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Immobilization of Peroxidase Glycoprotein on Gold Electrodes Modified with Mixed Epoxy-Boronic Acid Monolayers

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Abstract: The development of bioelectronic enzyme applications requires the immobilization of active proteins onto solid or colloidal substrates such as gold. Coverage of the gold surface with alkanethiol self-assembled monolayers (SAMs) reduces nonspecific adsorption of proteins and also allows the incorporation onto the surface of ligands with affinity for complementary binding sites on native proteins. We present in this work a strategy for the covalent immobilization of glycosylated proteins previously adsorbed through weak, reversible interactions, on tailored SAMs. Boronic acids, which form cyclic esters with saccharides, are incorporated into SAMs to weakly adsorb the glycoprotein onto the electrode surface through their carbohydrate moiety. To prevent protein release from the electrode surface, we combine the affinity motif of boronates with the reactivity of epoxy groups to covalently link the protein to heterofunctional boronate-epoxy SAMs. The principle underlying our strategy is the increased immobilization rate achieved by the weak interaction-induced *proximity effect* between slow reacting oxyrane groups in the SAM and nucleophilic residues from adsorbed proteins, which allows the formation of very stable covalent bonds. This approach is exemplified by the use of phenylboronates-oxyrane mixed monolayers as a reactive support and redox-enzyme horseradish peroxidase as glycoprotein for the preparation of peroxidase electrodes. Quartz crystal microbalance, atomic force microscopy, and electrochemical measurements are used to characterize these enzymatic electrodes. These epoxy-boronate functional monolayers are versatile, stable interfaces, ready to incorporate glycoproteins by incubation under mild conditions.

1. Introduction

The development of bioelectronic enzyme applications[1] requires the immobilization of active proteins onto solid or colloidal substrates such as gold. Proteins adsorb nonspecifically onto clean gold surfaces with denaturation and a reduction of their activity.[2] Direct adsorption does not discriminate among protein populations nor control their orientation, features sometimes desirable for an immobilization protocol. Coverage of the gold surface with alkanethiol self-assembled monolayers (SAMs)[3] has made it possible to drastically reduce nonspecific adsorption of proteins,[4] to direct the binding of proteins to gold supports, and to control their orientation by using SAMs with ligands complementary to specific binding sites on native proteins.[5] It is noteworthy to mention that many strategies for protein immobilization on SAMs have been based on previous developments of chromatography supports, which oriented immobilized proteins through charged, hydrophobic, or other affinity group interactions.[6] In this context, a particularly interesting application of SAMs has been the use of specific

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cofactor-apoprotein interactions to assemble enzymes on gold surfaces.[7] Also proteins have been modified, by genetic[8] or chemical[9] procedures, so as to acquire binding sites with affinity for ligands on the monolayer. Electrostatic[10] and hydrophobic[11] interactions have also been used in protein immobilization procedures on gold electrodes modified with SAMs of thiols. As a complement to these achievements, it is also of interest to explore and develop general approaches applicable to native proteins and based on different kinds of weak interactions that living systems use to form molecular complexes.

Hydrogen bonding, although widely and efficiently used in nature, is difficult to mimic in synthetic systems because water, the most widely used solvent, competes for hydrogen bond formation. In recent years, the interaction between boronic acids and saccharides has been proposed as an alternative to hydrogen bonding to keep synthetic molecular receptors bound to their guest molecules, since boronic acids rapidly and reversibly form cyclic esters with diols in both nonaqueous and aqueous media at room temperature. They form reversible bonds with 1,2- or 1,3-diols to generate five- or six-membered cyclic complexes.[12a] This boronic acid-saccharide interaction has recently been exploited for the development of aqueous sugar sensors,[12] synthetic sugar and ribonucleoside transporters through lipid membranes,[13] separation of sugars in liquid chromatography,[14] and capillary electrophoresis.[15] The interaction between sugars and boronic acid has also been used for affinity chromatography purification and detection of glycoproteins[16] and for orientation and reversible immobilization of glycoproteins in cellulose beads.[17]

There have been a few reports on boronic acid SAMs describing the properties of hydrophobic monolayers obtained by adsorption of ω-mercaptoalkyl boronic acids onto gold substrates.[18a,b] SAMs of conjugates of dithioaliphatic acids and aminophenylboronic acid on gold surfaces display specific sugar recognition.[18c] More recently electrical contacting of glucose oxidase with gold electrodes has been achieved by surface-reconstitution of the apo-protein on a boronic acid-FAD cofactor SAM.[19] Therefore the use of boronate SAMs looks like a promising alternative for the preparation of enzyme electrodes of glycoproteins. One advantage of this strategy is that immobilizing a glycoprotein through its carbohydrate moiety is not likely to affect its biological activity. The carbohydrate region is generally located in areas that are not involved in glycoprotein activity,[20] and therefore many glycoproteins, including hormones,[21] antibodies,[22] and enzymes,[23] retain most of their biological function even when their carbohydrate regions are modified or altered. However, a disadvantage is that since the reversibility of the bonds allows protein release, enzyme electrodes do not remain stable over time.

To overcome this instability and render this immobilization of practical interest, in the present study we have investigated the use of heterofunctional boronate-epoxy SAMs that combine the affinity motif of boronates with the reactivity of epoxy groups able to covalently link the protein to the SAM. Oxyrane groups are known to form very stable ether, thioether, or secondary amine bonds with exposed nucleophilic protein residues, although these reactions are extremely slow.[24] Boronates might specifically interact with glycosylated domains of a protein to

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form a monolayer of physically adsorbed enzyme, and the increase of protein concentration on the SAM would accelerate the formation of intramolecular chemical bonds between adsorbed protein and the epoxy groups on the SAM. Besides, the multiple weak forces involved in the physical adsorption of the protein molecules on the mixed SAM stabilize its native conformation and therefore could protect it from potential distortions introduced by the subsequent formation of covalent bonds.

