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Reagentless biosensors based on self-deposited redox polyelectrolyte-oxidoreductases architectures

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Abstract

Reagentless fructose and alcohol biosensors have been produced with a versatile enzyme immobilisation technique which mimics natural interactions and flexibility of living systems. The electrode architecture is built up on electrostatic interactions by the sequential adsorption of redox polyelectrolytes and redox enzymes giving rise to the efficient transformation of substrate fluxes into electrocatalytic currents. All investigated multilayer structures were self-deposited on 3-mercapto-1-propanesulfonic acid monolayers self-assembled on gold electrodes. Fructose dehydrogenase, horseradish peroxidase (HRP) and the couple HRP-alcohol oxidase were electrochemically connected with a cationic poly[(vinylpyridine)$Os(bpy)_2Cl$] redox polymer (RP) interface in a layer-by-layer self-deposited architecture. The dependence of the distance on the electrochemical response of this interface was also studied showing a clear decrease in the Faradaic current when the distance to the electrode surface was increased. The sensitivities obtained for each biosensor were 19.3, 58.1 and 10.6 mA M^{-1} cm^{-1} for fructose, H_2O_2 and methanol, respectively. The sensitivity values can be easily controlled by a rational deposition and manipulation of the charge in the catalytic layers. The electrostatic assembly of the electrochemical interface and the catalytic layers resulted in integrated biochemical systems in which mass transfer diffusion and heterogeneous catalytic and electron transfer steps are efficiently coupled and can be easily manipulated.

Keywords: Self-deposited multilayer architectures; Reagentless amperometric biosensor; D-Fructose dehydrogenase; Alcohol oxidase; Peroxidase; Cationic redox polymer

1. Introduction

The concept of an integrated chemical system, defined as 'a controlled assembly of chemical components resulting in a system which functions efficiently and effectively as specified by the designer' was recently introduced in the biosensor and molecular device technology (Bard, 1994), and it deserves increased attention.

When a redox enzyme is used as an active component in such an integrated chemical system, two basic aspects must be considered (Willner et al., 1997): (i) the method of assembly of the enzyme electrode; (ii) the electrical contact of the bioelectrocatalyst within this assembly. Different strategies were proposed to solve the problems arising when each of the above mentioned aspects was examined separately or simultaneously (Cass, 1990; Heller, 1990; Hall, 1991; Kuwabata et al., 1995; Mandler and Turyan, 1996; Scheller et al., 1997; Wink et al., 1997; Patolsky et al. 1999). Among these and in the last few years, a new approach based on multilayer self-deposited structures, built up on electrostatic interactions, has deserved special interest due to at least some of its basic advantages. It is a simple and versatile enzyme immobilization technique offering an effective multiplication of surface functionality, and it is

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largely independent of the nature, size and topology of the substrate (Decher, 1997). It was in 1991 when the possibility to build-up ordered multilayer structures by consecutive adsorption of polyanions and polycations was proved (Decher and Hong, 1991), but only in 1995 was this new method applied to immobilise negatively charged glucose oxidase (GOD) in a polyethyleneimine based multilayer structure (Lvov et al., 1995). One year later, it was described an oxygen mediated glucose biosensor based on GOD and poly(L-lysine) coadsorbed onto a negatively charged monolayer of mercaptopropionic acid, deposited on a Au electrode (Mizutani et al., 1996). However, the first reagentless electrocatalytical active structure based on this technique, was carried out in 1997 by the successive alternate deposition of ferrocene modified poly(allylamine) (cationic) polymer and anionic GOD, on a Au surface, initially thiolated with negatively charged sulfonic groups (Hodak et al., 1997). Following the same line, Shi-Feng et al. (1998) have used an Os-based redox polymer for the electrochemical communication of GOD controlling the analytical performance of the sensor by introducing multiple bilayers of GOD and redox polymer.

Consequently, and despite the fact that these supramolecular structures based on electrostatic interactions have been applied only to glucose oxidase, they seem to offer a controlled method of assembly where efficient electrochemical communication can be achieved after the rational design and deposition of the biorecognition and transducing layers. Additionally, their easy manipulation opens up the possibility of controlling each component at will, in other words, the overall response of the system can be customised.

The aim of this work is the demonstration of the versatility and potentiality of this technique for the construction of reagentless biosensors extending their suitability to different biorecognition elements. Thus, this work describes the use of layer-by-layer polyelectrolyte self-deposition on a negatively charged alkanethiolated gold electrode surface, interfacing different biocatalytic layers with a positively charged Os-based redox polymer (RP). First, the electrochemical behaviour of the adsorbed RP, incorporated in different multilayer structures, was investigated by cyclic voltammetry to elucidate the built up structures. Then, self-deposition of fructose dehydrogenase (FDH), horseradish peroxidase (HRP), and the enzyme couple HRP–alcohol oxidase resulted in amperometric reagentless biosensors for fructose, hydrogen peroxide and methanol, respectively. The electrocatalytic efficiency of the RP interface, the manipulation of the catalytic layers as well as the analytical parameters of the sensors are also presented.

