Simple design of cast myoglobin/polyethyleneimine modified electrodes

AURÉLIE PARDO, RÉGINE BASSÉGUY and ALAIN BERGEL*
Laboratoire de Génie Chimique – CNRS, 5 Rue Paulin Talabot, BP 1301, 31106, Toulouse, France
*(*author for correspondence, tel.: + 33-5-34615248, fax: + 33-5-34615253, e-mail: Alain.Bergel@ensiacet.fr)*

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Abstract

Negatively charged myoglobin (at pH values above its isoelectric point) was immobilized with the positively charged polyion polyethyleneimine (PEI) on pyrolytic graphite electrodes. A modified form of the common layer-by-layer technique was proposed, which consisted in forming non-ordered cast polyion films. The modified Mb electrodes obtained by both techniques were compared by cyclic voltammetry. The cast polyion technique gave less reproducible results but enable the immobilization of higher percentages of the total quantity of protein required to prepare the electrodes. The electrochemical properties of the negatively charged myoglobin were determined, and its capability to catalyze the electrochemical reduction of oxygen and the dechlorination of trichloroacetic acid was demonstrated. The mechanisms are discussed, and the modified pathway that has been proposed to take into account the influence of pH in the Mb-catalyzed reduction of oxygen was confirmed here.

1. Introduction

The wide family of the cytochromes P450 (CytP450) catalyzes numerous oxidations of different substrates including the difficult hydroxylation of inactivated C–H bonds in hydrocarbons [1, 2] and other compounds [3]. The ultimate goal of our research is the development of electrode coatings for industrial electro-synthesis catalyzed by cytochromes P450. The first steps of electrode design were implemented here using a model heme protein, myoglobin (Mb), which is easier to handle than CytP450 and commercially available. Myoglobin is a single polypeptide chain with an iron heme inside, which stores and transports oxygen in muscle cells in mammalians. Although it cannot be considered as an enzyme, the Mb protein is able to catalyze a few reactions in a similar way as more complex heme proteins such as CytP450.

Electron transfer with soluble Mb [4] or soluble cytP450 [5, 6] has been achieved only with freshly purified enzyme and at low temperature. In contrast, numerous studies have demonstrated that electron transfer is remarkably efficient when these heme proteins are immobilized on the electrode surface.

Surfactants have been commonly used [7–9] to form cast films that facilitate electron transfer between Mb and electrodes. The structure of the films obtained is governed by molecular properties and interactions which guide the self-assembly process. Proteins are randomly immobilized in films of a few micrometer thick [5, 10]. Another known technique, the layer-by-layer immobilization has also been successfully used. It consists in adsorbing successive layers of polyion and protein of opposite charge. The obtained architecture is well-ordered and stable due to the strong electrostatic attraction between the successive polyion and protein layers. The thickness of the layer-by-layer films is of the same order of magnitude than a few macromolecule layers, i.e. in the nanometer range [11]. The layer-by-layer films obviously contain smaller quantities of protein, but they have been observed to be more stable in stirring conditions than cast films [12].

Here, a new procedure is discussed, which combines the advantage of the surfactants cast film: larger quantities of protein immobilized, with the advantage of polyion layer-by-layer films: better resistance to hydrodynamic. The new so-called "cast polyion films" technique is simpler than the common layer-by-layer technique, and should be more suitable for scaling up to large surface areas for synthesis applications.

Most of the electrochemical studies devoted to Mb have used it at slightly acidic pH values (around 5.5), where Mb, which has a pI of 6.8 [13], is positively charged. Consequently, layer-by-layer Mb immobilization has commonly been achieved with polyanions, generally the poly(styrenesulfonate) (PSS) [13–15]. In contrast, the common cytochromes P450, with pI values around 4.5, are negatively charged, and have been

immobilized with polycations, generally poly(ethyleneimine) (PEI) [11, 12]. This difference may represent a main drawback when using Mb as a model for CytP450 to design immobilization techniques. In this work, it was decided to form "cast polyion films" with the polycation PEI, with the view to reach operating conditions that can be directly transferred to CytP450 in a further work, and consequently to use a pH value where Mb was negatively charged. The catalytic effectiveness of the Mb cast polyion films was tested with two different electrochemically driven reactions: reduction of oxygen, and dechlorination of trichloroacetic acid (TCA). It has been demonstrated that Mb and CytP450 are effective catalysts of both reactions [16, 17]. Checking Mb-modified electrodes with regard to catalysis of oxygen reduction is a basic test, because oxygen reduction constitutes the first mono-electronic step of the complex pathway that leads to the hydroxylation of organic compounds; reaction with TCA was aimed at testing a more complex pathway, which involves successive reactions of the same substrate molecule with two hemic catalytic sites [14].

