Combined ATR-SEIRAS and EC-STM Study of the Immobilization of Laccase on Chemically-Modified Gold Electrodes.

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1- Introduction

Covalent immobilization of biomolecules on functionalized surfaces has been demonstrated to be a useful tool for developing biosensors and catalytic bioelectrodes.¹ Covalent linkage of biomolecules onto electrodes requires the chemical modification of the surface to be modified with appropriate functional groups or binding motives. This modification can be achieved by different methods, the most popular being the formation of a thiol self-assembled monolayer (SAM) onto a metallic surface, particularly gold or platinum. Thiol SAMs are easily prepared and allow incorporating a broad variety of functional groups in the SAM. However, thiol SAMs lack stability at negative potentials and are not suitable for applications-attractive surfaces based on carbon materials.

Pinson and co-workers²⁻⁴ introduced the electroreduction of aromatic diazonium salts as a method to modify carbon electrodes with appropriate functionalities. With this procedure, Bourdillon et al.⁵ obtained a stable layer of 4-phenylacetic groups on glassy carbon electrodes, robust enough as to immobilize glucose oxidase. More recently, aromatic diazonium salts have been used for modification of gold and other metals with functionalized organic molecules⁶, yielding adlayers more stable than those functionalized with thiol SAMs.⁶ This method has been used for covalent immobilization of biomolecules both on carbon^{4,7,8} and gold⁹⁻¹¹ electrodes. Recently, a gold electrode was modified with a mixed monolayer of an aromatic diazonium salt derivative and 6-mercapto-1-hexanol, and was used as a platform onto which laccase (a Cu-metalloenzyme that catalyzes the four-electrons reduction of oxygen to water) can be linked covalently. This strategy was shown to yield the stable immobilization of the enzyme, and to favour its adequate orientation for direct electron transfer from the electrode.¹¹

In this work, we have characterized the sequential steps in the covalent immobilization of laccase on gold electrodes, modified either by the mixed mercaptohexanol (MHO) and aminophenyl monolayer or by 4-aminotiophenol (4-ATP). The techniques used to follow the surface evolution were surface-enhanced infrared absorption spectroscopy in the attenuated total reflection mode (ATR-SEIRAS) and *in situ* scanning tunneling microscopy (STM). The aim of this work is to provide (i) spectroscopic proof of the chemical changes associated to the successive steps of the electrode modification, (ii) information about the changes in the topography and the structure of the electrode surface associated to the successive chemical modifications,

and (iii) unambiguous spectroscopic and topographic proof of the enzyme attachment to the surface of the chemically modified gold electrode.

2- Experimental

Gold surface: The working electrode for the IR experiments was a thin (20-50 μm) gold film deposited following a previously reported chemical procedure¹² on a Si prism bevelled at 60°. In order to obtain a more robust gold film, a first gold film was dissolved using *aqua regia* (1 : 3 (v/v) HNO_3 : HCl), and a new gold film was formed onto the Si prism.¹³ The Au-covered Si prism was then attached to the spectroelectrochemical cell, using an O-ring to prevent the electrolyte from leaking. Electrical contact to the gold film was achieved by pressing against it a circular Au wire by means of an O-ring. Before the experiments, the Au surface was electrochemically cleaned by repetitive potential cycling between 0 and 1.4 V *vs.* RHE in 0.1 M H_2SO_4 (Merck, suprapur). A coiled gold wire and a Ag/AgCl (KCl_{sat}) electrode were used as counter and reference electrodes, respectively.

For STM experiments, the working electrode was the (111)-oriented face of a single-crystal gold disk (10 mm diameter) from MaTeck (Jülich, Germany). The gold disk was annealed in the flame of a Bunsen burner before chemical modification.

Chemical modification of the gold surface: Figure 1(a-c) represents the sequence followed during surface modification with a mixed aminophenyl-MHO adlayer. The first step is intended to attach nitrophenyl groups to the gold surface by cycling the electrode twice between 0.6 and -0.6 V *vs.* Ag/AgCl_{sat} at 0.2 V s⁻¹ in a solution of acetonitrile (Scharlau, anhydrous) containing 0.1 M BF_4NBu_4 (Aldrich, p.a.) and 2 mM p-nitrophenyldiazonium salt (Aldrich, p.a.). Then, the resulting modified Au electrode was rinsed with water, immersed in a 0.1 M solution of KCl (Scharlau, p.a.) in EtOH : H₂O (1 : 9), and cycled twice between 0 and -1.4 V *vs.* Ag/AgCl_{sat} at 0.1 V s⁻¹, with the aim of reducing the nitro groups to amino groups, thus yielding an aminophenyl-modified Au electrode. Finally, the electrode was left overnight immersed in a 1 mM MHO (Aldrich, 97%) solution in absolute Ethanol (Panreac), with the intention of covering the naked regions of the gold surface with the thiol, thus generating a mixed aminophenyl-MHO adlayer.

