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A glucose biosensor using methyl viologen redox mediator on carbon film electrodes

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Abstract

A new methyl viologen-mediated amperometric enzyme electrode sensitive to glucose has been developed using carbon film electrode substrates. Carbon film electrodes from resistors fabricated by pyrolytic deposition of carbon were modified by immobilization of glucose oxidase through cross-linking with glutaraldehyde in the presence of bovine serum albumin. The mediator, methyl viologen, was directly immobilised with the enzyme together with Nafion cation-exchange polymer. The electrochemistry of the glucose oxidase/methyl viologen modified electrode was investigated by cyclic voltammetry and by electrochemical impedance spectroscopy. The biosensor response to glucose was evaluated amperometrically; the detection limit was 20 μM, the linear range extended to 1.2 mM and the reproducibility of around 3%. When stored in phosphate buffer at 4 °C and used every day, the sensor showed good stability over more several weeks.

Keywords: Glucose; Glucose oxidase; Amperometric enzyme electrode; Carbon film electrode; Methyl viologen mediator; Nafion

1. Introduction

Amperometric enzyme electrodes combine the advantages of the specificity of the enzyme for recognising particular target molecules with the direct transduction of the rate of reaction into current [1]. Enzyme electrodes, and particularly the glucose electrode, have been subject to numerous studies devoted to their optimisation for both biomedical and bioprocess control applications [2]. For glucose sensors two approaches are commonly employed: (1) monitoring of the hydrogen peroxide produced and (2) measuring the consumption of oxygen. Since, in the case of hydrogen peroxide monitoring the response of the device can be affected by the ambient concentration of oxygen, a solution is to replace the natural electron acceptor of GOx (oxygen) by electroactive compounds that will act as redox mediators [3]. In this case the enzyme glucose oxidase performs the first redox reaction with its substrate, glucose, but is then re-oxidised by the mediator, the reduced mediator in its turn being re-oxidised at the electrode [1].

A good redox mediator for a biosensor has to fulfil characteristics such as: (1) an operating potential ideally ∼0 V (versus SCE), where oxidation of most electrochemical interferents is avoided; (2) a fast reaction rate with the enzyme; (3) fast electron transfer kinetics; (4) no reaction with oxygen; (5) stable oxidised and reduced forms, etc. Typical mediators investigated have included: ferrocene [4,5] and its derivatives [6,7], Prussian Blue [8,9,10] and other metal hexacyanoferrates [11,12], quinones [13] and methyl viologen [14–16].

Viologens, derivatives of 4,4′-bipyridine, play an important role as electron relays in systems in which electron transfer is initiated by photochemical or electrochemical processes [17]. They exhibit fast reversible electrochemical response at negative potentials, which makes them useful as redox mediators for numerous enzymatic reactions [15]. Of special significance are the chemical properties of their one-electron-reduction products, which include good stability and the ability to undergo catalysed reactions with protons to give hydrogen [17]. Since viologens are highly water soluble

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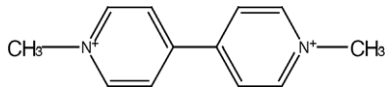

Fig. 1. The structure of methylviologen, MV^{2+}.

and toxic, any practical device containing these electron mediators must be based on immobilized viologens [15,16].

Methyl viologen's electrochemical behaviour involves reduction of MV^{2+} (see Fig. 1), by a reversible 1-electron reaction to a blue radical cation, which can be further reduced to the neutral form, that tends to adsorb on the electrode surface [18,19]. The structure of MV^{2+} consists of a hydrophobic part that is capable of hydrophobic-hydrophobic interaction with Nafion and two cationic pyridinium groups that undergo ion exchange with the sulphonate sites of Nafion polymer chains, according to:

$$MV_{aq}{}^{2+} + 2(SO_3{}^{-}Na^{+})_{film} \rightarrow [(SO_3{}^{-})_2MV^{2+}]_{film} + 2Na_{aq}{}^{+}$$

This interaction results in accumulation of MV^{2+} in Nafion films [17]. Nafion–methyl viologen complexes in the matrix possess excellent catalytic activity for the electroreduction of oxygen, and have high permeability to oxygen, but limited permeability to potential interferents such as uric acid, ascorbic acid and cystine [20].

