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A Biotinylated Conducting Polypyrrole for the Spatially Controlled Construction of an Amperometric Biosensor

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A new biotin derivative functionalized by an electropolymerizable pyrrole group has been synthesized. The electrooxidation of this biotin pyrrole has allowed the formation of biotinylated conducting polypyrrole films in organic electrolyte. Gravimetric measurements based on a quartz crystal microbalance, modified by the biotinylated polymer, revealed an avidin−biotin-specific binding at the interface of polymer−solution. The estimated mass increase corresponded to the anchoring of 1.5 avidin monolayers on the polypyrrole surface. In addition, the subsequent grafting of biotinylated glucose oxidase was corroborated by electrochemical permeation studies. Enzyme multilayers composed of glucose oxidase or polyphenol oxidase were elaborated on the electrode surface modified by the biotinylated polypyrrole film. The amperometric response of the resulting biosensors to glucose or catechol has been studied at +0.6 or −0.2 V vs SCE, respectively.

In recent years, there has been a growing interest in electrochemical microdevices owing to their potential application in clinical analysis and environmental control.[1] In particular, the development of miniaturized biosensors constitutes an attractive avenue for the ex vivo and in vivo measurements of metabolites. However, the reproducible immobilization of biological macromolecules on microsurfaces with a precise spatial resolution remains a crucial problem in automating the fabrication of miniaturized biosensors. Among the conventional methods of microtransducer functionalization, the immobilization of biomolecules on electropolymerized films is gaining importance.[2−4] One major advantage of electrochemical deposition procedures is the possibility of precisely electrogenerating a polymer film on conductive microsurfaces of complex geometry. In addition, the polymer films exhibit a good robustness in operation in aqueous and organic media. The electrochemical method involves, initially, the electrogeneration of functionalized conducting polymers. Then the attachment of biomolecules to the polymer surface can be obtained by covalent binding to adequate functional groups such as amino,[5−7] carboxylic,[8] and *N*-hydroxysuccinimide and *N*-hydroxyphthalimide esters groups.[9,10] The main advantage of this sequential procedure, namely, electropolymerization and covalent binding, lies in the possibility of using optimal conditions for each step. However, the covalent binding could induce chemical denaturation of the protein or influence protein flexibility. These effects may affect the catalytic activity or the recognition properties of the biomolecules.

To preserve their complete biological properties, the immobilization of proteins on electrogenerated polymer films has recently been attempted by the use of an affinity system.[11−15] Because of the high affinity of the avidin−biotin interaction (association constant $K_a = 10^{15}$ M^{-1}),[16] this coupling system has been used for protein immobilization on biotinylated polymer films. The attachment of proteins was achieved via the simple formation of an avidin−biotin bridge with biotinylated proteins or avidin-conjugated proteins.

The formation of biotinylated films was first attempted by electropolymerization of biotin derivatives functionalized by a phenol group.[11,12] Unfortunately, the electropolymerization provides nonconductive films which passivate the electrode surface

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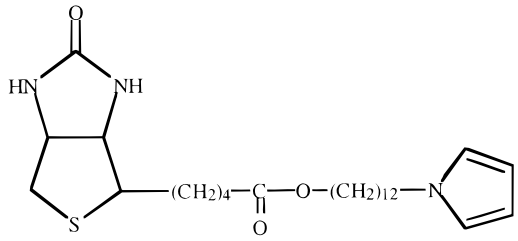

Figure 1. Structure of the pyrrole biotin **1**.

and prevent further film growth. In addition, their formation is restricted to very thin films. Consequently, the protein coupling could decrease the polymer stability.

An alternative consists of the electrogeneration of electronic conducting polymers functionalized by biotin moieties. In particular, their conductivity allows the reproducible formation of polymer layers of electrochemically controlled thickness. With this aim in view, biotin derivatives bearing an electropolymerizable pyrrole group have been recently synthesized and applied to the elaboration of electrically conducting polypyrrole films.[13−15] However, owing to the poor electropolymerization ability of these biotin derivatives, polymer formation requires a copolymerization process with free pyrrole,[14] a pyrrole ammonium,[15] or a polypyridinyl complex of ruthenium(II) containing three pyrrole groups.[13] As a consequence, the copolymerization has led to copolymer films that could exhibit a heterogeneous distribution of the polymerized biotin groups. This may induce a poor degree of control over the molecular architecture of the biorecognition layer at the interface of polymer−solution.

