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Construction of layer-by-layer self-assemblies of glucose oxidase and cationic polyelectrolyte onto glassy carbon electrodes and electrochemical study of the redox-mediated enzymatic activity

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Abstract

Build-up of enzyme–polyelectrolyte multilayer onto glassy carbon (GC) surfaces by electrostatic self-assembling method has been investigated. In order to functionalize GC surface by a starting negatively charged layer, two approaches have been carried out: (i) covalent linkage of phenyl acetic acid through electroreduction of 4-phenylacetic diazonium salt (GC_A surface), and (ii) formation of a glucose oxidase (GOD) monolayer through an affinity reaction between a GOD conjugated antibody and a previously adsorbed antigen monolayer (GC_B surface). GC_A and GC_B surfaces have been modified by a precursor film (PF) composed of one layer of poly(styrenesulfonate) (PSS) sandwiched between two layers of poly(dimethyldiallylammonium) (PDDA), which improves the further assembling of enzymes. GOD, used as a model enzyme, has been self-assembled with PDDA onto these GC/PF surfaces. Enzymatic activity of immobilized GOD has been electrochemically assessed layer-by-layer during the build-up of $(GOD–PDDA)_n$ multilayers. Cyclic voltammetry (CV) performed in the presence of excess glucose and using ferrocene derivatives as artificial redox mediators allowed to quantify the amount of electrically wired enzyme on the basis of kinetic models reported in literature. By using three mediators bearing different electrical charges we conclude that electrostatic interactions between the redox mediator and enzyme microenvironment play a key role in determining the rate of enzyme active site regeneration.

Keywords: Layer-by-layer electrostatic self-assembling; Enzyme multilayer; Polyelectrolyte; Enzyme wiring; Bioelectrode

1. Introduction

The alternating electrostatic adsorption of cationic and anionic polyelectrolytes at charged surfaces has proven to be an easy and efficient method for preparation of ultrathin organized polymer films [1,2]. The technique was originally developed by Decher and Hong [1] and Decher and co-workers [2] for linear polyelectrolytes and later extended to proteins, enzymes and nanoparticles. This process known as the layer-by-layer (LbL) electrostatic self-assembling (ESA) technique has recently attracted much attention due to its stricking simplicity, versatility and to potential applications resulting from these properties, in particular in the field of integrated molecular optics, electronics, biomaterials or biosensors. Film growth proceeds via electrostatic interactions by alternately exposing a solid substrate to different soluble polyelectrolytes of opposite charge. On each deposition cycle the charge on the exposed surface is compensated and reversed by adsorbed polymer. Up to date there are few papers devoted to the construction of biosensors using the LbL technique. The first example was carried out in 1997 by Hodak et al. [3] who described the redox mediation of glucose oxidase (GOD). Since that time several enzymes (GOD, cytochrome oxidase, fructose dehydrogenase, horseradish peroxidase, alcohol oxidase, lactate oxidase, soybean peroxidase, polyphenoloxidase, cholesterol

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esterase, urease) have been assembled with redox [4–13], non-redox [14–18] or conductive [19] polyelectrolyte in organized multilayers by electrostatic adsorption. These works demonstrated the feasibility of the LbL–ESA technique applied to biosensing.

Building electrostatically self-assembled multilayers onto an electrode surface implies first its reproducible functionalization by a monolayer of ionic groups that does not inhibit electron transfer process. Most of the work devoted to the area of LbL–ESA polyelectrolyte multilayers has been carried out by adsorption of polyelectrolyte onto thiol functionalized Au surfaces, in particular those connected to electrochemical applications. Using Au substrates allows a precise control of the interfacial properties of the starting surface and thus leads to an acceptable reproducibility of the assembled films. However, as the potential window of thiolated Au is limited, this can restrict the range of potential applications.

In the present paper, we have investigated the possibility of building polyelectrolyte–enzyme assemblies onto glassy carbon surfaces with the objective of producing stable and reproducible enzymatic films. In order to functionalize GC surface by a starting negatively charged layer, two approaches have been carried out and compared in term of stability, reproducibility and efficiency: (i) covalent linkage of phenyl acetic acid through electroreduction of 4-phenylacetic diazonium salt, the corresponding surface being called GC_A; and (ii) formation of a GOD monolayer through an affinity reaction between a GOD conjugated antibody and a previously adsorbed antigen monolayer, the corresponding surface being called GC_B. The GOD monolayer that is negatively charged at the working pH, is used as a starting negative layer. Both of these surfaces have been modified by a precursor film (PF) composed of one layer of poly(styrenesulfonate) (PSS) sandwiched between two layers of poly(dimethyldiallylammonium) (PDDA). Build-up of PF has been evidenced from cyclic voltammetry (CV) experiments using neutral, anionic or cationic redox probes. As will be further discussed, PF allows improving the further assembling of enzymes.

