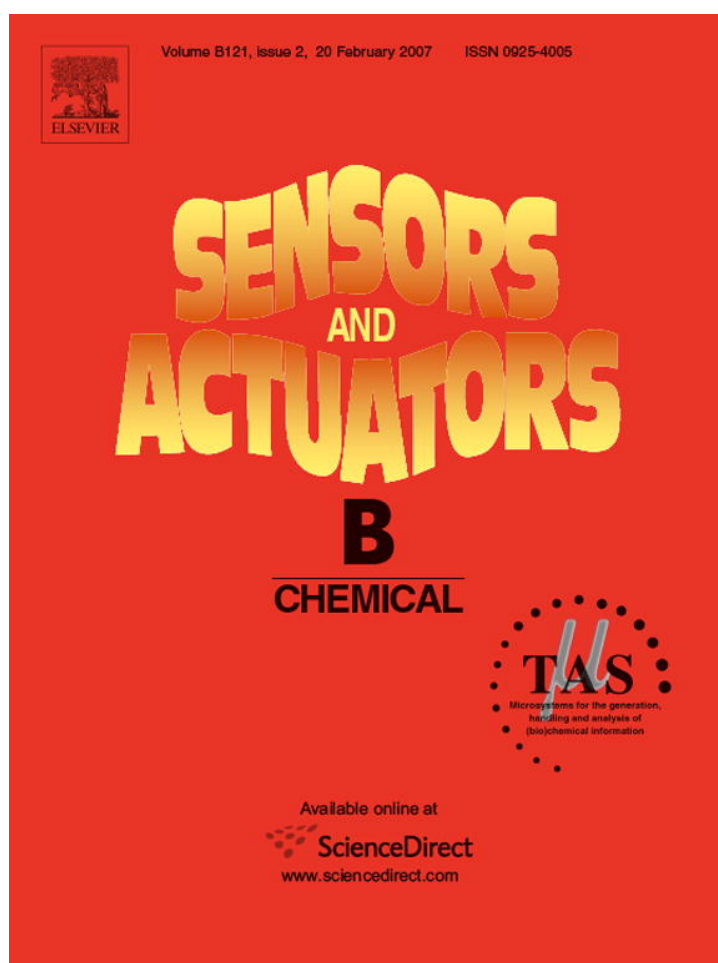


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Low potential detection of NADH with Prussian Blue bulk modified screen-printed electrodes and recombinant NADH oxidase from *Thermus thermophilus*

A. Radoi^a, D. Compagnone^b, E. Devic^c, G. Palleschi^{a,*}

^a Dipartimento di Scienze e Tecnologie Chimiche, Università "Tor Vergata", via della Ricerca Scientifica 1, 00133 Rome, Italy

^b Dipartimento di Scienze degli Alimenti, Università di Teramo, via C. Lerici 1, Mosciano S. Angelo, Teramo, Italy

^c G.T.P. Technology, Labège Cedex, France

Received 17 January 2006; received in revised form 1 April 2006; accepted 13 April 2006

Available online 30 May 2006

Abstract

A biosensor for the determination of the reduced coenzyme nicotinamide adenine dinucleotide (NADH) has been assembled using a recombinant enzyme NADH oxidase from *Thermus thermophilus* covalently immobilized on Prussian Blue bulk-modified screen-printed electrodes. Flow injection analysis (FIA) coupled with amperometric detection was used to detect NADH. Various parameters such as cofactor (FMN, flavin mononucleotide) concentration (2 mM), flow rate (0.35 mL/min), buffers (citrate–phosphate, phosphate and glycine–KOH), pH dependence (range 3.0–10.5), response time (12 s) and operational stability (120 injections) were evaluated and optimised. At pH 5.0, for which the biosensor showed the highest response, the detection and quantification limits were 1.1×10^{-7} and 3.6×10^{-7} M, respectively, and the linear working range was comprised between 1 and 400 μ M. The proposed biosensor was stable for 2 months (preserved in 50 mM phosphate buffer, pH 6.8, at 4 °C). The possibility to co-immobilize glycerol dehydrogenase (GDH) and the NADH oxidase in order to measure glycerol, a key target analyte during the alcoholic fermentation of grapes, was also investigated. Different dilutions of a complex matrix such as wine were tested to assess the interferences, the probe recovery and stability.

