Electrostatic-Driven Activity, Loading, Dynamics, and Stability of a Redox Enzyme on Functionalized-Gold Electrodes for Bioelectrocatalysis

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Supporting Information

ABSTRACT: The oxygen reduction reaction is the limiting step in fuel cells, and many works are in progress to find efficient cathode catalysts. Among them, bilirubin oxidases are copper-based enzymes that reduce oxygen into water with low overpotentials. The factors that ensure electrocatalytic efficiency of the enzyme in the immobilized state are not well understood, however. In this work, we use a multiple methodological approach on a wide range of pH values for protein adsorption and electrocatalysis to demonstrate the effect of electrostatic interactions on the electrical wiring, dynamics, and stability of a bilirubin oxidase adsorbed on self-assembled-monolayers on gold. We show on one hand that the global charge of the enzyme controls the loading on the interface and that the specific activity of the immobilized enzyme decreases with the enzyme coverage. On the other hand, we show that the dipole moment of the protein and the charge in the vicinity of the Cu site acting as the entry point of electrons drive the enzyme orientation. In case of weak electrostatic interactions, we demonstrate that local pH variation affects the electron transfer rate as a result of protein mobility on the surface. On the contrary, stronger electrostatic interactions destabilize the protein structure and affect the stability of the catalytic signal. These data illustrate the interplay between immobilized protein dynamics and local environment that control the efficiency of bioelectrocatalysis.

KEYWORDS: enzymes, catalysis, self-assembled-monolayers, electrochemistry, ellipsometry, surface plasmon resonance, PMIRRAS

INTRODUCTION

In an upcoming rising sustainable economy, fuel cells may play a role in energy production. One of their limitations is the low efficiency of the oxygen reduction reaction (ORR), imposing the study of a catalyst combining high performance, stability, and renewability.1−4 Redox enzymes are such catalysts that efficiently operate in microorganisms to convert substrates. Among them, bilirubin oxidase (BOD), which belongs to the multicopper oxidase family, is one of the most considered alternatives to platinum for oxygen reduction in enzymatic fuel cells.5−8 The global catalytic cycle performed by this enzyme immobilized on electrochemical interfaces is now well established.5−7 It involves four copper centers, the CuT1 being the one which accepts the electrons from the reductant and will therefore be the entry point of electrons from the electrode. Although enhanced catalytic performance has been achieved by entrapment of BOD in various carbon and metal nanomaterials,5−11 BOD-based bioelectrodes still suffer from low stability, precluding industrial use of the related biodevices. As an illustration, recent works in our laboratory showed that the half-life of a BOD-based bioelectrode incorporated in carbon felts was restricted to 1 week at room temperature.12 Furthermore, for direct wiring of a redox enzyme on a conductive support, it is mandatory to allow electron tunneling between the enzyme active site and the electrode and to permit substrate access.13−17 By varying the pH of BOD adsorption on a carbon nanotube (CNT) network, Mazurenko et al. demonstrated that electrostatic interactions were driving the adsorption process in an orientation favoring either this direct wiring (direct electron transfer, DET) or a connection via a diffusing redox mediator (MET).15 However, even after having defined the surface chemistry required for an efficient direct

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wiring, it was calculated that less than 10% of the loaded enzymes effectively participated in catalysis.\textsuperscript{12} A similar low percentage of electroactive enzymes was reported recently for laccase, another multicopper protein, on amorphous carbon nitride.\textsuperscript{18}

Understanding the factors that affect this low catalytic efficiency is thus required. While porous electrodes may enhance the loading of enzymes, planar electrodes are much more appropriate for fundamental studies of enzyme immobilization.\textsuperscript{14,19–22} In particular, planar gold surfaces are mostly used in methods allowing one to study loading of enzymes on solid supports (surface plasmon resonance (SPR), quartz crystal microbalance (QCM)) or enzyme conformation in the immobilized state (surface-enhanced infrared absorption (SEIRA), surface-enhanced Raman spectroscopy (SERS), polarization modulation infrared reflection absorption spectroscopy (PMIRRAS)).\textsuperscript{14,23,24} Coupling these methods to electrochemistry is crucial to being able to correlate electro-enzymatic activity to enzyme amount and conformation and to study the dynamics of the immobilized enzyme with the ultimate goal of proposing bioelectrode rationalization.\textsuperscript{25} Self-assembled-monolayers (SAMs) appear as fine tools allowing one to easily tune and control the chemistry and charge of a planar gold electrochemical interface while being rid of the complex surface chemistry and porosity of nanomaterials, which could influence the electrochemical response. Varying the pH may offer the additional advantage of changing the interactions between the surface and the enzyme by affecting both components in a controllable manner. However, the dynamics of the immobilized enzyme upon local pH change in the course of electrocatalysis has been rarely investigated. We cite the study by Jin et al., who investigated the pH-dependent interfacial electron transfer of cytochrome c electrostatically bound to a SAM.\textsuperscript{26}

In this work we bring new insight toward the comprehensive enzyme immobilization by the unprecedented coupling of electrochemistry to SPR, PMIRRAS, and ellipsometry. Myrothecium verrucaria BOD (Mv BOD) adsorption on negative and positive SAM layers on gold electrodes was explored. Both the pH of adsorption and the pH for electrocatalysis were systematically varied to modulate the charge of the SAM–gold electrode, the global charge of the protein, and the CuT1 vicinity charge. Modeling of cyclic voltammetry curves as well as analysis of DET and MET processes gave access to the distribution of enzyme orientations and enzyme dynamics as a function of pH conditions. Cyclic voltammetry was combined to SPR, PMIRRAS, and ellipsometry to correlate the loading and conformation of the biomolecules on the surface to their activity, giving access to a specific activity of the immobilized enzyme. Finally, electrocatalysis at different applied potentials as a function of pH was investigated to probe the effect of electric field on the stability of the bioelectrode. The key parameters obtained for enzyme functional immobilization on planar electrodes will allow determining the next mandatory steps for the development of efficient biotechnological devices.

