Electroactive cytochrome$P450_{BM_3}$ cast polyion films on graphite electrodes

Aurélie Pardo-Jacques, Régine Basseguy, Alain Bergel *

Laboratoire de Génie Chimique, CNRS, Basso Cambo, 5 rue Paulin Talabot, BP 1301, 31106 Toulouse, France

Abstract

Films of electrochemically active cytochrome $P450_{BM_3}$ were constructed on graphite electrodes using alternate assembly with polyethyleneimine (PEI). The original layer-by-layer adsorption method was slightly modified here to form so-called "cast polyion" films. The cast polyion films were elaborated by immobilizing two successive layers of PEI and protein in very large excess with respect to a monolayer, without any intermediate washing step. Following the immobilization steps by SEM showed that uniform films of a few micrometers were deposited on the graphite surface. The electrochemically activity of the immobilized cytP450 was tested with regard to the reduction of oxygen and the one-electron reduction of the heme. Cyclic voltammetry indicated surface concentration of electrochemically active cytP450 around 0.6 nmol/cm^2, which corresponded to 5% of the total amount of protein that was consumed by the immobilisation process. Adapting the procedure to a graphite felt electrode with the view of scaling up porous electrodes for large scale synthesis increased the concentration to 0.9 nmol/cm^2. Cast polyion films may represent a simple technique to immobilize high amount of electrochemically active protein, keeping the advantage of the electrostatic interactions of the regular layer-by-layer method.

Keywords: Cytochrome $P450_{BM_3}$; Bioelectrochemistry; Modified electrodes; Cast polyion films; PEI

1. Introduction

Cytochromes P450 are heme protein that catalyse numerous oxidation of natural and foreign substrates including the difficult hydroxylation of unactivated C–H bonds in hydrocarbons and other compounds [1]. Electrodes provide useful substitutes for the natural redox partners of the protein with the view to develop cytP450-catalyzed synthesis. Mediated electrode-driven biocatalysis with cytochrome P450 using cobalt Co^{III} sepulchrate as mediator has been demonstrated [2]. Direct electron transfer has been achieved with P450camphor on edge plane pyrolytic graphite electrodes, but it required very drastic conditions such as and freshly purified protein [3]. The most significant results have been obtained with P450 coated electrodes, because the immobilisation of P450 revealed to facilitate and to stabilize the electron transfer. Enzyme coated electrodes provide a wide basis of techniques for developing different bio-electrochemical devices such as biosensors, biomedical devices, biofuel cells and enzymatic reactors [4–7]. In the case of P450 two type of immobilisation techniques have been mainly investigated with cast film from surfactants and layer-by-layer alternate adsorption with polyions.

Surfactants have been used [8–10] to form cast films with cytochrome P450 or the simpler heme protein, myoglobin [10–12]. The structure of the films obtained is governed by molecular properties and interactions which guide the self-assembly process. The water-insoluble surfactants form ordered films featuring a stacked multi-bilayer structure, which is commonly compared to the biological membranes formed by lipids in living organisms. The resulting films are generally a few micrometers thick [11,13]. On the other hand, the layer-by-layer immobilisation technique consists in the alternate adsorption of polyion and protein monolayers of opposite charge [4,14–18]. The films are mainly stabilized because of the strong electrostatic interactions that occur between the opposite charges of the adjacent polyion and protein layers. It has been recently shown that proteins are able to strongly adsorb onto polyelectrolyte film whatever the sign of the charges of both polyelectrolyte

* Corresponding author. Tel.: +335 3461 5248; fax: +335 3461 5253.
E-mail address: Alain.Bergel@ensiacet.fr (A. Bergel).

and protein. Nevertheless, when the protein and polyelectrolyte are oppositely charged the adsorbed amounts are usually larger and the protein layer is thicker. Even if specific interactions are certainly involved, electrostatic interactions constitute the main motor of layer-by-layer buildup [19]. The well ordered architecture obtained gives a powerful tool for analytical purposes, such as studying electron transfer as a function of the distance of the protein molecules from the electrode surface [20]. The combination of electrostatic and specific interaction for film assembly allows for the preparation of molecular layered multicomposite films with a high degree of complexity, by tuning the space between alternate layers or incorporating different kinds of compounds. The thickness of the films is generally of a few tens nanometer only, it means that electrochemical studies are possible with very small quantities of protein. This technique is for instance very useful to determine the electrochemical behaviour of newly isolated proteins, when only small quantities are available.

