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Toxicity testing in environmental monitoring: the role of enzymatic biosensors

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Summary. - Biological toxicity testing is a rapidly expanding field involving numerous bioanalytical techniques. The enzymatic biosensors are valuable screening tools to identify pollutants and/or toxic agents in the environment and/or in food matrices, thus representing a valid alternative to animal testing in analytical toxicology. Inhibition based biosensors here presented have been proved to represent alternative assays for the toxicity evaluation of warfare agents and endocrine disrupting chemicals as well as algal toxins (phycotoxins) in the contamined sea foods (mainly clams and other mollusks). Results obtained by inhibition studies performed by means of several enzymatic biosensors indicate the reliability of the proposed method and the possibility to extend such an experimental approach to other toxicants as a simple, rapid and cheap biotest, to be used easily also "on the spot".

Key words: biological toxicity assays, animal testing, food matrices, environmental pollution, biosensors.

Riassunto *(Il ruolo dei biosensori nel monitoraggio ambientale e nel rilevamento di sostanze tossiche).* - Lo studio e lo sviluppo dei test biologici di tossicità sono in rapida espansione, comprese numerose tecniche bioanalitiche. I biosensori enzimatici sono validi strumenti di screening per identificare agenti tossici e/o inquinanti nell'ambiente e/o in matrici alimentari, rappresentando così una valida alternativa ai test di tossicità animale nel campo della tossicologia. I biosensori elettrochimici enzimatici ad inibizione, oggetto della presente nota, rappresentano saggi alternativi per la valutazione della tossicità, in campo ambientale, dovuta a sostanze chimiche impiegate per scopi bellici, così come di tossine algali (ficotossine) in molluschi contaminati ed altri campioni ittici, ma anche di una nuova classe di composti chimici di sintesi (EDC ossia *endocrine disrupting chemicals*) che hanno dimostrato di avere la capacità di interagire con sistemi endocrini e di alterarli o addirittura distruggerli. I risultati sperimentali ottenuti da studi di inibizione enzimatica condotti con diversi biosensori elettrochimici indicano la realizzabilità del metodo proposto e la possibilità di estendere tale approccio sperimentale ad altri agenti tossici, come biotest semplice, rapido, economico e facilmente utilizzabile anche *in situ.*

Parole chiave: saggi biologici di tossicità, test di tossicità animale, matrici alimentari, inquinamento ambientale, biosensori.

Introduction

The deliberate discharge and the accidental release of harmful chemicals into the environment have the potential to disrupt the structure and functioning of living organisms of the biosphere and to represent a real risk for human health.

The ability to detect adverse chemical activity is, therefore, a pre-requisite for an effective environmental management.

The conventional approach to the control of ecosystems is to use a set of monitoring procedures, to provide sufficient information on the potential harmful effects of different classes toxicants and/or contaminants. Since living organisms respond to the interactions with different physical, chemical and biological agents that lead to actual or potential damages, biosensor based assays have become important tools to assess activities of many toxic agents.

The alternative toxicity tests, which are now becoming an essential part of eco-toxicological assessments, have interesting features in comparison to standard toxicity tests: rapid response times, possibility to be used directly "on the spot" and to test many samples in very short times [1-10].

According to this, during last years biosensors have been applied as useful devices in monitoring and to improve the monitoring in the environmental programmes in a faster and cheaper way [11-16].

Our research programme is to employ specifically designed enzymatic biosensors as an alternative to the traditional toxicity tests for the screening of algal toxins, warfare agents and endocrine disrupting chemicals (e.g. atrazine, and other pesticides) in the environment.

Toxicity tests

Toxicity tests include standard aquatic tests, e.g. fish and *Daphnia* lethality tests [17] and microbiotests, e.g. a protozoan oxygen test [18, 19], algal and bacterial growth inhibition tests [19-21], urease and acetylcholinesterase enzyme tests [22, 23], Ames Salmonella mutagenicity test [24], toad embryo teratogenicity test [25] beside all the well known usual animal tests.

Biosensors

Biosensors are analytical devices incorporating biologically active materials, either intimately connected to, or integrated within, appropriate physico-chemical transducers for the purpose of detecting (reversibly and selectively) concentrations or activities of chemical species in any type of sample [26-30].