**EXPERIMENTAL SECTION**

**Chemicals and Materials.** Ethanol analytical grade 96% (v/v), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-mercaptopentanoic acid (6-MHA), 4-aminothiophenol (4-ATP), 11-mercaptoroundecanoic acid (11-MUA), sodium hydroxide 97% (NaOH), and sulfuric acid 95–98% (H₂SO₄) were purchased from Sigma-Aldrich. Phosphate–citrate (for pH 3.6 and 4.6) and phosphate (for pH above 5) buffer solutions were prepared by mixing Na₂HPO₄, NaH₂PO₄, and citric acid in an appropriate ratio to obtain pH in the range 3.6–7.5 and a final buffer concentration of 0.1 M. All solutions were prepared with Milli-Q water (18.2 MΩ cm). Bilirubin oxidase from Myrothecium verrucaria (Mv BOD) was a gift from Amano Enzyme Inc. (Nagoya, Japan). Fresh solutions of Mv BOD were prepared in 100 mM phosphate or phosphate–citrate buffers at the desired pH.

**Electrode Preparation.** A polycrystalline gold electrode (geometric surface of 0.008 cm\(^2\)) from Bio-Logic Science Instruments was used. The Au electrode was mechanically polished with a 1.0, 0.3, and 0.05 μm Al₂O₃ slurry prior to use, subsequently followed by intermediate washing with Milli-Q water. After polishing, the electrode was treated by cycling the potential between 0 and 1.35 V in 0.5 M H₂SO₄ at a scan rate of 100 mV s\(^{-1}\). Around 40 cycles were run for a stable voltammogram to be obtained. The electroactive surface area was obtained by integration of the gold oxide reduction peak given a charge of 390 μC cm\(^{-2}\) for a monolayer gold oxide reduction. The roughness factor, \(R_b\) defined as the ratio of electroactive surface area to projected geometrical surface area (\(R_b = \frac{A_{\text{electroactive}}}{A_{\text{geometric}}}\)), was determined for each electrode. Values between 2.7 and 3.2 are determined, allowing one to calculate the real electroactive surface. All of the currents in this work are reported versus this electroactive surface. Then the Au electrode was sonicated with a 1:1 (water–ethanol) solution for 10 min and rinsed twice extensively with water and later with ethanol. Finally, SAMs were formed by incubating the pretreated electrode in 5 mM ethanolic thiol solutions for 15 ± 5 h. The SAM modified electrodes are named according to the thiol molecule, i.e., 6-MHA-SAM, 4-ATP-SAM, etc. The surface was then cleaned with ethanol to remove all organic contaminants, washed with water, and dried under nitrogen flux. Prior to enzyme immobilization, one CV cycle was done as a blank for thiol–SAM electrode. A reproducible voltammogram at 5 mV s\(^{-1}\) with capacitive current in the range 0.04–0.06 μA cm\(^{-2}\) was observed which reflects that the Au electrode surface is well decorated by thiol molecules.

pH-dependent electrochemical response from adsorbed enzyme on thiol–SAM-modified gold electrode was realized by following two independent approaches. The first approach was based on the enzyme adsorption at different pHs on thiol–SAM electrodes. Unless otherwise indicated, the thiol–SAM electrode was incubated in 20 μM Mv BOD solution at the desired pH for 15 min at 4 °C. This bioelectrode is named as thiol–SAM/Mv BOD. Then the thiol–SAM/Mv BOD electrode was removed from enzyme solution, gently washed with the same buffer to remove the loosely adsorbed enzymes, and transferred to the electrochemical cell containing enzyme-free phosphate buffer (100 mM) saturated with O₂ at a fixed pH 6 as a supporting electrolyte solution for electrocatalysis experiments.

In another approach, a thiol–SAM/Mv BOD electrode was prepared by incubating a thiol–SAM electrode in 20 μM Mv BOD solution of fixed pH (100 mM buffer concentration) for 15 min at 4 °C. After washing, the thiol–SAM/Mv BOD electrode was transferred to the electrochemical cell containing 100 mM buffer of variable pH as supporting electrolyte solution for electrocatalysis experiments.

**Electrochemistry Measurements.** All electrochemical measurements (cyclic voltammetry (CV), chronoanamperom-
etry, and electrochemical impedance spectroscopy) were performed in a standard 3-electrode cell (comprising a polycrystalline gold as a working electrode, a Hg/Hg2SO4 reference electrode, and a Pt-wire auxiliary electrode) using a potentiostat from Autolab PGSTAT30 controlled by Nova software (Eco Chemie). All potentials are quoted vs Ag/AgCl reference electrode by adding 430 mV to the measured potential. The cell was thermostated at 25 °C, and oxygen was continuously bubbled into the cell throughout the experiments, unless otherwise specified. No significant differences in the magnitude and shape of the catalytic curves were observed when varying the scan rate (Figure S1), suggesting that the voltammograms are close to the steady state. At least 3–5 experiments were conducted in each condition, and only the bioelectrodes whose output current deviation was less than 10% of the average were considered. After DET signal was recorded, 50 μM ABTS was introduced in the solution to detect any MET process. The MET contribution was evaluated by the ratio between DET and (DET+MET) current.