Carbon materials are widely used in electrochemistry as electrodes and as substrates for modified electrodes. A wide variety of physical forms, relatively high chemical stability, low cost, and suitability for chemical modification, make them attractive in bioelectrochemical applications, i.e., interphase electron transfer involving solution or surface-attached biomolecules. The electrochemistry of different carbon materials has been much studied during the past few decades, and a number of excellent review articles and monographs have been published [21].

Recently, carbon film electrodes fabricated from cylindrical carbon film electrical resistors, made by pyrolytic deposition of carbon on ceramic cylinders, have been developed [22]. Electrochemical pretreatment by cycling in acid or at fixed applied potential leads to a widened potential window and permits direct use in a number of analytical applications. These have included anodic stripping voltammetry without mercury films, and cathodic stripping voltammetry [22,23]. Other applications investigated have been concerned with the development of enzyme biosensors by glutaraldehyde cross-linking [24] and with cobalt hexacyanoferrate mediator [25], the assembly being evaluated for glucose detection using glucose oxidase enzyme. Furthermore, the carbon film electrode surfaces have been investigated electrochemically in different electrolyte solutions by electrochemical impedance spectroscopy [26]. These carbon electrodes are easy to prepare and are envisaged for application as short-term or disposable sensors.

A current objective is to evaluate these carbon film electrodes using GOx enzyme with different types of redox mediator for application in food quality monitoring. The specific aim of this work concerned methylviologen mediator: the carbon film resistor electrodes were modified by methyl viologen (MV) mediator mixed with glucose oxidase by cross-linking with glutaraldehyde in the presence of bovine serum albumin and Nafion. The resulting enzyme electrode's performance was characterised by cyclic voltammetry and electrochemical impedance spectroscopy and the performance of the enzyme biosensor evaluated by the amperometric detection of glucose.

2. Experimental

2.1. Reagents

Glucose oxidase (GOx, EC 1.1.3.4, from *Aspergillus niger*, 24 U/mg) was from Fluka, α-D(+)-glucose, glutaraldehyde (GA) (25%, v/v) and bovine serum albumin (BSA) were purchased from Sigma, Nafion (5%, v/v) and methyl viologen were from Aldrich.

For electrochemical experiments, the supporting electrolyte was sodium phosphate buffer saline (NaPBS) (0.1 M phosphate buffer + 0.05 M NaCl, pH 7.0).

Milipore Milli-Q nanopure water (resistivity >18 MΩ cm) was used throughout for the preparation of solutions. A stock solution of 1 M glucose was prepared in supporting electrolyte one day before use to permit equilibration of α and β anomers of D-glucose and was kept in the refrigerator.

2.2. Apparatus

Cyclic voltammograms were registered using an μAutolab Type II running with GPES (General Purpose Electrochemical System) for Windows version 4.9, software PG (Echo-Chemie, Utrecht, The Netherlands), and impedance spectra were performed with a Solartron 1250 Frequency Response Analyzer together with a Solartron 1286 Electrochemical Interface (Solartron Analytical, UK), controlled by Zplot software. Amperometric measurements were carried out at −0.5 V using a Bioanalytical Systems (BAS, West Lafayette, IN) CV-50W electrochemical analyzer. The electrochemical three-compartment cell employed in these experiments consisted of carbon resistor/methyl viologen modified glucose sensor working electrode, a platinum auxiliary electrode and an Ag/AgCl (3 M KCl) electrode as reference.

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

2.3. Electrode preparation

Electrodes were made from carbon film resistors (2 Ω nominal resistance). These resistors are fabricated from ceramic cylinders by pyrolytic deposition of carbon [22]. The

resistor has two metal caps with an external contact placed over each end. One of the two caps was removed and the other insulated by plastic and protected by normal epoxy resin. In this way the exposed electrode geometric area was ~0.2 cm^2.

GOx was 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. Methyl viologen was deposited together with the enzyme and with Nafion in order to obtain a stable and active enzyme layer. A mixture of 5 μl of glutaraldehyde (2.5% in water), 4 μl of Nafion (5% in alcohol), 8 μl of methyl viologen (0.1 M in buffer) and 15 μl of enzyme solution was prepared. The enzyme solution contained 40 mg of bovine serum albumin (BSA) and 10 mg of GOx in 1 ml of 0.1 M NaPBS (pH 7.0). From this mixture 10 μl was placed onto the surface of the electrode and allowed to dry at room temperature for at least 1 h. Electrodes were kept at 4 °C in sodium phosphate buffer solution when not in use. Electrodes were used only on the second day after preparation when the current response was better.