Electrochemical biosensors here considered represent an alternative to traditional tests. They show selectivity towards classes of contaminants and/or toxicants, reduce procedures of sample pre-treatments, reduce response times, increase the number of detectable analytes and the number of sampling points, are suitable for miniaturization and for "on line" measurements and automation, are easy to use also for unexperienced personnel and, finally, their costs, as well as the costs of required instrumentation, are very modest.

In the analysis of toxicants, the signal production sequence is activated by an interaction between the toxicant and the biological component, immobilized on the tip of the biosensor (Fig. 1).

In a recent work Botrè and Mazzei [31] stressed how a worthwhile feature is that biosensors operate in an energy range of the same order of magnitude in which most biological processes and related interferences responsible for the toxic effects take place. In fact, the biological agent immobilized on the sensor represents, or at least it is strictly correlated, to the biological target of the toxicant *in vivo*, so that biosensors represent an adequate alternative to animal testing, since they lie in between the scale from physico-chemical tests (LC-MS, GC-MS), which are extremely specific and widely used as confirmation methods, and biological effect-based assays (primarily cellular toxicity tests or mammalian biotests), which are more universal screening tests.

Electrochemical enzymatic biosensors

In the case of electrochemical enzymatic biosensors, the effect of the interaction, Θ, between the toxicant and the enzymatic system is correlated to a variation in the enzymatic reaction rate and to a generation of a detectable electrical response, for instance, as a variation of the current intensity, ΔI, or as a variation of the potential difference, $\Delta\psi$, with respect to the recorded pre-existing electrical signals [31-34].

The enzymatic reaction responsible for the transformation of substrate in products is represented by the solid curve of Fig. 2, while the interaction toxicant-enzyme, that affects the trend of the enzymatic reaction, is represented by the dotted line. The arrows "S" and "In" indicate respectively the additions of the substrate and of the toxicant (dotted line), that acts as an inhibitor of the enzymatic activity.

The inhibition of the enzymatic system occurs shortly after the addition of the inhibitor.

Electrical variations are then correlated to the activities of all the chemical species involved in the interaction with the biochemical mediators and exactly measured after suitable calibration curves.

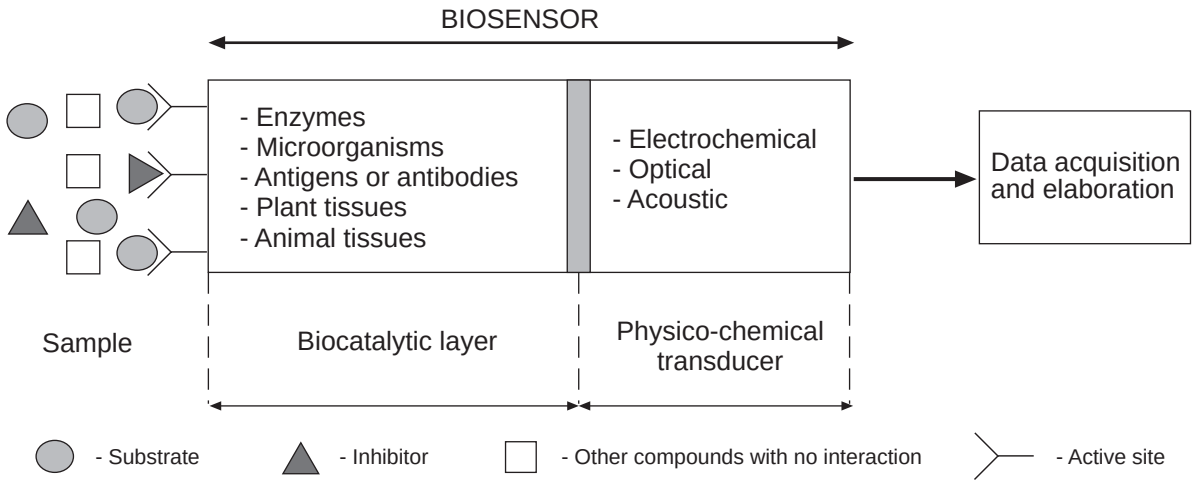

Fig. 1. - Scheme of a coupling of a biological agent with a physico-chemical signal transducer in a biosensor.

Due to the compact size and ruggedness of modern electrochemical detectors, most of the analytical methods based on the use of electrochemical biosensors are also suitable for the use "on the spot".