**Modeling of the Cyclic Voltammetry Curves.** The fitting of electroenzymatic curves was obtained by following the formalism developed by Armstrong and co-workers:

\[
j = \frac{j_{\text{lim}}}{\beta d_0} \left(1 + \frac{\beta d_0}{1 + c_1}\right) \left(\frac{p c_1 + (1 + c_1) \exp(-\beta d_0)}{p c_1 + (1 + c_1) \exp(-\beta d_0)}\right)
\]

where \(c_1 = \exp(\frac{n_F}{RT}(E - E_{\text{CuT}1}))\), \(c_2 = \exp(\frac{-n_F}{RT}(E_{\text{CuT}1} - E_{\text{CuT}2}))\), and \(p = (k_1 + k_2)/k_{\text{max}}\). \(E_{\text{CuT}1} = \text{CuT}1\text{redox potential}\). \(E_{\text{CuT}2} = \text{CuT}2\text{redox potential}\). \(E^{\text{eq}}_{\text{CuT}1} = E_{\text{CuT}1} + \text{CuT}1\text{redox potential}\). \(E^{\text{eq}}_{\text{CuT}2} = E_{\text{CuT}2} + \text{CuT}2\text{redox potential}\). \(n_F\) and \(n_D\) are the numbers of electrons transferred in the electrochemical and enzymatic reactions, respectively. \(\beta d_0\) represents the dispersion parameter. Each background was subtracted from the catalytic CVs prior to the fitting, and the formalism developed by Armstrong and co-workers was used to fit the measured and the calculated elliptic spectra of \(\tan(\Psi)\) and \(\cos(\Delta)\) to a Levenberg–Marquardt algorithm. The good agreement between the measurements and the calculations for all incident angles attested the dispersion models are robust. The obtained RMSE from the fits are over the whole spectral range and for all incident angles ranges between 0.01 and 0.018 for all samples.

The dielectric function of gold has been fitted using Drude–Lorentz oscillators combined with a Sellmeier model. Drude–Lorentz oscillators are suitable for the dielectric constant determination of metals. A Sellmeier model has been added in order to take into account the presence of H2O molecules in the gold porosities when the substrates are introduced in the solutions. The dielectric function of each gold substrate has been determined since it can weakly change from one sample to another. The same dielectric function model has been used for the 6-MHA-based SAMs and the Mv BOD layers. A Sellmeier model has been used to describe nonabsorbing dielectric materials. The obtained refractive index is quasi-constant around 1.48 as a function of the wavelength, which is very close to a previously reported value \((n = 1.45)\).

For the VASE measurements, the samples were first plunged overnight in thiol solutions in order to self-assemble the 6-MHA monolayers on gold and then in the enzyme solution to adsorb the Mv BOD on the 6-MHA-SAM at 4 °C for 15 min. The samples were washed with buffer and then with water. Finally, the samples were carefully dried under mild nitrogen flux before performing the VASE measurements in air. The thickness was measured at three different positions on the sample. The thicknesses of the 6-MHA-SAM and 4-ATP-SAM were measured as \(0.7 \pm 0.05\) and \(0.72 \pm 0.06\) nm, respectively.

**PMIRRS Measurements.** Gold mirrors from Optics Balzers were used for PMIRRS measurements. SAMs were formed by incubating the gold mirrors in 5 mM ethanolic thiol solutions for 1 night. The surface was then cleaned with ethanol to remove all organic contaminants, washed with water, and dried under nitrogen flux. The thiol–SAM functionalized gold surface was incubated in 20 μM Mv BOD solution at 4 °C and at the desired pH for 15 min. To
evaluate the effect of pH on the conformation of the immobilized enzyme, the thiol−SAM/Mv BOD gold surface was immersed in various pH buffers during 15 min. Protein adsorption can be attested by the presence of the amide I (mainly C=O stretching vibrational mode) and the amide II (mainly N−H stretching vibrational mode) at around 1660 and 1540 cm⁻¹, respectively. Subtraction of the thiol−SAM spectra from the thiol−SAM/Mv BOD was realized for PMIRRAS data analysis.

RESULTS AND DISCUSSION

SAMs and Mv BOD Charges as a Function of pH. Four different pHs were used throughout this work: 3.6, 4.6, 6, and 7.5. In this pH range, both the protein and the electrode charges vary. Concerning Mv BOD, the theoretical global charge of the protein is slightly positive (+10) at pH 3.6, almost neutral at pH 4.6, and negative at the other pHs. We calculated in this work a dipole moment around 800 D for the protein at pH 7.5, 6 and 4.6, while the value of the dipole moment decreases to less than 500 D for pH 3.6. The direction of the dipole moment points toward the CuT1 at pH 7.5 and 6, while its direction is shifted at pH 4.6 (Figure 1A). From our previous work, the charge in a sphere of 15 Å around the CuT1 is almost neutral at pH 7.5 and slightly positive at pH 6 (+2) and displays a net positive value at pH 4.6 and 3.6. Both charge distributions are important for Mv BOD adsorption. The protein global charge is expected to control the repulsive or attractive interaction between the enzyme and the electrode, i.e., the strength of adsorption, while the local charge around the CuT1 may control the orientation of the protein for DET. Two types of thiol-based SAMs were investigated in this work to tune the electrostatic interactions (Figure 1B): 6-MHA and 11-MUA both carry carboxylic end functions, and 4-ATP carries an amino group. As a function of pH, these SAM electrodes will present either positive or negative or neutral charges depending on the pKₐ of the chemical end function.

