New Trends in Biosensors for Organophosphorus Pesticides

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Abstract: Biosensors are sensitive and can be used as disposable sensors for environmental control. These biosensors are based either on inhibition of acyl cholinesterases acetylcholinesterase or butyrylcholinesterase) by organophosphorus compounds or on inhibition of enzymes phosphatases (acid or alkaline) or on direct detection of organophosphorus compounds by organophosphorus hydrolase. The state-of-the-art of the three types of biosensors will be presented in this paper and a particular development will be done on our work on ENFET (Enzymatic Field Effect Transistor) biosensors.

Keywords: Inhibition, Acetylcholinesterase, Butyrylcholinesterase, Phosphatases, Organophosphorus hydrolase, Multivariate correspondence analysis

Introduction

Organophophorus compounds are, among the known substances, the most toxic. They are used as pesticides, insecticides and chemical war agents. The high toxicity of organophosphorus neurotoxics and their large use in modern agriculture practices, has increased public concerns. They have incited the development of technologies to treat effluents generated at both the producer and consumer levels. Moreover, the recently ratified Chemical Weapons Treaty requires the participating nations to destroy all of their chemical weapons arsenal, including the organophosphorus-based nerve gases, within ten years. The use of any technology for detoxification of organophosphorus compounds, performed in laboratories, will need the development of analytical tools of high performance in order to control the concentration of neurotoxics.

The techniques of gas chromatography, liquid chromatography and thin film chromatography coupled with different detectors and the different types of spectroscopy are the most commonly used methods. However, these techniques are time consuming, expensive and demand a qualified and
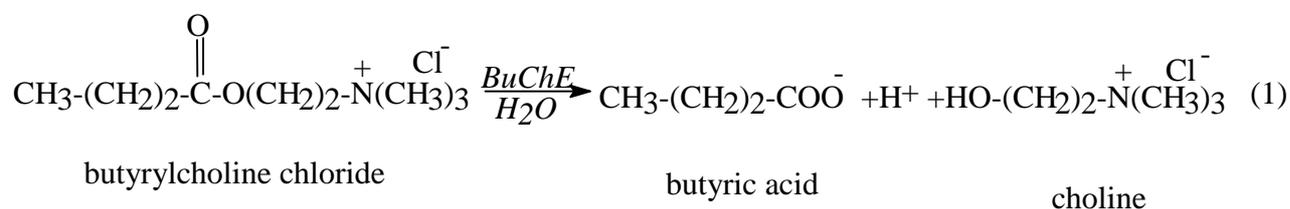
experienced staff and cannot be used for continuous monitoring. Biological techniques such as immunoassays and inhibition of cholinesterase activity by colorimetric techniques are also used for the determination of organophosphorus compounds. Immunoassays require long analysis time (one to two hours) and fastidious sample handlings (numerous washing steps), a considerable quantity of plastic well kits which are enough expensive and these techniques are not suitable for continuous monitoring.

Biosensors are sensitive and can be used as disposable sensors for environmental control. These biosensors are based either on inhibition of acyl cholinesterases acetylcholinesterase or butyrylcholinesterase) by organophosphorus compounds or on inhibition of enzymes phosphatases (acid or alkaline) or on direct detection of organophosphorus compounds by organophosphorus hydrolase. The state-of-the-art of the three types of biosensors will be presented in this paper and a particular development will be done on our work on ENFET (Enzymatic Field Effect Transistor) biosensors.

Biosensors based on the inhibition of acylcholinesterases

1. Principle

The enzyme, acetylcholinesterase or butyrylcholinesterase, is fixed on the surface of the transducer through different procedures (co-reticulation, entrapment, Langmuir-Blodgett films). The products of the following enzymatic reaction (1) are detected by the transducer:

$$CH_3\text{-}(CH_2)_2\text{-}\overset{\overset{\displaystyle O}{\|}}{C}\text{-}O(CH_2)_2\text{-}\overset{+}{N}(CH_3)_3 \;\; Cl^- \;\; \xrightarrow[H_2O]{BuChE} \;\; CH_3\text{-}(CH_2)_2\text{-}COO^- \; +H^+ \; +HO\text{-}(CH_2)_2\text{-}\overset{+}{N}(CH_3)_3 \;\; Cl^- \quad (1)$$

