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A probe for NADH and H_2O_2 amperometric detection at low applied potential for oxidase and dehydrogenase based biosensor applications

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Abstract

Modified screen-printed electrodes for amperometric detection of H_2O_2 and nicotinamide adenine dinucleotide (NADH) at low applied potential are presented in this paper. The sensors are obtained by modifying the working electrode surface with Prussian Blue, a well known electrochemical mediator for H_2O_2 reduction. The coupling of this sensor with phenazine methosulfate (PMS) in the working solution gives the possibility of measuring both NAD(P)H and H_2O_2. PMS reacts with NADH producing PMSH, which in the presence of oxygen, gives an equimolar amount of H_2O_2. This allows the measurement of both analytes with similar sensitivity (357 mA mol^{-1} L cm^{-2} for H_2O_2 and 336 mA mol^{-1} L cm^{-2} for NADH) and LOD (5×10^{-7} mol L^{-1} for H_2O_2 and NADH) and opens the possibility of a whole series of biosensor applications.

In this paper, results obtained with a variety of dehydrogenase enzymes (alcohol, malic, lactate, glucose, glycerol and glutamate) for the detection of enzymatic substrates or enzymatic activity are presented demonstrating the suitability of the proposed method for future biosensor applications.

Keywords: Modified electrode; Biosensor; Oxidase enzyme; Dehydrogenase enzyme; NADH

1. Introduction

The amperometric detection of nicotinamide adenine dinucleotide (NADH) has been a matter of investigation for many years in the biosensor field (Gorton, 1986; Bartlett et al., 1991). The reason for this interest is related to the fact that NAD(P)H is the side product of more than 300 $NAD(P)^+$ dependent dehydrogenase enzymes. By coupling the desired dehydrogenase enzyme with an effective amperometric detector of NADH, it would then be possible to easily detect many important biological analytes.

However, the problem associated with amperometric detection of NADH is the high overpotential required (Schmakel et al., 1975) and the electrode fouling due to the presence of radical intermediates produced during NADH oxidation (Moiroux and Elving, 1979). For this reason, redox mediators have found a wide use in order to decrease the overvoltage required for NADH oxidation and to avoid fouling problems (Hayes and Kuhr, 1999; Katakis and Domiguez, 1997). Quinones (Carlson and Miller, 1985), aromatic diamines (Kitani et al., 1981), oxometalates (Essaadi et al., 1994), poly-metallophtalocyanines (Xu et al., 1994) and ruthenium complexes (Somasundrum et al., 1994) have given encouraging results. Another class of NADH mediators is represented by organic dyes, such as phenazines, phenoxazines and phenothiazines. The phenoxazine group includes compounds, such as Meldola's Blue (Gorton, 1986; Gorton et al., 1984; Appelqvist et al., 1985) and Nile Blue (Huck et al., 1984); phenothiazines include methylene Blue (Ye and Baldwin, 1988) and Toluidine Blue (Persson, 1990); phenazines include the compound 5-methylphenazinium methyl sulfate (PMS) (Ye and Baldwin, 1988; Amine and Kauffmann, 1992; Amine et al., 1993). PMS has been extensively used as a NADH mediator and also as an electron-transfer catalyst with many dehydrogenase enzymes (Kulys et al., 1980; Kulys, 1981; Schuhmann et al., 1993; Gründig et al., 1995). The use of PMS as electron mediator has usually been accomplished by the adsorption onto an electrode surface. In such cases, PMSH produced after the reaction of PMS with NADH (Eq. (1)) is reoxidised at the electrode to PMS giving a detectable oxidative signal.

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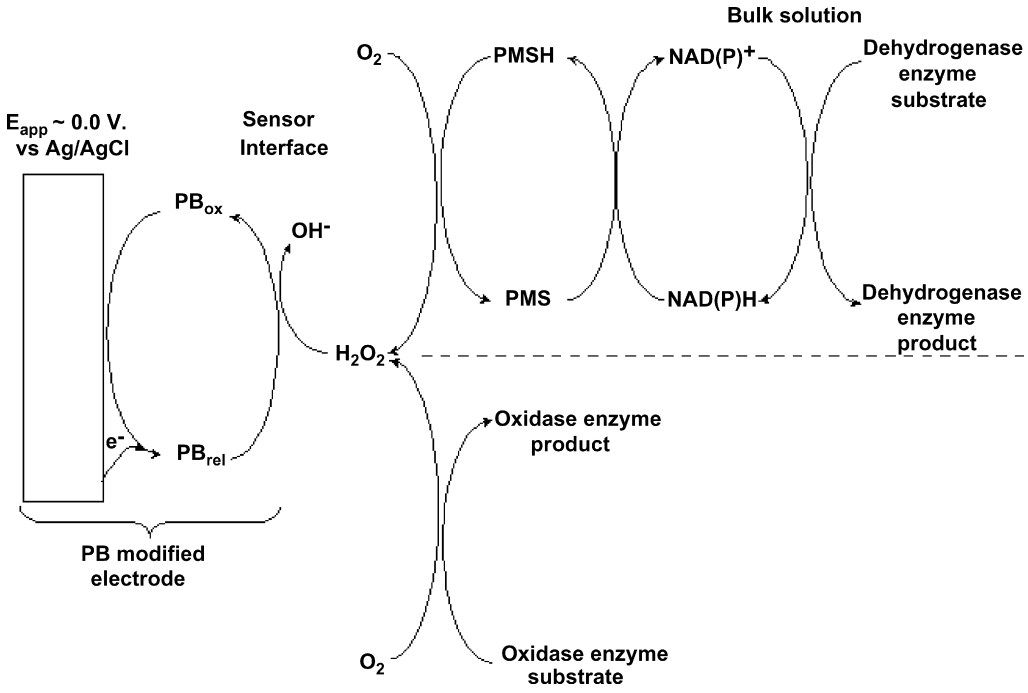

Scheme 1. Schematic representation of the system adopted for NADH detection. In the upper part of the scheme is shown the reaction chain for the detection of NAD(P)H as in the case of a NAD(P) dependent dehydrogenase enzyme. In the lower part of the scheme is shown the classic reaction utilised in the case of an oxidase enzyme. In both cases, the final product is H_2O_2, which is measured at the Prussian Blue modified electrode at an applied potential of ca. 0.0 V vs. Ag/AgCl.