2. Experimental section

2.1. *Chemicals*

Horse heart myoglobin (Mb) was purchased from Sigma (M-1882). Poly(ethyleneimine) (PEI) average Mn 1200, and trichloroacetic acid (TCA) were purchased from Aldrich. The buffer solutions were 10 mM Tris/HCl, pH 7.5. The pyrolytic graphite (PG) working electrodes (Le Carbone-Lorraine, France) were prepared by inserting 6.15 mm diameter graphite barrels into insulating epoxy resin.

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. The pyrolytic graphite (PG) electrodes were carefully polished before each modification. After polishing, each electrode was tested by cyclic voltammetry at 0.2 V s^{-1} in deoxygenated Tris buffer. Low-capacitive current values indicated that the electrode was suitable for further adsorption.

Following the classic layer-by-layer procedure, films were grown on PG by repeated alternate adsorption of PEI and Mb. Twenty microliters of PEI aqueous solution (7 mg mL^{-1} PEI, 0.5 M NaCl in water) were deposited on the electrode surface and the electrode was rinsed 20 min later by immersion in water for a few seconds. Twenty microliters of Mb solution (2 mg mL^{-1} in pH 7.5 Tris buffer) were then deposited and the electrode rinsed again 20 min later. Films were dried under a nitrogen stream after each adsorption step. The same procedure was repeated to obtain up to seven successive bilayers $\{PEI/Mb\}_7$.

Following the "cast polyion film" procedure, 20 μl of PEI aqueous solution (7 mg mL^{-1}, 0.5 M NaCl in water) were deposited and the electrode was kept in air until complete dryness of the solution, usually 1 h. Twenty microliters of Mb solution (2 mg mL^{-1} in pH 7.5 Tris buffer) were then deposited and dried for 1 h. Only two steps were performed and no washing was achieved between the two steps.

2.3. *Instruments and procedures*

A EG&G 263A electrochemical set-up was used for cyclic voltammetry, from 0.01 to 0.2 V s^{-1}, with a saturated calomel reference electrode (SCE), and a platinum wire as counter electrode. Voltammetry on electrodes coated with $\{PEI/Mb\}_n$ films was performed in buffer solutions that did not containing Mb, in a sealed Metrohm cell. The solution was purged with nitrogen for at least 20 min prior to experiments and a nitrogen flux was then kept flowing over the solution during the electrochemical experiments. All experiments were done at room temperature (22 ± 2 °C).

3. Results

3.1. *Comparison of the layer-by-layer and cast polyion film procedures*

In order to assemble the films with the polycation poly(ethyleneimine) (PEI), myoglobin (Mb) must get a negative global charge. Considering the Mb isoelectric point pI at pH 6.8 [13], all the experiments were consequently performed at pH 7.5 in 10 mM Tris/HCl buffer. The modified electrodes were built following either the classic layer-by-layer procedure, with successive steps of PEI adsorption, washing, Mb adsorption, washing, which were repeated up to 7 times, or according to the new "cast polyion films" procedure proposed here. The cast polyion film procedure consisted of only 1 deposition of PEI followed by 1 deposition of Mb, without intermediate washing. The electrodes obtained with the layer-by-layer procedure were noted $PG\{PEI/Mb\}_n$, where n is the number of successive PEI/Mb bilayers, while the electrodes resulting from the cast polyion film procedure were noted PG/PEI/Mb.

The modified electrodes were immersed in Tris/HCl buffer pH 7.5 free from Mb. After strictly deoxygenating the solution, cyclic voltammograms (CV) were recorded at 0.2 V s^{-1} from −0.75 V to 0.1 V/SCE (Figure 1). Generally, the first cycle exhibited a small cathodic current that revealed the presence of traces of oxygen, then all the cycles were perfectly reproducible. Reversible oxidation and reduction peaks of $MbFe^{III}$/$MbFe^{II}$ were observed at about −0.25 V and −0.4 V/SCE, respectively. A significant residual current was

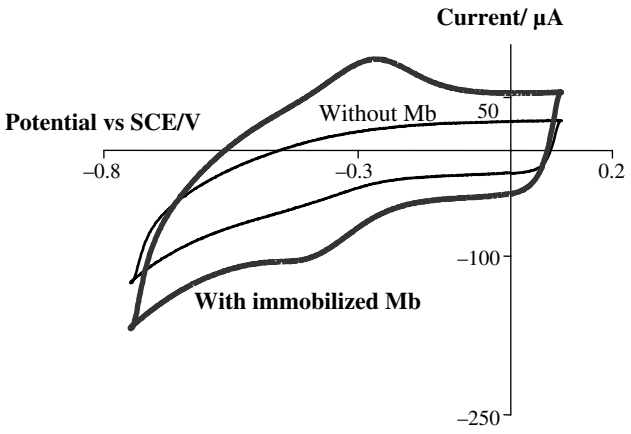

Fig. 1. Cyclic voltammograms on PG electrodes at 0.2 V s^{-1} in the absence of oxygen in Tris buffer pH 7.5 with a PG/PEI electrode (i.e. only with PEI, without immobilized Mb), and "cast polyion films" PG/PEI/Mb electrode.

