Bioanal Rev (2010) 1:159–176
DOI 10.1007/s12566-010-0012-z

Recent strategies for the biological sensing of pesticides: from the design to the application in real samples

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Received: 3 March 2010 / Accepted: 9 May 2010 / Published online: 3 June 2010

Abstract The rapid detection of pesticides has become in recent years a major issue both for environmental control and food safety. Bioassays have the potentiality to tackle the problem and to give valuable information depending on the nature of the biological element used. In this paper, the recent literature for the biosensing of pesticides via inhibition systems is reviewed. Particularly, inhibition assay based on acetylcholinesterase and photoystem II are reported. A brief description of the different characteristics of the biological elements and their variants are firstly given. Recent findings are then grouped according to the main contribution given in sensing design or application to real samples

Keywords Pesticides · Inhibitions biosensors · Ache · PSII · Organophosphates · Carbamates · Herbicides

Introduction

In the twentieth century, the use of pesticides became an integral, necessary and problematic practice of agriculture in the majority of the countries of the world. Starting from the 1940s, synthetic pesticides derived from organic chemistry, particularly the organochlorines and organophosphates have become widespread. Since then, numerous other kinds of pesticides have been developed and released into agricultural fields and the environment [1]. At the end of the last century, a decrease in the use of organophosphate (OPs) and carbamates (CMs) pesticides was registered in favor of piretroyds. The declining usage of the organophosphates is well illustrated by the fact that, in year 2000, imidacloprid, a neonicotinoid insecticide, was the top-selling insecticide worldwide [2]. Nevertheless, OPs and CMs still represents around 50% of the total pesticide production. The Food and Agriculture Organization inventory, estimated that more than 500,000 tons of unused and obsolete pesticides are threatening the environment and public health in many countries [3]. Therefore, they continue to represent a major concern for general public and regulatory agencies. Pesticides can be classified either according its target pest (insecticide, herbicide, fungicide) or on the basis of chemical structure (e.g., organophosphate, carbamate, organochlorine, etc). More interestingly, World Health Organization has produced a classification of pesticides by hazard [4] based on four classes: extremely hazardous (class Ia), highly hazardous (class Ib), moderately hazardous (class II), and slightly hazardous. According to this classification, organophophate and carbamate represent more than 50% of the extremely hazardous and more than 55% of the highly toxic pesticides. Herbicides acting of Photosystem II (PSII system; see later) are usually not responsible for acute intoxication but moderate toxicity and persistency can cause serious environmental problems. More than 600 single, active ingredients are applied by farmers on stable cultivated agricultural soil in Europe. The Water Framework Directive (Directive 2000/60/EC) marked a change in EU water policy towards a coherent and integrated framework for assessment, monitoring, and management of all surface waters and groundwater based on their ecological and chemical status [5]. The levels of pesticides found in different environmental compartments vary greatly, depending on vicinity of production factory, the type of usage on field (amount and frequency), aerial transport, and deposition

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and possible accumulation. The chemical nature of the compounds plays a crucial role in this respect as demonstrated by Dubus et al. in a survey of 28 European studies in ten European countries carried on the detection of pesticides in rainfalls [6]. The most frequently monitored pesticides, in descending order, were lindane (g-HCH; HCH: hexachlorocyclohexane) and its main isomer (a-HCH), atrazine, MCPA (2-methyl-4-chlorophenoxyacetic acid), simazine, dichlorprop, iso-proturon, mecoprop, DDT, terbuthylazine and aldrin. At the majority of the sites, concentrations were below 100 ng/L but occasionally hundreds of ng/L was detected. Organophosphates and carbamates are non-persistent pesticides not frequently found at high levels [7] in the environment; for this reason, the major concern has been over acute intoxication. However, metabolites of this pesticides have been recently and frequently found in human urine samples indicating that also chronic intoxication must be monitored [8]. Maximum residue limits (MRLs) or organophophates and carbamates in food samples are fixed by regulatory bodies and are also dependent on the the level of transformation that a sample has to undergo. For instance, for a "ready to eat" food as honey the EU Commission Regulation 508/1999/EC (and subsequent modifications) has established MRL for coumaphos at 0.1 mg/Kg [9], for durum wheat (used to make pasta) MRLs for dichlorvos is at 2 mg/Kg and for pirimiphos-methyl at 5 mg/Kg.

It appears then necessary to exert a close control starting from the production of "low impact" pesticides going through the implementation of best agricultural practices and ending to the monitoring of the residues in the final products (e.g., food, feeds) as well as in ecosystems close to the crops. Traditionally, assessment of contamination has comprised routine chemical analysis and characterization; considering the variety of chemicals to be detected this can be accomplished using gas-chromatographic of liquid-chromatographic techniques coupled with traditional or mass spectrometric detectors. Implementation and needs for this chromatographic method for the monitoring of surface waters has been suggested recently by Lepom et al. [10]. However, these techniques are difficult to be designed for rapid and in situ analysis. Bioassays, including biosensors, are screening assays that present the advantage of giving a rapid response and then can be used for real-time monitoring.

Moreover, they are able to give additional information depending on the nature of the biological element used (whole organism, part of it or an enzyme) and to the target for the analysis (e.g., toxicity) to better integrate the data found with classical chemical analysis. This is particularly true for the analytical systems presented in this work in which the inhibition assays for pesticides in the recent literature is reviewed. It should be pointed out that in this type of assays, the inhibition is not selective; and then, different analytes from the same group can inhibit the systems in a different way. For this reason, these systems have been proposed for the measurement of a "specific inhibitory power". This subject is of great interest as witnessed by works recently published, reviewing some aspects of inhibition-based pesticides detection using cholinesterases. Particularly, some of these works focused the attention on the potentialities of the use of such class of enzymes for the detection of new and relevant toxic analytes as nerve agents and toxins (aflatoxin) [11–14]. Arduini et al. [15] gave an overview of different analytical aspects of the use of reversible enzyme inhibition including the important parameters of the procedure affecting such systems and Peryasamy et al. listed a series of acetylcholinesterase biosensors based on nanomaterials [16]. In this review, we attempted to give an overview giving particular emphasis to the advancement in sensing design (including nanomaterials) aimed to the application of the inhibition-based assay in real samples. Because of the relevance of herbicides, we extended the review also to inhibition assays based on the PSII system. We did not treated affinity assays based on the selective recognition of pesticides via antibodies in this work (enzyme-linked immunosorbent assays (ELISAs), immunosensors, etc.) since the rationale behind the development of such affinity assays is different.

The biological elements

Acetycholinesterase Acetylcholinesterase (E.C. 3.1.1.7; AChE) is the most used enzyme for the detection of carbamates and organophosphates via enzyme inhibition. The hydrolysis reaction of acetylcholine, a neurotransmitter common to many synapses in the mammalian nervous systems, catalyzed by this enzyme makes it one of the crucial enzymes for nerve response and function. Over 100 X-ray structures of acetylcholinesterases from different sources and/or bound to different ligands are actually deposited and freely available on the PDB databank (www.pdb.org, Fig. 1). This clearly demonstrates the huge interest in studying these enzymes. The enzymatic reaction proceeds via nucleophilic attack of the carbonyl carbon with subsequent acylation of the active site of the enzyme and production of choline. Restoration of the esterasic site is obtained after hydrolysis of the acylated enzyme. Hydrophobic and electrostatic forces drive conformational changes favoring the access of the substrate to the active site; further conformational rearrangements are necessary to allow the release of the products of the reaction. The reaction mechanism presents a high degree of complexity and has been also recently object of modeling studies [17]. Due to the importance in the nervous transmission and the wide distribution in the animal reign, AChE represents the

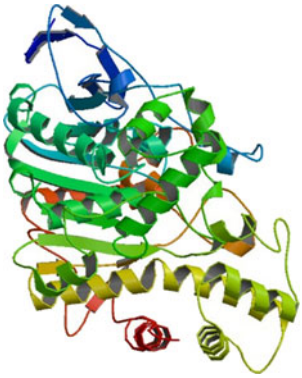
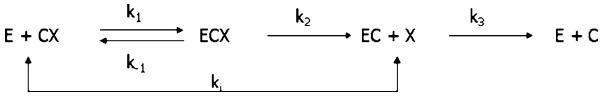


Fig. 1 Electrophorus electricus acetylcholinesterase ribbon structure. (10.2210/pdb1C2B/pdb from www.pdb.org). *E* enzyme, *CX* carbamate or organophosphate, *X* leaving group, K_d K_1/K_1K_2 carbamoylation or phosphorylation rate constant, K_3 decarbamylation or dephosphorilation rate constant, K_i bimolecular rate constant

selected "target" of specific actions directed against the living systems, starting from the use of toxic nerve agents (e.g., Sarin) ending to pest control with the use of CM and OP pesticides. The identification of the binding site of AChE has led to the knowledge of the inhibition mechanism [18, 19]. The amino acids involved in the AChE-pesticide binding are Ser 200, His 440, and Glu 327 the so-called "catalytic triad". Modeling studies of the binding of OPs and CMs vs. the active site of the enzyme allowed our group to develop peptide-based solid-phase extraction cartridges able to quantitatively extract from a solution Paraoxon and Carbaryl [20]. The interaction with OPs generally leads to phosphorylation of Ser while inhibition by CMs does not produce covalent modification of the active site. The inhibition scheme is reported in Fig. 2. For the reasons exposed AChe represents the ideal enzyme to be used for biological sensing of OPs and CMs.

The use of inhibition of an enzyme activity for the determination of an analyte is a non-selective measurement by its nature; this is particularly true in the development of AChe biosensors to detect pesticides because of the different inhibition pattern exhibited by enzymes from different sources. Electric eel AChe is commercially available and has been used for the majority of the applications. Interesting applications and a "tunable" selectivity can be obtained using recombinant AChe. A remarkable work on this side has been done by Fournier and co-workers [21, 22]. In a preliminary work, by comparison of the bimolecular rate constants calculated using a set of 19 OPs and CMs, Villatte et al., demonstrated that the enzyme from *Drosophila melanogaster* was more sensitive than AChes from other sources [23]. A mutant of the wild type from *Drosophila* (mutation in 408 position) resulted to be 12-fold more sensitive on average. This effect was explained by the increase in the π-π hydrophobic interaction induced by the mutation. However, it should be noticed that this is not the only mechanism that is responsible for an improved inhibition and that pendant groups of CMs and OPs varies greatly in terms of size and hydrophilic/hydrophobic characteristics. Thus, depending on the target inhibitor, different mutants can be used from a whole library. Biosensors based on genetically engineered enzymes have been very recently reviewed by Campas et al. [24] and mutant AChEs represent the majority of the enzymes reported. Mutations have been mainly addressed to alter some residues of the region accessing the active site. For example, the replacement of glutamic acid 69, located at the rim of the active site , by aminoacids with bulky side chains, such as tryptophan or tyrosine, has been demonstrated to increase significantly the inhibition constant, *k*i, for dichlorvos [25]. Very low detection limits (10^{-17}M) have been obtained using recombinant enzymes [26] A systematic evaluation of the possibility of "fine tuning" using recombinant enzymes has been reported [27] by Bucur et al. that studied the inhibition effect of carbaryl, carbofuran, and pirimicarb on biosensors assembled using different recombinant AChes immobilized via sol/gel techniques. Inhibition by methamidophos was instead investigated by De Oliveira Marques et al. [28]. These works and others following papers (see "Application to real samples" section) demonstrated the potential of using mutants for the improvement of selectivity and sensitivity. However, it should be kept in mind that, for any biosensing system, the architecture of the sensitive element, the type of immobilization (if applied), pretreatment of the sample (if any), time and format of the assay greatly influence sensitivity and selectivity of the assay that, then, should be specifically evaluated for the target (type of sample and of pesticide) and the purpose (quali-quantitative needs).

