Available online at www.sciencedirect.com

SCIENCE DIRECT®

Bioelectrochemistry 66 (2005) 29–34

Bioelectrochemistry

www.elsevier.com/locate/bioelechem

Electrochemistry of cytochrome *c* incorporated in Langmuir–Blodgett films of Nafion® and Eastman AQ 55®

Ligia M. Moretto, Paolo Bertoncello[1], Francesca Vezzà, Paolo Ugo*

Department of Physical Chemistry, University of Venice, Calle Larga S. Marta 2137, 30123 Venice, Italy

Received 16 February 2004; received in revised form 29 March 2004; accepted 30 March 2004
Available online 25 August 2004

Abstract

Ultrathin films of Nafion® and Eastman-AQ 55® loaded with cytochrome *c* (cyt *c*) were obtained and transferred on indium tin oxide (ITO) electrodes via the Langmuir–Blodgett (LB) technique. The pressure-area isotherms for mixed ionomer-protein films indicate that the miscibility of cyt *c* in the interfacial layer is better for Nafion® than for AQ 55. Interestingly, these composite films maintain the electroactivity of cyt *c* without requiring the addition of promoters or mediators. Both for AQ 55-cyt *c* and Nafion-cyt *c* films, the half-wave potential for the reversible reduction of ferricytochrome *c* corresponds to the value expected for the weakly adsorbed protein. The modified electrodes show electrocatalytic reaction with ascorbate anion. Comparison with previous literature reports indicate that for Nafion the LB coating procedure is unique in keeping the electroactivity of cyt *c*.

Keywords: Langmuir–Blodgett film; Nafion; Polyestersulfonated; Cytochrome *c*; Ion exchange; Voltammetry

1. Introduction

Langmuir–Blodgett (LB) techniques have attracted considerable attention, thanks to their capability to build up monolayers or multilayers ordered at a molecular level [1,2]. This is particularly interesting for tailoring surface properties and controlling electron transfer processes at the electrode/solution interface [3].

Since 1970s, the use of ionomers such as Nafion®, has received great attention because of their peculiar properties such as ion-exchange selectivity, good wetting properties, self-organization in hydrophobic/hydrophilic domains, chemical and biological inertness [4].

The capability of preconcentrating and determining trace concentrations of electroactive species even at nanomolar level has given rise to a new technique called ion-exchange voltammetry [3,5,6]. Traditionally, the ion-exchange preconcentration capabilities of modified electrodes have been exploited for the voltammetric determination of small (inorganic and organic) ions. However, this approach can be likewise employed for the immobilization into a polymeric matrix of electroactive species which display electrocatalytic properties [7] and ion-exchange interactions can be applied for the immobilization of redox proteins [8]. In this respect, some papers were devoted to the use of ionomers for ion-exchange immobilization of cytochrome *c* (cyt *c*) [9,10]. Cyt *c*, in fact, plays a central role both for its electrocatalytic capabilities and as a biological mediator able to shuttle electrons from electrodes to a variety of proteins such as cyt *c* oxidase [11], sulphite oxidase [12], some hydrogenases [13] and others [14,15]. Fundamentals on the electrochemistry and redox behavior of cyt *c* has been recently reviewed by Feducro [16].

At physiological pH values, cyt *c* is positively charged (p*I*=9.2) and tends to interact electrostatically with negatively charged species, like the redox proteins cited above. Positive ionic charges of cyt *c* are also the basis for electrostatic immobilization procedure in polyanions films. It was shown that cyt *c* is incorporated by ion-exchange in

* Corresponding author. Tel.: +39 041 2348503; fax: +39 041 2578 594.
E-mail address: ugo@unive.it (P. Ugo).
[1] Present address: Department of Chemistry and Biochemistry, The University of Texas at Austin, 1 University Station, Austin, TX 78712.

1567-5394/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioelechem.2004.03.007

films of polyestersulfonated ionomers such as the Eastman AQ 29 [10] and AQ 55 [9]. In a surprising contrast, attempts to observe typical cyt *c* electroactivity signals in Nafion® films loaded with cyt *c* have yielded unsuccessful results [17]. This was attributed to the heterogeneous nature of the Nafion® matrix and to the fact that the interactions between recasted Nafion® and cyt *c* develop in a unfavorable way for electron transfer with the electrode surface [17]. It was proposed that this might also be related to the strong hydrophobic interactions between Nafion® and the protein [18]. Consequently, it would be interesting to find a way to improve the control on such interactions.

