors in FIA systems have also been reported [15,19]. In this sense, Schöning et al. [19] developed and examined the applicability of a novel OPH amperometric/potentiometric biosensor chip for the determination of OPs. The amperometric and potentiometric transducers of the biosensor chips were prepared by thin-film techniques and OPH was immobilized by using a cysteamine/glutaraldehyde coupling technique on the amperometric gold electrode, and by means of a silane modification preparation on the potentiometric field-effect (EIS: electrolyte-insulator-semiconductor) sensor. Arranged in a serial set-up, the two biosensors were integrated in FIA system. Due to the two combined, but different electrochemical transducer principles, the developed amperometric/potentiometric biosensor chip enabled selective information for injections of the OPs paraoxon, parathion, dichlorvos and diazinon down to the lower μM concentration range. In this experiment, the biosensor set-up was operated for more than 4 weeks (one or two measuring cycles per day) without any mark able loss in biosensor performance. Although the OPH-based FIA-type biosensors did not provide the nano-molar detection limits (like those reported for AChE-modified electrochemical transducers), they did not require consumable supply of the enzyme substrate, and they had faster response time, were selective towards different OPs, and could simply be reused and thus suitable for continuous on-line monitoring. The authors suggested that the OPH biosensors could be combined with the inhibited AChE strategy to produce a multi-enzyme biosensor with the unique ability to discriminate between different pesticide classes. Moreover, the addition of a fluoride-sensitive part in the biosensor chip would offer the possibility to detect also neurotoxins containing P–F bonds such as soman or sarin. The higher discrimination by multiple transducers and/or enzymes opens the possibility to design powerful cross-reactive biosensor arrays [19].

Another flow injection amperometric detection method for OPs (paraoxon and methyl parathion) based on OPH biosensor was reported by Wang et al. [15]. The resulting flow system offered a fast, sensitive, selective, and stable response. The OPH-biosensor flow injection systems offered low detection limits (e. g. 0.1 μ mol L^{-1} paraoxon), along with a good precision (R. S. D. of 3.6% for 20 successive injections of a 1.0 μmol L^{-1} paraoxon solution). The OPH-biosensor flow detector was said to have offered great promise for rapid field screening of OP pesticides and nerve agents. The authors recommended that the attractive performance of the OPH detector could be combined with chromatographic separation of the target OP substances (along with minimization of interferences from co-existing easily oxidizable species).

To address the other drawbacks encountered by OPH sensors, OPH has been combined with the inhibited AChE strategy to produce a multi-enzyme biosensor with the unique ability to monitor pesticide mixtures and discriminate between different pesticide classes [12,65]. In this sense, Zhang et al. [65] reported for the first time, the development of a novel MWCNT-$(PEI/DNA)_2$/OPH/AChE bi-enzymatic biosensor for discriminative detection of OP and non-OP by electrochemical and optical methods. The detection limit was found to be ~0.5 μmol L^{-1} for the OP paraoxon and 1.0 μmol L^{-1} for non-OP carbaryl, in a wide linear range. The biosensor performance was also validated using real apple samples. Remarkable discriminative and straight forward detection between OP and non-OP neurotoxins was successfully achieved with this biosensor, showing its great potential for application in large scale screening of OPs and non-OPs in practical applications.

In other studies, detection selectivity of OPH has been enhanced by genetically modifying OPH enzyme substrate specificity [20]. Both directed evolution and rational design strategies based on informed structural data are very promising for developing enzyme variants that are able to hydrolyze OP compounds faster and more efficiently [60]. OPHs have been characterized from all domains of life including archaea. Recently, a phosphotriesterase showing 30% identity with mesophilic PTEs and activity against OP pesticides, including paraoxon and methyl paraoxon, was characterized from the hyperthermophile *Sulfolobus solfataricus* and more recently from *Sulfolobus acidocaldarius* [60].

4.1. Methyl parathion hydrolase biosensors

Methyl parathion hydrolase (MPH), a metal ion-dependent enzyme isolated from *Pseudomonas* sp. WBC-3, is a member of the organophosphorus hydrolase family. It has been found in several bacteria and it has attracted considerable attention due to its ability to specifically degrade methyl parathion [66,67]. The MPH specific substrate, methyl parathion, is still widely used as a potent insecticide and acaricide in agriculture. Since methyl parathion has high toxicity towards various organisms, developing simple, fast and sensitive methods by using MPH for the specific determination of methyl parathion is of great interest in environmental and food safety concerns. The MPH catalytic hydrolysis of methyl parathion produces dimethyl phosphate and 4-nitrophenol with a high turnover rate, and the latter is an electroactive compound that makes the construction of an amperometric biosensor possible [67]. However, limited information about the substrate preference and reaction mechanism of MPH is currently available.

