Abstract

A copper containing Prussian Blue analogue was incorporated into a conducting polypyrrole film. The modified electrode was synthesized through an electrochemical two-step methodology leading to very stable and homogeneous hybrid films. These electrodes were proved to show excellent catalytic properties towards H₂O₂ detection, with a performance higher than those observed for Prussian Blue and other analogues. Electrochemical impedance spectroscopy experiments demonstrated that the excellent performance of these hybrid films is strongly related to the electronic conductivity of the polymeric matrix that is wiring the copper hexacyanoferrate sites. A glucose biosensor was built-up by the immobilization of glucose oxidase; the sensitivity obtained being higher than other biosensors reported in the literature even in Na⁺ containing electrolytes.

Keywords: Prussian Blue analogues; Polypyrrole; Hybrid materials; Glucose biosensors

1. Introduction

In recent years the construction and application of modified electrodes has received much attention with a view to the enhancement of sensitivity and selectivity of electroanalytical techniques. The use of metal hexacyanoferrates in modified electrodes has been extensively studied [1]. These kinds of materials are becoming widely used as redox mediators in biosensors based on oxidase enzymes as the biological recognition element. These enzymes oxidize their specific substrates in the presence of molecular oxygen leading to the formation of hydrogen peroxide as side product [2,3]. Due to the fact that these materials proved to be excellent catalysts for H₂O₂ reduction at low potentials, minimizing the interference caused by reductant species, hexacyanoferrates have been widely studied. The possibility of selective detection mediated by Prussian Blue was first demonstrated by Karyakin et al. [4].

Besides Prussian Blue, other metal hexacyanoferrate used as mediators in oxidase based biosensors are copper hexacyanoferrate (CuHCNFe) [5–7], cobalt hexacyanoferrate (CoHCNFe) [8–11] and nickel hexacyanoferrates (NiHCNFe) [12–14]. Among the analogues of Prussian Blue, copper hexacyanoferrate shows a well-defined, reversible and reproducible electrochemical response in supporting electrolytes containing not only potassium but also other alkali metal cations such as lithium, sodium, rubidium or cesium [15–18], contrary to Prussian Blue that only shows good electroactivity in electrolytes containing K⁺.

In this work, CuHCNFe is incorporated into the structure of a polypyrrole network. In previous work [19], we reported the synthesis of an inorganic/organic hybrid, combining polypyrrole and nickel hexacyanoferrate, obtaining a material with enhanced electrocatalytic and mechanical properties compared to nickel hexacyanoferrate by itself. In that case, the composite was formed by cyclic voltammetry in solutions containing nickel(II), hexacyanoferrate(III) and pyrrole. In this case, the methodology was changed, forming a film of hexacyanoferrate(III)-doped polypyrrole in a first step, followed by a second step where the
insertion of Cu$^{2+}$ was performed with the concomitant formation of CuHCNFe inside the conducting polymer matrix.

2. Experimental

2.1. Reagents

Analytical grade CuCl$_2$·2H$_2$O, K$_3$Fe(CN)$_6$, KCl and NaCl were purchased from Merck. All solutions were prepared with Milli-Q nanopure water (resistivity 18 MΩ cm). Pyrrole (Merck) was distilled prior to use. Experiments were carried out at room temperature (24 ± 1 °C).

2.2. Instrumentation

Cyclic voltammetry (CV) and chronoamperometry (CA) experiments were performed with a µ-Autolab Type II potentiostat/galvanostat (EcoChemie, The Netherlands) driven by GPhes 4.9 software. All potentials are referred to a saturated calomel electrode (SCE), while a platinum foil was employed as the auxiliary electrode. As working electrode, a glassy carbon (GC) electrode (3 mm diameter) was used.

Electrochemical impedance measurements were carried out in the same electrochemical cell with a PC-controlled Solartron 1250 Frequency Analyser coupled to a Solartron 1286 Electrochemical Interface using ZPlot 2.4 software. A sinusoidal voltage perturbation of 10 mV (rms) was applied, scanning from 65 kHz to 0.1 Hz with 10 points per frequency decade and an integration time of 120 s.

