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Development and Characterization of Cobalt Hexacyanoferrate Modified Carbon Electrodes for Electrochemical Enzyme Biosensors

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ABSTRACT

Carbon film electrodes have been modified with films of cobalt hexacyanoferrate by potential cycling from solutions containing cobalt and hexacyanoferrate ions. The voltammetric characteristics of the films have been investigated in different electrolyte solutions and the properties related to insertion reactions within the crystal structure. The application of these modified electrodes as redox mediators in enzyme biosensors has been investigated using the mediated detection of hydrogen peroxide, demonstrated by the determination of glucose using glucose oxidase. Excellent detection limits in the micromolar region have been attained

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and the principle of measurement in real samples demonstrated by that of glucose in sweet wine.

Key Words: Carbon film; Cobalt hexacyanoferrate; Enzyme electrode; Glucose; Glucose oxidase.

1. INTRODUCTION

Currently, the most widely used amperometric biosensors are based on oxidase enzymes that generate H_2O_2, the transduction path being the electrochemical oxidation of the peroxide formed in an enzyme reaction.[1–3] A serious problem that must be overcome for the use of such biosensors in physiological or food samples is the presence of metabolites or other compounds that represent positive interference due to fact they are oxidized at the same potential as H_2O_2. In the case of glucose, the peroxide that is generated is harmful for glucose oxidase (GOX), limiting biosensor performance. One solution is to replace the natural electron acceptor of GOX (oxygen) by electroactive compounds that will act as redox mediators.[4] Such freely diffusing or immobilized artificial redox mediators can solve some of the difficulties.[5,6]

Carbon has become a commonly used solid electrode material due to its wide potential window, low cost, mechanical stability, and applicability to a wide range of redox systems.[7] Carbon film electrodes fabricated from electrical resistors have been developed which have a wider potential range than many other forms of carbon, especially after electrochemical pretreatment.[8] Their surface properties have been investigated in a number of electrolytes[9] and applications to the electroanalysis of trace metal ions in the negative and positive potential range have also been demonstrated.[8,10]

Such carbon film electrodes offer an inexpensive route for developing a glucose biosensor,[11] but require a high overpotential to detect H_2O_2, which reduces the selectivity of the sensors in complex matrices. Promising advances in improving the selectivity at carbon-based electrochemical sensors have been achieved through surface modification with redox mediators. These were obtained especially with glassy carbon,[12,13] graphite,[2] screen-printed,[14] and carbon rod electrodes.[15]

In second generation biosensors, the mediator exists in the soluble phase, while in third generation biosensors it is retained together with the enzyme close to the electrode, shuttling the electrons between redox center of enzyme and electrode.[16] Usually, methods to retain biomolecules on the electrode surface are also used to immobilize the redox mediators in order to assure an efficient electron transfer pathway.

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An ideal redox mediator has to fulfill characteristics required for the good performance of a biosensor, such as: (1) an operating potential (ideally $\sim$0.0 V vs. SCE), where oxidation of most electrochemical interferents is avoided; (2) a fast reaction rate with the enzyme; (3) electrochemical properties independent of pH; (4) fast electron transfer kinetics; (5) no reaction with oxygen; (6) absence of toxicity; (7) stable oxidized and reduced forms. The most widely-used mediators are ferrocene[17,18] and its derivatives,[19,20] Prussian Blue[12,14,21] and other metal hexacyanoferrates.[13,22]

Metal hexacyanoferrate films are interesting redox mediators. They are mixed-valence clusters possessing semiconductor characteristics and can transfer electrons during reduction and oxidation processes.[23,24] Attachment of these species to electrode surface can be achieved by controlled-potential electrodeposition, galvanostatic deposition, adsorption, or entrapping them into polymeric matrices and mechanically transferring them onto the electrode surface.