Glucose oxidase, used as a model enzyme, has been self-assembled with PDDA onto the GC_A/PF and GC_B/PF surfaces. Enzymatic activity of immobilized GOD has been electrochemically assessed layer-by-layer during the build-up of $(GOD–PDDA)_n$ multilayers. CV performed in the presence of excess glucose and using ferrocene (Fc) derivatives as artificial redox mediators allowed to quantify the amount of electrically wired enzyme on the basis of kinetic models reported in the literature [20]. Three mediators bearing various electrical charges were used in order to evaluate how the electrostatic interactions between mediator and enzyme multilayer control the GOD electrical wiring. These anionic, neutral and cationic mediators were, respectively, ferrocenecarboxylate ($FcCO_2^-$), ferrocenemethanol (FcMeOH) and ferrocenylmethyltrimethylammonium (FcN^+).

2. Experimental

2.1. Chemicals

Phosphate buffer (pH 8, ionic strength: $0.1\,mol\,l^{-1}$) was prepared by dissolving KH_2PO_4 and K_2HPO_4 from CAPPEL-ICN in deionized water.

Poly(dimethyldiallylammonium chloride) (PDDA) $M_W = 100{,}000–200{,}000\,g\,mol^{-1}$ in 20% aqueous solution was purchased from Aldrich.

Poly(styrenesulfonate) was purchased from Aldrich and subsequently dialyzed against pure water using dialysis membrane (cut-off: 14,000).

The 4-phenyl acetic acid diazonium fluoroborate salt has been synthesized following a published procedure [21].

Glucose oxidase (GOD) (EC 1.1.3.4. type VII from *aspergillus niger* $246\,U\,mg^{-1}$) and β-D-glucose were obtained from Sigma Co. Stock solutions of glucose (prepared in phosphate buffer, pH 8) were allowed to mutarotate overnight before use.

Mouse IgG (whole molecule) was purchased from Sigma. The affinity purified GOD conjugated antimouse IgG was from CAPPEL-ICN.

All other reagents used were obtained commercially and were of analytical grade.

2.2. Preparation of the $GC/PF/(GOD–PDDA)_n$ modified electrodes

2.2.1. Covalent grafting of 4-phenylacetate: GC_A electrodes

Covalent grafting of 4-phenylacetate groups onto GC surfaces has been carried out from a published procedure [21,22] based on the electrochemical reduction of 4-phenyl acetic acid diazonium fluoroborate salt. Clean and polished glassy carbon electrodes were first dipped under N_2-atmosphere in acetonitrile containing $5 \times 10^{-4}\,mol\,l^{-1}$ 4-phenyl acetic acid diazonium fluoroborate salt and $0.1\,mol\,l^{-1}$ tetrabutylammonium perchlorate. Electrogeneration and further grafting of 4-phenylacetic radicals was realized by cycling three times the electrode potential between 0 and $-1\,V$ versus Ag/Ag^+ reference electrode at a scan rate of $0.100\,V\,s^{-1}$. After rinsing with acetonitrile, the resulting GC_A modified electrodes were immersed in phosphate buffer pH 8 in order to ionize the carboxylic functions.

2.2.2. Adsorption of the immunological layer: GC_B electrodes

Solution of the antigen ($1\,mg\,ml^{-1}$), gelatin ($0.1\,mg\,ml^{-1}$), glucose oxidase conjugated antibody (dilution of the initial solution: /50) were prepared in a buffer composed of $0.01\,mol\,l^{-1}$ KH_2PO_4 and $0.15\,mol\,l^{-1}$ NaCl at pH 7.4 (PBS buffer solution). Sodium azide ($0.1\,mg\,ml^{-1}$) was added to the solution in order to prevent the formation of bacterial colonies.

The procedure for immobilizing the immunological layer was similar to that described by Bourdillon et al. [20]. Briefly, adsorption of the antigen resulted from a 2 h exposure of the electrode surface to the corresponding solution. The electrode was then thoroughly washed with the buffer and dipped for 10 min in a gelatine solution. After a thorough washing, the antigen–antibody reaction took place after overnight immersion of the electrode in the solution of glucose oxidase conjugated antibody. After a latter washing, the resulting GC_B modified electrode was ready for use.

2.2.3. Build-up of the (PDDA/PSS/PDDA) precursor film (PF)

PF was assembled by dipping the GC_A or GC_B electrodes alternately into PDDA or PSS solutions of $2\,mg\,ml^{-1}$ in phosphate buffer $0.1\,mol\,l^{-1}$ pH 8 for 20 min. After each adsorption step, the modified electrode was thoroughly rinsed with the same buffer.