Other enzymes for OPs and CMs Other enzymes have been reported to be used for the detection of CMs and OPs via inhibition assay but very few novel design and/or applications are found recently in the literature. The use of butyrrilcholinesterase (E.C. 3.1.1.8; BuChE; known also as pseudocholinesterase) that has a different substrates pattern and inhibitions mechanism and sensitivity versus pesticides has been investigated. This enzyme was initially



E= enzyme; CX= carbamate or organophosphate; X = leaving group; $K_d=K_1/K_1K_2$ carbamoylation or phosphorylation rate constant; K_3 decarbamylation or dephosphorilation rate constant; K_i= bimolecular rate constant

Fig. 2 AChE inhibition scheme. Depending on the complex dissociation rate constant either reversible or irreversible inhibition of AChE will be observed

used to vary the selectivity pattern of the measurement; however, the opportunities, already mentioned, given by genetically engeneering mutants of Ache appears more promising. Also tyrosinase (EC 1.14.18.1) has been reported to be inhibited by different pesticides. Tyrosinase is a binuclear copper containing metalloprotein, which oxidizes monophenols and *o*-diphenols into their corresponding *o*-quinones at the expense of oxygen reduction to water. The conversion of monophenols by tyrosinase proceeds in two consecutive steps; in the first step, monophenol is hydroxylated to its corresponding *o*-diphenol. In a second step, is then oxidized to its corresponding *o*-quinone, whereby the enzyme is oxidized by molecular oxygen back to its native form. This large family of enzymes widely distributes in nature and easy to purify from plants and fungi has been fully exploited for the development of biosensors for phenols in environmental and food matrices. Tyrosinase composite Co-phtalocyanine-based screen-printed sensors have been reported to have competitive inhibition in the ppb range for methylparathion, carbofuran, and carbaryl [29].

Photosystem II PSII is a multisubunit complex that uses light energy to catalyze a series of electron transfer reactions (Fig. 3). The electron transfer finally results in the splitting of water into molecular oxygen, protons, and electrons. PSII is present in the thylakoid membranes of higher plants and is responsible for the production of atmospheric oxygen. A major role of many of the subunits of PSII, and its light-harvesting antenna proteins, is to act as membrane scaffolds which provide the ligands that bind, and to organize a linked network of pigments and other cofactors. Together, these cofactors trap, transfer, and utilize solar energy to drive the reactions of water splitting and plastoquinone reduction [30]. A huge number of herbicides (derivatives of phenylurea, triazine, diazine, and phenolic types) that are currently in use inhibit the light reactions of photosynthesis usually inhibiting the PSII electron flow. PSII reaction center was isolated in 1987 and the isolated complex was found to maintain its herbicide-binding ability [31]. The D1 protein of the PSII reaction center protein is the main target of herbicides that inhibits photosynthesis. In an analogous way of Ache, it appeared useful to develop sensing systems based on PSII. Despite the apparent complexity of the use of such a system for an assay, clear advantages are represented by the potential simplicity of the transduction and by the extreme susceptibility and selectivity towards the binding of some agents. PSII specifically recognizes certain analytes or particular physico-chemical conditions inhibiting its electron transfer activity. The herbicide compounds can bind reversibly to the D1 subunit of PSII within its QB binding pocket, also called the herbicide-binding niche. Upon binding, the compounds alter or inhibit electron transfer by displacing the plastoquinone QB, thus blocking electron flow, oxygen evolution, and changing the fluorescence properties of PSII. The recognition of PSII photosynthetic herbicides is not highly specific, because other classes of compounds, such as heavy metals, are also detected, even though with lower sensitivity [32]. PSII preparations are suitable biological material for realization of devices because they exhibit light-induced electron transfer across lipid membranes. This property is very useful because the physiological activity can be easily monitored by amperometric/potentiometric systems. Major limitations on the use of biosensing systems appeared initially by the limited lifetime and stability of PSII immobilized on different electrode surfaces; however these problems can be solved using PSII systems from thermophilic species, PSII of selected mutants and adaptable strains from different algae [32].

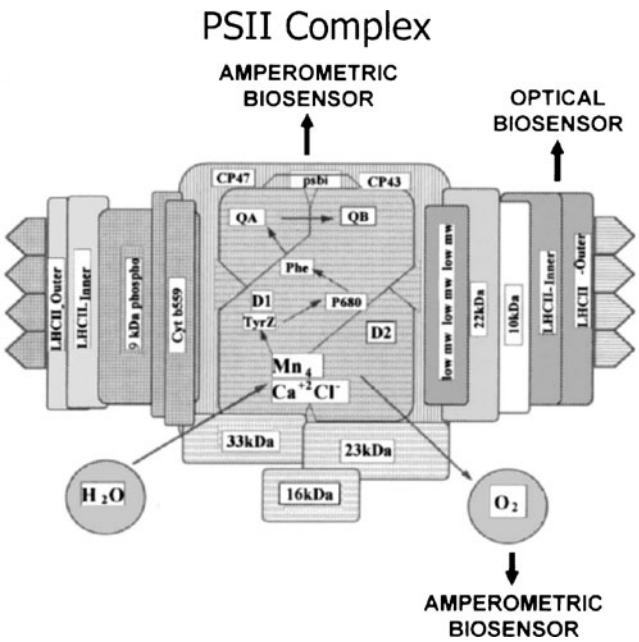


Fig. 3 Schematic drawing of the Photosystem II, the reaction center and the water-splitting system, also called the oxygen evolving complex is also sketched. Reprinted with permission from [30]

Sensing design

Ache sensors

Various types of electrodes and electrode materials have been reported in the literature for the fabrication of ChE biosensors. In a typical configuration, the enzyme can either be directly deposited onto the surface of the working electrode or embedded into a membrane which is casted on the surface of the working electrode, or it can be

incorporated in a composite which contain the electrode material. The enzyme activity is then measured by adding the substrate to the solution.

The original configuration of ChE biosensors uses a Clark oxygen electrode for the amperometric detection and a pH-sensitive electrode for the potentiometric detection. In both cases, the enzyme is physically trapped onto the surface of the electrode using oxygen or pH-sensitive membranes. The following generation was based on classical solid electrodes such as platinum, gold, glassy carbon, and laboratory-made carbon paste electrodes. Metallic working electrodes such as gold or platinum require careful cleaning and mechanical polishing in order to achieve a reproducible surface. In some cases, they are chemically and/or electrochemically activated to add functional groups onto their surface for further attachment of the enzyme. The different types of electrodes used for the fabrication of ChE biosensors are summarized in [33].

The use of solid electrodes offers good analytical performance for the determination of ChE activity but presents several important limitations for inhibition measurements.

The first limitation is due to the enzyme inactivation by the AChE inhibitors, the electrodes needs then a regeneration step or, alternatively, a new electrode is needed to perform the next measurement. Both these options are an evident limitation to the simplicity of the assay. In fact, regeneration and the preparation of a new electrode are time consuming and introduce a certain degree of variability in the following measurement. Due to these limitation, disposable screen-printed electrode, produced with thick film technology, were successfully introduced in the field of AChE biosensor in the late 1990s [34]. This technique allows mass production of disposable sensors with low cost and high reproducibility. At present, various screen-printed electrode designs are used in biosensor research laboratories. Usually, all the electrodes composing the sensing system (reference, auxiliary, and working) are deposited simultaneously onto a solid support. Typically, a conductive layer (Ag or carbon based paste) is printed to form the working, reference, and auxiliary electrodes. It is important to note that the screen-printed Ag/AgCl reference electrode is a pseudo-reference electrode, thus, in order to ensure a constant and stable potential, chloride (>1 mM) is needed. The enzyme can then be manually deposited or printed. Examples of different biocomposite pastes and screen-printed configurations for ChE biosensors and their applications for the detection of pesticides have been reviewed in [35].

Immobilization of Ache on screen-printed electrodes is still an open issue, the enzyme can be either encapsulated into a biocomposite that is placed on the working electrode surface or covalently linked to the electrode surface. In the latter option, it is possible to obtain sensitive biosensor due to the absence of diffusion barriers, which are usually present in other immobilization methods. Also, adsorption has been proposed as simple and rapid method [36].

Modification of screen-printed carbon electrodes with glutaraldehyde, terephthaldicarboxaldehyde, and then polyethyleneimine has been reported for production of pesticide biosensors based on acetylcholinesterase [37]. In order to improve the extent of dialdehyde modification, the electrodes were NH_2-derivatized by electrochemical reduction of 4-nitrobenzenediazonium to a nitroaryl radical permitting attachment to the carbon surface. *D. melanogaster* acetylcholinesterase was immobilized either covalently onto dialdehyde modified electrodes or non-covalently onto polyethyleneimine-modified electrodes. Internal diffusion limitations due to the dialdehyde and polyethyleneimine modifications increased the apparent Km of the immobilized enzyme. The sensitivity to thiocholine was reported to be about 90% for dialdehyde-modified electrodes and about 10% for polyethyleneimine-modified electrodes compared to non-modified carbon electrodes. The detection limit of the biosensors produced by non-covalent immobilization of acetylcholinesterase onto polyethyleneimine modified carbon electrodes was found to be about 10^{-10}M for the organophosphate pesticide dichlorvos.

An interesting approach for the realization of convection-free biosensors based on array technology developed on a screen-printed platform was proposed in [38].