The pKₐ of thiols involved in SAMs is a function of the number of carbons forming the linear chain. The pKₐ of 4-ATP was previously determined to be 6.9. In the case of the carboxylic-terminated alkanethiols, it is known that the pKₐ of the surface thiols is higher than that in solution as a result of the interactions between the thiol molecules in the SAM. The pKₐ of 11-MUA-SAM was reported to be around 6.26 In this work, we determined by impedance spectroscopy a pKₐ value close to 6 for 6-MHA-SAM (Figure S2). According to the statement made above, the comprehensive Figure 1C allows one to envision pH zones for repulsive or attractive interactions between the enzyme and the SAM layer expected to control enzyme adsorption. It also permits one to predict pH zones for which the orientation of the protein for DET is expected to be favored as a function of the SAM chemistry.

Influence of the pH of Adsorption of Mv BOD on 6-MHA-SAM. Mv BOD adsorption was carried out at 4 °C on 6-MHA-SAM at the 4 different pHs while recording the electroactivity at pH 6 and 25 °C. Following this protocol, the intrinsic activity of enzymes adsorbed on SAM is fixed during the electrochemical experiments. The CV responses should have a direct dependence on enzyme loading and on the enzyme−SAM/enzyme−enzyme interactions that come into play at the electrochemical interface, both during the adsorption step and upon transfer to pH 6. Not only DET but also MET were quantified for each pH of adsorption. In addition, SPR and ellipsometry measurements as well as PMIRRAS spectra were recorded after enzyme adsorption at
the four different pHs to correlate the activity with the amount and conformation of the enzymes (Figure 2).

Independently of the pH of adsorption, DET occurs when the bioelectrode is transferred from a given pH to pH 6 (Figure 2A and 2B). A sigmoidal wave develops in the presence of O$_2$, with an onset potential of around 0.55 V, suggesting catalysis driven by the CuT1. The value of DET current density depends however on the pH of adsorption in the range: $I_{DET}^{pH}$ 3.6 $< I_{DET}^{pH}$ 7.5 $< I_{DET}^{pH}$ 4.6 $≈ I_{DET}^{pH}$ 6. The DET current did not show any drastic variation between 1 and 15 min of adsorption, except for pH 3.6 where it is twice less after 15 min of adsorption compared to 1 min of adsorption. The CV shapes and modeling indicate that the enzyme orientation distribution on 6-MHA-SAM is more or less identical irrespective of the adsorption pH with enzyme orientation distribution on 6-MHA-SAM at pH 6 and electroactivity at di $\text{pH}$. The DET current did not show any drastic variation between 1 and 15 min of adsorption, except for pH 3.6 where the DET current is either zero or less than 5% (Figure 2A and 2B, red lines) in accordance with a narrow enzyme distribution.

Catalytic current relative magnitude can be ascribed either to a different amount of loaded enzymes with similar ET rates or to different ET rates of similar amount of proteins adsorbed. Change in enzyme orientation or modification of enzyme conformation can affect the ET. The appearance of the amide bands at the same wavelength in the PMIRRAS spectra irrespective of the adsorption pH demonstrates that there is no change in the secondary structure of the enzyme (Figure 2D). Similar values of $\beta_d$ further suggest that the loading of enzymes should be more critical than the orientation of the enzyme. Accordingly, PMIRRAS spectra and SPR signals indicate an increase in the enzyme amount adsorbed on the 6-MHA-SAM with decreasing pHs, which correlates with an increase of the enzyme layer thickness measured by ellipsometry (Figure 2D and 2E and Figure S3). After 15 min of adsorption, values of 2.7 ± 0.39, 6.2 ± 0.89, 10.3 ± 1.5, and 12.2 ± 1.78 pmol·cm$^{-2}$ were obtained from the SPR angle deviations at pH 7.5, 6, 4.6, and 3.6, respectively. Considering that a theoretical monolayer of Mv BOD should be between 4.6 and 10.4 pmol·cm$^{-2}$ depending on the conformation the enzyme takes upon immobilization (Mv BOD dimensions are 4 × 5 × 6 nm$^3$), a monolayer is not obtained at pH 7.5. More

Table 1. Values of the Parameter $\beta_d$ for Distribution of Enzyme Orientation

<table>
<thead>
<tr>
<th>$\beta_d$</th>
<th>SAM (6-MHA)</th>
<th>bare gold</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>adsorption at different pH</td>
<td>adsorption at pH 6</td>
</tr>
<tr>
<td>3.6</td>
<td>5.1 ± 0.15</td>
<td>12.8 ± 0.05</td>
</tr>
<tr>
<td>4.6</td>
<td>3.9 ± 0.15</td>
<td>10.6 ± 0.12</td>
</tr>
<tr>
<td>6.0</td>
<td>4.9 ± 0.19</td>
<td>4.9 ± 0.18</td>
</tr>
<tr>
<td>7.5</td>
<td>5.1 ± 0.16</td>
<td>6.8 ± 0.17</td>
</tr>
</tbody>
</table>

Values of $\beta_d$ are obtained from the modeling of CV curves in Figures 2 (adsorption of Mv BOD on 6-MHA-SAM at different pHs) and 4 (adsorption of Mv BOD on 6-MHA-SAM at pH 6 and electroactivity at different pHs). For comparison, $\beta_d$ values obtained by modeling of CVs of O$_2$ reduction by 20 $\mu$m Mv BOD adsorbed on bare gold at pH 6 are also given.

No DET due to low activity of enzyme.