However, if PMS is not in direct contact with the electrode, but solubilised in an aqueous solution, the PMSH formed has a very short-life time due to the rapid reaction with the oxygen present in solution (Eq. (2)). The reaction between PMSH and O_2 is well known and produces an equimolar concentration of H_2O_2 (Halaka et al., 1982).

$$PMS^+ + NADH \rightleftarrows PMSH + NAD^+ \quad (1)$$

$$PMSH + O_2 + H^+ \rightarrow PMS^+ + H_2O_2 \quad (2)$$

Previous papers have reported the amperometric detection of NADH based on the use of PMS in solution. NADH concentration was related to the final production of H_2O_2 (Eqs. (1) and (2)) which was measured via a peroxidase (POD) based sensor (Vreeke et al., 1992).

In this paper, we propose the use of a similar system where PMS, free in solution, reacts with NADH to rapidly produce H_2O_2 whose final detection will be accomplished by means of a Prussian Blue modified screen-printed electrode.

This latter probe has already been investigated and optimised as reported in a previous paper which described the stability, sensitivity and selectivity of PB modified screen-printed electrodes towards H_2O_2 detection at low applied potential (Ricci et al., 2003).

In our opinion this approach presents some interesting and new features. First of all, there is no problem due to lack of the NADH mediator since the mediator is in solution, and it also offers the possibility for the mediator to work in the proper way, as it is present under optimal conditions (Prieto-Simon and Fabregas, 2004) (high concentration with free mobility). Also the fact that the reaction occurs in bulk solution would avoid the fouling problem usually observed with NADH oxidation. The most positive feature is, however, represented by the fact that the final detector used in this case is an H_2O_2 probe which works at a low applied potential (0.0 V). It can be claimed that this probe could represent a sort of "universal" sensor for biosensor application (see Scheme 1). It would in fact be possible to easily couple the desired oxidase or dehydrogenase enzyme to this system in order to detect a number of important analytes with high sensitivity. In comparison to what is generally observed with peroxidase-based sensors, this approach provides a major advantage of lower cost, due to the inorganic nature of Prussian Blue; a higher stability and ease of modification (Ricci et al., 2003). Moreover, the Prussian Blue modified electrodes will overcome the interference problems encountered in the case of the peroxidase-based sensor.

The analytical features of this system for NADH detection will be presented in this paper. Optimisation of pH, concentration of the mediator and reaction time are illustrated and discussed. Moreover, some dehydrogenase enzymes applications, which demonstrate the suitability of this approach for substrate and enzymatic activity measurements will be discussed.

2. Experimental

2.1. Apparatus

Amperometric measurements were carried out using a VA 641 amperometric detector (Metrohm, Herisau, Switzerland), connected to a *X–t* recorder (L250E, Linseis, Selb, Germany).

Cyclic voltammetry (CV) was performed using an Autolab electrochemical system (Eco Chemie, Utrecht, The Netherlands) equipped with PGSTAT-12 and GPES software (Eco Chemie).

2.2. Electrodes

Screen-printed electrodes were produced in our laboratory using a 245 DEK (Weymouth, England) screen-printing machine as previously described (Ricci et al., 2003). Graphite-based ink (Elettrodag 421), silver ink (Electrodag 477 SS RFU) and insulating ink (Elettrodag 6018 SS) were used and were purchased from Acheson (Milan, Italy). The diameter of the working electrode was 0.3 cm, resulting in a geometric area of 0.07 cm^2.

2.3. Reagents

All chemicals from commercial source were of analytical grade. All solutions were prepared with 0.05 mol L^{-1} phosphate buffer + 0.1 mol L^{-1} KCl, pH 7.4 unless otherwise stated.

Phenazine methosulfate (PMS), L-glutammic acid sodium salt, L-lactic acid sodium salt, D-glucose, ethanol, malic acid, β-nicotinamide adenine dinucleotide phosphate (NADP), β-nicotinamide adenine dinucleotide (NAD), β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), β-nicotinamide adenine dinucleotide reduced form (NADH) and glycerol were purchased from Sigma–Aldrich (Sigma, Italy). Malic enzyme (EC 1.1.1.40, 26 U/mg), glucose dehydrogenase (EC 1.1.1.47, 216 U/mg), alcohol dehydrogenase (EC 1.1.1.1, 451 U/mg), glutamate dehydrogenase (EC 1.4.1.3, 39.5 U/mg), lactate dehydrogenase (EC 1.1.1.27, 626 U/mg), glycerol dehydrogenase (EC 1.1.1.6, 65 U/mg) were purchased from Sigma–Aldrich (Sigma, Italy).

2.4. Preparation of PB modified screen-printed electrodes

PB modification of SPEs was accomplished by placing a drop (10 μL total volume) of "precursor solution" onto the working electrode area. This solution is a mixture obtained by adding 5 μL of 0.1 mol L^{-1} potassium ferricyanide ($K_3Fe(CN)_6$) in 10^{-2} mol L^{-1} HCl to 5 μL of 0.1 mol L^{-1} ferric chloride in 10^{-2} mol L^{-1} HCl. Care was taken to apply the drop exclusively on the working electrode area. After 10 min, the electrodes were rinsed with a few millilitres of 10^{-2} mol L^{-1} HCl. The probes were then left 90 min in the oven at 100 °C to obtain a more stable and active layer of Prussian Blue (Ricci et al., 2003).