Recently, we have shown that it was possible to engineer at a molecular level ionomer films by resorting to Langmuir monolayer formation techniques. Langmuir monolayer of Nafion® at the air–water interface were obtained using electrolyte loaded subphases [19]. It was shown that Langmuir ultrathin films of ionomers can be transferred on electrodes surfaces by using Langmuir–Schaefer (LS) [19,20] and Langmuir–Blodgett (LB) deposition techniques [21]. These techniques can also be extended to other ionomer polymers such as Eastman AQ 55 [21] and polycationic Tosflex IESA 48 [3,21].

Although keeping the ion-exchange preconcentration capability and permselectivity typical of ionomer films recasted on electrode surfaces, mass and change transfer processes relevant to electroactive species incorporated in LS and LB films of ionomers are characterized by apparent diffusion coefficient smaller than in "conventional" films [20]. This is attributed to a denser film structure, with multilayering of hydrophobic and hydrophilic domains as a consequence of the 2D ordering of the interfacial film during Langmuir compression in the trough [21].

The present work studies the possibility of obtaining Langmuir films of polyanionic ionomers (namely AQ 55 and Nafion®) premixed with cytochrome *c*, and the role of the Langmuir–Blodgett deposition technique on the incorporation of cyt *c* and its voltammetric behavior.

2. Experimental

2.1. Materials

Horse heart cytochrome *c* (type VI, molecular weight 12,384) was purchased from Sigma and used without further purification. Note that both on the basis of our experience [9,22] and on previous reports [23,24], voltammetric responses obtained on this kind of commercial preparation do not differ significantly from those of purified samples.

All the chemicals were of reagent grade quality. Milli-Q water was used throughout to prepare all the solutions. Phosphate buffer solutions were prepared using suitable amounts of NaH_2PO_4 and Na_2HPO_4.

Nafion® 117 solution (5% w/v in a mixture of low molecular weight alcohol) was purchased from Sigma-Aldrich. AQ 55 pellets were kindly provided by Eastman-Italia. A dispersed polymeric solution of AQ 55 was prepared following the procedure of Brunetti and Ugo [25]; briefly, 15 g of AQ 55 were mixed in 100 ml of water; after vigorous and extended stirring at 45 °C a transparent and colorless solution was obtained. The exact concentration of the polymer in the dispersion was determined by drying and weighing a known volume of this dispersion.

2.2. LB films

The ionomers polymeric Langmuir monolayers were formed by using a Langmuir trough (Langmuir KSV 2000 trough, capacity 1.25 l; KSV Instruments, Finland) interfaced with a PC and controlled by the software KSV LB WIN 1.22 version. The surface pressure was measured by the Wilhelmy plate method, with an accuracy of 0.2 mN m^{-1}.

A mixture of cyt *c* and AQ 55 was prepared by dissolving 2 mg of cyt *c* in 0.03 M phosphate buffer (pH 7), 3 ml methanol and 30 μl AQ 55 15% (w/v) aqueous dispersion. A mixture of Nafion® and cyt *c* was prepared by dissolving 1 mg of cyt *c* in 3 ml 0.03 M phosphate buffer (pH 7.0), 3 ml methanol and 60 μl Nafion® 5% w/v solution. It was observed that the use of higher phosphate concentration or changing the water/methanol percentage from the above values caused the formation of a precipitate.

A total of 400 μl of Nafion-cyt *c* or AQ 55-cyt *c* solutions was added to the subphase (0.1 M phosphate buffer, 1.25 l) in the trough. An elapsed time of 15 min was allowed before the compression of the floating film, which was performed using 30 mm min^{-1} as barrier speed. The films were transferred onto solid substrate, namely indium tin oxide (ITO)-coated glass slides, at a deposition speed of 33 μm/s for the first layer (emersion) and at increased speed (830 μm/s) for the following emersion steps (odd numbered layers). A much higher speed (1670 mm/s) was used for immersion steps (even numbered layers), since it was observed that negative transfer ratios characterized these steps (see Section 3.1). A delay of time of 15 min was interposed between deposition of successive layers, during which the film was gently dried with a soft flux of nitrogen gas.