Sensors were fabricated via the electropolymerisation of a thin insulating polymer film at planar electrode surfaces and then ablated ultrasonically to expose microdiameter scale areas of underlying conductor. Conducting polymer protrusions of polyaniline with co-entrapped acetylcholinesterase were then electrodeposited in situ at these microelectrode cavities and together these form an enzyme micro-electrode array. The enzymatic product thiocholine was oxidized at a Cobalt-phtalocyanine/carbon electrode and the enzymatic response is inhibited after incubation with the pesticide. The combination of these transducer with genetically modified AChE resulted, according to the published data, in AChE biosensors able to detect as low as 1×10^{-17}M for Dichlorvos, Parathion, and Azinphos in standard solution. A biocomposite made of AChE and Al_2O_3 sol-gel provided encapsulation of the enzyme on screen-printed electrodes [39]. Al_2O_3 sol–gel has been proved to be an ideal material to couple with enzymes due to its hydrophilicity necessary for the retention of the tertiary structure of the enzyme and subsequent enzyme stability, but also because its efficiency to promote electron transfer, thus reducing the over-potential needed for thiocholine determination. The simple low cost self-made screen-printed electrodes covered with an enzymatic Al_2O_3 gel membrane were built in a homemade flow cell, and used to detect dichlorvos. The reported limit of detection

was 80 nM, with a working interval 0.1-80.0 μM. The authors also proposed a reactivation procedure based on substrate addition which was properly working within certain inhibition values and conditions. The theory behind such reactivation was also reported. A surface modification of gold electrode for the construction of AChE biosensor is reported in [40]. In this paper, potentiometric and amperometric enzyme sensors based on modified gold electrodes were compared for pesticide determination. PAMAM dendrimer (generation G4) stabilized with 1-hexadecanethiol used for the immobilization of acetylcholinesterase from electric eel and choline oxidase from Alcaligenes species in the assembly of amperometric sensor. Polyaniline-doped with camphorsulfonic acid was used to obtain potentiometric response. Trichlorfon, carbofuran, and eserine suppress the biosensor response due to their inhibitory effect. The detection limits of 0.003 and 200 nmol l^{-1} (trichlorfon), 0.04 and 6 nmol l^{-1} (carbofuran), and 0.1 and 700 nmol l^{-1} were obtained for the amperometric and potentiometric sensors, respectively. The biosensor behavior and the high sensitivity of the dendrimer-modified sensor to the inhibitors is attributed to the specific organization of protein layer at charged surface of the modifier macromolecules. Recently, the application of a sonogel-carbon electrode has been proposed [41]. The enzyme was immobilized on the electrode surface employing Al_2O_3 using a simple sol–gel technology which requires minimal preparation time. The enzyme was retained in sol–gel matrix and the direct contact with the sonogel-carbon allows a fast and sensitive electrochemical response. The biosensor was applied to measure paraoxon, dichlorvos, chlorpyrifos ethyl-oxon achieving a detection limit of 7.5×10^{-9}, 5.0×10^{-10}, and 2.5×10^{-10}M, respectively. Other oxide such as zinc oxide can be used for sol-gel preparation and entrapment of enzymes [42]. Zinc oxide has been used as a matrix for immobilization of AChE and detection of the pesticide paraoxon. The immobilized enzyme retained its enzymatic activity up to 3 months when stored in phosphate-buffered saline (pH 7.4) at 4°C. The biosensor detected paraoxon in the range 0.035–1.38 ppm.

Nanostructered material for AChE biosensor In this section, we will focus our attention on ChE biosensors that use nanostructured material as electrochemical sensing layer. Often, these nanosensors are assembled via deposition of the nanomaterials on the surface of a solid state electrode which works as contact layer. There are different ways for confining, for example, carbon nanotubes (CNT) onto electrochemical transducers. Most commonly, this is accomplished using CNT-coated electrodes [43] or using CNT/binder composite electrodes [44].

The solid state electrodes can be either classical graphite or metal electrodes or screen-printed electrodes. This latter options offer some advantages as no regeneration of the electrode surface is required as they can be used in a disposable fashion. Among nonostructured materials available CNT and gold nanoparticles (AuNPs) have been the most widely used for the assembly of ChE-based biosensors.

Carbon nanotubes Since the discovery of CNT, they have become the subject of intense investigation in different areas of material science including electrochemical sensors [45]. These materials are characterized by unique features including their remarkable electrical, chemical, mechanical, and structural properties. As electrode material, CNT can display metallic, semiconducting, and superconducting behavior. They possess a hollow core that in some cases may retain guest molecules and show a high elastic modulus [46]. CNT can be obtained in a variety of geometrical arrangement such as single wall carbon-nanotubes (SWCNT) and multi-wall carbonnanotubes (MWCNT); they are manufactured according to diverse strategies ranging from chemical vapor deposition, carbon arc methods, or laser evaporation. SWCNT are formed by rolling up a single graphite sheet in a tube resulting in a cylindrical nanostructure. MWCNT comprise of several layers of graphene cylinders that are concentrically nested with an interlayer spacing of 3.4 A. The unique properties of carbon nanotubes make them extremely attractive for the task of chemical sensors, in general, and electrochemical detection, in particular. Recent reviews on CNTs application for general biosensing purpose can be found in the literature [47, 48].

The noteworthy sensitivity of CNT as electrode material towards adsorbed analytes enables the use of CNT as highly sensitive nanosensors. To take advantages of the remarkable properties of these unique nanomaterials in such electrochemical sensing applications, the CNT need to be properly functionalized and immobilized. Recent studies demonstrated that CNT can enhance the electrochemical reactivity of important biomolecules [49, 50]. In addition to enhanced electrochemical reactivity, CNT-modified electrodes have been shown useful to accumulate important biomolecules (e.g., nucleic acids) [51] and to alleviate surface fouling effects [52]. Methods for the vertical attachment of CNTs on solid supports consist of either synthesis-induced alignment (e.g., chemical vapor deposition) or post-synthesis manipulation. One of the most interesting approaches for the post-synthesis alignment of CNTs is the self-assembly technique [53]. Vertical attachment can be achieved via disulfide modified ssDNA wrapped CNTs on a gold substrate by the self-assembly technique [54–56]. Due to their high electrochemical

catalytic activity, CNT are foreseen as a promising electrode material to enhance the sensitivity of ChE biosensors towards inhibitors. A disposable CNT-modified screen-printed electrode for the amperometric detection of organophosphorous compounds has been proposed in [57]. This relied on the inhibition of acetylcholinesterase and the CNT-promoted detection of the hydrogen peroxide produced by the co-immobilized choline oxidase. The catalytic effect of WCNT was investigated for the sensitive detection of thiocholine, which represents one of the product of acetylcholinesterase reaction. MWNTs promoted electron transfer reactions at a lower potential and catalyzed the electro-oxidation of thiocholine, thus increasing detection sensitivity [58]. In this paper, a simple method for immobilization of acetylcholinesterase on MWNTs–chitosan composite was described resulting in a sensitive, fast and stable amperometric sensor for quantitative determination of organophosphorous insecticide. Very interestingly, the electrode showed high working stability (up to 100 measurements with a loss of response of 20%), and an interesting shelf life (up to 1 month, loss of response 30%). Under optimal conditions, the inhibition of triazophos was proportional to its concentration in two ranges, from 0.03 to 7.8 μM and 7.8 to 32 μM with a detection limit of 0.01 μM. It is noteworthy that a 95% reactivation of the inhibited AChE could be regenerated using pralidoxime iodide within 8 min. More recently, CNT modified with streptavidin as substrate for the anchoring of biotinylated acetylcholinesterase have been reported in order to obtain an oriented immobilization of the enzyme. A glassy carbon electrode was coated with the modified CNT and the acetylcholinesterase product, thiocholine, was measured by amperometry obtaing high sensitivity [59]. A clear and interesting description of the layer by layer (LBL) modification of a glassy carbon working electrode by means of MWCNT, poly(diallyldimethylammonium chloride) (PDDA) and AChE is reported in [60]. By this technique, electro-deposition of MWNTs on glassy carbon electrode was achieved by magnetic stirring for 2 h, with the potential set at +1.7 V. The modified electrode was then dipped into alkaline solution in order to negatively charge surface group. Afterwards, by immersing the electrode in PDDA solution and 1 mg mL^{-1} MWNTs solution (pH9.2 borate buffer) alternately, a five-PDDA/MWNTs-bilayer sensor was obtained. AChE can then be immobilized directly by LBL self-assembly, being important that a PDDA membrane is fabricated at the solution side in order to avoid the release of the enzyme. Again, the direct oxidation of thiocholine is used to evaluate extension of AChE activity and, hence, its inhibition by carbaryl. However, despite a reported limit of detection of 10^{-12} g/L, the real detection limit (LOD) appears to be at least two orders of magnitude higher looking at the data shown. No information was given with respect to biosensor regeneration. Different properties of SWCNT that might be used to detect ChE inhibition have been investigated in [61]. The pH-sensitive behavior of a single-wall carbon nanotube-modified electrode was exploited using differential pulse voltammetry technique. A Nernstian and reversible response was found. This system was able to detect acetylcholinesterase activity via the pH shift provoked by the product of the reaction (acetic acid).

The same detection princicle (change in pH) was also used for the construction of an AChE inhibition biosensor in [62]. The authors report on the modification of a gold electrode with a vertically aligned ssDNA–SWCNT self assembled monolayer coated with thin polyaniline film (PANI). The incorporation of PANI, a conducting polymer, is justified by ease preparation, well-behaved electrochemistry, impressive signal amplification, and elimination of electrode fouling when used in biosensor applications. The PANI film resulted in superior charge transport properties than the ssDNA SAMs. The incorporation of SWCNTs not only provided the conductive pathways to promote the electron transfer, but also increased the surface area of flexible three-dimensional conductive supports for acetylcholinesterase enzyme. Thin PANI film on SWCNT acted as good sensor for enzyme by product acetic acid. The changes of local pH in the proximity of the electrode surface by enzymatic reaction increases the redox activity of PANI thin film on SWCNTs. The biosensor was tested in the determination of two common pesticides such as methyl parathion and chlorpyrifos, which belongs to the OPs family. The detection limit was found to be 1×10^{-12}M for both.