observed on the voltammograms, which was removed through numerical treatment of the experimental curves (Figure 2). This residual current was made up of two contributions: a capacitive current I_{cap}, and a residual Faradaic current I_{Far}. The capacitive contribution I_{cap} was expressed as:

$$I_{cap} = C_{dl} \cdot v \tag{1}$$

where C_{dl} is the double layer capacity (Farad) and ν is the potential scan rate (V s^{-1}). This contribution alone should give a constant value of the current over the whole potential range.

The residual Faradaic current observed on all the CV might be due to the reduction of surface species on the PG electrode, such as quinonic compounds for instance. This residual Faradaic contribution I_{Far} was expressed by a Tafel's law:

$$I_{Far} = -Ai_0 \exp\left[\frac{-(1-\alpha)\cdot n \cdot F}{R \cdot T}(E - E'^{\circ}s)\right] \tag{2}$$

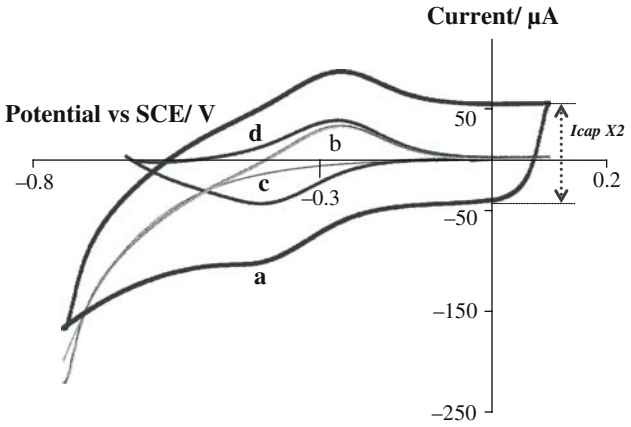

Fig. 2. Numerical fitting of cyclic voltammogram 0.2 V s^{-1} for PG/PEI/Mb: (a) raw experimental curve, (b) experimental oxidation current without capacitive residual, (c) theoretical Faradaic residual current, (d) current due to Mb electrochemistry only.

where A is the surface area (cm^2), i_0 is the exchange current density (A cm^{-2}), α and $E'^{\circ}s$ the electronic exchange coefficient and the apparent standard potential of the surface species respectively, R the gas constant (J mol^{-1} K^{-1}), T temperature (K) and E the applied potential (V). Recombining this equation gave:

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

The two constants:

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

$$C_2 = 1 - \alpha \tag{5}$$

were adjusted by fitting numerically the experimental curves. The oxidation scan (curve b Figure 2) 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. This order of magnitude confirmed that the Tafel approach used to model the residual Faradaic current physically makes sense (curve c). The total residual current $I_{Far} + I_{Cap}$ that resulted from this model was subtracted from the experimental CV to get the actual current corresponding to the Mb reduction/oxidation peak reported in Figure 2 (curve d).

A formal potential E'°_{Fe} of −0.33 V/SCE was derived from this new curve. This value matched the formal potential of −0.344 V/SCE that has been reported for the positively charged Mb-heme Fe^{III}/Fe^{II} redox couple in PSS/Mb assemblies [13]. In the same time, the experimental value of a formal potential E'°_{Fe} is measured at −0.375 V/SCE for $MbFe^{III}/Fe^{II}$ redox couple in layer-by-layer assemblies.