Prussian Blue (ferric ferrocyanide) has been the most widely used of the metal hexacyanoferrates to develop enzyme redox mediators. An example of another metal hexacyanoferrate, which has been investigated recently for various purposes, is cobalt hexacyanoferrate.[25,26] A cobalt hexacyanoferrate-modified microband gold electrode was used for catalytic oxidation of NADH,[27] modified glassy carbon for dopamine[28] and for morphine,[29] modified gold for several catecholamines,[30] modified graphite for hydrazine[31] and thiosulfate,[32] and a cobalt hexacyanoferrate-modified graphite organosilicate electrode for oxidation of thiosulfate.[33] Additionally, a cobalt hexacyanoferrate-coated glassy carbon electrode was used for preparing an enzyme biosensor for glucose detection[22] and the electrochemical and electrocatalytic activity of this mixed-valence compound thin film electrode, grown electrochemically and modified with ruthenium, is described.[34]

Regarding the use of other cobalt compounds as redox mediators, cobalt phthalocyanine was used successfully at screen printed carbon electrodes which were then coated with GOX as well as cellulose acetate to enhance selectivity and reduce possible interferences.[35]

In this work we study development and characterization of an amperometric GOX–enzyme electrode based on a cobalt hexacyanoferrate-modified carbon film electrode.

2. EXPERIMENTAL

2.1. Chemicals

Glucose oxidase (EC 1.1.3.4, II-type from *Aspergillus niger*, 35,600 units mg^{-1}), α-D(+)-glucose, glutaraldehyde (GA) 25% (v/v), and

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bovine serum albumin (BSA) were purchased from Sigma-Aldrich, UK. Potassium hexacyanoferrate(III) ($K_3Fe(CN)_6$) and $CoCl_2 \cdot 6H_2O$ were obtained from Merck, Germany. Nafion 5% (v/v) was from Sigma-Aldrich, UK. Millipore Milli-Q nanopure water (resistivity $> 18\,M\Omega\,cm$) was used throughout for the preparation and dilution of solutions. The supporting electrolyte was phosphate buffer saline (PBS) (0.1 M phosphate buffer + 0.05 M NaCl). Hydrogen peroxide solutions were calibrated by titration with acidified $KMnO_4$ solution. Glucose standard solutions were prepared by dilution of a 100 mM α-D(+)-glucose stock solution prepared in water. The stock solution was prepared 24 hr before use to establish the anomeric equilibrium between α and β forms of D-glucose; it was kept in the refrigerator and used within a week.

2.2. Electrochemical Measurements

Measurements were made in a one-compartment cell containing the carbon film electrode, a platinum auxiliary electrode and a saturated calomel electrode (SCE) as reference. Voltammetric and amperometric experiments were carried out using CV-50 W Voltammetric Analyzer from Bioanalytical Systems, West Lafayette, IN, controlled by BAS CV-2.1 software.

2.3. Preparation of Electrode

Electrodes were prepared from carbon film resistors (2 Ω nominal resistance), as described in Ref.[8] Briefly, these electrical resistors are fabricated by pyrolytic deposition of carbon at 1100°C in a nitrogen atmosphere containing a small amount of methane onto ceramic cylinders of length 0.40 cm and external diameter 0.15 cm. Tight fitting metal caps attached to external connecting wires are then press fitted to each end.

To make an electrode, one of the metal caps plus conducting wire was removed from one of the ends of a resistor. The remaining conducting wire was sheathed in plastic insulation up to the respective cap and the cap and plastic contact area was carefully covered in epoxy resin so that only the carbon film would be exposed to solution. After this assembly, the exposed electrode geometric area was $\approx 0.2\,cm^2$.

2.4. Electrochemical Modification of Carbon Film with Cobalt(II)-Hexacyanoferrate

The carbon film electrodes were modified by electrochemical deposition of cobalt(II)-hexacyanoferrate (CoHCF). This was accomplished by cycling

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the electrodes 15 times between 0.0 and +0.9 V at a scan rate of 50 mV sec^{-1} in a freshly prepared solution containing 0.5 M $CoCl_2$, 0.25 M $K_3Fe(CN)_6$, and 0.05 M NaCl at pH 3 (pH adjusted with HCl) with solution agitation by slow mechanical stirring. Subsequently, the CoHCF film electrodes were stabilized for 1 hr in 0.05 M NaCl, pH 3. After that they were left to dry at room temperature. Modification of the electrodes was always carried out employing the same, identical solution composition for obtaining reproducible results.