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Keywords: Biosensor; NADH; NADH oxidase; FMN; Prussian Blue; FIA

1. Introduction

Nicotinamide adenine dinucleotide, both in its reduced and oxidised forms (NADH/NAD⁺), is coenzyme for about 300 dehydrogenases, being involved in a wide range of enzymatic reactions [1,2].

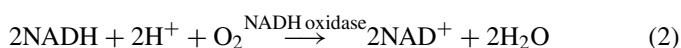
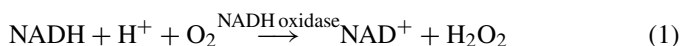
In part for this reason, the electrochemical detection of NADH is a matter of intense research activity. It is well established that the oxidation of NADH at bare electrodes occurs via radical cation intermediates with the consequent formation of highly reaction adsorptive products. Both the fouling process observed at ordinary electrodes (like graphite and its derivative forms, Pt, glassy carbon, etc.) [3], with the formation of inactive NAD⁺ dimers, and the relatively highly overpo-

tential [4] required for the oxidation of the NADH, are just two drawbacks that still await a viable and elegant solution. One way to overcome problems related to the high overpotentials required at bare electrodes is the use of chemically modified electrodes. Using a wide range of electron mediators, the redox potential of the system NADH/NAD⁺ is reduced to the formal redox potential of the mediator, with relatively improved efficiency regarding the electro-catalytic oxidation of NADH. Several mediators for NADH oxidation have been reported in the literature, among them quinines [5,6], oxometalates [7], ruthenium complexes [8], quinonoid redox dyes such as indamines [9], phenazines [10,11], phenoxazines [12,13] and phenothiazines [14]. These redox mediators are very efficient as electron shuttles for NADH oxidation, however they are not always suited for repeated measurements of NADH because of the water soluble mediator loss from the electrode surface. Moreover the majority of the electron transfers reactions involve exchange of protons, thus making the formal redox

* Corresponding author. Tel.: +39 06 72594423; fax: +39 06 72594328.
E-mail address: palleschi@uniroma2.it (G. Palleschi).

potential of the sensors highly pH dependent and prone to interferences.

The oxidation of NADH with the regeneration of NAD⁺ can also be obtained by enzymatic methods [15]. One example is the regeneration of NAD⁺ by NADH oxidase or diaphorase in the development of immunoassays with alcohol dehydrogenase as the label enzyme [16]. NADH oxidases have been purified from different bacterial sources [17–19]. This class of enzymes uses O₂ as electron acceptor forming hydrogen peroxide or H₂O as final product, as described in Eqs. (1) and (2):



A reagentless enzyme electrodes has also been also proposed [20] for the NADH based detection of malic acid. Although very appealing, this approach still presents limitations for discrimination of interferences in real samples and in terms of stability.

In this article we describe the features of a biosensor for NADH based on a newly produced recombinant NADH oxidase from *Thermus thermophilus* obtained by GTP Technology, France. This recombinant enzyme needs as cofactor the flavin mononucleotide, FMN, to catalyse the enzymatic reaction and yields hydrogen peroxide as final product of the enzymatic reaction. The H₂O₂ produced during the enzymatic reaction was detected amperometrically at a Prussian Blue bulk-modified screen-printed electrode polarized at –50 mV versus the Ag/AgCl pseudoreference electrode.

