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“No Calibration” Type Sensor in Routine Amperometric Bio-sensing - An Example of a Disposable Hydrogen Peroxide Biosensor

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1. Introduction

The predicted potential market size for the use of biosensors in the biomedical sector has correlated closely to the actual global market sales of biosensor devices. Unlike glucose measurement in blood, biosensors for environmental or food analysis are niche markets in which the demand varies and the sale volumes are low. Until now the use of biosensors in these markets has been limited. In contrast, spectrophotometric methods, and particularly enzyme-coupled assays, packaged in simple to use kit form, have proved successful in such fragmented markets and have become, in many cases, the recommended standard method for industrial analysis. The popularity of such kits is the result of disposable consumables, reliable detection methods and most importantly, no requirement for tedious calibration steps (if the Beer-Lambert law is applicable). Disadvantages include sample preparation, multiple and sequential addition of several reagents during the analysis process. We believe that within the environmental and agri-food industries, amperometric biosensor technology could be a valid competitor to the standard spectrophotometric methods; provided that cheap and easy to use methods are proposed. According to industrial users within these fragmented markets, the biggest obstacle for the take up of biosensor technology as a standard method is the requirement for complex pre-calibration. It seems that, to meet the demand of these markets, an essential improvement would be the removal of calibration steps before the use of the biosensor.

The electrochemical signal of a large number of amperometric biosensors is dependent upon enzymatic or chemical kinetics that are reliant on operating conditions (enzyme concentrations, temperature, pH) and so necessarily requires a calibration step prior to use. Despite their excellent analytical performances this makes them unattractive for industries that want to use these analytical tools in remote locations using unskilled operators. With spectroscopic methods these calibration steps are unnecessary because, for a given wavelength and a fixed light path, the measured absorbance is directly proportional to the concentration of sample material. This is true for a concentration range for which the Beer-

Lambert law is applicable. Theoretically, electrochemistry offers similar opportunities, providing certain parameters are set, where the amount of charge or the current passed through an electrochemical cell is proportional to the concentration of an electroactive product. This is particularly applicable in the case of amperometric biosensors using disposable screen printed electrodes as transducers: controlled-potential coulometry which obeys Faraday's law and chronoamperometry ruled by the Cottrell equation.

After evaluation of both electrochemical methods we have chosen chronoamperometry. This has allowed us to develop a new strategy to achieve the objective of preparing disposable biosensors that can be used without prior calibration. Our preference to validate this strategy was that of horseradish peroxidase (HRP) based biosensors for the analysis of hydrogen peroxide (H_2O_2).

This choice is motivated by the fact that hydrogen peroxide is widely used in many industrial applications and is also involved in very large number of analytical procedures. H_2O_2 is commonly used in fields such as sterilization, textile and paper bleaching, or food and pharmaceutical industries. In addition H_2O_2 is the product of many biological reactions involving oxidase enzymes. Therefore, it is the key product to the analysis of many oxidase substrates particularly when using electrochemical sensors and biosensors for health, environment and biosecurity.

2. Disposable amperometric biosensors

Typically, a biosensor may be defined as a measurement device that contains a biological element whose role is to recognize, often specifically, a product that is desired to detect or to quantify. This recognition is accompanied by changes which are converted by the transducer into a signal to be amplified, processed and displayed. The biosensor consists of all the components that make this chain, though very often the word is given to the single transducer associated with the biological element (as we will do in this article).

Various types of biosensors have been described and developed; they differ mainly according to their biological components, their transducers and to the physicochemical method that generates the signal. The most studied biosensors are undoubtedly those which associate enzymes with electrochemical transducers operating in amperometric mode. The main driving force for the development of this type of biosensors is certainly the great success of the very popular home-care device used by diabetics to measure glucose in blood. This successful development was due to the fact that the sensor met two keys needs: the disposable electrochemical transducers fully met the needs of the user and the mass production techniques met the low cost requirement for the producer. The way to associate the biological element to the transducer depends on the intended use of the biosensor. In many cases it is necessary to add other components to the active surface for the proper functioning of the biosensor (activator, buffer pH, redox mediator, membrane). The easiest way to accomplish this is via deposition onto the transducer surface, either in one shot or successively, via solutions of different components, followed by the evaporation of the liquid.

2.1 Screen printing transducers

2.1.1 Short history of screen printing

Several conflicting theories have been put forward relating to the screen printing process. One of them suggests that screen printing originated in China and was used for textile

printing. These prints, believed to have been produced using some form of stencil printing, are over 2000 years old; hence there is no corroborative evidence to prove how they were made.