Alternatively, the gold electrode was modified with 4-aminothiophenol (4ATP, Aldrich, 97%) by immersing the electrode into a 1 mM 4ATP solution in absolute ethanol during 16 hours. Both aminophenyl- and 4ATP-modified Au electrodes expose the same functional group ($-\text{NH}_2$) to the electrolyte, but in the former case the aminophenyl group is directly bonded to the gold surface via a Au-C bond,¹⁴ while in the latter case it is anchored to the metal surface via a Au-S bond.¹⁵

Figure 1. Sequence of the steps followed for the modification of a gold surface with *p*-nitrophenyldiazonium salt and subsequent immobilization of laccase: a) formation of nitrophenyl adlayer; b) aminophenyl adlayer formed by electroreduction of the nitro groups of the nitrophenyl adlayer; c) formation of a mixed aminophenyl/mercaptohexanol monolayer; d) covalent attachment of laccase to the mixed monolayer by formation of amide bonds; e) covalent attachment of laccase to the mixed monolayer by formation of imine bonds; f) covalent attachment of laccase to the mixed monolayer by formation of imine bonds and amide bonds.

Covalent immobilization of laccase: Covalent immobilization of laccase, illustrated in Figure 1(d-f), was achieved either by forming amide bonds between carboxylic acid groups of the enzyme and the amino groups of the functionalized surface, or by Schiff base reaction¹⁶ followed by formation of an amide bond. In the former case, 10 μL of *Trametes hirsuta* laccase (8.9 $\mu\text{g mL}^{-1}$ in 0.1 M phosphate buffer, pH 6.0) were added to 400 μL of a 10 mM MES solution (pH 6.0) containing 36 mM 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC, Fluka, $\geq 98\%$) hydrochloride and 17 mM N-hydroxysuccinimide (NHS, Sigma, 98%). The electrode was immersed in this solution and left reacting during 2 hours. In the latter case, 10 μL of *Trametes hirsuta* laccase (8.9 $\mu\text{g mL}^{-1}$ in 0.1 M phosphate buffer, pH 6.0) were added to 110 μL of a 10 mg mL^{-1} NaIO₄ (Sigma-Aldrich, 99.8%) solution and incubated during 30 min in order to oxidise the sugar residues of the laccase. Then, 180 μL of 0.1 M Na₂HPO₄ (Fluka, 99%) were added to the solution to increase the pH to 7. The gold surface was immersed in this enzyme solution and incubated during 30 min. After rinsing the gold surface with water, a 10 mM MES solution (Sigma, pH 6.0) containing 36 mM EDC hydrochloride and 17 mM NHS was added and left reacting during 2 hours. Finally, the enzyme modified gold surface was consecutively cleaned with 10 mM MES buffer and water.

ATR measurements: ATR-SEIRA spectra were recorded with a Perking-Elmer 1725-X or a Nicolet 6700 FTIR spectrometer equipped with a liquid nitrogen-cooled MCT detector, using p-polarized light. Each spectrum consisted of 40 interferograms, collected with a spectral resolution of 8 cm^{-1} . The differential spectra were calculated as $-\log(R_{\text{sample}}/R_{\text{reference}})$, where $R_{\text{reference}}$ and R_{sample} are the reference and sample spectra, respectively. Since the N-H stretching modes of the amine and hydroxylamine groups and the H-N-H bending mode of the amine group appear in the same region as the O-H stretching and H-O-H bending modes of water, respectively, we decided to record the spectra in the absence of water, either in air, or using CH_3CN as a solvent. In all spectra, the bare gold surface has been taken as $R_{\text{reference}}$.