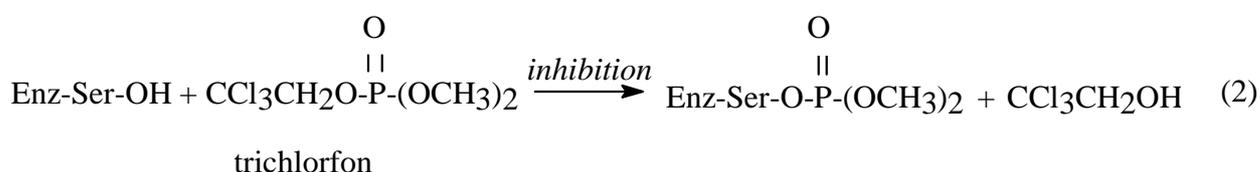   butyrylcholine chloride   butyric acid   choline

With an amperometric transducer, the substrate used is butyrylthiocholine, the current generated by oxidation of thiocholine is measured. If a bienzymatic system is used (acetylcholine esterase + choline oxidase), the hydrogen peroxide generated by the oxidation reaction of choline is detected with an amperometric transducer.

With potentiometric transducers (Ion Selective Electrode -ISE, Ion Sensitive Field Effect Transistor -ISFET), the variation of the local pH value is detected as well as with optical transducers.

A conductimetric detection is also possible.

The organophosphorus compounds partly inhibit the biological activity of acylcholinesterase through phosphorylation of the serin group, according to the reaction (2) (exemple of the organophosphorus compound trichlorfon):

$$Enz\text{-}Ser\text{-}OH + CCl_3CH_2O\text{-}\overset{\overset{\displaystyle O}{\|}}{P}\text{-}(OCH_3)_2 \xrightarrow{inhibition} Enz\text{-}Ser\text{-}O\text{-}\overset{\overset{\displaystyle O}{\|}}{P}\text{-}(OCH_3)_2 + CCl_3CH_2OH \quad (2)$$

         trichlorfon

The reactivation of the acylcholinesterase can be performed through a treatment with pyridine-2-aldoxime (PAM-2), according to the reaction (3):

$$
\text{PAM-2} + \text{Enz-Ser-O-P-(OCH}_3)_2 \xrightarrow{\textit{reactivation}} \text{Enz-Ser-OH} + \text{PAM-P-(OCH}_3)_2 \tag{3}
$$
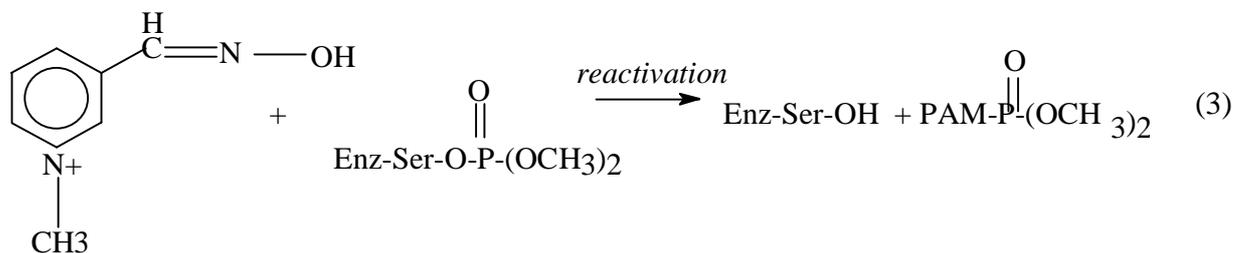
It is necessary to incubate the biosensor in medium containing the inhibitor in order to obtain a high sensitivity of detection and to treat the biosensor in a PAM-2 solution to obtain the reactivation of the enzyme. Inhibition of acylcholinesterase enzyme is obtained with organophosphorus pesticides but also with pesticides of carbamate types, thus this biosensor will not be specific. Numerous recent research works concerns this type of biosensor. They are based on different types of transducers and different procedures for immobilization of the enzyme.

2. Compared features of the different transducing systems

Amperometric transducers

They are based on amperometric detection of thiocholine or of hydrogen peroxide formed by oxidation of choline (with a second enzyme, choline oxidase) [1-13].

A recent review paper was presented by Everett et al [14].

Potentiometric transducers

The transducer measures the pH variation due to the acid formed during the hydrolysis reaction. The sensors are either ion selective electrodes (ISE) [1,15-19] or transistor based sensors, pH-ISFET [20-25]. A particular potentiometric transduction is LAPS (Light Adressable Potentiometric Sensor), it is presented in paper from Rogers et al. [26]. This technique is based on modulation of the silicon space charge through pH variation inducing a variation of the insulator surface charge, as for pH-ISFET.

A comparison of features of amperometric and potentiometric sensors for insecticides detection is presented by Marty et al. [27], as shown in Table 1.