2.2.4. Build-up of $(GOD–PDDA)_n$ multilayer

$(GOD–PDDA)_n$ multilayer were assembled by dipping the GC_A/PF and the GC_B/PF modified electrodes alternately into GOD and PDDA solutions of $2\,mg\,ml^{-1}$ in phosphate buffer $0.1\,mol\,l^{-1}$ pH 8 for 20 min. After each adsorption step, the modified electrode was thoroughly rinsed with 0.1 M phosphate buffer, pH 8. The GOD catalytic activity involved in the oxidation of glucose ($0.5\,mol\,l^{-1}$) was evaluated by CV in the presence of ferrocenemethanol (FcMeOH) $2 \times 10^{-4}\,mol\,l^{-1}$ in phosphate buffer $0.1\,mol\,l^{-1}$ pH 8 at 25 °C. FcMeOH was selected as single electron acceptor mediator. The catalytic current, i_{cat}, was evaluated from the difference between the current responses obtained in the presence and absence of glucose at a potential of 0.28 V. Similar procedures were used with FcN^+ and $FcCO_2^-$ mediators, respectively, at potential of 0.36 and 0.44 V.

2.3. Measurements and apparatus

2.3.1. Apparatus

All electrochemical studies were performed with a conventional three-electrode potentiostatic system. The equipment was a PAR model 273A potentiostat and a Sefram TGM 164 XY/t recorder. The working electrode was a 3 mm diameter glassy carbon disk (geometric area of $0.0707\,cm^2$). Preparation of electrode surfaces involves successively abrasion with grade 1200 grinding paper and polishing with 3 and 1 μm diamond paste with intermediate rinsing and ultrasonication. The reference electrode was Ag/AgCl/KCl $3\,mol\,l^{-1}$ in aqueous electrolyte. Bioelectrode measurements were carried out in a thermostated cell at 25 °C containing phosphate buffer solution under N_2-saturating conditions.

2.3.2. Measurements of GOD solution enzymatic activity

The enzymatic activities of GOD in solution were determined spectrophotometrically. The absorbance increase at 425 nm, resulting from the oxidation of *o*-tolidine (3,3′-dimethyl(1,1′-biphenyl)4,4′-diamine) in the presence of glucose through a peroxidase coupled system, was measured versus time. Spectrophotometric measurements were performed with a Varian Cary 1 UV-Vis spectrophotometer.

3. Experimental results and discussion

At first we present a comparison of the two approaches used in order to make GC surfaces suitable for the electrostatic assembling of $(GOD–PDDA)_n$ multilayer. Preliminary experiments, not reported here, demonstrated that the direct adsorption of PDDA onto bare GC surface was inefficient as evidenced by non-reproducible results and multilayer instability. We have also concluded that electrostatic self-assembling of GOD was a more efficient and reproducible process once the GC_A and GC_B surfaces have been previously modified by the PF film (PDDA/PSS/PDDA). This behavior, which will not be presented here, is relevant to previous work [23], where the authors show that a regular polyelectrolyte grow is obtained after three or four adsorbed layers, when the influence of the underlying surface is strongly attenuated. In this sense, PF adsorption allows a more regular charge distribution and facilitates further protein adsorption. Thus, in the following, we will only describe the construction and electrochemical characterization of GC_A/PF and GC_B/PF modified electrodes and compare their efficiency for binding to a further GOD layer.

In the second part of this section, we will study the GOD electrical wiring by redox mediators and the build-up of $(GOD–PDDA)_n$ multilayer onto GC_B/PF surfaces, which appears to be the most efficient one for this purpose.

3.1. GC_A/PF modified surfaces: covalent grafting of 4-phenyl acetic radical

As previously reported in literature [21], the electrochemical reduction of 4-phenyl acetic diazonium salt leads to the formation of 4-phenyl acetic radicals that can further graft on GC surfaces, through the formation of a C–C covalent bond. The resulting modified electrode exhibits a negatively charged surface when immersed in an aqueous electrolyte of pH higher than pK_a of the carboxylate functions (abbreviated COO^- in the following). It has been demonstrated that the surface coverage can be limited through varying the diazonium salt concentration as well as electrolysis time [22]. In this work, we have chosen a $5 \times 10^{-4}\,mol\,l^{-1}$ diazonium salt concentration and electrolysis conditions (see Section 2) that allow to functionalize GC surfaces without blocking access of diffusing species. Fig. 1 shows the CV responses recorded step-by-step during the building of GC_A/PF electrode in the presence of the following redox probes: (A) the neutral FcMeOH, (B) the cationic FcN^+ and (C) the anionic 2,2′-azino-bis(3-ethylbenzothiazole-6-sulfonate) (ABTS). FcMeOH exhibits on bare GC a Nernstian behavior with $E_{1/2} = 0.187$ V in good agreement with the literature [24].