STM measurements: the STM used was a PicoLE Molecular Imaging with a PicoScan 2100 Controller. Experiments were performed with tungsten tips, etched from a polycrystalline wire in 2 M NaOH (Sigma-Aldrich, $\geq 98\%$) and coated with electrophoretic lac or polyethylene glue (ethyl-vinylacetate copolymer) from a polyethylene glue gun, in order to reduce the faradaic current at the tip/electrolyte interface. A platinum wire was used as quasi-reference electrode, although the potentials in the text are referred to the Ag/AgCl (KCl_{sat}) electrode. All images were measured either in 0.1 M HClO_4 (Merck, p.a.) or in 0.1 M KClO_4 (Merck, p.a., recrystallized) + 0.05 M $\text{Na}(\text{CH}_3\text{COO})$ (Merck, pH 4.2), and recorded in the constant-current mode. The auxiliary electrode was a short platinum wire. Raw STM images were treated using WSxM software.¹⁷

3- Results

3.1. Laccase immobilization onto a mixed aminophenyl-MHO modified gold surface.

3.1.1. Surface functionalization with a mixed aminophenyl-MHO adlayer.

3.1.1.1. Infrared spectroscopy. The first step in the modification process consisted in the electroreduction of *p*-nitrophenyldiazonium cations, aimed at forming a gold-carbon bond and at creating a *p*-nitrophenyl-modified gold surface. The success of this first step is demonstrated by the observation of the characteristic bands of the nitrophenyl group in the ATR-SEIRA spectrum of the resulting surface, particularly the asymmetric NO_2 stretching ($\nu_{\text{a}}\text{-NO}_2$) at 1520 cm^{-1} and the symmetric NO_2 stretching ($\nu_{\text{s}}\text{-NO}_2$) at 1350 cm^{-1} (Figure 2, line a). The weaker band at 1600 cm^{-1} must correspond to the aromatic ring stretching. It should be accompanied by another band around 1500 cm^{-1} , which is most likely obscured by the $\nu_{\text{a}}\text{-NO}_2$ band. Our results are in good agreement with previous infrared studies of nitrophenyl-functionalized gold surfaces.^{18,19} The band at 1245 cm^{-1} , also observed by Ricci et al.¹⁸ when the modification of gold with nitrophenyl was done by means of cyclic voltammetry, must be due to silicon oxides that appear on the surface of the Si substrate during the electrochemical modification. The absence of a band at 2300 cm^{-1} indicates the absence of physically adsorbed diazonium cations¹⁹.

The second step consisted in the electroreduction of the nitro group in $\text{EtOH:H}_2\text{O}$ 1:9 containing 0.1 M KCl, yielding an aminophenyl-functionalized surface (Figure 1b) to which the enzyme can be chemically bonded *via* (i) a Schiff base reaction with aldehyde groups of the laccase obtained by oxidation of the sugar residues and (ii) amide bonds with the carboxylic acid groups of the laccase. The bands at 1520 and 1350 cm^{-1} due to the asymmetric and symmetric NO_2 stretching disappear after the second step of the surface modification (Figure 2, line b), a clear evidence of the successful electrochemical reduction of the nitro groups.

Figure 2. ATR-SEIRA spectra of the nitrophenyl-modified (a), the aminophenyl-modified (b), and the mixed monolayer (aminophenyl/MHO)-modified (c) gold surface in CH_3CN , obtained using the bare gold surface as reference.

Electrochemical reduction of the surface-anchored nitrophenyl groups to yield an aminophenyl-functionalized gold electrode (Au-Ar-NH_2) should result in an infrared spectrum very similar to that obtained with a 4-ATP-modified Au surface. However, a small portion of hydroxylamine (Ar-NHOH) and nitroso groups (Ar-NO) are also generated during the reduction process.²⁰ As a consequence, instead of two well-defined bands around 1600 cm^{-1} corresponding to the aromatic ring and to the amino group, as in the case of 4-ATP (see below), we observe a broad feature extending from 1558 to 1737 cm^{-1} that includes the ring stretching, the aminophenyl NH_2 scissoring and the hydroxylamine NH bending modes. A small band appears at 1507 cm^{-1} , that can be attributed to the ring band accompanying that at 1600 cm^{-1} and/or to the N=O stretching of nitroso groups.

The weak, broad symmetric band centered at 3250 cm^{-1} must include the N-H stretching mode of the NH_2 and the NHOH groups. The sharp band at 3733 and the band at 1425 cm^{-1} correspond to the stretching and bending modes, respectively, of hydrogen bonds-free OH groups of hydroxylaminophenyl moieties.

The third step consisted in the formation of a mixed aminophenyl-MHO adlayer by immersion of the aminophenyl-modified gold surface in a 1 mM solution of MHO in absolute ethanol (Figure 1c). The addition of the thiol molecules forms a SAM that prevents laccase denaturalization by blocking the gold surface. After this step, a new broad band appears around 3500 cm^{-1} , which we assign to the O-H stretching mode of the alcohol group of MHO (Figure 2, line c). The rest of the spectrum remains identical to that observed after reduction of the nitrophenyl groups.