2.5. NADH detection

NADH amperometric detection was performed using amperometric batch analysis in a stirred phosphate buffer solution 0.05 mol L^{-1} + KCl 0.1 mol L^{-1}, pH 7.4 (10 mL) with an applied potential of −50 mV versus internal reference electrode (int. ref.). The PB-SPE was dipped in the buffer containing a PMS concentration of 10^{-4} mol L^{-1}. After a stable background current is observed (20 s), NADH is added to the solution and the formation of H_2O_2 is followed until a stable signal is reached (ca. 2 min).

2.6. Enzymatic substrate measurement using cyclic voltammetry

When cyclic voltammetry (CV) was used for substrate measurement a much lower sample volume was needed. One hundred microliters were in fact sufficient to completely cover the three screen-printed electrodes. This allows the use of higher concentration of enzyme. Alcohol, phenylalanine and lactate dehydrogenase were tested with this technique. The enzymes (20 units) were dissolved in 100 μL buffer solution (phosphate buffer 0.05 mol L^{-1} + KCl 0.1 mol L^{-1} + PMS 10^{-4} mol L^{-1}, pH 7.4) containing 10^{-3} mol L^{-1} of NAD^+. A first CV (scan rate 2 mV/s) was recorded with this buffer composition. After this, the substrate was added and new CVs were recorded at different time intervals.

For alcohol and phenylalanine dehydrogenase 10^{-2} mol L^{-1} of substrate (ethanol and phenylalanine) were added, while for lactate dehydrogenase 5×10^{-2} mol L^{-1} of lactate was used.

2.7. Enzymatic substrate measurement using batch amperometry

Enzymatic substrate measurement with glutamate, glucose and glycerol dehydrogenases was also performed using amperometric batch analysis in a stirred phosphate buffer solution 0.05 mol L^{-1} + KCl 0.1 mol L^{-1} (10 mL) with an applied potential of −50 mV versus internal reference electrode. The PB-SPE was dipped in the buffer containing $NAD(P)^+$ and the dehydrogenase enzyme together with PMS (10^{-4} mol L^{-1}).

When a stable baseline was reached the substrate was added and the formation of NADH was measured as described above for NADH detection. For glutamate dehydrogenase, the enzyme concentration in the working buffer (pH 8.8) was 4 U mL^{-1}, NAD^+ was 10^{-3} mol L^{-1}. For glycerol dehydrogenase (pH 8.5), the concentration of the enzyme was 10 U mL^{-1} and NAD^+ was 10^{-3} mol L^{-1}. For glucose dehydrogenase (pH 7.4), the concentration of the enzyme was 1 U mL^{-1} and $NADP^+$ was 10^{-3} mol L^{-1}.

2.8. Dehydrogenase activity assay

Also in the case of enzymatic activity assay a batch amperometry ($V = -0.05$ V versus int. ref.) in a stirred solution was used. NAD^+ (10^{-3} mol L^{-1}) and PMS (10^{-4} mol L^{-1}) were first added to the buffer solution together with different concentrations of dehydrogenase enzymes (lactate, alcohol and malate dehydrogenase). When a stable baseline was reached, a saturing concentration of substrate (2×10^{-2} mol L^{-1} of lactate, 5×10^{-4} mol L^{-1} of ethanol and 10^{-3} mol L^{-1} of malic acid) was added and the signal due to the formation of NADH was measured after 10 min.

2.9. Glycerol biosensor

A glycerol biosensor was obtained by immobilising glycerol dehydrogenase on the surface of a PB modified SPE. This was accomplished using a cross-linking method with glutaraldheyde and nafion as already reported in Ricci et al. (2003). The final glycerol dehydrogenase content on the electrode was ca. 7 units. The glycerol biosensor was tested with a drop measurement. After placing a drop (100 μL) of phosphate buffer solution (pH 8.5) + KCl ($0.1\,mol\,L^{-1}$) + PMS ($10^{-4}\,mol\,L^{-1}$) and NAD^+ ($2 \times 10^{-3}\,mol\,L^{-1}$) on the screen-printed electrode held in horizontal position, a potential of −50 mV was applied. After achieving a stable baseline response, glycerol was added at different concentration and current responses as a function of time were recorded.

3. Results and discussion

3.1. NADH amperometric detection

To perform NADH amperometric detection the principle reported in Scheme 1 (upper part) is proposed. NADH, under certain conditions, readily reacts with the PMS present in solution to give NAD^+ and PMSH. As already pointed out in Section 1, PMSH reacts with oxygen giving H_2O_2, which could be easily measured at a low applied potential at the PB modified screen-printed electrode. Preliminary experiments were carried out in order to determine the best conditions for the reaction between NADH and PMS. Before this, however, it was important to study the possible interaction between PMS and the modified electrode surface in order to make sure that there are no secondary reactions occurring in the detection system that would interfere with the signal. Being a strong electron mediator and given the presence of PB, it is in fact possible that PMS could give some kind of signal at the electrode surface making the final measurement of H_2O_2 more difficult.