The oxidation peaks were integrated numerically to get the surface concentration of electroactive myoglobin Γ_{activ}. Integrating the CV oxidation peak gave the oxidation charge Q:

$$Q = \frac{1}{v}\int_1^2 I \cdot dE \tag{6}$$

where Q is the charge (C), ν is the scan rate (V s^{-1}), and $\int_1^2 I \cdot dE$ (A V) represents the area under the oxidation peak. Faraday's law gives the surface concentration of electroactive Mb, Γ_{activ} (mol cm^{-2}):

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

where $n = 1$ is the number of electrons exchanged by oxidation of Fe^{II} to Fe^{III}, F (=96,500 C mol^{-1}) Faraday's constant, and A the electrode surface area (cm^2). In Figure 3, the surface concentrations of electroactive Mb were calculated following the same numerical procedure. These values increased with the number of

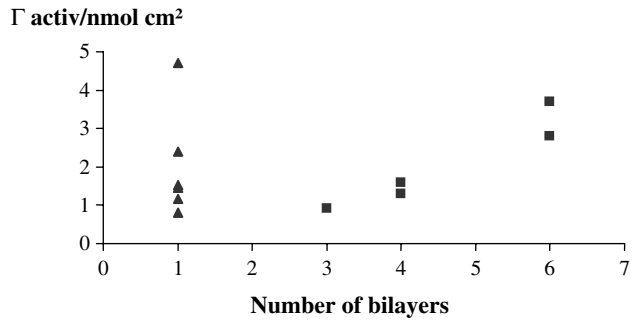

Fig. 3. Influence of the number of bilayers (n) for $\{PEI/Mb\}_n$ films on the surface concentration of electroactive Mb (nmol cm^{-2}) in Tris buffer pH 7.5: classical layer-by-layer (■) and cast polyion films procedure (▲).

bilayers when the layer-by-layer technique was applied, as already reported for poly(styrenesulfonate)/Mb films [14]. Values around 3.3 ± 0.4 nmol cm^{-2} were obtained with $PG\{PEI/Mb\}_6$ electrodes. The cast polyion film method gave Γ_{activ} values from 0.8 to 4.7 nmol cm^{-2}. In some cases Γ_{activ} obtained with the cast polyion technique was higher than the values obtained with 6 adsorbed bilayers following the classic layer-by-layer procedure. In spite of the rather poor reproducibility of the Γ_{activ} values obtained with the new polyion cast procedure, amounts of the immobilized active protein were of the same order of magnitude than with the layer-by-layer technique or in some cases even higher.

Table 1 reports the ratios of the immobilized electroactive Mb, which were calculated in Figure 3, with respect to the total quantity of Mb used during the immobilization; i.e. the Mb contained in the whole solution consumed during the successive deposition steps. The layer-by-layer technique led to immobilization of 3–6% of the total Mb required for electrode preparation. The cast polyion technique gave ratios from 7 to 57%, with an average value around 21%. The cast polyion technique avoided the significant loss of protein that occurred during the successive washing steps of the layer-by-layer procedure. The cast polyion technique should be effective when the high cost of the protein requires minimization of any possible loss during large scale immobilization procedure.

Table 1. Ratios of electroactive myoglobin (Γ_{activ}) to the total quantity of myoglobin used during the different steps of immobilization, according to the technique and the number of bilayers (Q is the total Mb quantity contained in the different solutions deposited on the electrode surface)

Technique	Number of bilayers/n	$(\gamma_{activ})/Q$/%
Layer-by-layer $PG\{PEI/Mb\}_n$	6	5.9
	6	4.5
	4	3.2
	4	3.8
	3	2.9
Cast polyion film PG/PEI/Mb	1	7.8
	1	11.4
	1	15
	1	57.3
	1	23.3
	1	14.4

3.2. *Catalytic reactions*

CVs were recorded at 0.01 V s^{-1} from −0.75 V to 0.1 V/SCE with PG/PEI modified electrodes with or without immobilized Mb, for both methods. Figures 4 and 5 were recorded with PG/PEI/Mb cast polyion films with two different Γ_{activ}, and Figure 6 shows CVs obtained with layer-by-layer $PG\{PEI/Mb\}_3$ electrode. For each figure, curves a and c were recorded with electrodes without immobilized Mb, and curves b and d corresponded to immobilized Mb. In each case, curves a and b were recorded in oxygen-free solutions, while curves c and d were recorded after oxygen was bubbled through the solution. Comparing curves a and b shows that the presence of Mb was not detectable at low scan rates in the absence of dissolved oxygen. Curves c indicate that oxygen was reduced on PG/PEI, mainly from −0.15 V/SCE as commonly observed on PG electrodes [18]. A

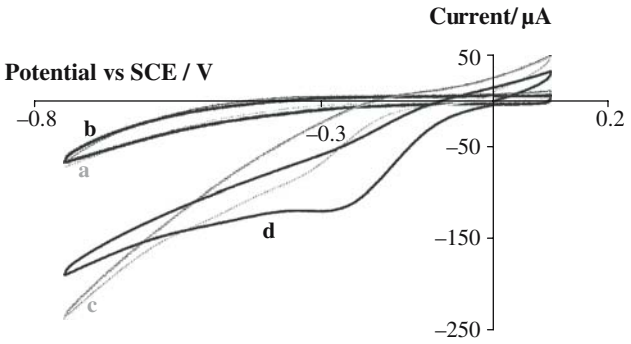

Fig. 4. Cyclic voltammograms for PG/PEI cast polyion films (a, c) and PG/PEI/Mb electrodes (b, d) with $\Gamma_{activ} = 1.52$ nmol cm^{-2} electroactive Mb, in the absence (a, b) and in the presence (c, d) of oxygen, pH 7.5, scan rate 0.01 V s^{-1}.