2.3. Electrochemical measurements

Electrochemical measurements were performed at room temperature under nitrogen atmosphere, using a Potentiostat CHI660B controlled via PC by its own software. In all cases, a standard three-electrode cell configuration was used. The working electrode was an ITO-coated glass plate (Delta Technologies, USA) with R_s=8–12 Ω, on which LB films were deposited. A platinum coil was used as the counter-electrode and a Ag/AgCl (KCl saturated) as the reference electrode. The working electrode area was kept equal to 0.07 or 0.126 cm^2 by using an insulating tape mask.

3. Results and discussion

3.1. Deposition of Langmuir–Blodgett films of Nafion®-cyt c and AQ 55-cyt c

Fig. 1 shows the surface pressure (Π) vs. trough area (*A*) isotherms of cyt *c*, AQ 55 and AQ 55-cyt *c* obtained in subphases containing phosphate buffer 0.1 M, pH 7.0. The *X*-axis plots the trough area instead of the area per molecule due to the fact that the exact solubility of the ionomers used here in phosphate buffer are unknown. Moreover, since we are dealing with a polymeric material, the area per molecule parameter should be correctly substituted by an area per monomeric unit value, which is a parameter of doubtful usefulness and definition, particularly for polymers which aggregate in domains, such as the studied ionomers [3]. The isotherm of pure cyt *c* (curve a) is characterized by a rather broad trend showing an increase in the Π/*A* plot with a collapse pressure of about 16 mN m^{-1}. This trend and the relatively low collapse pressure are typical of Langmuir isotherms of proteins and appear similar, for instance, to the isotherm recently reported in literature for cytochrome p450 [26].

The Π/*A* isotherms of AQ 55 (Fig. 1, curve b) shows an increase in the surface pressure when the area limited by the barriers is decreased. The trend of the isotherm is rather flat, typical for the compression of interfacial films of this ionomer in subphases containing added electrolytes [21], but steeper than the cyt *c* isotherm. The maximum surface pressure is about 23 mN m^{-1}, which is higher than the maximum Π value for cyt *c*, but lower than values obtained for Nafion® LB film [19,21]. The plot in Fig. 1 (curve b) confirms that the addition of strong electrolytes (in this case, phosphate buffer) to the subphase is a compulsory requirement for obtaining the formation of a stable ionomer layer at the air–water interface [3,19]. In the absence of dissolved salts the sulfonic groups of AQ 55 (and Nafion® as well, see below) repel each other hindering the formation of a stable interfacial film. On the other hand, the presence of dissolved cations minimizes these repulsive interactions, thanks to the neutralization of the negative charges of the sulfonic groups.

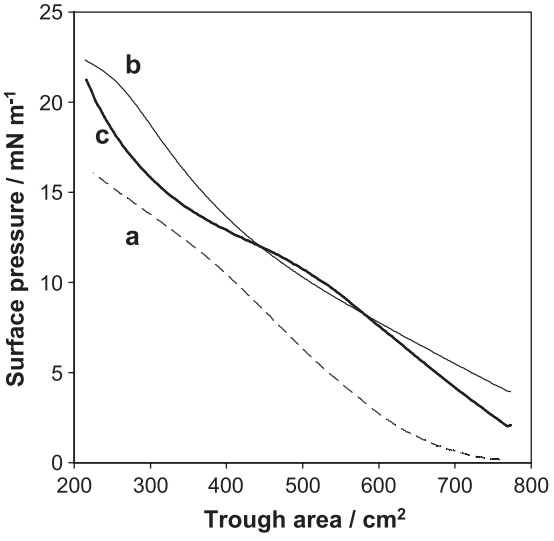

Fig. 1. Surface pressure–trough area isotherm curves recorded using a phosphate buffer 0.1 M (pH 7.0) subphase, at 22 °C, of: (a) cyt *c*, (b) AQ 55 and (c) AQ 55-cyt *c*; barrier speed 30 mm min^{-1}.

The isotherm relevant to the AQ 55-cyt *c* mixture is shown in Fig. 1 (curve c). It is characterized by a maximum surface pressure of 22 mN m^{-1}, with a change of slope in the 13–14 mN m^{-1} zone. Such a behaviour is typical of the isotherms relevant to mixtures of different compounds whose Langmuir films are characterized by different collapse pressure and limited miscibility [27]. In any case, a mixed interfacial film is formed in which positively charged cyt *c* interacts electrostatically with the negatively charged sulphonate groups of AQ 55.

