Revue Roumaine de Chimie, **2007**, *52*(10), 969–974

EVALUATION OF COBALT HEXACYANOFERRATE MODIFIED CARBON FILM ELECTRODES FOR ELECTROCHEMICAL GLUCOSE BIOSENSORS

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Received September 7, 2006

The evaluation and electrochemical characterisation of new glucose oxidase electrochemical enzyme biosensors on a carbon film electrode support using two types of enzyme immobilisation has been carried out, with a view to their application as short-time-use or disposable sensors.
Carbon film electrodes have been modified with films of cobalt hexacyanoferrate (CoHCF) redox mediator by potential cycling from solutions containing cobalt and hexacyanoferrate ions. Enzyme immobilisation onto the electrode surface was done using cross-linking methods with glutaraldehyde and oxysilane sol-gel polymers. Application of the modified electrodes as redox mediators in glucose enzyme biosensors was investigated using the mediated detection of hydrogen peroxide, with excellent detection limits in the micromolar region.

INTRODUCTION

The development of easy-to-use and robust biosensors is an important challenge in areas of application such as medical, environmental, agricultural and biotechnological.[1-8] The most widely used amperometric biosensors are based on oxidase enzymes (*e.g.*, glucoseoxidase, GOx) that generate H_2O_2, the transduction path being the electrochemical oxidation of the hydrogen peroxide formed. [9-11] A serious problem that must be overcome for using such biosensors in natural samples is the presence of metabolites or other compounds that represent positive interference due to the fact they are oxidized/reduced at the same potential as H_2O_2. One solution is to utilize an electroactive compound that will act as a redox mediator, decreasing the hydrogen peroxide reduction or oxidation potential close to 0.0 V.[12]

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.[13] 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 pre-treatment.[14] Their surface properties have been investigated in a number of electrolytes and applications to the electroanalysis of trace metal ions in the negative and positive potential range have also been demonstrated.[14,15] Such carbon film electrodes offer an inexpensive route for developing a glucose biosensor,[16] but require a high overpotential to detect H_2O_2, which reduces the selectivity of the sensors in complex matrices.

Transition metal hexacyanoferrates (MHCF) are becoming widely used redox mediators for biosensors because of their mixed-valence cluster organisation that can transfer electrons during reduction and oxidation processes.[17,18] Prussian Blue (ferric ferrocyanide) has been the most widely used of the metal hexacyanoferrates to develop enzyme redox mediators.[19] An interesting compound from this class is cobalt hexacyanoferrate that can be used to modify the surface of different electrodes in order to obtain very sensitive biosensors for: NADH,[20] dopamine,[21] morphine,[22] catecholamines,[23] hydrazine[24] and thiosulphate.[25] Additionally, for glucose detection a cobalt hexacyanoferrate-coated glassy carbon electrode was used for preparing an enzyme biosensor.[26]

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In this work we study the development and evaluation of new glucoseoxidase electrochemical enzyme biosensors on a carbon film electrode support with a bilayer configuration, using two types of enzyme immobilisation, with a view to their application as short-time-use or disposable sensors.

EXPERIMENTAL PART

1. Chemicals

Glucose oxidase (GOX, EC 1.1.3.4, II-type from Aspergillus niger, 35,600 units/mg), α-D(+)-glucose, glutaraldehyde (GA) 25% (v/v) and bovine serum albumin (BSA) were purchased from Sigma, USA. Potassium hexacyanoferrate (III) ($K_3Fe(CN)_6$) and $CoCl_2\times 6H_2O$ were obtained from Merck, Germany. Tetraethoxysilane (TEOS) from Fluka (Switzerland), 3-glycidoxypropyltrimethoxysilane (GOPMOS) and methyltrimethoxysilane (MTMOS) from Aldrich (Germany). Nafion 5% (v/v) was from Sigma-Aldrich, UK. Millipore Milli-Q nanopure water (resistivity > 18 MΩ 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). H_2O_2 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 h 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. Preparation of carbon film electrode

Electrodes were prepared from carbon film electrical resistors (2 Ω nominal resistance), as described.[14] Briefly, these electrical resistors are fabricated by pyrolytic deposition of carbon at 1100ºC in a nitrogen atmosphere containing a small amount of methane onto ceramic cylinders of length 0.60 cm and external diameter 0.15 cm. Tight fitting metal caps attached to external connecting wires are then press fitted to each end. The metal cap plus conducting wire was removed from one of the ends of a resistor, the remaining conducting wire sheathed in plastic insulation up to the respective cap and the cap and plastic contact area carefully covered with epoxy resin so that only the carbon film would be exposed to solution. After this assembly, the exposed electrode geometric area was ≈0.20 cm^2.