In this paper, we report the first example of the electrogeneration of a pure biotinylated conducting polypyrrole film from a novel pyrrole biotin (**1**) (Figure 1). Gravimetric measurements were carried out using a quartz crystal microbalance (QCM) to characterize the efficient coupling of avidin onto the poly **1** film. The potentialities of the biotinylated polypyrrole film (poly **1**) for the biomolecule grafting have been illustrated in connection with biotinylated glucose oxidase and polyphenol oxidase. The analytical characteristics of the resulting amperometric biosensors toward the measurement of glucose and catechol are described.

EXPERIMENTAL SECTION

Reagents. Avidin (chromatographically purified), polyphenol oxidase (PPO) (EC 1.14.18.1, from mushroom, 3800 units mg^{-1}), and glucose oxidase-biotinamidocaproyl labeled (GOx-B) (EC 1.1.3.4, from *Aspergillus niger*, 110 units mg^{-1}) were purchased from Sigma. Biotin-labeled polyphenol oxidase (PPO-B) was prepared as follows: 3 mg of PPO was dissolved in 0.6 mL of 0.1 M $NaHCO_3$. After complete dissolution of PPO, 45 μL of 0.25 M *N*-hydroxysuccinimidobiotin was added with constant stirring. The resulting solution was kept under constant stirring in the dark for an additional 60 min. The resulting biotinylated enzyme was purified in a Sephadex G25 column (1.0 × 12 cm) equilibrated and eluted with 5 mM Tris-HCl, pH 7.0, and lyophilized.

12-(1-Pyrrolyl)dodecanol. This compound was prepared as described previously.[17] The pyrrole biotin (**1**) was synthesized in the following manner: biotin (500 mg, 2 mmol) 12-(1-pyrrolyl)-dodecanol (417 mg, 1.66 mmol), 1,3-dicyclohexylcarbodiimide (413 mg, 2 mmol), and 4-(dimethylamino)pyridine (81 mg, 0.66 mmol) were dissolved in dry deoxygenated CH_2Cl_2 (10 mL). The mixture was stirred at room temperature for 5 days under argon. The resulting solution was filtered and evaporated. After extraction, **1** precipitated as a white solid. This crude product was then chromatographed on silica gel with a 95:5 CH_2Cl_2/Et_2O mixture as eluting solvent, yielding 618 mg of **1** (78% yield): FAB-MS (*m*/*z*) 478 ($[M + H]^+$), 476 ($[M - H]^+$); ^1H NMR (200 MHz) (DMSO) δ 1.30−1.70 (m, 26 H), 2.25 (t, 2 H), 2.58 (m, 1 H), 2.88 (m, 1 H), 3.08 (m, 1 H), 3.82 (t, 2 H), 4.00 (t, 2 H), 4.12 (m, 1 H), 4.30 (m, 1 H), 5.95 (m, 2 H), 6.35 (s, 1 H), 6.40 (s, 1 H), 6.70 (m, 2 H).

Water was doubly distilled in a quartz apparatus. All other reagents used were of analytical reagent grade. Stock solutions of glucose were allowed to mutarotate at room temperature for 24 h before use and were kept refrigerated.

Safety rules, accepted by the European Community, must be taken into consideration when handling with acetonitrile (S, 16-27-45) and catechol (S, 22-26-37).

Electrochemical Instrumentation and Procedure. Electrochemical investigations were performed with an EG&G Princeton Applied Research 273A potentiostat in conjunction with a Kipp and Zonen BD90 *XY*/*t* recorder. The amperometric measurements of glucose and catechol were performed with a Radiometer Analytical PRG-DL potentiostat.

The electropolymerization of **1** and the electrochemical characterization of **1** and poly **1** film were run at room temperature under an argon atmosphere in a conventional three-electrode cell. Acetonitrile (Rathburn, HPLC grade) was used as received. Tetrabutylammonium perchlorate (TBAP) was recrystallized from ethyl acetate/cyclohexane and vacuum-dried at 80 °C three days before use. A 10 mM Ag/Ag^+ in CH_3CN electrode was used as reference electrode in acetonitrile electrolyte, and a saturated calomel electrode (SCE) was used as reference electrode in 0.1 M phosphate buffer. The working electrodes were platinum or glassy carbon disks (diameter 5 mm) polished with 1-μm diamond paste.

