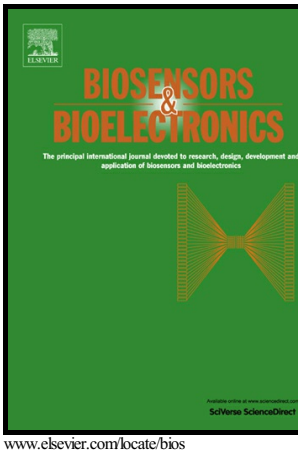

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RECENT ADVANCES IN BIOSENSORS BASED ON ENZYME INHIBITION

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Abstract

Enzyme inhibitors like drugs and pollutants are closely correlated to human and environmental health, thus their monitoring is of paramount importance in analytical chemistry. Enzymatic biosensors represent cost-effective, miniaturised and easy to use devices; particularly biosensors based on enzyme inhibition are useful analytical tools for fast screening and monitoring of inhibitors. The present review will highlight the research carried out in the last 9 years (2006-2014) on biosensors based on enzyme inhibition. We underpin the recent advances focused on the investigation in new theoretical approachs and in the evaluation of biosensor performances for reversible and irreversible inhibitors. The use of nanomaterials and microfluidic systems as well as the applications of the various biosensors in real samples is critically reviewed, demonstrating that such biosensors allow the development of useful devices for a fast and reliable alarm system.

Keywords: biosensor, enzyme inhibition, nanomaterials, analytical applications

1. INTRODUCTION

The enzymatic biosensors are based on enzymes in intimate contact with the transducers. A moltitude of research efforts was focused on the development of these biosensors for the detection of their substrates such as glucose oxidase biosensor for glucose monotoring. However, detection of compounds with biosensors based on enzyme inhibition is recently in growing progress.

The principle of this type of biosensors is based on the quantification of the inhibitor, measuring the enzymatic activity in absence and presence of the inhibitor. The study of inhibition is often a key point in clinical field, because some drugs are based on the inhibition of key enzymes of biological

pathways, while other inhibitors are considered toxic compounds; thus biosensors based on enzyme inhibition are reliable tools for the detection of a lot of toxic compounds (Figure 1).

From the historical point of view, the first biosensor based on enzyme inhibition was reported on *Analytical Chemistry* by Guilbault in 1962 (Guilbault et al., 1962). This biosensor for detection of nerve agents was constructed using cholinesterase enzyme as biocomponent, because this enzyme is inhibited by nerve agents. Since 1962, several biosensors were developed based on enzyme inhibition. The data on the number of publications in enzyme inhibition field from 1990 up to now are summarised in Figure 1. These data were obtained by searching the articles using as keywords "biosensor" and "enzyme" and "inhibition" on ISI web and Scopus. As shed light in Figure 2, there is an increasing trend during these years, which is also observed in the period analysed by this review (2006-2014).

The importance of this type of biosensors was also demonstrated by several reviews reported in literature (Amine et al., 2006; Andreescu and Marty, 2006; Pohanka et al., 2009; Periasamy et al., 2009; Arduini et al., 2009; Arduini et al., 2010).

In the present review we would like to underpin the recent advances in biosensors based on enzyme inhibition field, focusing on:

- *the investigation of a new theoretical approach* in order to easily understand the type of inhibition and calculate the kinetic parameters;
- *the evaluation of the performances of the biosensor based on enzyme inhibition in the case of reversible and irreversible inhibition*, in terms of time of analysis, detection limit, matrix effect
- *the use of nanomaterials* in order to improve the analytical performances of the biosensors;
- the development of biosensors based on enzyme inhibition *embedded in labs on a chip*;
- *the applications* of biosensors based on enzyme inhibition *in clinical, food and environmental samples.*

All these aspects will be critically reviewed in order to highlight the advances from 2006-2014 in this area.

2. NOVEL THEORETICAL APPROACH

2.1 Diagnosis of inhibition

Because the need of highly sensitive inhibitive biosensor, the optimization of key parameters such as concentration of enzyme, concentration of substrate and incubation time as well the diagnosis of inhibition type is highly required.

The types of inhibition can be classified as *irreversible* and *reversible* inhibition. Irreversible inhibition is characterized by a covalent link between the inhibitor and the active site of the enzyme. Consequently, the enzyme became permanently inactive (Kitz and Wilson, 1962). On the contrary, in the case of reversible inhibition, the inhibited enzyme can recover its original activity by a simple wash with buffer or water. Various kinds of reversible inhibition are reported in literature: the inhibitor may link the free enzyme (competitive inhibition), complex enzyme-substrate (uncompetitive inhibition), both free and complex enzyme-substrate with the same affinity (non competitive inhibition) or with different affinity (mixed inhibition). To quantify the inhibition, the term "degree of inhibition" is used, which is defined as the ratio of the difference of enzyme activity in absence and in presence of inhibitor divided by the enzyme activity in absence of inhibitor. Furthermore, the I_{50} is defined as the concentration of inhibitor which causes 50 % of inhibition and Ki as the inhibition constant that measures the affinity of enzyme to the inhibitor.

Recently, we proposed a novel graphical method for diagnosis of type of inhibition and determination of I_{50} and Ki, based on plot of degree of inhibition versus inhibitor concentration, (Amine et al., 2014). It was demonstrated that the equation of degree of inhibition (y) can be simplified to the equation 1 that is valid for all types of inhibition.

$$y = \frac{[I]}{[I]+[I_{50}]} \quad (1)$$

In this review, we show further studies of this equation. Indeed, by rearrangement of this equation in dimensionless form, we obtain:

$$y = \frac{\frac{[I]}{[I_{50}]}}{\frac{[I]}{[I_{50}]}+1} \quad (2)$$

Plotting the degree of inhibition versus I/I_{50}, and giving to I the values of 0.1 x I_{50} – 10 x I_{50}, the hyperbolae curve is obtained (Figure 3), similar to the traditional curve of Michaelis-Menten. The values of inhibition around 50% (20%-70%) can be used for rapid estimation of I_{50} (Table insight Figure 3). This approach empowers the scientist to perform only a single experiment of inhibition for an estimation of I_{50} without any prior knowledge of the substrate concentration, K_i or even the type of inhibition, offering unprecedent easy and fast approach for I_{50} calculation.

2.2 Biosensors based on reversible inhibition versus biosensors based on irreversible inhibition

This review is limited to biosensors based on direct enzyme immobilization on the transducer device. The enzyme and transducer elements are in close contact and incorporated in a single unit.

The immobilization of the enzyme increases the stability of the biosensor, reduces the time of analysis and can be used in flow systems allowing repetitive analyses that are highly required in the case of reversible inhibition. Several immobilization techniques have been reported in the literature. These techniques included physical entrapment, adsorption, covalent binding and covalent cross-linking (Sassolas et al., 2012).

Immobilization of enzyme is accompanied by modification of kinetic parameters such Km, Ki, V_{max} and I_{50} due to the formation of an enzymatic membrane which acts as diffusional barrier between substrate or inhibitor and the enzyme (Sassolas et al., 2012). Consequently, slight deviations from the theoretical behaviour of the free enzyme can be observed.

2.3 Measurement protocol employed for reversible and irreversible inhibition

In the case of reversible inhibition, the most frequent procedure is based on the measurement of enzyme activity in absence (I_0) and in presence of inhibitor (I_1) as indicated in Figure 4. The decrease of enzymatic activity can be evaluated by measuring the degree of inhibition as follows:

$$((I_0 - I_1)/I_0) \times 100 \qquad (3)$$

After inhibition, the biosensor is washed with buffer, and the enzyme activity is again evaluated. If the enzyme restores the initial activity, then the inhibition is reversible, otherwise the inhibition is irreversible. Furthermore, in the case of irrreversible inhibition, an incubation time (the time of reaction between the enzyme and inhibitor in absence of substrate) is required (Figure 4) (Arduini and Amine, 2014).

2.4 How to improve the analytical performance in the case of reversible or irreversible inhibition

2.4.1 Time of analysis

Long analysis time is often required for biosensor based on irreversible enzyme inhibition due to the necessary time of incubation. Moreover, after each single experiment, the enzyme looses a part of its initial activity, and thus the lifetime of such sensors is limited to only few cycles of reuse. The addition of a particular reagent can regenerate the enzyme activity (Du et al., 2007 b; Liu et al., 2006), but extends its lifetime for only few cycles, since the original activity of the enzyme can never be restored at 100% (Kartal et al., 2007). Therefore, a single experiment approach, like the use of screen-printed electrodes (Yang et al., 2006, Arduini et al. 2013, Arduini et al., 2007) is justified and highly desired in the case of irreversible inhibition. On the contrary, high frequency of

analyses can be reached when biosensors based on reversible enzyme inhibition are combined with flow injection analysis (Wang and Hasebe, 2011; Kochana et al., 2012, Zeravik et al., 2010).

The thickness of the enzymatic membrane can play also a role in the analysis time. A thicker film can result in increasing diffusion limitations of the substrate and the inhibitor, but an increasing incubation time can improve the degree of inhibition in the case of reversible inhibition. In the same way, the time of washing required to regenerate the initial response, can increase due to problems of diffusion. As an example, the immobilisation of cyclooxygenase in a gel-like carrageenan membrane had, as consequence, an increasing degree of inhibition with an increasing time of incubation, although the inhibition is reversible (Campanella et al., 2009). Ghica and Brett (2008) immobilized the glucose oxidase by cross-linking with glutaraldehyde, and observed that the activity of the inhibited enzyme is restored to about 90-96% after washing for 5 to 15 minutes in buffer solution.

In the case of irreversible inhibition, a reasonable incubation time of 10-20 minutes is generally adopted. Indeed, inhibition of lipase by methyl-parathion requires 15 minutes (Kartal et al., 2007), inhibition of invertase by mercury and silver needs 10-20 minutes (Soldatkin et al., 2012) and 10 minutes incubation time was sufficient to inhibit glucose oxidase by atrazine (Qing Yang et al., 2010).

2.4.2 Linear range, sensitivity and detection limit

The general curve of the degree of inhibition in the case of reversible inhibition (Figure 3), indicates that the upper limit of linear range is in the concentration range of the inhibitor and equal to I_{50}. We should keep in mind that when $[I] = 0.1I_{50}$, $y = 0.091$, and when $[I] = 10I_{50}$, then $y = 0.91$, in agreement with Equations 1 and 2. Thus, the dynamic range of inhibitor concentration can be considered as two decades. In the case of enzyme immobilized on transducer, a diffusional barrier can exist, which limit the access of substrate or inhibitor to the enzyme. Consequently, decreased sensitivity and extended linearity will be observed. For istance, in the case of cyanide detection, the range of linearity can be changed from 1-100 µM to 30-2000 µM of cyanide by increasing the peroxidase enzyme loading which increases the thickness of the enzymatic membrane (Ko et al., 2012).