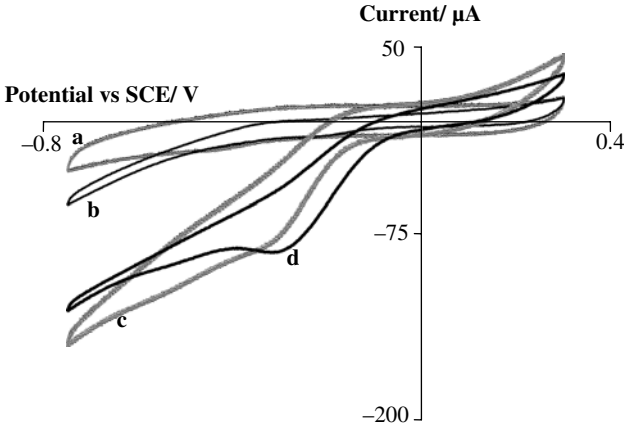

Fig. 5. Cyclic voltammograms for PG/PEI cast polyion electrodes (a, c) and PG/PEI/Mb electrodes (b, d) with $\Gamma_{activ} = 0.80$ nmol cm^{-2} electroactive Mb, in the absence (a, b) and in the presence (c, d) of oxygen, pH 7.5, scan rate 0.01 V s^{-1}.

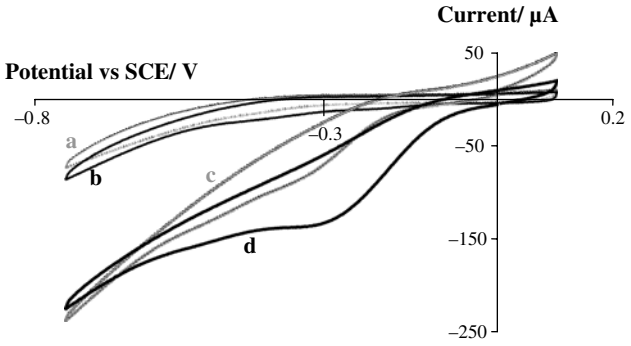

Fig. 6. Cyclic voltammograms for classical layer-by-layer PG{PEI} electrodes (a, c) and PG{PEI/Mb}$_3$ electrodes (b, d) with $\gamma_{activ} = 0.93$ nmol cm^{-2} electroactive Mb, in the absence (a, b) and in the presence (c, d) of oxygen, pH 7.5, scan rate 0.01 V s^{-1}.

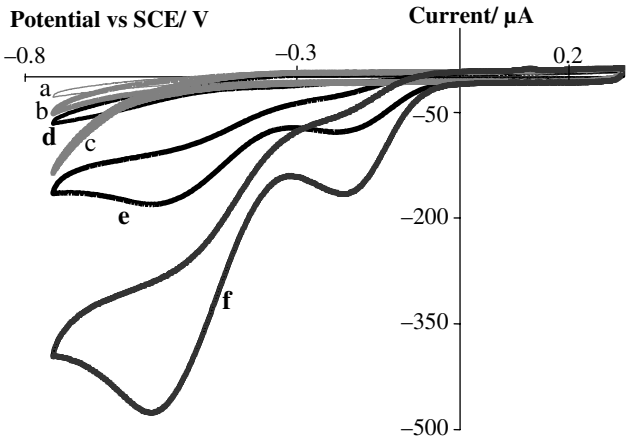

Fig. 7. Cyclic voltammograms on PG electrode, scan rate 0.01 V s^{-1}, in the absence of oxygen for (a) PG{PEI} in Tris buffer pH 7.5, (b) PG{PEI} in buffer containing 5 mM TCA, (c) PG{PEI} in Tris buffer containing 10 mM TCA, (d) PG{PEI/Mb}$_4$ films in Tris buffer, (e) {PEI/Mb}$_4$ films in Tris buffer containing 5 mM TCA, (f) PG{PEI/Mb}$_4$ films in Tris buffer containing 10 mM TCA.

significant increase in the oxygen reduction current was observed from 0.0 V/SCE when Mb was present in the film, for both PG/PEI/Mb and PG{PEI/Mb}$_3$ electrodes (curves d). These results clearly show the catalysis of oxygen reduction by immobilized Mb.