The electrochemical measurements were performed under stirred conditions in an air-saturated 0.1 M phosphate buffer solution (pH 6.5 or 7). These experiments were carried out in a conventional three-electrode cell thermostated at 30.0 ± 0.1 °C.

Enzyme Electrode Preparation. The poly **1** films were elaborated by controlled potential oxidation at 0.85 V of 2 mM monomer **1** in CH_3CN + 0.1 M TBAP. The thickness of the poly **1** films was controlled by integration of the electrical charge passed during the electropolymerization.

The modified electrodes were transferred in CH_3CN + 0.1 M $LiClO_4$ in order to replace the initially incorporated cation (TBA^+) by the electrolyte cation (Li^+) by repeatedly scanning the electrode potential at 0.1 V s^{-1} over the polypyrrole oxidation wave.[18] The polypyrrole electroactivity of the modified electrodes was destroyed by overoxidation at 1.3 V for 5 min.

The specific binding of avidin to the polymerized biotin sites was performed by depositing, for 30 min at 4 °C, 20 μL of avidin solution (2 mg mL^{-1}) on the poly **1** disk electrodes (diameter 5 mm). The resulting electrodes were carefully washed with phosphate buffer, and 20 μL of biotinylated enzyme solutions (1

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mg mL^{-1} in 1 mM phosphate buffer) was deposited on the electrode surfaces for 30 min at 4 °C. The resulting enzyme electrodes were carefully rinsed in distilled water and incubated 30 min in stirred phosphate buffer (pH 6.5 or 7) to remove the nonspecifically bound PPO-B or GOx-B molecules.

The modification of the polymer surface by multiple layers of the avidin-biotinylated enzyme system was carried out by the successive deposition of avidin and biotinylated enzyme solutions following the preceding procedure.

Gravimetric Measurements. AT-cut 9-MHz quartz crystals (CQE) coated with two identical gold layers (2000 Å thick) were modified by electrogeneration of a poly **1** film onto one gold electrode (surface area, 0.27 cm^2). Then, the crystal was mounted between two O-rings seals inserted in a plexiglass cell, the poly **1** electrode being in contact with the solution. The flow-through cell (volume, 50 μL) was associated with a micropump (P1, pharmacia) allowing a constant flow rate of 60 μL min^{-1}. The experimental setup was built by coupling a homemade 27-MHz QCM, by using the 9-MHz resonator on the third overtone, and a frequency counter (HP 53132 A).[19,20] This 27-MHz QCM transducer exhibits a sensitivity of 360 pg Hz^{-1}.[19,20] In the continuous-flow mode, the frequency shift was monitored for successive flow carriers: 0.01 M phosphate-buffered saline (PBS) (pH 7.4, 137 nM NaCl, 2.7 mM KCl), then PBS containing avidin (0.5 mg mL^{-1}), and PBS again.

The same procedure was applied to a crystal modified by an electropolymerized poly(*N*-methylpyrrole) for checking the specificity of the biotinylated polymer.

Assays. The catalytic oxidation of glucose by GOx in the presence of dioxygen produced H_2O_2. Consequently, the enzymatic activity of the immobilized GOx-B was determined by measuring amperometrically the increase in H_2O_2 concentration.[21] For this purpose, a Pt electrode was immersed in stirred 0.1 M phosphate buffer (pH 7) containing glucose (50 mM) and potentiostated at 0.6 V. Then, the electrode modified by GOx-B was soaked in the aqueous electrolyte, and the increase in anodic current vs time was recorded. The enzymatic activity of the biosensor was evaluated from the slope of the rectilinear part of the current vs time dependence, by comparison with the slope obtained in the same conditions with the free enzyme.