Table 1. Detection limits for insecticides (carbamates and organophosphorus compounds)

obtained with potentiometric and amperometric transducers.

Insecticides	Potentiometric (pH effect)	Amperometricn (thiocholine oxidation)
Aldicarb	1140 ppb	1.9 ppb
Carbaryl	1000 ppb	19 ppb
Carbofuran	6 ppb	0.02 ppb
Dichlorvos	300 ppb	22 ppb

In general, amperometric detection gives lower detection limits than that of potentiometric detection. Concerning potentiometric detection, paper from Campanella et al. [5], presents compared features of enzymatic pH-ISEs and pH-ISFETs for detection of simili paraoxon. These features are presented in Tables 2 (a, b, c).

Table 2 (a). Analytical features obtained for paraoxon with an enzymatic pH-ISE.

(concentration of butyrylcholine: 6.2×10^{-4}M)

Response time	30 min
Time of analysis	60 min
Lifetime	2 days
Linear regression	y = 1.295x + 2.777 (x=µg/l)
Correlation coefficient	0.9876
Linear range	2.5 - 40µg/l
Detection limit	1.0µg/l (ppb)
Accuracy	<7.1%

Table 2 (b). Analytical features obtained for paraoxon with an enzymatic pH-ISFET.

(concentration of butyrylcholine: 2.5×10^{-4}M)

Response time	<10 min
Time of analysis	<12 min
Lifetime	4 days
Linear regression	y = 5.011x – 2.875 (x=µg/l)
Correlation coefficient	0.9959
Linear range	3.3 - 12µg/l
Detection limit	3.3µg/l (ppb)
Accuracy	<8.1%

Table 2 (c). Analytical features obtained for paraoxon with an enzymatic pH-ISFET.
(concentration of butyrylcholine: 5.6×10^{-4}M)

Response time	<5 min
Time of analysis	<7 min
Lifetime	4 days
Linear regression	y = 1.023x – 3.684 (x=μg/l)
Correlation coefficient	0.9927
Linear range	16.6 - 82μg/l
Detection limit	10μg/l (ppb)
Accuracy	<13.2%

Conclusions of authors are that, taking into account response time and lifetime, ISFET is the most promising transducer.

Effect of the immobilisation technique on operational features of ENFETs

BSA enzymatic membrane: a mixture of 5mg BSA, 5mg BuChE, 10μl of glycerol in 90μl of phosphate buffer (1mM, pH 8.0) was prepared. Then glycerol was used as a plasticiser to avoid the formation of cracks in the enzymatic membrane during storage and also to result in a better homogeneity of the membrane and better adhesion to the surface of the transducer. 0.5μl of this mixture was deposited on the sensitive area of a FET. Then the sensor chips were placed in a saturated glutaraldehyde vapor for 30 min.

Enzymatic membrane prepared with PVA/SbQ (polyvinyl alcohol functionalized with methyl pyridinium methyl sulfate (cf. Fig.1)): a mixture of 45 mg PVA/SbQ, 5 mg BuChE in 50mg phosphate buffer (1mM, pH 8.0) was prepared. 0.5 μl of this mixture was deposited on the sensitive area of a FET. Then, the sensor chips were exposed under UV light for 25min.
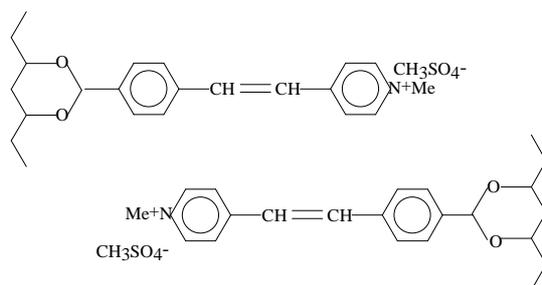

Figure 1. PVA-SbQ photocrosslinkable polymer.

