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A peroxidase-tetrathiafulvalene biosensor based on self-assembled monolayer modified Au electrodes for the flow-injection determination of hydrogen peroxide

Susana Campuzano, María Pedrero, José M. Pingarrón *

Dpto. Química Analítica, Facultad de CC. Químicas, Universidad Complutense de Madrid, E-28040 Madrid, Spain

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Abstract

The construction and performance under flow-injection conditions of an integrated amperometric biosensor for hydrogen peroxide is reported. The design of the bioelectrode is based on a mercaptopropionic acid (MPA) self-assembled monolayer (SAM) modified gold disk electrode on which horseradish peroxidase (HRP, 24.3 U) was immobilized by cross-linking with glutaraldehyde together with the mediator tetrathiafulvalene (TTF, 1 μmol), which was entrapped in the three-dimensional aggregate formed.

The amperometric biosensor allows the obtention of reproducible flow injection amperometric responses at an applied potential of 0.00 V in 0.05 mol L^{-1} phosphate buffer, pH 7.0 (flow rate: 1.40 mL min^{-1}, injection volume: 150 μL), with a range of linearity for hydrogen peroxide within the 2.0×10^{-7}–1.0×10^{-4} mol L^{-1} concentration range (slope: $(2.33 \pm 0.02) \times 10^{-2}$ A mol^{-1} L, $r = 0.999$). A detection limit of 6.9×10^{-8} mol L^{-1} was obtained together with a R.S.D. ($n = 50$) of 2.7% for a hydrogen peroxide concentration level of 5.0×10^{-5} mol L^{-1}. The immobilization method showed a good reproducibility with a R.S.D. of 5.3% for five different electrodes. Moreover, the useful lifetime of one single biosensor was estimated in 13 days.

The SAM-based biosensor was applied for the determination of hydrogen peroxide in rainwater and in a hair dye. The results obtained were validated by comparison with those obtained with a spectrophotometric reference method. In addition, the recovery of hydrogen peroxide in sterilised milk was tested.

Keywords: Self-assembled monolayers; Tetrathiafulvalene; Amperometric biosensor; Hydrogen peroxide

1. Introduction

The determination of hydrogen peroxide is of great importance in many fields including food, pharmaceutical, clinical, industrial and environmental analysis [1–3]. Moreover, hydrogen peroxide is also a product of photochemical reactions in the atmosphere, which means that it can be found at levels of mg dm^{-3} in drinkable, rain and sea waters, and in snow [4]. From the biosensing point of view, hydrogen peroxide is a reaction product of oxidases, and its measurement is the basis for the biodetection of many substances of biochemical significance such as glucose, cholesterol or uric acid [5].

Although many analytical methods can be found in the literature for the determination of hydrogen peroxide [6], most of them show limitations such as a lack of sensitivity, the susceptibility to interference by other substances in samples, or long times of analysis. The use of amperometric biosensors is of special usefulness to overcome these drawbacks because of their simplicity and high sensitivity [7,8].

Although peroxidase (HRP) electrochemical biosensors have been widely used for the detection of hydrogen peroxide, and many different approaches can be found in the literature, involving the use of carbon paste [3,6], glassy carbon [5,9], and Au electrodes [10,11], only a few of the reported HRP biosensors have been checked and applied under flowing conditions [12–14], with well-known advantages for automation, possibility of processes control and

* Corresponding author. Tel.: +34 913944315; fax: +34 913944329.
E-mail address: pingarro@quim.ucm.es (J.M. Pingarrón).

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coupling with liquid chromatography that the work under such conditions offers. Moreover, flow conditions need better stability requirements for enzyme immobilization in order to prevent leaching of the biomolecules by the flow stream.

Recently, we have reported on the construction of reliable enzyme electrodes for some biologically active molecules such as glucose and fructose, which are suitable to work both under batch and flow-injection (FI) conditions [15,16]. These biosensors are based on the use of 3-mercaptopropionic acid (MPA) self assembled monolayers (SAMs)-modified Au electrodes, and were constructed by immobilising the corresponding enzyme (glucose oxidase and fructose dehydrogenase, respectively) by cross-linking with glutaraldehyde atop the alkanethiol SAM together with the employed redox mediator (tetrathiafulvalene, TTF), which was entrapped in the three-dimensional aggregate formed. The dramatic reduction of the background current, with the subsequent increase in sensitivity, and the reproducibility and robustness that can be achieved are among the most important analytical advantages expected from immobilization of biomolecules in SAMs [1,17,18].

Some HRP biosensors using electrode SAM-modification, in which the enzyme is covalently attached, have been described [2,11,19–22]. This immobilization strategy also allows the incorporation of a redox mediator such as viologen [23], or thionine [21,22] to the monolayers. Nevertheless, none of these HRP biosensors were tested for the determination of H_2O_2 under flowing conditions, nor applied for the analysis of real samples.

In this article, the construction and performance under flowing conditions of an integrated amperometric biosensor for hydrogen peroxide is reported. The design of the bioelectrode is based on an MPA-modified AuE on which horseradish peroxidase is immobilized by cross-linking with glutaraldehyde together with TTF, which is entrapped in the three-dimensional aggregate formed.

2. Experimental

2.1. Apparatus and electrodes

Voltammetric and amperometric measurements were carried out with an ECO Chemie Autolab PSTAT 10 potentiostat using the software package GPES 4.7 (General Purpose Electrochemical System). Flow-injection experiments were carried out using a Gilson Minipuls-2 peristaltic pump, and a Rheodyne Model 5020 injection valve with variable injection volumes.

