Full Paper

Development of Novel Glucose and Pyruvate Biosensors at Poly(Neutral Red) Modified Carbon Film Electrodes. Application to Natural Samples

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Abstract

Amperometric biosensors based on the corresponding oxidase enzyme with poly(neutral red) redox mediator have been developed for the determination of glucose and pyruvate. The enzymes have been immobilized on top of poly(neutral red) modified carbon film electrodes with glutaraldehyde as the cross-linking agent. The biosensors were characterized by cyclic voltammetry and by electrochemical impedance spectroscopy. The glucose biosensor exhibited a linear response in the range 90 μM to 1.8 mM with a detection limit of 22 μM and the pyruvate biosensor in the range 90 to 600 μM with a detection limit of 34 μM. The relative standard deviations were found to be 2.1% ($n = 3$) and 2.8% ($n = 4$) respectively. The interference effects of various compounds were also studied. The glucose content of several types of wine and the amount of pyruvate in onion and garlic were determined and the results were compared with those obtained by standard spectrophotometric methods.

Keywords: Amperometric enzyme biosensor, Glucose oxidase, Pyruvate oxidase, Poly(neutral red), Carbon film electrode, Electrochemical impedance spectroscopy

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1. Introduction

Reliable analytical methods are increasingly needed in the food and beverage industry for the determination of specific components (e.g., sugars, proteins, vitamins, fats) and for detecting and quantifying chemical contaminants such as pesticides, heavy metals, antibiotics, and pathogenic microorganisms. The need arises from numerous regulatory actions and increased consumer concern about food composition and safety. Normally, analytical techniques such as spectrophotometry and chromatography are utilized, which do not always allow easy and continuous monitoring, are rather expensive, need well-trained operators and often require sample pretreatment steps, resulting in increased time and low cost-effectiveness. Biosensors offer a promising alternative: besides their good selectivity and low cost, they can easily be integrated in automation schemes and used to develop simple and portable equipment, allowing fast in-situ monitoring of the raw materials and food processing steps.

The determination of glucose is very important during fermentation processes, such as of grape must, when it is transformed into alcohol, and for final quality control. Biosensors for glucose determination in wine samples, using glucose oxidase, have been described [1].

Pyruvate is one of the most important metabolites for which a sensitive and fast detection method is required in clinical, bioprocess and food analysis [2]. Pyruvate oxidase has usually been used for pyruvate detection in electrochemical enzyme biosensors [3].

Electrochemically deposited polymers have considerable potential as components for the fabrication of enzyme biosensors, electropolymerization representing a powerful methodology for the development of modified electrodes. Electroactive polymers exposed to electrolyte solutions can exhibit both electronic and ionic conductivity. In addition, the electropolymerized materials usually possess some unique properties which the corresponding monomers do not have. The preparation of semiconducting films by electropolymerization from a monomer which is itself a redox mediator [4, 5] generates high catalytic currents in the electroenzymatic reaction.

Azines are a group of chemical compounds which has already found wide use as redox indicators and mediators, both as monomers and following electropolymerization. Graphite electrodes modified by electropolymerization of phenazine methosulfate and phenazine ethosulfate have been used for the electrocatalytic oxidation of NADH [5]. Platinum electrodes modified with a layer of poly(azur A) (poly(AA)) were able to give a quasireversible electrochemical response to hemoglobin [6]. More recently, an amperometric dehydrogenase biosensor based on poly-(methylene blue) (poly (MB)) on glassy carbon electrodes has been described [7].

The electropolymerization of azines occurs via binding of azine monomers without their destruction [8]. In particular,

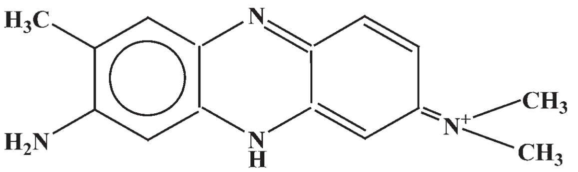

Fig. 1. Chemical structure of neutral red.

the phenazine neutral red (Fig. 1) has some similarities with the molecular structure of flavins. It is assumed that, upon its electropolymerization, structures which mimic the NADH–dehydrogenase activity are synthesized. Indeed, as already reported, NAD^+ can be reduced on poly(neutral red) (PNR) modified electrodes to enzymatically active NADH [9].

The characterization of the electropolymerization process of neutral red and the polymer formed from neutral aqueous solution on a glassy carbon electrode [10], and from acidic solution on an ITO electrode [11] has been described. Since then, electrochemical studies of PNR films formed on glassy carbon [8], graphite [12] or gold electrodes [13] were performed; PNR films were used for the development of sensors [14, 15] and NADH biosensors [9, 16].