Low concentrations of inhibitor can be detected if Ki is very low. This is the case of biosensors based on phosphatase PP2A, that allow the detection of microcystin (Campas et al., 2007) and okadaic acid (Volpe et al., 2009) at ppb levels. Biosensors based on elastase immobilized on SPR showed a detection limit of 0.97 ppb of aflatoxins B_1 (Cuccioloni et al., 2008). Furthermore, the sensitivity can be improved by combining the inhibited enzyme with another enzyme as in the case

of the co-immobilisation of acid phosphatase and polyphenol oxidase into layers of clay for specific detection of As (V) (Cosnier et al., 2006). Thanks to the amplification signal associated to the electrochemical generation of hydroquinone and to the high turnover of polyphenol oxidase, the detection limit of 2 nM for As (V) was reached.

In the case of irreversible inhibitors such as the case of organophosphorus pesticides and nerve agents using the cholinesterase enzyme, the sensitivity increases with increasing the incubation time until reaching a plateau. Furthermore, using many enzymatic units the sensitivity decreases, thus, instead of gel inclusion, an immobilization by adsorption and a covalent grafting are to be preferred, due to the low amount of enzyme per gram or per cm^2 fixed by these methods (Sassolas et al., 2012).

Another important aspect to be investigated concerns the concentration of substrate. In the case of irreversible inhibition, the concentration of substrate should be judiciously selected in order to achieve high rate of enzymatic activity, preferably at the starting point of plateau of the Michaelis-Menten curve. Furthermore, we should highlight that it is important to avoid very high concentrations of substrate, in order to avoid the reagent consumption and the inhibition due to high concentration of substrate. In addition, in the case of hydrolase enzymes the spontaneous hydrolysis of the substrate can contribute to a high value of the blank, and thus to the decrease of the detection limit of the inhibitor. Indeed, inhibition of invertase by mercury should take into account the non-enzymatic conversion of sucrose in solution (Mohammadi et al., 2002) or the inhibition of cholinesterase by organophosphorus pesticides should take into account the spontaneous hydrolysis of the enzymatic substrate (Arduini and Palleschi, 2012). Thus in the case of irreversible inhibition, we suggest to use a concentration of substrate equal to 2Km.

Even in the case of reversible inhibition, the effect of substrate on the degree of inhibition should be taken in consideration. For istance, in the case of competitive inhibition a low concentration of substrate should be preferred, because of the competitive reaction between substrate and inhibitor.

The existence of different suggestions to define the limit of detection (LOD) when used for inhibition study is a source of confusion. LOD is frequently defined as 3 x standard deviation (SD) of the blank (Mousty et al., 2007; Kochana et al., 2012). However, the value of the blank (V_0) is not near to zero, since V_0 corresponds to 100% of the signal response (in absence of inhibitor). Thus the SD can be high, and small variations of $V_0 - V_i$ are difficult to quantify precisely. It has been reported in literature that limit of detection (LOD) can be fixed at 5% (Yang et al., 2008), 10% (Campàs et al., 2007; Amine et al., 2006) and 20% of inhibition degree (Waibel et al., 2006).

In order to harmonize the published results, the authors of this review recommend that 10% of inhibition should be considered as a detection limit.

As demonstrated above, variation of the degree of inhibition from 10% to 90% corresponds to a dynamic range of two decades of inhibitor concentration. The sensitivity can be evaluated by measuring the slope of degree of inhibition versus inhibitor concentration in the range of inhibition from 10% to 50%. Thus, the determination of I_{10} and I_{50} highlights the analytical performances of the inhibitive biosensor.

2.4.3 Selectivity

Interfering species present in a matrix can give a non-enzymatic response signal. For example, the oxidation of electrochemical species found in real samples at an appropriate applied potential, leads to an under estimation of the inhibitor amount. It was reported that, in the case of irreversible inhibition, these interferences can be avoided if the "medium exchange method" is employed (Arduini et al. 2006). This method (schematised in the Figure 4) allows avoiding both electrochemical and enzymatic interferences. The electrochemical interferences are eliminated because the residual enzymatic activity is measured in a new buffered substrate solution, in absence of the real sample. Enzymatic interferences are avoided because, after the incubation step, the biosensor is washed with distilled water, and in this way only the decreased enzyme activity due to the inhibitor covalently linked to the enzyme is measured.

In the case of reversible inhibition, the medium exchange method cannot be employed, thus the electrochemical interferences cannot be eliminated.

Regarding the enzymatic selectivity, is well reported in literature that these biosensors are characterised by low specificity (i.e. capacity to detect selectively only one analyte). In fact, some enzymes are inhibited by a family of inhibitors, for instance cholinesterase is inhibited by several organophophorous and carbammic compounds: in a mixture of these compounds, only an anti-cholinesterase activity index can be calculated (Marinov et al., 2011). In this case, the inhibition assay can be considered as a "Family Doctor"; further analyses can be performed by a "Specialist Doctor", such as a HPLC method, able to identify and quantify each inhibitor. However, non contaminated samples do not show any inhibition when measured with biosensor, so they do not require further analysis with HPLC, saving time and money. Although the biosensor based on enzyme inhibition is not specific, it can be considered as an excellent fast and cost-effective device for screening methods. However, the selectivity can be improved using an array of enzyme electrodes for multi-analyte detection in combination with the use of chemiometric methods for interpretation and discrimination of experimental data (Alonso et al., 2012). Another interesting approach was reported by Korpan et al. for the detection of glycoalkaloids using acetylcholinesterase biosensor in presence of heavy metals and pesticides. To this regard, the

interference of heavy metals was removed by complexing them with ethylendiamminetetraacetate, while the interference of organophosphate pesticides was reduced using the phosphotriesterase enzyme, able to hydrolyse these pesticides (Korpan et al., 2006).

3 ENZYMES EMPLOYED IN BIOSENSORS BASED ON ENZYME INHIBITION

In the field of biosensors based on enzyme inhibition, the gold standard biocomponent is the cholinesterase enzyme, as shown in Figure 5. This trend is not only specific to the period analysed (2006-2014), but it appears also in our previous review (Amine et al. 2006).

The reason can be explained taking into account different factors:

i) Cholinesterase is characterized by high turnover;

ii) it is inhibited by several compounds such as organosphosphorus pesticides and nerve agents, and a fast and *in situ* detection for these analytes is very useful;

iii) inhibitors of cholinesterase such as pesticides are widely distributed in the environment;

iv) the substrate is soluble in aqueous solution and is not so expensive;

v) the enzymes is also irreversibly inhibited by a class of neurotoxic compounds, thus the biosensor is able to give useful neurotoxic index;

vi) this enzyme is present in the insects or humans, and is exactly the target of organophosphate and carbamate, thus the biocomponent was selected following the reactions that happen in nature, developing a *bio-inspired* biosensor.

On the other hand, among the different transducers, the electrochemical transducer is the preferred one (more than 90% of published biosensors based on enzyme inhibition are based on electrochemical transducers). This choice can be ascribed to several characteristics: robustness, cost-effectiveness, miniaturisability and capability to work in colored solutions. Furthermore, the possibility to use screen-printed electrodes renders this kind of sensors suitable for easy measurement in the case of reversible as well as of irreversible inhibitors, in the latter case avoiding the reactivation step due to their characteristic of single use biosensor.

Regarding acetylcholinesterase based biosensors, several compounds are able to inhibit this enzyme like organophosphorus and carbammic pesticides, nerve agents, aflatoxins, and heavy metals as reported in the Table 1.

The reaction catalysed by the acetylcholinesterase enzyme is:

Acetylcholine + H_2O → Choline + Acetic Acid

and the amount of inhibitor is quantified measuring the choline or the acetic acid production before and after the exposure of the inhibitor to the enzyme. For measuring the enzymatic activity, several

strategies have been reported in literature and shown in Table 2. When recording the pH variation, the AChE activity was measured by potentiometric or optical transducers. To this regard, classical enzyme-modified pH electrodes (Zhang et al., 2009a) or miniaturized pH-sensitive field effect transistors were used. For instance, pH-FETs was used as transducer with immobilized BChE for the detection of solanaceous glycoalkaloids in the concentration range of 0.1 μM to 0.1 mM (Benilova et al., 2006). In the case of optical biosensors, a chromoionophore sensitive to pH changes was used for nerve agents (Pohanka et al., 2010) or for the pesticide dichlorvos (detection limit of 0.5 mg/L)(Wong et al., 2006). The potentiometric transducer required a low buffer capacity, which could be a problem for application in real samples with high ionic strength, and is characterised by low sensitivity. However, ion-selective electrodes (ISE) with a novel polymeric membrane reached very low detection limit (0.05 ppb) (Ding and Qin, 2009). For the amperometric detection, two strategies were employed. The first one, which uses the natural substrate acetylcholine, requires choline oxidase (COx) enzyme in order to produce the electroactive hydrogen peroxide. For instance, AChE and COx were covalenty immobilised on the mercaptoproprionic acid self assembled monolayer on gold electrode to detect carbaryl at nM level (LOD=5.96 nM) (Hatefi-Mehriajrdi, 2013), or on a gold-platinum bimetallic nanoparticles for paraoxon ethyl and Sarin quantification and nM levels (Upadhyay et al., 2009) or on poly(dimethylsiloxane)-poly(diallydimethylammonium) /gold nanoparticles composite film (Zhao et al., 2009).

In the case of amperometric monoenzymatic biosensor, the non natural substrate acetylthiocholine can be employed, since the enzymatic product (thiocholine) is electroactive and in details, the acetylthiocholine with chloride as counter ions should be preferred in respect to iodide anions, to avoid the electrochemical interference of iodide as well demonstrated by Bucur et al. (Bucur et al., 2013). In order to reduce the applied potential, fouling problems, and electrochemical interferences, electrochemical mediators and/or nanomaterials were used. Electrochemical mediators in solution such as ferricyanide (Arduini et al., 2013), Ellman's reagent (usually employed for colorimetric detection (Dong et al., 2013)) or Cytochrome C (Zhang et al., 2009b) made possible to detect the thiocholine at low applied potential with satisfactory sensitivity. However for reagentless sensing, electrochemical mediators like cobalt(II) phthalocyanine (Laschi et al., 2007; Alonso et al., 2011), Prussian Blue (Arduini et al., 2007), 7,7,8,8-tetracyanoquinodimethane (Cortina et al., 2008) can be confined on the surface of the working electrode.