A Metrohm 6.1204.020 gold disk electrode (3 mm Ø) was used as electrode substrate to be coated with the MPA-SAM. A BAS MF-2063 Ag|AgCl|KCl 3 mol L^{-1} reference electrode and a Pt wire counter electrode, were also employed. A large volume (50 mL) home made glass wall-jet cell was employed for the measurements.

2.2. Reagents and solutions

0.5 mol L^{-1} hydrogen peroxide (Sigma) stock solutions were prepared daily in 0.05 mol L^{-1} phosphate buffer of pH 7.0. More dilute standards were prepared by suitable dilution with the same phosphate buffer solution, which was also used as the supporting electrolyte.

A 40 mmol L^{-1} mercaptopropionic acid (Research Chemicals Ltd.) solution, prepared in a 75/25% (v/v) ethanol/water mixture, was employed for the formation of the monolayers. The solutions used for the enzyme immobilization were a 12.15 U μL^{-1} solution of HRP (Sigma, EC 1.11.1.7 Type II: from Horseradish, 158 U mg^{-1} solid), and a 25% glutaraldehyde (Aldrich) solution. Moreover, a 0.5 mol L^{-1} tetrathiafulvalene (Aldrich) solution in acetone was prepared.

Other solutions employed were: a 2 mol L^{-1} KOH (Panreac) solution prepared in deionized water; 0.05 mol L^{-1} stock solutions of L-cysteine, uric acid, D-fructose (Sigma), 4-acetamidophenol (Aldrich), ascorbic acid (Fluka), urea (Merck), D-glucose (Panreac), prepared in 0.05 mol L^{-1} phosphate buffer of pH 7.0, for the interference study.

All chemicals used were of analytical-reagent grade, and water was obtained from a Millipore Milli-Q purification system.

2.3. Procedures

Before the deposition of the SAM, the gold disk electrode was pretreated as described previously [15,16]. MPA-SAMs were formed by immersion of the clean AuE in a 40 mmol L^{-1} MPA solution in EtOH/H_2O (75/25, v/v) for 15 h. Then, the modified electrode was rinsed with deionized water. Coimmobilization of the enzyme and the mediator was carried out by cross-linking of HRP with glutaraldehyde and entrapping of the mediator in the three-dimensional aggregate formed. The procedure was as follows: 2 μL of the 0.5 mol L^{-1} TTF solution were deposited on the SAM-modified AuE and let to dry at ambient temperature. Then 2 μL of a 12.15 U μL^{-1} HRP solution were also deposited on the electrode surface. Once the electrode had dried out at ambient temperature, it was immersed in a 25% glutaraldehyde solution for 1 h at 4 °C.

FI-measurements with amperometric detection were carried out at an applied potential of 0.00 V versus Ag/AgCl. The carrier stream was a 0.05 mol L^{-1} phosphate buffer of pH 7.0, with a flow rate of 1.40 mL min^{-1}. The sample injection volume was of 150 μL.

2.4. Determination of hydrogen peroxide in real samples

Hydrogen peroxide was determined in three different real samples: sterilized milk, rainwater and a hair dye. The only sample treatment required in all cases consisted on an appropriate dilution with the carrier solution.

Recovery tests of hydrogen peroxide in milk samples were carried out by adding hydrogen peroxide to sterilised milk at a concentration level of 5.0×10^{-5} mol L^{-1}.

In the case of the hair dye sample, 3.0 mL were dissolved in 100 mL of 0.05 mol L^{-1} phosphate buffer of pH 7.0, and the resulting solution was 10,000-fold diluted with the carrier solution. A 150 μL aliquot of this solution was then injected into the FI system.

Concerning the analysis of rainwater, once it was collected, only a 1:1 sample dilution with 0.05 mol L^{-1} phosphate buffer of pH 7.0 was necessary prior to the injection in the FI system.

The results obtained in the analysis of both, rainwater and the hair dye were compared with those achieved by an alternative method proposed by Mottola [24], which is based on the spectrophotometric detection of the reaction product formed in the reaction between H_2O_2 and *o*-dianisidine. As matrix effects were observed in the analysis of the two samples, the absorbance ($\lambda = 460$ nm) of solutions prepared as follows was measured:

- *Rainwater:* 500 μL of sample, 0–40 μL of a 10^{-4} mol L^{-1} H_2O_2 solution, and 50 μL of a 100 μg mL^{-1} HRP solution were added to a 5.0 mL volumetric flask and diluted to the mark with a 66 μg mL^{-1} *o*-dianisidine solution.
- *Hair dye:* A diluted sample solution was firstly prepared by a 100-fold dilution with 0.05 mol L^{-1} phosphate buffer (pH 7.0), of a solution obtained by diluting 3.0 mL of the hair dye sample in 100.0 mL of the same buffer solution; then, 20 μL of the diluted sample solution, 0–40 μL of a 1 mmol L^{-1} H_2O_2 solution, and 50 μL of a 100 μg mL^{-1} HRP solution were added to a 5.0 mL volumetric flask, and diluted to the mark with a 66 μg mL^{-1} *o*-dianisidine solution.

3. Results and discussion

3.1. SAM-based integrated biosensor for hydrogen peroxide

Prior to testing the performance of the SAM-based HRP integrated biosensor under FI conditions, cyclic voltammograms for 5.0 mmol L^{-1} hydrogen peroxide solutions were recorded at three different electrodes: a biosensor fabricated by immobilization of the enzyme atop the MPA-AuE (HRP-MPA-AuE), a MPA-AuE with no enzyme but with the mediator immobilized (TTF-MPA-AuE), and the biosensor in which both the enzyme and the mediator were immobilized atop the MPA-AuE (HRP-TTF-MPA-AuE) (Fig. 1).