This affinity-trap immobilization strategy has been tested with the well-known glycoprotein horseradish peroxidase (HRP). It is an oxidoreductase containing a heme group, calcium, and glycans and participates in a two-step enzymatic reaction with H_2O_2 and a large array of electron donors.[25] The catalytic activity of redox enzymes can be followed electrochemically by using a diffusible redox-mediator that shuttles electrons from the electrode surface to the enzyme active site.[26] Furthermore, the crystal structure and carbohydrate composition of similar peroxidases have been determined.[25c] The presence of mannose among the sugar residues[25d] in HRP_{native}, one of the sugars presenting good affinity toward immobilized boronic acid,[18c] contributes to making this redox glycoprotein an appropriate choice to test this immobilization procedure. The available deglycosylated recombinant protein (HRP_{recomb}) serves as a good control to test the effect of the sugar cover on the binding affinity.[27]

Peroxidases have a number of important features that make them suitable for amperometric biosensors,[28] an important factor being their good stability in water and organic solvents.[29] In addition, the reactions driven by oxidases used in amperometric biosensors for glucose, alcohols, amino acids, glutamate, L-lactate, cholesterol, etc.[28,30] release H_2O_2 that must subsequently be reduced. The importance of this bioelectrocatalytic reduction has stimulated studies of the performance of peroxidases on graphite, metal, and polymer-modified electrode surfaces.

In this study atomic force microscopy (AFM), electrochemical quartz crystal microbalance (EQCM), and cyclic voltammetry (CV) have been used to demonstrate that (i) bare gold surfaces bind nonspecifically glycosylated proteins as well as their genetically deglycosylated derivative; (ii) gold covered with a phenylboronate SAM specifically binds the glycosylated form in a reversible way; and (iii) a mixed SAM of epoxy and phenylboronate residues displays specific affinity for glycoproteins combined with the ability to covalently trap the physically adsorbed protein.

2. Experimental Section

2.1. Gold-Support Preparation. Gold wire electrodes (99.9% and 0.5 mm in diameter, 0.2 cm^2 area) were first exposed to the flame of a Bunsen burner and then polished with alumina slurry (Buehler number 3, 0.05 μm particle size). Finally, they were sonicated for 20 min in pure ethanol. AT-cut quartz crystals (5 MHz) of 24.5 mm diameter with gold-coated electrodes deposited over a titanium adhesion layer (Maxtek Co) were cleaned by exposure to "piranha" solution (3:1 concentrated H_2SO_4/30% H_2O_2) followed by exhaustive rinsing with distilled water and a final rinse with ethanol/water (2:1). *Caution: piranha solution reacts violently with most organic materials and must be handled with extreme care.* An asymmetric electrode format was used with the side having the larger gold area facing the solution. The electroactive working area (front side) was 1.370 cm^2 and the piezoelectric area (backside) was 0.317 cm^2. Glass supports (1.1 cm × 1.1 cm) covered with evaporated gold layers (0.2−0.3 μm) deposited over a chromium adhesion layer (1−4 nm) (Metallhandel Schröer GmbH) were used for atomic force microscopy (AFM); they were cleaned with piranha solution, rinsed in distilled water, and annealed for 2 min in a gas flame. Surface roughness of gold electrodes, estimated by integration of the gold oxide reduction peaks of cyclic voltammograms in sulfuric acid 0.1 M, were 1.5 and 1.1 for wires and EQCM electrodes, respectively. A value of 482 $\mu C\ cm^{-2}$ was used for a monolayer of chemisorbed oxide on polycrystalline gold.[31]

2.2. Synthesis of SAM-TOA Modified with Aminophenyl Boronic Acid (APBA). After the cleaning treatment the gold surfaces were covered with a monolayer of dithioctic acid (SAM-TOA) obtained after the substrates were immersed for at least 16 h in a 1×10^{-3} M solution of 6,8-dithioctic acid (Sigma) in ethanol/water (2:1). After this the electrodes were rinsed with ethanol/water (2:1) and dried in air. Activation of the carboxylic acid groups of the SAM-TOA electrodes was performed by immersion for 3 h in a dimethyl sulfoxide solution containing *N*-hydroxysuccinimide (Fluka; NHS) 0.1 M and 1-ethyl-3-[3′-(dimethylamino)propyl] carbodiimide (Sigma; EDC) 0.1 M. After this treatment, the electrodes were rinsed with dioxane and dried in air. Amidation of NHS-esters of SAM-TOA was effected by incubation in 3-aminophenyl boronic acid (Sigma; APBA) 75×10^{-3} M in dimethylformamide (Merck; DMF) overnight (SAM-TOA-APBA). To dilute APBA in the monolayer with ethanolamine (EA), different molar ratios of APBA and EA (Merck) were used in DMF as solvent (SAM-TOA-[APBA/EA]). In both cases, electrodes were rinsed with water and incubated in the corresponding protein solutions.