In this study, neutral red (NR) was polymerized electrochemically on a carbon film electrode surface. These low-cost carbon film electrodes have been recently developed and electrochemically characterized by our group [17–19]. They have a large potential window, are small and easy to fabricate and they have been applied to mediated glucose enzyme biosensors particularly using metal hexacyanoferrate, ferrocene and methyl viologen mediators [20–25]. The aim of the present investigation was to test the resulting polymer poly(neutral red) as an electrochemical mediator for oxidase enzymes. Glucose oxidase and pyruvate oxidase oxidize glucose or pyruvate with dioxygen and the product, hydrogen peroxide, is detected. When using poly(neutral red) as mediator, different reactions must be taken into account, depending on the enzyme substrate. In the case of glucose, there appears to be enzyme-catalyzed reaction of glucose at PNR. In the case of pyruvate the H_2O_2 produced in the enzyme reaction is reduced at the polymer film on the electrode. The poly(neutral red) film-modified electrodes possessed high stability and were used for the development of new biosensors for glucose and pyruvate.

2. Experimental

2.1. Reagents and Buffers

Glucose oxidase (GOx, EC 1.1.3.4, from *Aspergillus niger*, 24 U/mg) and glutaraldehyde (GA) (70% v/v) were from Fluka Chemie AG, Buchs, Switzerland, pyruvate oxidase (PyOx, EC 1.2.3.3, 8.9 U/mg), α-D(+)-glucose and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. St. Louis, USA. Nafion (5% v/v), pyruvic acid (98%) and neutral red (65% dye content) were from Aldrich Chemie GmbH&Co KG, Steinheim, Germany.

Other reagents used were: D(+) fructose, DL-lactic acid, L-ascorbic acid, L-glutamic acid and trizma hydrochloride (Sigma Chemical Co. St. Louis, USA), tartaric acid (PAHI, Lisbon, Portugal), citric acid, DL-malic acid and L-arginin monohydrochloride (Merck, Darmstadt, Germany), DL-lysine monohydrochloride and DL-*iso*-leucine (BDH, Poole, England), L-cysteine (Aldrich Chemie GmbH&Co KG, Steinheim, Germany); trichloroacetic acid was from Panreac, Barcelona, Spain.

Potassium phosphate buffer (KPB) (0.025 M KH_2PO_4/K_2HPO_4, pH 6.0) + 0.1 M KNO_3 was used as supporting electrolyte for the polymerization of neutral red and for amperometric measurements with the glucose biosensor. Amperometric measurements with the pyruvate biosensor used a trizma-based buffer solution (0.01 M trizma-HCl + 0.05 M KCl + 0.015 M K_2HPO_4, pH 7.2) containing 1 mM $MgCl_2$ and 1 mM thiamine pyrophosphate (TPP).

Solutions were prepared with ultrapure water obtained from a Millipore Milli-Q purification system (resistivity > 18 MΩ cm) and all reagents were analytical reagent grade, unless specified.

Stock solutions of 1.0 M pyruvic acid (98%) and 1.0 M glucose were prepared and were kept in the refrigerator. In the case of glucose, the stock solution was left one day before use to permit equilibration of the α and β anomers of D-glucose.

The concentrations of enzyme substrates in natural samples were validated by standard methods, for glucose by a spectrophotometric enzyme assay kit [26] (Cat 0 139 106, Boehringer, Mannheim, Germany) and for pyruvate by a modified Schwimmer and Weston procedure, by determination of total 2,4-dinitrophenylhydrazine-reacting carbonyls [27].

2.2. Instrumentation

Electrochemical polymerization of neutral red was performed using a μAutolab Type II potentiostat running with GPES (General Purpose Electrochemical System) for Windows version 4.9, software (EcoChemie, Utrecht, The Netherlands). Amperometric measurements were performed using a Bioanalytical Systems (BAS, West Lafayette, IN) CV-50W electrochemical analyzer. Electrochemical impedance spectroscopy (EIS) was carried out with a Solartron 1250 Frequency Response Analyzer together with a Solartron 1286 Electrochemical Interface (Solartron Analytical, UK), controlled by ZPlot 2.4 software. A sinusoidal voltage perturbation of 10 mV amplitude was applied, scanning from 65 kHz to 0.1 Hz with 10 points per frequency decade, auto-integration time 120 s. Fitting to equivalent electrical circuits was performed with ZView 2.4 software.

The electrochemical three-compartment cell employed in these experiments consisted of the poly(neutral red) / enzyme modified carbon film working electrode, a platinum wire auxiliary electrode and an Ag/AgCl (3 M KCl) electrode as reference. In the case of pyruvate, the reference electrode was saturated calomel electrode (SCE).

The pH measurements were made with a CRISON 2001 micro pH-meter at room temperature.

2.3. Electrode Preparation and Enzyme Immobilization

Carbon film electrodes were made from carbon film electrical resistors (2 Ω nominal resistance), which are fabricated from ceramic cylinders of length 6 mm and diameter 1.5 mm by pyrolytic deposition of carbon in an oven at 1100 °C [17]. The resistor has metal caps with an external contacting wire placed over each end. One of the two caps was removed and the other cap, together with external contact wire, insulated by plastic sheathing and epoxy resin. In this way the exposed cylindrical electrode geometric area was ~ 0.20 cm^2.