2.3. Prussian Blue deposition

Thin Prussian Blue films were prepared by polarizing a GC electrode (3 mm diameter) at 0.40 V in an aqueous solution containing 2.5 × 10$^{-3}$ mol L$^{-1}$ FeCl$_3$ + 2.5 × 10$^{-3}$ mol L$^{-1}$ K$_3$Fe(CN)$_6$ + 0.10 mol L$^{-1}$ KCl + 1.5 × 10$^{-2}$ mol L$^{-1}$ KCl + 1.5 × 10$^{-2}$ mol L$^{-1}$ KCl. After deposition, the film was activated in a 0.10 mol L$^{-1}$ KCl solution by cycling 50 times from 0.3 to 0.9 V versus SCE in a solution containing 2 × 10$^{-3}$ mol L$^{-1}$ CuCl$_2$ + 0.1 mol L$^{-1}$ KCl during 2 h. The second step consisted in placing the Fe(CN)$_6^{3−}$/PPy modified electrode in a solution containing 2 × 10$^{-3}$ mol L$^{-1}$ CuCl$_2$ + 0.1 mol L$^{-1}$ KCl and 0.10 mol L$^{-1}$ KCl during 2 h.

2.4. Copper hexacyanoferrate deposition

Thin CuHCNFe films were prepared by applying triangular potential sweeps (at scan rate 0.05 V s$^{-1}$) in the potential range −0.3 to 0.9 V versus SCE in solutions containing 2 × 10$^{-2}$ mol L$^{-1}$ K$_3$Fe(CN)$_6$ + 0.1 mol L$^{-1}$ KCl + 1.5 × 10$^{-2}$ mol L$^{-1}$ KCl + 1.5 × 10$^{-2}$ mol L$^{-1}$ KCl. The second step consisted in placing the Fe(CN)$_6^{3−}$/PPy modified electrode in a solution containing 2 × 10$^{-3}$ mol L$^{-1}$ CuCl$_2$ + 0.1 mol L$^{-1}$ KCl during 2 h. After that, the electrode was cycled 20 times in the same electrolyte, transferred to a solution containing 0.1 mol L$^{-1}$ KCl and cycled until stabilisation of the peaks corresponding to copper hexacyanoferrate oxidation/reduction. In the text this electrode will be referred to as GC/CuHCNFe/PPy.

2.6. Enzyme immobilization

Glucose oxidase (GOx) was immobilised onto the electrode surface by the cross-linking method, using a mixture of glutaraldehyde (GA), enzyme and BSA. To prepare 30 μL of this mixture, 10 μL of glutaraldehyde (2.5% v/v diluted in water) were added to 25 μL of enzyme solution. The enzyme solution was prepared by dissolving 40 mg of BSA and 10 mg of GOx in 1 mL of 0.1 mol L$^{-1}$ phosphate buffer solution, pH 7. From this mixture, 10 μL were placed onto the surface of the working electrode and allowed to dry at room temperature.

2.7. Amperometric measurements

The amperometric response of modified electrodes to H$_2$O$_2$ additions was carried out in a conventional three-electrode electrochemical cell (5 mL) containing stirred electrolytes. Before successive H$_2$O$_2$ additions, the electrodes were held at the operating potential until the background current stabilised.

Tests of the biosensor response to glucose were made in a three-electrode electrochemical cell (5 mL) containing stirred electrolytes. Before successive H$_2$O$_2$ additions, the electrodes were held at the operating potential until the background current stabilised.