The ultimate goal of our research is to develop electrode coating for industrial synthesis catalysed by the cytochrome from *Bacillus megaterium* triple mutant ($P450_{BM_3}$). This protein has been shown to catalyze regiospecifically the hydroxylation of several substrates like saturated fatty acids (*para*-nitrocarboxylic acids) [21,22] or the oxidation of octane to octanol [23–26]. In this paper it was attempted to combine the advantages of the cast surfactant films: simple construction procedure and capability to immobilise high quantity of protein, with those of the layer-by-layer films: high stability due to strong electrostatic interactions. For instance it has been demonstrated that myoglobin was more stable under synthesis conditions in layer-by-layer films than in cast films [10], which seem to undergo more important mechanical damage with stirring [27]. With the view to improve mechanical stability of cast surfactant films, hybrid constructions have been proposed introducing polyion backbones in the composition. The resulting surfactant–polyion composite films possess multi-layer structure and some characteristics of dispersed lipid membrane. They have shown better stability than the cast surfactant films [28,29]. These attempts confirm that electrostatic interactions are definitively the best motor for promoting film stability. Actually, the well ordered structure given by the layer-by-layer technique has been questioned in some papers that have revealed interpenetration up to four adjacent layers [30]. Moreover, a strict architecture seems not to be so suitable from the point of view of synthesis. It has been claimed that some disorder between the alternate layers may favour the electron transfer by electron hopping [14]. The modified method that was proposed here used alternate adsorption of oppositely charged polyion and protein, to keep strong electrostatic interaction, but in a non-ordered way. The classic layer-by-layer procedure is based on the successive adsorption of monolayers. A strict washing step is necessary between each adsorption step to remove any protein in excess with respect to a single monolayer. A careful examination of the assembly procedure has demonstrated that washing between the consecutive adsorption steps was effective for optimising the well-ordered architecture [31]. On the contrary, in the modified technique developed here, a high quantity of polyion was firstly adsorbed on the graphite electrode and then a high quantity of protein, without any intermediate washing between the adsorption steps. It was expected that this modified technique lead to so-called "cast polyion" films, associating the main advantages of the two basic techniques, it means high quantity of protein retained, strong electrostatic interactions that should favour stability, and a disordered assembly that should promote effective electron transfer.

With an isoelectric point p*I* at pH 4.6, $P450_{BM_3}$ mutant has a negative global surface charge at pH 7.5. The films were consequently assembled with the positively charged polycation poly(ethyleneimine) (PEI). The morphology of the cast polyion films were followed by scanning electron microscopy at each step of the formation, and the quantities of electrochemically active immobilized cytP450 were measured through cyclic voltammetry.

The cast polyion modified electrodes were checked on the catalysis of oxygen reduction, because oxygen reduction represents the second basic step in the cytP450 catalytic pathway of any hydroxylation. It was finally attempted to adapt the procedure to the immobilisation of cytP450 on a graphite felt electrode, which should me more convenient with the view of scaling up to industrial synthesis.

2. Experimental

2.1. Chemicals

Cytochrome $P450_{BM_3}$ mutant was supplied by Prof. R. Schmid (ITB, Stuttgart, Germany), and used as received. Horse heart myoglobin (Mb) was purchased from Sigma (M-1882). Poly(ethyleneimine), average Mn 1200, was purchased from Aldrich, and octane was purchased from Fluka. All experiments were performed in 20 mM Tris/HCl, pH 7.5. The pyrolytic graphite (PG) working electrodes (Le Carbone-Lorraine, France) were elaborated by inserting 6.15 mm diameter graphite barrels into isolating epoxy resin. Graphite felt electrodes RVG2000 (Le Carbone-Lorraine) 5 mm thick were used as received, without any preliminary treatment.

2.2. Film assembly

The pyrolytic graphite disk electrodes were firstly polished with successive abrasive disks (Lam Plan, France) of decreasing roughness P240, P400, P1200.

Following the classic layer-by-layer procedure, films were grown on PG by repeated alternate adsorption of PEI and $P450_{BM_3}$. Twenty microliters of PEI aqueous solution (7 mg/mL PEI, 0.5 M NaCl in water) were adsorbed first for 20 min, and the electrode was washed by immersion in water. Twenty microliters of $P450_{BM_3}$ solution (2 mg/mL in pH 7.5 Tris buffer) were then adsorbed for 20 min, and the electrode was washed again by immersion in water. When indicated, a last PEI layer was deposited repeating the same procedure. Electrodes were dried under a nitrogen stream after each adsorption step.

Cast polyion films were made as following: 20 μL PEI aqueous solution (7 mg/mL, 0.5 M NaCl in water) were adsorbed

first until complete adsorption, usually 1 h. Twenty microliters of $P450_{BM_3}$ solution (2–20 mg/mL in pH 7.5 Tris/HCl buffer) were then adsorbed for 1 h. When indicated, a last PEI layer was adsorbed identically. Neither washing nor drying was performed between each step. Myoglobin (Mb) immobilization was achieved following the same procedure with 20 μL Mb solution (2 mg/mL in pH 7.5 Tris buffer).

2.3. Instruments and procedures

A PAR 263A electrochemical set-up was used for cyclic voltammetry with a saturated calomel reference electrode (SCE), and a platinum wire as counter electrode. Voltammetry on modified electrodes was performed in Tris/HCl buffer pH 7.5 solutions that did not contain $P450_{BM_3}$ in a closed Metrohm cell. Solution was deoxygenated by nitrogen bubbling for at least 20 min before each experiment, and a nitrogen flux was then kept over the solution during electrochemical experiments. All experiments were done at room temperature ($22 \pm 2\,^{\circ}C$). Scanning electron microscopy (SEM) was done with an LEO 435VP microscope.

3. Results

3.1. Film morphology

The modified electrodes were built following either the classic layer-by-layer procedure or the modified cast polyion procedure. The layer-by-layer procedure consisted of successive steps of PEI and $P450_{BM_3}$ adsorption and intermediate washing. This intermediate washing are believed to ensure that the last remaining layer correspond to the strict monolayer that is required to balance the charge. The layer-by-layer procedure should result in the strict succession of alternated monolayers of PEI and protein. On the contrary, the cast polyion procedure proposed here consisted of complete adsorption and evaporation of the solution of PEI, followed by complete adsorption of the solution of $P450_{BM_3}$, without intermediate washing. The cast polyion procedure should result in higher amount of randomly adsorbed protein and PEI, because no intermediate washing removed the excess of protein or PEI with respect to a monolayer. The electrodes obtained following the layer-by-layer procedure were noted PG{PEI/$P450_{BM_3}$}, while the electrodes modified following the cast polyion technique were noted PG/PEI/$P450_{BM_3}$. In some cases, a last layer of PEI was added on the films with the view to minimize the further release of protein in solution, the electrodes obtained were noted PG{PEI/$P450_{BM_3}$/PEI} or PG/PEI/$P450_{BM_3}$/PEI.

