Chapter 8

Electrochemical Biosensor Technology: Application to Pesticide Detection

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Summary

In recent years, electrochemical sensors and biosensors are becoming an accepted part of analytical chemistry since they satisfy the expanding need for rapid and reliable measurements.

An area in which electrochemical biosensors perhaps show the greatest diversity and potential for development involves the measurement of environmentally significant parameters. The increasing number of pollutants in the environment calls for fast and cost-effective analytical requirements. In this context, biosensors appear as suitable alternative or complementary analytical tools.

The aim of this chapter is to review some basic concept concerning the electrochemical biosensors and to illustrate a protocol for the detection of environmental organic pollutants on the basis of electrochemical biosensors. In particular, a method based on the inhibition of the enzyme acetylcholinesterase (AChE) for the detection of organophosphorus and carbamate pesticides will be described in detail.

Key words: Electrochemical biosensor, Inhibition, AChE, Organophosphorus, Carbamate, Pesticide.

1. Introduction

1.1. General Overview

Despite the wide variation in biosensors and biosensor-related techniques that have been introduced, the consensus definition for these devices has remained fairly constant – an analytical device composed of a biological recognition element directly interfaced to a signal transducer, which together relate the concentration of an analyte (or group of related analytes) to a measurable response. Depending on the method of signal transduction, biosensors can be classified into five basic groups: optical, mass, electrochemical, thermal, and magnetic *(1)*.

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Electrochemical biosensors have some advantages over other analytical transducing systems, such as the possibility to operate in turbid media, comparable instrumental sensitivity, and possibility of miniaturization. As a consequence of miniaturization, small sample volume can be required. Modern electroanalytical techniques (i.e., square wave voltammetry, chronopotentiometry, chronoamperometry, differential pulse voltammetry) have very low detection limit (10^{-7}–10^{-9} M). In-situ or on-line measurements are both allowed. Furthermore, the equipments required for electrochemical analysis are simple and cheap when compared with most other analytical techniques *(2)*. Basically electrochemical biosensor can be based on amperometric and potentiometric transducers, even if some examples of conductimetric as well as impedimetric biosensor are reported in literature *(3–5)*.

1.1.1. Amperometric Transducers

L. C. Clark, Jr., is generally considered to be the pioneer of amperometric biosensors in 1962 *(6)*. Clark trapped an enzyme that reacted with oxygen against the surface of a platinum electrode, using a piece of dialysis membrane. He then followed activity of the enzyme, glucose oxidase, by changes in oxygen concentration. Because glucose oxidase is highly specific, it reacts only with glucose, producing hydrogen peroxide and gluconic acid. This analytical scheme became the concept for the basis of the commercial glucose analyzer.

Basically amperometric biosensors rely on an electrochemically active analyte that can be oxidized or reduced at a working electrode. This electrode is poised at a specific potential with respect to a reference electrode. The current produced is linearly proportional to the concentration of the electroactive product. A typical set of equipment for amperometric analyses consists of a three electrode cell, based on a working electrode, a reference electrode and an auxiliary electrode as well as the voltage source and devices for measuring current and voltage. If measurements of the current are carried out by scanning the potential, then we obtain a voltammetric system. In this case, measurements of changes in time (τ) in the current (I) flowing through the system of electrodes in relation to the potential (E) applied to the working electrode are performed. The registered changes in the current allow drawing the $I(\tau) = f[E(\tau)]$ relationship, which is called the voltammogram. In both amperometric and voltammetric biosensors the transducer is an electrode. Typical electrode material are platinum (Pt), gold (Au), carbon, and for some applications, also mercury. Like many other technologies, electrochemical sensors and biosensors have benefited from the growing power of new materials, design, and processing tools; thus many technologies are available to fabricate miniaturized, simple to operate and low cost devices. Among these, thick-film technology is one of the most used as the equipment needed is