Fig. 2 shows the Π/*A* isotherms of Nafion® (curve a) and Nafion-cyt c (curve b). The isotherm of Nafion® in phosphate buffer subphase (curve a) is similar to the isotherms obtained for this ionomer in different sodium containing subphases, such as, e.g., NaCl [19]. The shape of the isotherm relevant to the Nafion-cyt *c* mixture (curve b) is very different from the cyt *c* isotherm (see Fig. 1, curve b). It is characterized by an increase in surface pressure steeper than the one observed for the AQ 55-cyt *c* case. Moreover, the curve presents a less evident change in slope, now at about 18 mN m^{-1}, which suggests a better miscibility of the redox protein in the Nafion® interfacial layer. The higher collapse pressure (almost 28 mN m^{-1}) of Nafion-cyt *c* with respect to AQ 55-cyt *c* indicates that LB transfer of the mixed interfacial film, in this case, can be performed at surface pressures higher, i.e., compression level which corresponds to more condensed films. Interactions between Nafion® and cyt *c* in the interfacial film appear stronger than in the AQ 55-cyt *c* case. This agrees with the results reported in Ref. [18] for the case of solution mixture of Nafion® and the protein.

The transfer on solid substrates both of AQ 55-cyt *c* and Nafion-cyt *c* interfacial films were performed by using the LB deposition technique (vertical dipping) [2]. On the basis of the relevant isotherms discussed above, the deposition was performed using a constant surface pressure of 15 mN m^{-1} for AQ 55-cyt *c* and 22 mN m^{-1} for Nafion-cyt *c* layers.

An important parameter which allows one to evaluate the transfer of the interfacial film to the substrate is the *transfer ratio* (TR). It is defined as the decrease in the area occupied by the monolayer on the trough surface divided by the coated area of the substrate [27]. We examined this parameter in detail for the transfer of Nafion-cyt *c* films. Emersion steps were characterized by positive TR values, while for immersion steps TR values were close to zero or slightly negatives, which agrees with the hydrophilic nature of the ITO substrate. In order to favour the transfer of the

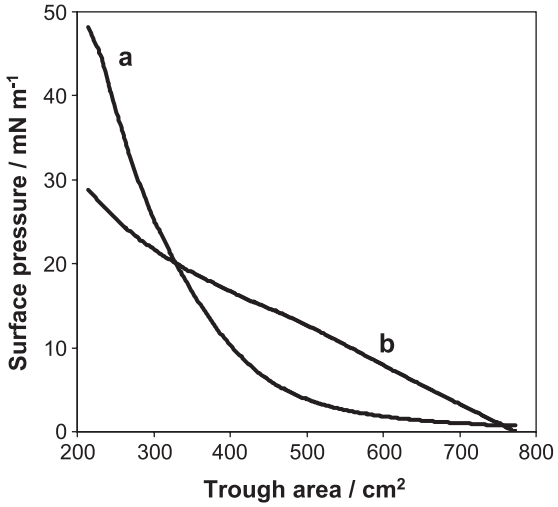

Fig. 2. Surface pressure–trough area isotherm curves recorded using a phosphate buffer 0.1 M (pH 7.0) subphase, at 22 °C, of: (a) Nafion and (b) Nafion/cyt *c*; barrier speed 30 mm min^{-1}.

film, the deposition speed during the immersion steps was kept high during emersion steps and slow for immersions (see Experimental). The TR for the first immersion layer was always the highest (up to 5), with other odd layers giving TR values in the range (2.0–1.2). Note that, since we were using asymmetric substrates (glass slides coated with ITO on one side), the ideal value of the TR equal to unity is improbable to obtain. In any case, the overall transfer of the film was characterized by positive values, indicating the successful transfer of the interfacial film on the substrate. The observation of a slightly reddish thin film on the ITO surface at the end of the process confirmed "visually" the successful transfer of the mixed polymer layer.