2.3. Synthesis of SAM-TOA Modified with Aminophenyl Boronic Acid and Epoxy Groups. Gold surfaces covered with an *N*-hydroxysuccinimide activated SAM-TOA were dipped overnight in undiluted 1,8-diamino-3,6-dioxaoctane (DADOO, Merck), after which the unreacted diamine was removed by washing with ethanol followed by dipping in undiluted 1-chloro-2,3-epoxypropane (epichlorohydrin, Merck). Unreacted epichlorohydrin (Epi) was eliminated by washing with ethanol. These SAM-TOA-DADOO-Epi electrodes were incubated in APBA 0.16 M in acetonitrile/water (1:5), pH 8.0 for different times. To obtain an estimation of the remaining epoxy groups on SAM-TOA-DADOO-[Epi-APBA], the electrodes were incubated overnight in β-aminoethyl ferrocene (Fc) 2 mM in DMF. Unreacted β-aminoethyl ferrocene was eliminated by washing with solvent. The integrated charge of the anodic CV peaks of bound ferrocene was used as an indirect measurement of the degree of APBA incorporation to the SAM-TOA-Epi surface (SAM-TOA-DADOO-Epi-[Fc/APBA]). β-Aminoethyl ferrocene was synthesized as described by Godillot et al.[32]

2.4. Detection of SAMs. The evaluation of SAM coverage was performed by cyclic voltammetry in 0.5 M KOH supporting electrolyte, under nitrogen, in the potential range −0.2 to −1.2 V, at a scan rate of 0.1 V s^{-1}. Reductive desorption of SAMs monitored by EQCM measurements were also used for coverage calculations.[33] Surface coverage by sulfur atoms was estimated from the peak charge of

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reductive desorption at −1.0 V and assuming a value of 1 electron per sulfur atom,. Characterization of the different monolayers (SAM-TOA, SAM-TOA-APBA, SAM-TOA-[APBA/EA], SAM-TOA-DADOO-[Epi-APBA]) was done by cyclic voltammetry in solutions of different pH values with two electrochemical probes: potassium ferricyanide (Aldrich) and ruthenium hexaamine chloride (Sigma).

2.5. Enzymatic Electrodes. These were prepared by incubation of the different SAM-gold electrodes in 0.2 mg mL^{-1} solutions of HRP_{native} or HRP_{recomb}. Bare, SAM-TOA-APBA, SAM-TOA-[APBA/EA] gold electrodes were incubated in phosphate buffer 50 mM, 0.15 M KCl, pH 7.5 for 60 min and SAM-TOA-DADOO-[Epi/APBA] gold electrodes in 50 mM bicarbonate buffer, pH 9 containing 0.2 mg mL^{-1} HRP for 24 h, at room temperature.

HRP_{native} used in this work was purified from a commercial source (HRP Sigma type I, lot 63H9512) in batch on boronate Sepabeads[24] as follows: 5 mL of HRP solution (20 mg mL^{-1} in Tris-HCl 50 mM pH 8.0) was added to 3 mL of wet gel and gently stirred for 20 min; after washing the gel with the Tris buffer, HRP was eluted with 5 mL of acetic acid 50 mM, pH 4.5, and immediately adjusted to pH 7.0 with 50 mM phosphate buffer. The purified protein had an activity of 370 U mg^{-1} protein toward pyrogallol (Sigma). Recombinant carbohydrate-free HRP (HRP_{recomb}) was produced in *E. coli* and refolded from inclusion bodies as described in ref 27. This recombinant HRP gave an activity of 330 U mg^{-1} protein toward pyrogallol.

The activity of HRP enzyme molecules incorporated to electrodes was analyzed by cyclic voltammetry at 25 °C under a nitrogen stream in appropriate buffer containing 0.1 M KCl, 10 μM thionine as soluble redox mediator[34] and 1 mM H_2O_2 as substrate.

2.6. Apparatus. Cyclic voltammetry data were obtained with an Autolab PGSTAT 10 (Eco Chemie). In situ mass changes were measured with a quartz crystal microbalance (Maxtek Co) with AT-cut quartz crystals (5 MHz) set in a probe made of Teflon (TPS-550, Maxtek) at 25.0 ± 0.1 °C as described by Takada and Abruña;[35] crystals sensitivity was 17.68×10^{-9} g Hz^{-1} cm^{-2}. Atomic force microscopy (AFM) was done with a Nanoscope III microscope (Digital Instruments) operating in tapping mode in solution.

3. Results and Discussion

3.1. Substrate Preparation. 3.1.1. Characterization of Functionalized SAM. Previous studies have demonstrated the suitability of TOA monolayers for electrochemical studies, in terms of stability and permeability to electrochemical probes.[36] The integrity of the monolayer after its functionalization was verified through its electrochemical reductive desorption measured in basic media.[37] The charge produced by a full SAM-TOA monolayer on gold corresponds to a thiolate surface coverage of 7.2×10^{-10} mol cm^{-2}.[36] After covalently linking the APBA to the SAM-TOA (SAM-TOA-APBA) the monolayer desorption current gave similar values, indicating that the monolayer remained intact (not shown). To minimize nonspecific adsorption of proteins,[4] mixed monolayers were prepared by diluting the APBA with different ethanolamine concentrations, and the stability of the resulting monolayer (SAM-TOA-[APBA/EA]) was tested. The results indicated that monolayers prepared with an APBA/ethanolamine molar ratio from 1:10 to 1:50 gave monolayers with 100% coverage (not shown). The response of the electrode to different electrochemical probes provides information about the degree of ionization of the exposed functional groups of the monolayer.[8a] Therefore it is possible to check the stepwise construction of the SAM-TOA-[APBA/EA, 1:50] by recording cyclic voltammograms of two differently charged electroactive compounds, potassium ferricyanide ($Fe(CN)_6^{3-}$) and ruthenium hexamine ($Ru(NH_3)_6^{3+}$), at different pH buffer solutions (Figure 1).