2. Materials and methods

2.1. Reagents

Sodium salt of 3-mercapto-1-propane sulfonic acid (MPS) and cystamine dihydrochloride were purchased from Aldrich and Fluka, respectively. The poly(styrene sulfonic acid) (PSS) (MW 70 000, Polyscience) was used as received. The cationic poly[(vinylpyridine)$Os(bpy)_2Cl$] redox polymer partially quaternised with bromoethylamine (RP) was synthesized as described elsewhere (Katakis and Heller, 1992). The positively charged polyelectrolyte, called Binder (B), has the same structure as RP except that no Os redox centres are present. Fructose dehydrogenase, from *Gluconobacter* sp., (EC 1.1.99.11, 34 U mg^{-1} solid), and horseradish peroxidase (EC 1.11.1.7, 290 U mg^{-1} solid; type VI) were supplied by Sigma. The alcohol oxidase from *Hansenula* sp. (EC 1.1.3.13, 4 U mg^{-1} solid) was purchased from Applied Enzyme Technology Ltd (Leeds, UK). D-Fructose, hydrogen peroxide (30%) and methanol (99.97%) were obtained from Fluka, Merck and Scharlau, respectively. Ethylene glycol and ferric chloride were purchased from Sigma. Taurine (99%) and 3-in ethyl-2-benzothiazolinone hydrazone hydrochloride hydrate (971/6) were obtained from Aldrich. All other chemicals used were of analytical grade. Water was obtained by means of a Millipore Milli-Q system.

2.2. Instrumentation

Cyclic voltammograms were recorded in a conventional three electrode electrochemical cell using a computer controlled BAS CV-50W voltammetric analyzer (Bioanalytical Systems, West Lafayette, USA). Potentials were measured against a potassium-saturated silver/silver chloride electrode (Ag/AgCl, KCl_{sat}) and a coiled Pt wire served as counter electrode. Surface plasmon resonance measurements (expressed as ΔRU units or relative variation of the refractive index at the Au/solution interface) were obtained with a BIACORE X™ instrument using PIONNER gold sensor chips J1. Phast System™ equipment (Pharmacia LKB Biotechnology) was used for the isoelectro-focusing experiments.

2.3. Monolayer preparation

Au wires (0.5 min diameter, geometrical area ca. 0.16 cm^2, 99.99% purity), used as working electrodes, were successively and manually polished on fine, wet emery paper and with graded (0.3–0.05 μm) alumina (Buehler Inc.). They were first treated with freshly prepared 'piranha' (7:3 mixture of concentrated H_2SO_4 and 30% H_2O_2; *caution*, *piranha reacts violently with organic com-*

pounds) for 30 min, and, finally, with a boiling saturated KOH solution for 2 h. The cleaned wire electrodes were stored in concentrated H_2SO_4. Prior to use, the Au electrodes were dipped in a concentrated HNO_3 solution for 10 min and, thoroughly washed with water. The negatively charged surface (Au/MPS) was prepared by immersing the cleaned gold wire into a 1 mM ethanolic solution of MPS for 12 h, and then rinsed with pure ethanol. The positively charged surface (Au/Cys) was obtained by keeping the cleaned gold electrode for 2 h in a 1 mM aqueous solution of cystamine, and then by rinsing it with water.

2.4. Preparation of self-deposited multilayers

The build-up of the supramolecular architectures was based on the sequential deposition of different polyelectrolytes by alternate immersion of the MPS monolayer covered Au electrode (Au/MPS) in the corresponding aqueous solutions, at room temperature while stirring vigorously (Decher et al., 1992; Lvov et al., 1995; Laurent and Schlenoff, 1997; Caruso et al., 1997a,b). In all experiments the RP was deposited for 2 h from a 20 μg ml^{-1} aqueous solution to guarantee a monolayer deposition. The PSS solution was *25* mg l^{-1} and the adsorption time 1 h. The Binder solution was 10 mg l^{-1} and the corresponding adsorption time was 2 h. All the polyelectrolytes were dissolved in pure water. The concentrations of the polyelectrolyte solutions used in this work were relatively high to assure that a number of charged groups remain exposed to the solution, and thus, the surface charge was effectively reversed.

2.5. Assembly of biocatalytic layers

In order to obtain the fructose bioelectrodes, FDH was immobilized on the multilayer modified electrodes by adsorption (2 h at 4°C without stirring) from a 1 mg ml^{-1} enzyme solution, dissolved in 0.1 M acetate buffer (pH 5.0). Similarly, the H_2O_2 bioelectrode was obtained by self-deposition (for 2 h at 4°C without stiffing) of the modified (vide infra) or native HRP from a 200 μg ml^{-1} enzyme solution, prepared in a 0.1 M phosphate buffer pH 6. The modified HRP was prepared as follows: 5 mg of HRP type VI was dissolved in 1 ml of 0.01 M carbonate buffer pH 9.5 and then 100 μl of 1% 2,4-dinitrofluorobenzene dissolved in ethanol were added. The mixture was gently rotated during 1 h at room temperature enabling protection of the amino groups of the enzyme. One millilitre of 0.08 M sodium periodate aqueous solution was added into the solution and after 30 min of rotation in dark conditions, a 0.6 M aqueous solution of ethylene glycol was used to stop the reaction. The activated enzyme solution was ultrafiltrated through 5000 MWCO membranes (Millipore) and extensively washed with carbonate buffer until complete elimination of the free aldehydes tested with the 3-methyl-2-benzothiazolinone hydrazone assay (Sawicki et al., 1961). The enzyme solution was collected in 2 ml of phosphate buffer 0.1 M pH 7 and taurine powder was added to achieve 0.1 M concentration and gently rotated during 3 h for the formation of a Schiff base between the amino and carbonyl groups of taurine and peroxidase, respectively. Ultrafiltration and extensive washing with phosphate buffer eliminated the excess of reagents. Reduction of the inline bonds was carried out by the addition of 4 μl of 30 mM $NaBH_4$ dissolved in 14 M NaOH. Finally, the modified enzyme containing covalently bound sulfonate groups was purified by ultrafiltration (5000 MWCO) and collected in 2 ml of phosphate buffer. The solution was stored at 4°C until use.