It was in the textiles printing industry that the first modern form of the process originated, in both England and France, about 1850. The details of the method are now unclear but it seemed to incorporate the use of a stencil system for the production of continuous lengths of printed fabric.

By the beginning of the 20th century, this process has become somewhat more refined, with use of the silk stretched on frames to support hand-made stencils. Ink was applied through the silk by means of a brush. This system was developed further by 1920, with the introduction of a rubber-bladed squeegee to force ink through the screen.

A major breakthrough was achieved in 1940 when the first photographic stencil was developed. The screen printing method used today relies on the same principle as these early systems.

2.1.2 Screen printing process

The basic process involves forcing an ink or paste through a screen comprising a mesh stretched over a frame. The mesh is left open in areas to be printed but occluded in areas where no deposit is required (Figure 1).

The size of the holes in the screen mesh is determined by the thread diameter of the mesh material and the spacing of the threads in the weave. During the print cycle, the squeegee pushes the ink through the mesh. The ink must pass freely through these holes in order to give a repeatable pattern and avoid problems with blocking. After pushing the ink into the open area, the surplus is removed by the edge of the squeegee. The mesh should peel away from the surface immediately behind the squeegee, leaving all the ink that was in the mesh deposited on the printing surface (Website 1).

Screen-printed carbon electrodes can be mass produced at low cost and are readily adaptable as base transducers. Nowadays the use of automatic and semi-automatic machines for screen printing has made the entire process fast and clean, allowing the manufacture of billions of screen printed sensors, per year, worldwide. Nearly the entire production of glucose strips for glucose monitoring in blood is manufactured by means of screen printing. There are also many other types of sensors being manufactured using this technique.

There are several advantages attributed to the use of screen printing process for high volume sensor manufacture. Some of them are related to fact that the technique is versatile in its ability to print on almost all materials in a variety of shapes. Moreover it is a relatively fast technique and precise, giving highly reproducible devices. There are a large number of functional inks (pastes) available and they can be used for manufacturing electrochemical base transducers.

2.1.3 Screen printing inks (pastes)

Many terms are used to describe the printing medium, for example: ink, paste, dye, conductive epoxy, adhesive, etc. However, the general terms 'ink' and 'paste' cover most needs. Ink is generally low in viscosity, paste is high, but the words are used interchangeably.

Ink formulation is a specialised field, and most inks are proprietary compounds.

All inks or pastes consist of two main ingredients: the pigment or other active element and a vehicle to convey the pigment throughout the process to its position on the printed surface.

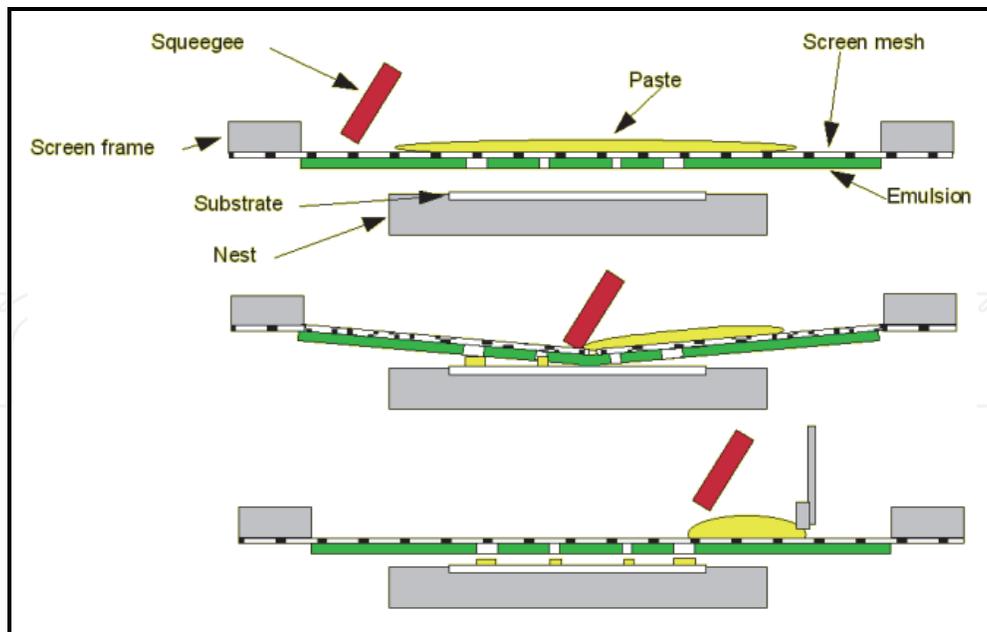


Fig. 1. Screen printing process – the basic.