Figure 2 shows that the degree of enzyme inhibition for BSA membrane depends on the trichlorfon concentration and incubation time (only results obtained with BSA membrane are presented because those obtained with PVA membrane are quite similar). Figures 3 shows a range of detected concentration comprises between 10^{-3}M (0.26 g.l^{-1}) and 10^{-6}M (0.26 mg.l^{-1}) which corresponds to the values found in the literature [23, 27]. To our knowledge, no exposure limits have been established for trichlorfon in water.
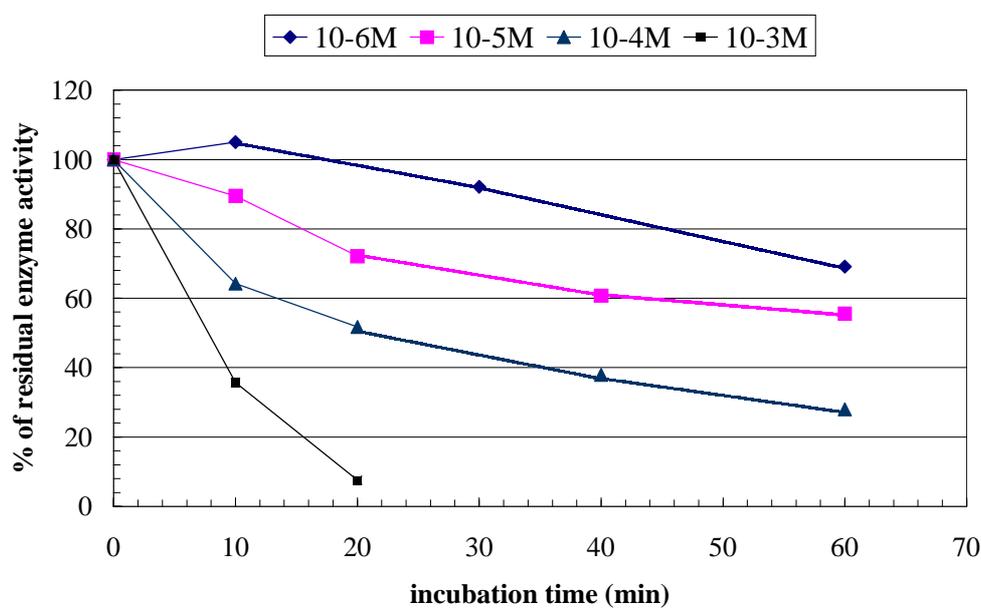

Figure 2. Inhibition effect on the BuChE-BSA FET sensor by trichlorfon. Percent of residual enzyme activity as a function of incubation time.
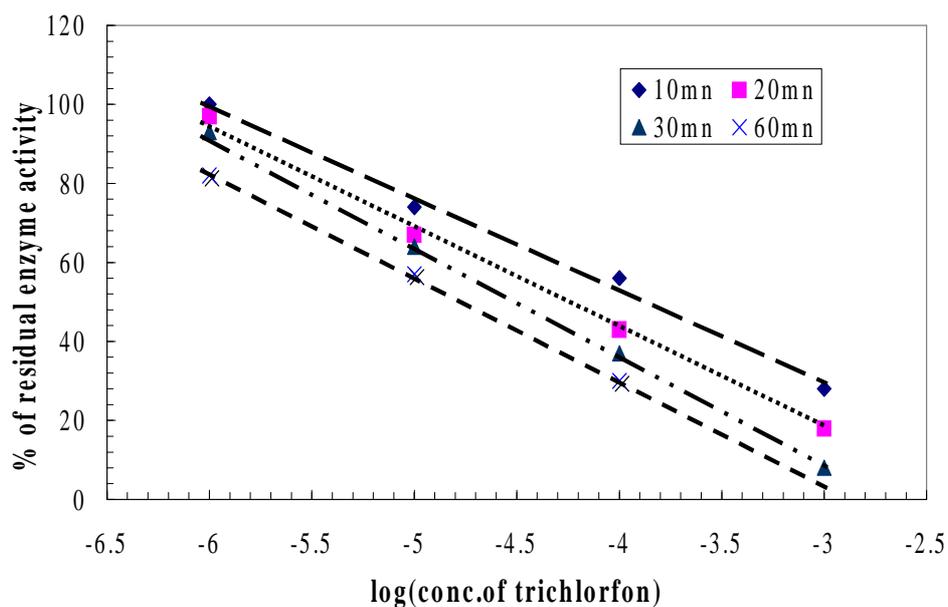

Figure 3. Inhibition effect on the BuChE-BSA FET sensor by trichlorfon. Percent of residual enzyme activity as fuction of trichlorfon concentration.