For the methanol configuration, the AOD was self-deposited on the top of a Au/MPS/RP/HRPmod/B structure by its immersion for 2 h at 4°C in 1 mg ml^{-1} of enzyme dissolved in 0.1 M phosphate buffer pH 7.

2.6. Surface plasmon resonance measurements

The gold chips were previously modified with 1 ml of 1 mM ethanolic solution of NIPS followed by addition of pure ethanol during 30 min to avoid evaporation. Finally the chip was rinsed with pure ethanol and dried. The same supramolecular architecture used in the electrochemical experiments was constructed in these MPS gold chips by injecting 100 μl of RP, 20 μg ml^{-1} and 50 μl of PSS, 10 mg ml^{-1} dissolved in pure water. The biocatalytic layers were deposited by 30 μl injections of 1 mg ml^{-1} of FDH enzyme solution dissolved in 0.1 M acetate buffer (pH 5.0), or 10 RL of 1 mg ml^{-1} HRP solution dissolved in 0.1 M phosphate buffer (pH 7.0). The flow rate and the temperature for all the SPR experiments were 20 μl min^{-1} and 25°C, respectively. Milli-Q water was used as a carrier.

2.7. Determination of pI

Polyacrylamide gels containing Pharmalyte(r) carrier ampholytes and IEF 3–9 media were used in the Phast System™ equipment. The isoelectric focusing method and electrophoretic titration curve were carried out according to the optimized procedure described by Pharmacia, using the calibration Kit (3–9) for p*I* determinations. A Phast Gel Silver Kit (Cat. No. 17-0617-01) was used for the visualisation of the bands.

3. Results and discussions

The new strategy recently proposed to obtain reagentless glucose amperometric biosensors, based on multilayer electrostatically self-deposited structures

built up on the electrode surface (Lvov et al., 1995; Mizutani et al., 1996; Hodak et al., 1997; Shi-Feng et al., 1998) was first checked for fructose dehydrogenase (FDH, MW 140 000), a pyrroloquinoline quinone membrane-bound enzyme completely inactive to oxygen as electron acceptor and specific for fructose (Ameyama and Adachi, 1982). Then extended to a horseradish peroxidase (HRP, MW 40 500) a ferriprotoporphyrin X protein widely used as an indicator enzyme, and finally used for a coupled bienzyme system including alcohol oxidase (HRP-AOD, MW_{AOD} 600 000). As mentioned previously, the aim of this work is the demonstration of the possibilities of this technology as a general procedure for the construction of integrated bioelectrocatalytic systems and for this reason three enzymes with a different molecular weight and p*I* were chosen. Our general approach consisted in two parts. First, work was carried out into the electrochemistry of the cationic Os-based redox polymer, electrostatically immobilized in different multilayer self-deposited structures, buit-up on alkanethiolated Au electrodes. Secondly, the Au/MPS/RP modified electrode was used as an electrical interface in order to achieve the electrochemical communication between the selected enzymes and the electrode surface.

3.1. Electrochemical characterisation of the transducing layer: Os-redox polymer adsorbed on multilayer self-deposited structures built up on alkanethiolated gold electrodes

The electrochemistry of a water soluble cationic poly[(vinylpyridine)$Os(bpy)_2Cl$] redox polymer (RP) adsorbed at graphite electrodes has been investigated previously (Katakis, 1994). Briefly, it was found that, in quasi steady-state conditions (potential scan rate of 1 mV s^{-1}), this redox polymer presents one electron surface wave ($E^{0'} = 295$ mV versus SCE) corresponding to the $Os^{III/II}$ complexed redox pair, reflecting the different orientation of reduced and oxidised species on the electrode surface ($\Delta E_p = 38$ mV) and affected by some weak interactions between the surface adsorbed species ($E_{HWFM,a} = 105$ mV, $E_{HWFM,c} = 123$ mV).

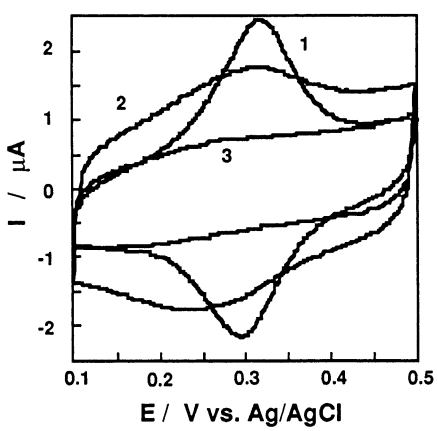

Fig. 1. Voltammetric response of the cationic Os-redox polymer (RP) adsorbed on gold electrodes: (1) Au/MPS/RP; (2) Au/RP; (3) Au/MPS. Experimental conditions: scan rate, 50 mV s^{-1}; initial potential, + 0.1 V versus Ag/AgCl, KCl_{sat}; RP was adsorbed from a 10 μg ml^{-1} aqueous solution; supporting electrolyte, 0.1 M phosphate buffer (pH 6.0) containing 0.15 M NaCl.