The vehicle must be of a sticky, elastic nature so that the ink can be pushed about, change shape, yet hold together as a body until the required amount is separated from the remainder by the squeegee. The vehicle contains a combination of solvents, resins, humectants, antifoaming agents, preservatives, thickeners etc. One role of the vehicle is to allow the dispersion of the pigment or functional element into the ink. The functional element, whether carbon powder or metallic powders, has to be evenly distributed in the vehicle without any agglomerates and present final particle sizes that would allow the ink to go through the screen printing process without blocking the screen.

There is a minimum of two processes used to ensure the dispersion of components in the screen printing ink preparation. Mixing of the ingredients allows good dispersion of the active particles in the vehicle and triple-roll milling ensures a reduced particle size and no agglomerates in the ink (Website 2).

The repeatability of the final screen printed transducer depends not only on the quality of the screen printing process but also on a thorough dispersion of the active ingredient into the ink. At Gwent Group we ensure that both ink manufacturing and screen printing processes are performed following the highest levels of quality control to provide the most repeatable electrochemical sensors.

2.2 Screen printed electrochemical transducers

2.2.1 Design and production

One of the advantages of manufacturing transducers by the method of screen printing is the ability to produce them in large numbers at once. Industrially speaking, mass production techniques significantly lower the fabrication costs. But such production can only be successful if it results in reproducible transducers. This is a crucial condition because of the nature of the single use of the biosensor and the impossibility to carry out calibration before its use to make the measurement.

The electrochemical transducers we have generally used in our work are 2 electrode systems with a working electrode (WE) in carbon /graphite and a common reference and counter

electrode in Ag/AgCl. A typical screen printing production batch was made of 20 cards (Figure 2) each containing 220 transducers. The WE is circular with a diameter of 6 mm surrounded by an Ag/AgCl ring electrode. The two electrodes are extended by connectors and are isolated using an insulating ink which creates a flat circular electrochemical cell of 10 mm diameter.

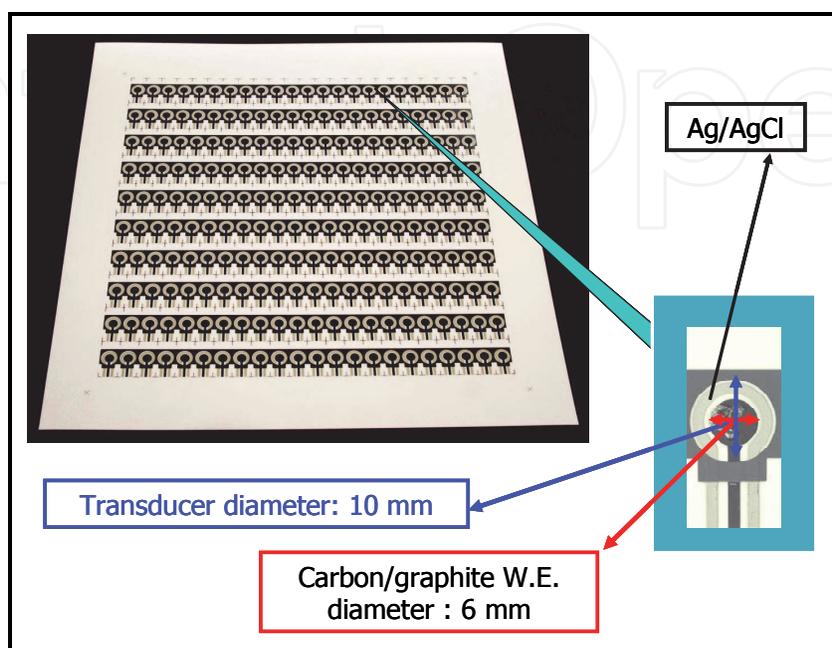


Fig. 2. Design of screen-printed electrochemical transducers

2.2.2 Reproducibility tests

One of the major challenges for the screen printing technique, in the mass production of transducers, is without doubt, that of reproducibility. This is even more crucial when producing single use transducers. Indeed, a good reproducibility offers the possibility to apply to the whole batch manufactured, with a minimum of error, the parameters of a calibration curve obtained either by measurements of standard solutions or imposed by a mathematical formula defined in advance.