To investigate this possibility, both batch amperometry and cyclic voltammetry were used. In the first case, PMS was added at different concentrations in a phosphate buffer solution and the signal obtained at a PB modified SPE was recorded after each injection of PMS. For a concentration up to $10^{-4}\,mol\,L^{-1}$ no appreciable signal was detected at the potential tested (−50 mV versus int. ref.). This means that the presence of PMS, at concentration as high as $10^{-4}\,mol\,L^{-1}$, would not affect the subsequent H_2O_2 measurements. The same behaviour was observed in a CV experiment, where no difference in the classic CV shape of a PB modified electrode was observed after the addition of PMS to the buffer solution. The CV experiment was also used to demonstrate that no interaction occurs between Prussian Blue and NADH. Prussian Blue is in fact an effective electron mediator that has not been used just for H_2O_2 detection. It has been found that Prussian Blue has catalytic activity toward the oxidation of several analytes (such as, for example, thiols) (Ricci et al., 2004; Ricci and Palleschi, 2005) and in this perspective it has found several applications. The CV obtained after the addition of NADH in the absence of PMS shows no difference with respect to the CV obtained in buffer solution, thus demonstrating that Prussian Blue has no catalytic activity towards NADH at least in this potential range (data not shown). The same result was also obtained in batch amperometry where no signal was observed for concentrations of NADH up to $5 \times 10^{-3}\,mol\,L^{-1}$ (pH 7.4, $V = -0.05$ V versus int. ref.).

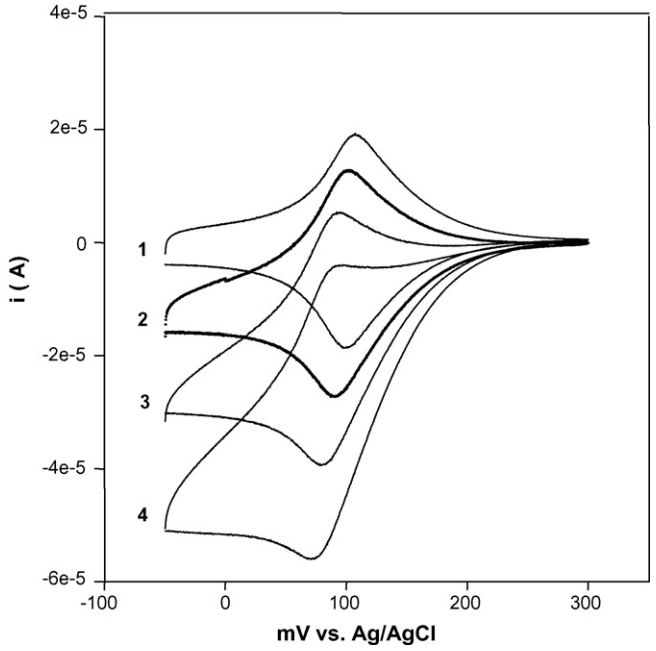

Fig. 1. Cyclic voltammetry of Prussian Blue modified electrode. Phosphate buffer $0.05\,mol\,L^{-1}$ + KCl $0.1\,mol\,L^{-1}$, pH 7.4. Scan rate 50 mV/s. CV obtained in buffer + PMS $10^{-4}\,mol\,L^{-1}$ in absence (1) and presence of $2.5 \times 10^{-4}\,mol\,L^{-1}$ (2), $5 \times 10^{-4}\,mol\,L^{-1}$ (3) and $10^{-3}\,mol\,L^{-1}$ (4) of NADH.

The CV recorded after the addition of different concentrations of NADH in a buffer solution containing PMS are shown in Fig. 1. In this case, the reduction current increases linearly with the concentration of NADH added and the CVs have the classic shape of a mediated reaction at the electrode surface. This is due to the formation of H_2O_2 via the NADH-PMS reaction as depicted in Scheme 1.

3.1.1. Study of the concentration of PMS

The NADH-PMS reaction was then studied in order to determine the best operative conditions relative to PMS concentration and pH of the buffer solution in a batch amperometric system.

First, the PMS was added in the buffer solution and, when a stable background was reached, two fixed concentrations (2×10^{-6} and $10^{-5}\,mol\,L^{-1}$) of NADH were added at different times. The production of H_2O_2 was measured after 2 min from the injection of NADH. For comparison the signal due to $10^{-5}\,mol\,L^{-1}$ H_2O_2 was also recorded. The concentrations of PMS tested varied in a range between 10^{-6} and $2.5 \times 10^{-4}\,mol\,L^{-1}$. At a concentration lower than $2.5 \times 10^{-5}\,mol\,L^{-1}$ the signal obtained 2 min after the injection of NADH is not as high as that obtained at higher concentrations of PMS and is still lower than that obtained for H_2O_2 under the same condition. This means that the reaction was still not complete and requires a longer time. At a PMS concentration of $10^{-4}\,mol\,L^{-1}$ the signal obtained after 2 min is the same as that

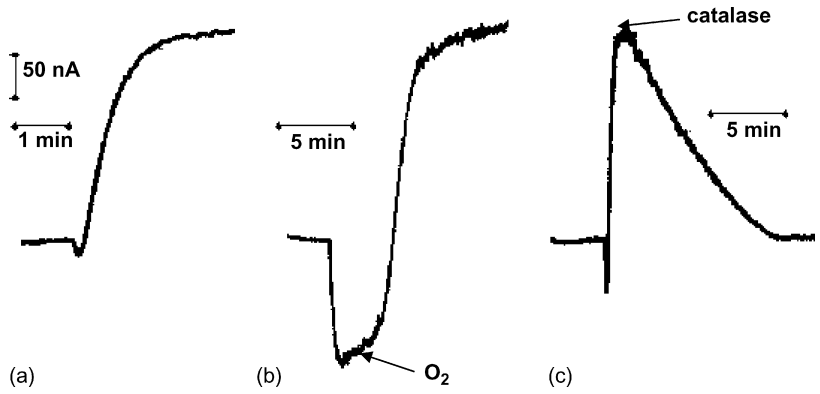

Fig. 2. Original recordings obtained using batch amperometry in phosphate buffer solution 0.05 mol L^{-1} + KCl 0.1 mol L^{-1}, pH 7.4 + PMS 10^{-4} mol L^{-1}. Applied potential = −0.05 V vs. int. ref. (a) Signal recorded after the injection of 10^{-5} mol L^{-1} NADH. (b) Signal recorded after the injection of 10^{-5} mol L^{-1} NADH in a deoxygenated solution; the arrow indicates when the N_2 flow was stopped allowing the entrance of oxygen into the working solution. (c) Signal recorded after the injection of 10^{-5} mol L^{-1} NADH; the arrows indicates when the catalase enzyme (1 U mL^{-1}) was added to the solution.

obtained for higher concentration of PMS and almost the same as that recorded for the same concentrations of H_2O_2, indicating that the reaction was complete. This is also confirmed by the fact that the signal is stable and does not increase further with time. At higher concentrations of PMS no appreciable improvement of the reaction rate was observed while an increased noise level was observed. For this reason, 10^{-4} mol L^{-1} of PMS was chosen for further studies.