3.1.1.2. STM. The process of modification of an $\text{Au}(111)$ electrode with a mixed aminophenyl-MHO adlayer was also followed using *in situ* STM. In order to proceed systematically, pure MHO adlayers were first studied in 0.1 M HClO_4 , and then in $0.05\text{ M Na}(\text{CH}_3\text{COO})$ (acetate buffer, $\text{pH } 4.2$). Then, mixed aminophenyl-MHO adlayers were studied in acetate buffer.

Figure 3. STM image ($30 \times 50 \text{ nm}^2$) of a MHO adlayer on Au(111) in 0.1 M HClO_4 (a); STM image ($20 \times 20 \text{ nm}^2$) of a MHO adlayer on Au(111) in 0.1 M KClO_4 + 0.05 M $\text{Na}(\text{CH}_3\text{COO})$ (pH 4.2) (b); STM image ($20 \times 20 \text{ nm}^2$) of a mixed aminophenyl/MHO adlayer on Au(111) in 0.1 M KClO_4 + 0.05 M $\text{Na}(\text{CH}_3\text{COO})$ (pH 4.2) (c).

Figure 3a shows an STM image of a MHO adlayer on Au(111), formed *ex situ* in an ethanol solution containing in 0.1 M HClO_4 . The image reveals the presence of ca. 0.25 nm deep holes that must correspond to monoatomic depressions (calculated height of a monoatomic step for Au(111): 0.235 nm), due to etching of the gold surface upon formation of a high-coverage thiol adlayer²¹⁻²⁷, and of small rotational domains of the same structure, which can be identified with the $c(3 \times 2\sqrt{3})$ MHO structure on Au(111) reported by Liu et al when examined with a higher resolution (not shown).²⁸ In this structure, the distance between individual spots (each attributable to an adsorbed thiolate) is $\sqrt{3}d_{\text{au}}$, but the unit cell contains two different kinds of spots. Distances of $\sqrt{3}d_{\text{au}}$ between adsorbed thiolates are typical of alkanethiol monolayers, and the different contrast of the spots within the unit cell has been attributed to different molecular conformations.^{21,28}

Interestingly, very noisy STM images are obtained (Figure 3b) when a MHO adlayer is formed *ex situ* in an ethanol solution on Au(111) and the electrode is then immersed in acetate buffer. An ordered $(\sqrt{3} \times \sqrt{3})R30^\circ$ structure, with all the spots in the unit cell having the same contrast, can however be observed on some small patches of the surface. We believe that the different images obtained in 0.1 M HClO_4 and in acetate buffer are a consequence of the interaction of the OH functional groups of the MHO adlayers with Na^+ and K^+ ions, present only in the latter case. It has been shown

recently that non-covalent interactions between surface-anchored functional groups and cations present in the electrolyte can affect significantly the structure and properties of the electrochemical double layer.^{29,30} In this case, the presence of Na⁺ and K⁺ at the double layer might increase the relative stability of the structure composed of only one conformer, as compared to that composed of two alternating conformers. Along this line of reasoning, we attribute the noise in the *in situ* STM images of MHO adlayers in acetate buffer to the rapid interconversion of one monomer into the other.

Modification of the Au(111) surface with 1 aminophenyl groups *via* the electroreduction of *p*-nitrophenyldiazonium cations and further reduction of the nitro functionality to amino groups does not result in any morphological change that can be appreciated with STM (not shown). However, after further modification of the electrode by immersion of the aminophenyl-modified gold surface in a 1 mM solution of MHO in absolute ethanol, the two structures previously observed with pure MHO adlayers can be observed on the electrode surface in acetate buffer using high-resolution EC-STM (Figure 3c): the ($\sqrt{3} \times \sqrt{3}$)R30° structure composed of only one of the two conformers covers most of the surface, while the c($3 \times 2\sqrt{3}$) structure containing two kinds of spots with different contrast, attributed to two different conformers, covers clearly smaller areas of the surface.

It is impossible to determine the aminophenyl coverage from STM images like that in Figure 3c, but they suggest that the surface is essentially covered by MHO. However, the clear difference between the STM image in Figure 3c and that of a pure MHO adlayer in the same electrolyte (Figure 3b), provides further support to our above-described hypothesis above that very subtle changes in the interaction between adsorbed species and between the adsorbed species and the constituents of the electrolyte can affect the structure and dynamics of the adlayer significantly. At pH 4.2, the -NH₂ groups of the aminophenyl moieties on the surface of a Au(111) electrode modified by a mixed MHO/aminophenyl adlayer must be protonated,³¹ and bear, hence, a positive charge that will screen the surface from the Na⁺ and K⁺ ions in the electrolyte, impeding their interaction with the OH groups of MHO. Under these conditions, the mobility of the MHO adlayer would be expected to resemble that found in 0.1 M HClO₄, as is indeed the case (compare Figures 3a and 3c).