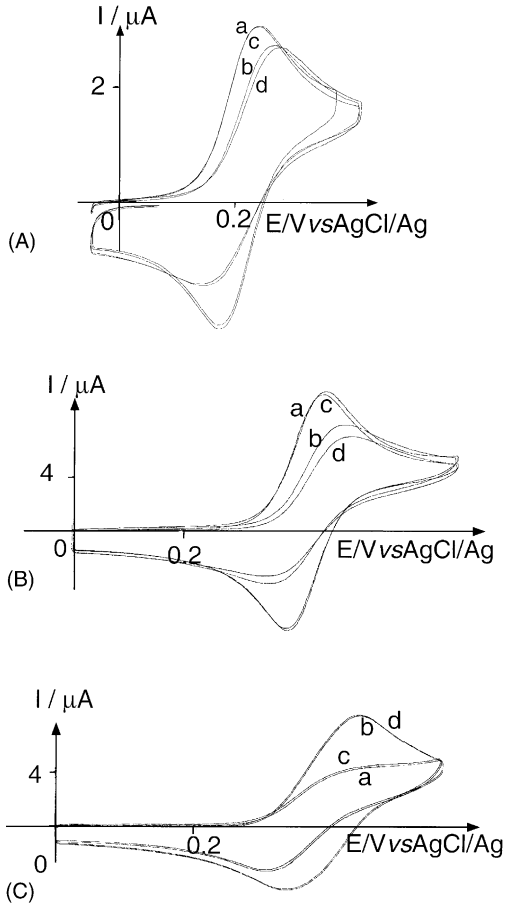

Fig. 1. Cyclic voltammograms recorded on a (a) GC_A, (b) GC_A/PDDA, (c) GC_A/PDDA/PSS, (d) GC_A/PDDA/PSS/PDDA (GC_A/PF), in the presence of: (A) FcMeOH 2×10^{-4} mol l^{-1}; (B) FcN^+ 8.8×10^{-3} mol l^{-1}; (C) ABTS 8.8×10^{-3} mol l^{-1}. All measurements were performed in pH 8 phosphate buffer (ionic strength: 0.1 mol l^{-1}) with a potential scan rate of 0.100 V s^{-1}.

This behavior remains Nernstian when the electrode bears a negative charge either after 4-phenyl acetate grafting or PSS adsorption (Fig. 1A, curves a and c) indicating a rapid electron transfer process. In contrast, ΔE_p increases from 0.065 to 0.125 V when the outer layer is composed of cationic PDDA (curves b and d in Fig. 1A). The increase of ΔE_p can be attributed to electrostatic repulsion effects developed between the radical–cation $FcMeOH^{\bullet+}$ and the positive PDDA outerlayer. Similar behavior has already been reported by several authors with $Fe(CN)_6^{3-}$ probe [25–27]. It was shown that the charge in the outer layer of a polyelectrolyte multilayer strongly influences the electron transfer kinetics of diffusing redox ionic probes. In agreement with these results, Fig. 1B and C present, respectively, the CV responses for the positive FcN^+ and the negative ABTS, respectively. As clearly shown, ΔE_p for FcN^+ increases when the outer layer is composed of positive PDDA (Fig. 1B, curves a and c), whereas a similar behavior is observed for ABTS when the outer layer is composed of COO^- or PSS (Fig. 1C, curves a and c). In an opposite way, the reversible

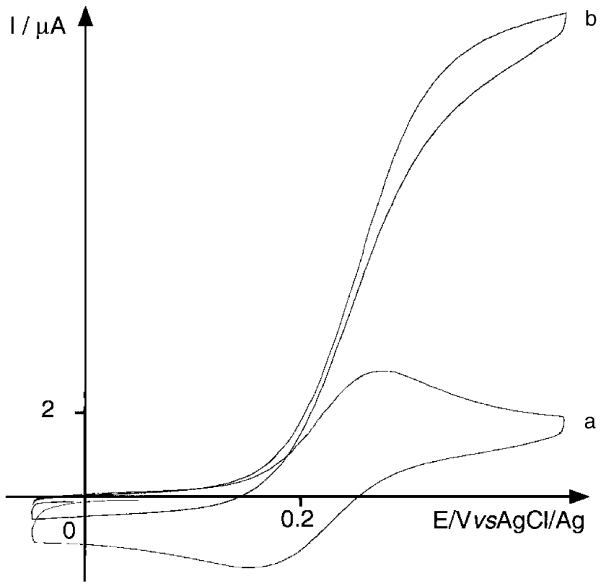

Fig. 2. Cyclic voltammetry of the catalysis of glucose oxidation with FcMeOH 2×10^{-4} mol l^{-1} as mediator on a GC_A/PF/GOD electrode in a pH 8 phosphate buffer (ionic strength: 0.1 mol l^{-1}) with a potential scan rate of 0.100 V s^{-1}: (a) in the absence of glucose; (b) in the presence of glucose 0.5 mol l^{-1}.

redox waves are restored when the outer layer exhibits a charge opposite to the one of the diffusing redox probe (curves a and c in Fig. 1B and curves b and d in Fig. 1C).