Recently, starting from 2009, a very interesting approach was also proposed, based on photoelectrochemical detection through photoactive electrodes, such as $PbO_2/TiO_2/Ti$ (Wei et al., 2009), bismuth oxyiodide flake array (Gong et al., 2012) and nitrogen and fluorine co-doped TiO_2

nanotubes (Huang et al., 2013). In presence of irradiation, electrons can be excited and move to the conduction band, leaving holes in the valence band. The thiocholine can act as a sacrificial electron donor, generating a photocurrent; thus the photocurrent is related to the concentration of the inhibitor allowing for its quantification. Another innovative biosensor based on the use $AuCl_4$ and liquid cristals was reported. The principle of the method is based on the enzymatic growth of gold nanoparticles (AuNPs) in presence of thiocholine, which is capable to reduce $AuCl_4$ to AuNPs. In presence of an inhibitor, a decrease of the catalytic growth of AuNPs was observed, with a reduction in the orientational arrangment of the liquid cristals (Liao et al., 2012).

Other AChE substrates can be employed, like indoxylacetate as a chromogenic or electrochemical substrate (Pohanka et al., 2012), or 3-indolyl acetate for electro-acoustic resonator transduction. In the last case, the AChE hydrolyzes the ester bond of the substrate, giving rise to the acetate and 3-hydroxyindole as products. The hydroxyl group tautomerizes, forming a ketone that in neutral or alkaline conditions causes dimerization with a water-insoluble indigo pigment product deposited on the Au surface, and consequent resonant frequency decreases during the course of the enzymatic reaction (Chen et al., 2012). The low detection limit achieved for the inhibitors, demonstrates that the artificial substrate can be successfully employed. The substrate was not required in the case of the use of surface plasmon resonance with immobilised acetylcholinesterase. The biosensor was based on the evaluation of the competitive binding between AFB_1 and the conjugated AFB_1-HRP allowing for the detection of AFB_1 at ppb level with the detection limit of 0.94 ppb (Puiu et al., 2012).

Looking at the strategies above reported, it is well evident that the monoenzymatic approach using the acetylthiocholine as substrate is the preferred one (around 97% of published papers uses the monoenzymatic approach).

Among the other enzymes reported in Table 1, phosphatase, peroxidase and polyphenoloxidase are the most employed in inhibitive biosensors. The activity of free phosphatase enzymes is widely measured by reaction with traditional substrate *p*-nitrophenyl phosphate and detection of *p*-nitrophenol at around 410 nm. Development of phosphatase biosensor for inhibitor determination requires the research of alternative substrate or the modification of the electrode surface. In the latter case, for instance the electrode surface was modified with gold nanoparticles for decreasing the fouling process due to the electropolymerisation of *p*-nitrophenol (Alvarado-Gámez et al., 2014). Other substrates were proposed, such as catechol monophosphate (Szydlowsk et al., 2006), riboflavin-5-monophosphate (Alvarado-Gámez et al., 2013), 2-phospho-L-ascorbic acid (Sanllorente-Mendez et al., 2012). These substrates showed less fouling on the electrode compared to phenol oxidation. Cosnier et al., 2006 reported the development of bienzyme electrode using

simultaneous immobilization of phosphatase and polyphenoloxidase (PPO). The strategy of this biosensor consists in the successive hydrolysis of phenyl phosphate into phenol by phosphatase, followed by the oxidation of phenol into o-quinone by PPO. Phosphatase activity was also succesfully evaluated using monofluoro phosphate as substrate and fluoride as product, measured with an ion selective electrode as sensor by Koncki et al., 2006.

In the case of free peroxidase enzyme, two substrates for its reaction in solution (hydrogen peroxide and phenol derivative such as hydroquinone) are required. The possible mechanism of peroxidase sensor is as follows:

$HRP(Fe^{3+}) + H_2O_2 \rightarrow$ Compound (I) + H_2O

Compound (I) + $QH_2 \rightarrow$ Compound (II) + BQ

Compound (II) + $QH_2 \rightarrow HRP(Fe^{3+}) + BQ + H_2O$

$BQ + 2e \rightarrow QH_2$

Where HRP is peroxidase, BQ is benzoquinone and QH_2 is hydroquinone, compound I and II denote enzyme intermediates in the reaction.

It should be noted that under some configurations of sensor, where intimate contact of enzyme and electrode surface is favourable, direct electrocatalytic reaction between compounds I and II and the electrode can occur. However, these sensors showed lower sensitivity than mediated peroxidase reaction with HQ. Table 2 showed some examples reported in literature of inhibitive biosensors based on mediated and unmediated peroxidase enzyme.

Polyphenoloxidase enzymes (PPO) catalyse in presence of oxygen the oxidation of *o*-diphenols to *o*-quinones, which is reduced on the electrode surface at around -0.2 V vs Ag/AgCl.

Inhibitors of PPO can be monitored either by measuring the consumption of oxygen with Clark electrode as reported by Asav et al, 2009 or by measuring produced o-quinones. The best substrates of PPO in terms of activity with enzyme and electrochemical reaction of the corresponding o-quinones are catechol and tyrosine. These substrates find wide application of inhibitive biosensors (Table 2). Reduced form of 1,2-naphtoquinone-4-sulfonate was proposed as substrate of PPO by Vidal et al. 2006. In this work, inhibitory effects of pesticides on PPO enzyme activity were monitored by chronocoulometry which is an integration of the chronoamperometric current decay at -0.15V Ag/AgCl.

In Figure 6 some novel strategies for inhibitor detection using inhibitive biosensors were reported.

4 NANOMATERIALS

Recently, there is a growing interest in nanomaterial-based biosensors (Zhang et al., 2014). Different materials were investigated in the preparation of biosensors based on enzyme inhibition such as carbon nanomaterials and metallic nanoparticles; among them, the carbon nanotubes and gold nanoparticles, represent the two most famous nanomaterials employed. Their easy functionalization represents a key point for their wide spread application. Drop casting is the most common technique adopted to produce nanomaterial modified sensors, due to its fast and simple procedure, although lacking in homogeneity of the film deposited.

Chitosan, Nafion and conducting polymeric films were usually employed as support for fixation of nanomaterials and enzymes (Oliveira et al., 2014; Moyo et al., 2014b; Can et al., 2012; Gong et al., 2009, Kesik et al., 2014). To this regards, other materials were also employed, like Cu–Mg–Al calcined layered double hydroxide (Zhai et al., 2014), Mg/Al layered double hydroxides (Gong et al., 2013) and the polyelectrolyte polydiallyldimethylammonium chloride (Liu et al., 2006).

4.1 Carbon nanomaterials

As reported above, without any doubt carbon nanotubes (CNTs) are the most employed carbon nanomaterial. One of the first biosensors utilizing CNTs was based on a self-assembled AChE on a CNT-modified glassy carbon electrode. The advantage to use CNTs was established on the detection of enzymatic product thiocholine at very low potential (+150 mV) without fouling problem when compared with the bare electrode (+750 mV) (Liu et al., 2006). Furthermore, the negatively charged CNT surface was exploited to immobilize the AChE, by alternatively assembling a cationic poly(diallyldimethylammonium chloride) layer and an AChE layer. The formation of layer by layer nanostructures on the CNT surface helped to immobilize the enzyme in a favorable microenvironment and to maintain the bioactivity of AChE. The suitable immobilisation adopted made the biosensor very sensitive, reaching a limit of detection of 40 pM for paraoxon.The CNTs were not only used alone as previously reported, but also combined with other materials in order to modify the sensor with novel nanocomposites such as 7,7,8,8-tetracyanoquinodimethane (Rotariu et al., 2012), chitosan–prussian blue–hollow gold nanospheres (Zhai et al., 2013), Co-phtalocyanine (Ivanov et al., 2011), β-cyclodextrin (Du et al., 2010a), gold nanoparticles (Du et al., 2010b), polypyrrole and polyaniline (Du et al. 2010c) and ionic liquids such as imidazolium-based ionic liquids (Zamfir et al., 2011). The use of nanocomposites allows for detecting the inhibitors at very low detection limit, but very often requires high potential, even higher than the sensor

modified with only carbon nanotubes. The CNTs were also used as a link between the cysteamine immobilised onto nanoporous gold film electrode and AChE (Ding et al., 2014). As expected, the detection of substrate occurred at a high potential (the peak of thiocholine oxidation was observed at at 913 mV in cyclic voltammetry), however the developed biosensor leads to detect the malathion with a detection limit of 0.5 ppb.

Graphene has received increasing attention during the recent years by virtue of its outstanding physical, chemical properties, and excellent electrocatalytic ability. In the sector of biosensors based on enzyme inhibition, some biosensors were recently assembled using graphene. A sensitive amperometric biosensor was fabricated through modifying glassy carbon electrode with AChE immobilized on porous reduced graphene oxide (pRGO) and chitosan. The biosensor allows for acetylthiocholine (ATCl) detection at 0.75 V with a Michaelis–Menten constant value of 0.73 mM and a detection limit of 0.5 ng mL^{-1} for carbaryl (Li et al., 2013). The use of graphene to increase the sensitivity was also demonstrated in the case of laccase carbon paste biosensors for carbamate pesticide detection (Oliveira et al. 2013a). Although the sensitivity was enhanced, the applied potential was high when compared with electrodes modified with electrochemical mediators or carbon nanotubes (Arduini et al., 2006, Zamfir et al., 2011; Liu and Lin, 2006). In order to reduce the applied potential, the graphene was also employed with other nanomaterials such as NiO nanoparticles (NiONPs) (Yang et al., 2013a), TiO_2 (Wang et al., 2011a), gold nanoparticles (Zhang et al., 2012), gold nanoparticles and polypyrrole (Yang et al., 2014), 3-carboxyphenylboronic and gold nanoparticles (Liu et al., 2011), CdS (Wang et al., 2011b), boronic acid functionalized Fe@Au magnetic nanoparticles (Dong et al., 2012), platinum nanoparticles (Yang et al., 2013b), and ZnO-decorated nanotubes (Nayak et al., 2013). In the carbon nanomaterial field, the advantage to use nanopowder was also reported as in the case of alkaline phosphatase immobilized on nano-powder paste electrode. This biosensor was capable to detect the carbofuran with a low detection limit of 10 µg/l (Samphao et al., 2013).