Poly(neutral red) films were made by potential cycling from a solution containing 1 mM neutral red in 0.025 M $KH_2PO_4/K_2HPO_4 + 0.1$ M KNO_3, pH 6.0.

GOx and PyOx were immobilized onto the electrode surface by the cross-linking method. A mixture of enzyme with glutaraldehyde, as cross-linking agent, and BSA carrier protein was used. Nafion was also used in the case of glucose biosensors to reduce interferences. This has previously been found to be beneficial in interference reduction and increasing sensitivity, e.g., in [24] and [25] using ferrocene and methylviologen mediators, respectively, at carbon film electrode – based biosensors. For this purpose, a mixture of 5 μL glutaraldehyde (2.5% in water), 4 μL Nafion (5% in alcohol), and 15 μL enzyme solution was prepared. The enzyme solution contained 40 mg of bovine serum albumin (BSA) and 10 mg of GOx in 1 mL of 0.025 M KPB (pH 6.0) for the glucose biosensor, and 4 mg of BSA and 1 mg of PyOx in 1 mL of trizma-HCl buffer (pH 7.2), respectively for the pyruvate biosensor. From this mixture 10 μL was placed onto the surface of the poly(neutral red) carbon film modified electrode and allowed to dry at room temperature for at least one hour. Pyruvate biosensors were incubated overnight in a humid environment, as in [32]. When not in use the biosensors were kept at 4^0C: after testing different storage conditions, the better ones were found to be glucose biosensors in potassium phosphate buffer solution and dry for pyruvate biosensors.

2.4. Analysis of Natural Samples

For the analysis of glucose in wine, aliquots of wine samples were added to 0.025 M KPB + 0.1 M KNO_3 (pH 6.0), and the standard addition method was used to determine the glucose concentration. Independent analysis of glucose concentrations was done using the standard spectrophotometric enzyme assay [26].

For testing the determination of pyruvate in natural samples, onion and garlic were used. Flavor in fresh onions is dominated by volatile sulfenic and thiosulfenic acids that are liberated when alk(en)yl cysteine sulfoxide (ACSO) flavor precursors are cleaved by the enzyme after tissue disruption. It was proved that there is a linear relationship between the content of ACSOs and pyruvate [28].

Duplicate batches of onion and garlic were chopped with a knife and homogenized for 2 minutes with water in a ratio of 1 mL of added water per gram of onion/garlic. The homogenates were allowed to stand for 10 minutes at room temperature, and then 1 mL of trichloroacetic acid was added per gram of onion/garlic. After a minimum of one hour, the slurry was filtered through four layers of cheesecloth and the filtrate was clarified by centrifugation at 2000 *g* for 20 minutes. Control duplicates were first heated in a microwave oven, power equal to 600 W, for a period of time in seconds equal to the number of grams of onion/garlic; this served to deactivate the enzyme aliinase [27]. Aliquots of this clarified onion/garlic were added to 0.01 M trizma-HCl (pH 7.2) and pyruvate was determined using the standard addition method.

Independent analysis of pyruvate was performed using a spectrophotometric method as described by Anthon and Barrett [27].

The amount of pyruvate produced by hydrolysis of the ACSOs was obtained by subtracting a measurement of the endogenous pyruvate, obtained from tissue where aliinase has been inactivated, from the total pyruvate generated after disruption of the tissue [28].

3. Results and Discussion

3.1. Electropolymerization of Neutral Red

The generation of a poly(neutral red) film on the carbon film working electrode was achieved by electropolymerization of the neutral red monomer. It is an acid – base indicator of $pK_a \approx 6.8$ and the mechanism of polymerization involves initiation by formation of a cation-radical upon electro-oxidation [8].

The peak potential corresponding to irreversible neutral red oxidation is pH dependent, becoming less positive by approximately 60 mV per unit increase in pH. However, in neutral solutions, the oxidized neutral red releases a proton which makes it uncharged and thus only slightly soluble. In order to compromise between these opposing tendencies, pH 6.0 was chosen for electropolymerization. In contrast to its analogue phenothiazines and phenoxazines, PNR showed redox activity at pH 6.0 in a similar potential region as for the neutral red monomer [8].

Prior to polymerization, electrodes were cycled in 0.025 M KPB + 0.1 KNO_3 (pH 6.0) in the potential range − 1.5 V to + 1.5 V at a sweep rate of 100 mV s^{-1} for approximately 5 minutes. After treatment a stable voltammogram of the carbon film electrode was obtained.