The morphology of the layer-by-layer films and the cast polyion films were followed step by step by scanning electron microscopy. Fig. 1 gives the pictures of the surfaces after deposition of first PEI layer, with 20 μL 7 mg/mL PEI, 0.5 M NaCl in water. Column (a) corresponds to the layer-by-layer electrodes PG{PEI} and column (b) corresponds to the cast polyion electrodes PG/PEI.

Only a few small dots where deposited PEI remained were observed on the PG{PEI} electrode surface. Washing obviously removed most of the PEI, as expected by the layer-by-layer theory. On the contrary, a significant coverage of the electrode surface was observed in the absence of washing (column (b)). Nevertheless, only a small part of the electrode surface area was covered. The pictures with magnification of ×5000 confirmed

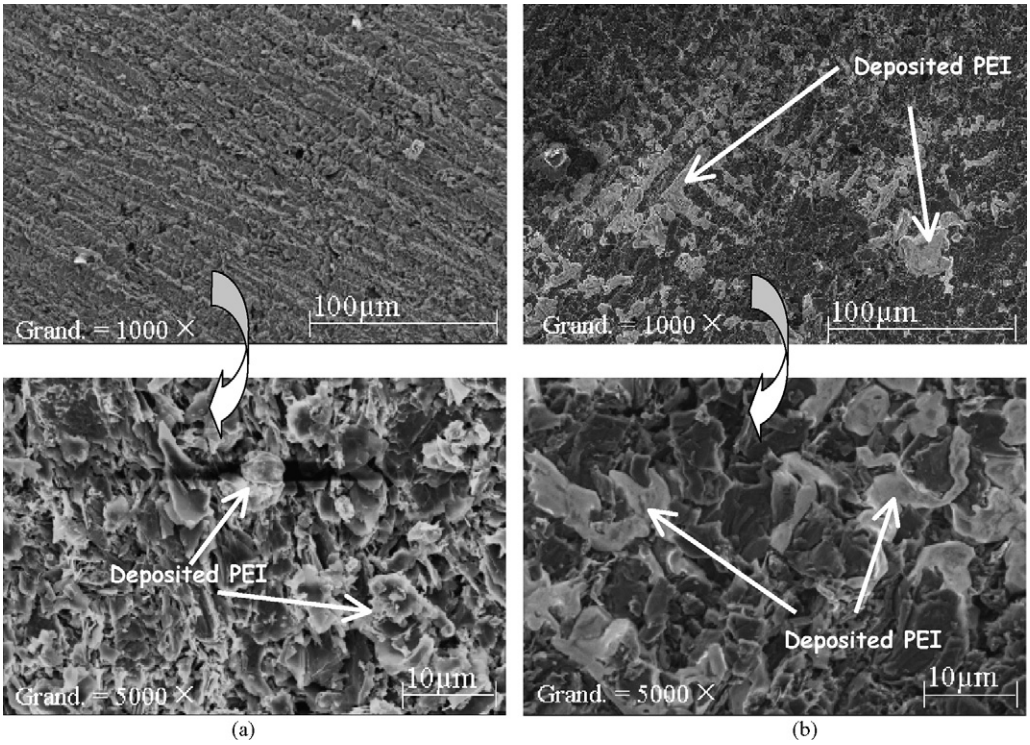
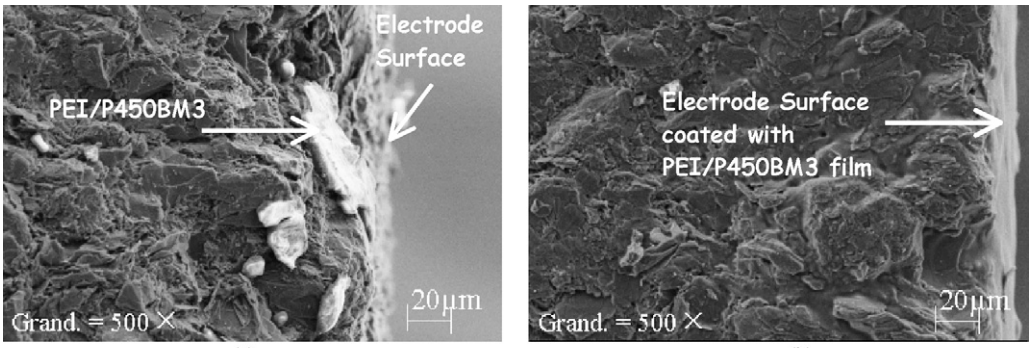


Fig. 1. SEM top views of coated PG electrodes, magnification of ×1000 and ×5000: (a) {PG/PEI} layer-by-layer film and (b) PG/PEI "cast polyion" film.