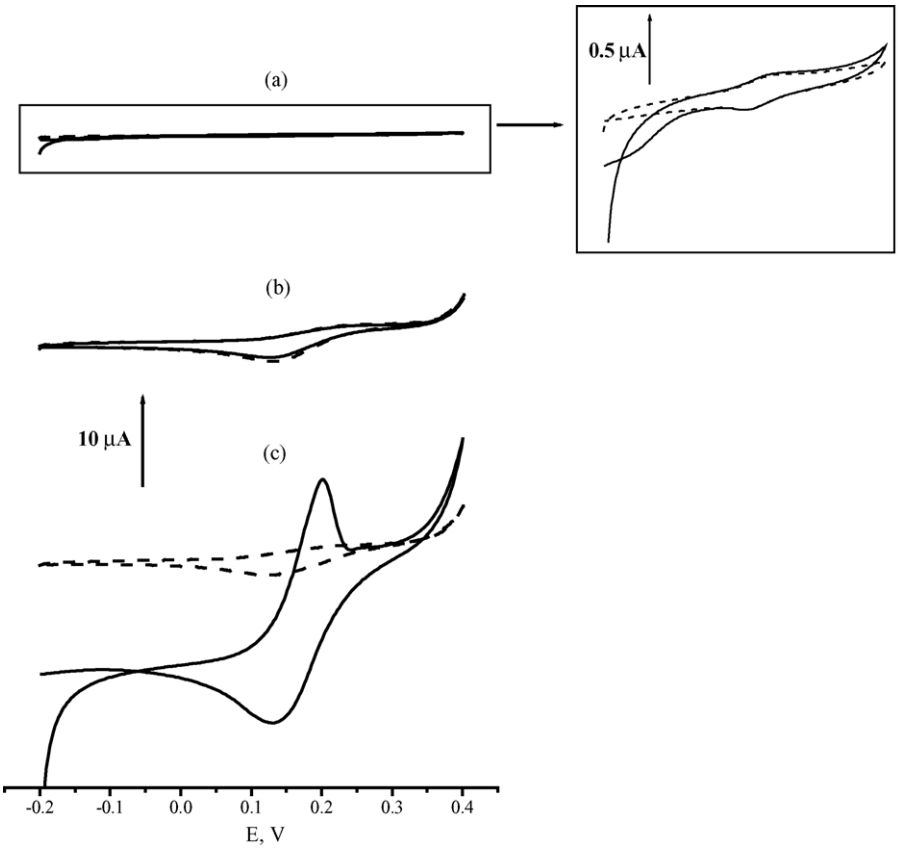

Fig. 1. Cyclic voltammograms obtained for 5.0 mmol L^{-1} hydrogen peroxide in 0.05 mol L^{-1} phosphate buffer of pH 7.0 at: (a) a HRP-MPA-AuE, (b) a TTF-MPA-AuE, and (c) a HRP-TTF-MPA-AuE; (- - -) background voltammogram; $V = 25$ mV s^{-1}.

As can be seen in Fig. 1a, only a small increase in the cathodic current was observed upon addition of hydrogen peroxide when compared with the background voltammogram at the HRP-MPA-AuE. This suggests a poor direct electron transfer between the immobilized HRP and the MPA-AuE. As expected, no electrocatalytic reduction of hydrogen peroxide was observed at the TTF-MPA-AuE (Fig. 1b). Fig. 1c displays cyclic voltammograms at the integrated biosensor, HRP-TTF-MPA-AuE. In the absence of H_2O_2 the redox waves due to the first reversible TTF redox process were observed. The addition of hydrogen peroxide to the solution produced a significant current increase, thus demonstrating that the TTF incorporated atop the SAM-modified electrode effectively enhances the electron transfer between the immobilized HRP and the electrode surface, according to the following reaction sequence:

$$H_2O_2 + 2H^+ + HRP_{red} \rightarrow HRP_{ox} + 2H_2O$$

$$HRP_{ox} + 2TTF \rightarrow HRP_{red} + 2TTF^+$$

The TTF^+ thus generated is then electrochemically reduced when the applied potential is more negative than the formal potential of the TTF/TTF^+ redox couple,

$$2TTF^+ + 2e^- \rightarrow 2TTF$$

The electron transfer pathway occurring at the biosensor can be assumed to be as follows: TTF and HRP are closely co-immobilized atop the MPA SAM. The SAMs constituted by this short-chain alkanethiol exhibit a high percentage of defects and/or pinholes [15], and in fact they can be used to prepare microarray electrodes for measuring very fast electron-transfer kinetic [25]. This architecture permits that the distance between the enzyme and the electrode surface was rather short thus improving the electron transfer. Consequently, the TTF^+ generated at the electrode detection potential, which is very scarcely soluble in aqueous solutions, and therefore, is not appreciably leaked from the electrode, allows the electron transfer to be achieved by a shuttle mechanism.

3.1.1. Optimization of the working variables under flow injection conditions

The influence of several parameters such as the composition of the biosensor, the applied potential, the working pH, and the hydrodynamic variables was tested.

Firstly, the effect of the enzyme and mediator order of immobilization on the FI amperometric response of the biosensor to H_2O_2 was checked. The obtained results demonstrated that the electron transfer was more efficient when the mediator was immobilized in the first place, which was attributed to its higher closeness to the electrode surface.