2.5. Enzyme Immobilization

The GOX was immobilized onto the CoHCF-modified electrode surface with the cross-linking method. A mixture of GA, enzyme, and BSA was used. In order to obtain a stable and active enzymatic layer,[5] a layer of Nafion membrane 1% was applied over the prepared enzyme-electrode, after that the sensor having been left in air to dry.

To prepare 35 μL of this enzyme mixture, 10 μL of GA (2.5% v/v diluted in water) were mixed with 25 μL of an enzyme solution. The enzyme solution was prepared by dissolving 40 mg of BSA and 10 mg of GOX in 1 mL of 0.1 M PBS, pH 7, (0.1 M phosphate buffer + 0.05 M NaCl).

From this mixture, 10 μL of enzymatic solution was placed onto the surface of the working electrode and allowed to dry for 1 hr at room temperature, and after that the biosensor was coated with 4 μL of 1% Nafion solution.

3. RESULTS AND DISCUSSION

3.1. Cobalt(II)-Hexacyanoferrate Modified Electrode Preparation and Characterization

The modification of carbon film electrodes by cobalt hexacyanoferrate was carried out by cyclic voltammetry, and voltammograms were continuously recorded. Figure 1 illustrates the formation of the CoHCF film onto the electrode which demonstrates the growth in thickness with each cycle, as revealed by the increasing charge under the deposition peak. The film formed during the above cycling was kept in pure supporting electrolyte of 0.05 M NaCl, in this way yielding a stable CV response.

Figure 2(a) shows typical cyclic voltammograms for the CoHCF-modified carbon film electrode in 0.05 M NaCl aqueous solution at different scan rates. The anodic and cathodic peak potentials shift symmetrically, resulting in an increase in peak separation with increasing scan rate: from 36 mV at 10 mV sec^{-1} to 190 mV at 190 mV sec^{-1} scan rate. Both current

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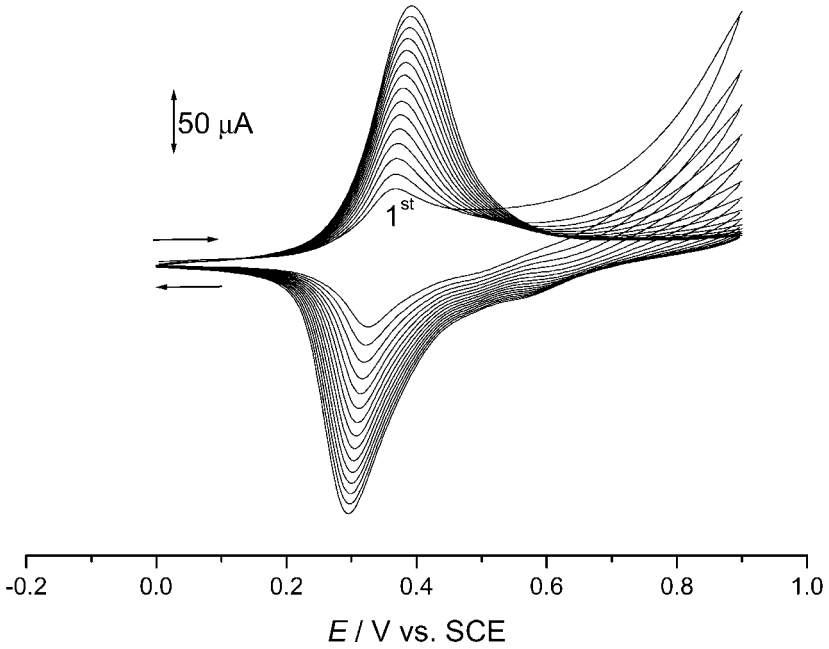

Figure 1. Cyclic voltammograms showing the continuous growth of CoHCF on carbon film electrode. Scan rate 50 mV sec^{-1}.

peaks increase linearly with scan rate up to 190 mV sec^{-1} [Fig. 2(b)] and the ratio of anodic and cathodic peak currents for different scan rate values is almost unity.