3. Electrochemical measurements

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

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 done by cycling the applied potential 15 times between 0.0 and +0.9 V at a scan rate of 50 mV s^{-1} in a freshly prepared solution containing 0.5 mM $CoCl_2$, 0.25 mM $K_3Fe(CN)_6$ and 50 mM NaCl at pH 3.0 (pH adjusted with HCl) with solution agitation by slow mechanical stirring. The CoHCF film modified electrodes were stabilised for 1 hour in 0.05 M NaCl, pH 3.0 and left to dry at room temperature. Modification of the electrodes was always carried out employing the same, identical solution composition in order to obtain reproducible results.

5. Enzyme immobilisation

a. Cross-linking method

A mixture of GA, enzyme and BSA was put on CoHCF-modified carbon film electrode. In order to obtain a stable and active enzymatic layer, 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 phosphate saline buffer solution (PBS), pH 7.0. Enzyme concentration solution was 1% and that corresponds to concentrations of approximately 0.7% in the resulting layer on the electrode surface. From this mixture, 10 µL of enzymatic solution was placed onto the surface of the working electrode and allowed to dry for 1 hour at room temperature, and after that the biosensor was coated with 4 µL of 1% Nafion solution. Electrodes were stored in PBS buffer electrolyte at +4 ºC when not in use.

b. Sol-gel method

In the optimised procedure, sol-gel solution was prepared by mixing oxysilanes and water in the ratios: GOPMOS:TEOS:H2O – 130:70:600 µL; and GOPMOS:MTMOS:H2O – 150:70:620 µL. A volume of 2 µL of 1 M HCl solution was added to each mixture to promote hydrolysis. The mixtures obtained were intensively stirred for a few minutes and then sonicated for 15 min. Following this, the mixtures were heated in a hot air stream (~70 ºC) to evaporate the alcohol formed during hydrolysis of the oxysilanes until the solutions lost 40 % of their volume; they were left for 10 min at room temperature to cool down and neutralized to pH 7.0 by addition of 0.1 M NaOH. Then 50 µL of each solution was carefully mixed with 15 µL of 4-15 % GOx solution in 0.1 M PBS solution pH 7.0. Following this, CoHCF-coated carbon film electrodes were immersed in the sol-gel-enzyme solutions for 5 min, removed and then left for sol-gel formation at +4 ºC for 3 days. Electrodes were stored in PBS buffer electrolyte at +4 ºC when not in use.

RESULTS AND DISCUSSION

1. CoHCF modified electrode preparation and characterisation

The modification of carbon film electrodes by cobalt hexacyanoferrate was carried out by cyclic voltammetry, and voltammograms were continuously recorded. Fig. 1 illustrates the formation of the

CoHCF film onto the electrode that demonstrates the growth in thickness with each cycle, as revealed by the increasing charge under the deposition peak.

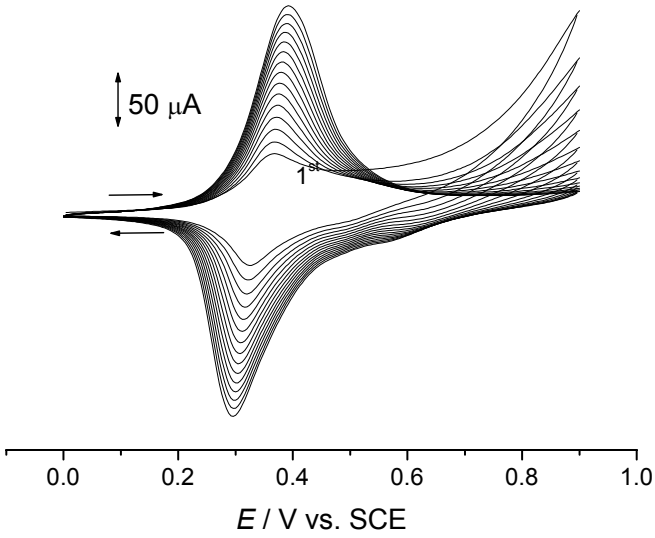

Fig. 1 – Cyclic voltammograms showing the continuous growth of CoHCF on carbon film electrode. Solution composition: 0.5 mM $CoCl_2$, 0.25 mM $K_3Fe(CN)_6$, 50 mM NaCl; pH 3.0. Scan rate 50 mV s^{-1}.