Since our aim is to develop amperometric biosensors for off-line or on-line detection, by coupling already existing or new recombinant dehydrogenases with the newly developed NADH biosensor, we tested the NADH biosensor and evaluated its stability and ability to work under different conditions, in both batch and flow injection mode. The possibility to couple two enzymes like NADH oxidase and a NADH/NAD⁺ dependent dehydrogenase enzyme, like glycerol dehydrogenase (GDH) is also reported.

2. Experimental

2.1. Reagents

All chemicals from commercial sources were of analytical grade.

Glutaraldehyde (GAD, 25%, v/v, aqueous solution) and glycerol dehydrogenase (EC 1.1.1.6, 250 units, from *Cellulomonas* sp.) were from Sigma, St. Louis, MO, USA. Nafion[®] 5% (v/v) solution, flavin mononucleotide (FMN, 85% HPLC), β-NAD⁺ (>95%, HPLC) and KOH pellets were purchased from Fluka Chemie, Buchs, Switzerland. Sodium and potassium dihydrogen phosphate and potassium chloride were from Carlo Erba Reagenti, Milano, Italy; potassium phosphate dibasic was supplied by Riedel-de Haen, Seelze, Germany.

Imidazole (approximately 99%), NaCl, glycerol (minimum 99%), glycine (>99%, titration), anhydrous citric acid,

Tris[hydroxymethyl]ammonimethane (99–99.5%) and all other reagents were from Sigma.

Lyophilised NADH oxidase (20 IU) has been supplied by GTP Technology, Labege, France (see Section 2.3).

Spherical glassy carbon Sigradur G (GC-G) particles (diameter 0.4–12 μm) were purchased from Sigradur, HTM Hochtemperatur-Werkstoffe GmbH (Gemeindewald, Germany).

2.2. Apparatus

Measurements were carried out using either a batch amperometric set up (magnetic stirrer, potentiostat, electrochemical cell and recorder) or a FIA system consisting of a peristaltic pump Gilson model Minipuls 3 (Villers-le-Bel, France) equipped with Tygon tubes, a home made thin-layer flow through cell (*d*_{int} = 15 mm) and a Rheodyne, Cotati, CA, USA, four-way injection valve (50 or 100 μL loop volume). A 641 VA Detector (Metrohm, Herisau, Switzerland), connected to an X–t recorder (L250E, Linseis, Selb, Germany) was used for amperometric measurements. A magnetic stirrer (Heidolph, model MR 3001) and a high performance oven (MOD 2100, F.lli Galli, Milan, Italy) were also used. Cyclic voltammetry (CV) and all other electrochemical investigations were performed using a μAutolab type II electrochemical system (Eco Chemie, Utrecht, The Netherlands) equipped with PGSTAT-12 and GPES software (Eco Chemie, Utrecht, The Netherlands).

2.3. Production of bacterial recombinant NADH oxidase

Bacterial NADH oxidase was produced by GTP TECHNOLOGY using an *Escherichia coli* ER2566 strain transformed with the 6His tagged NADH oxidase gene from *T. thermophilus* carrying a pGTPc101 plasmid (slightly modified pET28, Novagen). The strain was grown in a bioreactor containing a rich medium (12 g/L tryptone; 24 g/L yeast extract; 0.4%, v/v, glycerol; 2.3 g/L KH₂PO₄; 12.5 g/L K₂HPO₄; pH 7.2) at 37 °C, impeller setting of 500 rpm; aeration 1 vol/min per vol (v/vm). Expression of the recombinant NADH oxidase was induced at a density of 1.5 by addition of 0.4 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside). After 7 h, cells were harvested by centrifugation (6000 × *g*, Allegra[®] X-22R centrifuge; 10 min; 4 °C), washed in 50 mM Na₂PO₄ + 50 mM KCl, pH 7.4, and stored at –20 °C. Cell pellets from 1 L culture were resuspended in 100 mL, 50 mM Na₂PO₄ + 250 mM NaCl, pH 7.4 and sonicated. The lysis extract was recovered after centrifugation (18,000 × *g*; 15 min; 4 °C). After 30 min incubation at 65 °C, the supernatant was clarified by centrifugation (18,000 × *g*; 15 min) and filtered on a 0.45 μm size pore filter membrane (Sartorius AG).