RESULTS AND DISCUSSION

The electrochemical behavior of the pyrrole biotin was investigated by cyclic voltammetry at a platinum electrode. The cyclic voltammogram of **1** recorded in CH_3CN + 0.1 M TBAP displays, upon oxidative scanning, an irreversible peak at 0.96 V due to the oxidation of the pyrrole group (Figure 2). Electropolymerization of **1** was accomplished by controlled-potential oxidation at 0.8 V to prevent the overoxidation of the polypyrrole skeleton and hence to preserve its conductivity. The resulting electrode was transferred with thorough rinsing to a CH_3CN + 0.1 M TBAP solution free of monomer. The cyclic voltammogram of this electrode exhibited a reversible oxidation wave around 0.4 V, which is in good agreement with the reported $E_{1/2}$ values for poly(*N*-alkylpyrroles)[22] (Figure 3). This indicates the formation of an electropolymerized conducting film (poly **1**) on the electrode surface. In contrast with our previous attempts related to the electropolymerization of 1-(3-biotinylpropyl)pyrrole,[13,15] this behavior is quite typical of polypyrroles, which become electronically conducting in the oxidized state. Thus, it appears that the increase in the length of the flexible spacer from 3 to 12 methylene groups between pyrrole and biotin moieties improves markedly the electropolymerization ability of the pyrrole biotin. Moreover, this longer alkyl chain should facilitate the further coupling of biotin to avidin.[14] The apparent surface coverage of electropolymerized **1**, $\Gamma_1 = 7.3 \times 10^{-9}$ mol cm^{-2}, was determined from the charge recorded under the oxidation wave of the polypyrrole backbone. Owing to its conductivity, the amount of poly **1** film increases almost linearly with the charge passed during the electropolymerization step. Moreover, the electropolymerization yield is slightly better than those previously reported for electronically conducting N-substituted polypyrroles,[23] the mean value being 33% for poly **1** electrodes exhibiting apparent surface coverages

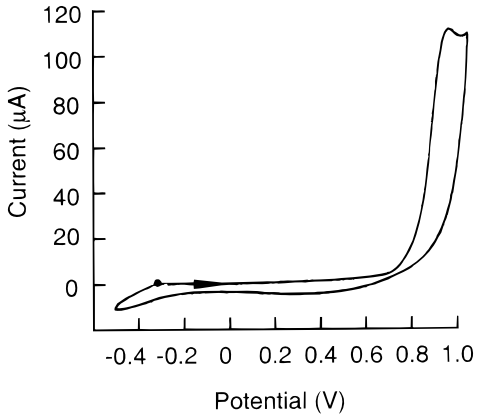

Figure 2. Cyclic voltammograms recorded at a Pt disk electrode (diameter 5 mm) of **1** monomer (2 mM) in CH_3CN + 0.1 M TBAP. Scan rate, 0.1 V,s^{-1}.

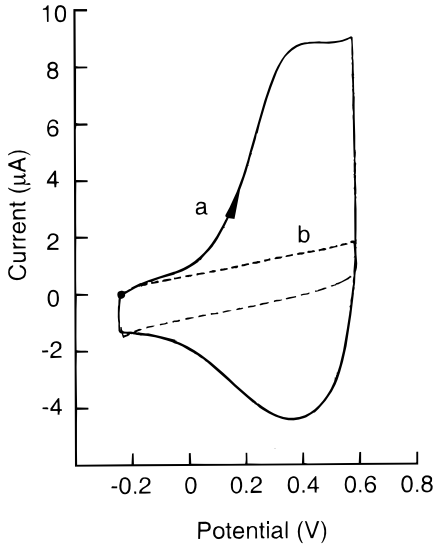

Figure 3. Cyclic voltammograms of a poly **1** electrode ($\Gamma_1 = 7.3 \times 10^{-9}$ mol cm^{-2}) (a) before and (b) after overoxidation of the polypyrrolic chains at 1.3 V for 5 min in CH_3CN + 0.1 M $LiClO_4$; scan rate, 0.1 V s^{-1}.