To examine the reproducibility of the electrochemical transducers that we prepared, we have taken at random three of the twenty cards of the same batch on which we conducted comparative studies by chronoamperometry. Twenty transducers were taken from each of the three cards and every ten were used to analyze the electrochemical behaviour of ferrocene derivative (Fc-R) solution of definite concentration. Figure 3 illustrates the results of ten chronoamperometry experiments for each ferrocene solutions. In the insert is shown the expansion of the current area showing that each ten curves are superimposed indicating excellent reproducibility of the electrochemical transducers.

The overall results of the statistical study on the reproducibility of fabrication of the electrochemical transducers are recorded in Tables 1 and 2.

In this study, the electrochemical experiments were performed using the transducers in a two electrodes configuration. 40 μL of the test solution is deposited on the surface of the transducer in the area delimiting the planar electrochemical cell, a potential of +0.5 V is imposed, using a potentiostat, on the surface of the WE and the collected current (i) is

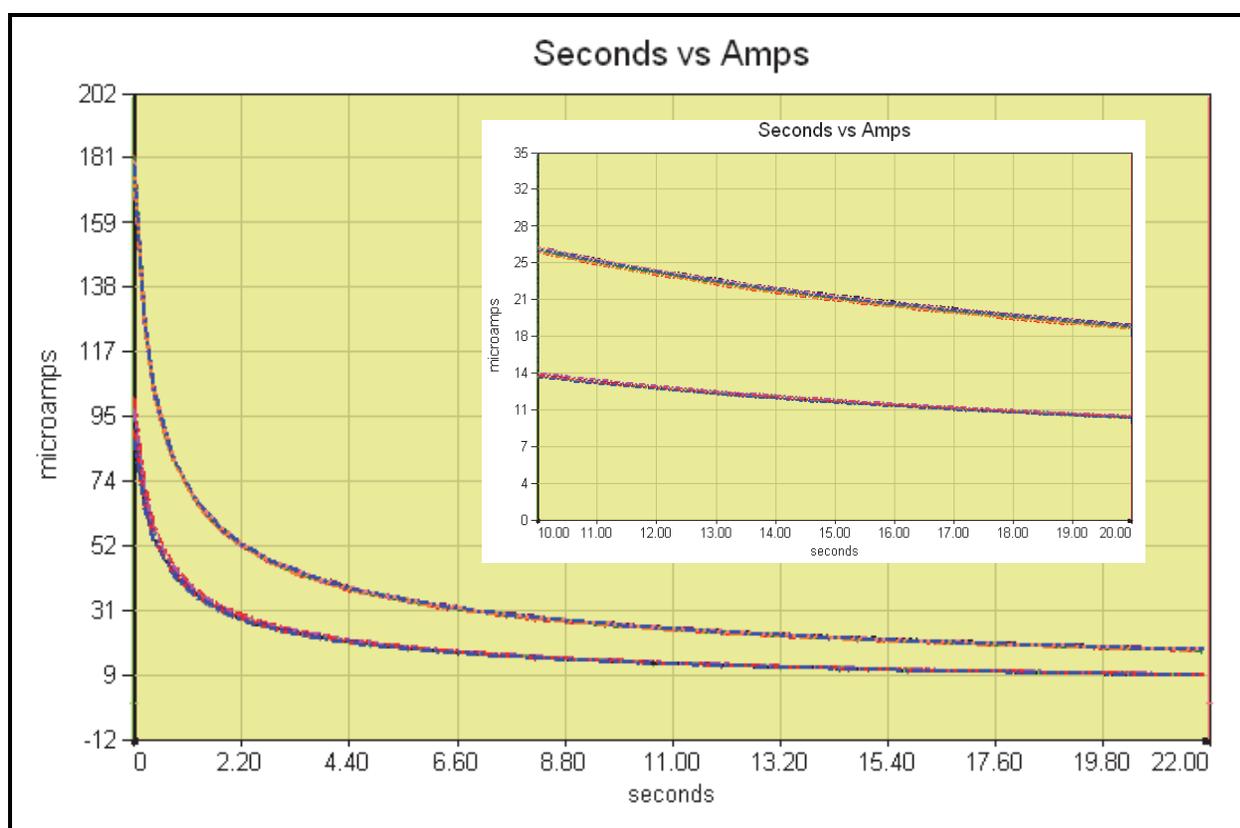


Fig. 3. Superposition of the signals obtained by chronoamperometry at +0.5 V *vs.* Ag / AgCl, with ten different transducers for two ferrocene derivative solutions.