The experiments reported in Figures 4–6 used electrodes with Γ_{activ} values of 1.52, 0.80, and 0.93 nmol cm^{-2}, respectively. For the cast polyion technique (Figures 4 and 5) the catalytic effect was almost proportional to the concentration of active Mb, Γ_{activ}. The layer-by-layer procedure (Figure 6) gave higher oxygen reduction current for a similar Γ_{activ} value. As recalled above, the cast surfactant films are claimed to be thicker (micrometer scale) [5, 10] than the layer-by-layer films (nanometer scale) [11]. It was expected that the cast polyion films obtained here had similar thickness as the common cast surfactant film. Consequently, oxygen diffusion limitation may occur inside the cast polyion films, resulting in smaller values of the reduction current compared with layer-by-layer technique. For industrial application an optimum should be found with high Γ_{activ} values but with film thickness as small as possible.

The electrochemical catalytic reduction of trichloroacetic acid (TCA) was investigated under the same operating conditions with PG{PEI/Mb}$_4$ modified electrodes. When 5 mM TCA was added to an oxygen-free pH 7.5 buffer, an increase of the $MbFe^{III}$ reduction peak was observed at about −0.20 V/SCE (Figure 7, curve e), compared to the reduction peak in the absence of TCA (curve d), and a second peak appeared around −0.55 V/SCE. No peak was observed with PG/PEI modified electrodes, i.e. without immobilized Mb inside the film (curve b and c, respectively, 5 and 10 mM of TCA). The reduction peak current obtained with PG{PEI/Mb}$_4$ electrodes had a proportional relationship with the TCA concentration up to 10 mM TCA (curve f). The same proportionality has been observed elsewhere with the common positively charged Mb [17].

The electrochemical catalytic dechlorination of TCA was also carried out with PG{PEI/Mb}$_4$ electrodes in oxygen-saturated solution (curve b Figure 8). Two peaks were observed at around −0.20 and −0.55 V/SCE, which were correlated to the presence of TCA in solution. The third peak at around −0.025 V/SCE was due to oxygen reduction while this peak was not observed in the absence of oxygen. When the experiment was performed under the same conditions (oxygen saturated, 10 mM TCA) but with PG/PEI electrodes, i.e. that did not contain Mb (curve a), the oxygen reduction wave appeared at potential values more negative than −0.2 V/SCE. The peak around −0.025 V/SCE that only appeared in the presence of the three compounds oxygen, Mb, and TCA demonstrated that Mb was involved in the catalysis of oxygen reduction.

In all cases the peaks progressively disappeared when several CV were recorded successively. When voltammograms were recorded a few minutes after the addition of TCA in solution the peaks no longer appeared, even during the first recording.

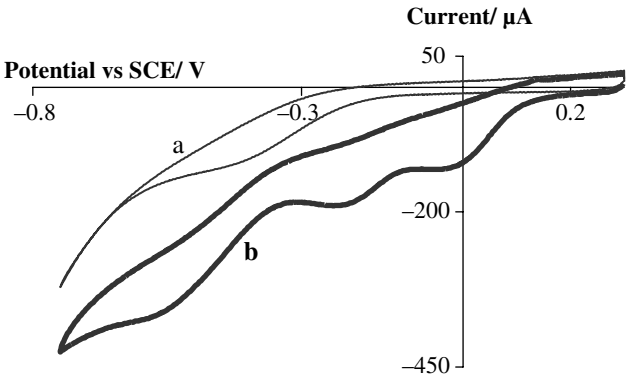

Fig. 8. Cyclic voltammograms on PG electrode, scan rate 0.01 V s^{-1}, in the presence of oxygen for (a) PG/PEI in Tris buffer containing 10 mM TCA, (b) PG{PEI/Mb}$_4$ electrodes in Tris buffer containing 10 mM TCA.

4. Discussion

4.1. *Cast polyion film procedure and film structure*

Figure 3 shows that the quantity of electroactive Mb increased with the number of deposited layers, but the 6-layer films revealed higher values of Γ_{activ} than expected by linear extrapolation of the values obtained with 3 and 4 layers. Actually, very different results have been reported in the literature:

- on smooth gold electrodes, polyion films typically featured a fully electroactive layer closest to the electrode surface, 20–40% electroactive layer for the second layer, and no electroactivity for additional protein layers [15];
- on rough pyrolytic graphite, the sixth and seventh electroactive PSS/Mb layers of films made of up to seven layers contained only 31.9 and 9% electroactive Mb with respect to the first layer closest to the electrode surface [10];
- films of Mb/DNA and P450/PEI made on rough pyrolytic graphite revealed an almost linear increase in electroactivity with the number of protein layers up to six layers [12].