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between 8.5×10^{-10} and 1.33×10^{-8} mol cm^{-2}. To determine the more appropriate thickness of the poly **1** film, its permeability has been investigated in phosphate buffer by studying the electrochemical response of a neutral molecule. Biotinylated glucose oxidase (GOx-B) and polyphenol oxidase (PPO-B), which catalyze the formation of H_2O_2 and *o*-quinones, have been chosen to illustrate the potentialities of biotinylated polypyrrole for the biomolecule grafting. Consequently, *p*-benzoquinone was chosen as the electroactive probe for the permeation measurements. The cyclic voltammograms of *p*-benzoquinone were recorded at a bare electrode and at poly **1** electrodes exhibiting different polymer thicknesses. The permeability was expressed as the ratio of the peak current for the reduction of *p*-benzoquinone at the poly **1** electrode and at a bare electrode. As expected, this ratio decreases for an increase in poly **1** thickness, namely, 0.53, 0.42, and 0.3 for 0.85×10^{-9}, 1.66×10^{-9}, and 3.00×10^{-9} mol cm^{-2}, respectively. Taking into account that the surface area of a (12-pyrrol-1-yldodecyl)triethylammonium (2 nm^2)[24] may be approximated to the surface area of **1**, a monolayer of poly **1** film would correspond to 8.3×10^{-11} mol cm^{-2}. This provides a crude estimation of the number of equivalent monolayers of the three poly **1** coatings, namely, 10, 20, and 36 equivalent monolayers for 0.85×10^{-9}, 1.66×10^{-9}, and 3.00×10^{-9} mol cm^{-2}, respectively. Therefore, to prevent pinholes or other defects, the value of polymer thickness $(1.6 \pm 0.1) \times 10^{-9}$ mol cm^{-2} was chosen as the best compromise between the less steric hindrances and an efficient coverage of the electrode surface.

To investigate the affinity of the biotinylated polypyrrole film for avidin, a tetrameric glycoprotein, gravimetric measurements were carried out using the QCM. In aqueous solutions, the QCM may characterize quantitatively the binding event between a sensitive layer coated on the quartz surface and biological macromolecules.[19,20,25−27] The QCM is an oscillating quartz crystal whose the frequency response to a change in the mass is described by the following equation:[28]

$$\Delta f = -f_o^2(2/Sn(\sqrt{\mu\rho}))\Delta m$$

where Δf is the measured shift in frequency (Hz), S is the active area of the crystal (cm^2), ρ is the quartz density (2.648 g cm^{-3}), μ is the shear modulus ($2.947\ 10^{11}$ g s^{-2} cm^{-1}), n the overtone number, f_o is the fundamental crystal frequency, and Δm is the mass change on the surface of the crystal (g). Thus, the employed 27-MHz QCM transducer exhibits a theoretical mass/frequency sensitivity of 360 pg Hz^{-1} ($S = 0.2$ cm^2). A poly **1** film ($\Gamma_1 = 1.33 \times 10^{-8}$ mol cm^{-2}) was electrogenerated onto the one side of Au electrode of the QCM. The latter was incorporated in a flow-through microcell and its frequency response to mass increase was recorded in continuous-flow mode (Figure 4). After stabilization of the QCM response (within 16 min), avidin (0.5 mg mL^{-1}) was added to the flow carrier. In the presence of avidin, a rapid decrease in microbalance frequency was observed, a steady-state value being reached within 6 min. Then, the avidin solution was replaced by the initial PBS flow carrier (Figure 4). Since this replacement does not have any effects on the QCM response, the preceding decrease in frequency was therefore attributed to the immobilization of avidin onto the biotinylated film. It should be noted that the same experiment was performed with an Au electrode of the QCM covered with a poly(*N*-methylpyrrole). No changes in the frequency response of the modified QCM were observed in the presence of avidin. This corroborates that the mass increase detected with the poly **1** film corresponds unambiguously to the specific binding of avidin to the polymerized biotin moieties. The frequency change was 307 Hz, which corresponds to an increase in mass of 550 ng cm^{-2} on the poly **1** electrode. Taking account that the maximum coverage corresponding to a close-packed one molecular layer of avidin was estimated at 3.3×10^{12} molecules cm^{-2},[29] the theoretical mass increase should be estimated to 362 ng cm^{-2}. It should be noted that the complete frequency variation was attributed to a mass variation; this hypothesis is valid only for thin and purely elastic added mass. Therefore, the poly **1** film can bind efficiently 1.5 equivalent avidin monolayers. It has been previously reported that conducting polypyrrole films exhibited strongly corrugated surfaces.[30] Consequently, the high amount of bound avidin may be due to the roughness of the surface morphology of the poly **1** film, which increases the real/geometric surface ratio.