less complex and costly than other; moreover, thick-film electrochemical transducers can be easily mass produced at low cost and thus used as disposable; in electrochemistry, a disposable sensor offers the advantage of not suffering from the electrode fouling that can results in loss of sensitivity and reproducibility *(7)*. Nowadays disposable thick-film electrochemical transducers were produced mainly by the screen-printing technique. Screen-printed electrodes are planar devices, based on different layers of inks printed on a plastic or ceramic substrate (**Fig. 1**). Many papers *(8–12)* in the recent years report the use of these devices for environmental as well as for clinical or food analysis, and many of these papers are related to the use of these electrodes in the field of electrochemical sensors and biosensors. One of the most used strategies in screen-printed electrode production is the use of carbon inks for the fabrication of the working electrode surface, since this material is relatively inexpensive, shows a wide working potential range, is inert, has good electrical conductivity, and has relatively high hydrogen overpotential. Moreover carbon screen-printed electrode surface can be easily modified using biomolecules, redox compounds, or catalytic particles, thus increasing the range of compounds detectable.

Limitations for amperometric and voltammetric transducer include potential interferences to the response if several electroactive compounds can generate false current values. These effects have been eliminated through the use of selective membranes, which carefully control the molecular weight or the charge of compounds that have access to the electrode.

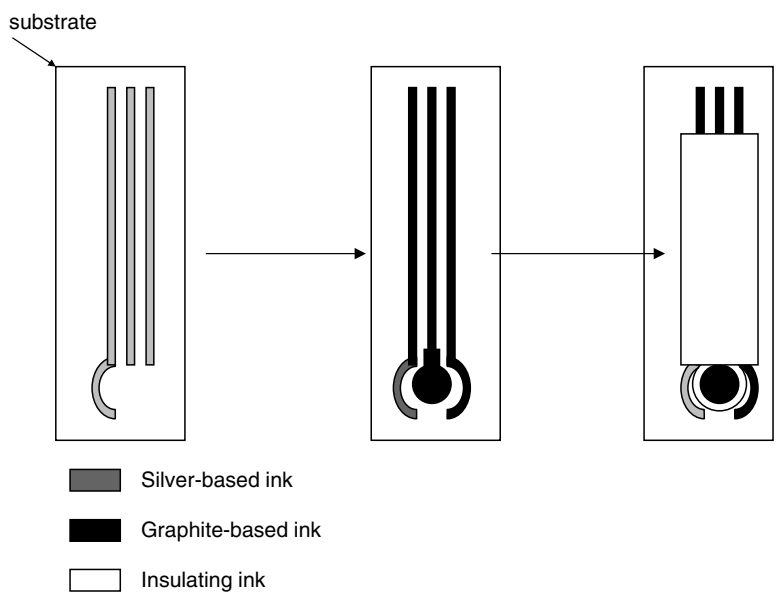

Fig. 1. Screen-printed sensor fabrication.

1.1.2. Potentiometric Transducers

The basic principle behind potentiometric measurements is the development of charge related to the analyte activity a_1 in the sample through the Nernst relation:

$$E = E_0 \pm (RT / nF) \ln a_i,$$

where E_0 is the standard potential for $a_i = 1$ mol/L, R is the gas constant, F is the Faraday constant, T is the temperature in K, n is the total number of charges on ion i, and the signal + and – are for cations and anions, respectively. Typically, a reference electrode and one working electrode both in contact with the sample are required.

The use of ion-selective membranes can make these transducers sensitive to various ions (e.g., H^+, F^-, I^-, Cl^-) in addition to gases such as CO_2 and NH_3, including enzyme systems that change the concentration of any of these ions or gases can result in biosensors that can measure substrates, inhibitors, or modulators of the enzyme *(13)*.

The main advantage of such devices is the wide concentration range for which ions can be detected, generally 10^{-6}–10^{-1} mol/L. Their continuous measurement capability is also an interesting possibility for environmental applications. The apparatus is inexpensive, portable, and is well suited for in situ measurements. The main disadvantage is that the limit of detection in some kinds of environmental samples can be rather high (10^{-5} mol/L) and the selectivity can be rather poor.