Gold nanoparticles Recently, nanoparticles, particularly AuNPs have received considerable attention in analytical electrochemistry due to their high conductivity. The detection of thiocholine as the target by-product of the AChE reaction has been pursued also with gold-based nanomaterial used as working electrode [63]. The AuNPs were assembled on a sol–gel derived silicate network (AuNPs-SiSG) which was also used for the immobilization of AChE. Based on the inherent conductive properties of AuNPs, the immobilized AChE exhibited a higher affinity to the substrate and produced detectable and fast response. The AuNPs-SiSG composite was deposited on the surface of a glassy carbon electrode and AChE was immobilized on the modified electrode. The inhibition of monocrotophos was proportional to its concentration ranging from 0.001 to 1 μg/ml and 2 to 15 μg/ml, with the correlation coefficients of 0.9930 and 0.9985, respectively. The detection limit was 0.6 ng/ml at a 10% inhibition. The same biosensor construction scheme was applied in [64], to analyze some anti AChE drugs, galantamine, and neostigmine. The

authors in this and other similar papers reports Km values for inhibitors to caompare their effect on sensors. This is, in our opinion is misleading and should be avoided; in fact, Ki (inhibition constants) are more appropriate and should be anyhow indicated as apparent since the enzyme is immobilized. We suggest, for analytical purposes, to compare sensitivities using I_{50} (concentration giving 50% of inhibition) in analogy with ELISA competition assays An advance reported in this paper is the stability of the biosensor to the regeneration step using pralidoxime iodide: 96% reactivation of the inhibited AChE could be regenerated. The authors suggested that due to the exhibited high sensitivity, low cost, and simplified procedures, the test should be considered as a promising tool for investigation of drug. In a following paper [65], the same authors developed an AChE biosensor by modification of a glassy carbon electrode (GCE) with CdTe quantum dots (CdTe), AuNPs, and chitosan microspheres (Fig. 4). A synergistic effect of CdTe quantum dots and AuNPs was observed in facilitating the electron transport, lowering the oxidation potential of thiocholine, and increasing the signal. Nevertheless, the improved electron transport reflected scarcely on the LOD of the method that was 0.3 vs. 0.6 ng/ml reported in [63]. An interesting feature of the developed biosensor relies in the shelf life of the sensor that after 30 days of storage retained 92% of its initial activity. According to the authors' interpretation, this phenomenon was due to the formation of covalent binding between amine groups of AChE and carboxyl groups of quantum dots thus preventing the leakage of the enzyme from the electrode. Very little improvement is reported in a further paper of the same authors [66], where the same biosensor preparation procedure (including the same voltammetric data) is published, and similar evidences are demonstrated. Another approach attempted consists in the electro synthesis of AuNPs onto a gold electrode followed by the creation of a self-assembled monolayer of 11-mercaptoundecanoic acid [67]. The carboxyl groups of the modification layer served also, after activation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide for immobilization of AChE. In the optimal experimental conditions, the inhibition of malathion on AChE biosensor was proportional to its concentration in two ranges, from 0.001 to 0.1 μg mL^{-1} and from 0.1 to 25 μg mL^{-1}, with detection limit of 0.001 μg mL^{-1}. This simple method exhibited good reproducibility and acceptable stability. In fact the inter-assay precision was estimated on a set of six biosensors and the coefficient of variation resulted lower than <3.1%. As long as concern stability, the enzyme electrode was stored at 4 °C in dry condition showing no decrease in the thiocholine measurements; at longer storage period (30 days), the biosensor retained 90% of its activity.

Another group from China [68] has recently published a paper on the entrapment of AuNPs in an electro-synthesized polypirrole matrix. The electrochemical biosensor was designed for organophosphate pesticides based on acetylcholinesterase immobilized onto Au nanoparticles–polypyrrole nanowires composite film modified glassy carbon electrode (labeled as AChE–Au–PPy/GCE). The generated Au nanoparticles were homogenously distributed onto the interlaced PPy nanowires matrix, constructing a three-dimensional porous network. This network-like nanocomposite not only provided a biocompatible microenvironment to keep the bioactivity of AChE, but also exhibited a strong synergetic effect improving the sensing properties of OPs. The Authors claimed that the combination of AuNPs and PPyNWs greatly catalyzed the oxidation of the enzymatically generated thiocholine product, thus increasing the detection sensitivity. On the basis of the inhibition of OPs on the enzymatic activity of AChE, the conditions for OPs detection were optimized using methyl parathion as a model compound. The inhibition of methyl parathion was proportional to its concentration ranging from 0.005 to 0.12 and 0.5 to 4.5 μg mL^{-1}. The detection limit was

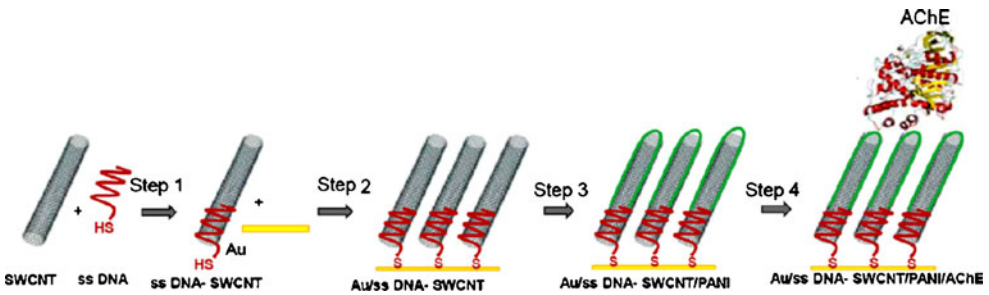


Fig. 4 Schematic representation of biosensor using thiolated DNA for SWCNT orientation. Step 1, SWCNT wrapping with thiol terminated ssDNA; step 2, ssDNA wrapped SWCNT self-assembled on gold electrode; step 3, controlled electrochemical polymerization of aniline on the Au/ssDNA–SWCNT layers; step 4, immobilization of AChE enzyme by glutaraldehyde [62], with permission

2 ng mL^{-1}. The developed biosensor exhibited good reproducibility and acceptable stability (60% of the response at 30 days storage, 4 °C).

A biosensor with increased stability was obtained by the use of silk fibroin (SF) as modifier in the AChE and AuNPs composite [69]. The SF provided a biocompatible microenvironment around the enzyme molecule to stabilize its biological activity and effectively prevented it from leaking out of platinum electrode surface. After storage for 6 weeks, the biosensor still retained 90% of its initial current response. The biosensor preparation included the realization of the composite and its direct immobilization on a Pt-working electrode. The AuNPs catalyzed the oxidation of thiocholine with signal amplification compared to the bare electrode. After characterization of the electrode material and optimization of the assay conditions, three pesticides were used as model analytes to characterize the biosensor response to AChE inhibitors: methyl paraoxon, carbofuran, and phoxim. The AChE inhibition was proportional to their concentration in the range: of 6×10^{-11}–5×10^{-8}M, 2×10^{-10}–1×10^{-7}M, and 5×10^{-9}–2×10^{-7}M, respectively. The detection limits were found to be 2×10^{-11}M for methyl paraoxon, 1×10^{-10}M for carbofuran, and 2×10^{-9}M for phoxim.

Other nano-materials The combination of self assembled monolayers and quantum dots (QDs) to prepare an organized sensing structure for successive modification with AChE to have a biosensor for the detection of carbaryl was attempted [70]. The authors initially obtained a cysteamine SAM onto a planar gold electrode; the SAM was used as anchoring layer, via the amino group of cysteamine, to immobilize CdTe QDs terminated with carboxyl groups through carbodiimide chemistry. QDs are semiconductor particles that have all three dimensions confined to nanometer length scales and appear particularly suited for bioconjugation. They led to highly oriented and organized structures and electrocatalytic activity promoted electron transfer reactions and catalyzed the electro-oxidation of thiocholine, thus amplifying the detection sensitivity. The biosensor was then used to detect the reversible inhibitor of AChE, carbaryl. The detection limit was 0.6 ng mL^{-1}. Sensor stability was similar to that reported for the other nanobiosensor developed by this group. It would be worthy to extend the stability study to a longer period as long as 90% of the signal was retained after 30 days.

An interesting selective nanobiosensor for methyl-parathion obtained using three different nanostructured materials have been recently reported in [71]. The selectivity of the biosensor was given by a methyl-parathion degrading enzyme (MPDE) obtained by purification from a commercial bacterial strain. The enzyme product was p-nitrophenol that was measured at the nanoelectrode surface. The biosensor was prepared by a layer-by-layer approach using MWCNT on a glassy carbon electrode followed by electrochemical deposition of gold nanoparticles by a multipotential step. On this AuNPs, a self-assembled monolayer of cysteamine was formed and via the amino group the free carboxylic groups of the CdTe quantum dots, loaded with MPDE, reacted to form the biosensors. The authors demonstrated that MWCNT and gold nanoparticles significantly increased the surface area and exhibited synergistic effects towards enzymatic catalysis. The role of CdTe QDs is, in this case, as carrier to load a large amount of enzyme. As a result of these two important enhancement factors, the proposed biosensor showed interesting analytical performances. The detection limit was 1.0 ng/mL. Moreover, since MPDE hydrolyzes pesticides containing the P–S bond, it showed high selectivity for detecting methyl-parathion also in presence of many interfering compounds, such as carbamate pesticides. An alternative approach in the preparation of the nanobiosensor is reported in [72]. The assembly of the sensor was a typical 80' biosensor with the enzyme membrane separated by the transducer surface that was retained in contact by an o-ring. The novelty of the device relies in the technology within the membrane preparation and formulation. Poly(acrylonitrile-methylmethacrylate-sodium vinylsulfonate) membranes were chemically modified and loaded with gold nanoparticles. Also, acetylcholinesterase immobilization was carried out according to two distinctive procedures, the first of which involved immobilization of the enzyme by convection and the other by diffusion. The prepared enzyme carriers were used for the construction of the amperometric biosensors for detection of acetylthiocholine. The biosensor was calibrated with acetylthiocholine in the concentration interval 10-400 μM, (linear interval 10-170 μM). The potential application of the biosensor for detection and quantification of organophosphate pesticides was investigated against sample solutions of Paraoxon. The biosensor detection limit for Paraoxon was found to be 7.39×10^{-11} g l^{-1}, and the concentration was 10^{-10}-10^{-7}g l^{-1}. It was demonstrated that the presence of gold nanoparticles was the crucial step to achieve sensitivity, a fast electrochemical response and operational and storage stability.

Detection of herbicides via inhibition of the PSII system

Due to the nature of PSII, different sensing formats have been designed to detect the herbicide or generally phytotoxic effect. The key role played by chlorophylls has led to the development of methods based on fluorescece measurement. In this respect, Schreiber et al. [73] have reported a measurement called Tox Y-PAM (Heinz Walz GmbH, Effeltrich, Germany) where a dual-channel fluorometer was specifically developed for detection of phyto-toxic substances in water samples. Effective quantum yield (DF/Fm)

is calculated for each channel from measures of minimum fluorescence (F1 or F 2, where the number denotes channel 1 or 2) and maximal fluorescence (Fm1 or Fm2) taken during a 0.4 s saturating pulse of more than 2,000 mmol photons $m^{-2}s^{-1}$. DF/Fm values for each channel are represented as Y1 or Y2 and these values are used to determine the response variable % PSII Inhibition, calculated as % inhibition Z100(Y2-Y1)/Y2, where Y1 is treatment and Y2 is the control chamber. The ToxY-PAM was developed for use with microalgal suspensions where the cells are assumed to remain in suspension for the duration of the measurements. As variable chlorophyll fluorescence originates almost exclusively from PSII, the method is particularly specialized, in the immediate term, for the detection of PSII herbicides, the family of herbicides that act by blocking electron flow at the primary plastoquinone acceptors of photosystem II. In [74], it was demonstrated that a chlorophyll based fluorescence measurements has definitive advantages in terms of sensitivity and assay time over a germination bioassay. In this paper, the effect of Diuron Irgarol and Bromacii on Hormosira banksii gametes was evaluated. Other authors [75] developed a method using fluorescence induction curves and mathematical programs to detect herbicides using the change in fluorescence signal of chlorophylls in thylacoids suspensions.