2.4. Analysis of wine samples

For analysis of wine samples, 10 μl aliquots were added to 10 ml of 0.1 M NaPBS, and the standard addition method was used to determine the glucose concentrations.

Independent analysis of glucose concentrations was done using the standard spectrophotometric enzyme assay kit [27] (Cat 0139106, Boehringer, Mannheim).

3. Results and discussion

3.1. Characterisation of methyl viologen-modified carbon film electrode

3.1.1. Cyclic voltammetry

The modified electrodes were characterized by cyclic voltammetry to confirm the electron flow from the carbon resistor electrode to GOx via the mediator. Fig. 2 shows voltammograms of unmodified and of methyl viologen/Nafion/enzyme-modified carbon film electrodes in air-saturated phosphate buffer solution at pH 7.0. Fig. 2A, the cyclic voltammogram of the unmodified electrode, does not show any peak. Fig. 2B shows cyclic voltammograms of the GOx/Nafion/methyl viologen-modified electrode with and without the addition of 10 mM glucose. The oxygen reduction peak can be seen at about −0.5 V versus Ag/AgCl in the absence of glucose, whilst it completely disappears in the presence of glucose.

The catalytic effect of the oxygen reduction by the methyl viologen-modified electrode is clearly observed. The mechanism of reduction of oxygen to hydrogen peroxide by methyl viologen-modified electrode can be described as [28]:

$$MV^{2+} + e^- \rightarrow MV^+$$

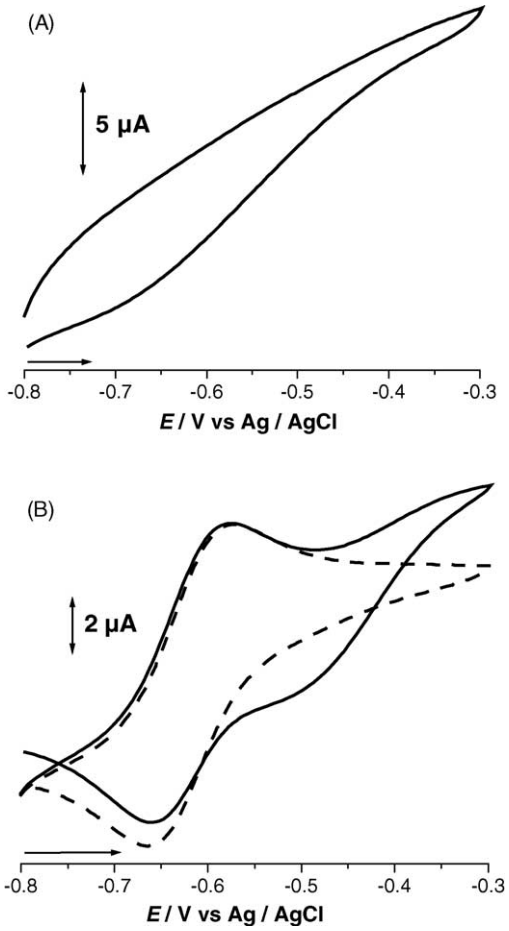

Fig. 2. Cyclic voltammogram of (A) carbon film electrode and (B) MV/Nafion/GOx-modified electrode in 0.1 M NaPBS buffer without (—) and with (- - -) addition of 10 mM glucose.

$$MV^+ + O_2 \rightarrow MV^{2+} + O_2^- \quad \text{(within the film)}$$

$$2O_2{}^- + 2H^+ \rightarrow O_2 + H_2O_2$$

In the presence of glucose, there is also the reaction:

$$\text{glucose} + O_2 + H_2O \rightarrow \text{gluconic acid} + H_2O_2$$

MV itself is not stable in water but by binding with Nafion become stable in aqueous solution so that this modified carbon resistor can be used as an amperometric sensor for glucose.

3.1.2. Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy is an extremely powerful and sensitive characterisation technique for probing the charge transfer and charge separation processes occurring at electrode/solution or modified electrode/solution interfaces as well as their variation in time.