2. Enzymatic substrate measurements

The present study deals with the development, evaluation and characterisation of glucose oxidase (GOx) electrochemical biosensors, based on a carbon film electrode support and with a bilayer configuration. Carbon film electrodes were first modified by deposition of cobalt hexacyanoferrate using cyclic voltammetry, as described previously. The second layer consisted of the film containing GOx enzyme, which was prepared using two methods: 1) cross-linking with GA and BSA and 2) sol-gel encapsulated employing GOPMOS with either MTMOS or TEOS. 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.

a. Cross-linking method

Measurements were performed at fixed potential, after stabilisation of the baseline, by injection of glucose into PBS solution containing the enzyme sensor with continuous stirring, detecting the produced H_2O_2 using the experimental conditions optimised in hydrogen peroxide detection with the CoHCF-modified electrode. 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.[26] 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. Kinetic studies of the immobilised enzyme were carried out and the electrochemical response to increasing concentrations of enzyme substrate was plotted. Fig. 2 shows the response 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 and data from analysis of the calibration plots are given in Table 1. Repetitive measurements over several days showed a decrease in response of 20% but detection limits were unaffected.

b. Sol-gel method

The sol–gel enzyme mixture was deposited onto the surface of the CoHCF-modified carbon film electrode by dipping the electrode into sol–gel solution, as described in Section 2.5, using optimised sol–gel formation conditions.[27] Concerning the three precursors used here and by studying the sol-gel morphology using AFM from previous work, it was demonstrated the hydrophobic character of GOPMOS compared with the less hydrophobic of MTMOS and hydrophilic of TEOS.[28] The combination of

GOPMOS and TEOS precursors was studied and compared with GOPMOS plus MTMOS in the development of glucose-oxidase biosensors. Different proportions of each precursor have been tested and the optimum composition was found to be GOPMOS : TEOS : H_2O – 130 : 70 : 600 μl and GOPMOS : MTMOS : H_2O – 150 : 70 : 620 μL. These compositions will be abbreviated to GT and GM, respectively, in the text that follows. The concentration of the entrapped enzyme has a big influence on sol-gel based-biosensor response, since it has been observed that an increase in the concentration of some entrapped enzymes can result in a marked decrease in specific activity that can form aggregates at higher concentrations.[29] For this reason different concentrations of enzyme in PBS solution were used: 4%, 10% and 15%, that correspond to concentrations of approximately 1.0%, 2.3% and 3.5% in the resulting dry gel on the electrode surface.