	Card N° 1		Card N° 2		Card N° 3	
	Solution 1	Solution 2	Solution 1	Solution 2	Solution 1	Solution 2
Current (μA) (mean of 10 measures)	9.44	18.44	9.78	18.69	9.52	18.64
Standard deviation (μA)	0.14	0.15	0.07	0.25	0.11	0.19
Coefficient of variation (%)	1.53	0.82	0.71	1.32	1.12	1.03

Table 1. Reproducibility of transducers from each screen-printed card

	Solution 1			Solution 2		
	Card N° 1	Card N° 2	Card N° 3	Card N° 1	Card N° 2	Card N° 3
Current means (μA)	9.44	9.78	9.52	18.44	18.69	18.64
Mean (μA)	9.58			18.59		
Standard deviation (μA)	0.15			0.11		
Coefficient of variation (%)	1.55			0.59		

Table 2. Reproducibility of transducers between screen-printed cards

recorded versus time (t). In the case of ferrocenes solutions the curve obeys the Cottrell equation which states that the product ($it^{1/2}$) is constant for a diffusion-controlled reaction at a planar electrode.

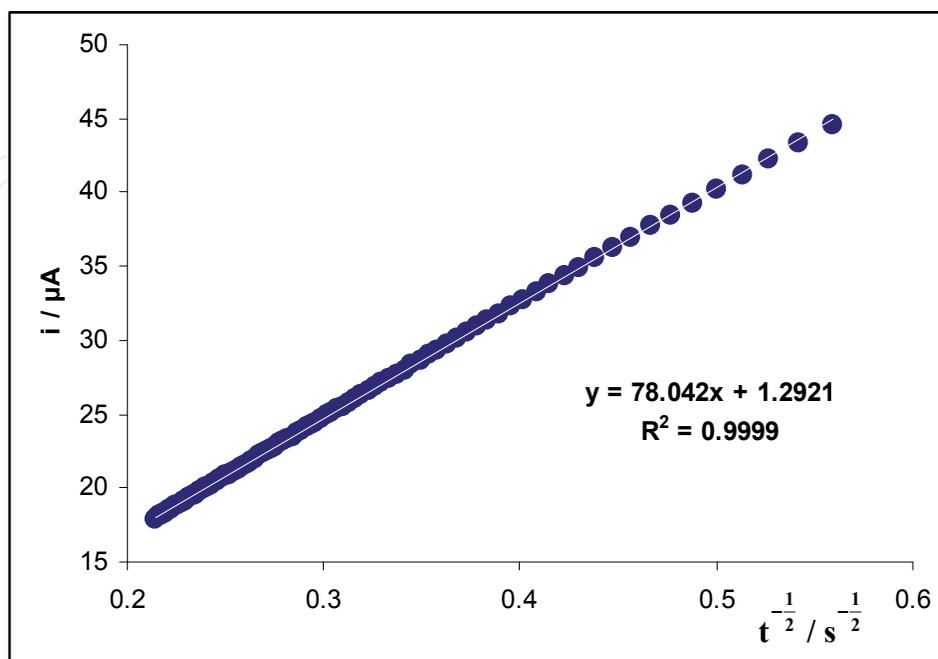


Fig. 4. Correlation curve as a function of $t^{-1/2}$ of the current (i) measured by chronoamperometry of a solution of ferrocene derivative

The results of one of the chronoamperometric experiments run with ferrocene derivative solution on the electrochemical transducer is shown in Figure 4, where the graphical representation of the relationship $i = f(t^{-1/2})$ shows a perfect linearity indicating that the electrochemical reaction (1), taking place at the surface of the transducer, is controlled by diffusion in accordance with the Cottrell equation.



2.3 Disposable amperometric peroxidase mediated biosensor

The concentration of hydrogen peroxide can be measured directly using amperometric detection. Electrochemical oxidation is commonly achieved at a potential of approximately +0.6 to +0.7 V *vs.* Ag/AgCl on a platinum electrode. A number of electroactive chemical species can interfere since they are oxidised within the same potential range. Several electrochemical sensors and biosensors have been described to minimize the interferences (Giannoudi et al., 2006) by modifying the transducers particularly by Prussian blue and its analogous (Karyakin, 2001; Ricci & Palleschi, 2005), carbon nanotubes (Wang, 2005; Zagal et al., 2009) and peroxidase enzymes. The latter are based on electron transfer reactions between the enzyme and the working electrode either directly (Ferapontova, 2004) or through redox mediators (Ruzgas et al., 1996). This last concept of mediated electron transfer reaction is the basis of our work on "no calibration" type biosensor.

