17.1 OBJECTIVES

To obtain a stable and easy to produce Prussian blue (PB, ferric hexacyanoferrate, Fe₄³⁺[Fe²⁺(CN)₆]₃⁻)-modified screen-printed electrode for the amperometric detection of hydrogen peroxide.

17.2 MATERIALS AND INSTRUMENTS

Screen-printed electrodes used for the PB modification were home-produced with a 245 DEK (Weymouth, UK) screen-printing machine using different inks obtained from Acheson Italiana (Milan, Italy). Graphite-based ink (Electrodag 421), silver ink (Electrodag 477 SS RFU) and insulating ink (Electrodag 6018 SS) were used. The substrate was a polyester flexible film (Autostat HT5) obtained from Autotype Italia (Milan, Italy). Each electrode is comprised of three printed electrodes, a carbon working and two silver electrodes, acting as pseudo-reference and counter, respectively. The diameter of the working electrode was 0.3 cm, which resulted in a geometric area of 0.07 cm².

Potassium ferricyanide and ferric (III) chloride hexahydrate were obtained from Sigma Chemical Company (Steinhein, Germany).

All the amperometric measurements were accomplished in a solution of 0.05 mol l⁻¹ phosphate buffer + 0.1 mol l⁻¹ KCl, pH 7.4. Hydrogen peroxide solutions were prepared using the same buffer solution.
17.3 SENSOR PREPARATION

Place a drop (40 μl of total volume) of precursor solutions onto the working electrode area. This solution is a mixture prepared directly onto the screen-printed electrode by adding 20 μl of 0.1 mol l⁻¹ potassium ferricyanide (K₃Fe(CN)₆) in 10 mmol l⁻¹ HCl to 20 μl of 0.1 mol l⁻¹ ferric chloride in 10 mmol l⁻¹ HCl. The drop has to be carefully placed exclusively on the working electrode area in order to avoid the formation of PB on the reference and counter electrodes, an event that could significantly increase the internal resistance of the system.

Gently shake the electrodes on an orbital shaker for 10 min and then rinse with a few millilitres of 10 mmol l⁻¹ HCl.

Leave the probes for 1.5 h in the oven at 100°C to obtain a more stable and active layer of PB.

Store the PB-modified electrodes dry at room temperature in the dark.

17.3.1 Sensor test with cyclic voltammetry

Verify the effectiveness of the PB deposition procedure onto the SPEs by cycling the modified electrodes from −0.05 to 0.3 V. Figure 17.1 shows the voltammograms of the classic PB-modified SPE obtained in phosphate buffer solution+0.1 mol l⁻¹ KCl. The typical two pairs of redox waves showing the oxidation as well as the reduction of PB are present.

Evaluate the two most important parameters: the coverage of the PB deposited on the electrode surface (Γ, mol cm⁻²), and the difference between the anodic and the cathodic peak potentials (ΔEp, mV) revealing the electrochemical reversibility of the interconversion between PB and Prussian white.

Obtain the total amount of deposited PB by using the following equation:

\[
i_p = \frac{n^2 F^2 Γ Av}{4RT}
\]

where \(v\) represents the scan rate (mV s⁻¹), \(A\) the electrode surface (cm²), \(i_p\) the peak current (A) and the others are the well-known constants.

Also, the charge density (\(Q\), C cm⁻²), calculated by dividing the area of the anodic wave (C), with the area of the electrode surface (cm²), is
directly proportional to the total amount of deposited PB:

\[ Q = nF\Gamma \]  

(17.2)

Both these parameters could be used in order to evaluate the deposition of PB and for statistical evaluation of the reproducibility of the deposition procedure.

17.3.2 **Hydrogen peroxide measurements**

H₂O₂ measurements are performed in a 0.05 mol l⁻¹ phosphate buffer + 0.1 mol l⁻¹ KCl, pH 7.4 and at an applied potential of −0.05 V using both batch amperometry and continuous flow amperometry. In the first case, measure H₂O₂ by dipping the electrode in 10 ml of a stirred phosphate buffer. Apply the potential and when a stable current background is reached (30–60 s), add the H₂O₂. The response obtained (cathodic current) is measured after 30 s.

When tested in continuous flow, insert the sensors into the wall-jet cell (Scheme 17.1C and D) and connect the cell with a peristaltic pump.
at a fixed rate. Apply the potential and let flow a buffer solution until a stable baseline is obtained. Then, change the solution with a hydrogen peroxide solution prepared in the same buffer and wait for the cathodic signal.