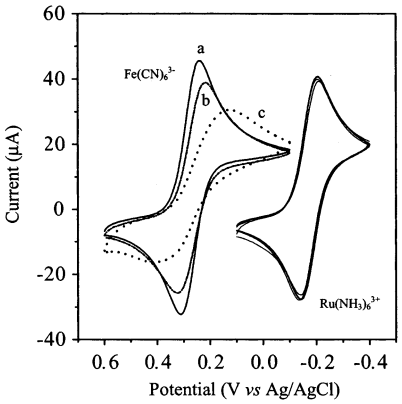

Figure 1. Cyclic voltammograms of 1 mM $Fe(CN)_6^{3-}$ and 1 mM $Ru(NH_3)_6^{3+}$, in 1 M KCl on gold electrodes covered with SAM-TOA-[APBA/EA, 1:50]: (a) pH 5.5, (b) pH 7.3, and (c) pH 11. The scan rate was 0.1 V s^{-1}.

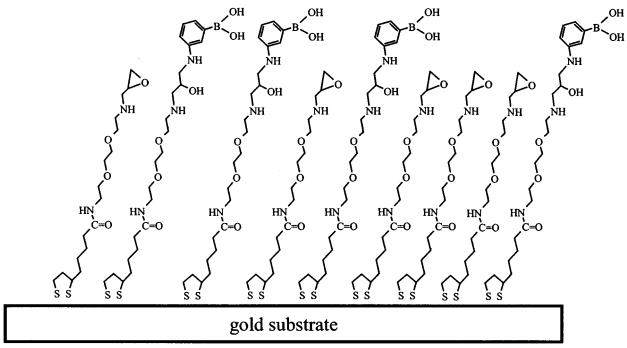

Figure 2. Schematic representation of a monolayer containing TOA-DADOO-Epi-APBA and TOA-DADOO-Epi chains (molecules sizes, relative amounts, and distribution of the chains are not accurate).

At pH 5.5 the negatively charged carboxylic acid residues of the TOA do not allow the oxidation−reduction cycle of the negatively charged ion ferricyanide but permit the redox cycle of the positively charged ruthenium hexaamine to take place.[8a] When SAM-TOA-[APBA/EA, 1:50] was analyzed the complete oxidation−reduction cycle of both mediators was observed at pH 5.5 (Figure 1). However, when the pH of the solution was raised to 11, the appearance of a negative charge in the boronic acid due to the presence of a third hydroxyl group bound to the boron atom at this pH[38] distorted the cyclic voltammogram of potassium ferricyanide while leaving the CV of the positively charged mediator unchanged (Figure 1). These results show that the ionic character of the surface changes in the expected direction after the incorporation of the APBA and ethanolamine.

The presence of reactive oxyrane groups in SAM-TOA-DADOO-[Epi-APBA/Epi] (Figure 2) was ascertained by reaction of these monolayers with β-aminoethyl ferrocene.

The CV in Figure 3 indicates the presence of immobilized electroactive ferrocene groups in the monolayer. The peak current densities increased linearly with the scan rate (inset of

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(37) Walczak, M. M.; Popenoe, D. D.; Deinhammer, R. S.; Lamp, B. D.; Chung, C.; Porter, M. C. *Langmuir* **1991**, *7*, 2687−2693.
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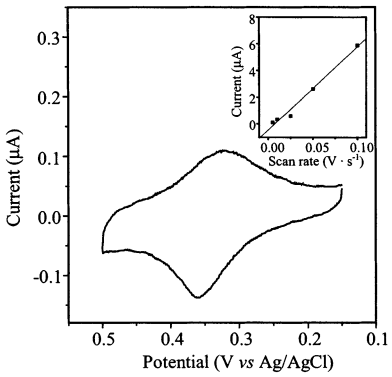

Figure 3. Cyclic voltammogram in 0.1 M $HClO_4$ of gold electrode covered with SAM-TOA-DADOO-[APBA/Fc]. The scan rate was 10 mV s^{-1}. The inset shows the linear correlation between the peak current and the scan rate of immobilized β-aminoethyl ferrocene.

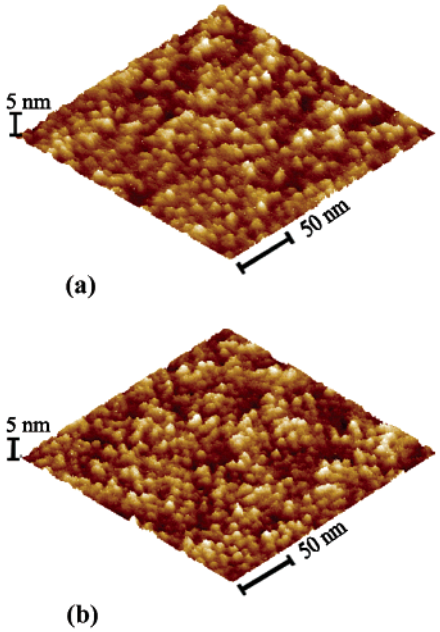

Figure 4. AFM tapping mode images of a bare gold surface incubated with 1.5 μM solutions of (a) HRP_{native} or (b) HRP_{recomb} in 0.05 M phosphate buffer, KCl 0.1 M pH 7.5 at room temperature for 60 min.

Figure 3). However the ΔE_p observed was 38 mV and the width of the peak at half-height was 71 mV, less than the theoretical value of 90.6/n ($n = 1$) expected for an ideal nernstian process of immobilized redox species, which suggests the existence of attractive interactions between neighbor molecules.[39]

Taken together these results support the scheme depicted in Figure 2 as representative of these monolayers.

3.2. Enzyme Adsorption on Bare Gold Electrodes. The adsorption of glycosylated and deglycosylated HRP onto a clean gold surface has been previously analyzed with the help of a quartz crystal microbalance by Ferantopova et al.[40] The authors found similar adsorption kinetics for native and deglycosilated HRP onto naked gold. In the present work we have observed adsorption on gold of HRP_{native} and HRP_{recomb} by atomic force microscopy.

Figure 4 shows AFM tapping mode images of bare gold surfaces incubated in the presence of native and recombinant HRP. In both cases the individual protein molecules can be distinguished on the gold surface, although no order was observed within this densely packed arrangement that completely covers the area analyzed.