Electropolymerization of NR was carried out by potential cycling from − 1.0 V to + 1.0 V at a sweep rate of 50 mV s^{-1} and the polymerization time was about 27 minutes (20 cycles). The neutral red concentration in the solution was

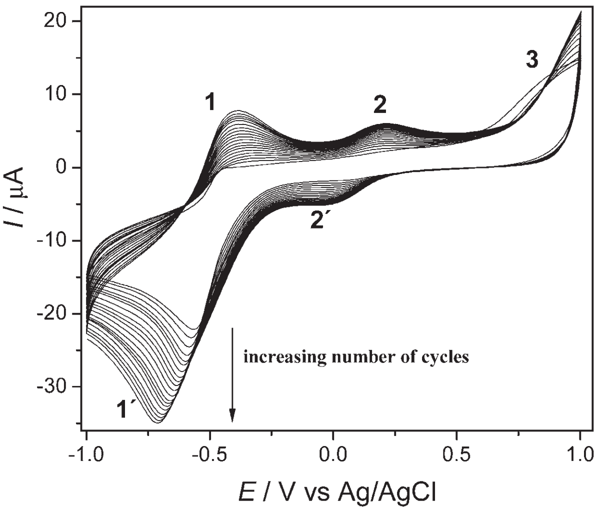

Fig. 2. Cyclic voltammograms of neutral red polymerization from 1 mM NR in 0.025 M potassium phosphate buffer, pH 6.0, with 0.1 M KNO_3 as supporting electrolyte, sweep rate 50 mV s^{-1}.

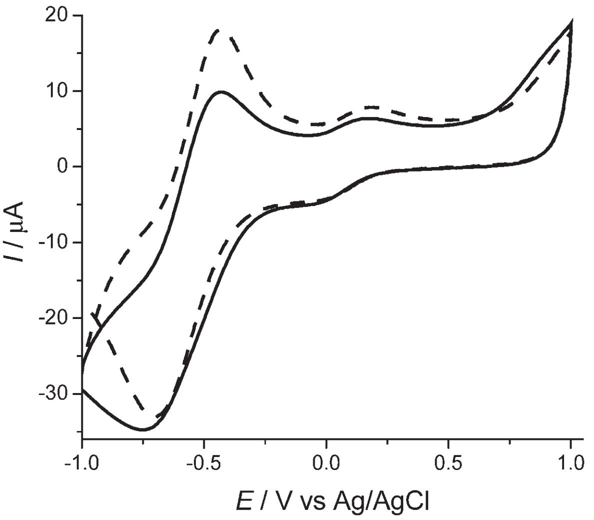

Fig. 3. Cyclic voltammogram of PNR/GOx modified electrode at 50 mV s^{-1} in 0.025 M potassium phosphate buffer (—) and with addition of 2 mM glucose (– – –).

1 mM in 0.025 M KPB + 0.1 M KNO_3 (pH 6.0). The addition of 0.1 M KNO_3 to the supporting electrolyte was done because of the previously observed catalytic effect of NO_3^- anions on the electropolymerization of phenazine and phenothiazine dyes [8, 29].

Figure 2 shows consecutive cyclic voltammograms of the poly(neutral red) film during formation. At high positive potentials (around 1.0 V) irreversible oxidation of the monomer occurs. The anodic current due to oxidation of the monomer to the cation radical (peak 3) increases from cycle to cycle similarly to the electropolymerization of pyrrole, aniline and other conducting polymer monomers. The redox pair that appears at negative potentials is due to the reduction/oxidation of the polymer film, but also overlaps with peaks due to the monomer (peaks 1 and 1′). There is rapid polymerization under the experimental conditions employed in the first 15 cycles (Fig. 2). During further cycling, the peak currents slowly increase until a maximum is reached after 20 cycles.

3.2. Voltammetric Behavior of the PNR/Enzyme Biosensors

Poly(neutral red)/enzyme modified electrodes were then prepared by glutaraldehyde cross-linking immobilization as described in the experimental section. Modified electrodes were characterized by cyclic voltammetry in buffer and after the addition of enzyme substrate. For the glucose biosensors, Figure 3 shows a typical cyclic voltammogram obtained in the absence and presence of glucose. With the addition of glucose to the buffer solution, a large increase in the oxidation peak at – 0.55 V vs. Ag/AgCl was observed, which can be explained by enzyme-catalyzed reaction of glucose at PNR:

$$\text{glucose} + \text{PNR}_{\text{ox}} \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + \text{PNR}_{\text{red}}$$

the PNR_{red} being reoxidized at the electrode surface. This is in agreement with the amperometric experiments in Section 3.5.

In the case of the pyruvate biosensor a decrease of the anodic peak at – 0.5 V vs. SCE was observed after addition of pyruvate to buffer solution (data not shown) suggesting a reduction step in the mechanism. This, and the fact that the amperometric response is an increase in cathodic current (see Sec. 3.5) can be explained by the following mechanism:

$$\text{pyruvate} + \text{phosphate} + O_2 \xrightarrow{\text{pyruvate oxidase}} \text{acetylphosphate} + CO_2 + H_2O_2$$

reduction of H_2O_2 leading to a cathodic current. Independent experiments with hydrogen peroxide at PNR films confirmed the reduction of hydrogen peroxide at these potentials.

3.3. Characterization by EIS

Complex plane plots of electrochemical impedance spectra obtained with the biosensors in buffer and after addition of the corresponding substrate are shown in Figure 4. The impedance spectra are shown at – 0.35 V (Fig. 4A) for glucose and at – 0.25 V vs. SCE (Fig. 4B) for pyruvate biosensors respectively, the potentials subsequently used for amperometric biosensor measurements.