As one can see from Fig. 4, the sensor response was almost reversible for a 10 minutes incubation if the concentration of the pesticide is close to 10^{-5} M, whereas for higher concentration (10^{-3} M) only a partial recovery of the sensor occurs.
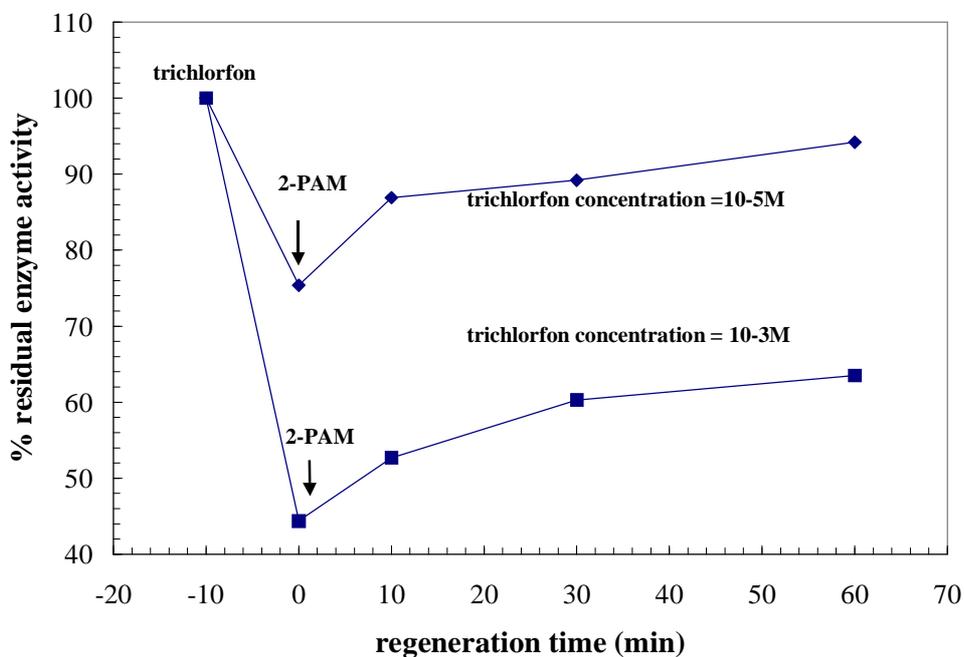

Figure 4. Recovery of the BSA-BuChE biosensor response after incubation by 0.1M of PAM-2b in a phosphate buffer after incubation for 10min at different concentration of trichlorfon.

Table 3. Compared analytical features of BSA and PVA/SbQ ENFETs.

Membrane	BSA	PVA/SbQ
Linear dynamic range Stationary mode calibration	0 – 10 mM 0 – 1 mM	0 – 10 mM 0 – 1 mM
Dynamic range Kinetic mode calibration	0.2 - 1 mM	0.2 - 5.8 mM
Apparent Km (Michaelis-Menten constant)	2 mM	3.8 mM
Life-time (dry storage)	35 days	> 9 months
Life-time (storage in buffer)	< 62 days	~4 months
Trichlorfon detection Dynamic range	10^{-3}M - 10^{-6}M (0.26g.l^{-1} - 0.26mg.l^{-1})	10^{-3}M - 10^{-6}M (0.26g.l^{-1} - 0.26mg.l^{-1})
Trichlorfon detection Regeneration	PAM-2 Inc. 10^{-5}M, 10 min Reg. 0.1M, 60 min	PAM-2 Inc. 10^{-5}M, 10 min Reg. 0.1M, 60 min

Immobilisation of the LB films containing enzyme onto ISFET to obtain enzymatic sensor: 22 μl stearylamine in chloroform of 1g.l^{-1} concentration was spread on an enzyme solution (4.0 mg.l^{-1}) subphase (22°C, pH=5.5). When the adsorption of enzyme molecules towards the air/water interface reached an adsorption-desorption equilibrium state, it took about 3 hours to obtain a surface pressure of 26 mN/m [28]. The stearylamine (ODA) monolayer containing enzyme was compressed with double movable barriers at 10 cm²/min until a certain surface pressure (40mN/m, for example). Under this fixed dipping surface pressure, the compressed enzyme containing films were transferred onto the hydrophobized ISFET surface as Y-type LB multilayers (30 layers in this work) by the vertical lifting method . The dipping arm rate was fixed at 4 mm/min.

After each upstroke, the substrate was maintained in air for 5 min to dry the films. The transfer ratio (TR) expressed as the ratio of the area of film transferred to the monolayer-coated area on the substrate was used as a parameter to characterize LB deposition.