Real biosensors systems in which PSII is immobilized onto a sensing surface have been also object of investigations. An attempt to develop surface plasmon resonance chip for the detection of atrazine has been reported in [76] using the photosynthetic reaction center (RC) from the purple bacterium, Rhodobacter sphaeroides. The heavy subunit histidine-tagged RCs were immobilized on an SPR sensor chip via nickel chelation and was challenged against atrazine. Changes in the resonance angle obtained using a BIACORE X versus increasing amount of atrazine were recorded, but, were not satisfactory. In fact, the dynamic range obtained was1_100 mg/ml well above the admissible level of the herbicide in waters. The detection of chlorophyll fluorescence emission light at 683 nm (excitation 482 nm) corresponding to the Soret band maximum absorption of chlorophyll b (present in large amounts in green algae) was exploited for the development a whole cell fiber optic biosensor for herbicides [77]. The biosensor was based on the physical entrapment into membranes of *Chlorella vulgaris* microalgae adsorbed on a quartz microfiber filter. The algal chlorophyll fluorescence modified by the presence of herbicides was collected at the tip of an optical fiber bundle and sent to a fluorimeter. A continuous culture was set up to produce algal cells in reproducible conditions. The biosensor was able to rapidly detect atrazine, simazine, isoproturon, and diuron reversibly and with detection limits at sub ppb level.

Oriented immobilization of the photosynthetic reaction center of non-sulfur bacteria on gold has been obtained by Trammel et al. [78] via Ni-NTA assembled monolayers. The Reaction Centre is composed of three polypeptides H, L, and M. A dimer of bacteriochlorophylls, so called the primary donor, two monomer bacteriochlorophylls, two bacteriopheophytins (HA and HB), two quinones (QA and QB), and one iron are all non-covalently bound to the protein. A poly-histidine tagged RC from R. sphaeroides was immobilized gold electrodes with the acceptor side toward the solution. This allowed the measurement of photocurrent produced at 800 nm and at open circuit potential demonstrating the mediatorless electron exchange between the RC and the electrode. The inhibiting effect of atrazine was also demonstrated. Considering the possibility to genetically engineer different RCs, the approach is very interesting for the development of multipurpose reagentless amperometric detector for herbicides. A similar approach was at the same time reported in [79] that claim the advantages of using cysteamine derived immobilization not only on gold but also on gold-printed electrode. An improved system was later reported by the same author [80]. The mediatorless biosensor described was achieved using poly-mercapto-p-benzoquinone layers on screen-printed gold electrodes and gold microelectrodes in an array on silicon substrate. PSII particles isolated from the thermophilic cyanobacteria *Synechococcus bigranulatus* physically adsorbed on the polySBQ generated a photocurrent at E=+250 mV (versus Ag/AgCl) without any additional mediator. The authors report an improved sensivity of this system vs. other immobilized PSII systems for diuron and a lifetime comparable to systems in solution. A viable and simple solution to the problem of stability and lifetime of the biological element was recently proposed by Bettazzi et al. [81] that used the already described thylacoid membrane modified screen-printed electrodes plus duroquinone in a disposable way. Thylacoid membranes were purified from *Spinacia vulgaris* for the realization of a very low cost device. Using this approach, in order to have a reliable measurement the major, effort has to be devoted to inter-electrode reproducibility. Twelve percent interelectrode RSD was reported together with LODs within the 10^{-6}-10^{-8} M range for the herbicides tested.

Screen printed electrodes as transduction material to detect PSII inhibition in a multi-electrode set-up in flow system have been also proposed [82]. Thylacoid membranes from wild type and mutant strains of *Senecio vulgaris* and *Spinacia olearacea* have been immobilized onto the grafite working electrode using a BSA-Glutaraldehyde procedure. The sensing system is contained in a parallelepiped—shaped box (size 20×15×13 cm weight of 1 kg). Sealed printed electrodes can be inserted in a black Perspex flow cell supplied with a high-intensity

LED of 650 nm, suitable to activate light reaction in Photosystem II. The measurement of the PSII activity was based on amperometric oxidation at proper applied potential of the electron acceptor duroquinone. The light pulse send to the thylakoids induce reduction of the duroquinone that is reoxidised at the electrode surface at an applied potential of +0.2 vs. pseudo Ag/AgCl. Evaluation of the response against herbicides using this multichannel device gave very interesting data, detection limits below 10^{-7} diuron, atrazine, simazine terbuthylazine, deethylterbuthylazine, and the possibility to distinguish between different subclasses of the analytes by comparison of thylacoids sensors from different sources. Lifetime of the probes was 10 h (one working day). The potentialities of coupling genetic engineering, computational modeling and sensing have been brilliantly exploited in a recent paper by Giardi et al. [83]. In this approach, modeling and docking simulations have been the basis to produce a library of functional mutations in the unicellular green alga *Chlamydomonas reinhardtii*. Modifications of only one amino acid within the QB binding pocket can, in fact, change photosynthetic activity and herbicide binding considerably. Three mutants were prepared by site-directed mutagenesis and characterized by fluorescence analysis. The whole algae cells were immobilized on a silicon septum. The biomediators were found to be highly stable, for at least 1 month at room temperature. A portable fluorometer, developed for the purpose was used for the measurement. Detection limits between 10^{-9} and 10^{-10} were observed for triazines and urea herbicides and the biosensor in the realized setup was able to distinguish among the two classes. The procedures described in the last two papers were objects of patents and the instruments described are commercially available, full info are available at the website (www.biosensori.it).

A microfluidic system has been also recently developed. The production of hydrogen peroxide by thylakoid membranes extracted from higher plants was investigated and detected using the chemiluminescence produced by the well-known horseradish peroxidase /luminal reaction [84]. The presence of herbicides in the thylakoid samples reduced the hydrogen peroxide production. The biological components were firstly immobilized of magnetic beads and then kept in place by magnets. The portable device realized was tested with diuron and atrazine that were detected at concentrations below 10^{-7}M. The device can be used to detect the potential toxicity of natural waters. A sol-gel technique was used by Pena-vazquez et al. [85] to immobilize three microalgal species (*Dictyosphaerium chlorelloides*, *Scenedesmus intermedius*, and *Scenedesmus* sp. (S.s.)) onto glass in order to detect the chlorophyll fluorescence signal. The optical biosensor showed response to those herbicides that inhibit the photosynthesis at photosystem II (triazines: simazine, atrazine, propazine, terbuthylazine; urea-based herbicides, linuron). However, no significant increases of fluorescence response was obtained for similar concentrations of 2,4-D (hormonalherbicide) or Cu(II). A lifetime up to 3 weeks was achieved for the immobilized algae. Mutants resistant to atrazine were also used, and this had a detection limit of about 20 times higher than wild type. Detection limits were in general higher than those reported previously using the TOXI PAM system probably because of a different intrinsic inhibiting sensitivity of free vs. immobilized algae.

Application to real samples

Despite the large number of biosensor formats and types developed, the applications to real samples are rare. Moreover, very few reports are devoted to the validation of the analytical method and to sample preparation that is a crucial step to prove measurement precision and accuracy; the potentialities of the systems are then not fully exploited. Similar considerations were given in [86]. The authors reported that "Despite the very large number of publications demonstrating enzyme inhibition, the majority of systems were unfortunately not challenged by real samples." Five years later, the scenario is not changed that much. Here, we will report about those researches where the developed systems are challenged versus real food or environmental samples.

Food samples The application of biosensors, or bioassays, requires a dedicated approach in terms of evaluation of matrix effect, extraction procedure, matrix matched calibration, and all the other factors that can bias the results once that the developed system is moved from standard solution to real samples.

Our group has developed biosensing strategies using AChE as the target for the detection of organophospate and carbamate pesticides in food samples. The common strategies adopted in all of these applications were the use of non-immobilized AChE coupled with the detection of the enzyme product either via chemically modified electrode [87] or via a choline oxidase biosensor [88–91]. The rationale behind this choice was that when facing real samples, matrices can greatly and not reproducibly affect the immobilized enzyme (AChE) thus increasing the bias of the measurement. The overall assay cost was not significantly affected by the use of AChE in solution due to the microliter scale (500 μl) and AChE concentration (0.01 U/ml) range of the assay volume. In [87], the negligible effect of egg, bovine meat, milk, and honey on the assay performance was reported. Moving to a vegetable sample as wheat grains a remarkable influence on the AChE inhibition assay was reported. The $IC_{50\%}$ for pirimiphos methyl was 360 ng/ml

when working in buffer, 640 ng/ml in a buffer extract obtained from non-milled wheat grains and finally 1,650 ng/ml in a buffer extract obtained from milled grains. Therefore, when analyzing real samples, often a matrix-matched calibration is also needed to avoid false positive samples. If the detection is directed toward organotiophosphate pesticides (precursors of the oxo-form of the pesticides), an activation procedure is needed; otherwise, the observed inhibition on AChE would result depressed. This must be optimized considering the matrix effect on the chemical reaction [88]. For example, measuring dichlorvos in wheat sample, the influence of the matrix on the assay sensitivity did not allow working in a buffer extract obtained from milled grains. Therefore, a medium exchange strategy was used where the pesticide was extracted according to a solvent extraction method. The sample extract was then dried and re-dissolved in the assay buffer for the AChE inhibition assay. An inter-laboratory validation, using GC as instrumental technique was also carried out with excellent results. Moreover, the proposed protocol allowed the determination of dichlorvos in milled wheat at levels as low as 0.05 μg/g which is 40 times lower than the European legal limit [89].

The use of recombinant enzyme specifically designed for a target pesticide can greatly facilitate the detection even when the sample matrix has not passed a clean-up step. In fact, as reported for a very high dilution factor can be used thus eliminating, or greatly limiting, the matrix effect. These characteristics allowed the accurate analysis of dichlorvos in wheat samples at the MRL, 2 mg/kg, and below that value. The mean recovery of the method was 75%, and neither false nor positive samples were detected. [90]. It should be mentioned that despite the lack of selectivity of the measurements, the bioassays developed on wheat can be applied to monitor the good agronomical practices that are generally regulated by contract rules between the producer and the food industry. The rules include the use of a specific product for pesticide treatment.