3.1.2. Bonding of laccase to a mixed aminophenyl-MHO modified gold surface.

3.1.2.1. Infrared spectroscopy. Bonding of laccase by a Schiff base reaction between the amino groups in the monolayer and the aldehyde groups of the laccase obtained by chemical oxidation of the sugar residues originally present on the surface of the enzyme (Figure 1e), is evidenced by the appearance of two bands at 1663 and 1526 cm⁻¹ (Figure 4), corresponding to the protein backbone vibrations amide I and amide II, respectively.³² Upon attachment of laccase to the electrode surface, the broad bands at 3500 and 3250 cm⁻¹, which we had attributed to the O-H stretching of MHO and to the N-H stretching of the aminophenyl groups respectively, are substituted by an asymmetric, more intense broad band with the absorption maximum at 3292 cm⁻¹ (Figure 4, line b). No decrease in the 3733 and 1425 cm⁻¹ bands is detected, as expected if they correspond to the OH vibrations of hydroxylaminophenyl groups, which are not involved in the covalent immobilization *via* formation of a Schiff base.

The last step, i.e., the treatment with NHS/EDC, aimed at catalyzing the formation of amide bonds between the amino groups of the aminophenyl moieties on the surface of the chemically-modified gold electrode and the carboxylic acid groups of the laccase (Figure 1f), results in the emergence of a new band at 1737 cm⁻¹, typical of a carbonyl

group, thus confirming the formation of covalent amide bonds between the chemically-modified surface and the laccase (Figure 4, line c).

Figure 4. ATR-SEIRA spectra in CH_3CN , calculated using the bare gold surface as reference, of the laccase immobilized onto the mixed monolayer (aminophenyl/MHO)-modified gold surface via amide bond formation (a), binding by Schiff base reaction (b), and binding by Schiff base reaction followed by amide bond formation (c).

In a control experiment, the mixed monolayer was treated in the same conditions used for laccase immobilization via the formation of a Schiff base and an amide bond, but in the absence of laccase. In this case, no change in the spectrum of the mixed monolayer occurred (Figure S1).

Some differences can be observed in the ATR-SEIRA spectrum when the Schiff base reaction does not precede the formation of an amide bond (Figure 4, line a): Attachment of the enzyme to the electrode surface is again evidenced by peaks at 1660 and 1547 cm^{-1} , corresponding to the protein backbone vibrations amide I and amide II, and a shoulder at 1736 cm^{-1} confirms the formation of a covalent amide bond between the chemically-modified surface and laccase, as described before. However, in this case the bands at 3733 and 1425 cm^{-1} , which we have attributed to the O-H vibrations of hydroxylaminophenyl groups, disappear after the reaction, and a broad band between 3680 and 3000 cm^{-1} , with an absorption maximum around 3500 cm^{-1} , substitutes the bands at 3500 and 3250 cm^{-1} , that we had attributed, respectively, to the O-H stretching of MHO and to the N-H stretching of the aminophenyl groups. These differences indicate that, although the two procedures used to immobilize the enzyme are successful, the resulting surfaces are different.

3.1.2.2. STM. Figure 5 shows STM images in acetate buffer of an aminophenyl/MHO-modified Au(111) electrode after having been treated with the solutions used for enzyme immobilization in the presence (Figure 5a) and in the absence (Figure 5b) of laccase. Although in both cases the treatment provokes changes in the surface topography, the presence of laccase affects the nature of these changes.

Figure 5. STM images ($100 \times 100 \text{ nm}^2$) of a mixed aminophenyl/MHO adlayer on Au(111) in $0.1 \text{ M KClO}_4 + 0.05 \text{ M Na}(\text{CH}_3\text{COO})$ (pH 4.2), after having been treated with the solutions used for enzyme immobilization in the absence (a) and in the presence (b) of laccase. Profiles along the lines marked on the STM images as 1 (c) and 2 (d) in the absence (black) and in the presence (red) of laccase.