3. Results and discussion

3.1. Electrochemical synthesis

The hexacyanoferrate/pyrrole matrix was prepared by potential cycling in an aqueous solution containing 2 × 10$^{-2}$ mol L$^{-1}$ K$_3$Fe(CN)$_6$ + 0.1 mol L$^{-1}$ KCl + 1.5 × 10$^{-2}$ mol L$^{-1}$ KCl. The first broad peak at 0.3 V corresponding to ferrocyanide oxidation and a second wave starting at 0.7 V, attributed to oxidation of the pyrrole monomer, can be observed. The electrode was cycled 10 times in the potential range −0.3 to 0.9 V. The positive potential limit chosen was lower than 1.0 V in order to avoid overoxidation of the polymer, which leads to loss of its conductivity [20,21]. As can be seen in Fig. 1a, during cycling an increase in the current in the region of 0.1 V occurs, corresponding to oxidation/reduction of the pyrrole polymer formed during the process, demonstrating the growth of the conducting polymer. After the deposition process, a homogeneous blue film can be observed on the electrode, with good mechanical stability.
The electrode was then cycled 20 times in the same electrolyte. Results are shown in Fig. 1b, where the appearance and growth of a new oxidation peak at 0.7 V and its complementary reduction peak at 0.4 V are seen. This redox couple corresponds to the oxidation/reduction of copper hexacyanoferrate [22] and the increase of current with the number of cycles indicates growth of the copper hexacyanoferrate inside the polymeric matrix.

When the electrode is transferred to an aqueous electrolyte containing only 0.1 mol L\(^{-1}\) KCl and cycled between –0.3 and 0.9 V, the peaks corresponding to oxidation/reduction of the copper hexacyanoferrate still increase, as illustrated in Fig. 1c. For this reason, the electrode was cycled until complete stabilisation of these peaks. All these results give strong evidence for the incorporation of copper hexacyanoferrate into the polymer network.

### 3.2. Electrocatalytic properties of the hybrid film

When held at a constant potential of 0.0 V, the GCE/CuHCNFe/PPy electrode shows a cathodic current response to added hydrogen peroxide. Fig. 2a depicts the response to peroxide, added in portions of 0.025 mmol L\(^{-1}\). As shown in Fig. 2b, the dependence of the catalytic current on the concentration of hydrogen peroxide was linear and reproducible up to 0.5 mmol L\(^{-1}\) of peroxide, with a sensitivity of 726 μA mmol\(^{-1}\) L cm\(^{-2}\) and a detection limit (three times the standard deviation of the slope) of 13 μmol L\(^{-1}\).

For comparison, peroxide detection tests were performed with both copper hexacyanoferrate and Prussian Blue modified glassy carbon electrodes, as described in Section 2. The sensitivities (summarised in Table 1) obtained were 0.14 and 340 μA mmol\(^{-1}\) L cm\(^{-2}\), respectively. The copper hexacyanoferrate/polypyrrole hybrid exhibits a sensitivity of 726 μA mmol\(^{-1}\) L cm\(^{-2}\), 5000 times higher than the copper hexacyanoferrate alone and, surprisingly, two times higher than the Prussian Blue modified electrode. This result is very striking due to the fact that it is well known from the literature that Prussian Blue analogues normally exhibit lower sensitivities for hydrogen peroxide detection than Prussian Blue itself [7,23]. This improvement can be related to the electronic conductivity of polypyrrole.

In the same way, in previous work [19], the high catalytic activity of a nickel hexacyanoferrate/polypyrrole hybrid was reported. When compared to nickel hexacyanoferrate alone, this hybrid material was found to be much more active to H\(_2\)O\(_2\).

![Figure 1](image_url)

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sensitivity (μA mmol(^{-1}) L cm(^{-2}))</th>
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<tbody>
<tr>
<td>CuHCNFe</td>
<td>0.14</td>
</tr>
<tr>
<td>CuHCNFe/PPy</td>
<td>726.00</td>
</tr>
<tr>
<td>Prussian Blue</td>
<td>340.00</td>
</tr>
<tr>
<td>NiHCNFe</td>
<td>9.61</td>
</tr>
<tr>
<td>NiHCNFe/PPy [19]</td>
<td>88.30</td>
</tr>
</tbody>
</table>