(a) (b)

Fig. 2. SEM cross-section views of coated PG electrodes, magnification of ×500: (a) {PG/PEI/P450$_{BM_3}$} layer-by-layer film and (b) PG/PEI/P450$_{BM_3}$ "cast polyion" film.

that only isolated islands of PEI were deposited, which did not mask the flaky structure of the pyrolytic graphite.

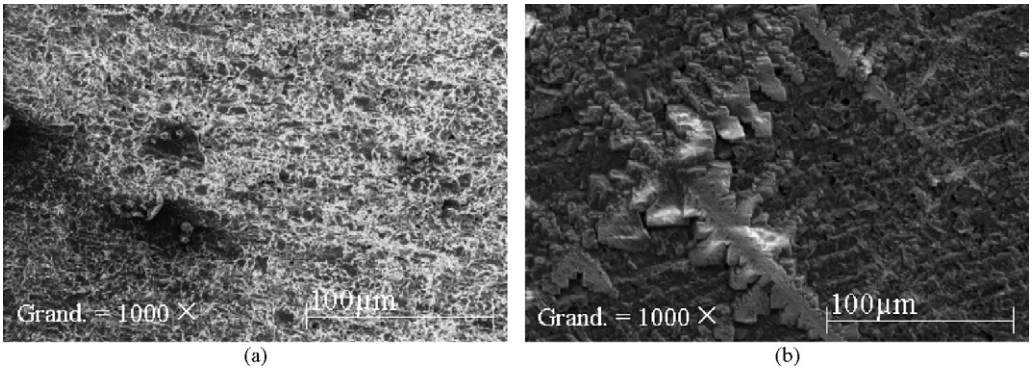
Layers of cytochrome P450$_{BM_3}$ were then deposited, following both procedures, with 20 µL solution of 2 mg/mL cytP450 in water. This concentration was similar to the values commonly used in other works [16,32]. Film depositing was achieved on PG electrodes that were previously cut with two faces at right angle to get the cross-sectional views reported in Fig. 2.

As expected for a layer-by-layer procedure, which assumed only nanometer-scale films, no modification of the electrode surface was observed on PG{PEI/P450$_{BM_3}$} electrodes. Only a few deposited dots were observed at the cut angle of the electrode. On the contrary, an almost uniform coverage was observed on the surface of the cast polyion PG/PEI/P450$_{BM_3}$ electrodes. Thanks to the cross-sectional views, the thickness of the film was estimated of the order of 5 µm approximately. Finally, a last PEI layer was added following both procedures (Fig. 3). The structure of the graphite remained visible under the {PG/PEI/P450$_{BM_3}$/PEI} layer-by-layer films, which did not induce a visible modification of the electrode surface (column (a)). On the contrary, the cast polyion films PG/PEI/P450$_{BM_3}$/PEI formed a uniform smooth film on the whole graphite surface area, which fully masked the initial structure of the graphite (column (b)). Crystal-like structures were also observed, which were certainly due to the crystallization of the salt contained in the buffer.

The SEM observations of the layer-by-layer modified electrodes were consistent with the theory. Washing after each adsorption steps was supposed to remove the PEI and protein that were in excess with respect to monolayer coverage. The molecular monolayer that remained adsorbed should not induce visible transformation of the surface. The cast polyion method firstly resulted in significant deposition of PEI, but with a non-uniform coverage. It may be inferred that the surface of the graphite did not present a uniform distribution of the negative surface charge. During the second step of the procedure the positive charged cytP450 were retained on the whole surface area, independently of the presence or not of PEI, resulting in a final uniform coverage. Non-uniformity of the first PEI layer that forms islands, and filling of the gaps by the successive layer, have already been described at the molecular scale using AFM and QCM for the regular layer-by-layer method [33–35]. The results obtained here with SEM on thick layers indicated that the cast polyion architecture seems to obey the same basic molecular behaviour as the regular layer-by-layer method. The thickness of the film may let hope that a significant amount of protein be retained.

3.2. Catalysis of oxygen reduction with cast polyion modified electrodes

The pyrolytic graphite (PG) electrodes were carefully polished before each experiment. After polishing the electrodes were systematically tested by cyclic voltammetry at 0.2 V/s in deoxygenated Tris/HCl buffer pH 7.5. Low capacitive current values of the order of 50 µA, which corresponded to capac-



(a) (b)

Fig. 3. SEM views of coated PG electrodes, magnification of ×1000: (a) {PG/PEI/P450$_{BM_3}$/PEI} layer-by-layer film and (b) PG/PEI/P450$_{BM_3}$/PEI "cast polyion" film.

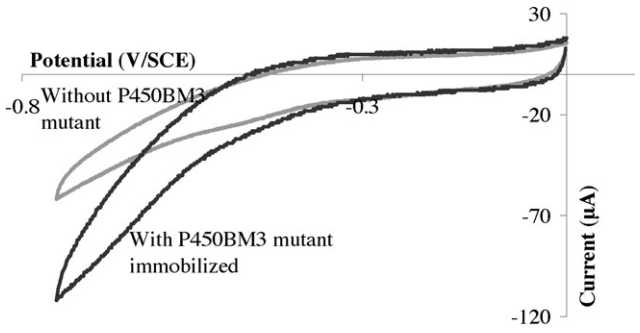


Fig. 4. Cyclic voltammograms at 0.01 V/s for PG/PEI/$P450_{BM_3}$/PEI and PG/PEI electrodes; 15 mL air in 20 mL buffer pH 7.5.