Finally, BuChE/ODA FET sensors were placed in a saturated glutaraldehyde vapor for 30 minutes, and stocked in dry at 4°C.
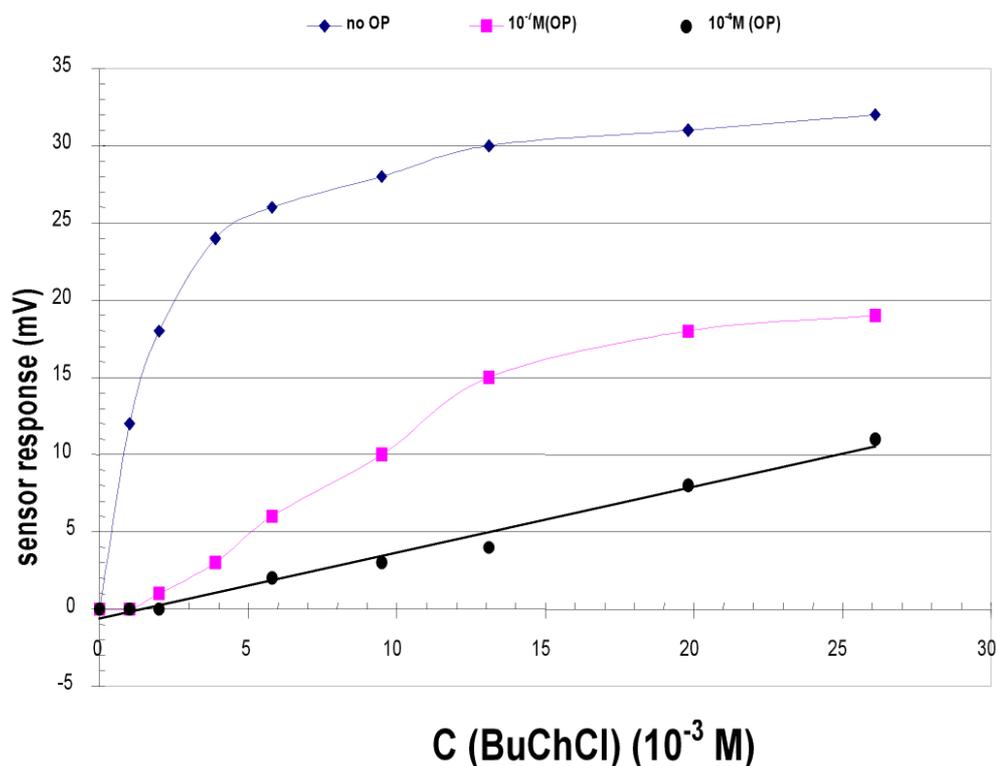

Figure 5. Inhibition effect on the response of the ENFET
with a mixed BuChE/ODA LB film by trichlorfon.

Figure 5 shows that after an incubation of 30 min in 10^{-7}M (26 ppb) trichlorfon solution, BuChE-FET lost about 44% of response (at 20mM BuChCl solution). A same measurement was done after the BuChE-FET was incubated in a 10^{-4}M trichlorfon solution for 30min, the result shows that enzyme lost most of its activity and the response in this case came from the "blank "signal output of the ISFET in individual mode.

Conductimetric transduction

This work was done by our laboratory [22, 29]; the second paper compares conductimetric transducers with ISFETs and show that their characteristics are comparable.

Optical fibre transduction

Local pH variation is followed by using a pH dye [30-35].

3. Conclusion

Biosensors based on inhibition of acylcholinesterases are sensitive and very suitable for use as disposable sensors. Inhibition of enzyme is made directly in effluent itself (water, mud) and detection is performed in a buffer solution where activity of enzyme is optimum.

New ways of detection of organophosphorus compounds appeared recently: reversible inhibition of acid and alkaline phosphatases by organophosphorus compounds and carbamates, which avoid reactivation treatment selective detection of organophosphorus compounds by organophosphorus hydrolase.

Biosensors based on inhibition of phosphatases

Inhibition of acidic phosphatase (AP) is used in bienzymatic sensor presented in paper from Mazzei et al. [36]. This biosensor is sensitive to glucose-6-phosphate (G6P), it is based on both following enzymatic reactions (4) and (5):

$$G6P + H_2O \quad \xrightarrow{\text{AP}} \quad glucose + inorganic\ phosphate \qquad (4)$$

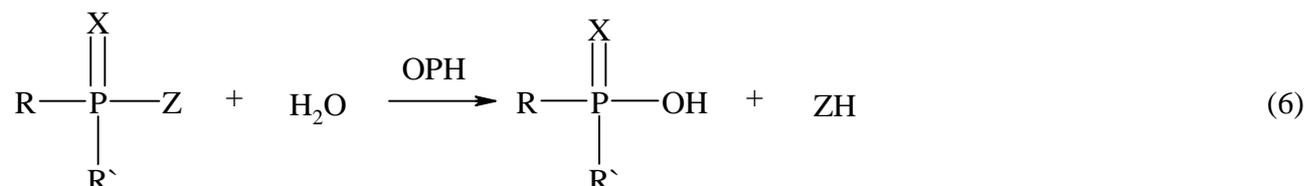$$Glucose + O_2 \quad \xrightarrow{\text{GOD}} \quad gluconolactone + H_2O_2 \qquad (5)$$

Both enzymes AP and GOD (Glucose Oxidase) are coupled on an electrode for amperometric detection of hydrogen peroxide. Detection limit of paraoxon is 5 ppb, detection limits of carbamate are higher. One sensor used for 4 or 6 determination per day keeps 80% of its initial activity after two weeks.