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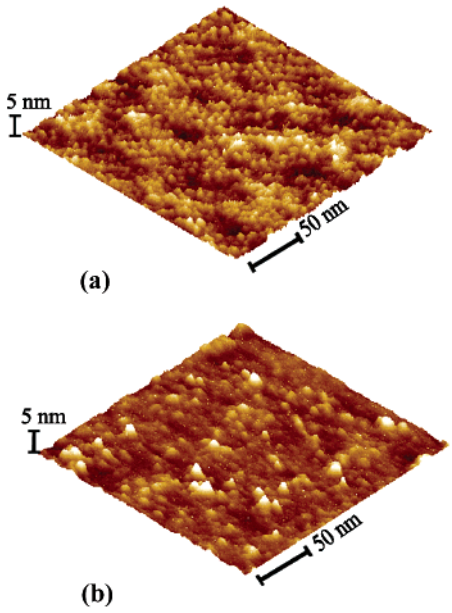

Figure 5. AFM tapping mode images of gold surfaces covered with SAM-TOA-[APBA/EA; 1:50] after incubation in solutions o: (a) HRP_{native} and (b) HRP_{recomb}. Other conditions as in Figure 4.

3.3. HRP Adsorption onto SAM-APBA Gold Electrodes. The effect of covering the gold surface with an APBA modified monolayer on the binding affinity to the glycosylated and deglycosylated proteins was then analyzed by AFM.

Figure 5 shows two areas of equivalent dimensions belonging to similarly modified electrodes but incubated in solutions of each type of HRP at similar concentrations. The surface of the electrode incubated with the glycosylated protein shows an ordered array of particles with dimensions corresponding to those expected for HRP X-ray data:[25c] 5.4 nm diameter, which yields a projection area of about 20 nm^2 per adsorbed molecule. The surface of the electrode incubated with the recombinant protein does not show this organized array of particles. At least three independent electrodes of each type were analyzed with different tips, all electrodes showing the same difference between the two proteins depicted in Figure 5. It appears that the amount of native protein on the surface is significantly higher than the amount of the deglycosylated one.

These results were further confirmed by quantification of the protein adsorbed on the surface by QCM.Figure 6 presents frequency changes of gold quartz crystals functionalized with SAM-TOA-[APBA/EA] as a function of time after injection of HRP_{recomb} or HRP_{native} at saturating concentrations of both proteins in the reaction chamber.

The resonant frequency decreases until reaching a steady state at different values. The mass adsorbed onto the electrode surface was calculated using Sauerbrey's[41] equation:

$$\Delta m = -C_f \Delta f$$

where Δm is the mass change (ng cm^{-2}), C_f (17.7 ng Hz^{-1} cm^{-2}) is a proportionality constant for the 5.0-MHz crystals used in this study, and Δf is the frequency change (in Hertz).

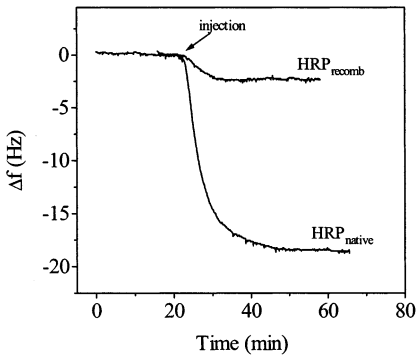

Figure 6. Time evolution of the resonance frequency of a QCM gold resonator covered with a SAM-TOA-[APBA/EA; 1:50] in 4.9 mL of 0.05 M phosphate buffer, KCl 0.1 M pH 7.5 solution, at 25 °C upon the addition of 100 μL of a stock solution (3.3 mg/mL) of HRP_{native}, final concentration 1.5 μM, or 100 μL of a stock solution (2.5 mg/mL) of HRP_{recomb}, final concentration 1.5 μM.

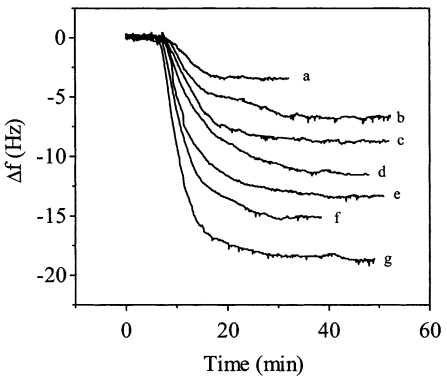

Figure 7. Time dependence of the frequency of a QCM gold resonator covered with a SAM-TOA-[APBA/EA; 1:50] for several final concentrations of added HRP_{native}: (a) 0.045; (b) 0.09; (c) 0.14; (d) 0.23; (e) 0.45; (f) 0.9, and (g) 2.3 μM. Other conditions as in Figure 6.

The results indicate that the amount of HRP_{native} on the surface is 7 times higher than the amount of the HRP_{recomb}, showing the effectiveness of the APBA/EA monolayer in discriminating between glycosylated and deglycosylated proteins.

Kinetic constants of association (k_{on}) and dissociation (k_{off}) of HRP_{native} to functionalized gold surfaces and equilibrium binding constant were obtained from QCM measurements carried out at different protein concentrations (Figure 7).

From these kinetic curves we obtained values of $k_{on} = 0.6 \times 10^4\ s^{-1}\ m^{-1}$ and $k_{off} = 1.2\ ms^{-1}$ using the equations described by Whitesides and co-workers.[4b] Both k_{off} and k_{on} are large, indicating that the interaction of APBA with the sugar residues is rapid but reversible.