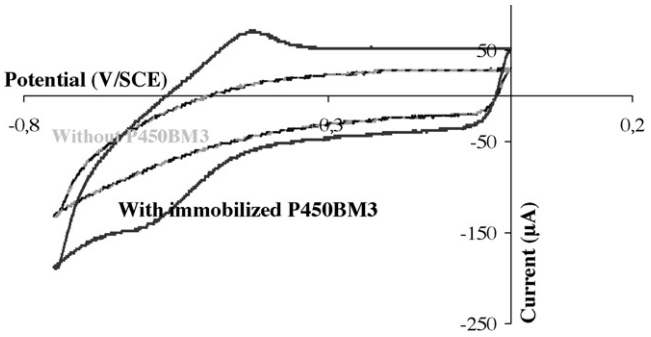


Fig. 5. Cyclic voltammograms on PG electrodes at 0.2 V/s in the absence of oxygen in pH 7.5 buffer with PG/PEI/$P450_{BM_3}$/PEI and PG/PEI selectrodes.

itance around 250 µF indicated that the electrode was suitable for further adsorption. In some cases higher values were obtained, which indicated a bad surface state, these electrodes were rejected.

Cyclic voltammograms were recorded at 0.01 V/s in the range −0.75 V to 0 V/SCE in 20 mL Tris/HCl buffer pH 7.5 with cast polyion PG/PEI electrodes (it means without $P450_{BM_3}$ immobilized on the electrode surface) and with PG/PEI/$P450_{BM_3}$/PEI modified electrodes. The solution was deoxygenated by 20 min nitrogen bubbling. The concentration of $P450_{BM_3}$ solution used for the immobilization was 20 mg/mL in water. The last PEI deposit was expected to reduce possible protein release from the film. The CV showed no differences in deoxygenated solution.

The CV reported in Fig. 4 were recorded after injection of 15 mL air into the solution. The nitrogen flux was stopped but the vessel remained hermetically closed. A significant increase in the current of oxygen reduction was observed from −0.35 V/SCE when $P450_{BM_3}$ was immobilised in the film. The catalytic pathway involves the initial reduction of cytP450 on the electrode [14]:

$$\text{cytP450-Fe}^{\text{III}} + \text{H}^+ + \text{e}^- \rightarrow \text{ cytP450-Fe}^{\text{II}} \qquad \text{(Reaction 1)}$$

then the reaction of reduced species with oxygen forms a complex:

$$\text{cytP450-Fe}^{\text{II}} + \text{O}_2 \rightarrow \text{ cytP450-Fe}^{\text{II}}\text{-O}_2 \qquad \text{(Reaction 2)}$$

which is reduced at the electrode to give hydrogen peroxide:

$$\text{cytP450-Fe}^{\text{II}}\text{-O}_2 + \text{e}^- + \text{H}^+ \rightarrow \text{ cytP450-Fe}^{\text{III}} + \text{H}_2\text{O}_2 \qquad \text{(Reaction 3)}$$

The current increase observed in Fig. 4 demonstrated that the immobilised CytP450Fe^{III} was able to ensure effectively the three first steps of the catalytic pathway. The catalysis showed that reduction of P450Fe^{III} started around −0.35 V/SCE. Previous studies have shown oxygen catalysis starting at less negative potential values, around −0.15 V/SCE with $P450_{CAM}$ immobilized in polyion films, e.g. [16]. It may be assumed that the graphite material used here had slightly different electrochemical properties or that the thick cast polyion film slowed oxygen transfer to the protein.

3.3. Electroactivity of the layer-by-layer and cast-polyion modified electrodes

The modified electrodes were immersed in Tris/HCl buffer pH 7.5. After strictly deoxygenating the solution by 20 min nitrogen bubbling, cyclic voltammograms were recorded at 0.2 V/s between −0.75 V and 0 V/SCE (Fig. 5). A nitrogen flux was maintained above the solution during potential scanning. In some cases, the first cycle exhibited a small cathodic current that revealed the presence of oxygen traces, then all cycles were perfectly reproducible. Reduction and oxidation CV peaks were observed at about −0.60 V and −0.42 V/SCE, respectively. The residual current was made up of two contributions: a capacitive current I_{cap}, and a residual Faradic current I_{Far}. The capacitive contribution I_{cap} was expressed as:

$$I_{cap} = C_{dl} \cdot \nu \qquad (1)$$

where C_{dl} is the double layer capacity (Farad) and ν is the scan rate (V/s). This contribution alone should give a constant value of the current on the whole potential range. The additional residual Faradic current, which was observed on all CV, was certainly due to the reduction of surface species of the PG electrode. This residual Faradic contribution I_{Far} was expressed by a Tafel's law:

$$I_{Far} = -A \cdot i_0 \cdot \left[\frac{-(1-\alpha) \cdot nF}{RT} \cdot (E - E'^{\circ}s) \right] \qquad (2)$$

where A is the surface area (cm^2), i_0 the exchange current density (A/cm^2), α the electronic exchange coefficient, R the gas constant ($J/mol\,K^{-1}$), T the temperature (K), n the number of electron exchanged, E the applied potential (V) and E'° is the formal potential of the suspected surface species. Recombining this equation gave:

$$I_{Far} = -C_1 \cdot \exp\left[-C_2 \cdot \frac{nF}{RT} \cdot E \right] \qquad (3)$$

where the two constants:

$$C_1 = A \cdot i_0 \cdot \exp\left[-(1-\alpha) \cdot \frac{nF}{RT} \cdot E'^{\circ}s \right] \qquad (4)$$

$$C_2 = 1 - \alpha \qquad (5)$$

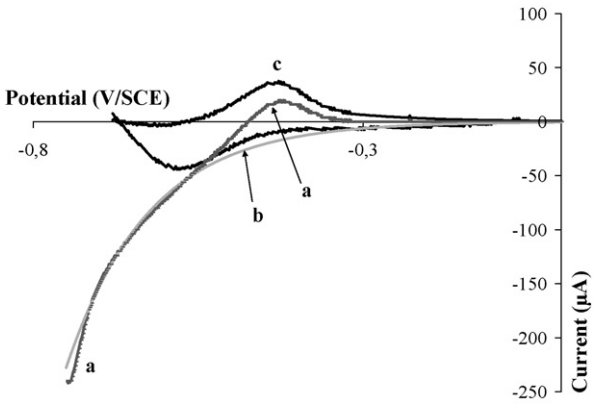


Fig. 6. Theoretical fitting of cyclic voltammogram 0.2 V/s for PEG/PEI/$P450_{BM_3}$/PEI electrodes: (a) experimental current; (b) theoretical residual current; (c) current due to $P450_{BM_3}$ electrochemistry only.

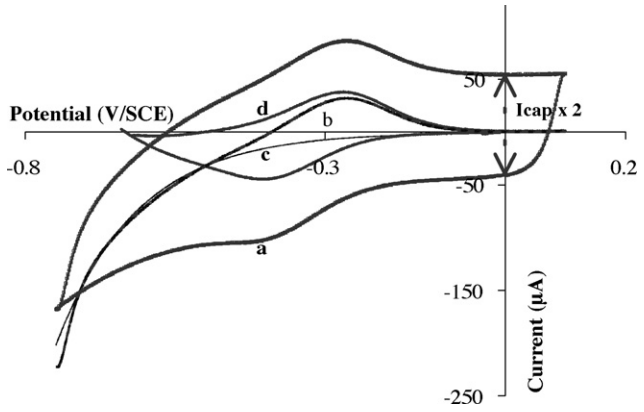


Fig. 7. Theoretical fitting of cyclic voltammogram 0.2 V/s for PEG/PEI/Mb electrodes: (a) experimental current; (b) oxidation scan corrected from capacitive current; (c) faradic residual current obtained by fitting; (d) CV due to Mb electrochemistry only.

were determined numerically. C_1 and C_2 were adjusted by fitting the experimental curves for the oxidation scan in the potential range from −0.65 V to −0.75 V/SCE, where the oxidation of $P450_{BM_3}$ did not occur (Fig. 6). Typical values of the constant C_2 (=1 − α) were around 0.19.This order of magnitude confirmed that the Tafel's approach used to model the residual Faradic current physically made sense.

The total residual current $I_{Far} + I_{Cap}$ that resulted from this model was then subtracted from the experimental CV to get the current that corresponded to the $P450_{BM_3}$ reduction/oxidation peaks. A formal potential $E'^{\circ}_{P450_{BM_3}Fe} = -0.51$ V/SCE was derived from this new curve, which corresponded to the one-electron reduction/oxidation of the hemic iron Fe^{III}/Fe^{II} of the cytochrome. This value was more negative than the values commonly found in literature for different types of cytP450: −0.31 V/ECS [4] and −0.35 V/ECS [17]. On the other hand, the reduction curve seemed quite dissymmetrical according to the oxidation curve, and the reduction peak started at potential values significantly more negative than may be expected from the curves obtained in the presence of oxygen.

The same experiments were repeated with myoglobin (Mb) instead of cytochrome $P450_{BM_3}$. Myoglobin is a simple hemic protein which shows an electrochemical behaviour quite close to those of cytochromes P450 [4,10]. The PEG/PEI/Mb coated electrodes were elaborated following the cast polyion procedure and CV were recorded and modelled in the same way (Fig. 7). The oxidation scan was fitted in the potential range from −0.65 V to −0.55 V/SCE, where the oxidation of Mb did not occur. Typical values of constant C_2 were around 0.15. A formal potential E'°_{Fe} of −0.33 V/SCE was derived. This value matched the formal potential values of −0.344 V/SCE that has been reported for the Mb–Fe^{III}/Fe^{II} immobilised in PSS/Mb assemblies [36].

The good match between the formal potential values derived here and the values coming from the literature in the case of myoglobin indicated that the discrepancy observed for the cytochrome $P450_{BM_3}$ was not due to the electrochemical characteristics of the graphite electrodes used here or to the immobilisation procedure. The discrepancy should be due to intrinsic properties of the specific cytochrome used here.