The results are different, depending on the nature of the electrode and the protein/polyion system and probably with a major influence of the operating conditions. Nevertheless, comparing the results obtained on gold and PG electrodes has led to the conclusion that the disorder induced by the roughness of the PG surface slightly disturbs the multi-layered architecture of the film and favors electron transfer with the upper protein layers. Here, the higher electroactivity obtained with the 6-layer films may be due to the negative-Mb/PEI system which was used for the first time. It may also be assumed that this system led to rather weak electroactive interactions (pH was only 0.7 unit greater than pI) and the successive deposition/washing steps may increase the disorder of the whole architecture when 6 layers were reached.

With the cast polyion film technique the concentration of immobilized electroactive myoglobin fluctuated. PEI and Mb were successively deposited in great excess with respect to mono-layer coverage: the film obtained consequently did not have a well-ordered structure, but probably had almost random internal distribution of PEI and Mb. In the layer-by-layer procedure, intermediate washing steps are aimed at removing the protein and PEI in excess with respect to a mono-layer. It is thus hoped to build successive well-ordered monolayers of PEI and protein. This well-ordered architecture was helpful in fundamental studies, but is not really useful with the sole aim of efficiency in large scale synthesis. The random packing of PEI and Mb was difficult to reproduce, even when the same procedure was strictly followed for each experiment. The significant variation in Γ_{activ} values may result from the poor control of the order/disorder of the assembly.

The peaks obtained after removing the residual current (Figure 2) were broader than peaks commonly reported in the literature for the layer-by-layer technique [15]. These broad peaks may be interpreted as the sum of several elementary 1-electron redox reactions that correspond to different $E'°$. The random immobilization of Mb in the cast polyion films should result in different distances and different orientations of the protein molecules with respect to the electrode surface and, consequently, in different electron hopping pathways. The broad peaks observed here may confirm the disordered structure of the cast polyion films which induced different local environments of the electroactive protein [19].

From the point of view of synthesis applications it can be concluded that the negatively charged Mb can be immobilized with PEI. These modified electrodes can now be used as an efficient model of the PEI/CytP450 system to design and scale up modified electrodes for synthesis. On the other hand, instead of the numerous successive steps required by the classic procedure, the new technique involved only two adsorption steps, without intermediate washing. This technique is consequently less suitable for analytical studies, where well-ordered layers are required, but enabled the immobilization of significant amounts of electroactive Mb in a simple way, which should be easily scaled up to large surface areas. Moreover, the ratio of the immobilized protein with respect to the total quantity of protein used was significantly better than for the layer-by-layer procedure. This drastic reduction of wasted protein is essential for the design of economically efficient synthesis procedures with costly proteins, such as CytP450.

4.2. *Catalytic reactions*

Cyclic voltammograms recorded in oxygen-saturated solutions showed that the catalysis of the electrochemical reduction of oxygen to hydrogen peroxide occurred with the negatively charged Mb used here (Figures 4–6). The commonly agreed pathway for the catalysis of the electrochemical reduction of oxygen by positively charged Mb involves the initial reduction of $MbFe^{III}$ to $MbFe^{II}$ at the electrode:

$$MbFe^{III} + e^- \longrightarrow MbFe^{II} \quad (8)$$

then the reaction of $MbFe^{II}$ with oxygen to form the complex $MbFe^{II}—O_2$:

$$MbFe^{II} + O_2 \longrightarrow MbFe^{II}—O_2 \quad (9)$$

which is reduced at the electrode to give hydrogen peroxide [9, 11]:

$$MbFe^{II}—O_2 + e^- + 2H^+ \longrightarrow MbFe^{III} + H_2O_2 \quad (10)$$

The currents obtained at the most negative potentials were slightly lower when catalysis occurred (curves d) than in the absence of Mb (curves c). The same behavior has been observed and modeled numerically elsewhere

with the catalysis of the electrochemical reduction of oxygen by catalase [18]. Actually, the rapid consumption of oxygen due to the catalytic effect in the first phase of the potential scan induced depletion of oxygen in the diffusion layer which reduced the current at the most negative potentials.