Spectra were fitted using two equivalent circuits. The first consists of the cell resistance, R_Ω, which was of the order of

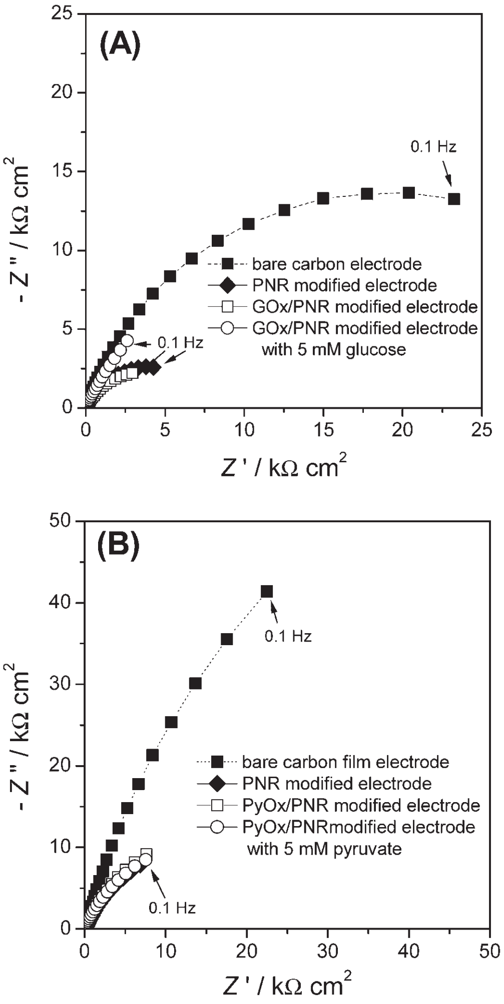

Fig. 4. Complex plane impedance spectra of different stages of electrode assembly for A) glucose biosensor in 0.025 M KPB (pH 6.0) at -0.35 V and B) pyruvate biosensor in 0.01 M trizma-HCl (pH 7.2) at -0.25 V vs. SCE.

10 Ω cm^2, and a combination of a constant phase element, *CPE*, modeled as a non-ideal capacitance:

$$CPE = -1/(Ci\omega)^{\alpha}$$

with C the capacitance, ω the frequency in rad s^{-1} and the CPE exponent α which can be interpreted as expressing the effects of surface roughness, in parallel with a charge transfer resistance, R_1. The second circuit was necessary to model the low frequency behavior in the case of PNR-modified electrodes without the enzyme layer, both at -0.25 V in trizma buffer (pH 7.2) and at -0.35 V in potassium phosphate buffer (pH 6.0) and comprises the first circuit with a second CPE in series. Such types of equivalent circuit are common for conducting-polymer modified electrodes [30]. The values of the circuit elements obtained are shown in Table 1. The charge transfer resistance values are higher for bare carbon electrodes than at the modified electrodes since very little electron transfer occurs at this interface; at the same time the capacity values significantly increase with the PNR film and the values of the CPE exponent are those expected for these types of electrode, as observed previously [18]. On addition of glucose the charge transfer resistance increases (Fig. 4A), suggesting some direct reaction of the PNR film whereas with pyruvate (Fig. 4B) it remains essentially unchanged; the conclusion is that the biosensor electron relay mechanism is different in each case and that there is direct reaction between glucose oxidase and PNR.

3.4. Basic Parameters of the Enzyme Biosensors

The amperometric response of the glucose biosensor as a function of glucose concentration was studied at several applied potentials from -0.45 V to $+0.25$ V. It was observed that the anodic current decreases with increasing applied potential in the studied potential range (data not shown). The higher current response is exhibited at the potential -0.45 V, and between -0.15 V and $+0.25$ V the current response remains almost constant. In order to minimize possible interferences, a potential of -0.35 V was selected for subsequent studies (see also next section). Nevertheless, more positive potential values can be used but with significantly lower current response.

For pyruvate biosensors, the effect of several basic parameters was studied. Figure 5 shows the pH dependence of the pyruvate response in trizma-HCl and phosphate buffer. Pyruvate can be detected in the pH range of 4–8 with maximum sensitivity around pH 5.5, corresponding to the optimum pH of the dissolved PyOx [31]. However, the highest signal for 0.4 mM pyruvate was measured at pH 7.2 in 0.01 M trizma-HCl buffer.

Table 1. Equivalent circuit fitting of impedance data for bare and coated carbon film electrodes in 0.025 M KPB pH 6.0 at -0.35 V (glucose) and in 0.01 M trizma-HCl (pH 7.2) at -0.25 V (pyruvate).