The enzyme and the mediator loadings were then optimized adopting as the criterion of selection the highest slope value obtained for the hydrogen peroxide calibration plot in the $(1.0–6.0) \times 10^{-5}$ mol L^{-1} concentration range. First, the enzyme loading was optimized using a constant TTF amount of 1.5 μmol. Fig. 2a shows that the slope value of the hydrogen peroxide calibration graph increased with the enzyme loading up to 24.3 U of HRP, after which the slope value practically leveled off. Consequently, this enzyme loading was selected for further studies. The influence of the amount of TTF on the electrode is shown in Fig. 2b; the calibration slope value increased with the mediator amount up to 1.0 μmol, after which a slight decrease in the slope was produced. This effect can be attributed to the non-conducting nature of TTF, which hinders the electron transfer in the presence of large amounts of this compound on the electrode.

Once the composition of the biosensor was optimized, the influence of the applied potential on the hydrogen peroxide FI amperometric response was tested. Fig. 3 shows the results obtained for a hydrogen peroxide concentration of 5.0×10^{-5} mol L^{-1}, using four different electrodes (HRP-TTF-MPA-AuE, HRP-MPA-AuE, TTF-MPA-AuE and MPA-AuE). As can be seen, the reduction current of H_2O_2 increased rapidly at the HRP-TTF-MPA-AuE as the applied potential varied from +0.25 to 0.00 V and almost reached a steady state in the potential range from 0.00 to −0.20 V. Since no significant differences in sensitivity were observed between applying 0.00 and −0.10 V, a working po-

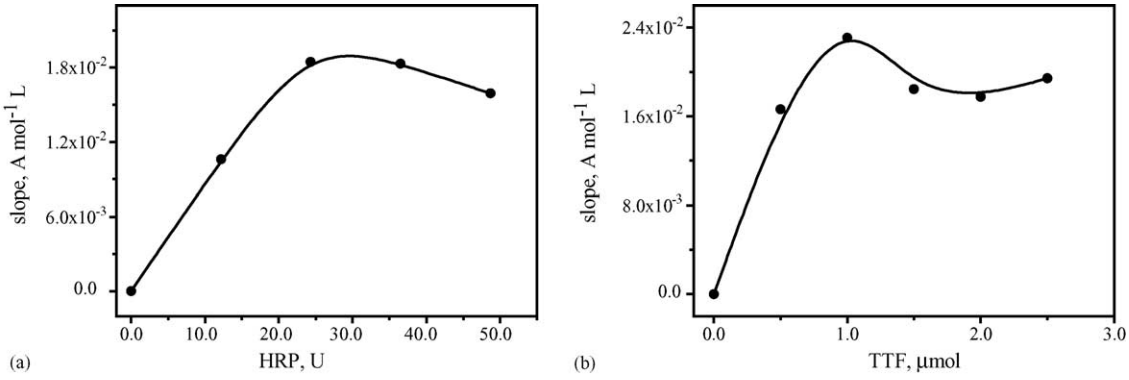

Fig. 2. Effect of the HRP loading (a) and the TTF amount (b) immobilized atop the MPA-AuE on the slope of the hydrogen peroxide calibration plot obtained by FI with amperometric detection in the 1.0×10^{-5}–6.0×10^{-5} mol L^{-1} concentration range. Carrier solution: 0.05 mol L^{-1} phosphate buffer (pH 7.0); q = 1.40 mL min^{-1}; V_i = 150 μL; E_{app} = 0.00 V.

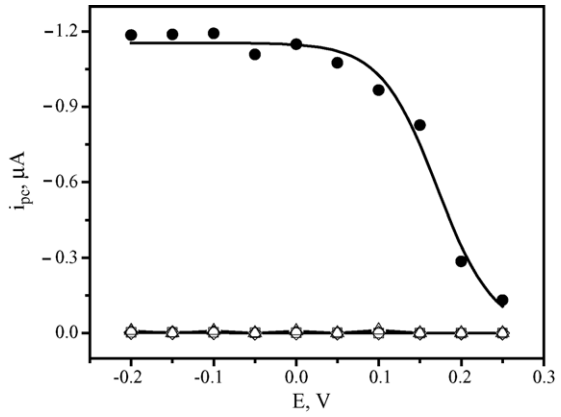

Fig. 3. Effect of the applied potential on the FI amperometric signal for 5.0×10^{-5} mol L^{-1} hydrogen peroxide at a HRP-TTF-MPA-AuE (●), a HRP-MPA-AuE (□), a TTF-MPA-AuE (△) and a MPA-AuE (◊). Carrier solution: 0.05 mol L^{-1} phosphate buffer (pH 7.0); q = 1.40 mL min^{-1}; V_i = 150 μL.

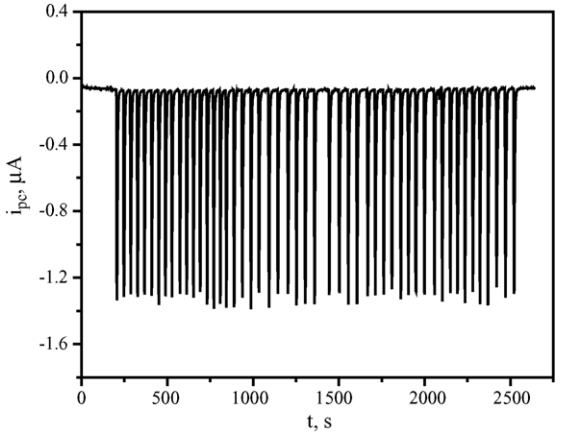

Fig. 4. Amperometric flow injection responses obtained for 50 repetitive injections of 5.0×10^{-5} mol L^{-1} hydrogen peroxide at the HRP-TTF-MPA-AuE as amperometric detector. Carrier solution: 0.05 mol L^{-1} phosphate buffer (pH 7.0); q = 1.40 mL min^{-1}; V_i = 150 μL; E_{app} = 0.00 V.

tential of 0.00 V was selected for further work in order to accomplish a sensitive detection and also to minimize the number of potential interferents able to be reduced at the electrode. Moreover, as expected, no amperometric signal was found in the whole potential range for the other three electrodes tested.