Fig. 2 shows the CV profiles of FcMeOH recorded on a GC_A/PF/GOD electrode in the absence (curve a) and in the presence (curve b) of glucose. Despite the fact that GOD is on average negatively charged at the present pH, FcMeOH, in the absence of glucose, exhibits a ΔE_p of 112 mV in contrast to the Nernstian behavior previously described on GC_A or GC_A/PDDA/PSS modified electrodes (curves a and c in Fig. 1A). In the presence of glucose, (Fig. 2, curve b), a well-defined S-shaped catalytic wave develops as a consequence of the GOD catalyzed oxidation of glucose. This reaction can be described by the well known following mechanism [20,24].

$$\mathrm{FAD} + \mathrm{G} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{FADG}$$

$$\mathrm{FADG} \xrightarrow{k_2} \mathrm{FADH_2} + \mathrm{GL}$$

$$\mathrm{FADH_2} + 2\mathrm{Fc}^{\bullet+} \xrightarrow{k_3} \mathrm{FAD} + 2\mathrm{Fc}$$

$$\mathrm{Fc} \rightarrow \mathrm{Fc}^{\bullet+} + \mathrm{e}^-$$

FAD and $FADH_2$: oxidized and reduced forms of the flavin adenine dinucleotide; FADG: enzyme–substrate complex; G and GL: glucose and gluconolactone; Fc and $Fc^{\bullet+}$: reduced and oxidized forms of the ferrocenyl mediator.

The i_{cat} obtained by subtracting the measured current in the absence of glucose to that measured in the presence of this substrate at 0.28 V was used to check the reproducibility of bioelectrode construction as well as the amount of electrically wired active enzyme sites (Γ_E^0). We used the kinetic

analysis developed by Moiroux and co-workers on the GOD multilayered assemblies involving antigen–antibody affinity reactions [20]. In the absence of mass transport limitations, the total amount of immobilized enzyme, Γ_E^0, can be derived from the cyclic voltammograms using the expression for i_{cat} presented in Eq. (1) and considering the same rate constant values than those determined in homogeneous solution for the same mediator ($k_2 = 700\,s^{-1}$, $k_3 = 1.2 \times 10^7\,M^{-1}\,s^{-1}$, $k_{red} = 1.1 \times 10^4\,M^{-1}\,s^{-1}$).

$$\frac{1}{i_{cat}} = \frac{1}{2Fk_3\Gamma_E^0}\frac{1}{[Fc^{\bullet+}]} + \frac{1}{2F\Gamma_E^0}\left(\frac{1}{k_2} + \frac{1}{k_{red}[G]}\right) \quad (1)$$

where F is the Faraday's constant, S the electrode area, Γ_E^0, the total amount of connected active enzyme, $[Fc^{\bullet+}]$ the concentration of the oxidized form of the mediator, [G] the glucose concentration in solution, and $k_{red} = k_1k_2(k_{-1}+k_2)$. We can consider that $[Fc^{\bullet+}] = [Fc]_{bulk}$ because i_{cat} was measured at high enough anodic potential.

The GC_A/PF/GOD electrodes exhibit a satisfying catalytic current with an acceptable reproducibility. The average i_{cat}, measured on 10 different electrodes was $(7.8 \pm 1.1)\,\mu A$ which leads to a variation coefficient of 13.5%. This value was used to extract Γ_E^0 according to the theoretical treatment described before. The total amount of electrically wired GOD was estimated 7.8×10^{-14} mol, that is $1.1 \times 10^{-12}\,mol\,cm^{-2}$. If we consider that a saturated monolayer of GOD corresponds to a surface concentration of $1.7 \times 10^{-12}\,mol\,cm^{-2}$, the determined Γ_E^0 corresponds to 65% of a monolayer.

3.2. GC_B/PF surfaces, functionalization by affinity reactions

Bourdillon et al. [20] have developed a method based on affinity reactions that allows to functionalize GC surface by a GOD monolayer. This method is very reproducible and the assembled structure very stable. The GOD monolayer has been used as a negatively charged underlayer for the further electrostatic assembling of polyelectrolytes. As previously reported [20], we observed that FcMeOH exhibits a perfectly Nernstian behavior on the GC_B modified electrode (GC/IgG/anti-IgG–GOD). The evolution of the CV profiles for anionic or cationic redox probes at GC_B electrode during PF building is similar to that reported for GC_A/PF, thus exhibiting electrostatic repulsion when the external layer of the film has the same charge as the soluble redox probe (not shown). The electrochemical response of FcMeOH at GC_B/PF/GOD presents a $\Delta E_p = 0.080$ V, significantly lower than the 0.112 V obtained on GC_A/PF/GOD. This difference points out a structural influence of the immunological layer onto the multilayer structure, which is currently under study. As shown in Fig. 3 (curve b), the catalytic current obtained in the presence of glucose is significantly higher than those observed on the previous architectures. The average i_{cat} measured with 12 electrodes was