The effect of electrolyte concentration on the CoHCF-modified carbon film electrode electrochemical behavior can be seen in Fig. 3. This figure shows comparative cyclic voltammograms of the modified electrode in three different concentrations of NaCl solution at a scan rate of 30 mV sec^{-1}. The peak currents decreased and redox peaks shifted in the negative direction simultaneously with decreasing electrolyte concentration. In 1.0 M NaCl the anodic–cathodic peak separation is 49.5 mV at a scan rate of 30 mV sec^{-1}, whereas in 0.05 M NaCl the separation is 81.8 mV and the reversibility decreased. This shows the importance of the rate of cation (i.e., counterion) exchange between the solution and film phases on film oxidation and reduction.

It was also important to characterize the behavior of the Co-HCF modified electrodes in phosphate buffer solutions, since this is the electrolyte commonly used for enzymatic experiments. Figure 4 shows voltammograms of the

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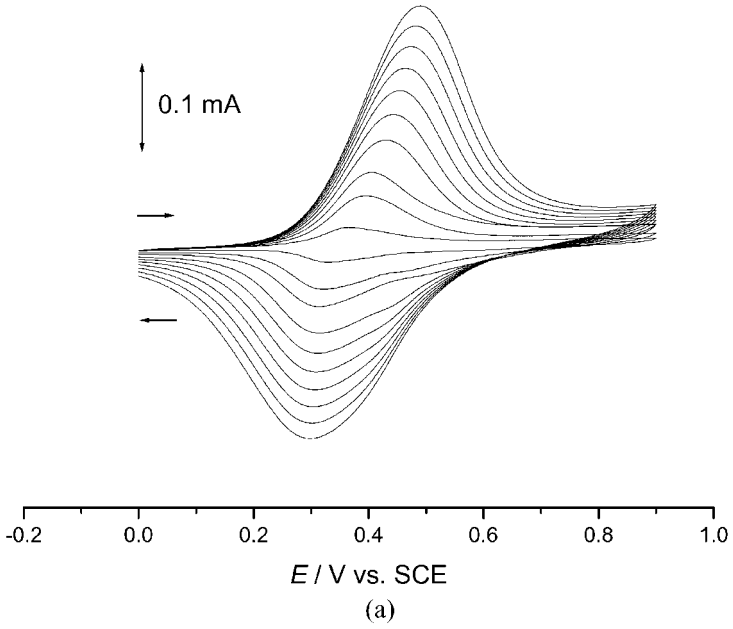

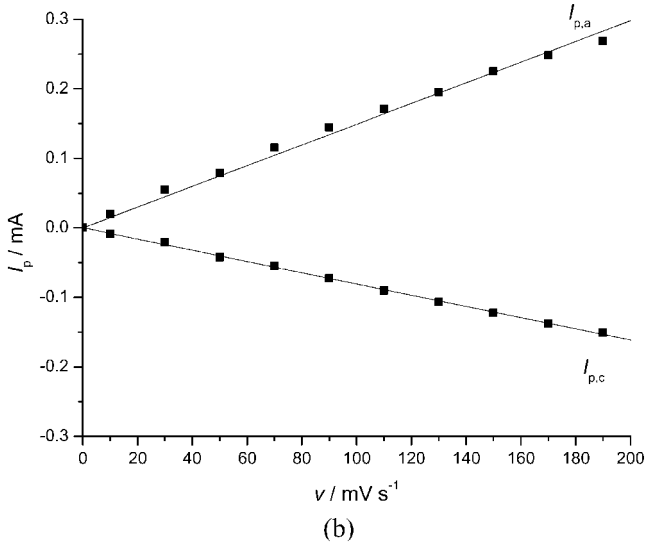

Figure 2. (a) Cyclic voltammograms of the CoHCF-modified electrode in 0.05 M NaCl aqueous solutions at different scan rates (from inner curve to outer curve: 10, 30, 50, 70, 90, 110, 130, 150, 170, and 190 mV sec^{-1}). (b) The dependence of anodic and cathodic peak currents on the scan rate for CoHCF-modified electrode in 0.05 M NaCl solution (pH = 7).