Characteristic flow-injection parameters, such as flow rate and injected sample volume were also optimized. Regarding the flow rate effect, the peak height obtained after injection of a 5.0×10^{-5} mol L^{-1} H_2O_2 solution increases with this parameter up to 2.13 mL min^{-1} and levels off for higher flow rates, indicating the fast response time of the biosensor. Furthermore, the peak width increased as the flow rate decreased. A flow rate of 1.40 mL min^{-1} was selected to ensure a good reproducibility of the signal. Concerning the injection volume, a sample volume of 150 μL was chosen taking into account the $i_p/W_{1/2}$ ratio, where $W_{1/2}$ is the peak width at half-height.

Finally, the effect of pH on the amperometric response was evaluated over the 3.0–10.0 range, for a hydrogen peroxide concentration of 1.0×10^{-5} mol L^{-1}. The current response increases between pH values of 3.0 and 6.5, and decreases from pH 7.0–10.0, the optimal pH range being between 6.5 and 7.0. Similar results were also observed for HRP in solution [26], indicating that the immobilization procedure does not alter the optimal pH value for the catalytic action of peroxidase. According to this, a 0.05 mol L^{-1} phosphate buffer carrier solution of pH 7.0 was chosen for further work.

3.1.2. Stability of the HRP-TTF-MPA-AuE biosensor

Different aspects regarding the stability of the HRP-TTF-MPA-AuE biosensor were considered:

Firstly, the reproducibility of the FI amperometric measurements was evaluated by constructing 10 successive calibration plots for hydrogen peroxide in the 2.0×10^{-5}–1.0×10^{-4} mol L^{-1} concentration range with the same biosensor. A relative standard deviation (R.S.D.) value of 5.2% was obtained for the slope of such calibration graphs, indicating a good reproducibility of the FI measurements with no need to apply a cleaning or pretreatment procedure to the HRP-TTF-MPA-AuE.

Secondly, the repeatability of the flow-injection responses was evaluated from a series of 50 repetitive injections of a 5.0×10^{-5} mol L^{-1} hydrogen peroxide solution. As can be seen in Fig. 4, a very good repeatability was obtained with a R.S.D. for i_p of 2.7%, thus demonstrating a good immobilization of both the enzyme and the mediator on the SAM-modified electrode in spite of the hydrodynamic conditions. According to this fiagram, when the HRP-TTF-MPA-AuE is employed as an amperometric detector in a FIA system, a sampling frequency of 75 samples/h is possible.

Thirdly, the reproducibility of the responses obtained with different HRP-TTF-MPA-AuEs was checked in order to characterize the performance of the SAM-based biosensors. Results from five different electrodes yielded a R.S.D. of 5.3% for the slope values of the corresponding calibration plots obtained for hydrogen peroxide in the 2.0×10^{-5}–1.0×10^{-4} mol L^{-1} concentration range. This demonstrated that the fabrication procedure of the HRP-TTF-MPA-AuE was reliable, thus allowing reproducible FI amperometric responses to be obtained with different biosensors constructed in the same manner.

Finally, the useful lifetime of one single HRP-TTF-MPA-AuE was checked by performing repetitive calibration graphs for hydrogen peroxide in the above-mentioned concentration range. After use, the biosensor was stored in phosphate buffer of pH 7.0 at 4 °C. Fig. 5 shows the control chart constructed, taking the mean value of the slopes of the 10 successive calibration plots obtained the first day of this study as the central value. The upper and lower control limits were set at ±3 × S.D. of this target value. From the second day, the mean values of the slopes of three successive calibration plots are plotted. As can be seen, the slope mean values remained within the control limits for around 312 h (13 days).

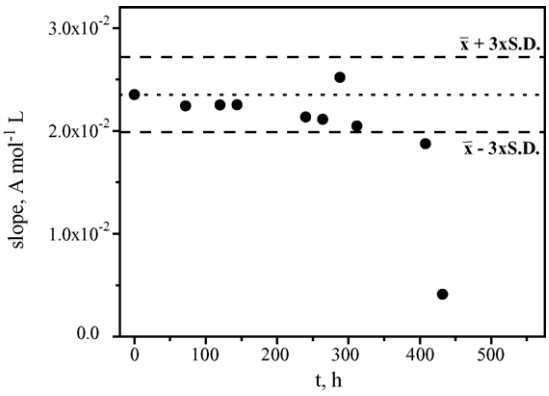

Fig. 5. Control chart constructed for one single HRP-TTF-MPA-AuE. Measurements correspond to the mean values of the slopes of three successive calibration plots for hydrogen peroxide in the 2.0×10^{-5}–1.0×10^{-4} mol L^{-1} concentration range. Carrier solution: 0.05 mol L^{-1} phosphate buffer (pH 7.0); $q = 1.40$ mL min^{-1}; $V_i = 150$ μL; $E_{app} = 0.00$ V.

After 17 days, the biosensor yielded a 80% of the original response, which can be attributed to the denaturation of the immobilized enzyme.