4.2 Metal nanoparticles

4.2.1 Gold nanoparticles

Gold nanoparticles are characterised by relevant electrochemical properties as reported for instance by Oliveira et al., 2014. In this case the gold nanoparticles were capable to reduce the charge transfer of the laccase/tyrosinase sensor, so a detection of carbamates at a concentration of nanomoles/L was achieved. A sensitive amperometric biosensor with gold nanoparticles was also developed for the detection of methyl paraoxon, carbofuran, and phoxim with AChE immobilised by means of gold nanoparticles and silk fibroin. In this case, the silk fibroin was used to provide a

biocompatible microenvironment around the enzyme molecule and to prevent the enzyme and the gold nanoparticles loss from the electrode surface. Under optimum conditions, the detection limits were found to be 20 pM for methyl paraoxon, 0.1 nM for carbofuran and 2 nM for phoxim (Yin et al., 2009). Another approach to prevent the leak of gold nanoparticles from the electrode surface, employs a sol–gel-derived silicate network incorporating gold nanoparticles. This modification provides a conductive pathway for electron transfer, able to improve the electrochemical reactions at around +600 mV (Du et al., 2007c). The use of gold nanoparticles to decorate the ionic liquid-doped polyaniline was reported by Teng et al. The designed AChE biosensor was successfully applied to evaluate the AChE inhibition induced by endogenous neurotoxin 1(R), 2N-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline ((R)-NMSal) (Teng et al., 2012). The gold nanoparticles were exploited dispersed in carbon nanotubes (Jha and Ramaprabhu, 2010) or together with CdTe quantum dots (QDs). In the latter case, the combination of CdTe QDs and gold nanoparticles was able to promote the electron transfer and catalyze the electro-oxidation of thiocholine, amplifying the detection. The biosensor was challenged with monocrotophos reaching a detection limit of 0.3 ppb (Du et al., 2008).

4.2.2. Other metallic nanoparticles

The CdTe semiconductor QDs have been integrated alone with AChE by the layer-by-layer assembly technique using QDs/poly(allylamine hydrochloride) and sodium polystyrenesulfonate. The detection limits of the proposed optical biosensor were 10.5 pM for paraoxon and 4.47 pM for parathion (Zheng et al., 2011).

Magnetic nanobeads were also used to develop an elegant way to screen tyrosinase inhibitors (Sima et al., 2011). Indeed, magnetic nanoparticles have a double function: i) have the capability of loading high concentration of enzyme, ii) can be employed in a real sample followed by confination onto the sensor surface using a magnet, minimising the matrix effect. Magnetic nanoparticles were also successfully used for horseradish peroxidase immobilization, and applied for amperometric detection of inhibitors such as thiol compounds (Yu et al., 2006).

4.3 Other nanomaterials

Among other nanomaterials different from graphene, AuNPs, and carbon nanotubes, SiO_2 nanosheets together with chitosan were used as a cross-linker to immobilize AChE. The AChE biosensor using SiO_2 nanosheets together with chitosan showed favorable affinity for acetylthiocholine calculated by amperometric mode at 0.65 V with an apparent Michaelis–Menten

constant of 134 μM. Under optimum conditions, the biosensor was capable of detecting methyl parathion, chlorpyrifos, and carbofuran with detection limits of 50 pM (Yang et al., 2013c). A composite based on iron oxide–chitosan and AChE was employed to modify glassy carbon electrodes. The nanocomposite-based biosensor could detect carbofuran as low as 3.6 nM by square wave voltammetry at around 0.6 V (Jeyapragasam et al., 2014). Calcium carbonate nano-material (nano-$CaCO_3$) was also explored in the inhibitive biosensor construction. Shan et al. developed a highly reversible and sensitive amperometric biosensor, based on the immobilization of tyrosinase by nano-$CaCO_3$ for benzoic acid determination. The inhibitive biosensor is characterised by a fast response to benzoic acid (<5 s) with a wide linear range of 0.56 to 92 μM, and a high sensitivity equal to 1061.4±13mA M^{-1} cm^{-2}. The authors claim that the good microenvironment of nano-$CaCO_3$ contributed to a considerable enhancement of sensitivity (Shan et al., 2008).

Taking into account the numerous papers reported above, it is evident that the nanomaterials display an enhancement of analytical potentialities of inhibitive biosensors because are capable to improve:

-sensitivity, by increasing conductivity and electron transfer reactions such as in the case of laccase biosensor for carbamic pesticides based on carbon paste prepared with graphene (Oliveira et al. 2013a);

-repeatability, by increasing antifouling capabilities such as in the case of acetylcholinesterase biosensor for organophosphorus pesticides based on glassy carbon modified with carbon nanotubes (Liu et al., 2006);

-working and storage stability, providing a better enzyme microenvironment such as in the case of tyrosinase immobilised by nano-$CaCO_3$ (Shan et al., 2008).

Among the different nanomaterials employed, carbon nanotubes in respect to graphene allow for the enzymatic product detection at lower potential, gold nanoparticles with fibroin lead for a better enzyme microenvironment maintaining the good electrochemical behavior, and other non conductive nanomaterials such SiO_2 nanosheets were successfully used for enzyme immobilisation.

5 MINIATURISED BIONSENSORS

The maturity of this type of biosensors was well demonstrated in the literature during the period studied in this review (2006-2014). We reported in 2007 the capability to detect Sarin in gas phase by means of a portable commercial available potentiostat (PalmSens) together with the butyrylcholinesterase enzyme immobilised on a disposable screen-printed electrode. Using a simple procedure of exposing the biosensor to the gas flow of Sarin (chemical warfare agent), the system was capable to detect Sarin gas at 0.1 mg/m^3 in 30s as incubation time, with a degree of inhibition of 34%, demonstrating the high sensitivity of the biosensor (Arduini et al., 2007) (Figure 7A). A

further step towards the development of a lab on a chip was reported by the Marty's research group. In their work, a tailored portable biosensor was developed for neurotoxic agent detection in water, using acetylcholinesterase from *Drosophila melanogaster* immobilised on a screen-printed electrode. The designed cost-effective user friendly and miniaturized potentiostat is able to perform amperometric measurements, adjusting the applied potential, and performing an internal calibration. The evaluation of the noise, drift, sensitivity, and repeatability was also reported demonstrating the suitability of this miniaturised device (Hildebrand et al., 2008). In the direction of automatisable systems, an interesting work was reported by the Hart's group; in this case, an array on six engineered acetylcholinesterase screen-printed electrodes was integrated in a novel automated instrument coupled with a neural network program. The system was successfully applied in several samples like water, food and vegetable extracts, demonstrating the possibility to cover the detection of several pesticides in a number of matrices by using an automatic and portable system (Crew et al., 2011). A portable set-up was also reported by Du et al. based on the use of screen-printed electrode modified with CNT combined with a microflow-injection device. This analytical system was able to assess the presence of AChE in saliva inhibited via regeneration of AChE, demonstrating the suitability for subclinical organophosphate exposure evaluation (Du et al., 2009) (Figure 7B). Another type of microfluidic device for organophosphorus detection using acetylcholinesterase and choline oxidase was developed by Han et al (Han et al., 2012). All components of the developed microfluidic device were integrated on 6" silicon wafer. This device was successfully applied for diazinon detection at ppm level.

The first example of lab-on-a-chip based on inhibitive biosensor was developed by Tan et al. This lab-on-a-chip was able to detect Sarin in a small volume (i.e. 1 mL) of blood using the cholinesterase enzyme. Many functions were integrated within the single chip by continuous-flow microfluidics. These include lysis of whole blood, regeneration of the free nerve agent from its complex with blood cholinesterase, protein precipitation, filtration, enzyme-assisted reaction and optical detection (Tan et al., 2008). The second example regards an integrated biofuel cell on microchip biosensor for detection of cyanide, and was developed by Deng et al., 2010. The chip was prepared using standard microfabrication on indium-doped tin oxide glass plates. It works as fully integrated biofuel cell and consisted of glucose dehydrogenase as anode and laccase as cathode. This microchip biosensor was successfully used to detect cyanide concentration in real samples at submicromolar concentrations. The third lab-on-a-chip was developed by us using butyrylcholinesterase immobilised on screen-printed electrodes. In this case, the system can be able to detect inhibitors like Sarin in gas phase using a microfun for air sampling. The integrated miniaturized circuit is able to apply the potential, to register the current, to turn on-off the fan and

eventually give an alarm switching on a led, demonstrating the readiness of the electrochemical biosensor for a rapid alarm in the case of nerve agent pollution in air (Arduini et al., 2012) (Figure 7C).

6 REAL SAMPLE APPLICATIONS

The availability and reliability of biosensors based on enzyme inhibition allows for their wide application in several matrices like environmental and clinical samples, as well as for food safety evaluation as highlighted in the Figure 8. In our previous review, the applications of these biosensors were restricted to a limited number of papers, while in the papers published from 2006 up to now the application in several food and environmental matrices is reported in more than the 50% of articles, demonstrating the utility of this type of biosensors for screening analysis in several samples. At least two thirds of the applications concern environmental samples and 25% of the applications involve the food analysis. However, clinical applications are still very scarce and do not exceed 4%. The determination of mercury in sewage water was successfully conducted with recovery between 94 and 107% using an urea biosensor based on self-assembled gold nanoparticles (Yang et al., 2006). Benzoic acid was determined in milk, yoghurt, sprite, and cola using a polyphenoloxidase biosensor (Shan et al. 2007). Milk and yoghurt were first centrifuged to remove insoluble residues, while the two soft drinks Sprite and Coca Cola were used without any treatment except dilution with the working buffer.The wide use of pesticides in the agrifood sector reflects the presence of pesticides in food and environmental samples. In the case of the food samples, a simple pre-treatment was required. For instance for pesticide detection in milk, a centrifugation at 5000 rpm followed by the removal of the supernatant and a filtration through a filter paper (Rodriguez et al., 2013) was carried out; in the case of olive oil, a liquid-liquid extraction with hexane was required, followed by heating at 50 °C and a centrifugation step at 134000 rpm (Ben Oujji et al., 2013); in the case of vegetables and fruits, the samples were first chopped, followed by the extraction with phosphate buffer (Zheng et al., 2011). For pesticide analysis in food, an interesting application was reported by Caetano and Machado. In this case, the insecticides were measured using an acetylcholinesterase biosensor without any previous manipulation of the sample; in fact, the biosensor was immersed directly in the tomato pulp obtaining a recovery of 83.4% and showing a very low interference of the matrix components (Caetano and Machado, 2008). The detection of pesticides in drinking or river waters was often only performed by filtration with disposable filter (Shi et al., 2006) or by dilution in buffer (Arduini et al., 2006). Methomyl and carbamate pesticides were quantified by laccase inhibition on sensor containing platinum nanoparticles. The quantification of methomyl was performed in tomato and carrot samples. The samples were mixed