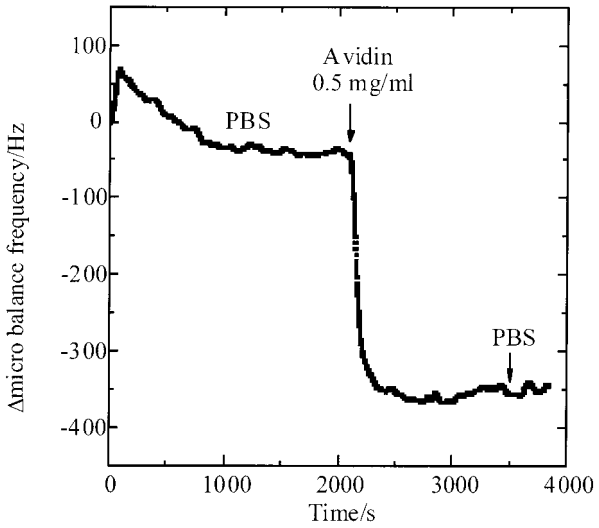

Figure 4. Time course of frequency response of a 27-MHz QCM (overtone 3) modified by an electrogenerated poly **1** film ($\Gamma_1 = 1.33 \times 10^{-8}$ mol cm^{-2}) to an aqueous avidin solution; flow rate, 60 μL min^{-1}. The zero microbalance frequency was arbitrarily set for the measurements.

The ability of the poly **1** film for the soft anchoring of biotinylated macromolecular biomolecules via the avidin−biotin bridge has been examined by permeation measurements. For this purpose, the evolution of the polymer permeability for the successive binding of avidin and GOx-B has been investigated by

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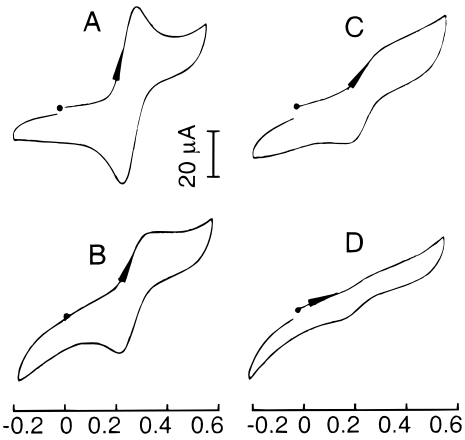

Figure 5. Cyclic voltammograms of 1 mM ferrocenecarboxylic acid in H_2O + 0.1 M $LiClO_4$ at (A) a bare Pt disk electrode (diameter 5 mm), (B) a poly **1** electrode, (C) a poly **1** electrode after reaction with an avidin solution, and (D) a poly **1** electrode after reaction successively with an avidin solution and a GOx-B solution; scan rate, 0.1 V s^{-1}.

recording the cyclic voltammogram of ferrocenecarboxylic acid (Fc) used as a permeant electroactive probe. Since the electrochemical responses of the probe and polypyrrole backbone are close together, the polypyrrole electroactivity was destroyed by overoxidation to avoid mixing of the two signals (Figure 3). The cyclic voltammograms for Fc at a bare Pt electrode and at a poly **1** electrode ($\Gamma_1 = 1.69 \times 10^{-9}$ mol cm^{-2}) show that the current peaks for the oxidation of Fc are similar, illustrating the good permeability of the polymeric film toward the Fc permeation (Figure 5A,B). In contrast, this signal was markedly reduced after the immersion of the poly **1** into an avidin solution and vanished quasi-totally after the subsequent immersion into a GOx-B solution (Figure 5C,D). This strong decrease in the oxidation on the Pt electrode of Fc, which diffused through the film, clearly indicates a permeability diminution attributed to an increase in the polymer layer. This illustrates successively the binding of avidin to the polymerized biotin moieties and that of GOx-B to avidin.

It has been previously reported that avidin−biotin interactions can be used successfully to build controlled molecular architectures composed of multienzyme layers.[31−34] Therefore, our immobilization procedure of biotinylated biomolecules has been applied to the construction of 1, 2, 4, and 7 layers of the avidin−GOx-B complex. The immobilized GOx-B catalyzes in the presence of dioxygen the oxidation of glucose with the production of H_2O_2. As a consequence, the enzymatic activity of the modified electrodes was evaluated by soaking these electrodes in phosphate buffer containing 50 mM glucose and recording amperometrically the time-dependent increase in H_2O_2 concentration (see Experimental Section). It appears that the enzymatic activity of the biosensors increases almost linearly with the number of successive depositions of GOx-B and avidin (Table 1). This illustrates the regular growth of the enzyme layers. On the other hand, taking into account that a GOx molecule occupies an area of 56 nm^2,[35,36] a compact enzyme monolayer corresponds to a coverage of 3×10^{-12} mol cm^{-2}. Consequently, a fully active GOx-B monolayer should exhibit a theoretical enzymatic activity of ~52 munits cm^{-2}, whereas the activity measured for a GOx-B/avidin/poly **1** electrode is 39 munits cm^2. This result suggests either a slight decrease in the specific activity of the immobilized enzyme or the formation of a noncompact monolayer, the mean area for a GOx-B molecule being 75 nm^2 instead of 56 nm^2. Nevertheless, in the case of a compact enzyme layer, the remaining activity of the immobilized enzyme (75%) is markedly higher than those previously reported for biosensors based on enzymes entrapped in conducting polymers.[4]