The extract prepared from a 1 L culture was applied to a column packed with 10 mL of chelating sepharose fast flow charged with 50 mM NiSO₄ and equilibrated in 50 mM Na₂PO₄ + 250 mM NaCl and 10 mM imidazole, pH 7.4. For elution of the 6His tagged NADH oxidase, an increasing concentration from 10 to 500 mM imidazole in 50 mM Na₂PO₄ + 250 mM NaCl, pH 7.4, was applied. Fractions of the 6His tagged NADH oxidase were collected in 500 mM imidazole. The purified pro-

tein was dialysed against 50 mM Na₂PO₄ + 250 mM NaCl, pH 7.4, and stored at 4 °C.

2.4. Prussian Blue bulk modified screen-printed electrodes (bPB-SPEs)

Screen-printed electrodes were produced with a 245 DEK (Weymouth, UK) screen printing machine using different inks obtained from Acheson Italiana (Milan, Italy). Graphite-based ink (Elettrodag 421), silver ink (Elettrodag 477 SS RFU) and insulating ink (Elettrodag 6018 SS) were used.

To obtain a PB bulk-modified screen-printed electrode (bPB-SPE), the graphite-based ink was mixed with 2.5% (w/w) of PB-modified glassy carbon powder. The Prussian Blue modification procedure is similar to one reported in literature [20]. Briefly, 1 g of glassy carbon powder was suspended in 10 mL solution of K₃Fe(CN)₆, 0.1 M in 10 mM HCl. Next, 10 mL of FeCl₃, 0.1 M in 10 mM HCl, were added and the resulting mixture was stirred for 30 min. Then the glassy carbon powder, with the adsorbed Prussian Blue, was filtered and washed on a porcelain Buchner funnel with 10 mM HCl, until the washing solution became colourless, and then dried in an oven for 1 h, at 100 °C. The modified glassy carbon powder was stored in a desiccator, at room temperature. The screen-printed electrode substrate was a polyester flexible film (Autostat HT5) obtained from Autotype Italia (Milan, Italy). The electrodes were produced in foils of 20 sensors, each one having a working electrode diameter of 3 mm. Each sensor consists of three screen-printed elements: a carbon working electrode and two silver electrodes, acting as counter and pseudoreference, respectively. After the printing step, the foils were allowed to dry for one day, at room temperature and then stored dry, at room temperature, in the dark. They were ready to use without any pre-activation step or electrochemical pre-treatment. The response of bPB-SPEs towards hydrogen peroxide and the applied working potential were evaluated by cyclic voltammetric (CV) investigations.

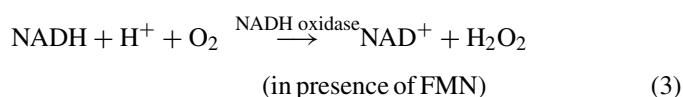
2.5. NADH biosensor assembling

Basically, 2 μL of 1% (v/v) of glutaraldehyde in distilled water was deposited on the working electrode area. Then, 2 μL of a mixed solution (33.6 IU/mL NADH oxidase and 1% neutralized Nafion[®], 1/1, v/v) were deposited and dried. The biosensor strips were preserved in 50 mM phosphate buffer, pH 6.8, at 4 °C until use.

2.6. Enzyme activity assay

2.6.1. Spectrophotometric assay

The reaction of NADH oxidation is based on the following reaction scheme (Eq. (3)):



The decay in absorbance of the NADH was measured, at 340 nm, using 10 mm light path precision cells, made of

quartz SUPRASIL[®] (Precision in Spectro-Optics. Worldwide, Hellma[®], Italy). To a volume of 400 μL, containing 0.1 mM FMN and 0.1 mM NADH, both dissolved in working buffer, 2 μL of NADH oxidase were added and the absorbance was recorded.