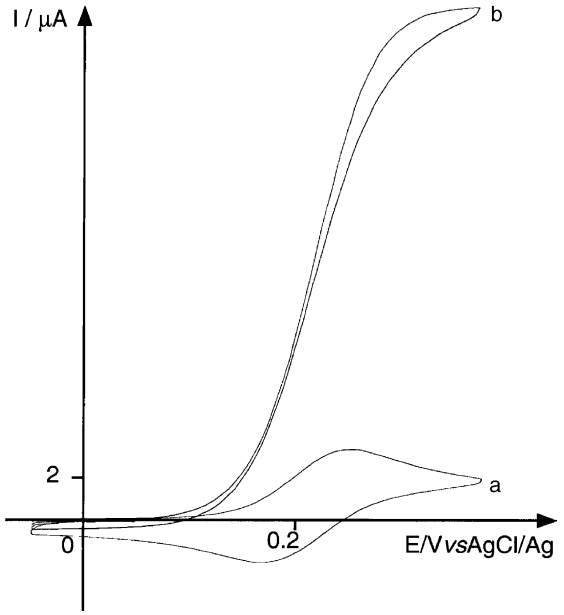

Fig. 3. Cyclic voltammetry of the catalysis of glucose oxidation with FcMeOH $2 \times 10^{-4}\,mol\,l^{-1}$ as mediator on GC_B/PF/GOD in a pH 8 phosphate buffer (ionic strength: $0.1\,mol\,l^{-1}$) with a potential scan rate of $0.100\,V\,s^{-1}$: (a) in the absence of glucose; (b) in the presence of glucose $0.5\,mol\,l^{-1}$; temperature: 25 °C.

$(24 \pm 3)\,\mu A$ which leads to a variation coefficient of 12.3%. This higher value is not a consequence of the additional enzymatic activity due to the underlying anti-IgG conjugated GOD because this commercially available conjugate exhibits an extremely low activity with i_{cat} lower than 1 μA. The high catalytic current has thus been attributed to a high amount of GOD at the outer layer of this assembly. From the average value of i_{cat}, Γ_E^0 was $3.3 \times 10^{-12}\,mol\,cm^{-2}$, which corresponds to a 1.95 times a densely packed monolayer of GOD.

The two procedures of carbon surface modification (GC_A and GC_B) lead to catalytic responses exhibiting similar reproducibility. Such reproducibility can be explained by the fact that in the two approaches, perfectly controlled procedures were carried out in each step of surface modification. A careful polishing of the electrode surface leads to a reproducible surface state on top of which it becomes possible to build reproducible anionic monolayer. Since higher amounts of GOD can be electrostatically assembled on GC_B/PF, it can be concluded that GC_B/PF constitutes a more efficient platform for the immobilization of enzyme and bioelectrode efficiency. Consequently, the following of this paper will be devoted to the building of $(GOD–PDDA)_n$ multilayer on top of a GC_B/PF electrodes.

3.3. Self-assembling of $(GOD–PDDA)_n$ multilayer on GC_B/PF electrodes

The first step involved in building $(GOD–PDDA)_n$ multilayered films consists to adsorb a PDDA layer on top of the