As can be seen in Fig. 1, the voltammetric response of the positively charged RP adsorbed at bare gold electrodes (voltammogram 2) showed a very poorly shaped peak, developed on a large capacitive current. In the same experimental conditions, the presence of an MPS negatively charged monolayer, self-assembled on the Au electrode surface, exerted a dramatic effect on the RP voltammetric response, giving rise to a well defined redox wave (voltammogram 1). The electrochemical parameters for this wave, recorded at a scan rate of 50 mV s^{-1} ($E^{0'} = 308$ mV versus Ag/AgCl, KCl_{sat}, $\Delta E_p = 13$ mV and $E_{HWFM} = 85$ mV), correspond to a surface confined redox couple, strongly adsorbed or presenting some low associative interactions (E_{HWFM}, slightly lower than the theoretical 90.6 mV value for a monoelectronic process) (Laviron, 1982) and a local non-equivalence of the reduced and oxidized species ($\Delta E_p \neq 0$) (Honeychurch and Rechnitz, 1998a,b). This is to be expected due to the change in charge between the two species. The number of electrons involved in the redox process was calculated from the Ip-v dependence (Murray, 1984; Merz, 1990; Murray, 1992), and it was found close to the expected value of 1 with an uncertainty below 25%. The presence of a spatially distributed negative charge between the Au electrode and the RP induced a standard potential shift (about 20 mV) towards more negative potentials, as was found before for the $Fe(CN)_6^{3/4-}$ redox system (Sayre and Collard, 1997), ferrocene-modified PAH (Hodak et al., 1997) and PBV (Laurent and Schlenoff, 1997) redox polymers (where PAH and PBV represent poly(allylamine) hydrochloride and poly(butanylviologen) dibromide, respectively).

It is worth mentioning the marked decrease of the background (capacitive) current induced by the presence of the MPS self-assembled monolayer (voltammogram 3). Similar behaviour was observed for a cationic poly(allylamine) modified with a ferrocene polymer and electrostatically deposited on an Au electrode modified by a negatively charged MPS self-assembled monolayer (Hodak et al., 1997). It appears that such behaviour is due to the passivation of the surface due to the MPS layer or the rearrangement of the double layer and due to the higher deposition of RP due to electrostatic attraction.

The electron transfer theory (Marcus and Sutin, 1985) predicts, and experimental studies on modified electrodes have proved (Murray, 1984, 1992; Merz,

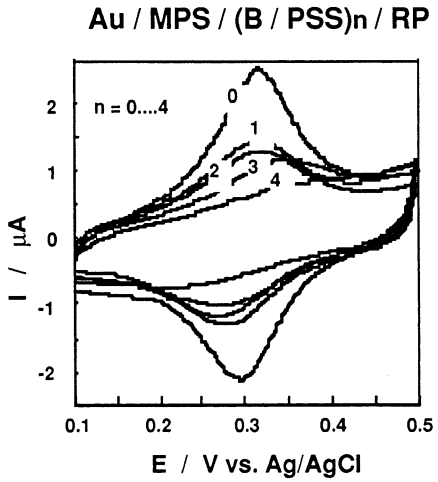

Fig. 2. Effect of the distance on the redox polymer voltammetric response. Experimental conditions as in Fig. 1; *n* denotes the number of B/PSS layers.

Table 1
Electrochemical parameters of the voltammetric response for the Os-redox polymer immobilized on different multilayer self-deposited structures on alkanethiolated Au surface[a]

Au/MPS/(B/PSS)$_n$/RP (n)	ΔE_p (mV)	E_{HWFM} (mV)	I_{ap}/I_{cp}	$E^{0'}$ (mV)
0	13	85	0.99	308
1	28	85	0.97	299
2	36	90	0.95	295
3	45	93	1.03	310
4	83	134	0.99	300

[a] Experimental conditions as in Fig. 1.

1990), that the electron tunnelling rate constant is strongly dependent on the distance between the donor and acceptor. Consequently, if after a sequential deposition of electrochemically inactive polyelectrolytes (B/PSS), an orderly supramolecular self-deposited structure was built up, the voltammetric response of the RP should progressively diminish when the distance between its real location and the electrode surface increases. The cyclic voltammograms recorded at 50 mV s^{-1} for Au/MPS/(B/PSS)$_n$[RP electrodes and presented in Fig. 2, prove, at least qualitatively, that for *n* varying between 0 and 4, the RP is really immobilised in its actual deposition layer. The corresponding electrochemical parameters, listed in Table 1, point out that, excepting ΔE_p up to $n = 3$ the adsorbed RP does not significantly change its electrochemical behaviour. As expected, the increase of the peak separation with distance indicates slower electron transfer due to certain contribution of the charge diffusion to the electron transfer mechanism. These results are similar to those recently reported by Laurent and Schlenoff (1997), which found that after four layers of insulating 'spacers' (PAR/PSS) the top PBV redox layer response was no longer detectable on the cyclic voltammogram recorded at 50 mV s^{-1}.

All the above work demonstrates an ordered self deposition, but it also proves the expected decrease of the Faradaic current with distance between donor and acceptor (in this case electrode and polymer). In other words, by introduction of a given number of layers, the redox polymer can be insulated, but ideally these architectures should offer the possibility of connecting multiple layers of redox polymers and, additionally, they should be implemented to other electron donors with additional biological activity.