Next, the Fe(CN)\(_6^{3−}\)/PPy modified electrode was placed in a solution containing 2 × 10\(^{-2}\) mol L\(^{-1}\) CuCl\(_2\) + 0.1 mol L\(^{-1}\) KCl during 2 h, in order to allow the insertion of Cu\(^{2+}\) ions into the polymer network. After this process, it is supposed that both Fe(CN)\(_6^{3−}\) and Cu\(^{2+}\) ions are present inside the polymer matrix.
because of the higher electronic conductivity of the hybrid owing to the presence of the conducting polymer matrix. As can be seen in Table 1, even though nickel hexacyanoferrate has a higher activity than the copper analogue, the improvement of the sensitivity provoked by the presence of polypyrrole in GC/CuHCNFe/PPy hybrid electrode is much more drastic than in the case of the hybrid material containing nickel hexacyanoferrate. This fact is an evidence of the relevant role played by the conducting polymer matrix in the enhancement of the catalytic properties of this hybrid material. The high catalytic performance of the hybrid containing copper must be related to the synthetic approach used to prepare the modified electrode due to the fact that the nickel containing hybrid is prepared in a simple one step method. The two-step procedure used to synthesize the copper hybrid seems to be much more efficient for the incorporation of copper hexacyanoferrate that would be in intimate contact with the conducting polymer chains.

3.5. Electrochemical impedance spectroscopy

With the aim of characterizing the electronic and transport properties of CuHCNFe/PPy hybrid films, electrochemical impedance experiments were performed. These experiments were performed at 0.0 V because this is the potential used in the H₂O₂ detection experiments. Fig. 3 shows complex plane spectra for CuHCNFe/PPy and CuHCNFe films. As can be seen, the presence of the conducting polymer dramatically modifies the impedance response of the system. In the case of hybrid material, a semicircle is clearly observed at high frequencies, the diameter of this semicircle being related to the resistance of the charge transfer reaction and the electronic resistance of the film. By analyzing the low frequency region, it is possible to observe a transition from semi-infinite diffusion, where the curve has a phase angle of 45°, to a finite diffusion process with the angle approaching 90°; this impedance diagram shows the typical shape expected for a thin conducting polymer film [24].
On the other hand, the impedance diagram of the CuHCNFe film shows a higher value of the film resistance (≈20 kΩ versus ≈20 kΩ) in the high frequency region whereas, from the low frequency region, we can conclude that the ionic movement inside the CuHCNFe film is slower than inside the hybrid one. An infinite diffusion process is observed in the same frequency range where the hybrid film exhibits a finite diffusion process. In other words, the presence of the conducting polymer improves both electronic and ionic conductivities of the film.

As mentioned above, the hybrid shows a better performance for the determination of H₂O₂ than CuHCNFe itself; this improvement being related to the increased electronic conductivity in the hybrid due to the polypyrrole network. The conducting polymer can act as a “wire”, connecting the redox sites of CuHCNFe, thus increasing the charge transfer rate. This can be confirmed by performing a simple experiment overoxidizing the polypyrrole matrix to reduce the polymer conductivity and evaluating the influence of the drop in conductivity on the analytical performance of H₂O₂ detection. The overoxidation process is characterized by an irreversible nucleophilic attack when the polymer is exposed to high positive potentials in aqueous solutions, with the subsequent loss of electroactivity and electronic conductivity [25].

First, the polypyrrole was partially oxidized by applying a potential of 1.0 V in 0.1 mol L⁻¹ KCl: Fig. 4a shows the impedance diagram after this process where an increase in the diameter of the high frequency semicircle can be observed, indicating that the resistivity of the film is higher and, consequently, also the charge transfer resistance. After that, the electrode was used to determine H₂O₂ in the same conditions as those used in Fig. 2. The resulting analytical curve is inserted in the figure and a sensitivity value of 295 μA mmol⁻¹ L⁻¹ cm² was obtained, demonstrating that the decrease in polypyrrole conductivity is reflected in a sensitivity loss towards H₂O₂ detection.