Among the different biosensors employed in environmental analysis, a leading role is played by these inhibition biosensors. The effect of interaction that occurs between chemical species (inhibitors) and the biocatalyst (enzyme, or poly-enzymatic system, or even a whole tissue) immobilized on the sensor surface is schematized in Fig. 2.

Data here presented have been obtained by monitoring, with this kind of enzymatic electrochemical biosensors, environmental toxicant classes, such as algal toxins, estrogen disrupting chemicals, pesticides and herbicides and specifically: cholinesterase - choline oxidase biosensor for organophosphate and carbamate pesticides [35] and poly-phenol-oxidase biosensor for herbicides [36] (Table 1); acid phosphatase - glucose oxidase biosensor for organophosphate pesticides [37] and for algal toxins [38] and alkaline phosphatase - glucose oxidase biosensor for herbicides [31] (Table 2).

Furthermore the data obtained by means of the AP/GOD-based inhibition biosensor for the detection and determination of several algal toxins e.g. okadaic acid were compared with the ones obtained by means of usual physical-chemical determination (reference HPLC technique) (Table 3).

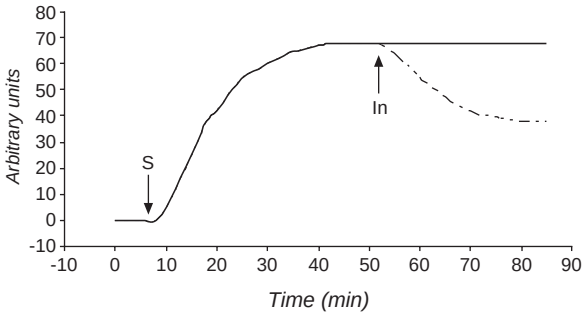

Fig. 2. -Typical behavior of inhibition based biosensor experiment. The first arrow, (S), refers to the addition of substrate; the second one, (In), refers to the addition of toxic agent. Solid line refers to the continuous monitoring of enzyme activity in the absence of inhibitor. Dotted line refers to the continuous monitoring of the same enzyme but in the presence of an inhibitor. Arbitrary units are respective to different interaction effects: (Θ/Θ_{max}) or (v/v_{max}) or (dc_p/dt) or ΔI (current intensity) or $\Delta\psi$ (electrical potential difference).

Discussion

The traditional analytical procedure is constituted by two stages: a preliminary testing (screening) of a representative population of samples to be assayed and the subsequent confirmation analysis of all samples which gave positive or even doubtful results after the first screening.

It follows that the "ideal" screening method should ensure the detection of all different toxicants of the same class (*i.e.* endowed with the same biological effect) without the risk of false negatives.

The solid curve of Fig. 2 is respective to a trend of different saturation phenomena of active sites responsible for several biochemical processes: e.g. transport phenomena across membranes by means of carriers (saturation operated by permeants), or enzymatic activity (saturation of active enzymatic sites operated by substrates), or receptor activity (saturations of receptor sites operated by agonists) [30]. The shape of such a curve is quite similar to the trend obtained in the case of potentiometric or amperometric measurements in model systems here represented by biosensors. Moreover in all such instances the role of interfering chemical species (inhibitors, antagonists, toxicants) is represented by the dotted line of the same Fig. 2 and is respective either of biological processes as well as to the behavior of the here considered electrochemical biosensors.

The extent of toxicity is proportional, in general and with good approximation, to the percentage of inhibition of the biocatalytic material immobilized on the biosensor, making an inhibition biosensor extremely useful not only from a merely analytical point of view, but also for toxicological evaluation.

To define the behavior of the biosensors, it seems useful to discuss several equations that have been proposed to clarify the signal production sequence of a biocatalytic electrochemical biosensor.