Preliminary studies using surface plasmon resonance were made not only to demonstrate the deposition of the non-electrochemically active PSS but also to see by an independent technique the ordered deposition of multiple layers of redox polymer. The almost constant ΔRU per layer found after deposition of 12 layers of redox polymers ($2319 \pm 13\%$, and $722 \pm 30\%$ for RP and PSS, respectively) demonstrated the possibility of assembling a large number of orderly redox layers. Whether all these layers are equally connected has to be demonstrated by electrochemical techniques.

Cyclic voltammograms recorded at 0.1 V s^{-1} in a 0.1 M acetate buffer solution pH 5.0 containing 0.15 M NaCl for Au/MPS/RP electrodes, assembling further RP layers through non-electrochemically active PSS, revealed an increase of the surface coverage with the number of RP layers at least up to $n = 3$ levelling of after this layer (data not shown). It has to be noted that in the case of sequential deposition of RP/RP type structures no charge build up was observed. The asymptotic behaviour could be due to the gradual change of the charge transfer mechanism induced by the increase of redox layers. In fact, the deposition of successive PSS/RP modules, provides an almost diffusion-free electron communication of the newly deposited redox layers with the electrode surface up to three RP layers. After this point, the distance from the surface is such, that the communication of the outer layers must be highly electron-diffusion limited. This change of the charge transfer mechanism through the successive layers is demonstrated by the shift from a direct scan rate dependence to a square root scan rate dependence of the peak currents (Fig. 3A and B, respectively) in the case of one and five redox layers.

3.2. Assembly of catalytic layers: a generic strategy to vary the donor–acceptor deposition tailoring biosensor characteristics

As previously mentioned, the versatility of these structures should open up the possibility of integrating other redox molecules. We propose to demonstrate this with three different oxidoreductases.

3.2.1. Fructose reagentless amperometric biosensor

FDH, a 140 kDa membrane-bound pyrrolo-quinoline quinone-containing oxidoreductase, immobilized on different electrode materials (glassy carbon, gold, platinum, carbon paste), has been used to construct a variety of amperometric biosensors for fructose. The necessary enzyme–electrode electrical communication was achieved either directly (Ikeda et al., 1991; Khan et al., 1991, 1992; Begum et al., 1993; Aizawa et al., 1994; Parellada et al., 1996; Yabuki and Mizutani, 1997) or by using different diffusional electron-transfer mediators as hexacyanoferrate (III) (Xie et al., 1991; Matsumoto et al., 1993; Khan et al., 1993), *p*-benzoquinone (Ikeda et al., 1990), ferrocene (Khan et al., 1993), coenzyme Q_6 (Kinnear and Monbouquette, 1997) or $Os(bpy)_2Cl_2$ (Paredes et al., 1997).

Despite the variety of all the above transducing chemistries, the loss of the mediator (recognised as the main disadvantage for the schemes based on diffusional electrochemical communication) and the low sensitivity, characteristic of the direct electron transfer, channelled our efforts to interface orderly FDH with the Os-based redox polymer described in the previous section. Preliminary experiments showed that no direct electron transfer could be observed in the presence of fructose between FDH and, either positively (cystamine) or negatively (MPS) charged modified Au electrode surfaces. However, FDH was electrically connected through the RP showing the bioelectrocatalytic currents depicted in Fig. 4A. Higher currents were observed immediately when FDH was self-deposited upon the RP layer (voltammogram 1). In fact the introduction of a negatively charged PSS layer between the redox polymer and this enzyme halved the electrocatalytic current (voltammogram 2). Several reasons could contribute to this dependence of the FDH electrocatalytic efficiency in the ordered structure. As electrostatic interactions between layers create the driving force for self-deposition in these architectures, its seems quite obvious to think that the overall charge of FDH at pH 5 is negative and thus, its deposition after a positive layer more favoured. Literature data about p*I* of FDH sup-

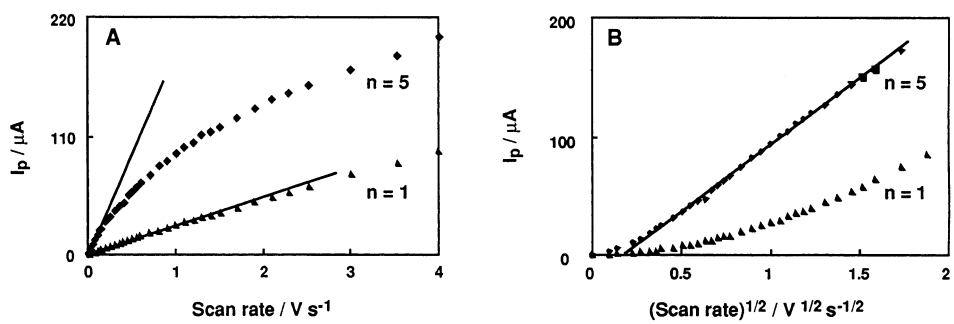

Fig. 3. Dependence of the anodic peak current on the scan rate (A) or on its square root (B) for $n = 1$ (▲) and for $n = 5$ (◆). Experimental conditions as in Fig. 1 n denotes the number of RP layers.