The calculated equilibrium protein coverages were represented versus protein concentrations and fitted to a Langmuir adsorption isotherm[42] (Figure 8):

$$\Gamma_e = \frac{\Gamma_s K_a C_s}{1 + K_a C_s}$$

where K_a is the thermodynamic binding constant, C_s is the bulk protein concentration, and Γ_e and Γ_s are equilibrium and saturation protein coverages. The calculated values for Γ_s and K_a were 7.3×10^{-12} mol cm^{-2} and $4.9 \times 10^6\ M^{-1}$ respectively.

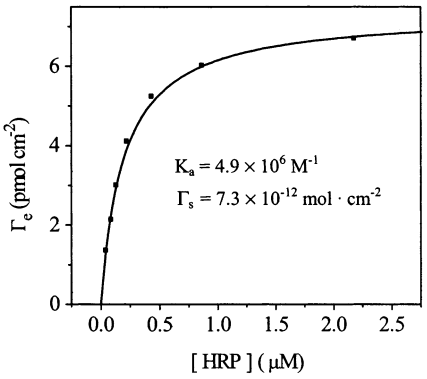

Figure 8. Equilibrium protein coverages of the adsorption curves in Figure 7 versus protein concentrations. The solid line is the Langmuir adsorption isotherm that best fits the experimental data.

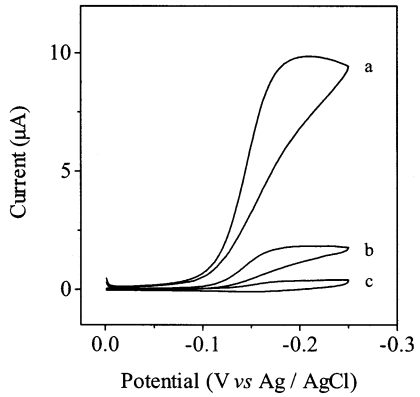

Figure 9. Electrocatalytic activity of SAM-TOA-[APBA/EA;1:50] modified gold electrodes incubated in (a) HRP_{native} and (b) HRP_{recomb} as in Figure 6. Cyclic voltammograms were recorded at 5 mV s^{-1} in the presence of 1 mM H_2O_2 and 10 μM thionine at 25 °C. Trace (c) is a control CV in the absence of proteins. Supporting electrolyte was 0.05 M phosphate buffer, KCl 0.1 M, pH 7.5.

It should be noted that the value obtained for the saturation surface coverage closely corresponds to that of 8.3×10^{-12} mol cm^{-2} estimated for a compact protein monolayer considering a projected area of about 20 nm^2 per molecule. The calculated binding Gibbs energy ($\Delta G = -RT \ln K_a$) was -38.2 kJ mol^{-1}, indicating a moderate binding strength of HRP_{native} protein to this modified surface gold electrode.

The catalytic current recorded in the presence of H_2O_2 and thionine for a SAM-TOA-[APBA/EA]-HRP_{native} electrode (Figure 9a) is 5.5 times the current measured with a SAM-TOA-[APBA/EA]-HRP_{recomb} electrode (Figure 9b). If we compare the catalytic currents with the amount of protein measured by QCM, it is possible to fairly match both results. All these results indicate that the [APBA/EA] monolayer favors the adsorption of native HRP over recombinant HRP and that this interaction is quite specific. The small extent of HRP_{recomb} binding to SAM-TOA-[APBA/EA] monolayers suggest that other interactions of phenylboronates with nonglycosilated proteins by charge transfer, hydrophobic and ionic bonding that have been observed[43] also take place in our system.

3.4. HRP Adsorption onto SAM-TOA-DADOO-[APBA/Epi] Gold Surfaces. As shown above, the boronic acid-saccharide interaction is reversible. As a result of protein release, the catalytic activity of the electrodes decreases to a fifth of

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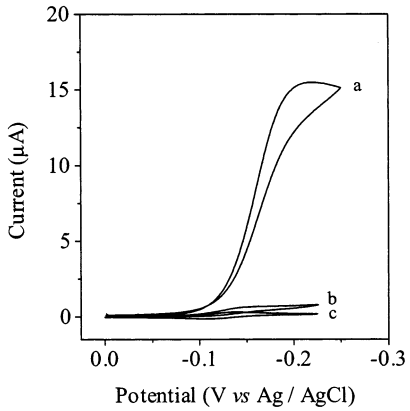

Figure 10. Electrocatalytic activity of gold electrodes modified with SAM-TOA-DADOO-[APBA/Epi] incubated for 24 h at room temperature in 50 mM bicarbonate buffer, pH 9.0, containing (a) 0.2 mg mL^{-1} HRP_{native} and (b) 0.2 mg mL^{-1} HRP_{recomb}. Other conditions as in Figure 9. Trace (c) was a control without enzyme.

their initial value after 2 h in solution (not shown). Moreover, the adsorbed protein is completely released after 30 min incubation in a 0.1 M sorbitol solution (not shown).

To fix the protein to the electrode surface while retaining the selectivity provided by the boronic acid interaction, we used a mixed SAM with terminal epoxy groups besides the aminophenyl boronic residues. First, epoxy groups were introduced on the monolayer to cover the whole surface of the electrode. The APBA was then bound to the monolayer through some of these epoxy groups, leaving the rest available for later interaction with the protein. The incubation time of a SAM-TOA-DADOO-Epi electrode in the boronic acid solutions determines the density of APBA molecules on the surface. Incubation for 8 h in a 0.16 M APBA solution resulted in a surface with exposed APBA molecules sufficient to provide specificity toward the glycosylated protein while leaving enough reactive epoxy groups to react with protein nucleophiles. Direct evidence of the presence of epoxy groups was obtained by reaction of the mixed monolayer with aminoferrocene and electrochemical analysis of the resulting monolayer (Figure 3). The surface concentration of the immobilized ferrocene calculated from the charge of the anodic CV peak was 1.2×10^{-10} mol cm^{-2} assuming a one-electron reduction process. This value indicates the presence of a concentration of remaining reactive oxyrane groups around 40% of the concentration expected for a full epoxy monolayer.