During this experiment, the dynamics of the reaction between PMS and NADH were also studied. The recordings obtained in batch amperometry show that immediately after the addition of NADH into the buffer solution containing PMS, there is a rapid oxidation peak-shaped signal (Fig. 2a). This signal rapidly disappears to give way to a reduction signal, which becomes stable after 2 min. This dynamic is related to the intermediates resulting from the overall reaction between PMS and NADH. The reaction proceeds rapidly to the production of PMSH (Eq. (1)) (second-order rate constant $k_1 = 2.6 \times 10^3$ mol^{-1} L s^{-1}) and with a slower rate PMSH reacts with oxygen to give H_2O_2 (second-order rate constant $k_2 = 160$ mol^{-1} L s^{-1}) (Eq. (2)) (Halaka et al., 1982). The oxidation peak observed after the addition of PMS can probably be attributed to the electroactive PMSH formed during Eq. (1). This is the reason why the oxidation peak is formed very rapidly (due to the rapid reaction between NADH and PMS) and then disappears with slower kinetics. In order to demonstrate this, the same experiment was carried out in a degassed solution with a constant nitrogen flow to prevent oxygen intrusion. After the addition of NADH in this solution, an appreciable oxidation signal was observed (Fig. 2b) and was stable, indicating that the species detected was not undergoing a secondary reaction. By stopping the nitrogen flow to allow oxygen to penetrate the solution, the oxidation signal disappeared slowly, making place for a slow reduction response (Fig. 2b). In this case, the time needed to reach a stable reduction signal was greater than 2 min, probably due to the fact that oxygen only slowly reaches its maximum concentration in the solution. However, the fact that a reduction signal is observed at the surface of the PB modified electrode after the addition of NADH in the presence of oxygen and PMS, is still not proof that this signal is due to the formation of H_2O_2. In order to demonstrate this, after the addition of NADH and the subsequent increase of the reduction signal till a stable value, a large amount of catalase enzyme was added to the solution (Fig. 2c). The fact that the reduction signal, after the injection of catalase, decreased to the initial background value provided confirmation. This series of experiments thus effectively showed that the NADH in presence of PMS goes to H_2O_2 through the formation of PMSH, which subsequently reacted with oxygen. At the concentrations chosen, the overall reaction was complete in 2 min.

The fact that there was an equal response to the same concentration of H_2O_2 and NADH in the presence of PMS (10^{-4} mol L^{-1}), as previously discussed, again demonstrates the equimolar conversion of NADH into H_2O_2 via the reaction with PMS.

3.1.2. Study of the pH dependence

It is well known that the reaction between PMS and NADH is highly dependent on the pH of the solution. The dependence of the NADH signal on the variation of pH in a range between 3 and 10 was then studied. For comparison, the signal due to the same concentration of H_2O_2 was compared. At low pH values (3–5) the reaction between PMS and NADH proceeds at a slow rate and either no or a low signal is observed after 2 min. At pH 6, the conversion of NADH into H_2O_2 calculated from the signal recorded after 2 min is about 70%. At pH 7, almost the same signal is observed for H_2O_2 and NADH, indicating that NADH is totally converted to H_2O_2 during the reaction time (2 min). The same behaviour is observed for higher pH values but, given the fact that the rate limiting step (Halaka et al., 1982) is the reaction with oxygen (Eq. (2)), only a small change in the time needed to get a stable signal is observed. Although this system is not suitable for use at acid pH solution, it still has a wide application given the fact that the majority of dehydrogenase enzymes work at neutral or basic conditions.

3.1.3. NADH amperometric detection and interference study

A NADH calibration plot was then recorded in a batch amperometric mode in pH 7.4 phosphate buffer solution. The analytical parameters obtained for NADH and hydrogen

Table 1
Analytical parameters of NADH and H_2O_2 batch amperometric response using PB modified screen-printed electrode

	Linear range (mol L^{-1})	LOD (mol L^{-1})	Sensitivity (mA mol^{-1} L cm^{-2})	Response time (s)	Time to stabilise the baseline current (s)
NADH	10^{-6} to 10^{-4}	5×10^{-7}	336	95	15
H_2O_2	10^{-6} to 10^{-3}	5×10^{-7}	357	5	15

$V = -0.05$ V vs. int. ref. Phosphate buffer 0.05 mol L^{-1} + KCl 0.1 mol L^{-1}, pH 7.4 + PMS 10^{-4} mol L^{-1}.

peroxide are summarised in Table 1. While the range of linearity for H_2O_2 extends up to 10^{-3} mol L^{-1}, in the case of NADH, due to the fact that a chemical reaction is involved and that the concentrations of PMS and also O_2 are limiting, the linear range is only up to 10^{-4} mol L^{-1}. In spite of this, the sensitivity (357 mA mol^{-1} L cm^{-2} for H_2O_2 and 336 mA mol^{-1} L cm^{-2} for NADH) is almost the same, thus once again confirming the total conversion of NADH into H_2O_2. Also the response time, as expected, is different. While for H_2O_2 5 s are sufficient to obtain 90% of the steady state signal, 95 s are needed in the case of NADH. In this context, it can be noted that NADPH has also been tested under the same conditions optimised for NADH, and identical behaviour was observed. This means that the reaction between NADPH and PMS has the same rate and proceeds in a comparable way of that between NADH and PMS.