Figure 2. Correlation between electroactivity, orientation, conformation, and loading of Mv BOD adsorbed at different pHs on carboxylic-based-SAMs. CVs of O$_2$ reduction at pH 6 and 25 °C by Mv BOD adsorbed on 6-MHA-SAM at different pHs at 4 °C after (A) 1 and (B) 15 min of adsorption. Black lines and red lines are obtained before and after 50 $\mu$M ABTS addition in solution, respectively; 0.1 M phosphate buffer, $v = 5$ mV·s$^{-1}$. (C) Modeling of the electrochemical signal. Electrocatalytic CV curves at pH 6 obtained at different adsorption conditions (solid line) and curves fitted according to eq 1 (see Experimental Section) (dotted line): pH 3.6 (blue), 4.6 (purple), 6 (green), and 7.5 (red). (D) PMIRRAS spectra of Mv BOD adsorbed on 6-MHA-SAM. Gold-modified surface was placed in 20 $\mu$m Mv BOD solution at pH 7.5 (black line), 6 (blue line), 4.6 (red line), and 3.6 (green line) for 15 min and then immersed in phosphate buffer at pH 6 for 30 min at 4 °C. Gold-modified electrodes were rinsed and dried in order to record PMIRRAS spectra. (E) Enzyme coverage and enzyme layer thickness as a function of pH after adsorption of 20 $\mu$M Mv BOD at RT for 15 min on 6-MHA-SAM. Enzyme coverage (blue bars) was obtained by SPR, and enzyme layer thickness (red diamonds) was measured by ellipsometry. (F) Increase in the consecutive CV catalytic currents at pH 6 after adsorption of 20 $\mu$M Mv BOD on 6-MHA-SAM in pH 4.6 buffer for 15 min at 4 °C.
than one monolayer is formed at pH 3.6, which is traduced in an enzyme layer thickness larger than the protein dimension.

The magnitude of the catalytic current and the loading of enzymes observed as a function of the pH of adsorption can be explained based on the respective charges of the protein, the environment of the CuT1, and the SAM. At pH 7.5, repulsive electrostatic interactions between the negatively charged protein and the negative SAM should prevent BOD adsorption. In accordance, less than one monolayer of enzyme is obtained. Nevertheless, the neutral environment around the CuT1, associated with a high dipole moment (764 D) pointing toward the CuT1, enables BOD adsorption via the CuT1. Hence, DET but no MET is observed. The enzyme thickness obtained by ellipsometry (2.7 ± 0.1 nm) (Figure 2E) suggests however some flattening of the enzyme. Compared to pH 7.5, the most prominent change at pH 6 is the lowest negative charge of the SAM. As a consequence, the amount of molecules adsorbed is more than two times higher than at pH 7.5, the enzyme layer thickness is increased (3.3 ± 0.2 nm), leading also to a higher direct catalytic current than when adsorption is made at pH 7.5. At pH 4.6, the amount of adsorbed proteins is enhanced compared to pH 6 because the repulsive interactions are now weak between the protonated SAM and the neutral Mv BOD. A coverage close to the maximum theoretical coverage is obtained. Ellipsometry gives an enzyme thickness of 3.9 ± 0.2 nm, very close to the geometrical enzyme dimension. The direction of the dipole moment at pH 4.6 which does not point anymore to the T1 should lead to a higher distribution of orientation. However, the MET contribution is low, and the $\beta d_0$ value suggests a narrow distribution of orientation, very similar to pH 6 (Figure 2 and Table 1). Two main hypotheses can be proposed: either the electrostatic interactions between the CuT1 and the SAM, although weak, are sufficient to induce a major DET orientation of the enzyme on the surface or the mobility of the protein allows it to adopt a favorable orientation for DET upon transfer to pH 6. The later hypothesis is supported by the increase in the DET current during the first three cycles before reaching the maximum current as a consequence of progressive reorientation (Figure 2F).

The second main conclusion from our experimental results is that a higher enzyme loading does not translate directly in a higher catalytic activity. The specific activity defined as the ratio of the DET current by the enzyme coverage has been calculated at all of the pHs of investigation. It is reported in Figure 3 as a function of the enzyme coverage. This analysis underlines that the highest specific activity is obtained for the lowest coverage. As developed by Blanford and co-workers, less steric hindrance because of lower coverage may be the reason for a higher specific activity.

The case of pH 3.6 is noteworthy to be discussed apart from the other investigated pHs. At this pH, the weak interaction between the positively charged enzyme and the protonated SAM in addition to a much lower dipole moment (477 D) is expected to yield a high degree of mobility of the enzyme, which can adopt many orientations. Although this is the only case where MET contributes to the whole catalytic signal (Figure 2), DET remains the major process and $\beta d_0$ value reflects a narrow distribution of orientations (Table 1). Protein dynamics upon transfer of the bioelectrode from pH 3.6 to pH 6 may explain a favored DET process. Both the amount of protein and the layer thickness measured by ellipsometry are indicative of the formation of more than one monolayer (Figure 2E). The occurrence of a MET process at pH 3.6 can thus be attributed to enzyme multilayers rather than to a distribution of orientation. Despite the highest amount of proteins, the lowest catalytic current is obtained. The stability of the enzyme is also the lowest at this pH, as highlighted by the homogeneous activity reported in Figure S4, where only 20% of the activity is recovered after 1 h of storage. Acidic pH conditions, mostly below pH 3, are known to cause protein unfolding as a result of intramolecular charge repulsion. In this work, aggregate formation was effectively observed at pH 3.6 and RT (Figure S5). However, similar enzyme layer thickness values (around 7.2 nm) were obtained by ellipsometry after adsorption at pH 3.6 either at 4 °C or at RT, and PMIRAS spectra indicated that there is no change in the secondary structure of the protein adsorbed at pH 3.6. Thus, the aggregation process might not be the major contribution to the low direct catalytic current when adsorption is made at pH 3.6, which would be more related to steric hindrance between proteins in the layer. Interestingly, when adsorption was made at RT at pH 3.6, conditions favoring protein aggregation, the direct electrochemical signal magnitude recorded at pH 6 was four times higher than when the adsorption was made at 4 °C (Figure S5). Although it is reported that cross-linked enzyme aggregates (CLEA) of laccases may remain active and stable, control experiments in this work showed that unfolded or denaturated proteins do not induce any electrocatalytic signals (Figures S6 and S7). The following hypotheses could thus explain this particular behavior: (i) protein aggregation occurring at RT might remove some BOD population not well folded and consequently increases the specific catalytic electroactivity, (ii) the presence of aggregated proteins adsorbed on the electrode could optimize the enzyme wiring, playing the role of cross-linkers.