3.2. Electrochemical characterization

Fig. 3 shows the cyclic voltammograms recorded at different scan rates at an LB Nafion-cyt *c*-coated ITO dipped in 10^{-2} M phosphate buffer pH 7.0. They are characterized by cathodic peak with a peak potential of −0.14 V at 5 mV s^{-1} and −0.18 V at 100 mV s^{-1}. The associated anodic process is characterized by peak potentials of −0.09 V at 5 mV s^{-1} and −0.08 V at 100 mV s^{-1}. The process involved is expected to be the quasi-reversible reduction of ferricytochrome *c* according to reaction (1).

$$[\text{cyt } c\text{-Fe(III)}]+e^- \leftrightarrows [\text{cyt } c\text{-Fe(II)}] \quad (1)$$

The $E_{1/2}$ value, calculated as $E_{1/2}=(E_{pa}+E_{pc})/2$ [28], is indeed constant with the scan rate and equal to −120 mV. This value is more negative than $E_{1/2}$ of −45 and 80 mV measured for cyt *c* at bare electrode in the presence [29] and in the absence [30] of promoters, respectively, and of 50–80 mV obtained at electrodes modified with recasted film of AQ 29 [10] and AQ 55 [9] loaded with cyt *c*. No signal was observed for ITO dipped in LB films of cyt *c* alone. The $E_{1/2}$ value obtained for LB–Nafion-cyt *c* films is close to the value of −165 mV reported in literature by Sagara et al. [31] for the case of electrochemical reduction of cyt *c* slightly adsorbed on gold surfaces. Sagara et al.'s data were obtained by controlling cyt *c* adsorption via a competition with small amounts of added 4–4′bipyridyl, where the electrode surface was covered by both 4–4′bipyridyl and few cyt *c* molecules. This situation appears quite similar to the one expected in our case, where the electrode surface interacts with Nafion® molecules with interposed some cyt *c* molecules. As shown in Fig. 3b, the cathodic peak currents increase linearly with the scan rate indicating a thin-layer-like behavior, in the range of scan rates explored here; halving the number of layers from 10 to 5 reduces roughly to a half the peak current recorded at a certain scan rate (not shown).

Very similar voltammetric patterns with comparable $E_{1/2}$ values and peak current behavior were observed in the same experimental conditions also using LB–AQ 55-cyt *c*-coated ITO (not shown).

In the case of Nafion®, it is worth noting that there had been a previous attempt to incorporate cyt *c* in Nafion® coatings by premixing the protein with the ionomer and then spreading the mixture on the electrode surface [17]. However, the coated electrode did not show any voltam-

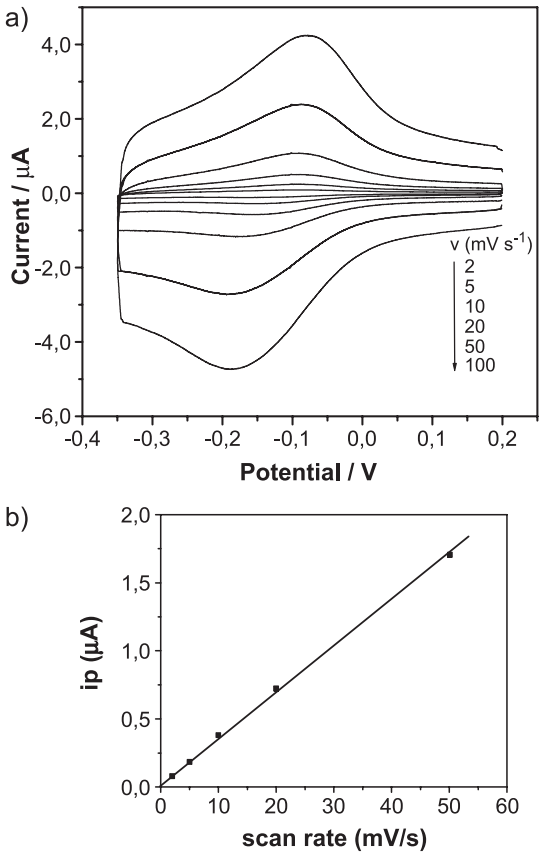

Fig. 3. (a) Cyclic voltammograms recorded at 10 layers LB Nafion-cyt *c* coated ITO in 10 mM phosphate buffer (pH 7.0), at different scan rates: 2, 5, 10, 20, 50, 100 mV s^{-1}. (b) Dependence of the anodic peak current on the scan rate.

metric signal. To explain such a behavior, it was proposed that highly positively charged cyt *c* bind too tightly to Nafion® and hence cannot exchange electrons with the electrode surface. We used the LB approach to overcome this limitation. On one hand, the fact that the thickness of the film is very low, in the range of 10 nm for 5 LB layers of Nafion® [19], can facilitate the electron transfer between the heme group and the electrode surface. On the other hand, it may be taken into account that in the Langmuir film the hydrophilic regions both Nafion® and cyt *c* are directed towards the aqueous subphase. After transfer on the ITO surface (hydrophilic), this ionic–hydrophilic regions should be in contact with the electrode surface, thus allowing the electron transfer. To the best of our knowledge, this is the first example of electroactivity of Nafion-cyt *c* films, which appears rather relevant in the application of the modified electrodes for biosensing purposes.