GC_B/PF/GOD electrode. We observe that this step strongly inhibit glucose oxidation since i_{cat} was only 30% of the value measured in the absence of the external PDDA layer. Several interpretations could explain the decrease in activity. (i) The first one is related to a possible partial removal of GOD from the electrode surface to the solution where PDDA is present during the adsorption step. A portion of enzyme could be peeled out by PDDA and transferred to the bulk in the form of GOD–PDDA complex. This problem has been addressed by Lvov et al. [28] in a study focusing onto the assembly of protein–polyion alternate multilayer with different proteins and polyelectrolytes. Specifically for GOD, they reported that PDDA did not produce this effect at low ionic strength. For this reason, desorption process can be assumed to be negligible. (ii) A second possible interpretation could be that deposition of the PDDA layer over the GOD layer strongly decreases its specific activity. The linear PDDA polyelectrolyte is expected to cover the protein molecules and to penetrate between them acting as an "electrostatic glue". This complexation process could induce limitations in the flexibility or accessibility to the enzyme active site. This possibility was readily ruled out from spectrophotometric enzymatic assay using O_2 as the natural mediator. These assays demonstrate that in homogeneous electrolytic medium, the addition of PDDA up to a GOD–PDDA ratio 1:20 does not induce any significant decrease of enzymatic activity. However, taking into account the confinement effect of the enzyme molecules sandwiched between the flexible chains of the polyelectrolyte and maintained in close contact with the electrode surface, a slight decrease of the enzymatic activity cannot be excluded a priori. (iii) A third interpretation has finally been underlined from CV experiments onto a bare GC electrode using GOD in solution; FcMeOH as artificial mediator and glucose in excess, Fig. 4 presents the results obtained. Addition of PDDA to the electrochemical cell induces an important decrease of the catalytic current, down to 55% of the initial value recorded in the absence of PDDA. This result is in contrast with those previously described using O_2 as the natural mediator showing no decrease in activity upon PDDA addition. The decrease of i_{cat} has been ascribed to electrostatic repulsions developed between the active oxidized form of the mediator, $FcMeOH^{\bullet+}$, and the cationic complex formed between PDDA in excess and GOD. This repulsive electrostatic interaction constitutes an additive energy barrier that slows down the accessibility to GOD and the regeneration of FAD active site. A similar effect upon addition of PDDA should operate when GOD is immobilized onto an electrode surface. Thus, the external PDDA layer in the GC_B/PF/$(GOD–PDDA)_n$ electrode acts as an electrostatic barrier that prevents the access of the cationic $FcMeOH^{\bullet+}$ to the GOD active site. In good agreement with this interpretation, a subsequent deposition of PSS on the latter assembly leads to a negatively charged surface which induces a 15% enhancement of i_{cat}. The initial i_{cat} obtained with GC_B/PF/GOD was not completely recovered, probably because of diffusional constraints induced by the additional PDDA/PSS bilayer. However, this experiment demonstrates clearly that electrostatic interactions between the artificial mediator and the surface of the LbL assembly constitute a key step that governs the enzyme electrical connection by soluble mediators. These electrostatic effects can be observed whatever is the electrical charge of the mediator and the multilayer assembly. Table 1 presents the average i_{cat} measured on five GC_B/PF/GOD electrodes using ferrocene carboxylate (FcO_2^-), FcMeOH and ferrocenylmethyltrimethylammonium (FcN^+) mediators, as well as the corresponding Γ_E^0 calculated from Eq. (1). It appears that Γ_E^0 decreases when going from $FcCO_2^-$ to FcMeOH and FcN^+ while the corresponding mediator charges increase, respectively, from 0 to +1 and +2. The lowest amount of connected GOD is obtained with FcN^+ although it was expected that electrostatic attraction between this mediator and negatively charged GOD outerlayer will induce an efficient wiring. In fact the highest amount of connected GOD is obtained with FcO_2^-. This seems to indicate that in a GC_B/PF/GOD assembly, the underlying polymer chains of PDDA exert electrostatic repulsion towards cationic mediators, even the assembly is made of an outerlayer of GOD. This strongly suggests that the protein molecules have been complexed and embedded by the underlying cationic polymer chains of PDDA. According to the generally accepted model, LbL assemblies are stratified, but do not consist of well separated distinguishable alternating layers [29]: the flexible linear polyions penetrate between protein globules acting as an "electrostatic glue" and adjacent layers interpenetrate more or less having a smeared structure along the film normal.

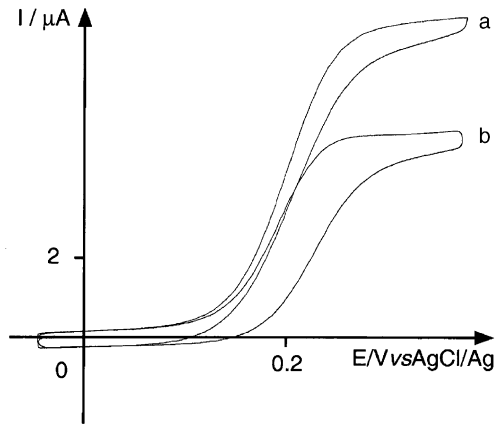

Fig. 4. Cyclic voltammetry onto a bare GC electrode of the catalysis of glucose oxidation with FcMeOH 2×10^{-4} mol l^{-1} as mediator and glucose (0.5 mol l^{-1}) in the presence of dissolved GOD (0.1 mg ml^{-1}) in a pH 8 phosphate buffer (ionic strength: 0.1 mol l^{-1}) with a potential scan rate of 0.100 V s^{-1}: (a) in the absence of PDDA; (b) in the presence of PDDA (2 mg ml^{-1}); temperature: 25 °C.