An equation, proposed by Tran-Minh and Beaux [39], "correlates the percentage of inhibition to the ratio of the product concentration in the active layer close to the electrode surface with and without inhibitor for the same concentration" of the substrate. The degree of inhibition, *I%*, is calculated as

$$I\% = \left[1 - \frac{[P]_e^I}{[P]_e^0}\right] \times 100 \qquad (1)$$

where $[P]_e^I$ and $[P]_e^0$ indicate, respectively, the concentrations of a product of the considered reaction in the presence and in the absence of an inhibitor. Apart from the formalism chosen, that causes slight deviation in the estimation of the actual percentage value of enzymatic inhibition (the background concentration of the considered reaction product is neglected), the equation reported above appears useful only in a very narrow range of experimental conditions, since (a) time is not explicitly considered, assuming that values of $[P]_e^I$ and $[P]_e^0$ correspond to a "stable" biosensor signal, and (b) the value of the ratio $[P]_e^I / [P]_e^0$ is constant for any value of and $[P]_e^0$, and it is widely recognized that this is not the case of most enzyme-catalyzed reactions.

Table 1. - Cholinesterase (ChE)/choline oxidase (ChO) biosensor for organo-phosphate and carbamate pesticides: determination of malathion, paraoxon and aldicarb (inhibition of ChE). Poly-phenol-oxidase (PPO) biosensor for herbicides: determination of atrazine

	ChE/ChO biosensor			**PPO biosensor**
	Malathion	**Paraoxon**	**Aldicarb**	**Atrazine**
Temperature of analysis	30 °C	30 °C	30 °C	25 °C
pH	7.5	7.5	7.5	6.6
Buffer	Phosphate 0.1 M	Phosphate 0.1 M	Phosphate 0.1 M	Phosphate 0.1 M
Incubation time	40 min	40 min	40 min	20 min
Equation of the calibration graph X = [Pesticide] in ppb	Y = 12.45 + 0.50 X	Y = 10.70 + 0.29 X	Y = -0.46 + 48.7 X	Y = 7.4 + 0.5 X
Linearity range	10 - 100 ppb	32 - 240 ppb	0.4 - 1.3 ppm	20 - 130 ppb
Correlation coefficient	0.9967	0.9951	0.9964	0.9987
Lower detection limit	1 ppb	10 ppb	100 ppb	10 ppb
Pooled standard deviation (in the linearity range)	2.8%	3.0%	3.2%	0.95%

Table 2. - Acid phosphatase (AP)/glucose oxidase (GOD) biosensor for organophosphate pesticides and algal toxins: determination of malathion, methyl parathion and paraoxon (inhibition of AP) and okadaic acid (OA) (inhibition of AP). Alkaline phosphatase (AlP)/glucose oxidase (GOD) biosensor for herbicides: determination of 2,4 dichloro-phenoxy-acetic acid (2,4 D) (inhibition of AlP)

	AP/GOD biosensor				**AlP/GOD biosensor**
	Malathion	**Methyl parathion**	**Paraoxon**	**Okadaic acid**	**2,4 D**
Temperature of analysis	30 °C	30 °C	30 °C	37 °C	37 °C
pH	6.0	6.0	6.0	6.0	8.0
Buffer	Citrate 0.1 M	Citrate 0.1 M	Citrate 0.1 M	Citrate 0.1 M	Glycine 0.1 M
Incubation time	20 min	20 min	20 min	20 min	30 min
Equation of the calibration graph X = [Pesticide] in ppb	Y = -0.4 + 2.3 X	Y = 4.0 + 5.2 X	Y = 1.8 + 1.6 X	Y = 6.8 + 1.8 X	Y = 0.09 + 0.51 X
Linearity range	3.0 - 20 ppb	0.7 - 8 ppb	3.1 - 20 ppb	2 - 20 ppb	0.035-4.2 ppm
Correlation coefficient	0.9940	0.9977	0.9966	0.9948	0.9984
Lower detection limit	1.5 ppb	0.5 ppb	1.5 ppb	1 ppb	0.05 ppm
Pooled standard deviation (in the linearity range)	3.2 %	3.4 %	3.0 %	2.5 %	2.0 %

Mascini *et al*. [40] calculate the percentage of inhibition of acetylcholinesterase, measured by an amperometric enzymatic biosensor, by means of the following equation:

$$I\% = \frac{I_1 - I_2}{I_1} \times 100 \qquad (2)$$

where it is stated "I_1 is the initial current and I_2 is the current after 20 minutes".