Measurements were carried out in sodium phosphate saline pH 7 at 0.0 and −0.5 V versus Ag/AgCl with and without addition of glucose. There are clear differences between the spectra obtained at the two potentials concerning both the shape of spectra, evidencing the changes to the

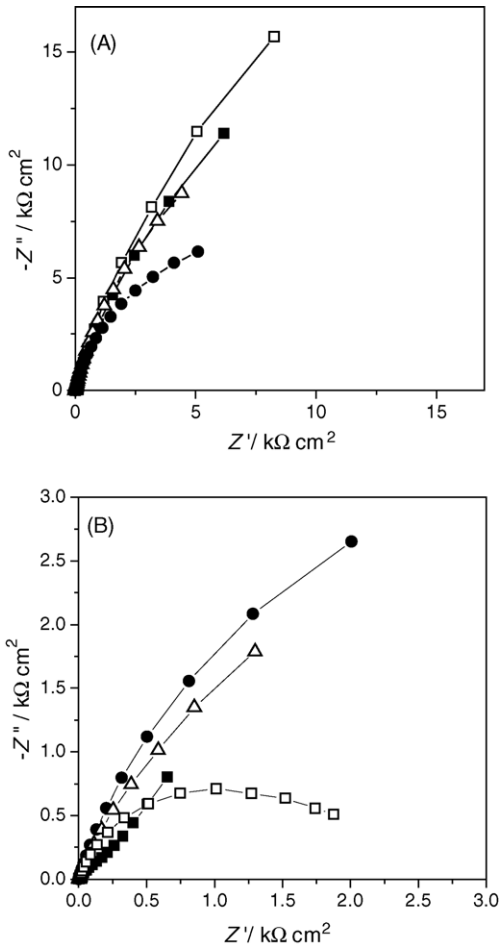

Fig. 3. Impedance spectra at (A) 0.0 and (B) −0.5 V at in pH 7.00.1 M NaPBS buffer for bare carbon film electrode, MV/Nafion modified electrode and MV/Nafion/GOx modified electrode without and with addition of 5 mM glucose.

electrode surface caused by MV/Nafion modification or by GOx/MV/Nafion modification. From Fig. 3 it can be seen that effect of modification of the electrode surface at −0.5 V (Fig. 3B) is different than at 0.0 V (Fig. 3A).

Spectra were fitted with one of two equivalent circuits to model the system, which are shown in Fig. 4. The first of these comprises the cell resistance, R_Ω, in series with a constant phase element, CPE_1, representing a distributed capacitance

$$CPE_1 = -\frac{1}{(C_1 i\omega)^\alpha}$$

in parallel with a resistance, R_1. The CPE describes the charge separation on the electrode film and through the enzyme layer, whilst R_1 represents charges transfer processes occurring at the carbon film interface; the necessity for the CPE arises from the electrode roughness and non-homogeneity of the mediator/enzyme layer.

The second circuit is the same as the first but with a second CPE, CPE_2, in series with R_1. The physical meaning of this is charge separation through the enzyme layer above the sites

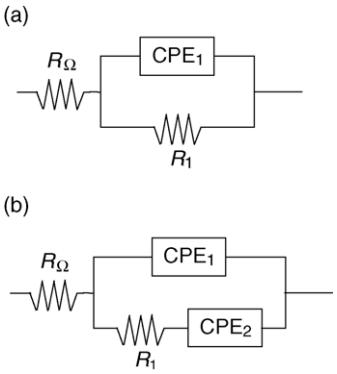

Fig. 4. Equivalent circuits for modelling impedance data of (a) GOx/MV and (b) Nafion/MV modified electrodes.

on the electrode substrate where charge transfer occurs. This type of circuit is a simplified version of that often used for coated electrodes [29]. The use of CPE_2 was only justified for one of the experimental conditions and electrode assemblies, namely MV/Nafion at −0.5 V without GOx. This can be ascribed to the fact that in these conditions oxygen can diffuse into the coating layer to the immobilised MV molecules and be reduced, whereas this does not occur easily in the presence of GOx. The consequences of this are that the presence of oxygen should have less influence on the experimental results than might be expected as is demonstrated below in the following section.

The values of the circuit elements obtained are shown in Table 1. The value of R_Ω was around 1.4 Ω cm^2 in all cases both for 0 V and for −0.5 V applied potential.

General conclusions are as follows. At an applied potential of 0.0 V, the resistance is significantly diminished in the presence of glucose, showing that some reaction of glucose can occur. The presence of GOx in the modifier film significantly increases the capacity values. However the values of the α_1 exponent are hardly changed from the value at the bare carbon film electrode, suggesting that its value is mainly due to surface roughness. At −0.5 V, the charge transfer resistances are much lower and the charge separation higher, owing to the occurrence of the mediated enzyme reaction. Note that the values of MV/Nafion modifier film are different since CPE_2 has to be invoked in this case, as discussed above.