2.6.2. Amperometric assay

Based on the same reaction scheme (Eq. (3)), the hydrogen peroxide generation was followed, using a batch amperometric detection scheme. In this case, a screen-printed electrode bulk-modified with Prussian Blue was polarized at −50 mV versus the Ag/AgCl pseudoreference electrode for the measurement of the activity. To a final volume of 5 mL, containing 2 mM FMN and 2 μL of NADH oxidase, additions of 40 μL of 10^{−2} M NADH were added and the current generated by the reduction of the H₂O₂ was recorded.

2.7. FIA analysis

FIA measurements of NADH were performed using the working buffer pumped at 0.35 mL/min (optimised flow rate) through the electrochemical cell by the peristaltic pump until a constant baseline current was reached. Standard solutions of NADH were injected into the flow stream using a manual injection valve, and the signal was recorded. The final product of the reaction was detected at an applied potential of −50 mV versus the Ag/AgCl pseudoreference electrode. Calibration curves were constructed by measurement of the peak current height generated by injection of NADH standard solutions after stabilization of the background signal.

3. Results and discussion

3.1. FMN study

NADH oxidase requires as cofactor the flavin mononucleotide (FMN) or the flavin adenine dinucleotide (FAD) in order to oxidise NADH, its natural substrate, to the reduced form, NAD⁺. Since the cofactor is not bound to the active site of this enzyme, it is essential to ensure a saturated and constant level of cofactor to maintain a rapid and efficient transfer of electrons from NADH to oxygen. This is particularly important when a flow analysis has to be carried out. The majority of NADH electrodes reported in the literature do not add the coenzyme (FAD, FMN) in solution because they are configured as single use detectors; regeneration of the surface or use of a new probe is requested for the analytical procedure. This is confirmed by the work of Matsumoto et al. [21] that developed a NADH oxidase based flow injection system for malate using the cofactor FAD in solution.

The best concentration of cofactor to be used for analytical determination of NADH was assessed using a flow-injection system. First attempts were focused on the co-injection of NADH and FMN into the loop, both dissolved in the working buffer, using the same buffer in the flow stream (50 mM + 0.1 M KCl phosphate buffer, pH 7.2, 0.35 mL/min). Under these conditions

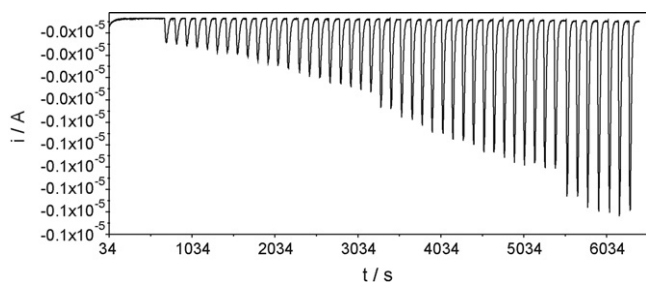


Fig. 1. FIA analysis performed on μ Autolab type II electrochemical system; -50 mV vs. Ag/AgCl pseudoreference electrode, 0.1 mM NADH into the loop, 1 mM FMN into the flow stream; flow rate 0.35 mL/min, loop volume: 50 μ L; 50 mM potassium phosphate buffer + KCl 0.1 M, pH 7.2 .

we observed that the biosensor response was not constant, since for a given injected concentration of NADH (0.1 mM), the signal increased exponentially with time. Also we observed a lag period (8 s) before the reaction started, probably due to the time necessary for the enzyme to bind the cofactor at the electrode surface.

We then optimised the amount of FMN by the introduction of the cofactor into the flow stream. For concentrations of FMN, ranging from 0.1 to 1 mM, keeping the concentration of NADH constant (0.1 mM), an increasing signal was also observed as reported in Fig. 1. Signal stability was reached at level of 2 mM of FMN (saturating conditions), and this concentration was used for batch amperometric investigations as well as for FIA.