New protrusions appear after immobilization of laccase on an aminophenyl/MHO-modified Au(111) electrode (Figure 5b). Although their height (ca. 0.3 nm) is lower than that expected for individual laccase units, this can be affected by electronic factors and is simply proportional to the local probability of tunneling (or, in other words, to the local density of electronic states). On the contrary, the lateral dimensions of the spots agree very well with those of individual laccase units ($6.5 \times 5.5 \times 4.5 \text{ nm}^3$),³³ to which we assign them. From the STM images we estimate an average density of $(7.27 \pm 1.93) \times 10^{11}$ laccase units per cm^2 , in good agreement with previous estimations of the coverage of laccase immobilized by other procedures on chemically-modified gold electrodes,³⁴ or of the coverage of other enzymes immobilized by a procedure similar to that used here.³⁵

In order to obtain further support to our assignment of the bright features in Figure 5b to individual laccase units, we submitted the aminophenyl/MHO-modified Au(111)

electrode to exactly the same treatment, but in the absence of laccase. Under these conditions, ellipsoidal protusions ca. 4 nm wide, ca. 8 nm long and ca. 0.2 nm high appear, and the number of protrusions per unit area is nearly half that found in the presence of laccase (Figure 5a).

3.2. Laccase immobilization onto a 4-ATP modified gold surface.

3.2.1. Surface functionalization with 4-ATP.

3.2.1.1. Infrared spectroscopy. The ATR-SEIRA spectrum of the 4-ATP-modified gold surface (Figure 6, line a) is similar to that reported for a 4-ATP-modified platinum electrode.³⁶ The absence of a band around 2550 cm^{-1} indicates the loss of the S-H bond and the formation of a S-Au bond. Bands corresponding to the stretching and scissoring modes of the amino group can be clearly observed at 3370 and 1630 cm^{-1} , respectively. The broad band at 3020 cm^{-1} is typical of the C-H stretching modes of the aromatic ring, and the bands at 1585 and 1488 cm^{-1} correspond to the C-C aromatic ring stretch. The band at 1290 cm^{-1} is due to the C-N stretching and the band at 1178 cm^{-1} could correspond to the C-S stretching.

Figure 6. ATR-SEIRA spectra of a gold surface in CH_3CN after modification with 4-ATP (a), after immobilization of laccase via Schiff base reaction (b), after immobilization of laccase via Schiff base reaction followed by amide bond formation (c), and after immobilization of laccase via amide bond formation (d), calculated using the bare gold surface as reference.

3.2.1.2. STM. Figure 7 shows an STM image of a Au(111) electrode covered by an *ex-situ* formed 4-ATP adlayer. Although some degree of order can be guessed in the darker areas of some images, there is a very high surface mobility (see Supporting Information), that prevented us from obtaining high-resolution images of reasonable quality. This is due to the well-known tendency of 4-ATP to form multilayers on Au(111)^{37,38} and, consequently, we attribute the darker areas in Figure 7 to the first 4-ATP layer and the very mobile islands to a second layer.

Ex-situ STM images of 4-ATP-modified Au(111) electrodes, obtained in air, revealed a $(\sqrt{3} \times \sqrt{3})R30^\circ$ structure with a coverage of 1/3 ML.³⁹ We assume that this must be the coverage of the first 4-ATP adlayer, whereas the total coverage, which includes the second layer, must be obviously larger than this. It has been recently proposed that the second 4-ATP adlayer attaches to the first one via S...H hydrogen bonds between the sulfur atom of the thiol group of the second-layer molecules and the hydrogen atoms of the amino functional groups of the molecules in the first layer.³⁸ This weak interaction between the first and the second 4-ATP adlayer explains the high mobility of the latter.

Figure 7. STM image ($100 \times 100 \text{ nm}^2$) of a 4-ATP adlayer on Au(111) in 0.1 M KClO₄ + 0.05 M Na(CH₃COO) (pH 4.2).

3.2.2. Bonding of Laccase to a 4-ATP-modified gold surface.

3.2.2.1. Infrared spectroscopy. Immobilization of laccase by formation of a Schiff base onto a 4-ATP modified gold surface (Figure 6, lines b and c) is evidenced by the appearance of two bands due to the protein backbone vibrations amide I (1650 cm^{-1}) and amide II (1545 cm^{-1}), as described above for the immobilization onto a mixed aminophenyl-MHO modified gold electrode. Other new bands appear at 1400 , 1320 and 1240 cm^{-1} . The 1400 and 1320 cm^{-1} bands can be attributed to the amide III mode of the protein backbone ($1200\text{-}1400 \text{ cm}^{-1}$)³². The band at 1320 cm^{-1} is broad because it includes that at 1285 cm^{-1} , attributed to the C-N single bond of the amino group. The band at 1240 cm^{-1} is possibly due to silicon oxides that appear on the surface of the Si substrate during the modification. A small shoulder appears at about 1720 cm^{-1} , that we assign to the C=N double bond formed after the Schiff base reaction. In the high frequency region, a band centered at 3350 cm^{-1} emerges, probably due to a shift of the stretching mode of the amino group after laccase immobilization. This shift is clearer in lines c and d of Figure 6. In line b of Figure 6 this band appears as a shoulder included

in a wide band at 3500 cm^{-1} , which is attributed to some residual water present in the surface. This water band also appears in the other spectra if the surface had only been dried with N_2 , but if the surface is first rinsed with ethanol and then dried with N_2 , the band at 3500 cm^{-1} disappears and the band at 1650 cm^{-1} decreases. Formation of the amide bond is evidenced by the shoulder at 1736 cm^{-1} (Figure 6, lines c and d).