1.2. Environmental Application of Electrochemical Biosensors

An area in which electrochemical biosensors perhaps show the greatest diversity and potential for development involves the measurement of environmentally significant parameters *(14, 15)*. These biosensors are composed of biological assays that have been interfaced with various signal transducers and measure the different parameters. In a number of cases, the interface of a biological assay to a signal transducer has been shown to reduce the time and complexity involved with these assays.

In this chapter, we limited the description of a procedure for the detection of environmental organic pollutants, and namely organophosphorus and carbamic pesticides, on the basis of inhibition of the enzyme activity. This inhibition is evaluated by means of an electrochemical biosensor.

1.2.1. Disposable Electrochemical Biosensors for Organophosphorus and Carbamate Pesticide

Organophosphorus and carbamate compounds have come into widespread use in agriculture, since they show low environmental persistence; nevertheless, they exert a high acute toxicity. The principal effect of these compounds is the inhibition of the enzyme acetylcholinesterase (AChE), which is essential for terminating the action of the neurotransmitter acetylcholine (ACh). Actually, the intoxication by these compounds results in

an accumulation of endogenous ACh and continual stimulation of the nervous system. Because of their toxicological activity, some of these compounds have been used also as chemical warfare agents (CWAs) *(16, 17)*.

The most frequently used methods for the unambiguous identification of organophosphorus and carbamate compounds are based on gas chromatography (GC) in combination with mass spectrometry (GC-MS) and/or tandem mass spectrometry (GC-MS/MS), liquid chromatography (LC) coupled with MS, and nuclear magnetic resonance (NMR) spectrometry *(18)*.

An alternative to the chromatographic determination of these compounds is the use of biosensor-based techniques. The bioanalytical detection of organophosphate and carbamate pesticides using cholinesterases (ChEs), either free in solution or immobilized as a biorecognition element in biosensors, has a long tradition. The promising results obtained in this research field have allowed the use of ChE in combination with a variety of transducers, such as potentiometric *(19, 20)*, amperometric *(21–23)*, or optical transducers *(24)*. If a salt of acetyl or butyrylthiocholine (ATCh and BTCh, respectively) is used as substrate for the ChE enzymes, thiocholine (TCh) is produced during the enzymatic reaction. Thiol-containing compounds are known as oxidable at the surface of solid electrodes, but the oxidation generally requires high potential values on a suitable electrode *(19, 25)*. This can be overcome using chemically-modified carbon electrodes *(26–29)*.

In this chapter, screen-printed carbon electrodes (SPCEs) were modified by incorporating in the ink an optimised percentage of cobalt(II) phthalocyanine (CoPC). As reported in the literature, among the electrochemical mediators, CoPC was indicated as one of the most suitable for the detection of thiol-containing molecules *(26)*, and the resulting oxidation signals occur at lower voltages, thus limiting the electrochemical interference of other oxidable compounds. Using these modified SPCEs, under optimized chronoamperometric conditions, it is possible to detect pesticides, such as Carbofuran, through the study of the AChE activity as reported in *(30)*.

In the optimized conditions, the dynamic range for Carbofuran detection was 10^{-10}–10^{-7} M with a detection limit of 4.9×10^{-10} M, for an analysis time of 15 min. This is an important feature, considering that the immobilization can determine a loss of the activity of the enzyme that influence the sensitivity as well as dynamic range of the pesticide detection *(31)*. Moreover, the proposed method was less prone to electrochemical interferences since the incubation and measurement were performed in two separate steps.

In the following sections is reported the methodology:

(a) To detect organophosphorus and carbamate pesticides using an (AChE)-based electrochemical biosensor. The inhibitory effect of the pesticide determines a decrease of the catalytic activity of AChE; as a consequence, less thiocholine (TCh) was produced from acetylthiocholine (ATCh), the enzymatic substrate. Therefore, the current value, due to the oxidation of TCh at the modified carbon screen-printed electrodes (SPCEs), is lower than that recorded in a blank solution. This current decrease is correlated with the pesticide concentration.

(b) To test a Cobalt(II)-phthalocyanine (CoPC) modified screen-printed carbon electrodes (SPCEs) as transducer of an (AChE)-based biosensor.

(c) To test biosensor in standard pesticide solutions.