Chen et al. [67] reported the fabrication of a nanocomposite biosensor for the sensitive and specific detection of methyl parathion. The square wave voltammetric responses displayed a detection limit of 0.3 ng mL^{-1} for methyl parathion. The application of this biosensor in the analysis of spiked garlic samples was also evaluated. It was proposed that the protocol can be used as a platform for the simple and fast construction of biosensors with good performance for the determination of enzyme-specific electroactive species [67].

Zhao et al. [68] developed an MPH biosensor for the detection of methyl parathion, based on self-assembly of MPH on the Fe_3O_4 @Au nanocomposite. Under optimal conditions, the biosensor showed rapid response and high selectivity for the detection of methyl parathion, with a linear range of 0.5–1000 ng/mL and a LOD of 0.1 ng/mL.

5. Organophosphorus acid anhydrolase biosensors

Organophosphorus acid anhydrolase is an enzyme that has been identified and classified as a prolidase (EC 3.4.13.9) enzyme, a type of dipeptidase hydrolyzing dipeptides with a prolyl residue in the carboxyl-terminal position [20]. Prolidases were first isolated from halophilic bacteria (*Alteromonas haloplanktis* and *Alteromonas* sp. JD6.5) and the prolidase from *Alteromonas* sp. JD6.5 was known as organophosphorus acid anhydrolase (OPAA; E. C. 3.1.8.2). OPAAs have also been isolated from squid, protozoa, clams, mammals and soil bacteria though OPAAs isolated from *Alteromonas* species: *Alteromonas haloplanktis* and *Alteromonas* sp. JD6.5 have been the most extensively studied. OPAA is a well characterized hydrolytic enzyme, which has been investigated for direct detection of

organophosphate compounds and it has demonstrated high levels of diisopropyl fluorophosphate (DFP) hydrolysis [20]. The bacterial isolate was identified as a species of the genus *Alteromonas* and DFP hydrolyzing activity has been observed in several *Alteromonas* species. Subsequently, the gene responsible for the DFP-hydrolyzing capability of the JD6.5 strain was isolated, cloned into *Escherichia coli* and sequenced [20]. OPAAs have also been shown to hydrolyze a variety of OP agents including, soman (GD; *O*-pinacolyl methylphosphonofluoridate), sarin (GB; *O*-isopropylmethylphosphonofluoridate), GF (*O*-cyclohexylmethylphosphonofluoridate) and cyanide containing tabun (GA; ethyl-*N,N*-dimethylphosphoramidocyanidate). These enzymes are highly active against the OP nerve agents, soman and sarin [69].

OPAA is an enzyme found in many species, and many of them are effective in hydrolysis of OP neurotoxins with a PF bond (such as diisopropyl phosphorofluoridate and G agents) [70]. Since OPAA is not capable of effectively hydrolyzing OPs with P-O, P-S bonds or P-CN bonds, such as paraoxon, demeton-S, tabun, including most OP insecticides [11], it may be used for highly selective detection of G-type agents. Several enzyme-based biosensors for the direct detection of G-type neurotoxins have been reported [9,12,13]. The OPAA enzyme is capable of effectively hydrolyzing the P-F bond (G agents) of OPs (such as sarin and soman), thus providing a pathway for their detection. This enzyme also provides specificity towards G agents. As such, OPAA appears to be the most attractive alternative in the catalytic sensing of OPs. In an interesting study, Simonian et al. [20] reported the use of OPAA for the detection of fluorine containing OPs (sarin GB and soman GD). The detection mechanism was based on hydrolysis of the P-F bond in fluorine containing OPs. The method was specific and did not respond to non-fluorine OPs [71].

The effect of buffer choice, pH, temperature, storage conditions and reconstitution on enzyme activity has also been reported [23]. Significant effort has focused upon development of an OPAA overproducing recombinant cell-line and use of OPAA for enzyme mediated nerve agent decontamination [24,25].