Varying the pH of Electroactivity. The adsorption of Mv BOD on 6-MHA-SAM was alternatively carried out at pH 6 for 15 min at 4 °C; then the Mv BOD/6-MHA-SAM was transferred to buffers at the different pHs 3.6, 4.6, 6 or 7.5, respectively. The typical CVs for electroenzymatic O2 reduction are shown in Figure 4A where both DET and MET signals are overlaid.

![Graph](image_url)
Switching the pH of the electrolyte changes simultaneously two parameters, i.e., the intrinsic activity of the enzyme as well as the interaction between Mv BOD preadsorbed at pH 6 and the SAM. A first observation is that the catalytic current mainly reflects the activity of the enzyme in solution measured by UV–vis spectroscopy (Figure S4). Hence, much lower activity is obtained at pH 7.5 compared to the other pHs. As expected, the onset for O₂ reduction decreases as pH increases, displaying a slope close to 60 mV. The second observation is that the shape of the CV curve is markedly different at pH 3.6 and 4.6 compared to pH 6 and 7.5, suggesting a larger distribution of ET rates, linked to a distribution of enzyme orientation. The modeling of the CV curves gave access to the orientation parameter βd, which takes values of 12.8, 10.6, and 4.9 for pH 3.6, 4.6, and 6, respectively, showing a larger distribution of ET rates at pH 6 and pH 4.6, and a narrow one at pH 6 (Figure 4B and Table 1). In accordance, MET currents were only observed at pH 3.6 and 4.6, although it cannot be excluded that the magnitude of the MET signal reflects the better affinity of BOD toward ABTS at low pH as attested by the decrease of the Michaelis–Menten constant (Figure S8). These results can be explained based on the weak interactions between the SAM and the protein at acidic pHs as discussed above. But this implies also some mobility of the enzyme when transferring the bioelectrode from pH 6 to lower pHs. Accordingly, the catalytic current increased during the first three cycles before reaching the maximum current (Figure 4C). Protein dynamics is further confirmed by experiments involving multiple transfer steps from one pH to another pH. As seen in Figure 4D, the changes in the CV shapes and current magnitude clearly reflect the reversible changes in the distribution of orientation between pH 6 and pH 3.6. A little less current output at the end of the process could be related to loss of some enzymes in the successive transferring steps. The full range of pH was finally investigated after Mv BOD adsorption at pH 6, showing that the electrochemical response can be easily tuned and reflects enzyme activity and dynamics yielding favorable/unfavorable interaction between the enzyme and the SAM layer for DET (Figure 4E).

Influence of the SAM Chemistry. To confirm the electrostatic model established from the electrocatalysis on 6-MHA-SAM, we performed adsorption of Mv BOD on other surfaces: (i) 11-MUA-SAM, a carboxylic-thiol with 11 carbons in the alkane chain (pKₐ of 11-MUA on SAM has been reported to be 6), and (ii) 4-ATP, an amino-thiol. After Mv BOD adsorption at pH 6 on 11-MUA-SAM, a DET process is observed when the activity is measured at pH 6, with a lower ET rate than on 6-MHA-SAM, as a consequence of the decrease of the electron tunneling rate with the length of the alkane chain (Figure 4F). As the chemical functions are identical on 11-MUA and 6-MHA, the charges as a function of pH are also similar. Then the occurrence of DET over MET process on 11-MUA is based on the same assumption as for 6-MHA.

The pKₐ of 4-ATP was reported to be 6.9. Hence, the SAM is positively charged at pH 3.6, 4.6, and 6 and neutral at pH 7.5. Except at pH 3.6, electrostatic interactions with the globally negatively charged Mv BOD must favor enzyme approach. As revealed by the SPR data and confirmed by PMIRRAS and ellipsometry measurements (Table 2, Figure 5A and Figure 5B), Mv BOD is adsorbed at pH 6 or at pH 4.6 on 4-ATP-SAM, with similar amounts, reaching a full coverage after 15 min of adsorption. As expected, the amount of loaded is the lowest at pH 7.5 as a result of lower electrostatic interactions but higher than on 6-MHA-SAM where repulsive interactions took place. A high amount of proteins is loaded at pH 3.6 despite repulsive interactions which may be ascribed to some aggregation process. The band corresponding to the