On the other hand, a biosensor prepared by direct immobilization of the enzyme and the mediator onto a bare AuE (non-MPA modified) showed a poorer stability. Thus, a R.S.D. of 7.1% was obtained for the slopes of 10 successive calibration plots, and the initial sensitivity decreased to an 80% of its initial value after 13 days. The worse stability can be attributed to a faster denaturation of proteins directly immobilized onto metal surfaces [27].

3.1.3. Kinetic constants and analytical characteristics

The enzyme reaction for hydrogen peroxide at the HRP-TTF-MPA-AuE obeyed a Michaelis-Menten behavior. Thus a linear log $[(i_{max}/i) - 1]$ versus log $[H_2O_2]$ graph was obtained with a slope of 0.97 ± 0.03, indicating that the immobilization procedure did not alter the Michaelis-Menten type kinetics. Then, calculation of the apparent Michaelis-Menten constant and the maximum rate of the reaction was accomplished from the Lineweaver-Burk plot. Values of K_M^{app} (1.0 ± 0.3) mmol L^{-1}, and $V_{max} = (41 \pm 9)$ μA were obtained, with the confidence intervals calculated for a significance level of 0.05 ($n = 3$). The K_M^{app} value for the HRP-TTF-MPA-AuE was smaller than that obtained for the free enzyme ($K_M = 11$ mmol L^{-1}) [7,28]. This can be attributed to that the high degree of enzyme reticulation promoted by glutaraldehyde probably occurred with a resulting limited substrate access to the enzyme active sites [29].

Under the optimized FI working conditions, a typical calibration curve for an enzyme system was obtained for hydrogen peroxide. A linear calibration graph was obtained over the 2.0×10^{-7}–1.0×10^{-4} mol L^{-1} concentration range ($r = 0.999$, slope = $(2.33 \pm 0.02) \times 10^{-2}$ A mol^{-1} L, intercept = $(2.2 \pm 0.7) \times 10^{-8}$ A). The limits of detection and determination were calculated, according to the $3s_b/m$ and 10 s criteria, respectively, where m is the slope of the linear calibration plot and s_b was estimated as the standard deviation ($n = 10$) of the amperometric signals from 1.0×10^{-7} mol L^{-1} hydrogen peroxide. These values were 6.9×10^{-8} and 2.3×10^{-7} mol L^{-1}, respectively. It should be noted that the sensitivity attained with a biosensor prepared in the absence of the MPA monolayer (TTF-HRP-AuE) was a 30% lower (slope = $(1.601 \pm 0.008) \times 10^{-2}$ A mol^{-1} L).

On the other hand, when a biosensor prepared in the absence of the redox mediator (HRP-MPA-AuE) was used under the same experimental conditions, the lowest detectable signal corresponded to a 2.0×10^{-4} mol L^{-1} H_2O_2 concentration, with a range of linearity extended up to 1.0×10^{-2} mol L^{-1} ($r = 0.996$), and a slope value of $(2.46 \pm 0.08) \times 10^{-6}$ A mol^{-1} L, which was four orders of magnitude lower than that obtained with the electrode containing TTF. This demonstrates again that the electron transfer through the TTF incorporated to the electrode is much more efficient than the direct transfer between the enzyme and the electrode [30].

3.1.4. Interference study

The selectivity of the HRP-TTF-MPA-AuE biosensor was checked under the experimental conditions specified above. The potential interferents tested were glucose, fructose, urea, L-cysteine, acetamidophenol and uric and ascorbic acids. The degree of interference was evaluated by calculating the relative error in the slope value for the hydrogen peroxide calibration graph in the absence and presence of the potential interferent at different concentration levels. Table 1 shows the hydrogen peroxide-to-interferent molar ratio (in the solution used to construct the calibration graph) for which a relative error lower than 10% was obtained. As can be seen, no interference was found from glucose, fructose, urea, acetamidophenol and uric acid even at a 1:20 concentration ratio. However, ascorbic acid and L-cysteine did affect the slope value of the hydrogen peroxide calibration plot for the ratios shown in Table 1. The interference from ascorbic acid is due to the electrochemical oxidation of this compound at the applied potential. Although the presence of the SAM inhibits in a high extent this oxidation process, as it was demonstrated by recording cyclic voltammograms at Au and MPA-Au electrodes, it has been described that the presence of TTF catalyses the ascorbic acid oxidation [31], and therefore a

Table 1
Hydrogen peroxide-to-interferent molar ratio for which a relative error lower than 10% was obtained for the slope of the hydrogen peroxide calibration graph in the 2.0×10^{-5}–1.0×10^{-4} mol L^{-1} concentration range

Interferent	Hydrogen peroxide-to-interferent molar ratio
D-Glucose	<0.05
D-Fructose	<0.05
Urea	<0.05
Acetamidophenol	<0.05
Uric acid	<0.05
Ascorbic acid	2
L-Cysteine	2

Table 2
Determination of hydrogen peroxide in real samples with a FI system and amperometric detection at the HRP-TTF-MPA-AuE, and comparison with the results obtained by the Mottola's spectrophotometric method

Sample	HRP-TTF-MPA-AuE	Mottola's method
Rain water (mg dm^{-3}) ($n = 5$)	(0.066 ± 0.003) R.S.D.$_{n=5}$ = 3.7%	(0.069 ± 0.009) R.S.D.$_{n=5}$ = 10.4%
Hair dye (%, v/v) ($n = 5$)	(6.9 ± 0.6) R.S.D.$_{n=5}$ = 6.5%	(7.1 ± 0.8) R.S.D.$_{n=5}$ = 9.1%

significant interference was produced. On the other hand, the interference of L-cysteine can be explained taking into account the oxidation of aliphatic and aromatic thiols by HRP [32].