The stability of the signal due to the formation of H_2O_2 in the presence of PMS is comparable with that obtained in a simple buffer solution with H_2O_2 demonstrating that no secondary reactions take place between H_2O_2 and PMS. The only problem relative to the system stability could be due to the fact that PMS is photosensitive (Halaka et al., 1982; Marzotko et al., 1973). At high pH values the spontaneous photochemical oxidation of PMS leads to the formation of equimolar amounts of pyocyanine and PMSH (Halaka et al., 1982). This latter will produce a detectable amount of H_2O_2 not derived from the reaction with NADH, thus interfering with its final detection. However, this reaction is slow and the formation of H_2O_2 due to the photochemically produced PMSH is not detectable until 30–60 min.

Also no problem of electrode fouling was observed and the electrode could be reused without any loss of sensitivity, maintaining its original stability characteristic already discussed and studied in a previous paper (Ricci et al., 2003).

The fact that PMS could react with species other than NADH producing H_2O_2 calls for a special study of the possible interferents of this system. While uric acid, thiols and acetaminophene give no signal at the selected conditions (pH 7.4 + PMS 10^{-4} mol L^{-1}), a very high signal, comparable to those obtained with H_2O_2 and NADH is observed for ascorbic acid. Given the fact that PB modified electrodes have already been demonstrated to be almost interference free (Ricci et al., 2003), this signal has to be ascribed to the reaction between ascorbic acid and PMS. This seems to be confirmed by the fact that the signal after the addition of ascorbic acid develops very slowly and reaches its maximum value after 15 min. This means that the signal observed is not due to a direct interaction between ascorbic acid and the electrode surface and that the reaction between ascorbic acid and PMS proceeds with a slower rate relative to that observed for the reaction between PMS and NADH. In addition, sodium sulfite show a strong interaction with PMS giving a signal 40% of that from NADH. Thiols were also tested since it is well known that PMS easily reacts with these species but no response was observed for cysteamine, glutathione and cysteine. In the eventual coupling with dehydrogenase enzymes, the test solution will have to be added with NAD^+ and its effect on the system has to be carefully evaluated. For NAD^+ concentrations up to 2×10^{-3} mol L^{-1}, no appreciable signal was observed at the Prussian Blue modified electrodes, demonstrating the suitability of using such a system coupled with dehydrogenase enzymes.

3.2. Dehydrogenase enzymes substrates measurement

The system proposed here for NADH amperometric detection at low applied potential has been then preliminarily tested in conjunction with a series of dehydrogenase enzymes. The enzymes, together with their principal cofactor $NAD(P)^+$, were initially used free in solution at their optimum pH.

3.2.1. Cyclic voltammetry experiments

Cyclic voltammetry experiments were first adopted to give only a qualitative response, which indicates whether or not this system could be used with the selected dehydrogenase enzymes.

In Fig. 3 are reported the CVs obtained using alcohol dehydrogenase with ethanol as substrate and recording the CV at different intervals from the addition of the substrate. The increase of the reduction wave indicates that NADH formation follows the addition of the enzyme substrate (10^{-2} mol L^{-1}) and proceeds with a similar rate for 15 min. Similar behaviour was observed for lactate, phenylalanine and glutamate dehydrogenase showing that, also for these enzymes, a coupling with PMS is possible.

To demonstrate this, three enzymes were selected as model enzymes to be studied in batch amperometric mode.

3.2.2. Batch amperometric mode

In this case, the classic stirred batch amperometry mode with a 10 mL solution was used and the enzyme was suspended at high concentrations in the buffer together with its cofactor. The addition of different concentrations of substrate was followed by the enzymatic formation of NADH, which, via the reaction with PMS led to equimolar formation of H_2O_2. Glutamate (pH 8.8), glucose (pH 7.4) and glycerol (pH 8.5) were detected using glutamate, glucose and glycerol dehydrogenase, respectively. Fig. 4a shows the original recording obtained for glutamate. The enzymatic reaction proceeds up to a limiting value of 8×10^{-5} mol L^{-1}. The detection limit calculated (S/N = 3) is 10^{-6} mol L^{-1} with the sensitivity being

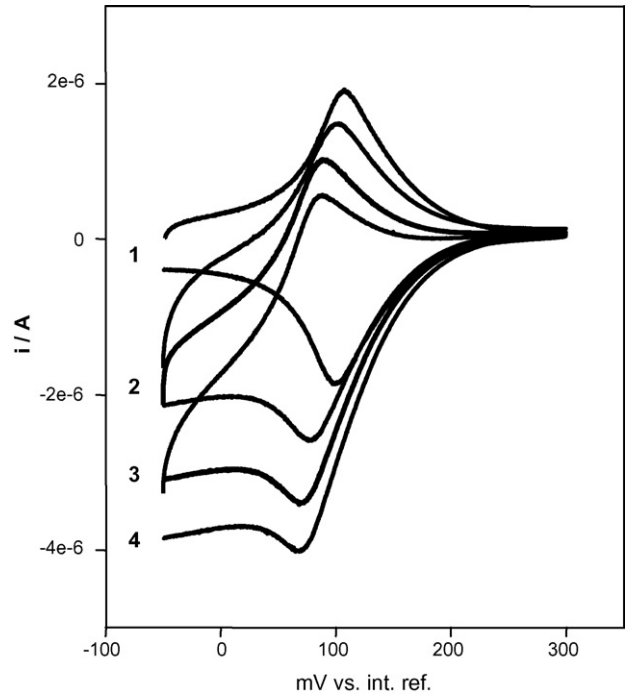

Fig. 3. Cyclic voltammetry revealing dehydrogenase activity with PMS. Prussian Blue modified electrode in phosphate buffer 0.05 mol L^{-1} + KCl 0.1 mol L^{-1}, pH 7.4. Scan rate 2 mV/s. CV obtained in buffer + alcohol dehydrogenase (20 units/100 μL) + PMS 10^{-4} mol L^{-1} + NAD^+ 10^{-3} mol L^{-1} (1) and after 5 min (2), 10 min (3) and 15 min (4) from the addition of 10^{-2} mol L^{-1} ethanol.

