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Oriented Immobilization of a Fully Active Monolayer of Histidine-Tagged Recombinant Laccase on Modified Gold Electrodes

Véronique Balland,*[a] Christelle Hureau,[a] Angela Maria Cusano,[b] Yingli Liu,[b] Thierry Tron,[b] and Benoît Limoges*[a]

Abstract: The formation of a dense monolayer of histidine-tagged recombinant laccase on gold electrodes by using a short thiol-NTA linker is described, as well as a kinetic analysis of the process by cyclic voltammetry. From a detailed analysis of the catalytic reduction of dioxygen by laccase in the presence of a one-electron redox mediator it can be concluded that the immobilized enzyme remains as active as in homogeneous solution.

Keywords: electrochemistry · enzymes · immobilization · redox mediators · self-assembled monolayers

Introduction

Controlling the molecular structure, organization, and/or orientation of proteins on a solid surface without distressing their native functionality is a key issue in the development of many biotechnological applications, such as biosensors,[1,2] biomolecular electronics devices,[3] or biofuel cells.[4] Initially developed for the biochemical purification of genetically engineered proteins, the formation of ternary metal–chelate complexes between nitrilotriacetic acid (NTA) and histidine-tagged recombinant proteins has been shown to be a promising strategy for controlling protein orientation at interfaces, requiring only mild protein modification.[5] Moreover, the specific immobilization is reversible, which allows the release and recovery of the immobilized protein under mild conditions. Such a strategy has proved to be useful for improving the recognition between a receptor immobilized on a surface and a ligand in solution[6–8] or for modulating the electrical communication between an electrode and a redox protein site-specifically oriented on its surface.[2,9,10]

[a] Dr. V. Balland, Dr. C. Hureau, Dr. B. Limoges
Laboratoire d'Electrochimie Moléculaire, UMR CNRS 7591
Université Paris Diderot, 2 place Jussieu
75251 Paris Cedex 05 (France)
Fax: (+33) 144-277-625
E-mail: veronique.balland@univ-paris-diderot.fr
limoges@univ-paris-diderot.fr

[b] Dr. A. M. Cusano, Y. Liu, Dr. T. Tron
BiosCiences, Institut Sciences Moléculaires Marseille (ISM2)
UMR CNRS 6263
Aix-Marseille Université Centre Scientifique Saint Jérôme
13397 Marseille Cedex 20 (France)

Self-assembled monolayers (SAM) of NTA-terminated alkanethiols on a gold surface are by far the most investigated platform in the applications mentioned above. Two approaches are currently used: 1) a direct method in which the NTA-terminated alkanethiol is separately synthesized before being self-assembled in a single step on a gold surface and 2) a two-step method in which a SAM of alkanethiol terminated by a functionalizable group is first chemisorbed on gold and subsequently chemically coupled to the desired NTA ligand. The second method is the most widely investigated and involves, for instance, the coupling of a NTA-containing primary amine with an activated carboxylate-terminated SAM.[2,9–11] The main inconveniences of this latter approach is a rather lengthy and laborious preparation of the protein-immobilizing ligand on gold surfaces and a poor control of the ligand coverage due to the low coupling yields reported for amide bond formation.[11] SAMs prepared by the direct method should provide modified gold surfaces that are better defined and characterized, but this has only been reported for long NTA-terminated alkanethiols,[6–8] which are generally not compatible with electrochemical detection (i.e., long alkyl chains may slow down or impede electron transfer). One objective of this work was thus to design, by the direct method, a short-length NTA SAM on the surface of a gold electrode for site-specific immobilization of a recombinant histidine-tagged redox enzyme and for efficient transduction of its catalytic activity into an electrochemical response. Another important objective was to characterize quantitatively all the steps in the immobilization procedure and to determine precisely the efficiency of the immobilization strategy and the extent to which the His-tagged enzyme is preserved from deactivation

through partial denaturation, steric hindrance, and/or conformational changes.