A first drawback of eq. (2) is that the rate of uncatalyzed reaction is neglected (and hydrolysis reactions, including the hydrolysis of esters, also occur in the absence of the relevant enzymes). Furthermore, the current intensity values, I_1 and I_2, should more appropriately be indicated as "variations of current intensity values" (between t = 0 and t = 20 min): according to eq. (2) the complete (100%) inhibition of the enzyme would indeed be reached only when $I_2=0$ and such an event, even if actually recorded, would be a sign of an unreliable measurement, since it would correspond to an open electrical circuit (with no current flow).

Another equation proposed for the "determination of inhibitors" by Tran-Minh [41] by means of potentiometric measurements

$$I\% = \frac{E_0 - E_1}{E_0} \times 100 \qquad (3)$$

and formally quite similar to the one (2) proposed by Mascini, appears hard to be accepted because the Nernstian relationship which links the "variation in potential" (or, more precisely, in the electromotive force) to the concentration of a chemical species is not linear but logarithmic!

Although the definition of a universal model capable of describing the kinetic behavior of an enzymatic inhibition biosensor is an extremely complex task, our opinion is that when it must be defined a decrease of an enzymatic activity, the percentage of such a decrease should be expressed in terms of enzymatic units (or enzymatic activity) and that any measurement should be traced, by means of suitable calibrations or correlation coefficients, to enzymatic units or enzymatic activity values.

As far as the enzyme inhibition is concerned one must take into account that being the enzymes biological catalysts, this affects the rate of both forward and backward reactions, so that the equilibrium constant must be unaffected by their influence for a reaction that, in principle and from a thermodynamic point of view, can take place also in their absence.

Carbonic anhydrases, catalase and esterases are some examples of enzymes that sharply affect reaction rates that can occur also in their absence.

Therefore, in the most general experimental conditions, like the ones mentioned in the work of Mascini *et al.* [40], more reactions can coexist:

$$[InE''] + [E'] \leftarrow [In] + [E] + [S] \rightarrow [ES] \rightarrow [Products]$$
$$[S] \rightarrow [Products]$$

where: [InE''], [E'], [E] and [ES] indicate respectively the concentrations of enzyme-inhibitor complex, free enzyme, total enzyme and enzyme-substrate complex, while [S] and [In] indicate the concentration of free substrate and of free inhibitor respectively.

Owing the relatively high concentration of the enzyme immobilized on the "membrane layer" of the biosensor with respect to the relatively low amount of both substrate and inhibitor capable of reaching such a layer, ([E] >> [In]), [E'] is an entity that could be not negligible.

Also the contribution of the uncatalyzed reaction, like for instance the uncatalyzed hydrolysis of an ester in an alkaline medium, cannot be neglected.

The situation, as far as the theoretical treatment and the relative equations to be applied, becomes even more complicated in the case of bienzymatic biosensors. In these cases, like it is for the bienzymatic cholinesterase/choline oxidase biosensor, as well as for the acid phosphatase/glucose oxidase biosensor, consecutive reactions, schematized as follows, take place:

	Biochemical mediators		**Sensor**
Sample solution containing [S]	Enzyme 1 [E1] $S \rightarrow P_1$	Enzyme 2 [E2] $P_1 \rightarrow P_2 + P_3$	[P3] is a detectable species (e.g. H_2O_2)

On the evidence of such an open and complex thermodynamic system, we are doubtful that simple equations that correlate an electrical signal to a percentage of inhibition can be correctly applied.

The same approach described in the present work is being followed also for the determination of other classes of toxicants different environmental matrices.

Table 3. - Comparison between the results obtained by the proposed AP/GOD-based inhibition biosensor and by a reference HPLC technique, on samples of the acetonic extract of the hepatopancreas of artificially contaminated mussels. Values of OA concentration are given in ppb

no. sample	Inhibition biosensor (a)	HPLC (b)	(a-b)/b (%)
1	2.2	2	+ 10
2	2.8	2.5	+ 12
3	1.6	1.5	+ 0.7

AP/GOD: acid phosphatase/glucose oxidase. OA: okadaic acid. HPLC: high performance liquid cromatography.

On the basis of this discussion, it seems important to conclude that biosensors represent a compromise between toxicity tests and physico-chemical determinations. Furthermore, the experimental data, referring to the kinetics of enzymatic biosensors, always require preliminary accurate calibrations and careful interpretations, in order to avoid several inconveniences mainly connected with the activities of both mono- or poly-enzymatic systems used [42].

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