250 mA mol^{-1} L cm^{-2}. With glucose dehydrogenase the sensitivity was almost the same (220 mA mol^{-1} L cm^{-2}) but with a higher detection limit (2×10^{-6} mol L^{-1}) and a more limited linear range (up to 4×10^{-5} mol L^{-1}). In the case of glycerol measurement, the sensitivity was lower (190 mA mol^{-1} L cm^{-2}) and a detection limit of 5×10^{-6} mol L^{-1} was calculated.

In all the cases, the time needed to reach a stable signal was longer than two minutes due to the fact that an enzymatic reaction was also involved. In the case of glutamate (4 U mL^{-1}) and glycerol (10 U mL^{-1}), 5 min were needed to reach a stable signal while for glucose (1 U mL^{-1}) 8 min were required.

It is important to stress the fact that these results have been obtained under conditions (enzyme and cofactor concentrations, pH buffer, etc.), which have not been thoroughly investigated. These preliminary investigations are in fact only designed to demonstrate the possibility of integrating and coupling a dehydrogenase enzyme with PMS and an effective H_2O_2 sensor for the eventual measurement of many important analytes.

The confirmation that this system could be used with dehydrogenase enzymes opens up to wide number of possible biosensor applications. As depicted in Scheme 1, the fact that the H_2O_2 probe used here could be adopted both with an oxidase and a dehydrogenase enzyme makes it suitable for almost all of the enzymatic biosensors reported to date. We have then tried to demonstrate this possibility by using two different enzymes, one of the oxidase (glucose oxidase) and the other of the dehydrogenase class (glutamate dehydrogenase) in the same buffer solution. After the background current was stabilised, glucose (2×10^{-5} mol L^{-1}) was first added to the solution and the signal due to the formation of H_2O_2 was continuously recorded (Fig. 4b). When a stable signal was observed, glutamate was added (2×10^{-5} mol L^{-1}) and the signal due to the formation of NADH was recorded. The fact that a similar signal was observed for both analytes demonstrates that the enzymes were not affected by the buffer composition (presence of NAD^+ and PMS and the non-optimum pH for GOx) and that the H_2O_2 sensor utilised here is suitable for coupling with both oxidase

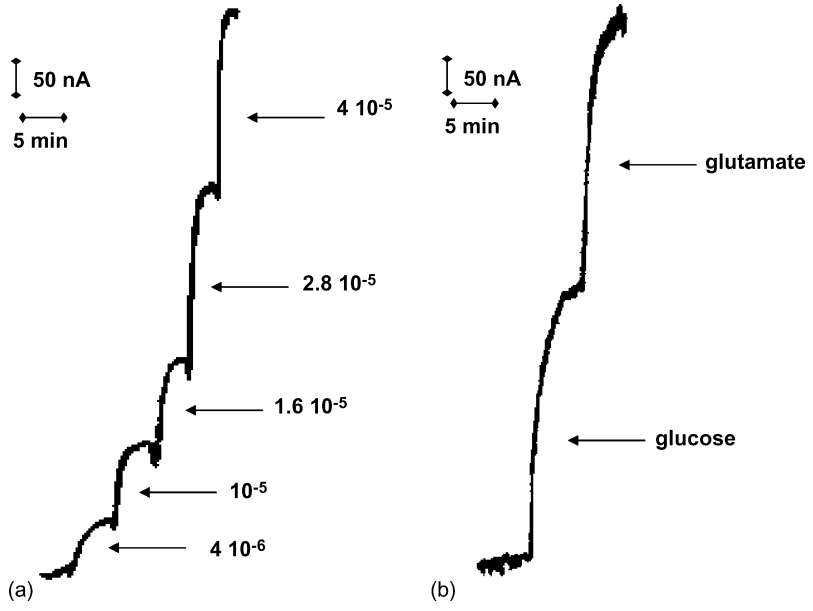

Fig. 4. Original recordings obtained in stirred batch amperometry: $V = -0.05$ V vs. int. ref. Phosphate buffer 0.05 mol L^{-1} + KCl 0.1 mol L^{-1} + NAD^+ 10^{-3} mol L^{-1} + PMS 10^{-4} mol L^{-1}, pH 8.8. (a) Glutamate detection. Glutamate dehydrogenase concentration, 4 U mL^{-1}. With the arrows are indicated the final concentrations of glutamate in the buffer. (b) Glutamate and glucose detection. Glutamate dehydrogenase concentration, 4 U mL^{-1}. Glucose oxidase concentration 1.0 U mL^{-1}. Glucose and glutamate concentration added 2×10^{-5} mol L^{-1}.

and dehydrogenase enzymes. This demonstrates the possibility of making simultaneous determinations of mixtures of oxidase and dehydrogenase substrates in the same sample.