2. Materials

2.1. Chemical and Biochemical Reagents

1. AChE from Electric Eel (EC 3.1.1.7); catalogue number C3389 Sigma (Milan, Italy).
2. ATCh chloride; reagent grade 99%; catalogue number A6625 Sigma (Milan, Italy).
3. Bovin Serum Albumin (BSA); catalogue number A2153 Sigma (Milan, Italy).
4. Nafion® (perfluorinated ion-exchange resin) 5WT % in mixture of lower aliphatic alcohols and water; catalogue number 527084 Aldrich (Milan, Italy).
5. Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzo-furanol N-methylcarbamate): Aldrich (Milan, Italy) catalogue number 426008.
6. Glycine (aminoacetic acid); catalogue number G7126 Sigma (Milan, Italy).
7. Glutaraldehyde 25% v/v in water; catalogue number G5882 Sigma (Milan, Italy).
8. Acetonitrile HPLC grade: Merck (Milan, Italy). Catalogue number 1,00030
9. Sodium dihydrogenophosphate, disodium hydrogenophosphate, KCl: Merck (Milan, Italy). Catalogue number 1.03345, 1.6580, 73911
10. NaClO 14%: Merck (Milan, Italy). Catalogue number 1.05614
11. All the electrochemical measurements are performed in phosphate buffer 0.05 M pH 8.0 with 0.1 M KCl (measuring buffer). ATCh, AChE, Carbofuran, and other dilutions are prepared in the measuring buffer.

12. The silver-based ink (Electrodag PF 410) and the graphite-based ink (Electrodag 423 SS): Acheson (Milan, Italy). The insulating mono-component ink (Vinylfast 36–100): Argon Italiana (Milan, Italy).
13. CoPC: Sigma-Aldrich Italiana (Milan, Italy).

2.2. Electrodes

1. CoPC modified screen-printed electrodes (Ecobioservices and Researchers S.r.l, http://www.ebsr.it).
2. A scheme of the CoPC modified screen-printed electrodes is shown in **Fig. 1**: the inks were deposited onto the polyester substrate (thick 350μm) in a film of controlled pattern and thickness to obtain overlapping layers. At first the silver tracks were printed, then the CoPC-graphite pad was positioned over part of the silver track, to obtain the working electrode; then the counter electrode is printed using a graphite ink. Finally, the insulating layer with openings that allow the electrical contact with the circuit at one end, and the analyte solution at the other end was deposited. Thus, the screen-printed electrochemical cell consists of graphite working electrode, graphite counter electrode, and silver pseudo-reference electrode. In addition, the silver electrical contacts were covered by a graphite layer to prevent oxidation phenomena, during storage.

2.3. Electroanalytical Instrumentation

Chronoamperometric measurements are carried out using a Palmsens, portable electrochemical sensor interface, obtained from Palmsens (Houten, BV, The Netherlands). The complete measuring system is illustrated in **Fig. 2**.