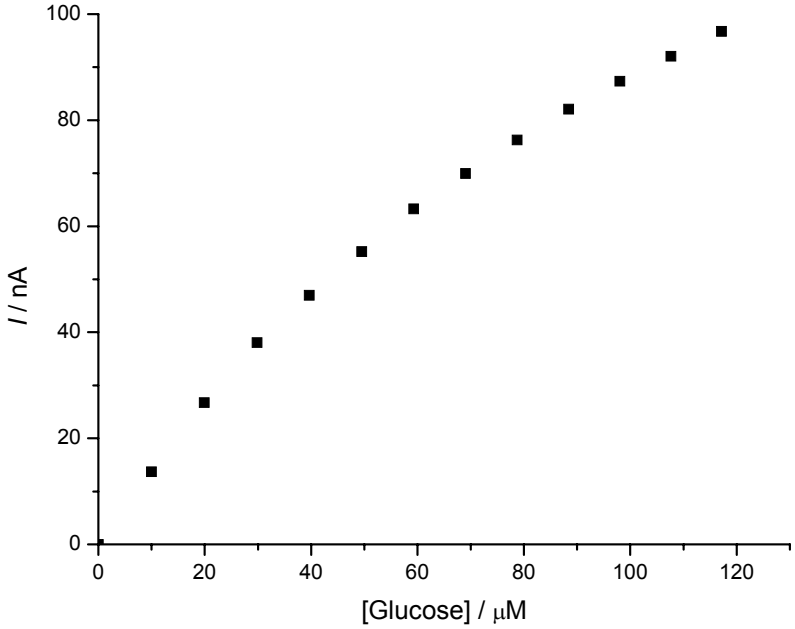

Fig. 2 – Glucose calibration curves for GOx CoHCF-modified electrode at applied potential 0.0 V vs SCE for successive additions of 10 μM glucose in PBS, pH=7.

Table 1

Data from calibration plots of CoHCF-mediated glucose biosensors

Method	[Enzyme] %	Sensitivity / nA mM^{-1}	Intercept / nA	DL/ μM	Linear range / mM	K_M / mM
Cross-linking	0.7	1280 ± 1.2	0.51 ± 0.3	1.9	0 – 0.03	0.16
GM	1.0	47.6 ± 0.3	0.64 ± 0.70	59	0 – 0.4	0.97
Sol-gel						
	2.3	39.7 ± 0.19	0.51 ± 0.30	34	0 – 0.4	1.28
	3.5	138.8 ± 0.1	1.09 ± 0.90	24	0 – 0.2	0.59
GT	1.0	19.7 ± 0.1	0.56 ± 0.30	71	0 – 1.0	1.62
Sol-gel						
	2.3	29.9 ± 0.1	0.33 ± 0.20	30	0 – 0.25	0.79
	3.5	57.4 ± 0.1	0.53 ± 0.50	46	0 – 0.5	2.73

[Enzyme] = enzyme concentration on the electrod surface
Sensitivity = the sensitivity of the biosensor (the slope of linear part of the calibration plot)
DL = detection limit
Linear range = the glucose concentration of linear part of the calibration plot
K_M = Michaelis-Menton constant of enzyme-substrate complex

Measurements were performed at fixed potential, after stabilisation of the baseline, by injection of glucose into PBS solution containing the enzyme sensor with continuous stirring, detecting the produced H_2O_2 at an optimal potential of 0.0 V vs. SCE. Kinetic studies of the immobilised enzyme were also carried out. The electrochemical response to increasing concentrations of enzyme substrate was plotted. Fig 3 shows the curve for an electrode in PBS pH=6.94 at a measurement potential of +0.0 V for successive additions of glucose. Glucose detection was done with both type of sol-gel mixture CoHCF-mediated biosensors and data from analysis of the calibration plots are given in Table 1.

These results can be explained taking into account that in the case of sol-gel immobilisation the concentration of the encapsulated enzyme has a big influence on its activity. The accessibility of the analyte to the enzyme is determined largely by the pore size and the electrostatics of the material, which can be tuned by various methods. For GM-based biosensors the combination of GOPMOS with MTMOS leads to a pore structure of a dry gel that is favourable for the higher concentration of immobilised enzyme. These biosensors present a high sensitivity but the linear response range is short, caused by the big accessibility of the glucose to the entrapped enzyme through the pores of the gel obtained by the combination of two hydrophobic precursors with different extents of hydrophobicity. According to the results obtained, the pore dimensions of the dry gel seem to be big enough to permit easy access of the glucose to the enzyme and to its active sites. Using a high concentration of the enzyme solution also ensures the presence of a sufficient quantity of immobilised enzyme on the biosensor surface, even allowing for enzyme leakage due to the big dimensions of the pores. In the case of GT-based biosensors the glucose reaches the immobilised enzyme over a longer time period that can be caused by the small dimensions of the dry gel pores due to the combination of the hydrophobic GOPMOS with the hydrophilic TEOS. In this case the enzyme substrate has more difficulty in reaching the enzyme, the active site of which can be buried in a non-accessible way inside the nano-cages where the enzyme is immobilised.

The reproducibility of three different biosensors was tested and a RSD of 5.1% for GM-based biosensors and 4.3% for GM-based biosensors was obtained. Results of testing newly prepared biosensors after different time intervals showed for both type of biosensors that the sensitivity values increased during 24 h after the first testing, which can be due to reorganisation of the sol-gel network and has been noted previously,[30] and then began to decrease.