Recently we also validated, by comparison with liquid chromatography-mass spectrometry (LC-MS) measurement, a biosensing approach to detect coumaphos in honey samples [91]. A simple procedure for the oxidative oxidation of coumaphos via N-bromosuccinimide and AChE inhibition was optimized. A calibration curve for coumaphos (8–1,000 ng/ml) was obtained in buffer; the intra electrode CV ranged between 8% and 12% whereas the inter electrode CV was comprised between 12% and 14%. A detection limit of 8 ng/ml was achieved, with an $I_{50\%}$ of 105 ng/ml. The assay was then applied to detect coumaphos in honey samples. Despite the solubility of the samples in buffer, the assay was affected by many electrochemical interference present in this sample matrix. A simple C18 based solid-phase extraction procedure has been then optimized and used for the assay. This allowed to eliminate all the electrochemical interferences with a satisfactory coumaphos recovery (around 86%) for a final LOD of 33 ng/g. Often, in papers dealing with real samples, the information related to sample preparation (e.g., spiking procedure, extraction procedure, matrix matched calibration) are poor. For example, detection of trichlorfon was achieved in celery, cauliflower, and leek samples in [92] and the authors claimed excellent recoveries, but the full analytical procedure was not clearly stated so that it is not easy, for the reader, to fully evaluate the performance of the proposed biosensor [92]. On the contrary, an interesting and throughout explanation of fruit samples preparation was reported in [93]. A direct immersion of a SAM/AChE biosensor assembled on a gold electrode in triturated samples or in fruit juice, was reported achieving reproducible and accurate measurement of carbaryl and parathion. The measurement recovery was between 77% and 101%. The same biosensor was also used to evaluate water samples obtained from urban creeks. No organophosphate and carbamate contaminants were found, therefore samples were spiked at different intervals and the samples used to build a matrix matched calibration. No matrix effect was observed comparing these calibrations with those obtained used distilled water. The same authors [94] reported on another biosensor for the detection of carbaryl directly in tomato pulp without any previous sample manipulation. The same paper reports also a simplified liquid extraction procedure that appears useful also for other matrices. It is noteworthy that the biosensor design is quite “old fashion” being a carbon paste electrode. However, the performance appears of great value being the recovery above 92% the liquid extraction procedure, whereas lower recoveries, around 80%, were obtained by direct immersion of the biosensor into the tomato pulp. The authors did not clearly stated if the biosensor was regenerated after each inhibition with a chemical procedure, or was physically renewed by mechanical polishing of the surface; in both cases, precision data are not reported.

Another simplified extraction approach for the detection of dichlorvos in apple skin is reported in [26]. In this paper, the authors used an AChE biosensor obtained using screen-printed electrode. In this work, genetically engineered AChE and wild type from electric eel were compared. The limit of detection for dichlorvos, obtained for both were comparable with those obtained by other authors for the two enzymes, 10^{-10} and 10^{-8} M, respectively. The apple skin sample were directly immersed into acetonitrile used as extraction solvent, and the same solution, diluted 1:20 in measuring buffer used for the inhibition and measurement steps. This approach appears, in our opinion one of the most suitable to balance between extraction recovery and

speed of measurement. Recently, different matrices were tested for the detection of chlorpyrifos-oxon using a portable AChE biosensing system. The biosensor was based on the inhibition of the acetylcholinesterase enzyme using screen-printed electrodes and a self-designed potentiostat. The lower concentration detected was of 2 μg/L for an enzymatic charge of 10 mU, the linearity was near to one order of magnitude, the repeatability was of 4.7% (n= 5) at 4 μg/L. Spiked and non-spiked water samples, beverages, and vegetable extracts were used to validate the system and for pepper extracts, the response was compared to HPLC-MS [95]. Zhang et al. [96] have developed a rapid biosensor based on disposable screen-printed electrodes, which is suitable for monitoring organophosphate and carbamate residues in milk. In this work, three engineered variants of Nippostrongylus brasiliensis acethylcholinesterase were used to obtain enhanced sensitivity.

Environmental samples All the applications related to the use of AChE biosensors to environmental samples that can be found in the literature in the last 6 years concern water samples. From the literature, it appears that no, or limited, matrix effect are reported for water samples. Filtration and pH correction appear in most cases as the only sample treatment needed before biosensor measurement. In [97] an FIA system equipped with enzymes obtained from different sources is reported, results showed the importance of carefully selecting the source of the enzymes used for the biosensor manufacturing. Additionally, the use of enzymes from different sources not only substantially improves the LOD, but also enlarges the spectrum of insecticides that can be detected using this kind of biosensors.

Two flow-injection biosensor systems using semi-disposable enzyme reactor have been developed to determine carbamate pesticides in water samples [98]. Acetylcholinesterase was immobilized on silica gel by covalent binding. pH and conductivity electrodes were used to detect the ionic change of the sample solution due to hydrolysis of acetylcholine. The biosensors were used to analyze carbaryl in water samples from six wells in a vegetable growing area. Both systems could detect the presence of carbaryl in the samples and provided good recoveries of the added carbaryl, i.e., 80–106% for the potentiometric system and 75–105% for the conductimetric system. The presence of carbaryl in water samples analyzed by the biosensors was confirmed by gas chromatography–mass spectrometric system. Another flow injection analysis system for water analysis is reported in [99]. In this application were combined: the use of AChE from different sources immobilized on screen-printed electrodes to obtain a sort of array, a multiple port flow injection apparatus and an artificial neural network (ANN) to discriminate between different possible contaminants in water samples (dichlorvos and methylparaoxon). When applied to real samples, the two pesticides could be determined with low error. This approach can be used to develop an on-site screening methodology. A parallel work consisting in discrete measures carried out with screen-printed biosensor carrying AChE from different sources is reported in [100].

This paper confirms that despite the detection strategy (FIA vs. batch analysis), the use of an array of biosensors obtained varying the enzyme source combined with ANN enables to discriminate between two pesticides simultaneously present in a sample. In this paper, water samples were analyzed for chlorpyrifos and chlorfenvinfos.

For validated systems, the research activity is currently active not only in the biosensor design field but also in the development of instrumentation with dedicated software both in environment and food areas [101, 102]. Examples of the most significant advances in the recent realization of Ache bioassays are given in Table 1.

Detection of herbicides with PSII system Altough the PSII system present very high potential for environmental control; applications in real samples are really scarce. In our opinion, this is a clear indication that there are problems that need still to be solved (i.e., operational stability, selectivity, pretreatment of the sample, etc) before a full validation is carried out. In this respect, to have an idea of the potential of the system we should mention the very interesting study carried out by Bengtson et al. [103]. In this paper, it is clearly demonstrated how the incorporation of bioassays into routine monitoring programs is likely to give valuable information. Comparison of the chemical vs. the TOX I PAM bioassay data, in fact, revealed the possibility to predict chemical contamination data. The data obtained demonstrated similarities of the chemical profiles for the eight herbicides tested in the two river estuaries selected for the monitoring (Brisbane and Thames). However, the similarities between herbicide profiles were not reflected by the toxicity observed in microalgae exposed to estuary surface water extracts. The authors, using comparative analysis of the data obtained, convincingly attributed this discrepancies to the presence of species inhibiting the PSII system in the River Thames not targeted by the chemical analysis.

In the already cited work of Bettazzi et al. [81], a comparison of the assay on different environmental samples was carried out. Blank and spiked atrazine solutions of river water samples, were compared with the data obtained with a Luminotox® assay. A comparable

Table 1 Selected examples of Ache bioassay formats

Enzyme	Electrode material/ mediator	Immobilization	Electrochemical technique	Analyte/matrix/ LOD	Reference
AChE/ChOx	Gold	SAM- PA-MAM dendrimer	Potentiometry amperometry	Trichlorfon, Carbofuran, Eserine	[40]
	-			No real samples	
				0.003 nM	
				0.04 nM	
				0.1 nM	
AChE	Sonogel carbon electrode	Al_2O_3 sol-gel entrapment	Amperometry	Paraoxon, Dichlorvos Chlorpyrifos-ethyl-oxon	[41]
				No real samples	
				0.75 nM	
				0.50 nM	
				0.25 nM	
AChE	MWCNT on GC	Glutaraldeyde crosslinking on chitosan MWCNT composite	Amperometry	Triazophos	[58]
				No real samples	
				30 nM	
AChE	SWCNT on gold	Acetylcholinesterase immobilized via Glutaraldeyde on polyaniline deposited on vertically assembled SWCNT wrapped with ssDNA	Square wave voltammetry	methyl parathion chlorpyrifos	[62]
				Spiked river water	
				0.001 nM	
				0.001 nM	
AChE	QD AuNPs on GC	Absorption of AChE on CdTe-AuNPs-CM/GCE	Voltammetry	Monocrotophos	[65]
				Garlic samples	
				0.3 ng/ml	
AChE	AuNPs on gold	Cross linking on MUA activated monolayer	Voltammetry	Malathion	[67]
				No real samples	
				1.0 ng/ml	
AChE	AuNPs on platinum	AChE crosslinking via glutaraldeyde with SF modified AuNPs	Amperometry	Methyl paraoxon Carbofuran	[69]
				Phoxim	
				No real samples	
				0.02 nM	
				0.10 nM	
				2.00 nM	
AChE (in solution) ChOx	Prussian Blue/G-SPE	AChE in solution	Chronoamperometry	Coumaphos	[91]
				Honey	
				8 ng/ml	
AChE	Gold electrode	AChE crosslinking via glutaraldeyde	Voltammetry	Parathion Carbaryl	[93]
				Water/tomato/apple/ orange	
				9.0 ng/ml	
				9.3 ng/ml	
AChE	Carbon paste/cobalt phtalocyanine	Carbon paste composite	Amperometry	Carbaryl	[94]
				Tomato	
				3.2 ng/ml	
AChe/rAChE	G-SPE/ cobalt phtalocyanine	Physical entrapment in a PVA-based photopolymer	Amperometry	Dichlorvos Methyl parathion	[99]
				River and bottled water	
				Simultaneous determination via ANN	
AChe/rAChE/ Phosphotriesterase	G-SPE/ cobalt phtalocyanine	Physical entrapment in a PVA-based photopolymer	Amperometry	Chlorpyrifos oxon Chlorfenvinfos	[100]
				Water lake	
				Simultaneous determination via ANN	

decrease of inhibition was recorded. Sample coming from polluted mining area also gave comparable inhibition data; however, major discrepancies were observed attributed to the presence of heavy metals in these polluted areas.

Also, Giardi et al. [83] reported that in real water matrix (control without herbicide) no inhibition of PSII activity was activity was recorded and in some cases even induced activation by 5–21%, measured as oxygen evolution. The authors state that low concentrations of pollutants affect living algal mutants by altering physiological processes. The biosensors can, in this respect, resembling real conditions, be applied for the evaluation of the real physiological impact of active herbicide compounds.

Conclusions

We have focussed in this paper our attention on inhibition-based bioassays for pesticides detection. Two biological systems that are targets for the pesticides are mainly used for this purpose, Ache and the PSII system. Bioassays and biosensors based on Ache are the object of a large interest; this was demonstrated by the increasing number of paper published on different sensors format based on Ache. The use of nanomaterial and/or nanostructured layers on sensors can improve efficiency of the bioassay (1) amplifying the electrochemical detection of the product, (2) facilitating a reproducible deposition of the enzyme and/or the regeneration of the inhibited Ache. On the other hand, genetic engineering of the enzyme has been proved to be extremely useful to design a biological element “fit for the purpose”. The use in the future, of a “nano- mutant” approach for the design of Ache sensors, possibly in an array format, is in our opinion, highly desirable and can improve the performances increasing the number of applications on real samples.