Numerous small redox active molecules exchanging one or two electrons with the oxidized form of peroxidase have been used to construct peroxidase-modified electrodes that can

measure H_2O_2 in a cathodic way (Ruzgas et al., 1996). The ferrocene derivatives are typical examples of one-electron mediators that are used for the bio-electrocatalytic reduction of peroxidases. In earlier research, we published the preparation of modified electrode with HRP and ferrocenes for hydrogen peroxide measurement (Charpentier & El Murr, 1995a), which can be combined with oxidase enzymes to permit the detection of their substrates in cathodic mode (Charpentier & El Murr, 1995b; Rondeau et al., 1999; Guémas et al., 2000; Serban et al., 2004; Serban & El Murr, 2006; Dai et al., 2007). The ferrocene mediated hydrogen peroxide biosensor is based on the reactions scheme shown in Figure 5.

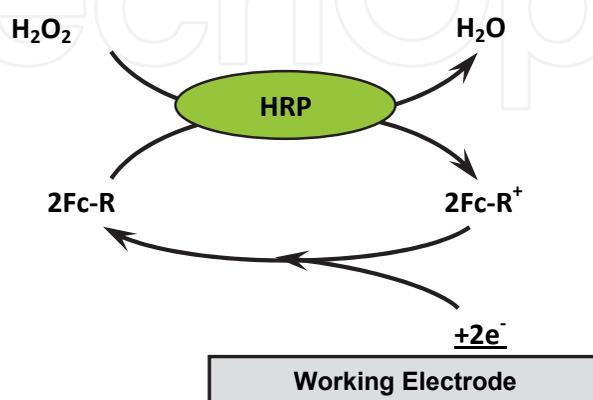


Fig. 5. Reactions scheme of ferrocene mediated peroxidase biosensor

2.3.1 Biosensor preparation

Starting from screen printed electrochemical transducers the general procedure for preparation of biosensors is as follows: three aqueous solutions were prepared separately, the first containing the horseradish peroxidase (HRP) enzyme, the second the ferrocene derivative (Fc-R) and the third a 0.4 M phosphate buffer solution + KCl. A total of 10 μL of each of the three solutions was deposited on the surface of the working electrode of screen-printed electrochemical cells, which were dried and stored at 6°C. In general, biosensors batches were made consisting of 100 to 200 units. The surface of each biosensor was usually modified by 0.5 U HRP, 16.10^{-8} moles ferrocene derivative, and a mixture of K_2HPO_4 , KH_2PO_4 , and KCl, which after addition of 40 μL of test solution produces a 0.1 M buffered solution at pH 7.2.

2.3.2 Choice of redox mediator

The ferrocenes are very popular as redox mediators particularly for reactions involving glucose oxidase or peroxidases. This popularity is mainly the result of the large number of derivatives previously synthesized, the commercial availability of many of them, the stability both in reduced and oxidized forms of a great number of derivatives, and particularly because they can cover a full range of redox potential depending on the nature and the number of substituents carried by the two cyclopentadienyl rings. During our work on the behaviour of hydrogen peroxide biosensors, we considered several ferrocene derivatives mono- or di-substituted by organic groups with varied electronic effects. Finally, the derivative that was selected is the carboxymethyl ferrocene; $(\text{C}_5\text{H}_5)\text{Fe}(\text{C}_5\text{H}_4\text{-CH}_2\text{-COOH})$. The reasons for this choice include the high solubility of its both reduced and oxidized forms and the electronic effect brought by its substituent which results in an adequate redox potential which guaranty high reactivity towards the enzyme. Very good reactivity and solubility of the mediator are essential requirements for the preparation of "no calibration" type biosensor.

2.3.3 "No calibration" type hydrogen peroxide biosensor

For amperometric biosensors, the detection method most commonly used is chronoamperometry. In practice, measurements can be performed in various modes depending on the positioning of the working electrode with respect to the sample solution: the most frequently used are (1) stationary electrode in a relatively large volume of solution, (2) stationary electrode in a stirred or flow solution (e.g. flow injection analysis), and (3) stationary electrode with a very low volume of solution. For single-use biosensors, the third method is the one usually implemented. Very often the area of the working electrode in such a configuration is relatively large compared to the volume of substrate added. The measured current, related to the concentration of the substance to be analyzed, depends on the method applied and on the kinetics that take place in solution as well as on the surface of the working electrode during the chronoamperometric detection. Thus currents controlled by chemical kinetics are different from those controlled only by the diffusion of electroactive substances and undergo differently the effects of physicochemical parameters (temperature, reagents concentrations, enzymes activities)