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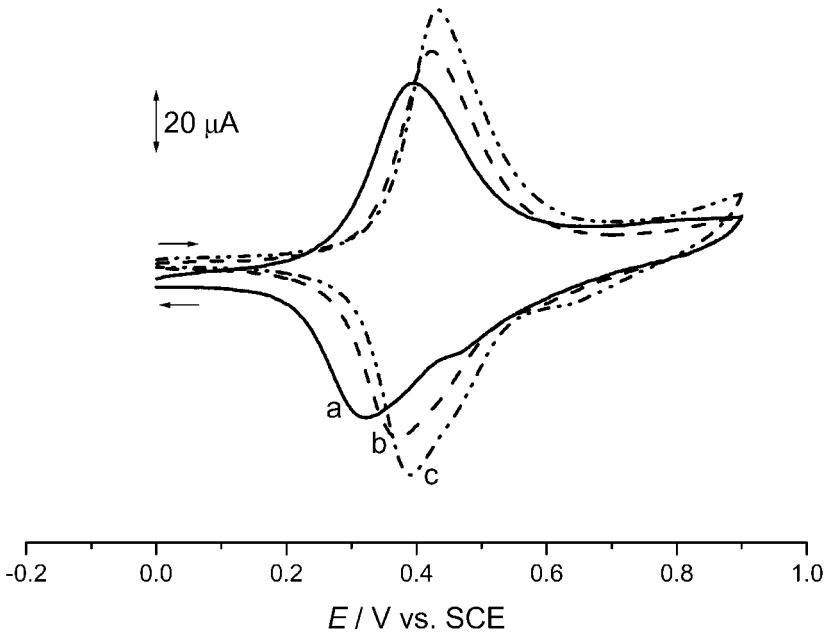

Figure 3. Cyclic voltammograms of the modified electrode in: (a) 0.05 M, (b) 0.5 M, and (c) 1.0 M NaCl solution at 30 mV sec^{-1} scan rate (pH = 5.26).

CoHCF-modified carbon film electrode in 0.1 M sodium phosphate buffer (NaPB) solution (pH = 7) at various scan rates. Well-defined anodic and cathodic peaks can be seen. No separation between these peaks is observed at low scan rate ($<$10 mV sec^{-1}), but at higher scan rates the peaks begin to separate: at 30 mV sec^{-1} the peak separation is 33 mV, while at 190 mV sec^{-1} the peak separation increases up to 164 mV.

The effect of the electrolyte cation on the CoHCF-modified carbon film electrode can be seen by comparing Figs. 4 and 5, that shows cyclic voltammograms of a modified electrode in 0.1 M potassium phosphate buffer (KPB) solution (pH = 7.4) at different scan rates. In this case the oxidation and reduction peaks are less well-defined and broader, which can be explained in terms of the different transfer rate of Na^+ and K^+ ions in CoHCF. The transport of K^+ in CoHCF film is more difficult than that of Na^+ because it has a larger ionic radius.[27]

The surface concentration of electroactive species, Γ_c, can be approximately calculated by the equation:

$$Q = nFA\Gamma_c$$

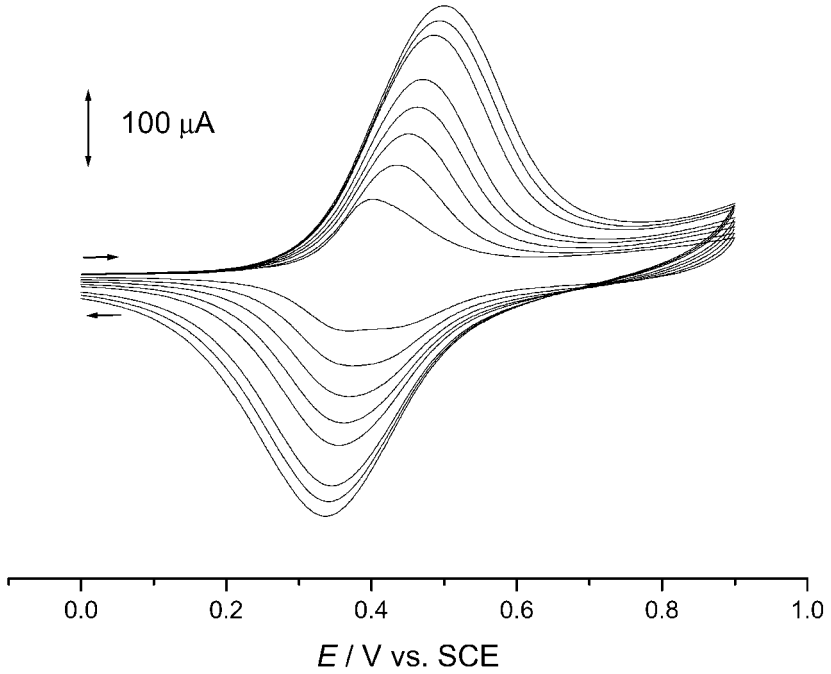

Figure 4. Cyclic voltammograms of the CoHCF-modified electrode in 0.1 M NaPB solution (pH = 7.0) at different scan rates (from inner curve to outer curve: 30, 50, 70, 90, 110, 150, 170, and 190 mV sec^{-1}).

where Q is the background-corrected charge obtained by integration of the anodic peaks ($\nu < 100\,mV\,sec^{-1}$), A is the electrode geometric surface area, F is the Faraday constant, and n is number of electrons. In the present case Γ_c is $9.9 \times 10^{-8}\,mol\,cm^{-2}$. This value is of the same order of magnitude as in Refs.[32,33] which suggests that the film structure is very similar.

3.2. Hydrogen Peroxide Detection

Since CoHCF-based carbon film electrodes will be used to fabricate a sensor for detection of hydrogen peroxide (enzymatic reaction product), the CoHCF-modified electrode response to the injection of H_2O_2 was also studied. A potential was applied to the electrode and the baseline was allowed to stabilize. Aliquots of H_2O_2 and 100-mM glucose stock solution in water were added to the cell such that each addition resulted and 10 μM, increment

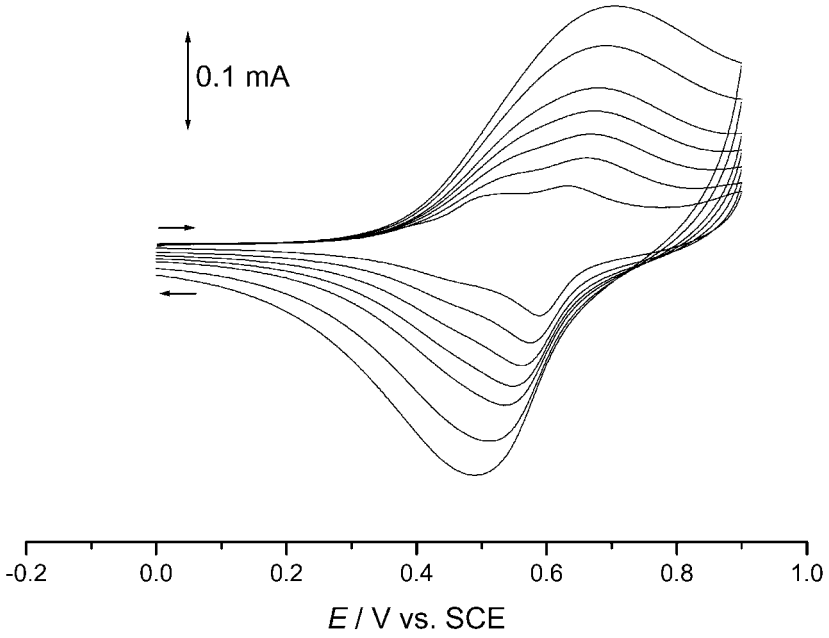

Figure 5. Cyclic voltammograms of the CoHCF-modified electrode in 0.1 M KPB solution (pH = 7.4) at different scan rates (from inner curve to outer curve: 30, 50, 70, 90, 110, 150, and 190 mV sec^{-1}).

concentration in glucose and in 1 μM H_2O_2, respectively; the corresponding increase in current was recorded, from which the baseline was subtracted.