3.2. pH study

Considering the over 300 NAD^+ dependent dehydrogenases potentially useful for biosensing applications, it is highly desirable that there be a wide optimum pH range for the NADH oxidase activity. For this reason, we investigated the optimum pH activity for both soluble and immobilized NADH oxidase. Spectrophotometric assays showed an almost constant enzymatic activity in the 4.0 – 9.0 pH range, with a 50% decrease at pH 3.0 and 10.0 (data not shown).

Amperometric detection of NADH oxidase (solubilised in the working buffer) activity was found to be maximum at pH 6.5 (Fig. 2). In fact, one has to consider the pH dependency of the Prussian Blue towards hydrogen peroxide. Immobilized NADH oxidase (assayed in FIA) exhibited an optimum pH shifted toward more acidic values (pH 5.0).

We then investigated the analytical range of the biosensor at different pHs. As can be seen in Fig. 3, the linearity of the NADH electrode was up to 0.4 mM NADH, in the pH range 5.0 – 6.0 , while in the pH range 7.0 – 8.0 the linear range was reduced by 10 . A similar reduction was observed in the range pH 3.0 – 4.0 (data not shown).

This behaviour confirms the results already reported in literatures [22,23], that is that the mediator used for the fabrication of Prussian Blue bulk screen-printed electrodes has a higher catalytic activity at acidic pHs, maintaining however some activity at alkaline pHs. The analytical parameters of the biosensor are described in Table 1.

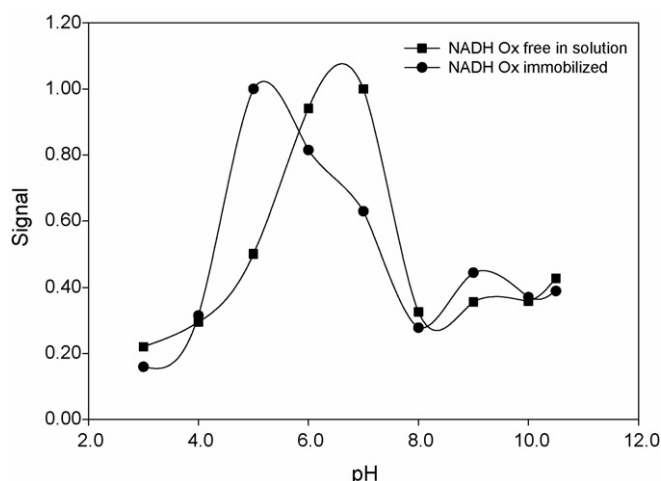


Fig. 2. Normalised response of the enzyme free in solution and immobilized as function of pH; -50 mV applied potential vs. Ag/AgCl pseudoreference electrode; $n = 3$; [(50 mM citrate–phosphate buffer + 0.1 M KCl, pH 3.0 , 4.0 and 5.0); (50 mM phosphate buffer + 0.1 M KCl, pH 6.0 , 7.0 and 8.0); (0.1 M Tris buffer + 0.1 M KCl, pH 9.0); (50 mM glycine KOH + 0.1 M KCl, pH 10.0 and 10.5)].

3.3. Real matrix investigation

Since our aim is to develop new amperometric biosensors for measurement of key target analytes, like glycerol, during the alcoholic fermentation of grapes, we tested the NADH biosensor and evaluated its stability and ability to work in a complex matrix such as wine.