Cyt *c*-mediated transformations can be exploited, in fact, for biocatalytic processes which are of interest in the biosensors field [11–15,32,33]. The possible application of the LB technique to immobilize cyt *c* on the electrode surface, thus keeping its typical electrocatalytic activity, is preliminarily tested here, using differential pulse voltammetry (DPV) as the detection technique and ascorbate as the substrate/analyte.

The electrocatalytic process examined is the known oxidation of ascorbate catalyzed by ferrocytochrome *c*, according to the reaction scheme [9]:

$$[\text{CytC-Fe(II)}] \leftrightarrows [\text{CytC-Fe(III)}] + e^- \quad (2)$$

$$[\text{CytC-Fe(III)}] + \text{Asc}^- \leftrightarrows [\text{CytC-Fe(II)}] + P \quad (3)$$

where Asc^- is ascorbate and *P* represents the relevant oxidation products. Reaction (3) between ascorbate and cyt *c* is used for performing the chemical reduction of cyt c-Fe(III) in homogeneous solutions (see, e.g., Prutz et al. [34], for recent applications).

Fig. 4 (curve a) shows the DPV pattern recorded at an ITO-coated with 5 LB layers of Nafion-cyt *c* in 10 mM phosphate buffer pH 7.0, after poising the electrode potential at −0.3 V for 60 s. This was done in order to perform the pre-reduction of ferricytochrome *c* to ferrocytochrome *c*. In the anodic scan, an oxidation peak is observed at about −0.14 V (reaction (2)). This peak is more negative than the DPV peak observed for cyt *c* dissolved in homogeneous solution and recorded at gold nanoelectrodes ensemble [30]. This shift confirms the weakly adsorbed state for cyt *c* at the interface between LB–Nafion-cyt *c* film in the ITO surface. Curves b–d in Fig. 4 show that the addition of ascorbate in the mM concentration range causes an increase in peak current, which is directly proportional to the ascorbate concentration (see insert). Note that the direct oxidation of ascorbate at a bare ITO in the same experimental conditions gave an anodic peak at much more positive potential values, namely at 0.420 V. These data confirm the occurrence of the electrocatalytic processes (2)–(3) at the LB–Nafion-cyt *c*-coated electrode.

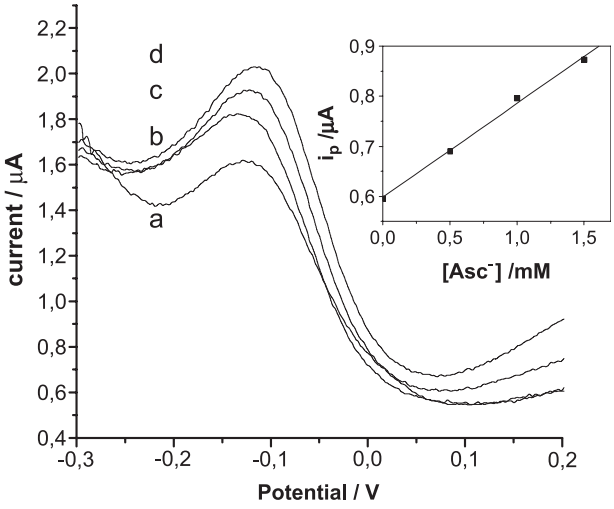

Fig. 4. Differential pulse voltammetries recorded at 5 layers LB Nafion-cyt *c* coated ITO in phosphate buffer 10 mM (pH 7.0) before (curve (a)) and after the addition of ascorbate (Asc) 0.5 mM (b), 1.0 mM (c), 1.5 mM (c). Experimental conditions: pulse height 50 mV, scan rate 5 mV s^{-1}. Inset: relevant calibration plot.