The influence of applied potential on H_2O_2 detection at CoHCF modified carbon films was studied and an optimal potential of 0.0 V vs. SCE was found (results not shown). This is in agreement with previous work at glassy carbon substrate.[22] This applied potential ensures a minimizing of interference effects when the electrode is used in real and complex matrices, such as beverages or food, and oxygen reduction also does not occur.

At the same time, the influence of pH on sensor activity was studied. It was found that in the pH range 5–7, the sensor response is very similar for small concentrations in the linear part of calibration curves. H_2O_2 calibration curves showed a good linearity in the range between 0 and 5 μM, with a detection limit (three times the signal to noise ratio) of 0.27 μM (Fig. 6). The corresponding regression equation of the linear plot for PBS, pH = 7, was $I/nA = 2.97 + 17.2c$, with $R = 0.9994$, where c is the H_2O_2 concentration in μM.

In order to study the stability of the CoHCF film on carbon film resistor electrodes, six successive calibration curves of H_2O_2 response in the

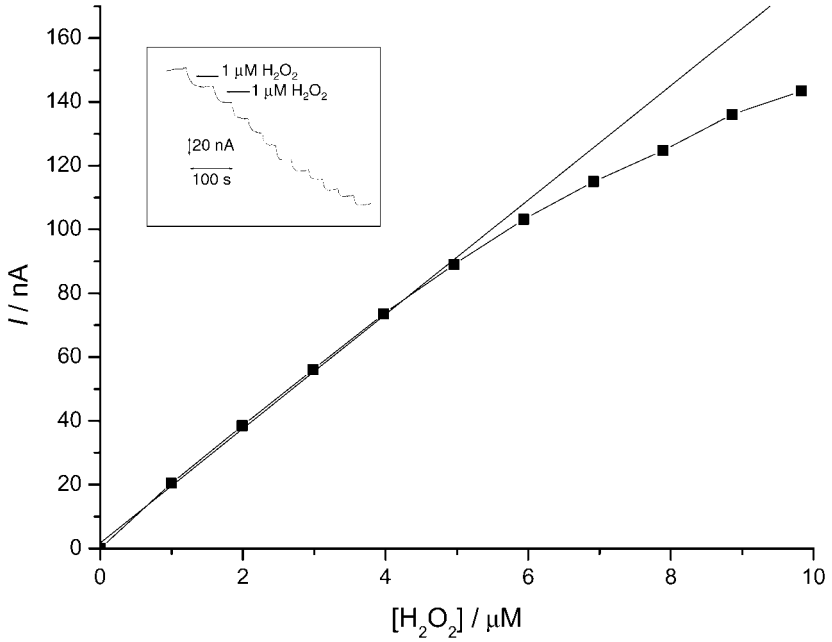

Figure 6. Typical H_2O_2 calibration curves for CoHCF-modified electrode at applied potential 0.0 V vs. SCE for successive additions of 1 μM H_2O_2 in PBS, pH = 7. In the insert is shown the response curve for H_2O_2 addition.

concentration range 0–15 μM were made with the same CoHCF-modified electrode. A large decrease in sensitivity of 40% after every calibration curve was found with a simultaneous increase in the linear range. Nevertheless, this loss of sensitivity is not a problem for short-term use sensors and was also found not to be a drawback in the measurement of hydrogen peroxide produced by enzymatic reaction using the standard addition method with enzymes, as will be seen below.

3.3. Enzymatic Substrate Measurements

The enzyme biosensor response was now evaluated. The enzyme layer was prepared on top of the CoHCF film, as described in the "Experimental" section. The cylindrical shape of the carbon film sensors means that a good and reproducible contact between the enzymatic layer and the electrode surface is needed as well as a good adhesion of this layer over the whole electrode.

Physical adsorption immobilization methods of GOX were investigated in order to obtain a stable and active enzymatic layer. Glutaraldehyde and Nafion were used for this purpose with a view to combining the cross-linking properties of GA with the ability of Nafion to fix the enzymatic membrane on the carbon film surface, in a similar approach to that in Ref.[11] The optimized composition and procedure are described in the "Experimental" section. Results were very similar with and without coating the biosensor with Nafion. Using Nafion to fix the enzyme on the carbon film electrode gave a more physically robust sensor. It was also confirmed that no response to the injection of glucose was observed in the absence of immobilized GOX.