3.2. Enzyme substrate measurements

Before performing each amperometric measurement, one cyclic voltammogram was recorded to activate the enzyme and to verify the stability of the mediator. Amperometric measurements were performed at fixed potential, after stabilization of the baseline, with injection of glucose into air saturated NaPBS solution containing the GOx/methyl viologen modified carbon film electrode, with continuous stirring, detecting the hydrogen peroxide formed. The response to glucose was observed within 30 s and the current reached a plateau in 2 min.

Table 1
Equivalent circuit fitting from impedance data for bare and coated electrodes in 0.1 M NaPBS pH 7.0 at 0 V and at −0.5 V

E/V vs. SCE	Electrode coating	R_1 (kΩ cm^2)	C_1 (μF cm^{-2})	α_1
0.0	Bare carbon film	68.9	73.3	0.84
	MV/Nafion	67.7	86.3	0.85
	MV/Nafion/GOx	59.6	141.3	0.87
	MV/Nafion/GOx/5 mM glucose	14.4	145.1	0.86
−0.5	Bare carbon film	2.5	164.5	0.78
	MV/Nafion[a]	0.34	493.9	0.71
	MV/Nafion/GOx	2.5	350.6	0.82
	MV/Nafion/GOx/5 mM glucose	5.0	578.8	0.81

Data from Fig. 3.
[a] Here CPE_2 was introduced in series with R_1, values $C_2 = 1.0$ mF cm^{-2} and $\alpha_2 = 0.61$.

The electrochemical response to increasing concentrations of enzyme substrate was plotted. Fig. 5 shows a typical calibration curve for an electrode in pH 7.0 NaPBS at an applied potential of −0.5 V versus Ag/AgCl. In this situation the methyl viologen-modified electrode showed a linearity range up to 1.2 mM. The corresponding detection limit (signal-to-noise ratio = 3) was 20 μM. The regression equation of the linear plot was I (μA) = 0.04983 + 3.47119c, with $R = 0.9998$, where c is the glucose concentration in mM.

Kinetic studies of the immobilized enzyme on freshly/prepared electrodes led to an apparent Michaelis–Menten constant from the Lineweaver–Burk plots of 4.6 ± 0.3 mM (five electrodes). The value obtained remained the same after repeated use, but sensitivity and linear range decreased.

In order to evaluate the long-term stability of this sensor, one calibration curve was recorded every day and the current at a fixed glucose concentration of 0.7 mM was measured. There is a rather large initial decrease of response magnitude (Fig. 6). After 6 days there was a decrease of 40% from the initial response. The sensor gave a stable response current after this period, which was retained for more than 2 weeks.

The reproducibility of the methyl viologen-modified carbon resistor electrodes was evaluated from calibration curves response plots using five different electrodes. The results showed relative standard deviations of around 3%. Thus the sensor showed a good, reproducible behaviour and can be used for reproducible measurements.

It has been reported that the oxygen interference is not negligible at the applied potential used in these measurements [30]. To investigate the influence of dissolved oxygen, nitrogen was bubbled through the cell to deoxygenate during 10 min before the measurements were made and the potential was applied. It was found that although the signal measured is higher in air-saturated buffer than in N_2-saturated buffer, the oxygen signal is almost constant so that there is only a small proportional increase of ∼1–1.5% in the presence of oxygen, which is not significant.

An alternative strategy for producing an enzyme sensor was without the inclusion of Nafion. The same immobilization method as before was used. A mixture of mediator and enzyme using glutaraldehyde for cross-linking was placed onto the electrode surface and left to dry at room temperature for at least 1 h. Parameters determined were quite different from those of immobilized enzyme using Nafion. A lower sensitivity of 2.36 μA/mM and higher detection limit of 32 μM were obtained with this sensor. The linear range was up to 1.67 mM and Michaelis–Menten constant $K_M = 3.16$ mM. On successive calibrations, a loss of 7% of