The electrode was then cycled between −0.3 and 1.2 V in aqueous 0.1 mol L⁻¹ KCl electrolyte, in order to cause full overoxidation of the polypyrrole and total loss of polymer electroactivity. The complex plane impedance plot obtained after this process is shown in Fig. 4b. A drastic increase in the impedance values to the order of MΩ can be observed, showing the complete loss of electronic conductivity of polypyrrole. After this treatment, the electrode was almost unable to detect H₂O₂, responding to successive additions of H₂O₂ only when the increase in the concentration was made as high as 0.25 mmol L⁻¹ per addition. Thus, it can be concluded that the influence of the electronic conductivity of the polypyrrole in the analytical performance of the hybrid in H₂O₂ determination is crucial.

3.4. Biosensors based on the PPY/CuHCNFe hybrid

The hybrid film was used for constructing a biosensor for the determination of glucose by immobilizing the enzyme glucose oxidase onto a hybrid modified glassy carbon electrode. Fig. 5a illustrates a typical current–time plot for a GO/CuHCNFe/PPy/GOx electrode that was obtained by injecting successive aliquots of stock glucose solution. The measurements were performed at 0.0 V, by injection of 10 μL of 0.2 mol L⁻¹ glucose solution into 20 mL of 0.1 mol L⁻¹ PBS + 0.05 mol L⁻¹ NaCl solution under continuous stirring. Fig. 5b displays the analytical curve, where the linear range extends from 0.02 to 1.0 mmol L⁻¹, the sensitivity obtained being 34.9 μA mmol⁻¹ L⁻¹ cm² and the detection limit 5.4 μmol L⁻¹.

It should be stressed that the biosensor is working in Na⁺-containing buffer and even so, the sensitivity is 2.1 times higher than that reported for a glucose sensor based on layer-by-layer deposition of GOx onto Prussian Blue ITO-modified electrodes [26] in K⁺ electrolytes. Consequently, the CuHCNFe hybrid composite not only overcomes Prussian Blue’s limitation for working in Na⁺ containing solutions but also leads to a better sensitivity. Other glucose biosensors reported...
in the literature can be compared and it can be confirmed that the performance of the GC/CuHCNFe/PPy/GOD electrode is better than that obtained for other electrodes such as GOx co-immobilized with ferrocene from ethanol/water mixtures [27] (1.5 μA mmol⁻¹ L⁻¹ cm⁻²), GOx immobilized in an osmium-based three-dimensional redox hydrogel [28] (5.2 μA mmol⁻¹ L⁻¹ cm⁻²), GOx trapped inside an electropolymerised film [29] (0.28 μA mmol⁻¹ L⁻¹ cm⁻²), GOx immobilized onto carbon film electrodes [30] (0.25 μA mmol⁻¹ L⁻¹ cm⁻²) or 4.0 μA mmol⁻¹ L⁻¹ cm⁻² for GOx within a polypyrrole film electrogenerated onto mesoporous TiO₂ [31].

4. Conclusions

The synthesis of a new organic/inorganic hybrid material was performed by combining polypyrrole and a hexacyanometalate (CuHCNFe), aiming to obtain an electrocatalyst for H₂O₂ reduction in the presence of either Na⁺ or K⁺ ions. The use of this material as a redox mediator in a glucose biosensor based on the immobilisation of glucose oxidase enzyme was also discussed. The electrochemical properties of the hybrid material were investigated and compared with those of the copper hexacyanoferrate film by itself. The electrocatalytic properties of the hybrid are better than those of the CuHCNFe film due to the presence of polypyrrole chains that enhance the electronic conductivity of the material.

Acknowledgements

Brazilian agencies FAPESP (Procs. 03/07568-0 and 03/10015-3) and CNPq-CTEnerg (Proc. 400562/2003-0) are gratefully acknowledged for financial support. P.A.F. thanks FAPESP (Proc. 01/04957-2) and Alfa ADEMAT network (EU) for fellowships granted. C.B. thanks ICEMS.

References