E (V vs. SCE)	Electrode coating	R_1 (kΩ cm^2)	C_1 (μF cm^{-2})	α_1
-0.35	–	35.5	27.9	0.82
	PNR	7.2	160.8	0.76
	PNR/GOx	6.8	251.6	0.74
	PNR/GOx + 5 mM glucose	34.6	260.8	0.73
-0.25	–	228.0	28.7	0.82
	PNR	29.8	109.8	0.75
	PNR/PyOx	36.8	100.4	0.76
	PNR/PyOx + 5 mM pyruvate	31.6	101.9	0.76

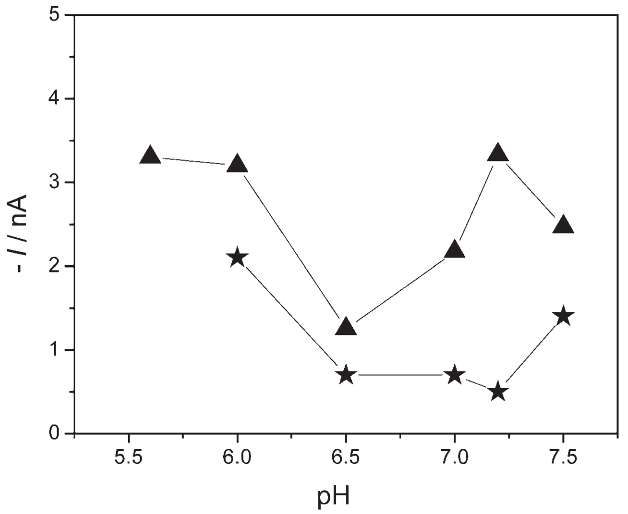

Fig. 5. pH dependence of the biosensor response to 0.4 mM pyruvate in (–★–) 0.1 M phosphate buffer and (–▲–) 0.01 M trizma-HCl buffer. The buffers contain 1 mM $MgCl_2$ and 1 mM TPP.

Concerning the working potential for the pyruvate biosensor, the studies over the same range of values as for glucose biosensors showed the same behavior. However, in this case the working potential was chosen as – 0.25 V vs. SCE, in order to avoid possible interferences in the natural samples to be analyzed. This represents an advantage compared with previous work where pyruvate was determined amperometrically at: + 0.65 V (vs. Ag/AgCl) [32]; + 0.4 V (vs. Ag/AgCl) [3, 33] or + 0.3 V (vs. Ag/AgCl) [34].

It is well known that PyOx is a tetrameric flavoenzyme with FAD, TPP and magnesium as cofactors [32]. The presence of cofactors in the enzymatic determination of pyruvate is necessary for catalytic activity of the PyOx [35, 36]. TPP is a pyrophosphate ester of thiamine (vitamin B1) and a cofactor [37] of pyruvate oxidase, acting as an aldehyde carrier in an enzyme system [38]. Since PyOx contains FAD, the effect of TPP and $MgCl_2$ was investigated. The influence of increasing concentration of TPP from 0.2 to 1 mM was an increase in enzyme electrode sensitivity. A higher concentration might increase biosensor sensitivity but also the cost of the biosensor. Keeping the same concentration of TPP the influence of $MgCl_2$ was studied. It was observed that when increasing the concentration of $MgCl_2$ from 1 to 5 mM the effect was a decrease in biosensor sensitivity. Therefore, the concentration of cofactors used to measure pyruvate was chosen as 1 mM both for $MgCl_2$ and TPP.

3.5. Glucose Biosensor

The sensor based on PNR/GOx was applied to the determination of glucose in amperometric mode. The response to glucose was first tested at – 0.2 V, addition of glucose leading to an increase in current. The sensitivity obtained at this potential was 0.29 μA mM^{-1} cm^{-2}, with detection limit 35 μM (signal to noise ratio of three, i.e. three times the standard deviation of the current divided by the slope of the calibration curve), and the Michaelis – Menten constant was 4.07 mM. At more negative potentials the sensitivity increased further, but the response to interferents can also increase, therefore the operating potential was chosen as – 0.35 V.

A typical calibration curve corresponding to the electrochemical response of the PNR/GOx electrode to increasing concentrations of glucose is plotted in Figure 6A, showing that the linear range extends up to 1.8 mM glucose. The sensitivity obtained at – 0.35 V is 3.5 μA mM^{-1} cm^{-2}, a factor of 12 greater than at – 0.2 V, and the detection limit was 22 μM glucose. The apparent Michaelis – Menten constant was determined from the corresponding Lineweaver – Burk plot and was 2.43 ± 0.35 mM (3 sensors).