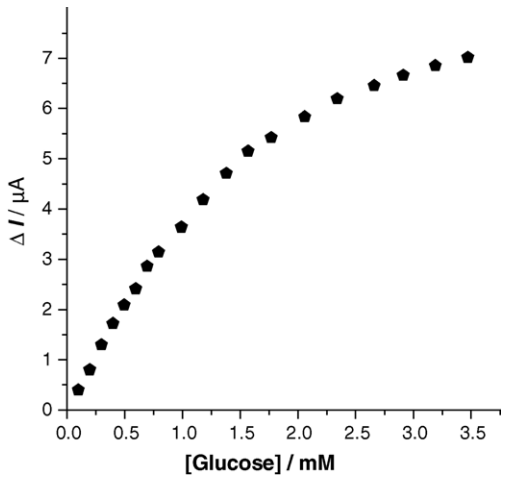

Fig. 5. Typical calibration curve for MV/Nafion/GOx-modified electrode at −0.5 V vs. Ag/AgCl for increasing glucose concentrations.

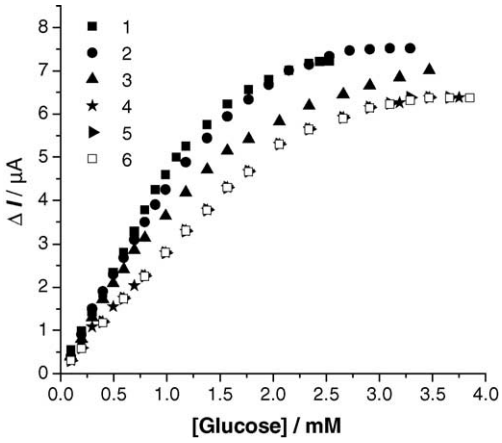

Fig. 6. Six sequential calibration curve measurements with fresh electrodes. The number indicates the order of the measurements.

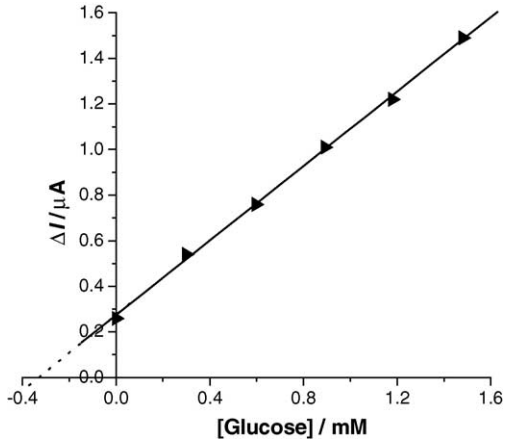

Fig. 7. Determination of glucose concentration in Port wine by the standard addition method.

the initial value was observed. Comparing the two sensors, with and without Nafion included, it is clear that using Nafion a sensor with higher performance was obtained, as seen previously using other electrodes [15].

3.3. Determination of glucose in port wine

In order to test the applicability of the developed GOx/Nafion/MV sensor to food and beverages, a sweet port wine was analysed by the standard addition method (Fig. 7). The wine sample was diluted 1000 times in 0.1 M NaPBS, and the analyte was spiked with 0.3 mM aliquots of glucose. Results obtained were $59.5 \pm 0.9\,g\,l^{-1}$ glucose in agreement with values obtained by the standard spectrophotometric method [27], which was $56.5 \pm 1.0\,g\,l^{-1}$.

Experiments were also carried out in the absence of oxygen and the results were $58.7 \pm 1.2\,g\,l^{-1}$ glucose in Port wine. This means that in absence of oxygen the response is slightly, but not significantly, smaller in better agreement with the independent reference method.

This augurs well for application of the methyl viologen-mediated glucose electrode, as a robust, cheap and disposable sensor for glucose monitoring in wines and during grape fermentation processes as well as in other foods.

4. Conclusions

A novel amperometric mediated biosensor for glucose determination has been developed employing carbon film resistor electrodes. Glucose oxidase was immobilized together with the redox mediator, methyl viologen, and Nafion by meaning of cross-linking with glutaraldehyde in presence of BSA. An alternative construction method without Nafion was less stable.

Characterization by cyclic voltammetry showed reduction of oxygen by MV-modified electrodes. Electrochemical impedance spectroscopy measurements clearly demonstrated the changes undergone on the electrode surface by modification and highlighted the mechanism of the processes occurring.

The sensor has been demonstrated to be successful for the measurement of hydrogen peroxide produced by the enzymatic oxidation of glucose and with low, micromolar detection limits, good reproducibility around 3% and good stability for over more than 1 week after daily use.

Analysis of Port wine led to values in agreement with standardised spectrophotometric determinations.

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