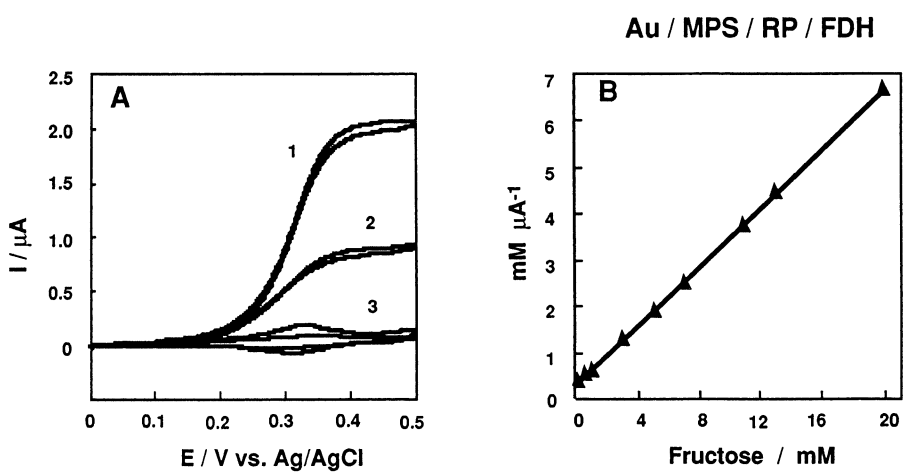

Fig. 4. (A) Dependence of the electrocatalytic current on the distance between the catalytic layer and the electrochemical interface: (1) Au/MPS/RP/FDH; (2) Au/MPS/RP/PSS/FDH; (3) Au/MPS/RP shown as control of each configuration when no fructose is present. Experimental conditions: scan rate, 2 mV s^{-1}; initial potential, 0 mV versus Ag/AgCl, KCl_{sat}; 0.1 M acetate buffer (pH 5.0) containing 0.15 M NaCl and 20 mM of fructose. (B) Hanes–Woolf linearisation of the experimental data for Au/MPS/RP/FDH configuration. Experimental conditions: 0.1 M acetate buffer (pH 5.0) containing 0.15 M NaCl; working potential +450 mV versus Ag/AgCl, KCl_{sat}.

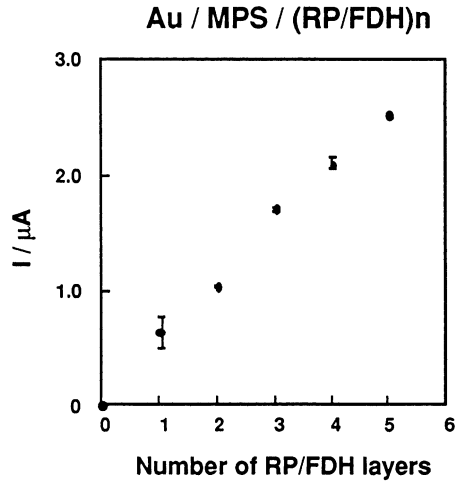

Fig. 5. Dependence of the electrocatalytic current on the number of catalytic and redox layers: Au/MPS/(RP/FDH)$_n$. Experimental conditions: 0.1 M acetate buffer (pH 5.0) containing 0.15 M NaCl and 20 mM of fructose, working potential +450 mV versus Ag/AgCl, KCl_{sat}. Error bars represent the standard deviation for three measurements.

port this idea (Ameyama et al., 1981). However, isoelectric focusing experiments with this enzyme showed six bands corresponding to p*I* 8.32, 8.07, 7.82, 5.30, 5.18, and 4.78 (underlined bands being quantitatively more important) thus, at pH 5.0, the enzyme could be considered as positively charged. This is also supported by the results obtained when deposition of FDH was monitored with SPR. A ΔRU value of 2545 was found when adsorbed upon the positively charged RP while a value of 3276 was observed if FDH was adsorbed upon the negatively charged PSS. It seems clear then that a greater amount of enzyme is self-deposited on a negative layer, despite the higher current found when deposited immediately on the RP. In the same manner that we have previously showed the importance of the distance between donor and acceptor, the electron transfer rate between FDH and the RP is also strongly influenced by the distance.

The dependence of the steady-state catalytic current on fructose concentration for the Au/MPS/RP/FDH electrode fits the Michaelis–Menten kinetics (data not shown). The $I_{max} = 3.15$ μA and $K_m = 1.02$ mM parameters, calculated from the Hanes–Woolf linearisation of the experimental data (the correlation coefficient 0.9999, for nine experimental data, Fig. 4B), give an estimated electrode sensitivity of 19.3 mA M^{-1} cm^{-2}. This value is very close to those reported for a biosensor based on carbon paste incorporating FDH and $Os(bpy)_2Cl_2$ as mediator (15.2 mA M^{-1} cm^{-2}) (Paredes et al., 1997), and for a membrane mimetic Q_{-6}/FDH immobilised on the gold electrode surface modified with a mixture of octadecyl mercaptan, cystamine dihydrochloride and 3, 3′ dithio-dipropionic acid (15 mA M^{-1} cm^{-2}) (Kinnear and Monbouquette, 1997).

The repeatability of this kind of structures showed a relative standard deviation of 6.3% after six consecutive measurements while the reproducibility of six different electrodes showed a value of 16%.

Considering that these architectures are limited by the catalytic activity of FDH as demonstrated by the observed current increase when FDH in solution was added to the Au/MPS/RP/FDH sensor, they offer an easy way to manipulate the overall response. If higher currents are required, successive deposition of layers would yield the desirable conversion. As an example, the electrocatalytic current intensity, measured at 450 mV versus Ag/AgCl KCl_{sat} for Au/MPS/(RP/FDH)$_n$ supramolecular structures showed a linear dependence for (at least) $n = 1–5$ resulting $I_{cat}(\mu A) = 0.51n + 0.07$ with a correlation coefficient of 0.997 (Fig. 5).