Besides sensitivity and selectivity, stability is also a very important factor for the sensor. Our investigation showed that the PNR film is stable, cyclic voltammograms in KPB (pH 6.0) (data not shown) after deposition showing a small decrease of current peak 1 after 20 scans. Regarding the operating stability with time and the effect of the storage period, some tests were carried out with the PNR/GOx

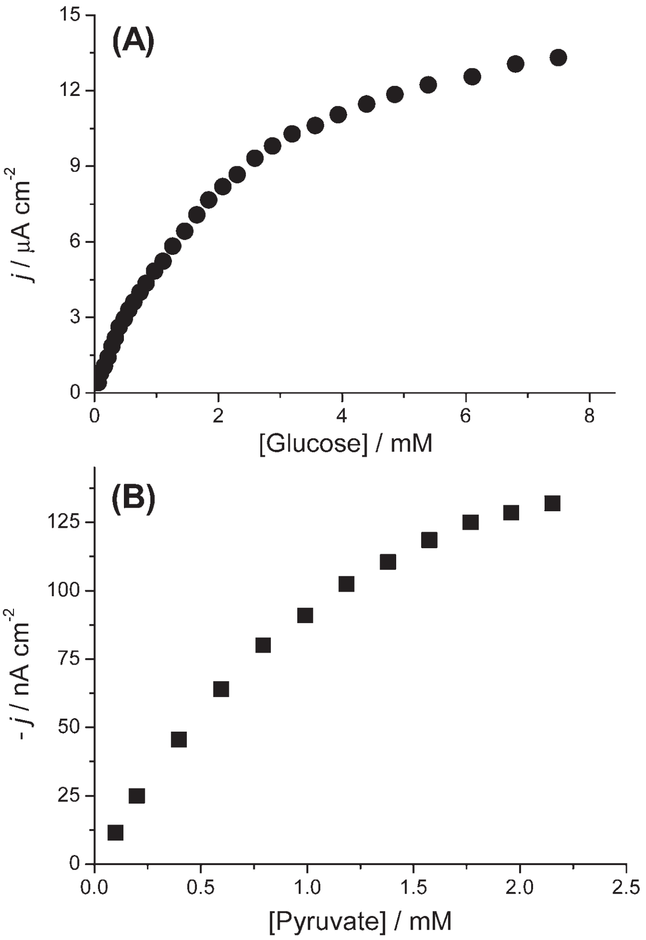

Fig. 6. Calibration curve for A) glucose at PNR/GOx-modified electrode at – 0.35 V vs. Ag/AgCl and B) pyruvate at PNR/PyOx-modified electrode at – 0.25 V vs. SCE.

biosensor. A calibration curve was recorded every day during two weeks. During the first three days, a loss in current sensitivity of ca 7% was observed and after two weeks the electrode lost 18% of its initial response. Electrodes stored in potassium buffer solution (pH 6.0) at 4 °C retained 69% of the initial current response after 42 days.

The reproducibility of the PNR/GOx modified carbon resistor electrodes was evaluated from calibration curve responses using three different electrodes. The relative standard deviation at these electrodes was 2.1%. Thus the sensor showed a good, reproducible behavior.

A study of interferences of compounds usually present in wines was also performed and the results are shown in Table 2. Substances tested as potential interferents for glucose were: fructose, lactate, tartaric acid, citric acid, and ascorbic acid.

Under the experimental conditions used for glucose only small interferences were observed from fructose and lactate even for a glucose-to-interferent concentration ratio of 1:5. However, citric acid, tartaric acid and ascorbic acid did affect the amperometric response of glucose yielding relative errors of 5.2%, 10.7% and 15%, respectively, for a glucose-to-interferent concentration ratio of 1:1. Ascorbic acid decreases the signal from glucose due to reaction with hydrogen peroxide, and it was found in independent experiments that tartaric acid is reduced directly at the PNR electrode. However, such interferences should not play a significant role for the determination of glucose by standard addition in wine samples.

These results demonstrated the good selectivity of the PNR/GOx electrode for the determination of glucose in wine samples.

3.5.1. *Glucose Determination in Wine Samples*

The PNR/GOx electrode was applied to the determination of glucose in sweet and dry wines in order to demonstrate its suitability for the analysis of natural samples containing glucose both at high and low concentrations.

Commercial wine samples were analyzed in batch injection mode. No sample pretreatment prior to measurements was necessary for white or red wines. Amperometry in stirred solution was carried out at − 0.35 V and determination of glucose was done by applying the standard addition method in order to minimize the matrix effect. The results obtained from three glucose determinations of each sample are summarized in Table 3. As can be seen, all the results are within the usual concentration range for glucose in this type of wines. In order to evaluate the accuracy of the proposed method, spectrophotometric determination of glucose was carried out using the standard spectrophotometric enzyme assay kit [26]. Good results were obtained, in agreement with the amperometric method.

3.6. Pyruvate Biosensor

Pyruvate was determined with the optimized PNR/PyOx biosensor, using chronoamperometry at − 0.25 V vs. SCE. The calibration plot for pyruvate standard solutions is presented in Figure 6B. The stationary-state current dependence was linear up to 0.6 mM pyruvate, with a slope of 0.13 μA mM^{-1} cm^{-2}. The detection limit of the pyruvate enzyme electrode was calculated as 34 μM, with signal to noise ratio of three, which is comparable to or lower than the detection limit previously reported for PyOx based biosensors [3, 32]. The calibration plot has a Michaelis–Menten dependence over the whole concentration range,

Table 2. Interference of some compounds on response to glucose at PNR / GOx carbon film electrodes.