Paper from Danzer et al. [16], uses acid and alkaline phosphatase and also acetylcholinesterase immobilised on potentiometric pH sensors in order to realise a sensor network allowing , when using statistical data processing, to evaluate toxicity of solutions containing mixture of pesticides and heavy metal ions.

Biosensors based on organophosphorus hydrolase

Organophosphorus hydrolase is an enzyme which catalyses hydrolysis of organophosphate esters, of pesticides such as parathion, coumaphos, acephate and chemical war agents such as soman, sarin and tabun. Catalytic hydrolysis of each molecule leads to the production of two protons according to the following reaction (6):

$$
\begin{array}{ccccccc}
\underset{\underset{R`}{\overset{\overset{X}{\parallel}}{\underset{|}{R-P-Z}}}}{} & + & H_2O & \xrightarrow{\text{OPH}} & \underset{\underset{R`}{\overset{\overset{X}{\parallel}}{\underset{|}{R-P-OH}}}}{} & + & ZH & \qquad (6)
\end{array}
$$

where X is an oxygen or a sulphur atom, R is an alkoxy group (methoxy to butoxy), R' is an alkoxy or a phenyl group and Z is a phenoxy group, a thiol specie, a cyanide or a fluoride group.

Principle of detection is based on monitoring of products of hydrolysis of organophosphorus compounds, catalysed by organophosphorus hydrolase. It offers advantages of measurements simpler, more direct and faster of neurotoxics of the single family of organophosphorus, when compared to method of inhibition of acylcholinesterases which detect all the neurotoxics.

This enzyme is immobilised on transducers either by cryoimmobilisation of *Escherichia coli* cells entrapped in poly(vinyl)alcohol gels [37] or by co-reticulation of isolated enzyme with bovine serum albumin [38-41]. The different transducers used are:

– potentiometric pH sensors, detection limits being around 1μM (0.3ppm) for paraoxon

– microelectrodes (detection of p-nitrophenol), detection limit being around 10^{-7}M (27ppb)

– transducers based on optical fibres including pH sensitive dye, detection limit being around 1μM (0.3ppm) [41].

This approach presents a lot of advantages concerning selectivity of detection and response time. Nevertheless, detection is done directly in the real sample which could no be convenient for enzymatic reaction and detection limit is much higher than that of inhibition methods.

Multisensor system based on enzyme inhibition analysis for determination of different toxic substances

This multisensor system is constituted of three ENFETs with three different enzymes (urease, BuChE and AcCHE) differently inhibited by different types of toxics (organophosphorus pesticides, carbamates and heavy metal ions). The multisensor is first calibrated in an equimolar mixture of substrates of the different enzymes, then inhibited and then again calibrated in the mixture of substrates [42]. The data on the enzyme inhibition by definite toxins and their mixtures are presented in Table 4.

Table 4. Level of inhibition of enzymes by different toxic substances (%).

	Urease	BuChE	AcChE
10 µM trichlorfon	0	50	5
50 µM trichlorfon	0	70	25
1 mM trichlorfon	0	100	85
100 µM carbofuran	0	100	50
10 µM Ag^+	0	3	25
50 µM Ag^+	10	7	70
10 µM Hg^{2+}	15	3	10
50 µM Hg^{2+}	40	7	70
Mixture No 1	20	100	30
Mixture No 3	95	100	90
Mixture No 4	100	100	100
Mixture No 2	30	100	35

Annotation: containing of toxins in mixtures:

Mixture No 1 - 10 µM Ag^+ + 10 µM Hg^{2+} + 10 µM trichlorfon + 10 µM carbofuran

Mixture No 3 - 50 µM Ag^+ + 20 µM Hg^{2+} + 50 µM trichlorfon + 20 µM carbofuran

Mixture No 4 - 50 µM Ag^+ + 50 µM Hg^{2+} + 50 µM trichlorfon + 50 µM carbofuran

Mixture No 2 - unknown sample

For semi-quantitative determination of mixed toxic components in a sample, the multivariate correspondence analysis [43, 44] is used to extract the important characteristics of these data. The important feature of this method is the simultaneous representation of objects (concentrations of different toxics presented in rows in Table 4) and variables (nature of enzyme presented in columns in Table 4). Correspondence analysis defines the axis, which provides the best fit to both the row points and the column points. A second axis is determined, which best fits the data subject to being orthogonal to the first. Best fit is in the least square sense, relative to the χ-squared distance. This can be viewed as a weighted Euclidean distance between profiles. Multivariate correspondence analysis is well tailored for inhibition data processing because they are coded data, between 0 and 100. Principal component analysis which is a correlation analysis would be less adapted to this type of coded data. The free statistical software R from cran.r-project (equivalent to S-Plus) was used. Two factors were extracted and represented along orthogonal axis in Figure 6 and data from Table 4 were represented in the plane defined by these two axes. As can be seen from this figure, the composition of the unknown sample is close to that of the mixture 1.

It proves that despite a rather small amount of experimental points for each toxic substance and their mixtures, the method described allows to perform half-quantitative analysis of the sample composition quite reliably. More accurate and reliable results could be obtained using a more different sensors (enzymes) and more experimental data for each of them.

The advantages of such a system is the simplicity of the procedures for inhibition measurement and data processing and its applicability for monitoring toxicity of natural waters.
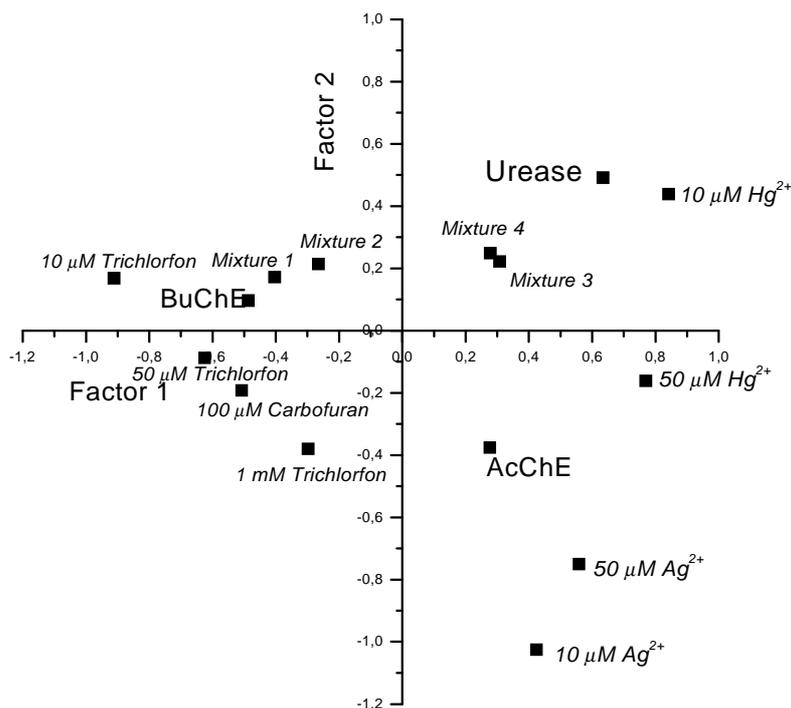
Figure 6. Representation of data from Table 4 by using multivariate correspondence analysis.

Conclusion

Biosensors based on inhibition of acylcholinesterases are very sensitive and very suitable for use as disposable sensors. Inhibition of enzyme is made directly in effluent itself (water, mud) and detection is performed in a buffer solution where activity of enzyme is optimum. Their limitation is the necessity to reactivate enzyme by a PAM-2 treatment.

New ways of detection of organophosphorus compounds appeared recently:

– reversible inhibition of acid and alkaline phosphatases by organophosphorus compounds and carbamates, which avoid reactivation treatment

– selective detection of organophosphorus compounds by organophosphorus hydrolase. This approach presents a lot of advantages concerning selectivity of detection and response time. Nevertheless, detection is done directly in the real sample which could not be convenient for enzymatic reaction and detection limit is much higher than that of inhibition methods.

An example is given of a multisensor system constituted of three ENFETs with three different enzymes (urease, BuChE and AcCHE) differently inhibited by different types of toxics (organophosphorus pesticides, carbamates and heavy metal ions). Experimental data are treated by multivariate correspondence analysis. The advantages of such a system is the simplicity of the procedures for inhibition measurement and data processing and its applicability for monitoring toxicity of natural waters.

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