with ethanol and the extract was filtred before the analysis. Results agreed well with those measured by an HPLC official method, confirming the accuracy of the laccase biosensor (Zapp et al., 2011). These results demonstrated that in the case of food samples, a facile pretreatment was required, that is well conjugated with the easyness of the biosensor use. In the case of water or saliva, a simple dilution of sample makes the biosensor well suitable for on-line measurement of the inhibitor in automatisable flow systems (Alonso et al., 2012, Wang et al., 2008). In the case of soil, the sample was centrifuged and filtered with a vacuum pump (Csiffary et al., 2013). For evaluation of the accuracy of these biosensors in real matrices, the samples were spiked with the tested inhibitors, and the results found in unspiked samples were compared with the ones obtained with the analytical official method (i.e. HPLC), and in all cases satisfactory results were obtained (e.g. recovery percentage equal to 96.7, 89.5, 110.7, 95.9 (Vastarella et al., 2007), 89.28, 98.33, 77.59 (Shi et al., 2006), 87, 90, 120, 83, 93 (Di Tuoro et al., 2011)).

7 CONCLUSIONS AND FUTURE PERSPECTIVE

Since 2006, a considerable research activity devoted to biosensors based on enzyme inhibition was accomplished. Analytical applications in real matrices such as food and environmental samples received recently considerable attention. The use of nanomaterials in the preparation of the biosensors improves some analytical features including sensitivity and stability. The sensitivity can be also improved by the knowledge of the type of inhibition (reversible or irreversible) and tuning the parameters like incubation time and enzyme amount. Although improved sensitivity is usually observed, the selectivity still represents the weak point of the biosensors. Simple pre-treatment of sample by solid phase extraction column would considerably improve the selectivity. For example, the use of column for separation of inorganic molecules such as heavy metals from pesticides will be helpful for screening them with cholinesterase, tyrosinase and lipase enzymes. The low selectivity, however, does not prevent the application of these biosensing devices if they are intended as on site alarm systems; in fact, due to the ability to detect classes of compounds, these biosensors are well suited for screening analysis. Despite a huge number of papers published in this field, and particularly on cholinesterase enzymes, the commercialization of biosensors based on enzyme inhibition is still untimely, thus the challenge of next years is how to convert the actual developed biosensors to commercially attractive devices, boosting the inhibitive biosensor technology from bench to the market.

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Figure Captions

Figure 1 Scheme of biosensor for substrate detection and inhibitor detection.

Figure 2 Number of published papers during the period 1990–2014. These data were obtained by searching papers using as keywords "biosensor" and "enzyme" and "inhibition" on ISI Web of Knowledge database, Thomson Reuters and Scopus.

Figure 3 General inhibition curve for the value of I varied from 0.1 x I_{50} to 10 x I_{50}.

Figure 4 Different protocols of reversible and irreversible inhibitor detection using biosensor based on enzyme inhibition.

Figure 5 Distribution of enzymes used for the design of inhibitive biosensors.

Figure 6 Examples of novel inhibitive biosensors (A) Photoelectrochemical biosensor. In presence of irradiation, electrons can be excited and move to the conduction band, leaving holes in the valence band. The enzymatic product acts as a sacrificial electron donor, generating a photocurrent which is correlated to the inhibitor concentration. Reprinted with permission from ref (Huang et al., 2013). (B) The detection of AFB_1 was based on competition of acetylcholinesterase enzyme reaction with AFB_1–HRP and AFB_1 monitored using SPR. Reprinted with permission from ref (Puiu et al., 2012). (C) Liquid crystal based biosensor. In presence of enzymatic product, the enzymatic growth of AuNPs is observed. In presence of an inhibitor, a decrease of the catalytic growth of AuNPs was observed, with a reduction in the orientational arrangment of the liquid cristals. Reprinted with permission from ref (Liao et al, 2012). (D) Cyanide detection is based on its inhibitive effect on laccase present in O_2 biofuel cell. The figure shows polarization of the uninhibited control (up) and polarization in presence of 1 mM cyanide. Reprinted with permission from ref (Deng et al., 2010).

Figure 7 **A** Portable commercial available instrument (PalmSens) employed with an inhibitive biosensor for nerve agent detection using drop measurement (5A) (Arduini et al., 2007). **B** Portable instrument commercial available coupled with a microflow-injection system and employed with inhibitive biosensor for biomonotoring of organophosphorus agents (Du et al., 2009). **C** Inhibited biosensor integrated into a lab-on-a-chip. The prototype is composed of a cell in which is inserted the biosensor, a little fan able to sampling air and an electronic circuit. The circuit is able to apply the potential, to register the current, to turn on-off the fan and eventually to give an alarm by led or wireless at a personal computer thorught the datalogger (Arduini et al., 2012).

Figure 8 Distribution of published papers reporting the main application fields.

Table 1 Immobilised enzymes employed in biosensors reported in literature (2006-2014) for inhibitors determination

Table 2 Several strategies reported in literature for inhibitor detection of cholinesterase, phosphatase, peroxidase, and polyphenoloxidase.