For the reagentless disposable peroxidase biosensor described above, all reagents and enzymes are on the surface of the electrochemical transducer. The assay procedure is as follow: a total of 40 μL of the test solution is added to the biosensor surface and left to react for a given time (reaction time: t_r) before applying the potential at the working electrode in chronoamperometry mode for 20 seconds. The reaction time (t_r) is very important and can be critical on the current result. It should be long enough when a chemical reaction is required prior to the electrochemical one. This is the case when the measure is based on the detection related to an end-point reaction. Considering the reaction scheme shown in Figure 5, if this time (t_r) is very short H_2O_2 will remain in solution so a homogenous enzymatic reaction will accompany the heterogeneous electron-transfer process occurring at the electrode-solution interface. A catalytic regeneration mechanism (EC') takes place for which the resulting current will be dependent on the enzyme reaction kinetics (Savéant & Vianello, 1967). If sufficient time is allowed so that the enzymatic reaction is completed, H_2O_2 will be totally consumed and the equivalent of twice its concentration of ferricinium cation Fc-R^+ will be generated in solution. No enzymatic reaction can then take place and the electrochemical response $i_{(t)}$ of the planar transducer is controlled by the diffusion of Fc-R^+ according to the Cottrell equation (2) where F is Faraday's constant, A the electrode area, n the number of electrons involved in the electrochemical process, D the diffusion coefficient of the electroactive ferricinium species and $[\text{Fc-R}^+]$ its concentration. A comprehensive study has allowed us to show that, for a time (t_r) equal to 30 seconds, it was possible to achieve an end-point reaction for H_2O_2 concentrations above 2 mM. A comprehensive study showed that the end-point of the enzymatic reaction shown in Figure 5 could be reached within 30 seconds for concentrations above 2 mM H_2O_2 . Therefore, for all tests with hydrogen peroxide biosensors, a time (t_r) = 30 s has been fixed before running the chronoamperometric experiments.

$$i_{(t)} = \pi^{-1/2} \cdot F \cdot A \cdot n \cdot [\text{Fc-R}^+] \cdot D \cdot t^{-1/2} \quad (2)$$

Because the hydrogen peroxide tests are carried out with disposable biosensors using reproducible transducers (as already shown above) the electrode area A is always constant. As the electrochemical reaction involved is always the same (reaction 1), the diffusion coefficient D is always constant and the number of electrons n equal to 1. Thus, for the same

batch of transducers the current obtained by chronoamperometry at a time t (20 s) depends only on the ferricinium concentration $[Fc-R^+]$ and equation (2) becomes:

$$i_{(t)} = k \cdot [Fc - R^+] \quad (3)$$

Considering the equation (3) k is simply the slope of the calibration curve that can be obtained beforehand with standard solutions of Fc-R using transducers from the same batch. Provided that the enzymatic reaction, shown in Figure 5, is completed (end-point reaction) in the time (t_r) the initial concentration of hydrogen peroxide is then equal to half of that of Fc-R⁺. The final equation to calculate the concentrations of hydrogen peroxide solutions using the biosensor, without prior calibration, is:

$$[H_2O_2] = i_{(t)} / 2k_{(t)} \quad (4)$$

where $i_{(t)}$ is the chronoamperometric current measured at the time (t), typically 20 seconds, with the biosensor and $k_{(t)}$ is the slope of the calibration curve obtained by chronoamperometry with standard ferrocene mediator solutions at the same time (t) using transducers from the same batch that was used for the preparation of disposable hydrogen peroxide biosensors.

2.3.4 Prior determination of $k_{(t)}$ for a given batch of transducers

Several standard solutions of carboxymethyl ferrocene Fc-CH₂-COOH covering the range from 0 to 4 mM were prepared. After deposition of 40 μ L of each on the surface of a transducer of the same batch as those used to prepare the biosensors, a potential of +0.5 V *vs.* Ag/AgCl was applied to the working electrode and the faradic current generated was measured at $t = 20$ s. The slope ($k_{(t)} = 11.64 \mu\text{A}/\text{mM}$ of Fc-CH₂-COOH) of the calibration curve (Figure 6) resulting from these measurements transforms the equation (4) in:

$$[H_2O_2] = i_{(t)} / 23.28 \quad (5)$$

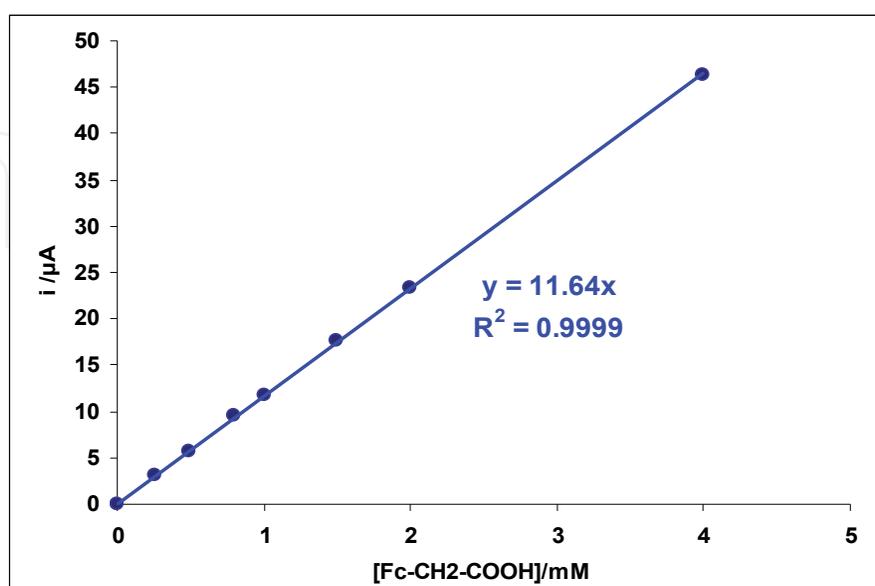


Fig. 6. Calibration curve of carboxymethyl ferrocene

For the batch of transducers used in this study, equation (5) enables by a simple calculation to determine the concentration of H₂O₂ solutions by chronoamperometric tests using disposable hydrogen peroxide biosensors.

2.3.5 Validation of the "No calibration" concept

To validate the concept, based on equation (5), to measure hydrogen peroxide without a preliminary calibration step with H₂O₂ standard solutions, we prepared from a stock solution titrated with KMnO₄ five H₂O₂ solutions of known concentrations, that we measured with the disposable biosensors.

In Table 3 are collected the results of this study, which shows that the deviations observed when using the equation (5) are low and remain anyway in the same order of magnitude as measurement errors obtained by biosensors in general. This shows that the proposed method provides reliable measurements, without the need to perform a tedious calibration step before each test.

[H ₂ O ₂] (mM)	i (μA)	[H ₂ O ₂] (mM) calculated with equation (5)	Deviation (%)
0.158	-3.78	0.162	2.69
0.316	-7.389	0.317	0.44
0.553	-12.49	0.537	-3.07
0.790	-18.54	0.796	0.80
0.988	-22.75	0.977	-1.05

Table 3. Results of measurements of H₂O₂ solutions by means of "no calibration" type biosensors

3. Conclusion

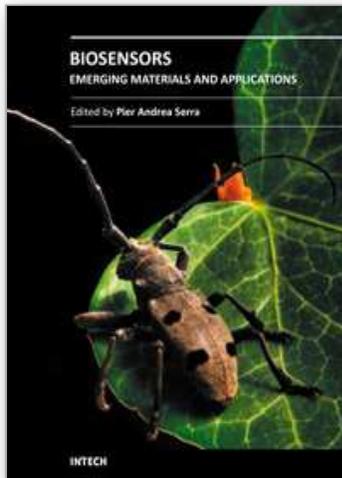
Amperometric enzyme based biosensors using redox mediators capable of shuttling electrons from the redox centre of the enzyme to the surface of the electrode are by far the most popular and the most studied. In addition, this type of biosensor is the one that has had the greatest commercial success, following the launch into the market of glucose biosensors devices for the control of diabetics' glycemia. From the perspective of electrochemistry, these biosensors are based on the measurement of a kinetic current controlled by the enzymatic reaction that detects the substrate. This current depends on the activity of the enzyme and is therefore sensitive to several physicochemical factors that may influence the kinetics of the reaction. For this, a calibration step is necessary to obtain, in the operating conditions, reliable measurements. This calibration step, often considered tedious and time consuming, makes these biosensors unattractive for industries that want to use these analytical tools in remote locations utilising unskilled workers. The development of "no calibration" type biosensor concept could be considered as the important step to overcome this difficulty. We have validated this concept in producing reliable and reproducible disposable biosensors for H₂O₂ that operate with horse radish peroxidase and carboxymethyl ferrocene as redox mediator which are commercially available, cheap and stable. Such a "no calibration" type H₂O₂ biosensor will serve as a general platform for a very large number of biosensors that use enzymes such as oxidases or combination of dehydrogenases and NADH oxidase.

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A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 19 different countries. The book consists of 27 chapters written by 106 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

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