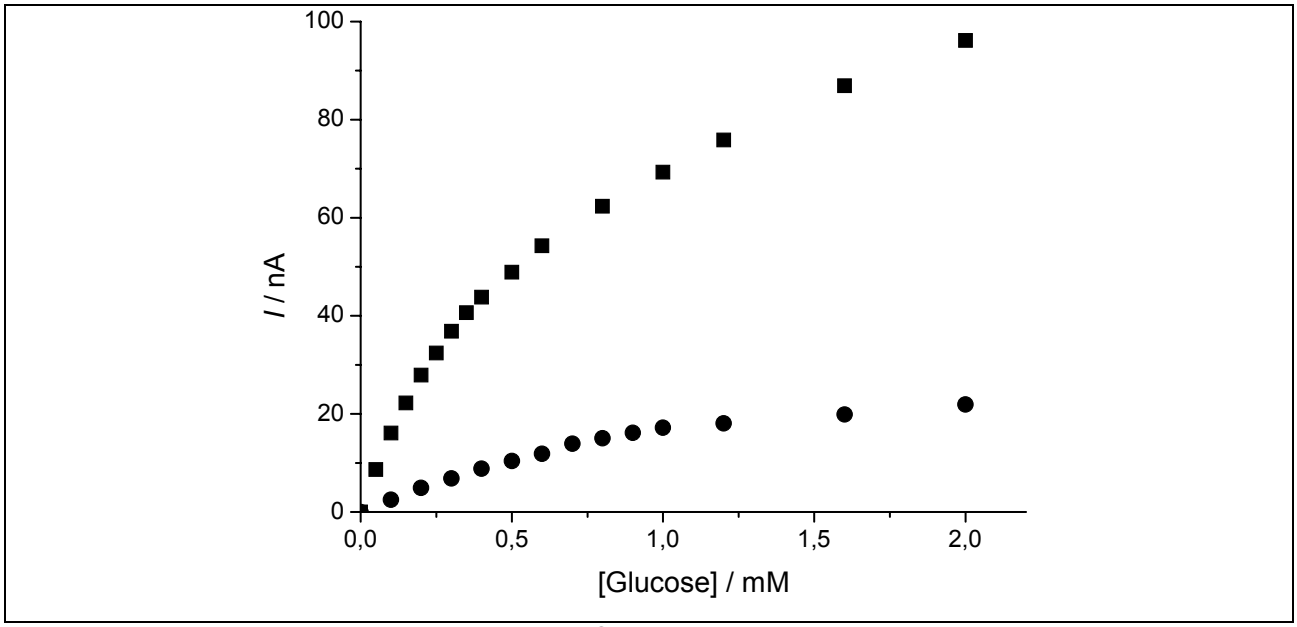

Fig. 3 – Calibration curves at (■)GM-based biosensor and (●) GT-based biosensor at applied potential 0.0 V vs SCE for successive additions of glucose to 0.1 M PBS pH 6.94.

CONCLUSIONS

Carbon film electrodes have been successfully modified with cobalt hexacyanoferrate films followed by enzyme immobilisation and successfully used as biosensors at low applied potential. CoHCF have been demonstrated to be successful redox mediators in the measurement of H_2O_2 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. Two methods for enzyme immobilisation were used: cross-linking with GA and BSA and in a sol-gel layer from a sol-gel precursor mixture. For the sol-gel-based biosensors the changes in proportions of hydrophilic and hydrophobic surface groups of sol-gel precursors influence the performance of gel-based biosensors due to the internal structure of dry gel that can affect the concentration and the activity of immobilised enzyme on the biosensor surface. The characteristics of cross-linked-based biosensors were better but for a very small linear range. That situation can be explained by the pore dimensions of the dry layer obtained by cross-linking that seem to be big enough to permit easy access of the glucose to the enzyme and to its active sites, even for a small concentration of immobilised enzyme on electrode surface.

ACKNOWLEDGEMENTS: Financial support from European Project HPRN-CT-2002-00186 (Novtech) and Fundação para a Ciência e Tecnologia (FCT), ICEMS (Research Unit 103), Portugal are gratefully acknowledged. Prof. H. D. Liess is thanked for the gift of the electrical resistors.

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