Development of sensors based on the inhibition of the PSII systems reflects a slightly different situation. The potentialities of this system are even greater considering the options that can be exploited. The choice of different algal species, the selection of mutants, reactions centers, and genetically engineered reaction centers give a very high degree of flexibility for the development of very useful devices. Particular care must be devoted in the development of analytical devices, considering the low stability of the biological elements. However, in order to evaluate the robustness of the approach we think that the devices developed should be inserted in official programs of environmental control as for other bioassays used for toxicity tests (e.g., Luminotox assay).

References

1. Ryan EG (2008) Beyond the circle of poison: significant shifts in the global pesticide complex 1976–2008. Glob Environ Change 18:786–799
2. Nauen R, Ebbinghaus-Kintscher U, Elbert A et al (2001) Acetylcholine receptors assites for developing neonicotinoid insecticides. In: Ishaaya I (ed) Biochemical sites of insecticide action and resistance. Springer, New York, pp 77–105
3. FAO/UNEP/OECD/SIB, Baseline Study on the Problem of Obsolete Pesticides Stocks, Rome, 2001
4. Various Authors (2005) The WHO recommended classification of pesticide by hazard and guidelines to classification: 2004, WHO Ed. 3–16
5. Guzzella L, Pozzoni F, Giuliano G (2006) Herbicide contamination of surficial groundwater in Northern Italy. Environ Pollut 142:344–353
6. Dubus IG, Hollis GM, Brown CD (2000) Pesticides in rainfall in Europe. Environ Pollut 110:331–344
7. Bjørling-Poulsen M, Andersen HR, Grandjean P (2008) Potential developmental neurotoxicity of pesticides used in Europe. Environ Health 7:50
8. Lacasaña M, López-Flores I, Rodríguez-Barranco M, Aguilar-Garduño C, Blanco-Muñoz J, Pérez-Méndez O, Gamboa R, Bassol S, Cebrian ME (2010) Association between organophosphate pesticides exposure and thyroid hormones in floriculture workers. Toxicol Appl Pharmacol 243:19–26
9. Council Regulation No. 2377/90/EEC of 26 June 1990 (OJ L 224 18.08.1990, p 1)
10. Lepoma P, Brown B, Hankec G, Loosc R, Quevauviller P, Wollgast J (2009) Needs for reliable analytical methods for monitoring chemical pollutants in surface water under the European Water Framework Directive. J Chromatogr A 1216:302–315
11. Pohanka M, Musilek K, Kuca K (2009) Progress of biosensors based on cholinesterase inhibition. Curr Med Chem 16:1790–1798
12. Manco G, Nucci R, Febbraio F (2009) Use of esterase activities for the detection of chemical neurotoxic agents. Protein Pept Lett 16:1225–1234
13. Pohanka M (2009) Cholinesterase based amperometric biosensors for assay of anticholinergic compounds. Interdisc Toxicol 2:52–54
14. Arduini F, Amine A, Moscone D, Palleschi G (2010) Biosensors based on cholinesterase inhibition for insecticides, nerve agents and aflatoxin B1 detection. Microchim Acta. doi:10.1007/s00604-010-0317-1
15. Arduini F, Amine A, Moscone D, Palleschi G (2009) Reversible enzyme inhibition-based biosensors: applications and analytical improvement through diagnostic inhibition. Anal Lett 42:1258–1293
16. Prakash Periasamy A, Umasankar Y, Chen S-M (2009) Nanomaterials - acetylcholinesterase enzyme matrices for organophosphorus pesticides electrochemical sensors: a review. Sensors 9:4034–4055
17. Nemukhin AV, Lushchekina SV, Bochenkova AV (2008) Characterization of a complete cycle of acetylcholinesterase catalysis by ab initio QM/MM modeling. J Mol Model 14:409–416
18. Kovarik Z, Radić Z, Berman HA et al (2003) Acetylcholinesterase active centre and gorge conformations analysed by combinatorial mutations and enantiomeric phosphonates. Biochem J 373:33–40
19. Millard CB, Kryger G, Ordentlich A et al (1999) Crystal structures of "aged" phosphylated *Torpedo californica* acetyl-

cholinesterase: nerve agent reaction products at the atomic level. Biochemistry 38:7032–7036
20. Mascini M, Sergi M, Monti D et al (2008) Oligopeptides as mimic of acetylcholinesterasefrom the rational design to the application in solid-phase extraction for pesticides. Anal Chem 80:9150–9156
21. Boublik Y, Saint-Aguet P, Lougarre A et al (2002) Acetylcholinesterase engineering for detection of insecticide residues. Prot Eng 15:43–50
22. Silva Nunes G, Montesinos T, Brasil O et al (2001) Acetylcholine enzyme sensor for determining methamidophos insecticide. Evaluation of some geneticallymodified acetylcholinesterases from *Drosophila melanogaster*. Anal Chim Acta 434:1–8
23. Villatte F, Marcel V, Estrada-Mondaca S et al (1998) Engineering sensitive acetylcholinesterase for detection of organophosphate and carbamate insecticides. Biosens Bioelectron 13:157–164
24. Campàs M, Prieto-Simónc B, Marty JL (2009) A review of the use of genetically engineered enzymes in electrochemical biosensors. Semin Cell Dev Biol 20:3–9
25. Sotiropoulou S, Fournier D, Chaniotakis NA (2005) Genetically engineered acetylcholinesterase-based biosensor for attomolar detection of dichlorvos. Biosens Bioelectron 20:2347–2352
26. Valdés-Ramírez G, Fournier D, Ramírez-Silva MT et al (2008) Sensitive amperometric biosensor for dichlorovos quantification: application to detection of residues on apple skin. Talanta 74:741–746
27. Bucur B, Fournier D, Danet A et al (2006) Biosensors based on highly sensitive acetylcholinesterases for enhanced carbamate insecticides detection. Anal Chim Acta 562:115–121
28. de Oliveira Marques PR Brasil, Silva Nunes G, SantosTC Rodrigues dos, Andreescu S, Marty J-L (2004) Comparative investigation between acetylcholinesterase obtained from commercial sources and genetically modifiedDrosophila melanogaster. Application in amperometric biosensors formethamidophospesticide detection. Biosens Bielectron 20:825–32
29. de Albuquerque YDT, Ferreira LF (2007) Amperometric biosensing of carbamate and organophosphate pesticides utilizing screen-printed tyrosinase-modified electrodes. Anal Chim Acta 596:210–221
30. Hankamer B, Barber J, Boekema EJ (1997) Structure and mebrane organization of photosystem II in green plants. Annu Rev Plant Phys Mol Biol 48:641–467
31. Giardi MT, Koblizek M, Masojıdek J (2001) Photosystem II-based biosensors for the detection of pollutants. Biosens Bioelectron 16:1027–1033
32. Giardi MT, Piletska E (eds) Biotechnological applications of photosynthetic proteins: biochips, biosensors and biodevices. Landes Bioscience, Springer Publishers, Georgetown, TX 78626, ISBN 0-387-33009-7
33. Andreescu S, Marty JL (2006) Twenty years research in cholinesterase biosensors: from basic research to practical applications. Biomol Eng 23:1–15
34. Palchetti I, Cagnini A, Del Carlo M et al (1997) Determination of anticholinesterase pesticides in real samples using a disposable biosensor. Anal Chim Acta 337:315–321
35. Albareda-Sirvent M, Merkoci A, Alegret S (2000) Configuration used in the design of screen-printed enzymatic biosensors. A review. Sens Actuators B 69:153–163
36. Bonnet C, Andreescu S, Marty JL (2003) Adsorption: an easy and efficient immobilisation ofacetylcholinesterase on screen-printed electrodes. Anal Chim Acta 481(209):211–213
37. Vakurov A, Simpson CE, Daly CL et al (2004) Acetylcholinesterase-based biosensor electrodes for organophosphate pesticide detection. Modification of carbon surface for immobilization of acetylcholinesterase. Biosens Bioelecton 20:1118–1125
38. Law KA, Higson SPJ (2005) Sonochemically fabricated acetylcholinesterase micro-electrode arrays within a flow injection analyser for the determination of organophosphate pesticides. Biosens Bioelectron 20:1914–1924
39. Shi M, Xu J, Zhang S et al (2006) A mediator-free screen-printed amperometric biosensor for screening of organophosphorus pesticides with flow-injection analysis (FIA) system. Talanta 68:1089–1095
40. Snejdarkova M, Svobodova L, Evtugyn G et al (2004) Acetylcholinesterase sensors based on gold electrodesmodified with dendrimer and polyaniline. A comparative research. Anal Chim Acta 514:79–88
41. Zejli H, Hidalgo-Hidalgo de Cisneros JL, Naranjo-Rodriguez I et al (2008) Alumina sol–gel/sonogel-carbon electrode based on acetylcholinesterase for detection of organophosphorus pesticides. Talanta 77:217–221
42. Sinha R, Ganesana M, Andreescu S et al (2010) AChE biosensor based on zinc oxide sol–gel for the detection of pesticides. Anal Chim Acta 661:195–199
43. Wang J, Musameh M, Lin Y (2003) Solubilization of carbon nanotubes by nafion towards the preparation of amperometric biosensors. J Am Chem Soc 125:2408–2412
44. Wang J, Musameh M (2003) Carbon nanotube/teflon composite electrochemical sensors and biosensor. Anal Chem 75:2075–2079
45. Rao CNR, Satishkumar BC, Govindaraj A et al (2001) Nanotubes. Chem Phys Chem 1:78–105
46. Davis JJ, Coleman K, Azamian B et al (2003) Chemical and biochemical sensing with modified single walled carbon nanotubes. Chem Eur J 9:3732–3742
47. Jacobs CB, Peairs MJ, Venton BJ (2010) Review: carbon nanotube based electrochemical sensors for biomolecules. Anal Chim Acta 662:105–127
48. Agüí L, Yáñez-Sedeño P, Pingarrón JM (2008) Role of carbon nanotubes in electroanalytical chemistry: a review. Anal Chim Acta 622:11–47
49. Lyon JL, Stevenson KJ (2008) Electrochim Acta. Electron transfer of peroxidase assemblies at tailored nanocarbon electrodes 53:6714–6721
50. Cui HF, Ye JS, Zhang WD et al (2009) Modification of carbon nanotubes with redox hydrogel: improvement of amperometric sensing sensitivity for redox enzymes. Biosens Bioelectron 24:1723–1729
51. Arias P, Ferreyra N, Rivas GA et al (2009) Glassy carbon electrodes modified with CNT dispersed in chitosan: analytical applications for sensing DNA–methylene blue interaction. J Electroanal Chem 634:123–126
52. Deng C, Chen J, Chen XL et al (2008) Boron-doped carbon nanotubes modified electrode for electroanalysis of NADH. Electrochem Commun 10:907–909
53. Mclean RS, Huang X, Khripin C et al (2006) Controlled two-dimensional pattern of spontaneously aligned carbon nanotubes. Nano Lett 6:55–60
54. Zheng M, Jagota A, Semke ED et al (2003) DNA-assisted dispersion and separation of carbon nanotubes. Nat Mater 2003:338–342
55. Pomales GS, Rivera-Vélez NE, Cabrera C (2006) DNA-wrapped carbon nanotubes assembled on gold substrates ECS. Trans 3:21–26
56. Pomales GS, Cabrera CR (2007) Vertical attachment of DNA-CNT hybrids on gold. J Electroanal Chem 606:47–54
57. Lin Y, Lu F, Wang J (2006) Disposable carbon nanotube modified screen-printed biosensor for amperometric detection of organophosphorus pesticides and nerve agents. Electroanal 16:145–149
58. Du D, Huang X, Cai J et al (2007) Amperometric detection of triazophos pesticide using acetylcholinesterase biosensor based