The epoxy groups are known to react slowly with protein nucleophiles and form stable bonds.[24,26,44] We found that SAM-TOA-DADOO-[APBA/Epi] electrode wires incubated for 24 h in 0.2 mg ml^{-1} HRP_{native} solution in a 50 mM bicarbonate buffer, pH 9, showed catalytic activity while in the case of HRP_{recomb} almost no activity could be detected (Figure 10).

These results were confirmed by EQCM measurements of the immobilization of the two proteins at the SAM-TOA-DADOO-[Epi-APBA] surface along with the estimation of activity from the voltammetric studies (Table 1).

It can be seen that the amount of immobilized HRP_{native} protein after 6 h of experimental observation is much higher than that of HRP_{recomb}. In addition, the ratio between the amount of native to recombinant protein bound is consistently lower

Table 1. Mass and Electrocatalytic Activity of HRP_{Native} and HRP_{Recomb} Enzymes Immobilized on a Gold Resonator Modified with a SAM-TOA-DADOO-[APBA-Epi][a]

		electrocatalytic activity (μA)	
	mass (ng cm^{-2})	6 h incubation	24 h incuation
HRP_{native}	274	33.6	48.2
HRP_{recomb}	65	3.6	5.9

[a] The results are average of two independent experiments run at 25 °C at the same conditions as those of Figure 10.

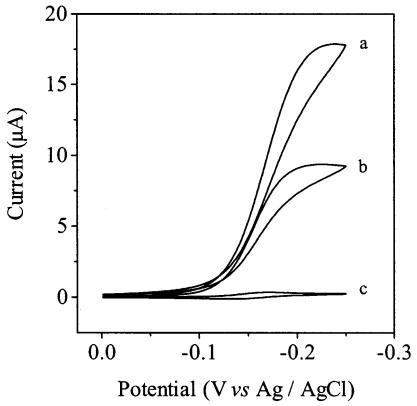

Figure 11. Electrocatalytic activity of gold electrodes modified with SAM-TOA-DADOO-[APBA/Epi] incubated for 24 h at room temperature in 50 mM bicarbonate buffer, pH 9.0, containing 0.2 mg mL^{-1} HRP_{native}: (a) electrode washed 5 min in 50 mM phosphate buffer pH 7.5, 0.1 M KCl; (b) electrode as in (a) but further incubated 30 min in the same buffer containing 0.1 M sorbitol. Other conditions as in Figure 9. Trace (c) corresponds to a control electrode treated as in (a) and without H_2O_2 addition.

than the corresponding ratio of catalytic activities, which could be due to stabilization of the HRP_{native} by the carbohydrate upon the immobilization.[45]

Further experiments on modified gold wires showed that the HRP_{native} electrodes retained more than half of activity after prolonged incubation in 100 mM sorbitol (Figure 11).

Complementary AFM experiments on gold surfaces also modified with SAM-TOA-DADOO-[APBA/Epi] and incubated with HRP_{native} showed an homogeneous distribution of protein molecules even after 24 h of washing with buffer (Figure 12a). The similarity in the height of the protein molecules in the image is consistent with an uniform orientation due to specific binding by the carbohydrate motif.

Figure 12b shows an AFM image of a similar surface but washed with buffer containing sorbitol, which clearly shows high protein coverages, although as expected it is less ordered as a result of the removal of the fraction of noncovalently trapped molecules. An AFM image of gold covered with [APBA/Epi] SAM is also presented for comparison (Figure 12c). Control experiments with HRP_{recomb} gives AFM images (Figure S1 available in the Supporting Information), which show a certain amount of isolated proteins together with larger aggregates that could represent denatured bonded protein. A similar denaturation effect of bovine seroalbumin upon binding to carboxy-ended SAMs, revealed by AFM, has been reported by Wadu-Mesthrige et al.[46]

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(45) We thank one reviewer for drawing our attention to this inactivating effect of the immobilization of the recombinant carbohydrate protein on epoxy-boronate monolayers.