3.2.1.2. STM. As in the case of aminophenyl/MHO-modified Au(111) electrodes, the immobilization of laccase on 4-ATP-modified Au(111) electrodes was also studied using STM (Figure 8). After immobilization of the enzyme by formation of a Schiff base and of amide bonds, the mobility of the surface is drastically reduced, as expected, since the second layer of 4-ATP is bonded to the first one via weak hydrogen bonds, and will be displaced by laccase, which is covalently bound to the $-\text{NH}_2$ groups of the first 4-ATP layer. Furthermore, the number of $-\text{NH}_2$ groups must have decreased after covalent immobilization of the enzyme, and thus fewer will remain for attaching a second 4-ATP layer.

Figure 8. STM image ($100 \times 100\text{ nm}^2$) of the 4-ATP/laccase modified gold surface in $0.1\text{ M KClO}_4 + 0.05\text{ M Na}(\text{CH}_3\text{COO})$ (pH 4.2).

The lateral size of the islands on the surface has also decreased, and their number and height has increased. Taking into account the similitude of their dimensions with those of the features observed upon immobilization of laccase on an aminophenyl/MHO-modified Au(111) electrode (Figure 5), we attribute the protrusions in Figure 8 to laccase units. Although it is difficult to estimate the laccase coverage from the STM images, it is evident that the coverage is higher in the case of a 4-ATP-modified Au(111) electrode than in the case of an aminophenyl/MHO-modified Au(111) electrode, as was to be expected from the larger amount of $-\text{NH}_2$ groups exposed to the solution in the former case.

4- Discussion

The results above provide clear spectroscopic evidence of the success of the chemical modification steps illustrated in Figure 1, and of the immobilization of laccase on gold surfaces modified by a mixed aminophenol/MHO or by a 4-ATP adlayer.

In both cases, the immobilization of laccase was achieved by formation of amide bonds between appropriate residues of the laccase units and the amino groups on the surface of a gold electrode covered by a NH_2 -terminated organic adlayer, preceded by formation of a Schiff base. It has been found that a high activity for direct electrocatalytic reduction of oxygen results upon immobilization of laccase by formation of imine bonds in a Schiff base reaction, followed by amide bond formation on a Au surface modified by a mixed aminophenyl/MHO adlayer.¹¹ This has been attributed to the correct orientation of the immobilized laccase units, with the Cu T1 center of the enzyme facing the electrode surface, thus allowing fast direct electron transfer at low overpotentials. However, if this previous step of Schiff-base formation is absent during laccase immobilization, a very low activity for direct electrocatalysis was measured, although significant mediated electrocatalysis was detected.¹¹ Similarly, immobilization of laccase on 4-ATP-modified gold electrodes yielded poorer results for direct electrocatalysis via the Cu T1 site,⁴⁰ despite the expected higher coverage by NH_2 functionalities in the latter case. We will attempt to find an explanation to these differences based on our combined spectroscopic and STM study.

The ATR-SEIRA spectra of laccase immobilized onto a mixed aminophenyl/MHO adlayer by formation of amide bonds with and without a previous Schiff base reaction are identical, except for the region corresponding to the O-H stretching mode (Figure 4). These differences indicate that, although the enzyme has been successfully immobilized in both cases, the resulting surfaces are different. This is probably due to the different laccase residues involved in both immobilization methods. Schiff bases formation involves sugar residues, which are mostly placed next to the Cu T1 site, whereas carboxylic residues are more abundant in the opposite region of the laccase.⁴¹ Therefore, a different orientation of laccase with respect to the gold surface is expected when laccase is immobilized only *via* amide bonds instead of the two-step method. As a consequence, the interaction between the OH groups of the MHO adlayer and the laccase residues is different in these two cases, generating the observed differences in the spectra. The ATR-SEIRA results reported here are, hence, in agreement with previous electrochemical results, which suggested that the formation of imine bonds *via* a Schiff base reaction prior to the amide bonds formation promotes a specific laccase orientation that facilitates a better communication between the T1 center and the metal surface and, consequently, a higher activity for low-overpotential, direct electrocatalytic reduction of oxygen to water.¹¹ As we will show below when comparing 4-ATP- with aminophenyl/MHO-modified gold electrodes, the presence on the surface of MHO also appears to be important in this regard.