on multiwall carbon nanotube–chitosan matrix. Sens Actuators B 127:531–535
59. Gao Y, Kyratzis I, Taylor R et al (2009) Immobilization of acetylcholinesterase onto carbon nanotubes utilizing streptavidin-biotin interaction for the construction of amperometric biosensors for pesticides. Anal Lett 42:2711–2727
60. Zhang SP, Shan LG, Tian ZR et al (2008) Study of enzyme biosensor based on carbon nanotubes modified electrode for detection of pesticides residue. Chin Chem Lett 19:592–594
61. Xu Z, Chen X, Qu X et al (2004) Single-wall carbon nanotube-based voltammetric sensor and biosensor. Biosens Bioelectron 20:579–584
62. Viswanathan S, Radecka H, Radecki J (2009) Electrochemical biosensor for pesticides based on acetylcholinesterase immobilized on polyaniline deposited on vertically assembled carbon nanotubes wrapped with ssDNA. Biosens Bioelectron 24:2772–2777
63. Du D, Chen S, Cai J et al (2007) Immobilization of acetylcholinesterase on gold nanoparticles embedded in sol–gel film for amperometric detection of organophosphorous insecticide. Biosensors and Bioelectronics 23:130–134
64. Du D, Chen S, Cai J et al (2007) Comparison of drug sensitivity using acetylcholinesterase biosensor based on nanoparticles–chitosan sol–gel composite. J Electroanal Chem 611:60–66
65. Du D, Chen S, Song D (2008) Development of acetylcholinesterase biosensor based on CdTe quantum dots/gold nanoparticles modified chitosan microspheres interface. Biosens Bioelectron 24:475–479
66. Du D, Chen S, Cai J et al (2008) Electrochemical pesticide sensitivity test using acetylcholinesterase biosensor based on colloidal gold nanoparticles modified sol–gel interface. Talanta 74:766–772
67. Du D, Ding J, Cai J et al (2008) In situ electrodeposited nanoparticles for facilitating electron transfer across self-assembled monolayers in biosensor design. Talanta 74:1337–1343
68. Gong J, Wang L, Zhang L (2009) Electrochemical biosensing of methyl parathion pesticide based on acetylcholinesterase immobilized onto Au–polypyrrole interlaced network-like nanocomposite. Biosens Bioelectron 24:2285–2288
69. Yin H, Ai S, Xu J et al (2009) Amperometric biosensor based on immobilized acetylcholinesterase on gold nanoparticles and silk fibroin modified platinum electrode for detection of methyl paraoxon, carbofuran and phoxim. J Electroanal Chem 637:21–27
70. Du D, Chen W, Cai J et al (2008) Development of acetylcholinesterase biosensor based on CdTe quantum dots modified cysteamine self-assembled monolayers. J Electroanal Chem 623:81–85
71. Du D, Chen W, Cai J et al (2010) Covalent coupling of organophosphorus hydrolase loaded quantum dots to carbon nanotube/Au nanocomposite for enhanced detection of methyl parathion. Biosens Bioelectron 25:1370–1375
72. Marinov I, Ivanov Y, Gabrovska K et al (2010) Amperometric acetylthiocholine sensor based on acetylcholinesterase immobilized on nanostructured polymer membrane containing gold nanoparticles. J Mol Catal B: Enzymatic 62:67–75
73. Schreiber U, Muller JF, Haugg A et al (2002) New type of dual-channel PAM chlorophyll fluorometer for highly sensitive water toxicity biotests. Photosynth Res 74:317–330
74. Seery CR, Gunthorpe L, Ralph PJ (2006) Herbicide impact on *Hormosira banksii* gametes measured by fluorescence and germination bioassays. Environ Pollut 140:43–51
75. Hiraki M, Vredenberg WJ, van Rensen JJS et al (2004) A modified fluorometric method to quantify the concentrationeffect (pI50) of photosystem II-inhibiting herbicides. Pest Biochem Physiol 80:183–191
76. Nakamura C, Hasegawa M, Nakamura N et al (2003) Rapid and specific detection of herbicides using a self-assembled photosynthetic reaction center from purple bacterium on an SPR chip. Biosens Bioelectron 18:599–603
77. Vedrine C, Leclerc JC, Durrieu C et al (2003) Optical whole-cell biosensor using *Chlorella vulgaris* designed for monitoring herbicides. Biosens Bioelectron 18:457–463
78. Trammell SA, Wang L, Zullo JM et al (2004) Orientated binding of photosynthetic reaction centers on goldusing Ni–NTA self-assembled monolayers. Biosens Bioelectron 19:1649–1655
79. Maly J, Di Meo C, De Francesco M et al (2004) Reversible immobilization of engineered molecules by Ni-NTA chelators. Bioelectrochem 63:271–275
80. Maly J, Masojidek J, Masci A et al (2005) Direct mediatorless electron transport between the monolayer of photosystem II and poly(mercapto-p-benzoquinone) modified goldelectrode—new design of biosensor for herbicide detection. Biosens Bioelectron 21:923–932
81. Bettazzi F, Laschi S, Mascini M (2007) One-shot screen-printed thylakoid membrane-based biosensor for the detection of photosynthetic inhibitors in discrete samples. Anal Chim Acta 589:14–21
82. Touloupakis E, Giannoudi F, Piletsky SA et al (2005) A multibiosensor based on immobilized Photosystem II on screen-printed electrodes for the detection of herbicides in river water. Biosens Bioelectron 20:1984–1992
83. Giardi MT, Scognamiglio V, Rea G et al (2009) Optical biosensors for environmental monitoring based on computational and biotechnological tools for engineering the photosynthetic D1 protein of *Chlamydomonas reinhardtii*. Biosens Bioelectron 25:294–300
84. Varsamis DG, Touloupakis E, Morlacchi P et al (2007) Development of a photosystem II-based optical microfluidic sensor for herbicide detection. Talanta 77:42–47
85. Vázquez EP, Maneiro E, Pérez-Condec C et al (2009) Microalgae fiber optic biosensors for herbicide monitoring using sol–gel technology. Biosens Bioelectron 24:3538–3543
86. Amine A, Mohammadia H, Bourais I et al (2006) Enzyme inhibition-based biosensors for food safety and environmental monitoring. Biosens Bioelectron 21:1405–1423
87. Del Carlo M, Mascini M, Pepe A et al (2004) Screening of food samples for carbamate and organophosphate pesticides using an electrochemical bioassay. Food Chem 84:651–656
88. Del Carlo M, Pepe A, Mascini M et al (2005) Determining pirimiphos-methyl in durum wheat samples using an acetylcholinesterase inhibition assay. Anal Bioanal Chem 381:1367–1372
89. Longobardi F, Solfrizzo M, Compagnone D et al (2005) Use of electrochemical biosensor and gaschromatography for determination of dichlorvos in wheat. J Agric Food Chem 53:9389–9394
90. Del Carlo M, Pepe A, De Gregorio M et al (2006) An electrochemical bioassay for dichlorvos analysis in durum wheat samples. J Food Protect 69:1406–1411
91. Del Carlo M, Pepe A, Sergi M et al (2010) Detection of coumaphos in honey using a screening method Talanta doi:10.1016/j.talanta.2009.11.038 (in press)
92. Wei Y, Li Y, Qu Y et al (2009) A novel biosensor based on photoelectro-synergistic catalysis for flow-injection analysis system/amperometric detection of organophosphorous pesticides. Anal Chim Acta 643:13–18
93. Pedrosa VA, Caetano J, Machado SAS et al (2008) Determination of Parathion and Carbaryl Pesticides in Water and Food Samples Using a Self Assembled Monolayer /Acetylcholinesterase Electrochemical Biosensor Sensors 8:4600-4610
94. Caetano J, Machado SAS (2008) Determination of carbaryl in tomato "in natura" using an amperometric biosensor based on the inhibition of acetylcholinesterase activity. Sens Actuat B 129:40–46

95. Hildebrandt A, Bragos R, Lacorte S et al (2008) Performance of a portable biosensor for the analysis of organophosphorus and carbamate insecticides in water and food. Sens Actuat B 133:195–201
96. Zhang Y, Muench SB, Schulze H et al (2005) Disposable biosensor test for organophosphate and carbamate insecticides in milk. J Agric Food Chem 53:5110–5115
97. Bucur B, Dondoi M, Danet A et al (2005) Insecticide identification using a flow injection analysis system with biosensors based on various cholinesterases. Anal Chim Acta 539:195–201
98. Suwansa-arda S, Kanatharana P, Asawatreratanakula A et al (2005) Semi disposable reactor biosensors for detecting carbamate pesticides in water. Biosens Bioelectron 21:445–454
99. Valdés-Ramírez G, Gutiérrez M, del Valle MT et al (2009) Automated resolution of dichlorvos and methylparaoxon pesticide mixtures employing a flow injection system with an inhibition electronic tongue. Biosens Bioelectron 24:1103–1108
100. Istamboulie G, Cortina-Puig M, Marty JL et al (2009) The use of artificial neural networks for the selective detection of two organophosphate insecticides: Chlorpyrifos and chlorfenvinfos. Talanta 79:507–511
101. Hildebrandt A, Ribas J, Bragos R et al (2008) Development of a portable biosensor for screening neurotoxic agents in water samples. Talanta 75:1208–1213
102. Compagnone D, Van Velzen K, Del Carlo M, Mascini M, Visconti A (2007) Rapid detection of organophosphates, Ochratoxin A and Fusarium sp. in durum wheat via screen printed based electrochemical sensors in Comprehensive Analytical Chemistry vol, 49 Electrochemical Sensor Analysis S. Alegret, A. Merkoci (eds) Cap.29 pp 687-715
103. Bengtson SM, Nasha J, Goddard JF, Muller (2006) Phytotoxicity of surface waters of the Thames and Brisbane River Estuaries: a combined chemical analysis and bioassay approach for the comparison of two systems. Biosens Bioelectron 21:2086–2093