Electrostatic effects can be unambiguously evidenced by measuring with each mediator the i_{cat} variations induced by the successive additions of PDDA and PSS layers on the

Table 1
Evolution of the percentage of catalytic response of GC_B/PF/GOD electrode upon adding a first layer of PDDA and a second layer of PSS using $FcCO_2^-$, FcMeOH or FcN^+ artificial mediator

	$FcCO_2^-$	FcMeOH	FcN^+
GC/PF_2/GOD	100 (2 μA)[a] $\Gamma_E^0 = 3.2 \pm 0.3 \times 10^{-13}$ mol[b]	100 (24 μA)[a] $\Gamma_E^0 = 2.2 \pm 0.2 \times 10^{-13}$ mol[b]	100 (17 μA)[a] $\Gamma_E^0 = 1.7 \pm 0.1 \times 10^{-13}$ mol[b]
GC/PF_2/GOD–$PDDA^+$	60	30	40
GC/PF_2/GOD–$PDDA^+$/PSS^-	20	45	60
Nature of the oxidized mediator	Zwitterionic	Cationic	Dicationic

The catalytic response recorded on the GC/PF_2/GOD electrode is taken as 100%.
[a] Average value of i_{cat} on five electrodes.
[b] Superficial amounts of connected active GOD (Γ_E^0) determined from Eq. (1) using the average measured catalytic current of five electrodes and the kinetic constants from the literature [24] (FcMeOH, $k_3 = 1.2 \times 10^7\ M^{-1}\ s^{-1}$; $FcCO_2^-$, $k_3 = 2.2 \times 10^5\ M^{-1}\ s^{-1}$ and FcN^+, $k_3 = 10^7\ M^{-1}\ s^{-1}$).

surface of a GC_B/PF/GOD electrode. Taking i_{cat} obtained on this last electrode as 100%, Table 1 shows that addition of a PDDA layer induces a systematic decrease of i_{cat}, whatever is the mediator charge. This decrease appears more intense with FcMeOH and FcN^+ than with $FcCO_2^-$. More interestingly, adding a PSS layer on the $GC_B/PF/(GOD–PDDA)_1$ electrode induces i_{cat} to decrease again with $FcCO_2^-$ and to increase with FcMeOH and FcN^+. These results clearly underline the influence of the electrostatic interactions (attractive or repulsive) that can develop between the charged mediator and the overall charge surrounding GOD in the multilayer assembly.

A similar behavior was observed during the building of $GC_B/PF/(GOD–PDDA)_n$ electrodes showing a systematic decrease of i_{cat} upon adsorbing the PDDA layer. However, a linear increase of the catalytic activity of the assembly was observed from the first to the seventh (GOD–PDDA) bilayer as shown in Fig. 5. A slope of (6.1 ± 0.2) μA with a correlation of 0.997 was determined, indicating a regular growing of the multilayer assembly.

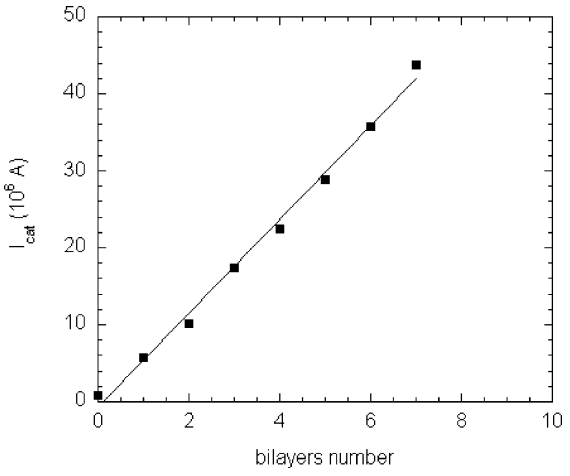

Fig. 5. Plot of i_{cat} vs. $(GOD–PDDA)_n$ bilayer number measured from CV experiments on $GC_B/PF/(GOD–PDDA^+)_n$ in the presence of FcMeOH 2×10^{-4} mol l^{-1} in a pH 8 phosphate buffer (ionic strength: 0.1 mol l^{-1}) at a temperature of 25 °C. The i_{cat} was evaluated from the difference between the current responses obtained in the presence and the absence of glucose (0.5 mol l^{-1}) at a potential of 0.28 V.

4. Conclusion

Our results lead to the conclusion that the immunological modification of GC surface by a protein monolayer constitutes an efficient starting platform for the further electrostatic assembling of enzyme multilayer onto GC surfaces. This work gives also additional information concerning the electrical wiring of enzymes in ESA multilayer films. Electrostatic interactions between the redox mediator and enzyme microenvironment play a key role in determining the rate of enzyme active site regeneration. This study also suggests that in a PDDA–GOD multilayer system where the top layer is constituted of GOD, the enzyme should be embedded inside the underlying film. As a consequence, and despite the fact that GOD is globally negatively charged, it is embedded in a positively charged microenvironment that induces electrostatic repulsive or attractive effects towards, respectively, positively or negatively charged mediators. This behavior is typical of globular GOD protein and does not occur with linear polyanion, such as PSS that is able to develop strong negative charges at the surface of the ESA assembly. $(GOD–PDDA)_n$ multilayer film can be efficiently built onto GC_B/PF surfaces with a linear dependence of GOD activity with bilayer number.

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