An evident difference between aminophenyl/MHO and 4-ATP adlayers is that the aminophenyl groups are bonded to the surface directly through an Au-C bond in the former case, while in the latter a S atom is intercalated between the gold surface and the aromatic ring. This might affect the electronic communication between the enzyme's T1 center and the electrode surface, and could be the reason for the drastic hampering of low-overpotential direct electrocatalytic activity when laccase is immobilized on a 4-ATP-modified gold electrode. However, this possibility must be discarded, because catalytic activity for the electroreduction of oxygen to H_2O_2 at higher overpotentials has been obtained after immobilization of laccase on 4-ATP-modified Au electrodes,⁴⁰ and direct electrocatalytic activity for the 4-electron reduction of oxygen has been obtained for laccase immobilized on anthracene-2-methanethiol-modified Au electrodes.³⁴

STM measurements show that the 4-ATP-functionalized surface presents a high mobility (Figure S2) as a consequence of the formation of bilayers in which the second layer is attached to the first one via S...H hydrogen bonds between the sulfur atom of the thiol group of the second-layer molecules and the hydrogen atoms of the amino functional groups of the molecules in the first layer.³⁸ This high mobility, or attachment of laccase to the second 4-ATP adlayer, could be invoked as the reason for the poorer direct electrocatalytic activity, but this possibility must also be discarded, because laccase should displace the 4-ATP molecules in the second layer upon covalent binding with the NH₂ groups of the first layer. This is confirmed by the much lower surface mobility observed by STM after immobilization of laccase on a 4-ATP-modified Au(111) electrode (Figure 8).

Amide III bands could only be observed in the ATR-SEIRA spectra when laccase is immobilized onto the 4-ATP-modified surface. The similar intensities of the amide I and amide II bands in the spectra in Figures 4 and 6 suggest that this is not due to a higher coverage by immobilized laccase onto the 4-ATP-modified gold surface (as could be expected from the larger amount of amino groups), and that, on the contrary, the spectral differences might be due to a different enzyme orientation. Since the functional groups to which the enzyme was anchored and the immobilization procedure (Schiff base reaction followed by amide bond formation) was the same on the two chemically-modified Au surfaces, the different orientation must be the result of the presence of a large amount of MHO on the surface of the aminophenyl/MHO-modified gold electrodes. The important role of MHO in adequately orienting the laccase units is supported by the fact that significantly lower activity for direct electrocatalytic reduction of oxygen was obtained when thiols with different terminal functional groups, particularly cystamine and mercaptopropionic acid, were used instead of MHO.¹¹

5. Conclusions

We have provided spectroscopic evidence of the success of all the steps involved in the immobilization of laccase on NH₂-terminated chemically-modified gold electrodes, and have examined the corresponding changes in the surface morphology using *in-situ* STM. Our results provide additional support to the extended idea that efficient communication between the T1 center of laccase and the electrode surface requires an adequate orientation of the immobilized enzyme, and that this orientation is governed not only by the covalent bonds formed, but also by subtle non-covalent interactions with the functional groups surrounding the binding site.

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Supporting information

SI Figure 1. ATR-SEIRA spectra, calculated using the bare gold surface as reference, of the mixed monolayer (aminophenyl/MHO)-modified gold surface in 50 mM acetate buffer pH 4.0 containing 100 mM HClO_4 , before (black) and after (red) treating the surface with $\text{NaIO}_4/\text{Na}_2\text{HPO}_4$ and NHS/EDC in the absence (a) and the presence (b) of *Trametes hirsuta* laccase. In the absence of laccase, there is no change in the spectrum after treatment. The band at 1237 cm^{-1} is due to the formation of silicon oxides during the gold modification as explained in the text; the negative band at 1400 cm^{-1} is due to the desorption of acetate adsorbed on the bare gold surface during the surface functionalization; and the band centered at 1650 cm^{-1} is due to water. When the mixed monolayer is treated in the same way in the presence of laccase some changes appear in the spectrum: an increase in the band at 1650 cm^{-1} , due to the contribution of amide I stretching; a shoulder at 1540 cm^{-1} due to amide II stretching of the laccase; and a shoulder at 1730 cm^{-1} due to the amide bond between the carboxylic groups of the laccase and the amide groups of the surface.