The NADH biosensor was challenged in red and white wine, in order to assess recovery and stability. Red and white wine samples were spiked and analysed to assess the matrix effect (Table 2). Recoveries ranging from 77% to 100% were found for dilutions of samples ranging from $1/100$ (v/v) to $1/400$ (v/v) for all the pHs investigated, demonstrating that the biosensor is viable for use in these real samples. As expected, recovery

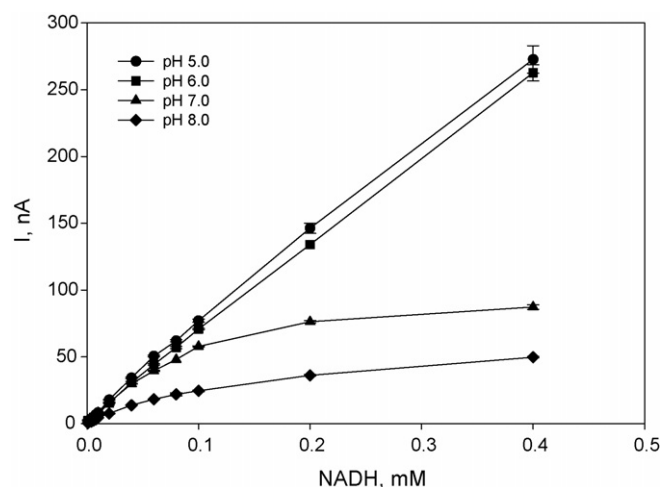


Fig. 3. FIA calibration curves for NADH oxidase electrode, at different pHs; -50 mV applied potential vs. Ag/AgCl pseudoreference electrode; 2 mM FMN into the flow stream; flow rate 0.35 mL/min, loop volume: 50 μ L; $n = 4$ [(50 mM citrate–phosphate buffer + 0.1 M KCl, pH 5.0), (50 mM phosphate buffer + 0.1 M KCl, pH 6.0 , pH 7.0 and pH 8.0)].

Table 1

FIA analysis performed on μ Autolab type II electrochemical system at an applied potential of -50 mV vs. Ag/AgCl pseudoreference electrode; 2 mM FMN into the flow stream; flow rate 0.35 mL/min, loop volume: 50 μ L

pH	Detection limit (LOD)	Limit of quantification (LOQ)	Linear working range (μ M)	Linear regression equation	R.S.D. (%) (100 μ M, $n=4$)	Time to stabilize baseline current (s)	Time to reach 90% of the signal (s)
5.0	1.1×10^{-7}	3.6×10^{-7}	1–400	$y = 4.05 + 687582x$; $r^2 = 0.9962$	2.8	140	12
6.0	2.5×10^{-7}	8.5×10^{-7}	1–400	$y = 2.42 + 656280x$; $r^2 = 0.9988$	4.5	140	12
7.0	3.0×10^{-7}	9.9×10^{-7}	1–60	$y = 2.30 + 603465x$; $r^2 = 0.9896$	4.8	140	18
8.0	5.7×10^{-7}	1.9×10^{-6}	1–40	$y = 0.43 + 341011x$; $r^2 = 0.9940$	4.8	140	18

The LOD and LOQ were calculated as follows: $\text{LOD} = 3s_b/S$; $\text{LOQ} = 10s_b/S$, where s_b is the standard deviation of the blank signal and S is the sensitivity.

Table 2

Recovery data evaluated from FIA analysis performed on μ Autolab type II electrochemical system at an applied potential of -50 mV vs. Ag/AgCl pseudoreference electrode; 2 mM FMN into the flow stream; NADH: 5×10^{-5} M, flow rate 0.35 mL/min, loop volume: 50 μ L, $n=4$; [50 mM citrate–phosphate buffer + 0.1 M KCl, pH 5.0; 50 mM phosphate buffer + 0.1 M KCl, pH 6.0, 7.0 and 8.0]