Table 1. Analytical Characteristics of the Poly 1 Electrodes Modified by Different Cycles of Sequential Deposition of Avidin and GOx-B, for the Determination of Glucose

no. of supposed monolayers	biosensor sensitivity[a] (mA M^{-1} cm^{-2})	enzymatic activity[b] (munits cm^{-2})
1	0.15	39
2	0.32	114
4	1.32	251
7	3.36	373

[a] Determined as the slope of the linear part of the calibration curves. [b] Determined amperometrically from the time-dependent increase in H_2O_2 concentration at saturating glucose conditions.

The analytical capabilities of the different biosensors for glucose determination were also examined in 0.1 M phosphate buffer. The biosensors were potentiostated at +0.6 V in order to detect amperometrically the enzymatically generated H_2O_2. It appears that the response time of the biosensors slightly increases from 5 to 10 s with the increase in enzyme layers. Figure 6 depicts the amperometric response of the biosensors as a function of glucose concentration. The calibration curves were quasi-linear with glucose concentration up to 2 mM and curved gradually at higher concentrations. As expected, the biosensor sensitivity (determined as the slope of the initial linear part of the calibration curve) increases with the supposed number of enzyme layers (Table 1). In addition, the K^{app}_M of the biosensor for glucose was determined from a Michaelis−Menten analysis of the glucose plot for each biosensor configuration. It appears that the K^{app}_M values remain almost constant (2−4 mM). This indicates the successive immobilization of enzyme layers exhibiting a similar kinetic behavior. However, it should be noted that the amperometric response of the biosensors is lower than their enzymatic activity. For instance, the maximum current density at saturating glucose conditions for the 4-layer enzyme is ~5 μA cm^{-2}. Taking into account that the oxidation of H_2O_2 and hence glucose consumes two electrons per molecule, the velocity of the enzyme reaction should be 1.56×10^{13} molecules s^{-1} cm^{-2}. This leads to a calculated enzymatic activity of 1.55 munits cm^{-2} instead of 251 munits cm^{-2} (Table 1). This difference clearly suggests that the major part of enzymatically generated H_2O_2 diffuses into the bulk solution rather than through the hydrophobic poly **1** film. A possible approach for overcoming this problem consists of

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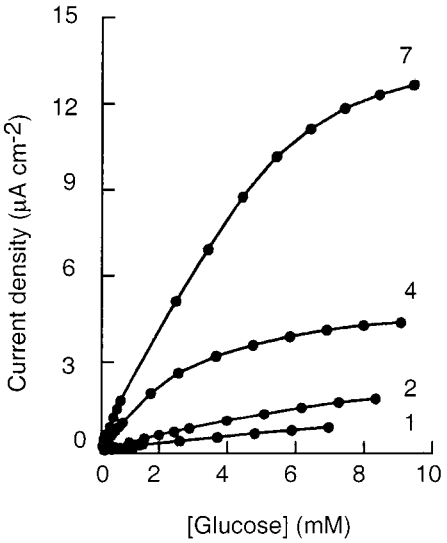

Figure 6. Calibration curves for glucose obtained at poly **1** electrodes ($\Gamma_1 = 1.6 \pm 0.1 \times 10^{-9}$ mol cm^{-2}) modified by enzyme layers constructed by 1, 2, 4, or 7 cycles of the successive deposition of avidin and then GOx-B. Applied potential, 0.6 V vs SCE; air-saturated 0.1 M phosphate buffer (pH 7) kept under stirring.