3.1.5. Analysis of real samples

The analytical usefulness of the developed biosensor was evaluated by determining hydrogen peroxide in three different samples: sterilised milk, rainwater and in a hair dye.

Concerning milk, hydrogen peroxide is used in the cleaning of instruments and equipment employed for cooling, mixing, transporting, bottling, and packing milk. If the subsequent washing and drying is incomplete, the foodstuff is (illegally) contaminated [33]. Hydrogen peroxide is also used as stabilizer in milk, in well-defined concentrations, in some European and American countries, and particularly in certain preparations of hard cheeses as an alternative to pasteurisation. Sufficient sterilization is obtained at 0.1% H_2O_2, and usually by adding 1–2 mL per litre of 33% H_2O_2 milk remains in good conditions till reaching the processing plant. Nevertheless, the residual hydrogen peroxide has to be eliminated before milk consumption due to its adverse effects.

After corroborating the absence of endogenous hydrogen peroxide in the sterilized milk sample analyzed, the performance of the biosensor for the analysis of hydrogen peroxide in this sample was tested by carrying out recovery studies.

The matrix effect observed was minimized by interpolating the peak current obtained after injection of the spiked (with 5.0×10^{-5} mol L^{-1} H_2O_2) and diluted (dilution factor, 1:10) sample in a standard calibration graph constructed by injection of a solution of the diluted (10-fold) non-contaminated sample, and then by injecting solutions prepared with the same amount of non-contaminated sample and growing amounts of 2.0×10^{-6} mol L^{-1} H_2O_2. A mean recovery ($n = 5$) of (99 ± 2)% (R.S.D. = 1.9%) was obtained, thus demonstrating a good accuracy of the hydrogen peroxide determination in this kind of sample in a few minutes, without any preventive treatment of the sample. The limit of detection in milk was calculated according to the same $3s_b/m$ criterion, where m was the slope of the linear standard calibration graph constructed as mentioned above ($(1.07 \pm 0.04) \times 10^{-2}$ A mol^{-1} L), and s_b was estimated as the standard deviation ($n = 10$) of the FI responses from 5.0×10^{-7} mol L^{-1} H_2O_2 spiked samples. The value obtained was 7.2×10^{-7} mol L^{-1}.

On the other hand, hydrogen peroxide is also used in the cosmetic industry. For example, discoloring hair solutions, containing 2–7% H_2O_2, are essential in the color developing process when mixed with the permanent hair dyes. In the case of the hair dye sample analysis no matrix effect was observed, and therefore the peak current obtained after injection of the sample diluted as described in the Experimental section, was

Table 3
Comparison of the performance of amperometric HRP biosensors for the determination of hydrogen peroxide by flow-injection

Electrode	Redox mediator	Sample	E_{app} (V)	L.R. (mol L^{-1})	Sensitivity	LOD (mol L^{-1})	Useful lifetime	Reference
CPE	Toluidine blue *O*-acrylamide redox polymer	–	−0.05 vs. Ag/AgCl	1.0×10^{-6}–3.0×10^{-5}	180 mA mol^{-1} L cm^{-2}	5.0×10^{-8}	30 measurement days	[13]
SPE	Dimethylferrocene	Waters from disinfection processes	−0.30 vs. Ag/AgCl	–	–	49.5×10^{-6}	After 4 days losses initial sensitivity: −45.6% for 0.03 μmol L^{-1} H_2O_2; −3.5% for 50.0 μmol L^{-1} H_2O_2	[14]
CPE	Ferrocene	Milk	+0.05 vs. Ag/AgCl	1.0×10^{-8}–2.0×10^{-6}	6.47×10^{-7} nA mol^{-1} L	7.0×10^{-9}	Retains 57% of signal after 7 days	[33]
MPA-AuE	TTF	Milk, rain water and hair dye	0.00 vs. Ag/AgCl	2.0×10^{-7}–1.0×10^{-4}	2.33×10^{-2} A mol^{-1} L	6.9×10^{-8}	13 days	This work

AuE: gold electrode; CPE: carbon paste electrode; LOD: limit of detection; L.R.: linear range; MPA: 3-mercaptopropionic acid; SPE: screen-printed electrode; TTF: tetrathiafulvalene.

Table 4
Comparison of the performance of amperometric HRP biosensors based on SAMs for the determination of hydrogen peroxide