Since PB-modified SPEs represent the support for enzyme immobilisation, carefully study their performances with respect to $\text{H}_2\text{O}_2$ response (detection limit, linearity range and reproducibility).

17.4 DISCUSSION

The procedure presented in this protocol for PB deposition is based on the spontaneous reaction between the two precursors of PB and does not require any electrochemical steps, which have typically been employed in the most common procedures for PB deposition. In Scheme 17.1.

Scheme 17.1. Schematic diagram of biosensor and wall-jet cell. (A) Screen-printed electrode front-view: (1) silver ink acting as reference electrode, (2) graphite ink acting as working electrode successively modified with PB and (3) silver ink acting as counter electrode. (B) PB-modified screen-printed electrode side-view: (1) polyester film as support for printing step, (2) graphite ink and (3) PB layer. (C): Wall-Jet flow cell side-view: (1) inlet of the flow, (2) outlet, (3) cell made of Teflon and (4) glucose biosensor. (D) Wall-jet flow cell front-view: (1) outlet, (2) inlet of the flow, (3) O-ring, (4) flow-cell and (5) glucose biosensor. Reprinted from Ref. [4] with permission from Elsevier.
Preparation of Prussian blue-modified screen-printed electrodes

**ELECTROCHEMICAL DEPOSITION**

- Constant Potential $+0.4$ V vs. Ag/AgCl for 60 sec in 2.5 mmol l$^{-1}$ FeCl$_3$, 2.5 mmol l$^{-1}$ K$_3$Fe(CN)$_6$, 0.1 mol l$^{-1}$ KCl and 0.1 mol l$^{-1}$ HCl.
- Activation through cycling between $+350$ mV and $-50$ mV (25 cycles) at a sweep rate of 50 mV/sec into a supporting solution (0.1 mol l$^{-1}$ KCl + 0.1 mol l$^{-1}$ HCl).
- Electrochemical cycling 10 times between $+350$ mV and $-50$ mV vs Ag/AgCl.
- Electrochemical conditioning at $-50$ mV for 600 seconds into phosphate buffer $0.05$ mol l$^{-1}$ + $0.1$ mol l$^{-1}$ KCl, pH=5.5.

**CHEMICAL DEPOSITION**

- Injection of 20 µl of 0.1 mol l$^{-1}$ FeCl$_3$ in 10 mmol l$^{-1}$ HCl + 20 µl of 0.1 mol l$^{-1}$ K$_3$Fe(CN)$_6$ in 10 mmol l$^{-1}$ HCl onto the working electrode area.
- Washing with 10 mmol l$^{-1}$ HCl after 10 minutes.
- 1.5 h at 100°C

Scheme 17.2. Schematic representation of the two procedures usually adopted for PB deposition.
17.2 are compared and summarised the two procedures. In the case of the electrochemical procedure, the electrode is first placed in a cell containing FeCl₃ and K₃Fe(CN)₆ and after deoxygenation (10 min) a constant potential of +0.4 V for 60 s is applied. After this, the electrode is washed and activated with a constant potential (−50 mV for 600 s) and a CV (25 cycles between +350 and −50 mV) experiment and then dried for 1 h at 100°C.

In the case of the chemical deposition, a drop of FeCl₃ is placed on the working electrode and followed by the injection of a drop of K₃Fe(CN)₆. After 10 min the electrode is washed and dried at 100°C for 1 h.

The advantage in terms of the time needed and of the procedures employed of using the chemical deposition, with respect to the electrochemical one, can be easily understood from Scheme 17.2.

The chemical deposition of PB leads to an effective modification of the electrode surface, which then shows positive features as to hydrogen peroxide detection with an effective rate constant very similar to that measured for the peroxidase enzyme (2 × 10⁴ M⁻¹ s⁻¹) [1]. Another major advantage of the chemical deposition is that it involves a more convenient and shorter procedure that avoids long electrochemical procedures during modification [2,3]. Moreover, the chemical deposition gives a more stable PB layer, which is only slightly affected by alkaline pH.

In our opinion, this procedure provides a new stimulus for the development of PB-modified biosensors, with the opportunity of applying the PB in an industrial process making possible the mass production of modified electrodes in a simple, automatable and cost-effective way.

SELECTED LITERATURE