![Figure 4. pH-induced dynamics of Mv BOD on 6-MHA-SAM. (A) CVs of O₂ reduction by 20 μM Mv BOD adsorbed on 6-MHA-SAM at 4 °C and pH 6 for 15 min and transferred to different pHs for catalysis measurement (black curves). Red curves are obtained after 50 μM ABTS addition, and gray dotted curves correspond to the SAM alone. (B) Modeling of the electrochemical signal. Electrocatalytic CV curves at different pHs after adsorption at pH 6 (solid line), and curved fitted according to eq 1 (dotted line) (see Experimental Section). Mv BOD was adsorbed on 6-MHA-SAM at pH 6, and electrochemistry was recorded at different pHs: pH 3.6 (blue), 4.6 (purple), 6 (green), and 7.5 (red). (C) Increase in the consecutive CV catalytic currents at pH 4.6 after 20 μM Mv BOD was adsorbed on 6-MHA-SAM in pH 6 buffer for 15 min at 4 °C. (D) CVs for O₂ reduction by Mv BOD adsorbed on 6-MHA-SAM at 4 °C after multiple steps of transfers: adsorption at pH 6 for 15 min, measurement at pH 6 (green), washing step then transfer and measurement at pH 3.6 (black), washing step then transfer back to pH 6 (blue). (E) CVs of direct O₂ reduction on a full pH range by 20 μM Mv BOD adsorbed at pH 6 for 15 min at 4 °C. Phosphate citrate buffer (pH 3.5) or phosphate buffer (pH 6–7.5). v = 5 mV/s⁻¹.](image-url)
and 4.6 (Figure 5D). This behavior has never been reported. Adsorption is made at pH 7.5, DET markedly occurs at pH 6. Also, in good agreement with the model, a DET signal is obtained at pH 7.5 thanks to the dynamics of the protein when transferred to a pH where 4-ATP-SAM is neutral. When the adsorption is made on the positively charged 4-ATP-SAM at pH 3.6, 4.6, and 6, a MET current develops with a similar magnitude whatever the pH of adsorption, underlining that the total amount of electroactive enzymes is similar (Table 2). Also, in good agreement with the model, a DET signal is obtained at pH 7.5 thanks to the dynamics of the protein when transferred to a pH where 4-ATP-SAM is neutral. The DET catalytic process is not stable with time however, as a result of mobility of the protein upon transfer to pHs where the SAM becomes positively charged (Figure 5E). Catalytic Stability and Effect of Applied Potential.

One main issue when dealing with enzyme-based bioelectrodes is the long-term stability. A decrease in electrocatalytic signals may be associated to different phenomena including the stability of the enzyme itself, enzyme leakage from the electrochemical interface, changes in orientation, and/or in the conformation of the enzyme in the immobilized state. Recent reports coupling electrochemistry to QCM41 or SPR16 established that the decrease of the catalytic signal for O2 reduction by Mv BOD adsorbed on SAM layers was not linked to enzyme loss from the electrode. Although the electric field effect on bioelectrode efficiency and stability is not well established, some other works concluded that a decrease in the catalytic activity could be related to changes of the enzyme layer upon applied potential.

To evaluate the effect of electrostatic interactions on the stability of the DET signal, we cycled for 45 min at different pHs the bioelectrode built by Mv BOD adsorbed at pH 6 on 6-MHA-SAM (Figure 6A).

The percentage of activity loss within 45 min of continuous cycling at RT between 0.6 and 0 V vs Ag/AgCl was 30%, 2%, and 4%, respectively at pH 3.6, 4.6, and 6. Compared to the stability obtained in solution at RT (Figure 6C and Figure S4), enzyme immobilization onto the electrode surface greatly enhances the stability, except in the case of pH 7.5, where heterogeneous or homogeneous catalytic stability is similar (20% against 25%). This latter pH is the case where the repulsive interactions between the enzyme and the SAM are the highest, yielding possible losses of proteins by desorption. SPR measurements confirm this hypothesis as 25% of the adsorbed proteins are removed away after the rinsing step at pH 7.5 against 5% at pH 3.6 and around 10% at pH 4.6 and 6. However, pH 7.5 is also the condition where the electrostatic interactions between the CuT1 and the SAM are the highest.

Table 2. SPR Data for Different pH of Mv BOD Adsorption for 15 min on 4-ATP, and Values of the Ratio of DET/DET + MET at Different pHs

<table>
<thead>
<tr>
<th>pH of ads.</th>
<th>ΓSpr (pmol·cm⁻²)</th>
<th>pH 3.6</th>
<th>pH 4.6</th>
<th>pH 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6</td>
<td>10.97 ± 1.6</td>
<td>0.10</td>
<td>0.36</td>
<td>0.50</td>
</tr>
<tr>
<td>4.6</td>
<td>9.5 ± 1.4</td>
<td>0.04</td>
<td>0.23</td>
<td>0.31</td>
</tr>
<tr>
<td>6</td>
<td>8.0 ± 1.2</td>
<td>0.06</td>
<td>0.30</td>
<td>0.58</td>
</tr>
<tr>
<td>7.5</td>
<td>5.7 ± 0.8</td>
<td>0.38</td>
<td>0.80</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Table 2: The ratios are measured at E = +120 mV vs Ag/AgCl.

Figure 5. Correlation between electroactivity, conformation, and loading of Mv BOD adsorbed on amino-based SAM. (A) Enzyme coverage obtained by SPR (blue bars) and enzyme layer thickness obtained by ellipsometry (red diamonds) on 4-ATP-SAM as a function of pH after 15 min of adsorption of 20 μM Mv BOD at RT. (B) PMIRAS spectra of Mv BOD adsorbed on 4-ATP-SAM. Gold-modified electrode was placed in a solution of 20 μM Mv BOD at pH 7.5 (black line), 6 (blue line), 4.6 (red line), and 3.6 (green line) for 15 min at 4 °C and then immersed in phosphate buffer at pH 6 for 30 min at 4 °C. Catalytic O2 reduction (C) at pH 4.6 after Mv BOD adsorption in the different pH buffers or (D) in different pHs after 20 μM Mv BOD adsorption at pH 7.5. (E) Decrease in the catalytic current at pH 6 along with CV cycling for bioelectrodes prepared in experiment D. Black curves and red curves are obtained before and after 50 μM ABTS in solution, respectively. v = 5 mV·s⁻¹.
Progressive irreversible change in the structure of the enzyme cannot be excluded as suggested by the higher variability of amide I/amide II ratio in PMIRRAS measurements and further revealed by the lower thickness obtained by ellipsometry, which suggested some flattening of the enzyme.