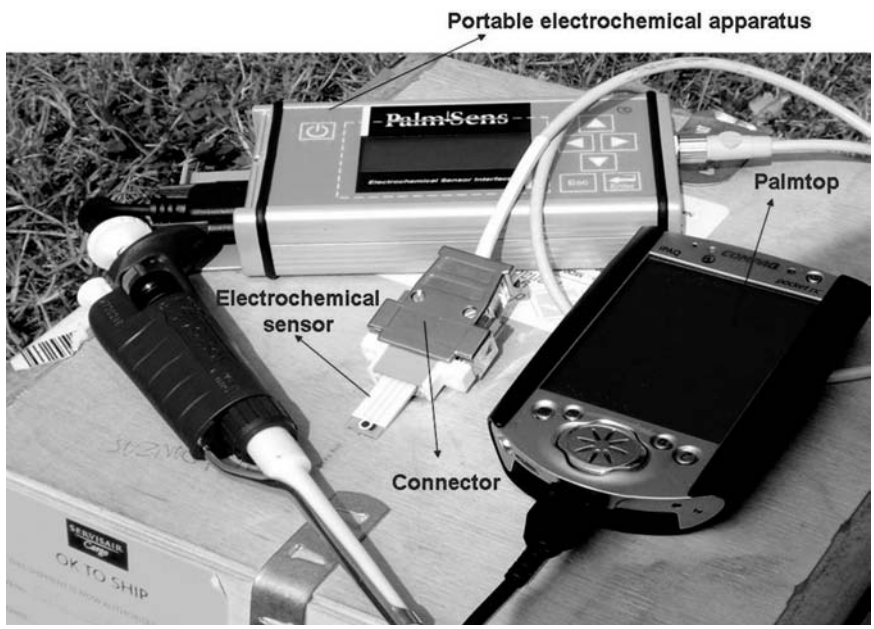

Fig. 2. The measuring system. The CoPC sensor was housed inside the connector.

The analytical data are the current value recorder at 30, after the potential application (0.1 V vs. Ag/AgCl).

3. Methods

3.1. Modified SPCEs Preparation

A scheme of electrochemical cell and electrode design is reported in (**Fig. 1**). The sensors can be obtained from Ecobioservices and Researches S.r.l. (Florence, Italy). A typical electrode modification formulation is reported in **Note 1**. Further explanations regarding electrode composition are reported in **Note 2**. Before use, the pseudo Ag reference electrode is oxidized using NaClO 14% solution, in order to avoid the oxidation of the Ag pseudo-reference by thiols during measurements. For storage conditions *see* **Note 3**.

3.2. Immobilization of AChE onto CoPC-Modified SPCEs

AChE is immobilized onto the CoPC-modified working electrode surface by cross-linking with glutaraldehyde, BSA, and Nafion®. A first enzyme solution was prepared by mixing 3.3 mL of the measuring buffer with 50 µL of AChE solution and 132 mg of BSA. Then, to 300 µL of this mixture are added 6 µL of glutaraldehyde 25% v/v and 90 µL of Nafion® 5% w/w. The final reagent concentrations are AChE 7.5 U/mL, BSA 3% w/w, glutaraldehyde 0.25% v/v, and Nafion® 0.25% w/w, respectively.

Finally, 7 µL of this mixture are casted onto the working area of a CoPC-modified electrode. When the enzymatic layer is dried, electrodes are dipped in a glycine solution 0.1 M for 30 min. This is a blocking treatment, necessary to saturate the surface sites not involved in the enzymatic immobilisation. Biosensors were then stored at +4°C until use.

3.3. Blank Measurements

The enzyme-modified working electrode is covered with 10 µL of buffer; after 5 min, the solution is removed. Then, 200 µL of ATCh chloride (enzymatic substrate) solution (1 mM) are casted onto the cell; after 10 min, the potential was applied and the current response at 30 s is evaluated. Chronoamperometric measurements are performed at the applied potential of +0.1 V vs. pseudo Ag/AgCl reference electrode (*see* **Fig. 3**). All potentials are referred to the screen-printed Ag/AgCl pseudo-reference electrode; the experiments are carried out at room temperature (25°C).

3.4. Inhibition Measurements

The enzyme-modified working electrode is covered with 10 µL of buffer containing the inhibitor (sample), *see* **Note 4**; after 5 min, the solution is removed and the biosensor washed with the buffer (*see* **Note 5**). Then, 200 µL of enzymatic substrate

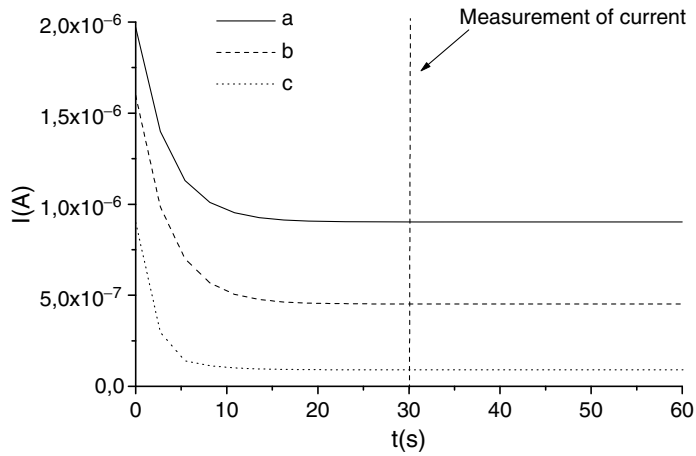

Fig. 3. Typical chronoamperograms obtained after incubation with different concentrations of Carbofuran: (a) 0 M [blank]; (b) 10^{-9} M; (c) 10^{-7} M.