Electrode	Redox mediator	Sample	E_{app} (V)	Immobilization procedure	Analytical characteristics	Useful lifetime	Reference
Gold disk	Viologen	–	–	Enzyme is adsorbed onto a thiol functionalized viologen monolayer deposited on the electrode	–	–	[23]
Gold disk	–	–	0.00 vs. Ag/AgCl	Covalent with EDC onto a MPA-SAM	–	–	[19]
Gold wires (Ø = 0.5 mm)	–	–	–	Covalent with EDC onto cystamine-SAM or onto 3,3′-dithiopropionic acid bis-(*N*-hydroxysuccinimide)	–	–	[20]
Gold polycrystalline wires (Ø = 4.5 mm)	Thionine	–	−0.20 vs. SCE	Covalent immobilization of the enzyme and the mediator with GA onto a cysteamine-SAM	L.R.: 8.0×10^{-7}–4.0×10^{-5} mol L^{-1}; LOD: 8.0×10^{-7} mol L^{-1}	Retains more than 90% of activity after 1 week	[21]
Gold polycrystalline wires (Ø = 1.08 mm)	Thionine	–	−0.18 vs. SCE	Covalent immobilization of the enzyme and the mediator with GA onto a L-cysteine-SAM	L.R.: until 4.0×10^{-5} mol L^{-1}	L-Cysteine-SAM is stable during 1 week	[22]
Gold disk (Ø = 2.0 mm)	[Os(phen-dione)$(phen)_2$]$(PF_6)_2$	–	–	Covalent onto a DTSP-SAM	L.R.: until 4.0×10^{-4} mol L^{-1}	–	[11]
Gold polycrystalline wires	Catechol	–	−0.10 vs. SCE	Covalent inmobilization with GA onto a cysteamine-SAM	L.R.: 0.39×10^{-6}–0.33×10^{-3} mol L^{-1}; LOD: 0.15×10^{-6} mol L^{-1}	–	[2]
Gold polycrystalline wires	Thionine	–	−0.10 vs. SCE	Entrapment of the enzyme into poly(thionine) film on a thionine-SAM	L.R.: 6.2×10^{-6}–9.4×10^{-3} mol L^{-1}; LOD: 3.0×10^{-6} mol L^{-1}	Retains more than 85% of activity after 2 weeks	[28]
Polycrystalline gold	–	–	−0.30 vs. SCE	Adsorption of the enzyme on nanometer-sized Au colloids quimisorbed onto cysteamine-SAM	L.R.: 1.4×10^{-6}–9.2×10^{-3} mol L^{-1}; LOD: 5.8×10^{-7} mol L^{-1}	Retains more than 87% of activity after 2 weeks	[34]
Gold electrodes sealed in Kel-F	–	–	+0.05 vs. Ag/AgCl	Electrostatic interaction between the enzyme and a mixed SAM of 3-carboxypropyl disulfide or hexamethyl cystamine and 3,3′-dithiopropionic acid di-(*N*-succinimidyl ester)	Sensitivity: 0.023 mA mol^{-1} L	–	[35]
Gold wire	–	–	−0.25 vs. Ag/AgCl	Adsorption of the enzyme on nanometer-sized Au particles quimisorbed onto MPS-SAM	L.R.: 5.0×10^{-6}–1.0×10^{-2} mol L^{-1}; LOD: 2.0×10^{-6} mol L^{-1}	120 days	[36]
MPA-AuE	TTF	Milk, rain water and hair dye	0.00 vs. Ag/AgCl	Coimmobilization of the enzyme and the mediator with GA onto a MPA-SAM	L.R.: 2.0×10^{-7}–1.0×10^{-4} mol L^{-1}[a]; sensitivity: 2.33×10^{-2} A mol^{-1} L[a]; LOD: 6.9×10^{-7} mol L^{-1}[a]	13 days	This work

AuE: gold electrode; CPE: carbon paste electrode; DTSP: 3,3′-dithiobis-sulphosuccinimidylpropionate; EDC: 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; GA: glutaraldehyde; LOD: limit of detection; L.R.: linear range; MPA: 3-mercaptopropionic acid; MPS: (3-mercaptopropyl)-trimethoxysilane; SCE: saturated calomel electrode; TTF: tetrathiafulvalene.
[a] FIA measurements.

interpolated in an external calibration plot prepared in the 2.0×10^{-6}–1.0×10^{-5} mol L^{-1} H_2O_2 concentration range.

Concerning rainwater, no matrix effect was either observed, so interpolation of the signal obtained for the sample 1:1 diluted with the carrier solution in an external calibration plot (prepared between 2.0×10^{-7} and 1.0×10^{-5} mol L^{-1} H_2O_2) could be used.

The results obtained from five replicates of each sample are summarized in Table 2, where the confidence intervals were calculated for a significance level of 0.05. The results were compared with those obtained by using the Mottola's method [24]. As can be seen in Table 2 no significant differences between the results obtained by both methods were found, thus confirming the accuracy of the analytical methods involving the use of the HRP-TTF-MPA-AuE biosensor.

3.2. Comparison with other HRP-biosensors

The analytical performance of the HRP-TTF-MPA-AuE biosensor has been compared with that of other HRP electrochemical biosensors, capable to work also in flow systems, reported in the literature. Characteristics such as the type of electrode and mediator used, measurement potential, range of linearity of the corresponding calibration graph, sensitivity of this calibration plot, limit of detection achieved, and stability of the biosensors are summarized for all of them in Table 3.

As can be observed, the detection potential used with the HRP-TTF-MPA-AuE was the second less negative, which implies an improvement in selectivity, and the range of linearity achieved was as wide as the best ones reported previously. Moreover, only a ferrocene-CPE gave a better sensitivity [34], but the useful lifetime of the HRP-TTF-MPA-AuE was considerably longer.

Moreover, Table 4 shows the comparison of the HRP-TTF-MPA-AuE biosensors based on SAMs. Although the lack of data concerning characteristics such as linear range, limit of detection, sensitivity and useful lifetime avoids a wide comparison, it can be clearly deduced that, in general, the HRP-TTF-MPA-AuE compares advantageously with respect to other biosensor designs. Moreover, it should be noted that this is the first time that an amperometric HRP-biosensor based in the use of SAMs is used for the determination of hydrogen peroxide in flow systems and applied for the analysis of real samples.

4. Conclusions

All the results discussed above allow us to conclude that the new hydrogen peroxide biosensor based on a MPA-SAM in which the enzyme HRP and the redox mediator TTF were coimmobilized, exhibited, under FI conditions, a good sensitivity, stability and reproducibility when compared with other biosensor designs. This good analytical performance allowed its use for the amperometric flow injection determination of hydrogen peroxide in several samples to which only very simple and rapid treatments needed to be applied. Moreover, the rapid time of response allowing a sampling frequency of 75 samples/h, constituted another important advantage for applications in the food industry.

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