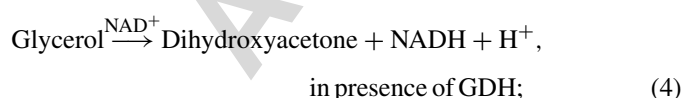
pH	Percentage recovery red wine			Percentage recovery white wine		
	1/100 (v/v)	1/200 (v/v)	1/400 (v/v)	1/100 (v/v)	1/200 (v/v)	1/400 (v/v)
5.0	83	89	93	85	91	95
6.0	81	90	95	89	92	98
7.0	88	93	98	84	91	98
8.0	77	85	98	85	89	100

increased for more diluted samples. Stability was tested at pH 5.0 by spiking red and white wines (diluted 1/100, v/v, in 50 mM citrate–phosphate buffer + 0.1 M KCl) with a known amount of NADH (5×10^{-5} M) for FIA. After 120 samples of Fontamara (Rubino, I.G.T., Terre di Chieti, Italy) spiked red wine, the biosensor response still retained 70% of the initial signal. However the 120 samples of Jidvei (Dry Muscat, D.O.C.-C.M.D., Tarnave, Romania) spiked white wine showed a 20% reduction of the initial response signal.

4. Glycerol biosensor

Just to demonstrate that this NADH probe can be successfully coupled with a dehydrogenase, a preliminary experiment was carried out: glycerol dehydrogenase (GDH) was co-immobilized with the NADH oxidase using the same procedure reported for the construction of the NADH biosensor: 0.017 IU of NADH oxidase and 16 IU of glycerol dehydrogenase were immobilized on the electrode.

The enzymatic reactions involved are:



the produced NADH is then reoxidised by the NADH oxidase to NAD^+ :

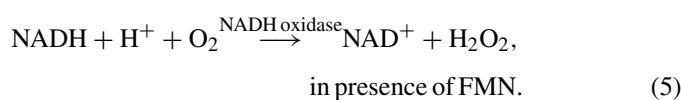


Table 3

FIA analysis of glycerol

pH	Detection limit (LOD)	Linear working range (mM)	Linear regression equation
10.0	2.2×10^{-6}	0.01–1.0	$y = -3.8 + 163348x$; $r^2 = 0.9910$

Applied potential of -50 mV vs. Ag/AgCl pseudoreference electrode; 2 mM FMN; 0.35 mL/min, loop volume: 100 μ L. The LOD was calculated as $3s_b/S$, where s_b is the standard deviation of the blank signal and S is the sensitivity.

For glycerol investigations the already optimised analytical parameters reported for the NADH oxidase based sensor were used. An important factor taken into consideration was the amount of the NAD^+ coenzyme to be used for glycerol determination. A suitable amount of reduced coenzyme was determined to be 1 mM. No response was observed when only 1 mM of NAD^+ was injected, nor when glycerol alone was injected, without the coenzyme, into the loop. The working pH was 10.0 and the buffer used was 0.1 M glycine–KOH, containing 0.1 M KCl. The loop volume was 100 μ L. Finally, a calibration plot for glycerol, in working buffer was generated, and the results are summarized in Table 3.

5. Conclusions

Due to its remarkable stability and ability to regenerate the coenzyme NAD^+ over a wide range of pH, the recombinant NADH oxidase from *T. thermophilus* appears to be well-suited for assembling and use with dehydrogenase/NADH-oxidase-

based electrodes. The possibility of coupling different dehydrogenases would appear at this stage to only be limited by their operational stability, which can be maintained using appropriate immobilization procedures. Considering the characteristics of the Prussian Blue bulk-modified screen-printed electrodes, these are comparable in sensitivity to classical platinum based hydrogen peroxide electrodes, but superior in terms of rejection of interferences without the use of any additional membrane. In fact they are able to operate at a very low potential (-50 mV versus Ag/AgCl reference electrode). Thus the combination of these probes with the recombinant NADH oxidase appears promising for the development of a flow injection detection of NADH in complex matrix without other pre-treatment than dilution.

The demonstration that co-immobilization of glycerol dehydrogenase and NADH oxidase is feasible is a further encouraging sign relative to the future development of sensors based on this approach.

Acknowledgement

The authors wish to thank the NOVTECH European project [NOVTECH No. HPRN-CT-2002-00186] for financial support.

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