4. Conclusions

The formation of LB films of ionomers recently introduced to deposit ultrathin films on electrode surfaces [3,19–22] proved to be suitable also for the preparation of mixed coatings incorporating biocatalytic species such as cyt *c*.

The small thickness of these films, together with the capability of LB techniques to control the 2D order and hydrophilic/hydrophobic interactions, showed to be useful for obtaining electroactive biocatalytic films, overcoming previous limitation encountered when using recasted ionomer films. This can lead to interesting prospects for further developments in electrochemical biosensing and related fields.

Acknowledgements

Financial support by MIUR (Rome) is acknowledged.

References

[1] G. Roberts, Langmuir–Blodgett films, Plenum, New York, 1990.
[2] A. Ulman, An Introduction to Ultrathin Organic Films from Langmuir–Blodgett to Self-Assembly, Academic Press, London, 1991.
[3] P. Ugo, L.M. Moretto, F. Vezzà, Ionomer-coated electrodes and nanoelectrode ensembles as electrochemical environmental sensors: recent advances and prospects, ChemPhysChem 3 (2002) 917–925.

[4] R.S. Yeo, H.L. Yeager, Structural and transport properties of perfluorinated ion-exchange membranes, in: B.E. Conway, R.E. White, J.O'M. Bockris (Eds.), Modern aspects of electrochemistry, vol. 16, Plenum Press, NY, 1985, Chap. 6.
[5] P. Ugo, L.M. Moretto, Ion-exchange voltammetry at polymer-coated electrodes: principles and analytical prospects, Electroanalysis 7 (1995) 1105–1113.
[6] M.W. Espenscheid, A.R. Ghatak-Roy, R.B. Moore III, R.M. Penner, M.N. Szentirmay, C.R. Martin, Sensors from polymer modified electrodes, J. Chem. Soc., Faraday Trans. I 82 (1986) 1051–1070.
[7] A.R. Hillman, Reactions and applications of polymer modified electrodes, in: R.G. Linford (Ed.), Electrochemical Science and Technology of Polymers, vol. 1, Elsevier, London, 1989.
[8] J.F. Rusling, Z. Zhang, in: A. Brajter-Toth, J.Q. Chambers (Eds.), Polyion and Surfactant Films on Electrodes for Protein Electrochemistry, Electroanalytical Methods for Biological Material, Marcel Dekker, NY, 2002, Chap. 6, and references therein.
[9] P. Ugo, V. Zangrando, L.M. Moretto, B. Brunetti, Ion-exchange voltammetry and electrocatalytic sensing capabilities of cytchrome *c* at polyestersulfonated ionomers coated glassy carbon electrodes, Biosens. Bioelectron. 17 (2002) 479–487.
[10] E. Lojou, P. Luciano, S. Nitsche, P. Bianco, Poly(ester-sulfonic acid) modified carbon electrodes for electrochemical study of cyt *c*-type cytochromes, Electrochim. Acta 44 (1999) 3341–3352.
[11] H.A.O. Hill, N.J. Walton, I.J. Higgins, Electrochemical reduction of dioxygen using a terminal oxidase, FEBS Lett. 126 (1981) 282–284.
[12] E.E. Ferapontova, T. Ruzgas, L. Gorton, Direct electron transfer of heme- and molybdopterin cofactor-containing chicken liver sulfite oxidase on alkanethiol-modified gold electrodes, Anal. Chem. 75 (2003) 4841–4850.
[13] V. Fridman, U. Wollenberger, V. Bogdanovskaya, F. Lidstat, T. Ruzgas, A. Lindgren, L. Gorton, F.W. Scheller, Electrochemical investigation of cellobiose oxidation in the presence of cytochrome *c* as mediator, Biochem. Soc. Trans. 28 (2000) 63–70.
[14] W. Jin, U. Wollenberger, F.F. Bier, A. Makower, F.W. Scheller, Electron transfer between cytochrome *c* and copper enzymes, Bioelectrochem. Bioenerg. 39 (1996) 221–225.
[15] H.A.O. Hill, N.J. Walton, Investigation of some intermolecular electron transfer reactions of cytochrome *c* by electrochemical methods, J. Am. Chem. Soc. 104 (1982) 6515–6519.