The oxidation peaks were then integrated numerically to get the oxidation charge Q (C):

$$Q = \frac{1}{\nu}\int_1^2 I \cdot \mathrm{d}E \tag{6}$$

where $\int_1^2 I \cdot \mathrm{d}E$ (A V) represents the area under the oxidation peak. The Faraday's law gave the surface concentration of the electroactive $P450_{BM_3}$ in the film Γ_{active} (mol cm^{-2}):

$$\Gamma_{activ} = \frac{Q}{n \cdot F \cdot A} \tag{7}$$

where $n = 1$ is the number of electron exchanged by the Fe^{III}/Fe^{II} redox couple, F (=96500 C/mol) the Faraday's constant, and A is the electrode surface area (cm^2). The surface concentrations Γ_{activ} measured with six different electrodes elaborated independently gave values in the range from 0.42 nmol/cm^2 to 0.96 nmol/cm^2 with an average value of 0.6 nmol/cm^2. The surface concentrations reported in the literature for layer-by-layer films are generally less than 0.2 nmol/cm^2, even for seven successive cytP450-PEI bilayers [17]. The maximal surface concentration that could be obtained if the whole initial amount of $cytP450_{BM_3}$ dissolved in the 20 µL solution used for immobilisation was retained on the electrode surface would be here $\Gamma_0 = 12$ nmol/cm^2. Dividing the actual surface concentration by the theoretical maximal surface concentration Γ_0 gave the ratio Γ_{activ}/Γ_0, which is essential from the point of view of industrial applications, because it measures the quantity of electrochemically active protein with respect to the total amount of protein that is consumed by the immobilization procedure. Here the average ratio was of 5%. It was not possible to derive the Γ_{activ}/Γ_0 ratios from the literature data, but the comparison should certainly highlight the interest of the cast polyion procedure. The original layer-by-layer procedure requires several successive adsorption and washing steps in order to increase the amount of immobilized protein, but it multiplies thus the amount of protein that is wasted. Nevertheless, it is difficult to attribute this good result to the cast polyion procedure alone, as the high roughness of the graphite surface used here, as shown

by the SEM micrographs, may also favour the adsorption of high amounts of proteins. The disorder created by the surface roughness may also increase the number of protein molecules that are available for electron transfer. It is generally agreed that the surface concentration of electrochemically active proteins are higher on rough surfaces as graphite than on flat surfaces as gold (less than 0.02 nmol/cm^2 [4]), because of the disorder induced in the upper layers by the roughness of the electrode surface. For instance, only the first two layers of myoglobin deposited on a gold surface have been demonstrated to be active, while up to seven layers were active on pyrolytic graphite [14]. Nevertheless, it should be noticed that the preliminary layer of mercaptopropanesulfonic acid that is necessary to condition the gold surface has also been suspected to exert a detrimental effect on electron transfer.

3.4. Stability of cast-polyion modified electrodes

The stability of modified cast polyion PG/PEI/P450$_{BM_3}$/PEI electrodes was studied during several days in different storage conditions. The electrodes were stored during 9 days (i) at room temperature and at air, (ii) at room temperature and in Tris–HCl buffer, (iii) at 4 °C and at air (iv) at 4 °C and in Tris–HCl buffer. The electrodes were periodically analyzed by cyclic voltammetry at 0.2 V/s and at room temperature. The intensity of the oxidation peak was reported as a function of the storage time in Fig. 8.

All modified electrodes were stable during at least one day. It was then difficult to distinguish a possible effect of temperature and of the storage conditions in air or in solution. The electrode stored at 4 °C in solution exhibited a higher electrochemical activity after one and two day storage. This may be explained by a reorganization of the layers, resulting in increasing the quantity of protein able to achieve electron transfer with the electrode surface. A "maturation" period might be favourable to the effectiveness of the modified electrodes.

Moreover, the results obtained with the electrodes dipping in solution, either at room temperature or at 4 °C, indicated that there was no significant protein release during 2 days.

Layer-by-layer films have already been demonstrated to be stable under storage conditions for long period, at least 3 weeks with myoglobin [4,14,37] or about 1 month with immobilised glucose oxidase [34,38]. Obviously the stability depends on the stability of the protein itself. In our case the 3 day stability observed in cast polyion films was consistent with the stability of the catalytic property of our cytP450$_{BM}$ in solution. This comparison may be discussed, as the stability in solution was measured with regard to the rate of octane hydroxylation in the presence of the natural electron donor NADPH. The stability of the hydroxylation activity may be quite different from the stability of the one-electron electrochemical reduction of the Fe^{III}/Fe^{II} heme (Reaction 1).

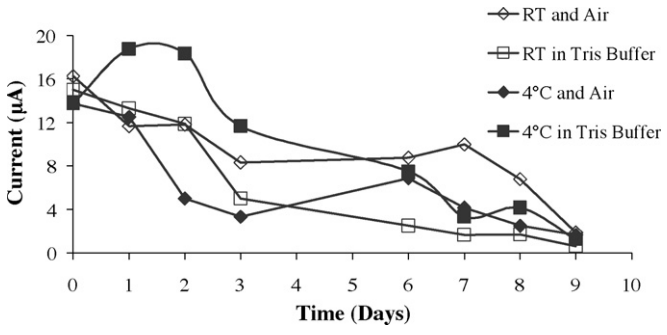


Fig. 8. Influence of storage conditions on the current of the oxidation peak recorded at 0.2 V/s.

This first approach of stability only concerned storage conditions, as most the studies in the literature. Nevertheless, it has been demonstrated that stability under real synthesis conditions may be drastically different. The reduction peak of myoglobin adsorbed in polyion films has been shown to lost 20% in 1 h under electrolysis in the presence of air and up to 30% under oxygen [10]. Mechanical damage with stirring has been evoked or also protein damage due to the hydrogen peroxide formed by oxygen reduction (Reaction 3).