Measurements were performed at fixed potential, after stabilization of the baseline, by injection of glucose into PBS solution containing the enzyme sensor with continuous stirring, detecting the produced hydrogen peroxide using the experimental conditions optimized in hydrogen peroxide detection with the CoHCF-modified electrode.

Kinetic studies of the immobilized enzyme were also carried out. The electrochemical response to increasing concentrations of enzyme substrate was plotted. Figure 7 shows the curve for an electrode in PBS pH = 7 at a measurement potential of +0.0 V for successive additions of ~10 μM glucose. In this situation CoHCF-modified carbon film glucose biosensors showed a linearity range up to 30 μM. Corresponding detection limits were 1.9 μM. The regression equations of the linear plots were $I/nA = 0.51 + 1.28c$ with $R = 0.9992$, where c is the glucose concentration in μM. Michaelis–Menten constant was 160 μM. Repetitive measurements over several days showed a decrease in response of 20% but detection limits were unaffected.

3.4. Analysis of Glucose in Wine

In order to test the applicability of the sensors to foods and beverages, a sweet port wine vas analyzed by the standard addition method. The wine sample was diluted 10^4 times in 0.1 M PBS, and the analyte was spiked five times with 10 μM aliquots of glucose to construct the analytical curve. Results obtained from four repetitive measurements were $42.3 \pm 4.6\,\mathrm{g\,L^{-1}}$ glucose. This augurs well for application in wines during the fermentation process as well as in other foods.

4. CONCLUSIONS

Carbon film electrodes have been successfully modified with cobalt hexacyanoferrate films and their properties characterized in different electrolytes.

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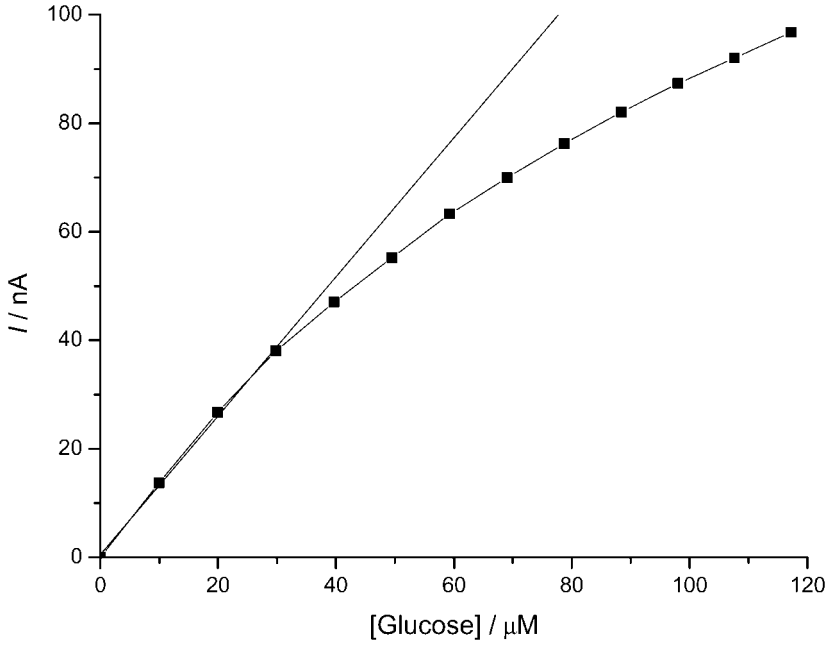

Figure 7. Glucose calibration curves for GOX CoHCF-modified electrode at applied potential 0.0 V vs. SCE for successive additions of 10 μM glucose in PBS, pH = 7.

They have been demonstrated to be successful redox mediators in the measurement of hydrogen peroxide produced by the enzymatic oxidation of glucose and with low, micromolar detection limits at 0.0 V where little interference from other electroactive interferents in complex matrices can be expected. Application to analysis of wines has been shown.

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