Immobilized Mb also proved able to catalyze the reduction of TCA with PG{PEI/Mb}$_4$ electrodes. The catalytic reduction of TCA with Mb has been schematized by Ma et al. [14]:

$$MbFe^{III} + e^- \rightleftarrows MbFe^{II} \quad (11)$$

$$MbFe^{II} + Cl_3CCOOH \longrightarrow Cl_2^{\cdot}CCOOH + MbFe^{III} + Cl^- \quad (12)$$

$$MbFe^{II} + Cl_2^{\cdot}CCOOH \longrightarrow MbFe^{III} + Cl_2^{-}CCOOH \quad (13)$$

$$Cl_2^{-}CCOOH + H^+ \longrightarrow Cl_2CHCOOH \quad (14)$$

The peak at −0.20 V/SCE obtained under our conditions (Figure 7) showed this catalytic phenomenon, as observed elsewhere [17, 20]. Panchagnula et al. [20] showed that TCA catalytic reduction is pH dependent and is more significantly observed at acidic pH, as is the case here, since the pH of the solution was 2.3 after addition of TCA. The mechanism proposed does not explain the occurrence of the second peak observed at around −0.55 V/SCE (Figure 7). To our knowledge no paper has presented CVs with cathodic limits sufficiently negative to observe this second peak, except in the supporting information available in [20] (but no comment was given). Experiments carried out with the product of dechlorination ($Cl_2HCCOOH$) added in solution demonstrated that this second peak was not induced by this species, as might be supposed. The reaction pathway (reactions 11–13) generally agreed is quite complex. It involves two successive reactions of TCA species with one or two Mb catalytic site(s), and involves an intermediate TCA radical species. It may be assumed that this radical species is the source of uncontrolled side-reactions, such as dimerization. In this work TCA reduction was only a model reaction implemented to check the reactivity of the modified electrodes; it would require specific mechanistic studies to clearly decipher its behavior at negative potential values.

The CVs recorded for PG{PEI/Mb}$_4$ electrodes in oxygen-saturated solution and in the presence of TCA showed a shift of around 0.275 V for oxygen reduction (peak at −0.025 V/SCE on curve b Figure 8) compared to catalysis without TCA (Figures 4–6). The pH of the solution after addition of TCA matched the calculation done with a pK_a of 0.89 [13]. The potential shift of around 0.275 V from pH 7.5 to pH 2.3 was close to the theoretical value of 59 mV pH^{-1} which is expected for a one-electron/one-proton electrochemical reaction. Nevertheless, no significant pH-induced potential shift was observed with a PG/PEI electrode in the absence of immobilized Mb (curve a Figure 8). In this case the presence of the PEI layer on the electrode surface certainly slowed down oxygen reduction. The pH-induced potential shift, which only occurred in the presence of immobilized Mb, was not due to the direct reduction of oxygen but to the mediation of this reduction by Mb. This potential shift suggests that the reduction of the myoglobin heme is not a single one-electron exchange, as schematized by reaction (8), but that a simultaneous proton exchange is likely involved. A shift in formal potential has already been observed as a function of pH, with values less than 59 mV pH^{-1} by different authors. It has been proposed either to include a term 'mH^+' in reaction (8) [21], or to accept an approximate overall one-electron/one-proton exchange [14]:

$$MbFe^{III} + e^- + H^+ \longrightarrow MbFe^{II} \quad (15)$$

instead of reaction (8), and to correct the Mb-catalyzed reduction of oxygen (reaction 10) into:

$$MbFe^{II}{-}O_2 + e^- + H^+ \longrightarrow MbFe^{III} + H_2O_2 \quad (16)$$

This scheme appears more appropriate in explaining the pH-induced potential shift of oxygen reduction observed here, than a first pH independent $MbFe^{III}$ reduction (reaction 8) followed by a 1-electron/2-protons $MbFe^{II}{-}O_2$ reduction (reaction 10).

The disappearance of the current peaks as a function of time, or their absence when the CV was recorded several minutes after the addition of TCA, can be explained in two ways. Because of the pH decrease due to TCA the surface charges of Mb in the films may become positive. The electrostatic interactions with the polycation PEI should consequently become repulsive and the electrostatic assembly may be destroyed. On the other hand, it has been shown that at acid pH values Mb may loose the heme group and, consequently, loose its electrochemical properties [14].

5. Conclusion

It was demonstrated that the negatively charged Mb can be efficiently immobilized on PG electrodes with the polycation PEI using the cast polyion procedure proposed. Negatively charged Mb has electrochemical characteristics similar to the positively charged Mb commonly studied, and it remains an effective catalyst of oxygen reduction and TCA dechlorination. The negative PG/PEI/Mb electrode seems perfectly suited to carry out the further experiments necessary to design and scale up modified electrodes with CytP450.

The simplified cast polyion technique proposed here made it possible to immobilize large quantities of proteins in a simple way. It did not ensure a well-ordered structure of the assembly, but a high proportion

of protein was immobilized with respect to the total quantity required for electrode preparation. This parameter will be essential for the design of economically efficient protein-catalyzed electrochemical synthesis systems.

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