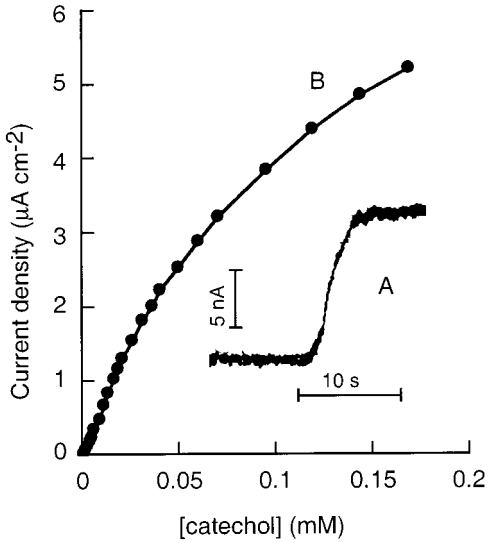

Figure 7. (A) Steady-state current−time response of a poly **1** electrode ($\Gamma_1 = 1.7 \times 10^{-9}$ mol cm^{-2}) modified by 5 PPO-B layers for an increase in catechol concentration (1 μM); (B) calibration curve for catechol. Applied potential, −0.2 V vs SCE; air-saturated 0.1 M phosphate buffer (pH 6.5) kept under stirring.

preparing an amphiphilic biotin monomer in order to confer a lesser hydrophobic character to the resulting biotinylated polypyrrole.[4] This may improve the swelling phenomenon of the polymer in aqueous electrolytes and hence the permeation of H_2O_2.

The storage stability of the poly **1** electrodes modified by 2, 4, and 7 enzyme layers, stored at 4 °C in 0.1 M phosphate buffer, was also examined. For this purpose, the current response of the biosensors with increasing concentration of glucose in the linear part of the calibration curve was periodically recorded. It appears that the three biosensors exhibit the same behavior, namely, a marked decrease in biosensor sensitivity to 50% of their initial values followed by a stabilization in the range 20−25% for 15 days. These values remain almost constant after 38 and 64 days.

Another example of macromolecule immobilization has been developed with a biotinylated polyphenol oxidase (PPO-B). PPO catalyzes the oxidation of several monophenols and *o*-diphenols to *o*-quinones while dioxygen is reduced to water. Consequently, PPO-based microbiosensors can be useful analytical tools in the fields of environmental control and medicine for the determination of pollutants and catecholamine neurotransmitters.[37] Following the preceding procedure of GOx-B immobilization, a biosensor configuration containing 5 layers of enzyme was elaborated by successive deposition of avidin and PPO-B on a poly **1** electrode. The analytical characteristics of the biosensor have been investigated in 0.1 M phosphate buffer (pH 6.5) by potentiostating the electrode at −0.2 V vs SCE. Thus, the enzymatically produced *o*-quinones were reduced at the electrode surface, generating an amperometric signal. Figure 7A presents the steady-state current response of the biosensor to an increment (1 μM) of catechol concentration illustrating its fast response time ($\cong$ 3−4 s). Figure 7B shows the amperometric current response of the biosensor as a function of catechol concentration. The calibration curve was linear with catechol concentration up to 30 μM and curved gradually at higher concentrations. The sensitivity of the biosensor and its detection limit (based on a signal-to-noise ratio of 3) are 65 mA M^{-1} cm^{-2} and 2 $\times$ 10^{-7} M, respectively. This catechol sensitivity is markedly lower than that (1.5 A M^{-1} cm^{-2}) obtained with biosensors based on PPO entrapped in a functionalized polypyrrole matrix.[38] However, it should be noted, that the PPO amount entrapped in the polymer matrix (210−230 μg)[38] was strikingly higher than that of PPO-B (0.47 μg estimated from a theoretical maximum coverage of 5 (PPO-B) layers) immobilized on the poly **1** surface. The K^{app}_M of the biosensor for catechol was determined from a Michaelis−Menten analysis of the catechol plot. The K^{app}_M value (0.15 mM) is in good accordance with that reported (0.24 mM)[39] for the free enzyme, illustrating the nondenaturating character of the procedure of enzyme anchoring.

CONCLUSION

In this report, we have described the successful electrogeneration of a biotinylated conducting polypyrrole film. By use of biotinylated polyphenol oxidase and glucose oxidase, we have illustrated the attractive potentialities offered by this conducting polymer for the fabrication of biosensors. It is expected that this simple immobilization method of biotinylated molecules such as enzymes, antibodies, antigens, and oligonucleotides will be useful for the development of microbiosensors.

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