Overall, the design of a reagentless fructose sensor with the desirable properties seems feasible with this technology and it has been shown in the case of an enzyme with a rather low catalytic turnover rate. Multiple deposition of RP/FDH modules results in increased sensitivities but other parameters (e.g. response time, dynamic range) could be easily manipulated at will taking advantage of simple prediction electron tunnelling theory.

3.2.2. Hydrogen peroxide and methanol reagentless amperometric biosensors

Many efforts have been focused on the electrochemical communication of HRP, since this enzyme is used routinely in many immunoassays for transducing immunoreactions, and it is used as the indicator enzyme in many H_2O_2 producing oxidases.

The experience gained with FDH was transferred to HRP, a rather cationic protein at pH 7.0 (Isoenzyme Q having a p*I* of 7.8. Previously, the absence of direct electrical communication was observed between this heme protein and Au/MPS after addition of hydrogen peroxide. Surprisingly, it was also found that no significant electrocatalytic currents could be detected with either Au/MPS/RP/HRP or Au/MPS/RP/PSS/HRP electrodes at pH 7.0. Surface plasmon resonance experiments showed that this lack of electrocatalytic current could be due to the low amount of enzyme deposited onto the layers (data not shown). Consequently, the manipulation of the overall charge of HRP seemed necessary. This enzyme (Isoenzyme C, wild type) is a monomeric heme protein of 308 residues containing two structural Ca^{2+} ions and eight N-linked neutral glycan sites positioned in the external loop regions (Gajhede et al., 1997) that can be easily oxidised. Thus, chemical derivation introducing sulfonate groups covalently bound to the enzyme was carried out to manipulate the overall charge of the enzyme in order to achieve significant deposition of the biomolecule.

A comparison of the electrocatalytic currents obtained with the native and modified HRP is presented in Fig. 6A. An increase of almost six fold is observed after chemical derivation, despite the loss of catalytic activity observed by the introduction of sulfonate groups (comparison of activities was measured spectrophotometrically in solution). The strong electrostatic interactions between the RP and the modified HRP enabling adsorption of higher amounts of enzyme may be the reason for the efficient electrochemical communication. SPR experiments confirmed that the amount of protein deposited upon the RP layer was 40 times higher with the chemically modified peroxidase than in the case of the native enzyme ($\Delta RU = 400$ for the modified enzyme and $\Delta RU = 10$ for the native enzyme).

The steady-state response curve for H_2O_2 under vigorous stiffing is presented in Fig. 6B with a maximum current density of 12.7 μA cm^{-2} and an apparent K_m, of 56 μM, calculated from Hanes–Woolf linearisation with a correlation coefficient of 0.997, and 25 experimental data (inset in Fig. 6B). This configuration also offers very good repeatability with a RSD of 12% for $n = 3$. The sensitivity obtained was of 58.1 mA M^{-1} cm^{-2} being comparable to 60 mA M^{-1} cm^{-2} at pyrolytic graphite electrodes with HRP adsorption (Wollemberger et al., 1990), to 73 mA M^{-1} cm^{-2} with HRP electropolymerised in *o*-phenylenediamine (Deng and Dong, 1994), but still far from the 1 A M^{-1} cm^{-2} obtained with redox hydrogel (Vreeke et al., 1992). Nevertheless, the multilayer configuration presented here easily allows further increase of the sensitivity by a rational design of the donor–acceptor assembly.

Having connected the peroxidase, this electrode configuration opens up many possibilities for the construction of biosensors based on H_2O_2 producing oxidases, particularly those that currently do not electrically communicate at all with electrode surfaces, such as alcohol oxidase. A schematic representation of the multilayer configuration including AOD is depicted in Fig. 7A. Preliminary control experiments in the absence of HRP confirmed the lack of electrochemical communication between AOD and the Au surface. The enzyme used is a large molecule of 600 kDa with a p*I* of 6.3 and consequently rather anionic at the working pH (pH 7.0) demanding a positive layer for electrostatic deposition. This was achieved by deposition of a positively charged polyelectrolyte on the modified peroxidase. Optimally, the hydrogen peroxide produced after addition of methanol will diffuse to the peroxidase layer that will transduce the peroxide flux into electrons through the RP interface. The feasibility of this supramolecular structure is demonstrated in Fig. 7B showing the steady state response curve at 100 mV versus Ag/AgCl. The maximum current densities obtained with these electrodes were 0.91 μA cm^{-2} with a sensitivity of 10.6 mA M^{-1} cm^{-2}. The relative response for methanol in relation to hydrogen peroxide remained in the order of 18% showing a limitation in this first catalytic step. Chemical modification of this enzyme is being developed in our laboratories in order to introduce an overall positive charge that will permit deposition on the peroxidase layer and will also increase the amount of adsorbed enzyme.