The stability of the 6-MHA-SAM/Mv BOD bioelectrodes apparently differs from the previous measurements that we made on SPR chips at pH 6,16 where we observed a decrease of more than 30% of the catalytic signal during similar duration. The only difference between the two experiments is that in the current work we are continuously cycling the electrode potential, while in the previous one, we made one cycle every 1000 s and held the electrode at OCP the rest of the time. We used this protocol in the present work and observed a decrease of the catalytic current of 50%, 15%, 25%, and 28%, respectively, at pH 3.6, 4.6, 6, and 7.5 (Figure 6B). We undertook comparative chronoamperometry experiments with 6-MHA-SAM modified by Mv BOD adsorbed at pH 6 at two different potentials: one situated on the plateau for catalytic O$_2$ reduction, i.e., +0.13 V vs Ag/AgCl, and the other close to the OCP, i.e. + 0.53 V vs Ag/AgCl. Four different pHs (i.e., pH 4.6, 5.5, 6, and 6.5) above and below the pK$_a$ of the SAM and in which the enzyme activity is high and comparable were investigated. In the pH range investigated, the electrostatic interactions between Mv BOD and the SAM are either weak or repulsive. Typical curves are provided for the bioelectrodes stabilized at pH 4.6 (Figure 6D) or 6 (E) at +0.13 (blue lines) or +0.53 V (green lines) with the sequence denoted within brackets.

Figure 6. Stability of 6-MHA-SAM/Mv BOD: effect of applied potential. Stability of the catalytic O$_2$ reduction in different pHs by Mv BOD adsorbed on 6-MHA-SAM at pH 6 and 4 °C for 15 min. (A) Continuous CV cycling for 45 min. (B) One cycle every 1000 s holding the electrode at OCP between the cycles. (C) Comparative activity loss between (blue diamonds) continuous cycling, (red squares) one cycle every 1000 s, and (gray circles) homogeneous catalysis. (D and E) Effect of applied potential on the stability of the catalytic current. Mv BOD adsorbed on 6-MHA-SAM at pH 6, and chronoamperometry recorded at pH 4.6 (D) or 6 (E) at +0.13 (blue lines) or +0.53 V (green lines) with the sequence denoted within brackets.

CONCLUSION

The practical use of devices such as biosensors, bioreactors, or biofuel cells based on redox enzyme activity relies on the controlled, efficient, and stable immobilization of the protein on solid conductive supports. The results obtained in this work provide key parameters to propose new solutions to improve the process. Thanks to a multidisciplinary approach coupling electrochemistry to SPR, ellipsometry, and PMIRRAS, we demonstrated the correlation between enzyme loading, conformation, and catalytic activity. The results have been rationalized according to an electrostatic model, where the global charge of the protein influences the rate of adsorption, while the enzyme dipole moment and the charge in the vicinity of the CuT1, the entry site of electrons, influences the enzyme orientation and then the electron transfer rate. We also demonstrated that a strong electrostatic field on the electrode boundary at potentials far from zero point charge deteriorates enzyme stability. Whether this is a general rule for enzymes on electrochemical interfaces and whether this could induce changes in the enzyme conformation should be an interesting matter of future discussion.

One objective of this work was to evaluate to which extent the hypothesis and main conclusions made on planar surfaces can be extended to porous carbon nanotube networks. Actually, Mazurenko et al. studied the consequences of BOD adsorption on carbon nanotubes presenting different surface chemistry on the electrocatalytic activity. The experiments conducted in the current work show that the main parameters for enzyme orientation for direct electrical wiring which are determined on planar electrodes are conserved on carbon nanotube networks. That means that rationalization of other
enzyme-based bioelectrodes should be gained by the examination of enzyme behavior on planar electrodes taking mainly into account dipole moments, both the direction and the value, and the environment of the entry/exit site of electrons on the protein. However, we also highlighted in this work the dynamics of the protein on SAM–gold electrodes upon changes in the local pH environment which affects the efficiency of the catalysis. Even if immobilization on a porous material with multiple points of contact should restrict protein mobility, our results provide one explanation of the low efficiency of redox proteins in most bioelectrodes. Local variation of pH occurs in the course of electrocatalysis, and the effect on enzyme conformation, stability, or orientation in the immobilized state require in-depth investigations using combination of techniques. This will open avenues toward new material and architecture design to protect enzymes against local pH variation.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.8b03443.

CV catalytic signal as a function of sweep rate; pK determined of 6-MHA-SAM; SPR angle variation as a function of pH; homogeneous activity at RT or 4 °C as a function of pH; temperature and pH dependency of Mv BOD aggregation; control experiments using unfolded and denatured BOD; electrochemical behavior of ABTS and Mv constant current; electrocatalysis on 11-MUA and butanethiol (BT); control experiments on 6-MHA-SAMs; effect of Mv BOD concentration on the electrocatalytic activity; electroactivity loss with time as a function of applied potential at different pH (PDF)

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Author Contributions

V.P.H. performed electrochemistry, homogeneous enzyme activity measurements, and ellipsometry experiments and contributed to the redaction of the manuscript. I.M. initiated the modeling of the electrochemical curves. R.C. was in charge of protein purification and protein modeling. M.T. performed the PMIRRAS experiments, and S.C. and S.L. performed the PMIRRAS analysis. D.D. performed the ellipsometry analysis. M.T. realized the aggregation experiments and analysis of the data. I.M., M.I., and A.P. participated in the discussion of the results. E.L. is the initiator and director of the project and participated in all steps.

Notes

The authors declare no competing financial interest.

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