Figures

Figure 1

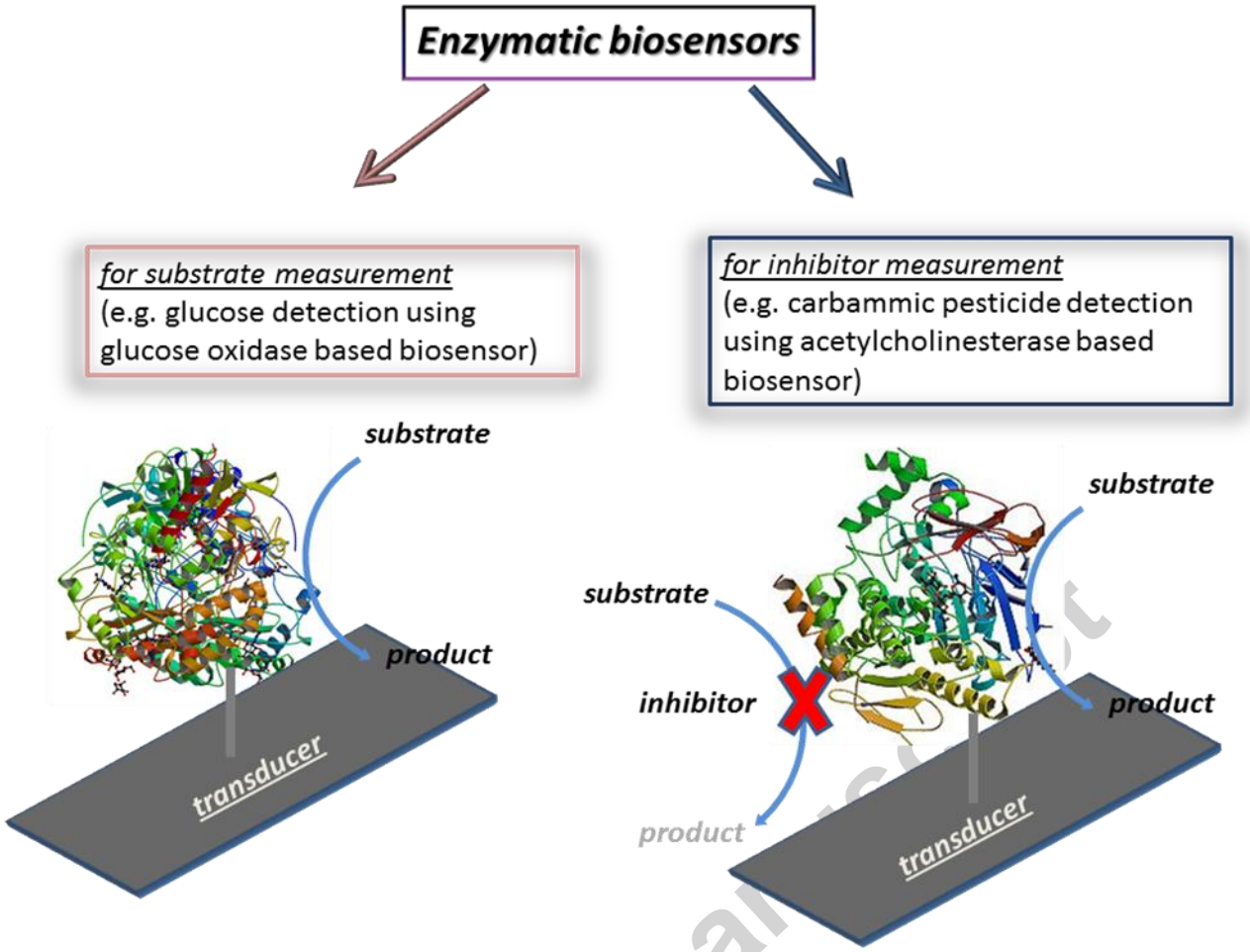


Figure 2

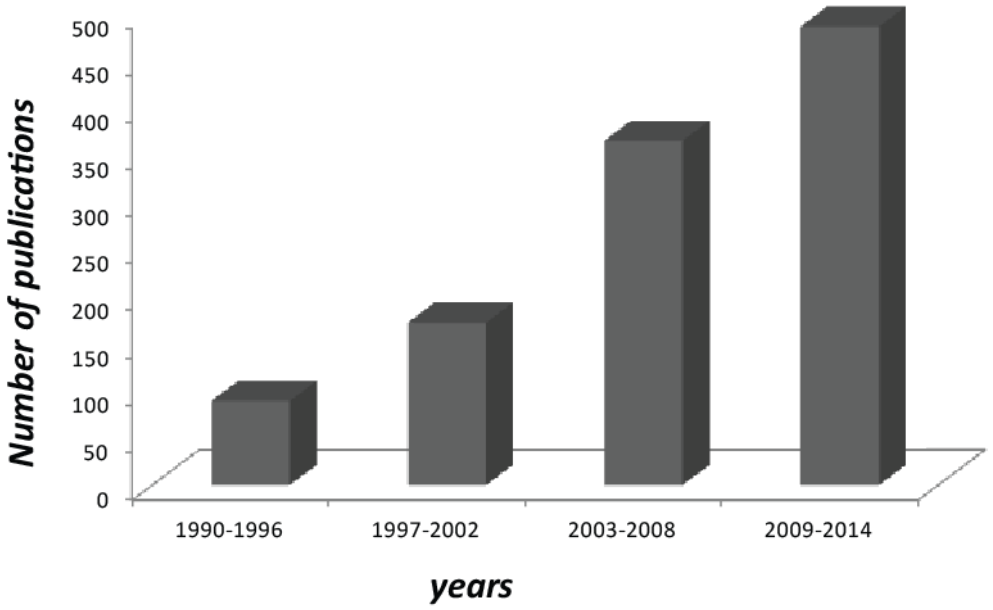
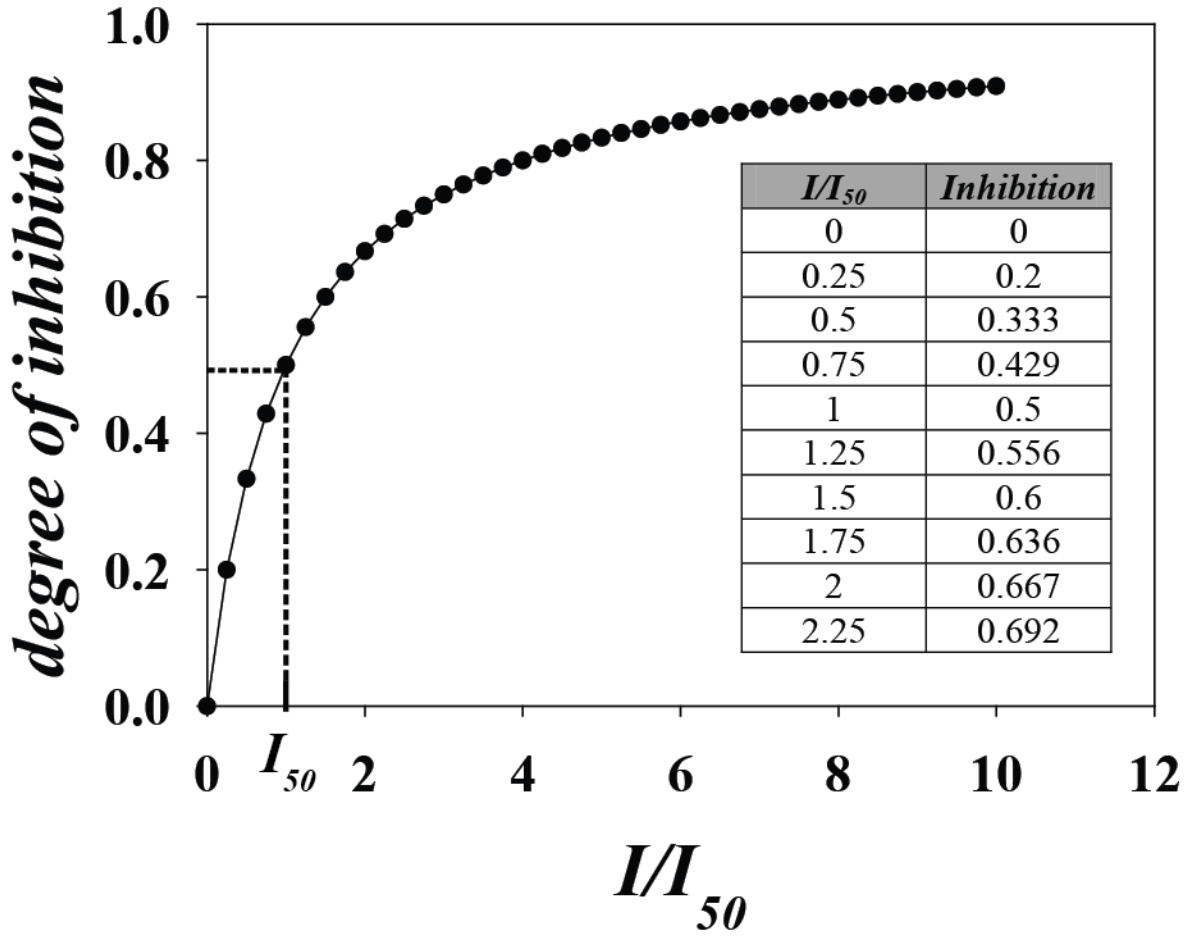


Figure 3



I/I_{50}	Inhibition
0	0
0.25	0.2
0.5	0.333
0.75	0.429
1	0.5
1.25	0.556
1.5	0.6
1.75	0.636
2	0.667
2.25	0.692

Figure 4

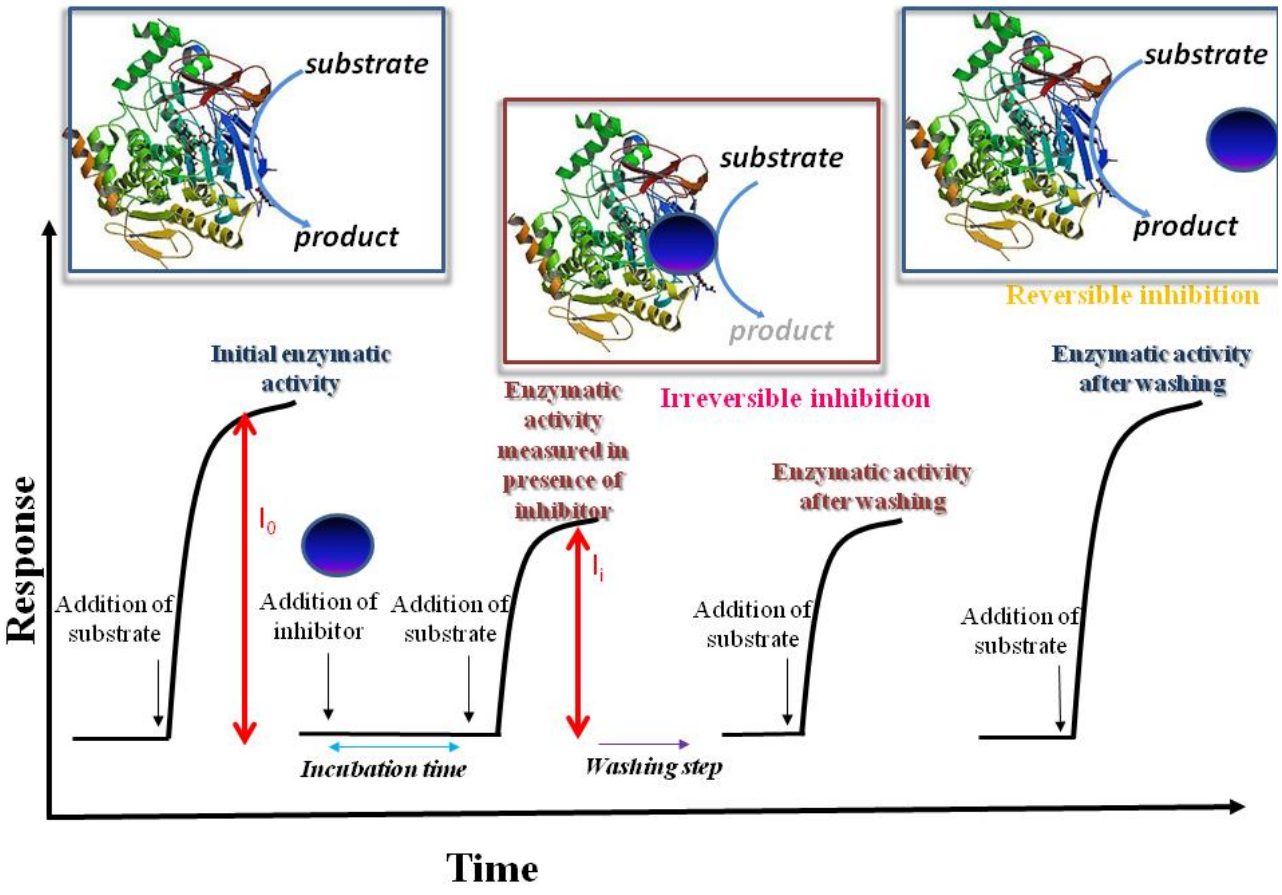

substrate
product
substrate
product
substrate
product
Reversible inhibition
Irreversible inhibition
Initial enzymatic activity
Enzymatic activity measured in presence of inhibitor
Enzymatic activity after washing
Enzymatic activity after washing
I0
Ii
Addition of substrate
Addition of inhibitor
Addition of substrate
Addition of substrate
Addition of substrate
Incubation time
Washing step
Response
Time