3.5. Cast polyion films on graphite felt electrodes

The cast polyion immobilisation procedure was adapted for 5 mm thick graphite felt electrodes. Because of the high porosity of these electrodes it was expected to increase the quantity of immobilised electrochemically active cytP450. The volume and concentration of the PEI and cytP450 solutions used for immobilisation were adjusted by successive trials and errors, to prepare felt electrodes with 2 cm^2 geometric surface area. The cast polyion procedure was firstly achieved with 800 μL PEI (7 mg/mL), and 400 μL cytP450 (20 mg/mL). After the cast polyion modification, the felt electrode was checked by immersion for 5 h in 5 mL Tris/HCl buffer containing 80 μL pure octane with gentle stirring and under constant polarisation at −0.55 V/SCE. Octane was added into the solution, and the electrode was polarized to test the electrode under conditions as close as possible to actual synthesis conditions. The bulk solution became trouble very fast, indicating a significant release of PEI from the electrode. The same trouble was obtained when a few microliters PEI was added into the initial Tris/HCl–octane mixture. Modification of the felt electrode was repeated with smaller volume of PEI solution (200 μL), and always 400 μL cytP450 solution. The same check experiment showed a slight yellow colour of the aqueous phase after 5 h immersion. The UV spectrophotometric analysis of the aqueous phase showed significant adsorption at 280 nm, indicating the presence of protein in solution. In this case the cytP450 concentration was toot high, and was significantly released from the electrode.

After a few such trials and errors, the optimal immobilisation procedure for the 2 cm^2 felt graphite electrode was: from 60 μL to 75 μL PEI 7 mg/mL, and 50 μL cytP450 from 0.20 mg/μL to 25 mg/μL. The cyclic voltammogram recorded at 0.2 V/s with an electrode modified following this procedure is reported in Fig. 9.

The general shape of the curve was similar to the shape obtained with planar graphite electrodes, but gave a formal potential value slightly different of $E'^{\circ}_{P450_{BM_3}} = -0.48$ V/ECS. It seems that the nature of the electrode material affected the

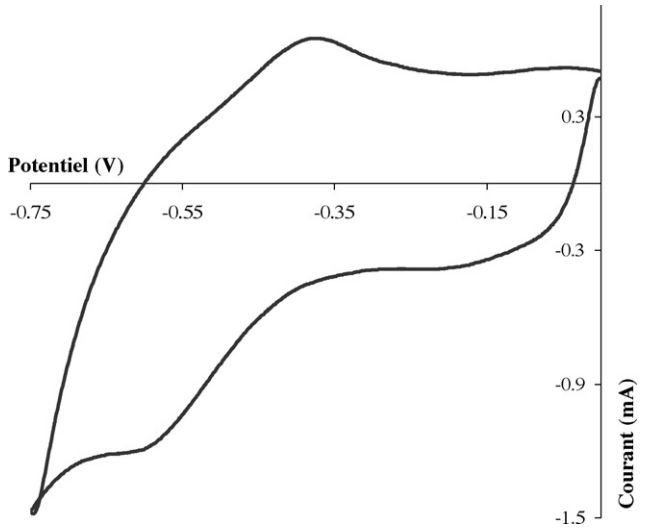


Fig. 9. Cyclic voltammogram at 0.2 V/s for a felt RVG/PEI/P450$_{BM_3}$/PEI electrode in Tris–HCl pH 7.5.

value of the apparent standard potential derived from the CV. The quantity of immobilised electroactive cytP450 was derived by integration of the oxidation peak: $\Gamma_{activ} = 0.9\,nmol/cm^2$. In this case, the quantity of cytP450 initially used for immobilisation was 88 nmol/cm^2 (21 mg dissolved in 100 μL for 2 cm^2), it means that 1% only of the initial quantity of cytP450 was retained under electroactive form. The surface concentration of electroactive cytP450 obtained on the 5 mm thick felt electrode (0.9 nmol/cm^2) was only slightly higher than the average value obtained with the planar graphite electrode (0.6 nmol/cm^2) and the ratio Γ_{activ}/Γ_0, around 1%, was significantly less than for the planar graphite electrode (around 5%). The high roughness of the planar graphite electrode certainly favoured an effective immobilisation of the protein. Comparing with the felt electrode proved that the available area of the electrode was not the sole parameter to be considered. From commercial information, the felt electrode should have an adsorption active area around 0.7 m^2/g, it means around 7×10^{-2} m^2 for 2 cm^2 of geometric surface area. It may be concluded that the material properties such as the accessibility of the surface in the case of porous electrode or the capability to induce non-coulombic interactions [4], may have a preponderant influence on the effectiveness of immobilisation.

4. Conclusion

The cast polyion procedure proposed here allowed the immobilisation of high quantities of electrochemically active cytP450$_{BM_3}$ in a fast way that should be easy to scale up to electrodes with large surface area. The average value of 5% of the ratio of the electrochemically active protein immobilized with respect to the whole amount of protein consumed is very promising, but this parameter still needs to be increased to get a real economic interest with expensive proteins as cytochromes P450. This ratio is a key parameter in assessing the economic interest of the protein coated electrodes in synthesis. The stability of the electrode was excellent during at least 3 days immerged in solution at low temperature. Stability must now be tested under real electrolysis conditions, keeping in mind the crucial role of the concentration of dissolved oxygen, either for the selectivity of the reaction and for possible damage of the protein. The immobilisation parameters were adapted to carbon felt electrodes. These first attempts highlighted the role in adsorption of other parameters than the sole value of the surface area, which have now to be investigated to progress in the direction of more efficient synthesis electrodes.

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