3.3. Glycerol biosensor

The possibility of adopting the proposed method for biosensor applications has also been demonstrated with a glycerol biosensor obtained by immobilising the enzyme glycerol dehydrogenase on the surface of the PB modified SPE. In this case, the amount of the enzyme immobilised on the surface of the electrode is ca. 7 units. However, the immobilisation via a crosslinking method always leads to a partial inactivation of the enzyme thus decreasing the overall enzymatic activity. This means that in a 10 mL solution the enzymatic activity will be too low to observe the formation of NADH in a reasonable time. To overcome this problem, the probe was tested with a drop amperometry technique as reported in Section 2. The biosensor showed a linear response to glycerol in the concentration range of 5×10^{-5}–2×10^{-3} mol L^{-1} with a detection limit of 5×10^{-5} mol L^{-1}. In this case, the sensitivity (7.5 mA mol^{-1} L cm^{-2}) is lower with respect to that observed for the batch amperometry mode due to the different technique adopted and also, due to the low volume employed, the time needed to reach a stable signal is higher (5 min).

3.4. Dehydrogenase activity assay

The system proposed in this paper has also been adopted for the determination of the enzymatic activity of some dehydrogenase enzymes. Some assay kits based on a similar approach are already commercially available (Fairbanks and Beutler, 1962; Hirono et al., 1997; Fujii et al., 1984). These systems are always based on the final spectroscopic detection of enzymatically produced NADH at 340 nm or on the coupling of PMS with tetrazolium salt. Similarly, in this work the rate of production of NADH is measured via the reaction of Eq. (2) with the final detection of H_2O_2. Given the fact that with low concentrations of enzyme the production of NADH proceeds at a relative slow rate, the reaction between NADH and PMS and between PMSH and oxygen is not rate limiting. This assures the possibility of relating the rate of NADH production to the rate in H_2O_2 formation. The working buffer solution was prepared with different concentrations of dehydrogenase enzymes and with a high amount of cofactor ($NAD(P)^+$). When a stable baseline was reached, a saturating concentration (two to three times the K_m value) of substrate was added to the solution and the signal was continuously recorded for 10 min. Fig. 5 shows the calibration plots obtained for malic enzyme, alcohol and lactate dehydrogenase. For all the enzymes tested, good sensitivity on the order of mU mL^{-1} has been achieved.

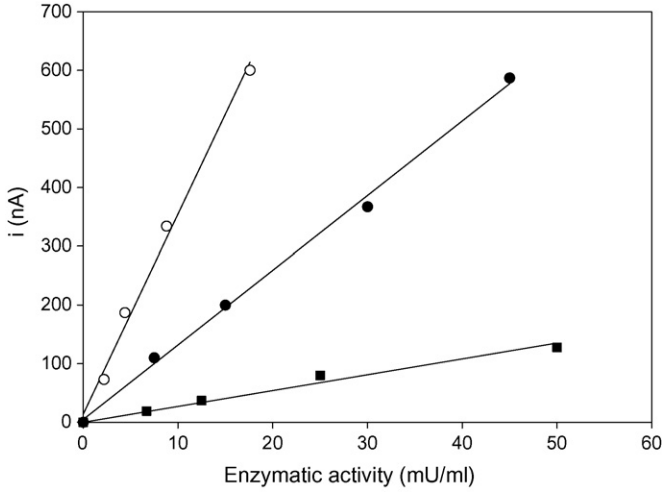

Fig. 5. Measurement of enzymatic activity. $V = -0.05$ V vs. int. ref. Phosphate buffer 0.05 mol L^{-1} + KCl 0.1 mol L^{-1}, pH 7.4 + PMS 10^{-4} mol L^{-1} + NAD^+ 10^{-3} mol L^{-1}. Signal recorded after 10 min from the addition of the substrate (see below for concentrations). (■) Lactate dehydrogenase: 2×10^{-2} mol L^{-1} of lactate. (●) Alcohol dehydrogenase: 5×10^{-4} mol L^{-1} of ethanol. (◯) Malate dehydrogenase: 10^{-3} mol L^{-1} of malic acid.

4. Conclusion

In this paper, a system based on the use of PMS in solution coupled with an H_2O_2 probe based on Prussian Blue modified electrodes has been demonstrated for the final detection of NADH at low applied potential. A detection limit of 5×10^{-7} mol L^{-1} together with a linear range up to 10^{-4} mol L^{-1} and a sensitivity of 336 mA mol^{-1} L cm^{-2} was obtained for NADH.

CV experiments were adopted to show the suitability of the system for use with phenylalanine, alcohol and lactate dehydrogenases.

The probe was demonstrated to be suitable for detection of the substrates of dehydrogenase enzymes with batch amperometry as in the case of glucose, glycerol and glutamate using as model enzymes glucose, glycerol and glutamate dehydrogenases. Detection limits were, respectively, 2×10^{-6}, 5×10^{-6} and 10^{-6} mol L^{-1} with a sensitivity of 220, 190, 250 mA mol^{-1} L cm^{-2}. In the case of glycerol, the enzyme was also tested as immobilised on the PB modified electrode surface. The results demonstrated the possibility of applying this method for biosensor purposes.

In addition, the use of PMS coupled with Prussian Blue modified electrodes was tested for the detection of the enzymatic activity of some dehydrogenase enzymes (i.e. alcohol, lactate and malic enzyme) and it gave detectable signals at the mU mL^{-1} range.

The most significant aspect of this work, in our opinion, is represented by the fact that an effective H_2O_2 probe based on the use of PB has allowed the amperometric detection of NADH at low applied potential. This makes the Prussian Blue modified electrode a sort of "universal" probe which could be adopted in a very wide range of biosensor applications. When an oxidase enzyme is used, the H_2O_2 enzymatically produced will be directly monitored by the means of the mediator.

On the other hand, in the case of a dehydrogenase enzyme, the production of NADH will be followed using its reaction with PMS and the subsequent production of H_2O_2 to generate the electrochemical signal. This is the first time, to our knowledge,

that the same ultimate H_2O_2 detector has been used with oxidase and dehydrogenase enzymes at the same time. This opens the future to a wide number of possible applications in the biosensor field and these are presently under investigation.

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