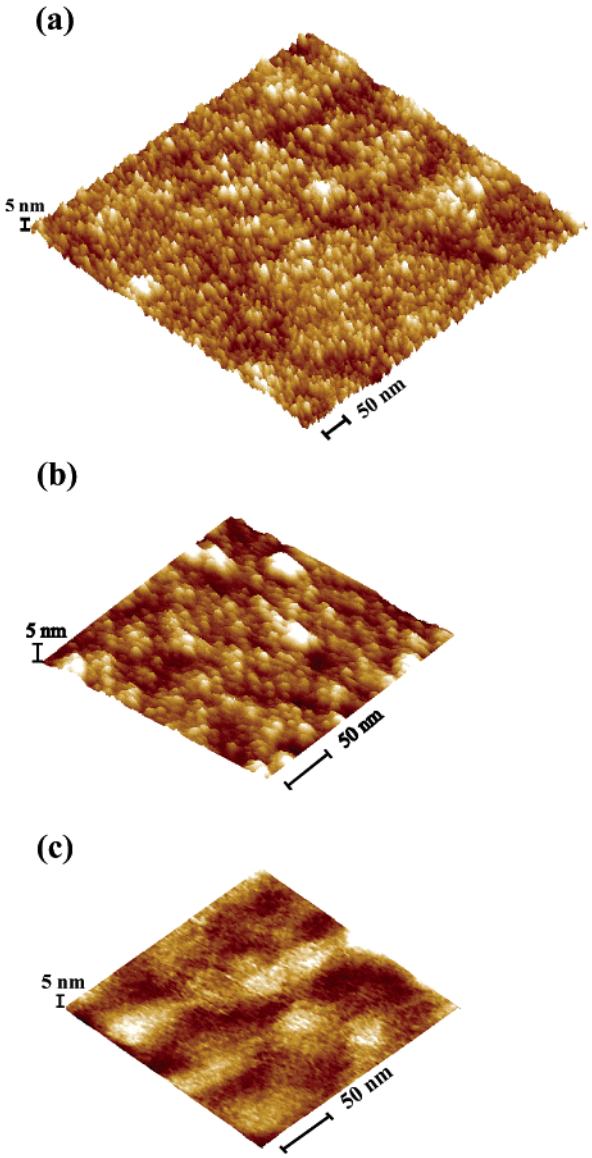

Figure 12. AFM tapping mode image of gold surface (a) covered with SAM-TOA-DADOO-[APBA/Epi] incubated for 24 h at room temperature in 50 mM bicarbonate buffer, pH 9.0, containing 0.2 mg mL^{-1} HRP_{native}; (b) similar experiment but after incubation (30 min) in 50 mM phosphate buffer pH 7.5, KCl 0.1 M containing 0.1 M sorbitol; (c) gold SAM surface incubated in buffer without protein.

Similar results obtained with different gold surfaces (wires, covered quartz crystals, and AFM supports) indicate that the specific binding of HRP_{native} depends of the glycan and the boronate-SAM and is independent of the type of gold surface used.

The requirement of the simultaneous presence of APBA and epoxy groups in the monolayer to obtain stable glycoenzyme electrodes is illustrated by the following experiment: SAM-TOA-DADOO-Epi electrodes were incubated in solutions of HRP_{native} or HRP_{recomb} under the same experimental conditions described above. Only the HRP_{native} electrode displayed catalytic activity toward H_2O_2 oxidation mediated by thionine (Figure 13a); however, this protein was easily released after incubation

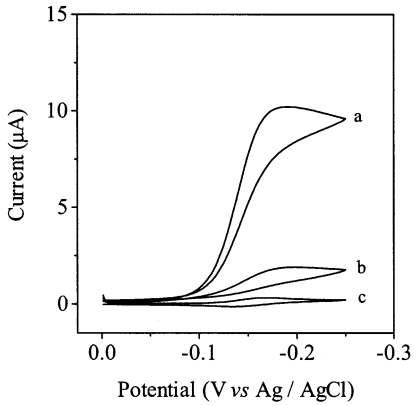

Figure 13. Electrocatalytic activity of gold electrodes modified with SAM-TOA-DADOO-Epi incubated for 24 h at room temperature in 50 mM bicarbonate buffer, pH 9.0, containing 0.2 mg mL^{-1} HRP_{native}: (a) electrode washed 5 min in 50 mM phosphate buffer pH 7.5, 0.1 M KCl; (b) electrode as in (a) but further incubated 30 min in the same buffer containing 0.1 M sorbitol. Other conditions as in Figure 9. Trace (c) corresponds to a control electrode treated as in (a) and without H_2O_2 addition.

in 100 mM sorbitol (Figure 13b). Interestingly enough, adsorption of HRP_{native} on this full epoxy monolayer did not occur in the presence of 100 mM KCl (not shown). These results strongly suggest that under these experimental conditions noncovalent links hold the protein on the surface. These results could be explained by considering the competition of water solvent molecules with nucleophilic residues of the protein for reaction with epoxy groups. After oxyrane ring opening by solvent molecules, the resulting monolayer offers a lawn of hydroxyl groups ready to establish weak hydrogen bonds with the glycosylated part of the native enzyme, these weak interactions being suppressed at high ionic strength.

We conclude that only the increase of the protein concentration on the epoxy monolayer competes efficiently with solvent molecules for attack on the oxyrane rings to form covalent links involving the carbohydrate moiety. After covalent trapping of the protein, the resulting enzyme monolayer becomes stable even under conditions which, although inadequate for the stability of boronate complexes, e.g., acidic or neutral pHs, are optimal for the activity of many enzymes.

The outstanding feature of this immobilization system is the enhancement in the rate of the reaction between enzyme molecules and epoxy groups achieved by adsorbing the protein on the surface. The intramolecular rate of the reaction in this configuration is much faster than the bimolecular rate governing the reaction when the protein molecules are in solution and have to diffuse to the activated electrode surface in order to attack the oxyrane rings. A similar mechanism, but implying hydrophobic interactions at high ionic strength, has been proposed for protein immobilization on hydrophobic epoxy-acrylic resins.[47] More recently this strategy has been extended to the specific immobilization of his-tagged enzymes to heterofunctional chelate-epoxy supports.[48]

It is expected that this strategy could be generalized to other glycosylated proteins. It is possible to introduce specific glycosylation sites in a protein by site-directed mutagenesis combined with chemical modification[49] or by specific glycosi-

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dation of the C-terminal amino acid residue.[50] Therefore, the possibility of using sugars as binding motifs is an interesting alternative to other immobilization procedures that compromise amino acid residues. This system can be easily extended to combinations of other types of weak interactions to specific ligands and reactive oxyrane groups SAM-TOA-DADOO-[Ligand/Epi]. These structures are very stable when stored dry. Therefore, this system represents an attractive *ready to use* custom-built support to immobilize native or ex novo designed proteins, e.g., glyco, poly-his, or biotin modified enzymes, by simple incubation of the electrodes in rather dilute protein solutions.

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Supporting Information Available: Figure S1, containing AFM tapping mode images of a "bare" gold surface (a) and a gold surface covered with SAM-TOA-DADOO-[APBA/Epi] incubated in HRP_{recomb} (b) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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