Figure 5

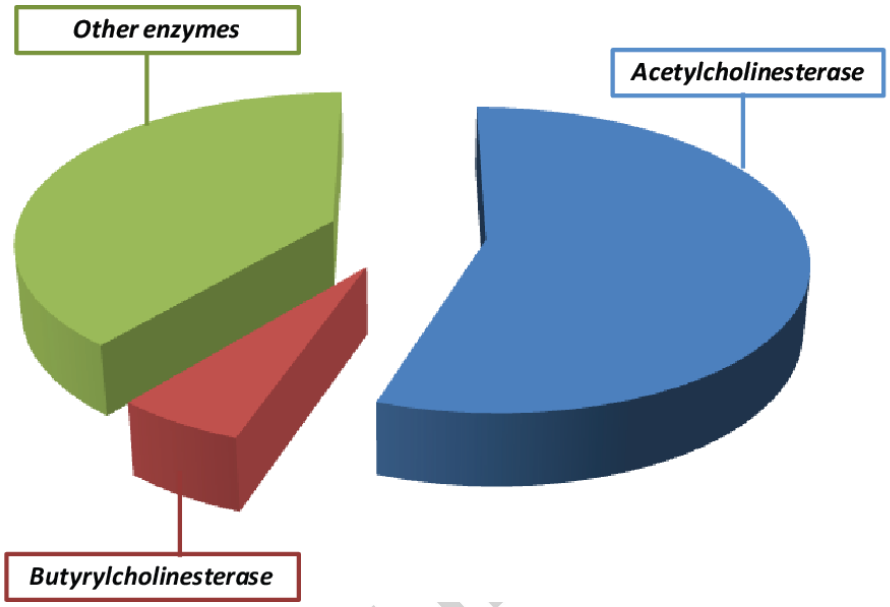


Figure 6

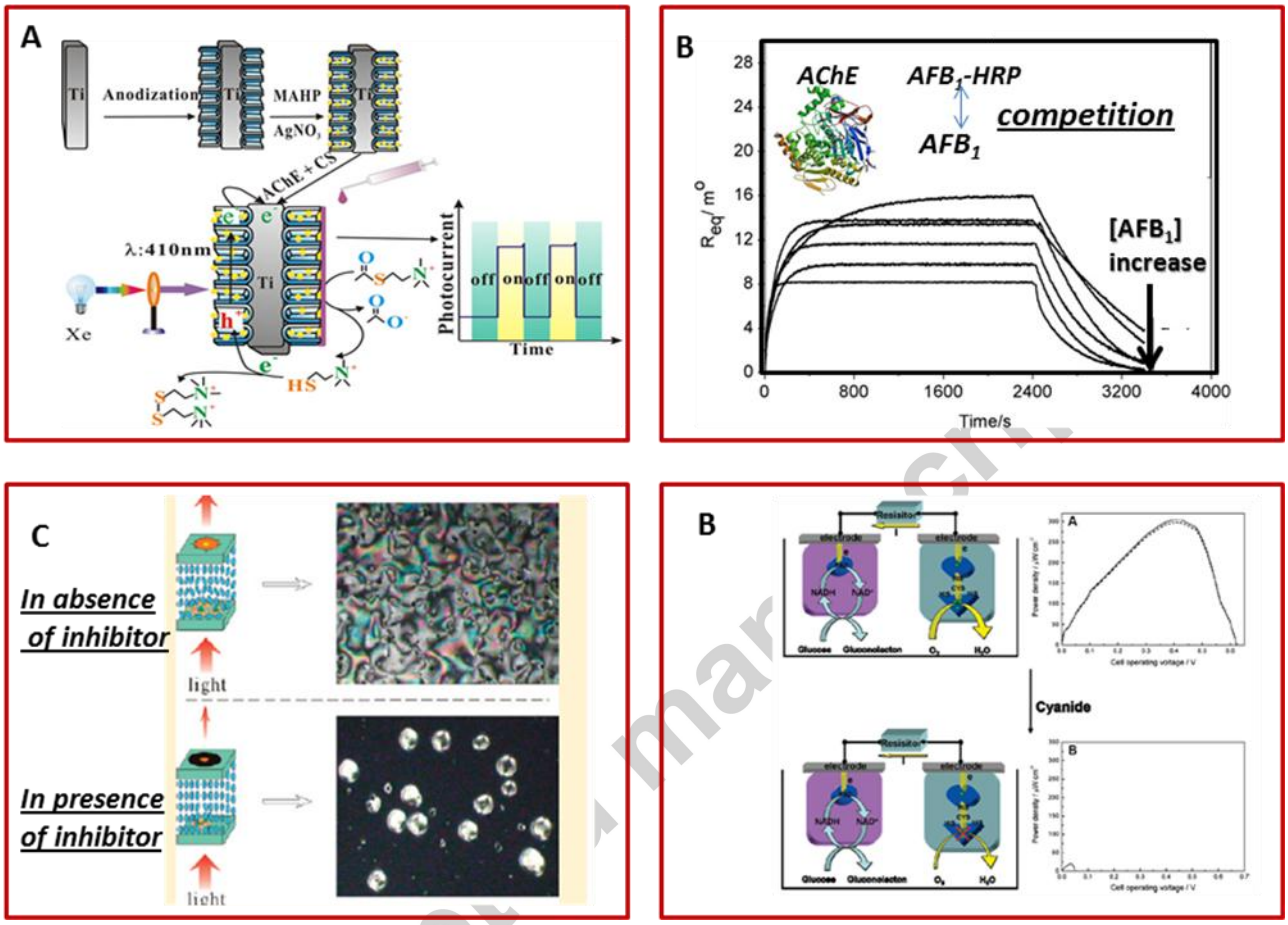
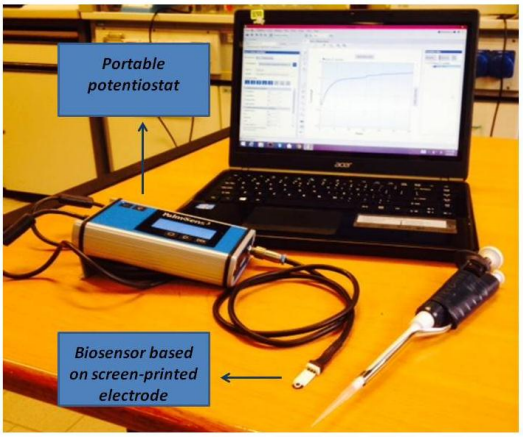
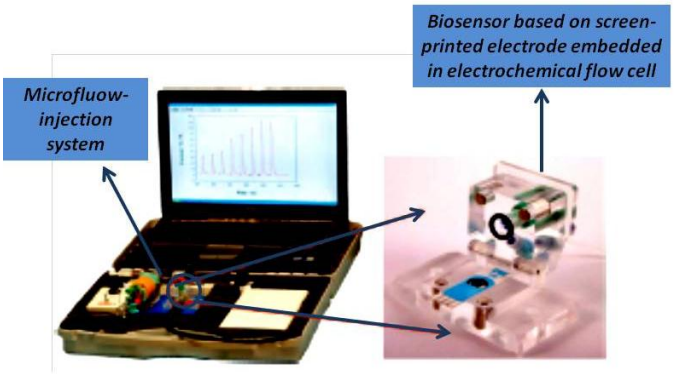


Figure 7

A


Portable potentiostat
Biosensor based on screen-printed electrode

B


Biosensor based on screen-printed electrode embedded in electrochemical flow cell
Microfluow-injection system

C

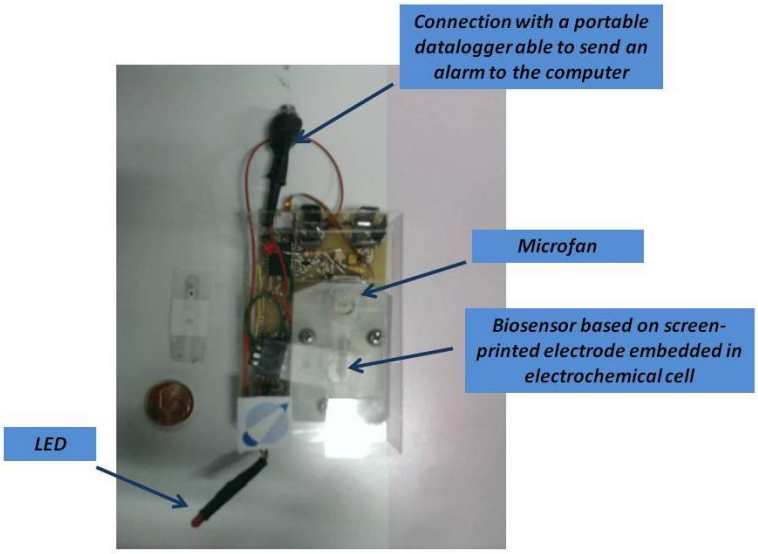

Connection with a portable datalogger able to send an alarm to the computer
Microfan
Biosensor based on screen-printed electrode embedded in electrochemical cell
LED

Figure 8

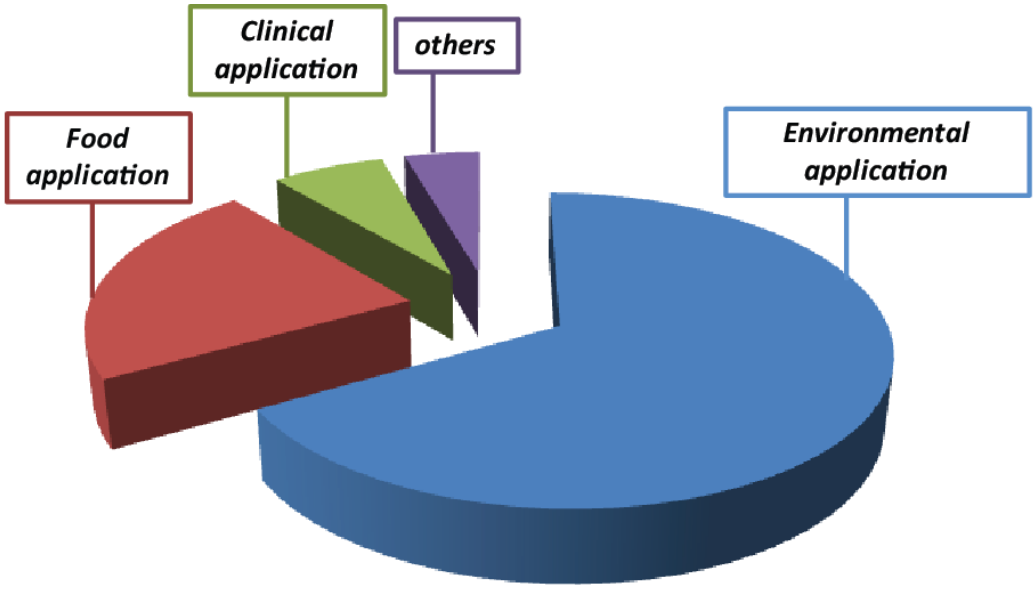


Table 1

Acetylcholinesterase	**Organophosphorus insecticides:**	
	malathion (0.5 ppb),	Ding et al., 2014
	chlorpyriphos (0.5 ppb)	Zhai et al., 2014
	paraoxon (0.5 nM)	Yang et al., 2014
	monocrotophos (0.1 nM)	Zhai et al., 2013
	dichlorvos (4.49 nM)	Liang et al., 2014
	methyl parathion (0.5 pM)	Yang et al., 2013c
	malaoxon (0.05 pbb)	Evtugyn et al., 2011
	monochrotophos (10.8 pM)	Marinov et al., 2011
	dimethoate (2 nM)	Du et al., 2010a
	phoxim (2 nM)	Yin et al., 2009
	chlorpyriphos-oxon (2 ppb)	Hildebrandt et al., 2008