4. Conclusions

A generic technology for the modular construction of biosensors based on the layer-by-layer built up of transducing and biorecognition chemistries has been demonstrated. The technique is modular, not only because it allows the choice of enzyme systems and combinations

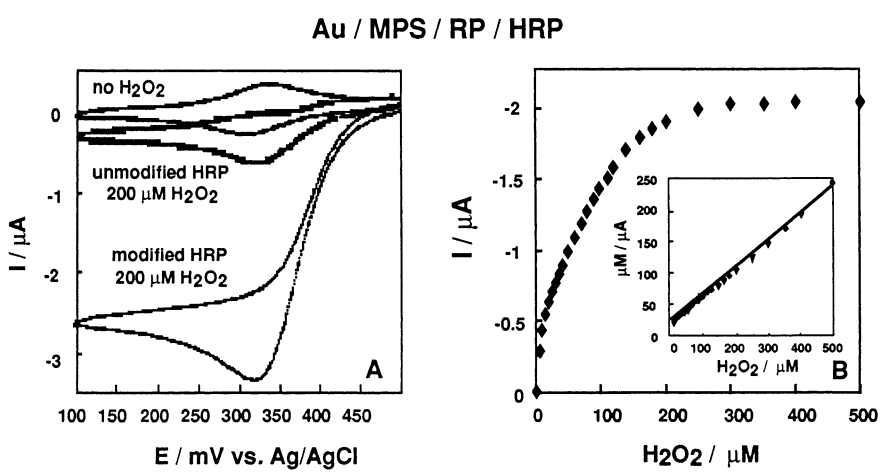

Fig. 6. (A) Electrocatalytic response for wild type HRP and modified HRR Experimental conditions: 0.1 M phosphate buffer containing 0.15 M NaCl (pH 7.0); initial potential, + 500 mV versus Ag/AgCl, KCl_{sat}; scan rate 2 mV s^{-1}. (B) Steady-state response curve for H_2O_2. The inset in B represents the Hanes–Woolf plot of these values. Experimental conditions: 0.1 M phosphate buffer containing 0.15 M NaCl (pH 7.0); working potential, + 100 mV versus Ag/AgCl, KCl_{sat}.

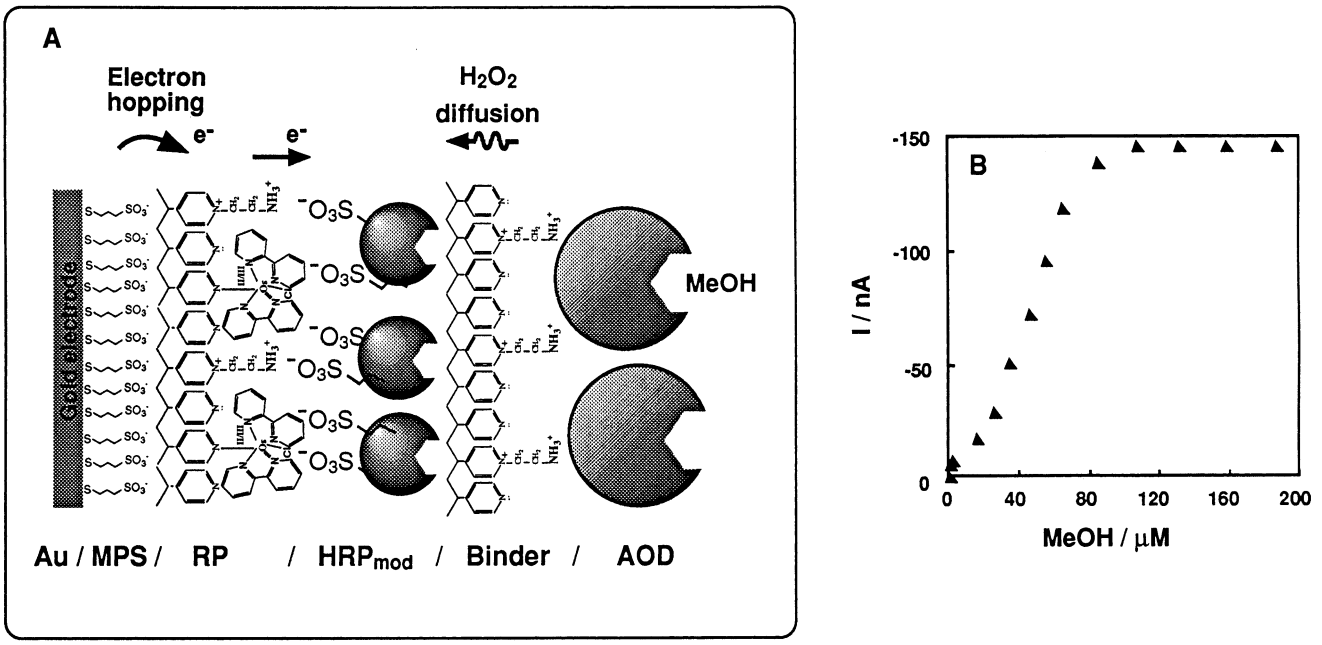

Fig. 7. (A) Schematic representation of the reagentless biosensor for the detection of methanol and (B) steady-state response curve for methanol. Experimental conditions: 0.1 M phosphate buffer containing 0.15 M NaCl (pH 7.0); working potential, +100 mV versus Ag/AgCl, KCl_{sat}.

(shown here with protein-bound cofactors) but also the rational modulation of their analytical properties. This generic technology has been demonstrated for fructose dehydrogenase, horseradish peroxidase and the couple HRP–alcohol oxidase being electrically connected to the electrode by a fast electrochemical interface. The feasibility of the methanol electrode configuration including coupled catalytic reactions, substrate and product diffusion and heterogeneous electron transfer steps demonstrates the suitability of this technology for the construction of integrated biochemical systems which is expected to be of general applicability to affinity sensors as well.

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