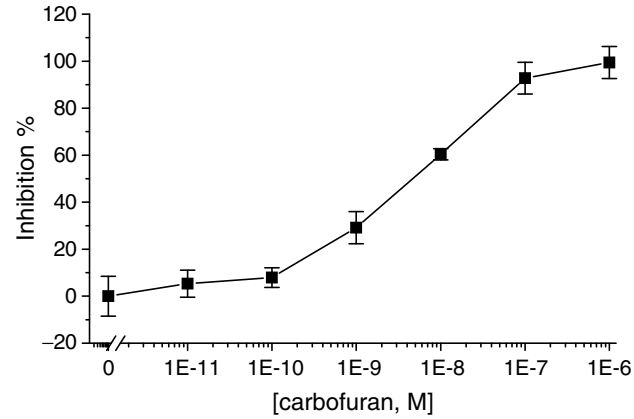

Fig. 4. Typical inhibition plot of carbofuran onto AChE-based biosensor.

solution (1 mM) are casted onto the cell; after 10 min, the potential was applied and the current response at 30 s is evaluated. Chronoamperometric measurements are performed at the applied potential of +0.1 V vs. pseudo Ag/AgCl reference electrode (*see* **Fig. 4**). All potentials are referred to the screen-printed Ag/AgCl pseudo-reference electrode; the experiments are carried out at room temperature (25°C).

Inhibition percentage ($I\%$) can be calculated as follows:

$$[I\% = 100[(I_1 \square I_2) / I_1],$$

where I_2 was the oxidation current obtained for the sample solution, and I_1 the oxidation current obtained for a blank measurement (incubation without pesticide).

By plotting the inhibition percentage (*I*%) vs. the pesticide concentration, a typical inhibition plot can be obtained (*see* **Fig. 4**). For concentration range see **Note 6**.

4. Notes

1. CoPC-modified carbon screen-printed electrodes are prepared by mixing the carbon ink with CoPC powder in an amount equivalent to 5% w/w of the total carbon in the printing ink. The mixture is then homogenized.
2. As reported in literature *(28)*, among the electrochemical mediators, CoPC was indicated as one of the most suitable for the detection of thiol-containing molecules and the resulting oxidation signals occur at lower voltages, thus limiting the electrochemical interference of other oxidable compounds. Using CoPC-modified SPCEs, under optimized chronoamperometric conditions, it is possible to detect pesticides, such as Carbofuran, through the study of the AChE activity.
3. The sensors were preferably stored in the dark at room temperature.
4. The term inhibitor used in this paragraph is referred to the carbamic pesticide Carbofuran. A stock solution of Carbofuran 10^{-3} M in acetonitrile was prepared; working solutions were prepared daily by diluting it in the measuring buffer. To evaluate the pesticide inhibition effect, 10 µL of carbofuran (diluted solution) were added to 500 µL of the buffer containing AChE to get the concentration range 10^{-11}–10^{-6} M.
5. An important feature of the proposed method is that the sample incubation and the electrochemical measurement were performed in two separate steps, and among them a washing procedure was also included. This guaranteed that the proposed method was less prone to electrochemical interferences, since oxidable substances, eventually present in the sample, were washed off before the electrochemical measurement. Washing step is carried out by depositing and removing 10 µL of the measuring buffer onto the working electrode, for three times.
6. The investigated pesticide concentration range is 10^{-11}–10^{-6} M. For concentrations higher than 10^{-6} M, an *I*% of 100 is generally obtained. The detection limit (dl) can be calculated by substituting the blank – 3× Standard Deviation in the equation of the linear portion of the inhibition curve.

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