	Carbammic insecticides: carbofuran (0.12 ppb) aldicarb (10 nM) carbaryl (0.5 ppb) pirimicarb (20 nM)	Dutta and Puzari, 2014 Evtugyn et al., 2014 Li et al., 2013 Bucur et al., 2006
	Other pesticides: permethrin (8.1 µM)	Dominiguez-Renedo et al.,2012
	Surfactants benzalkonium chloride (0.75 ppm), sodium dodecil sulphate (0.75 ppm), hexadecylpyridinium (2.5 µM).	Kucherenko et al., 2012
	Heavy metals Aluminum (2.1 µM)	Barquero-Quiros et al., 2013
	Nerve agents Sarin (7.41 pM), Soman (6.31pM), Tabun (61.7 pM), VX (21.9 pM)	Pohanka et al., 2013
	Toxins 1(R)-Sal (0.1 nM), (R)-NMSal (0.2 nM) AFB1 (0.94 ppb)	Huang et al., 2013 Puiu et al., 2012
	Glycoalkaloids α-chaconine (15 nM), α-solanine (23 nM)	Espinoza et al., 2014
	Drugs Donezepil (0.027 ppb) Codeine (20 µM) Galantamine and Neostigmine (Ki= 0.14 µM and 0.19 µM)	Turan et al., 2014 Asturias-Arribas et al., 2013 Du et al., 2007a
Acid Phosphatase	As(V) (0.11 µM)	Cosnier et al., 2006; Sanllorente-Méndez et al., 2012
Alkaline Phosphatase	Cd^{++} (0.1 aM), Hg^{++} (10 aM)	Tekaya et al., 2013
	Carbofuran (10 µg/l)	Samphao et al., 2013
	Caffeine (0.1 µM)	Akyilmaz et al., 2010
	Vanadium (0.4 µM)	Alvarado-Gámez et al., 2014 ; Alvarado-Gámez et al., 2013
Ascorbate oxidase	hydrogen sulphide (0.5mg/l)	Neda Zia Mottalebi poor et al., 2014
Butyrylcholinesterase	**Pesticides**	

	Ethion (22 ppb) Chlorpyrifos-methyl oxo (0.5 ppb) Paraoxon (2 ppb)	Khaled et al., 2014 Arduini et al., 2006
	Nerve agents Sarin 12 ppb, VX 14 ppb, Sarin in vapour (0.1 mg/m^3)	Arduini et al., 2007
	Glycoalkaloids α-chaconine (15 nM),α-solanine (40 nM) tomatine (0.1 µM)	Espinoza et al., 2014 Benilova et al., 2006
	Others: Nicotine (solution 10 µM, in gas phase 10 ppb)	Mitsubayashi et al., 2006
Catalase	CN^- (6 µM)	Bouyahia et al., 2011
	Nitrite (4 µM)	Chen et al., 2008
α-Chymotrypsin	Al^{3+} (3.3 µM)	Barquero-Quirós et al., 2012
Cyclooxygenase	Diclofenac (50 nM), naproxen (50 nM), ibuprofen(5 nM), tolmetin (5 nM)	Campanella et al., 2009
Cytochrome P450-3A4	Indinavir (61 ppb)	Ignaszak et al., 2009
Elastase	Aflatoxin B_1 (0.97 ppb)	Cuccioloni et al., 2008
Glucose oxidase	Cd^{2+} (1.2 µM), Co^{2+} (0.9 µM), Cu^{2+} (1.4 µM), Ni^{2+} (3.3 µM)	Ghica et al., 2013
	Hg^{++} (0.5 nM)	Chey et al., 2012
	Hg^{++} (7.4 µM) Hg^{++} (0.5 ppm)	Cosnier et al., 2011 Samphao et al., 2012
	Cadmium (1 ppb), copper (6 ppb), lead (3 ppb) and zinc (9 ppb)	Ghica and Brett 2008
	Hg^{++} (2.5 ppm), Ag^+ (0.05 ppm)	Guascito et al.,2008
	Atrazine (0.39µM)	Qing Yang et al., 2010
Invertase	Hg^{++}(2 5nM) , Ag^+(100 nM)	Soldatkin et al., 2012
	Hg^{++} (0.5 nM)	Bagal-Kestwal et al., 2008
Laccase	Carbamates (1 ppb)	Oliveira et al., 2013b
	Pirimicarb (0.2 µM)	Oliveira et al., 2013a
	Methomyl carbamate (0.2 µM)	Zapp et al., 2011
	CN- (0.1 µM)	Deng et al., 2010
	N^{3-} (5.5 nM), CN^- (6.2 nM), F^- (6.9 nM)	Mousty et al., 2007
	N^{3-} (2.5 µM)	Liu and Dong, 2008
Lipase	Chlorfenvinphos (84 µM)	K. Gangadhara Reddy et al., 2014
	Methyl-parathion (5 nM)	Kartal et al., 2007

Lipoxygenase	Caffeic acid (2 µM)	Dilek Odaci Demirkol et al., 2012
Neuropathy target esterase	phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride (10 µM)	Kohli et al., 2007
Nitrate reductase	Phenol (1 ppb)	Can et al., 2012
Peroxidase	Cu^{++} (2.5 ppb) Cd^{++} (2.0 ppb) Pb^{++} (0.033 ppb) Cr (VI) (0.09 µM) Cyanide (0.43 µM) Cyanide (1 µM) Cyanide (0.04 µM) Phenylhydrazine (0.5 mM) Glyphosate (30 ppb)	Moyo et al., 2014b Moyo et al., 2014a Nomngongo et al., 2011 Attar et al., 2014 Ghanavati et al., 2014 Ko et al., 2012 Wang and Hasebe, 2011 Huang et al., 2012 Oliveira et al., 2012
Phosphatase (Serine, threonine and tyrosine phosphatases)	Okadaic acid (1 nM)	Stenlund et al., 2006
Protease (bromelain,ficin, chymopapain)	Cystatin (0.1 ppb)	Gorodkiewicz et al., 2012
Phosphatase PP2A	Okadaic acid (30 ppb)	Volpe et al., 2009
	Microcystin (0.1 ppb)	Campàs et al., 2007; Szydłowska et al., 2006
Protease (bromelain,ficin, chymopapain)	Cystatin (0.1 ppb)	Gorodkiewicz et al., 2012
Pyranose oxidase	Glutathione (0.04 mM) and ethanol (3 mM)	Yazgan et al., 2008
Sarcosine oxidase	Carboxylic acids (0.5 mM)	Jiri Zeravik et al., 2010
Secreted aspartic proteases	HIV-1 protease inhibitors (195 nM)	Backman et al., 2006
Tyrosinase (polyphenol oxidase)	kojic acid, azelaic acid and benzoic acid (<2.5µM).	Sima et al., 2011
	Carbaryl (0.01 ppb)	Shim et al., 2009
	Dichlorvos (0.1 ppb)	Vidal et al., 2006
	Fluoride (1 µM)	Asav et al., 2009
	Organophosphates (0.01 µM) and carbamates (0.01 µM)	Campanella et al., 2007
	Benzoic acid (0.01 µM)	Amine et al., 2014; Kochana et al., 2012 ; Shan et al., 2008 ; Shan et

		al., 2007
Tyrosinase + Glucose oxidase	Cr(III) (2 µM), and Cr(VI) (0.09 µM)	Calvo-Pérez et al., 2014
Tyrosinase + Laccase	Carbaryl(20 nM), formetanate hydrochloride (0.2 µM), propoxur (0.2 µM) and ziram (1.7 nM)	Oliveira et al., 2014
Urease	Hg^{++} (2.5 ppb)	Kuralay et al., 2007; Yang et al., 2006
Xanthine oxidase	Allopurinol (20 µM)	Shan et al., 2009

Table 2

Enzyme	***Substrate employed***	***Detection mode***	***References***
acetylcholinesterase	acetylcholine	✓ Conductimetric detection	Soldatkin et al., 2013
		✓ pH electrode	Ding et al., 2009
		✓ Visual colorimetric detection using pH indicator	Pohanka et al, 2010
		✓ Amperometric detection of H_2O_2 using choline oxidase	Del Carlo et al., 2010
	acetylthiocholine	✓ Oxidation of thiocholine at electrode modified with nanomaterials	Liu et al., 2006
		✓ Electrochemical oxidation of thiocholine using mediator	Arduini et al, 2012
		✓ Colorimetric or amperometric detection of thiocholine using Ellman's reagent	Dong et al, 2013
		✓ Photocurrent generated in presence of thiocholine	Chen et al., 2013

	indoxylacetate	✓ Optical detection (Vis) ✓ Electrochemical detection	Pohanka et al., 2013 Pohanka et al., 2012
	3-indolylacetate	✓ Electro-acoustic resonator	Chen et al, 2013
phosphatase	monofluoro phosphate	✓ Potentiometric detection of fluoride with ion selective electrode	Koncki et al., 2006
	p-nitrophenyl phosphate	✓ Chronoamperometric/Voltammetric detection of *p*-nitrophenol	Alvarado-Gámez et al., 2014; Akyilmaz et al., 2010
	catechol monophosphate	✓ Amperometric detection of catechol	Szydlowsk et al., 2006
	phenyl phosphate	✓ Amperometric oxidation of phenol using polyphenol oxidase ✓ Direct oxidation of phenol on carbon paste electrode	Cosnier et al., 2006 Samphao et al., 2013
	riboflavin-5-monophosphate	✓ Direct oxidation of riboflavin at high potential (1.45V)	Alvarado-Gámez et al., 2013
	2-phospho-L-ascorbic acid	✓ Amperometric detection of ascorbic acid	Sanllorente-Mendez et al., 2012
peroxidase	hydrogen peroxide	✓ Amperometric detection	Shan et al. 2010
	hydrogen peroxide + hydroquinone	✓ Amperometric detection	Savizi et al. 2012
polyphenoloxidase	catechol	✓ Amperometric detection of enzymatic product ✓ Clark type oxygen electrode	Kochana et al. 2012; Asav et a., 2009
	tyrosine	✓ Amperometric detection of enzymatic product	Sima et al. 2011,
	catechol, *p*-cresol, *m*-cresol, phenol, *p*-chlorophenol	✓ Amperometric detection of enzymatic product	Shan et al., 2008
	reduced form of 1,2 naphtoquinone-4-sulfonate	✓ Chronocoulometric detection	Vidal et al. 2006

Highlights

1. Novel theoretical approach of enzyme inhibition
2. Analytical performances of biosensors based on enzyme inhibition
3. The use of nanomaterials and microfluidic in inhibitive biosensors