One of the main parameters which characterize a biosensor is analyte specificity. Enzymes with broad substrate specificity are suitable for detection of multiple analytes belonging to the same chemical class, while enzymes with high specificity for a single substrate are best for discriminative detection of a single target analyte [20]. Initial investigations of OPAA substrate selectivity indicated that the enzyme is capable of cleaving P-F bonds of OPs, while P-O or P-S bond hydrolysis is minimal. Since OPs, such as paraoxon and demeton-S, typically possess P-O and P-S bond structures, such unique substrate preference makes OPAA very attractive for discriminative detection of fluorine containing organophosphates. Based on this theory, Simonian et al. [20] developed an OPAA biosensor for specific detection of fluorine containing OPs on the basis of the ability of OPAA to selectively hydrolyze the P-F bond of these OPs. The hydrolysis rate of DFP, paraoxon and demeton-S, by soluble and immobilized OPAA was measured. These compounds were selected as representative substrates of OPAA hydrolysis of P-F, P-O and P-S bonds, respectively. The results indicated that hydrolysis of phosphofluoridates such as DFP was dominant while hydrolysis of phosphotriesters such as paraoxon or of phosphorothioates such as demeton-S, was negligible. Concentrations of DFP down to 25 μM with the glass electrode and 20 μM with the pH-FET were readily detected. No sensor response was observed with paraoxon or demeton-S indicating that such OPAA-based biosensors could be useful for direct and discriminative detection of fluorine containing organophosphorus compounds even in samples containing multiple OPs.

The results obtained in this study demonstrated the ability of the enzyme OPAA to serve as a highly discriminative biorecognition element for OP biosensors. OPAA could uniquely hydrolyze fluorine containing OP, such as DFP without responding to non-fluorine containing OPs. Such excellent discrimination between compounds with such dramatic differences in application yet within the same chemical class is highly desirable. The authors recommended the use of OPAA in combination with other organophosphate hydrolyzing enzymes, such as OPH, so as to enable construction of an array of biosensors able to discriminate between several classes of neurotoxins.

6. Conclusions and future perspectives

AChE inhibition-based biosensors have emerged over the years as the strongest candidates for screening OP residues and are becoming more and more relevant in environmental and food analyses. The majority of AChE biosensors are developed using *ee*AChE. This enzyme has demonstrated good characteristics for synthetic samples but it is not robust in real samples and also does not offer adequate selectivity (i.e. it is not usually able to discriminate various toxic compounds in the same sample). In addition, it has been very difficult to find a native AChE enzyme with all the features required to construct an ideal biosensor. Nevertheless, the biosensors developed with the native AChE enzymes can be used as alarm systems; they would provide either quantification of one contaminant when this analyte is present alone or an indication of total contamination of particular samples.

Current research studies are directed towards the development of AChE biosensing systems with improved sensitivity, selectivity and analytical performance in order to be able to monitor a wide range of pollutants in environmental and food samples. Protein engineering is helping researchers to produce tailor-made biorecognition molecules for integration in biosensing platforms to improve the biosensor sensitivity. The careful selection of the location and type of mutations gives rise to AChE enzymes with enhanced analytical performance. Effectively, multi-sensor arrays that are combined with the data processing of ANNs have already been shown to be useful adjunct for resolving binary mixtures of OPs in real samples and for interpretation of experimental data. Another important area of research in AChE biosensors is directed toward the development of automated and continuous systems for measuring AChE inhibitors in flow conditions. Single use test strips, especially with SPE have also been developed in order to avoid problems related to fouling of the electrode which generally involves a chemical or electrochemical deactivation of the working electrode surface. SPEs offer additional advantages including low cost, being amenable to both mass production and instrument miniaturizations.

OPH and OPAA enzymes seem to be superior to other protocols used for OP detection in view of the fact that OPH- and OPAA-based biosensors directly detect OP compounds in a single step in comparison to the AChE-inhibition mode biosensors which require a two-step operation. However, the number of publications reporting the use of OPH and OPAA enzymes is much lower than those reporting the use of AChE inhibition-based biosensors. This suggests that certain parameters of the OPH- and OPAA-based biosensors still require improvement in order to warrant their application in real samples. One such example would be the site-directed mutagenesis of OPH enzymes to improve their sensitivity and analytical performance.

A merit of electrochemical biosensors for OP detection is that compact and portable devices can easily be designed for *in-field* analysis. The easy operation of biosensors is another merit as compared to classical analytical methods. Therefore, biosensors have high potential for fast on-site screening of OPs. Indeed, the combination of engineered enzymes (double and triple mutants) with SPE, nanomaterials and automated flow systems could

improve the efficiency of enzyme based biosensors. Considerable progress is expected in the production and commercialization of genetically engineered OPH and AChE enzymes (to note that these enzymes are not currently commercially available). The development of cheaper and disposable array biosensors for rapid screening of OPs is desirable.

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