[16] M. Fedurco, Redox reactions of heme-containing metalloproteins: dynamic effects of self-assembled monolayers on thermodynamic and kinetics of cytochrome *c* electron-transfer reactions, Coord. Chem. Rev. 209 (2000) 263–331.
[17] C.E.W. Hahn, H.A.O. Hill, M.D. Ritchie, J.W. Sear, The electrochemistry of proteins entrapped in Nafion, J. Chem. Soc., Chem. Commun. (1990) 125–126.
[18] E. Sedlak, M. Antalik, J. Bagelova, M. Fedurco, Interaction of ferricytochrome *c* with polyanion Nafion, Biochim. Biophys. Acta 1319 (1997) 258–266.
[19] P. Bertoncello, M.K. Ram, A. Notargiacomo, P. Ugo, C. Nicolini, Fabrication and physico-chemical properties of Nafion Langmuir–Schaefer films, Phys. Chem. Chem. Phys. 4 (2002) 4036–4043.
[20] P. Bertoncello, P. Ugo, Preparation and voltammetric characterization of electrodes coated with Langmuir–Schaefer ultrathin films of Nafion, J. Braz. Chem. Soc. 14 (2003) 517–522.
[21] P. Ugo, P. Bertoncello, F. Vezzà, Langmuir–Blodgett films of different ionomeric polymers deposited on electrode surfaces, Electrochim. Acta 49 (2004) 3785–3793.
[22] L.M. Moretto, N. Pepe, P. Ugo, Voltammetry of redox analytes at trace concentrations with nanoelectrode ensembles, Talanta 62 (2004) 1055–1060.
[23] F.N. Buchi, A.M. Bond, Interpretation of the electrochemistry of cytochrome *c* at macro and micro sized carbon electrodes using a microscopic model based on a partially blocked surface, J. Electroanal. Chem. 314 (1991) 191–206.
[24] A. Szucs, G.D. Hitchens, J.O'M. Bockris, Ellipsometry of cytochrome *c* on gold surfaces: effects of 4,4′-dipyridyl disulfide, Electrochim. Acta 37 (1992) 403–412.
[25] B. Brunetti, P. Ugo, Factors influencing the ion-exchange preconcentration and voltammetric behaviour of redox cations at polyestersulfonated ionomer coated electrodes in acetonitrile solutions, J. Electroanal. Chem. 460 (1999) 38–45.
[26] C. Nicolini, V. Erokhin, P. Ghisellini, C. Paternolli, M.K. Ram, V. Sivozhelezov, P450scc engineering and nanostructuring for cholesterol sensing, Langmuir 17 (2001) 3719–3726.
[27] M. Petty, Langmuir–Blodgett films—An Introduction, Cambridge Univ. Press, Cambridge, 1996.
[28] A.J. Bard, L.R. Faulkner, In Electrochemical Methods, Fundamentals and Applications, 2nd ed., Wiley, New York, 2001.
[29] M.J. Eddowes, H.A.O. Hill, Electrochemistry of horse heart cytochrome *c*, J. Am. Chem. Soc. 101 (1979) 4461–4464.
[30] P. Ugo, N. Pepe, L.M. Moretto, M. Battagliarin, Direct voltammetry of cytochrome *c* at trace concentrations with nanoelectrode ensembles, J. Electroanal. Chem. 560 (2003) 51–58.
[31] T. Sagara, H. Murakami, S. Igarasho, H. Sato, K. Niki, Spectroelectrochemical study of the redox reaction mechanism of cytochrome *c* at a gold electrode in a neutral solution in the presence of 4,4′-bipyridyl as a surface modifier, Langmuir 7 (1991) 3190–3196.
[32] M. Lion-Dagan, E. Katz, I. Willner, A bifunctional monolayer electrode consisting of 4-pyridyl sulfide and photoisomerizable spyropan: photoswitchable electrical communication between the electrode and cytochrome *c*, J. Chem. Soc., Chem. Commun. (1994) 2741–2742.
[33] J.M. Cooper, K.R. Greenough, C.J. McNeil, Direct electron transfer reactions between immobilized cytochrome *c* and modified gold electrode, J. Electroanal. Chem. 347 (1993) 267–275.
[34] W.A. Prutz, R. Kissner, T. Nauser, W.H. Koppenol, On the oxidation of cytochrome *c* by hypohalous acids, Arch. Biochem. Biophys. 389 (2001) 110–122.