Compound	Ratio glucose : interfering compound	Relative response (%)
Fructose	1:1	102.5
	1:3	102.9
	1:5	103.2
Lactate	1:1	99.1
	1:3	97.9
	1:5	95.4
Citric acid	1:1	94.8
Tartaric acid	1:1	89.3
Ascorbic acid	1:1	85.0

Table 3. Determination of glucose in wine samples at poly(neutral red)/glucose oxidase coated carbon film electrodes.

Sample	Glucose (g L^{-1}) (amperometric)	Glucose (g L^{-1}) (spectrophotometric)
Port wine	53.0 ± 1.0	56.5 ± 1.0
White wine	0.36 ± 0.05	0.31 ± 0.0003
Red wine 1	0.38 ± 0.03	0.39 ± 0.003
Red wine 2	0.12 ± 0.001	0.11 ± 0.0002

Table 4. Determination of pyruvate in onion and garlic at poly(neutral red)/ pyruvate oxidase coated carbon film electrodes.

Sample	Pyruvate (μmol g^{-1}) (amperometric)	Pyruvate (μmol g^{-1}) (spectrophotometric)
Onion	14.5 ± 0.9	13.8 ± 0.3
Garlic	47.2 ± 0.7	46.4 ± 0.3

which allowed the calculation of the apparent kinetic constant $K_m = 0.84 \pm 0.05$ mM ($n = 5$). This value is about twice the value of 0.34 mM of the dissolved enzyme, indicating that the electrooxidation of pyruvate was not kinetically but diffusion-limited [33].

Pyruvate oxidase is a relatively unstable enzyme, probably due to the gradual loss of its cofactor flavin adenine dinucleotide (FAD), during the immobilization procedure and during the measurements [39]. The enzyme electrode's long-term stability was tested over a period of two weeks by performing one calibration curve each day. After this period the sensitivity of the biosensor decreased to 62% of the initial value. The storage stability of the pyruvate biosensor was assessed by storing the enzyme electrodes dry at 4 °C. The enzyme electrode showed a 19% loss of sensitivity compared with a one-day-old device after 7 weeks of storage. With repeated use involving 11 measurements over a six-hour period, the current decreased to about 86% of the initial current sensitivity (data not shown).

PNR/PyOx modified carbon film electrodes showed good reproducibility. The relative standard deviation calculated from calibration curves using four different electrodes was 2.8%.

Concerning the selectivity of the PNR/PyOx biosensor, several compounds usually present in onions and garlic were tested as possible interferents: potassium ions, glucose, fructose, malic acid, citric acid, ascorbic acid, glutamic acid, arginine, lysine, cysteine and leucine. Equimolar concentrations of potassium ions, glucose and fructose caused no interference to pyruvate. Pyruvate can be determined in the presence of equimolar concentrations of glutamic acid and leucine with an error of 8% and 15% respectively. The pyruvate signal decreased drastically in the presence of ascorbic acid, malic acid and citric acid. Addition of equimolar concentrations of cysteine, lysine and arginine leads to a large increase in the amperometric signal. It was reported in [15] that the PNR film has electrocatalytic properties with respect to carboxylic acids. In onions only small amounts of ascorbic acid, citric acid and lysine are present, so that these compounds will not interfere in the determination of pyruvate [40]. In order to overcome interferences produced by cysteine, homogenization in trichloroacetic acid can be performed.

3.6.1. Determination of Pyruvic Acid in Onion and Garlic

In order to demonstrate the suitability of the PNR/PyOx biosensor, it was applied to the determination of pyruvate in onion and garlic using the standard addition method. Onion and garlic macerates were prepared as described in the experimental section. Independent measurements were also performed using a modified Schwimmer and Weston procedure, by determination of total 2,4-dinitrophenylhydrazine-reacting carbonyls [27]. The results are shown in Table 4 as the average value of three determinations; good agreement between the electrochemical biosensors and reference methods is seen. Thus the carbon-film based biosensor can be easily applied to analysis of pyruvate in these vegetables.

4. Conclusions

Amperometric biosensors for the electrochemical determination of glucose and pyruvate at poly(neutral red) modified carbon film electrodes have been developed. These biosensors were characterized by cyclic voltammetry and by electrochemical impedance spectroscopy to study the changes that occur on the modified electrode surface. The response of the biosensors to enzyme substrates was tested using amperometry under stirring conditions. Glucose biosensors had a linear range from 90 μM to 1.8 mM with a limit of detection of 22 μM. Pyruvate biosensors had the linear range from 90 to 600 μM with 34 μM detection limit. These biosensors were shown to have good reproducibility, the relative standard deviation calculated was 2.1% ($n = 3$) for glucose biosensors and 2.8% ($n = 4$) for pyruvate biosensors. The biosensors showed a good working stability when used daily: after two weeks glucose biosensors maintained 82% of their initial sensitivity and pyruvate biosensors 62%. Also the good storage stability of the biosensors indicates good attachment of the enzyme to the polymer.

All the above-mentioned results demonstrate that PNR/enzyme carbon film electrodes constitute robust amperometric biosensors and can be successfully applied to the amperometric determination of glucose and pyruvate in natural samples.

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