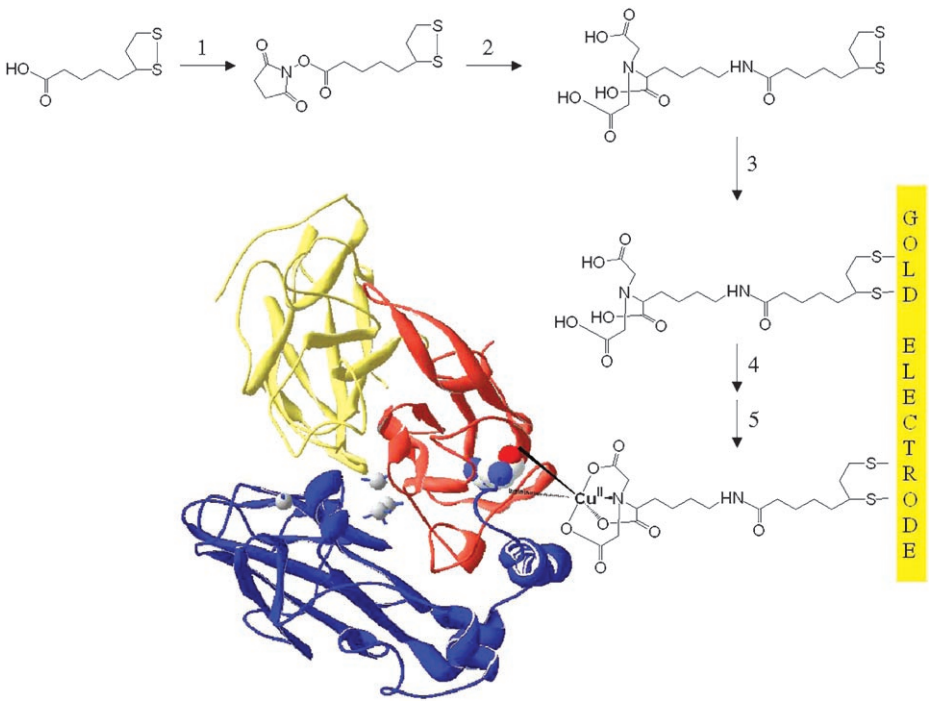
As a His-tagged redox enzyme model, we selected recombinant laccases (LAC3) from a *Trametes sp.* strain C30[12] with a 6×His-tag at their N- or C-terminal extremities. Laccases are multi-copper oxidases capable of catalyzing the four-electron reduction of dioxygen to water with a concomitant oxidation of a broad range of substrates, which makes them of special interest not only in bioremediation[13] and biosensing applications,[14–16] but also as cathodic electrocatalysts in biofuel cells.[4,17,18] Laccases are also characterized by the presence of a blue type-1 copper site, which acts as a primary electron-acceptor relay from reductant substrates, and a trinuclear copper site, which is responsible for the reduction of dioxygen. Although some attempts have been made to evaluate the kinetic properties of laccases immobilized on electrodes,[19–22] the rate constants determined were always apparent values because of the difficulty of precisely knowing the amount of fully active enzyme attached to the electrode and of appraising the effects of diffusion-restriction and partitioning within the more or less thick films of laccase usually deposited on the electrode surface.

With the aim of quantitatively characterizing the proposed enzyme immobilization strategy, it was necessary to address several interconnected issues. One was to establish the relationships that link the voltammetric catalytic current response to the surface concentration of affinity-bound laccase through a comprehensive overview of its mechanism. The second was to determine the true kinetic rate constants for the activity of the immobilized enzyme (i.e., equivalent to those obtained in homogeneous reactions) and to compare them with their homogeneous counterparts. For such a purpose, the amount of active enzyme immobilized on the electrode was carefully quantified by an indirect method. Finally, the last issue was to relate the electrochemical response to the affinity-binding isotherm of the His-tagged laccase towards the thiol-NTA electrode, thus offering a way to evaluate the efficiency of the proposed immobilization strategy.

The poor substrate specificity of laccases allows natural electron donors to be replaced by artificial redox substrates (mediators), which enables electrocatalytic reduction of dioxygen by laccases in cyclic voltammetry.[23] In this work, we selected a fast one-electron redox couple, $[Os(bpy)_2pyCl]^{2+}/[Os(bpy)_2pyCl]^{+}$ (bpy = bipyridine, py = pyridine), which proved to be a particularly efficient mediator of the catalytic reduction of O_2 by laccase in cyclic voltammetry.

Results and Discussion

A short-length NTA-terminated thiol, *N*-[5-(1,2-dithiolan-3-ylpentanoylamino)-1-carboxypentyl]iminodiacetic acid (Scheme 1), was synthesized by coupling thioctic acid with *N*-(5-amino-1-carboxypentyl)iminodiacetic acid. The SAM coating was achieved by immersion of a gold electrode in an ethanol solution of the thiol-NTA (1 mg mL^{-1}) for 12 h at 4 °C. The surface concentration of the thiols grafted onto the gold electrodes was estimated by oxidative desorption in a 0.5 M solution of KOH by scanning voltammetrically from −0.2 to 0.7 V versus SCE.[24] After correcting for oxidation of the gold surface, and assuming a three-electron oxidation peak, a sulfur atom surface concentration of $(7\pm0.5)\times10^{-10}$ mol cm^{-2} was found. This value is close to the value of $(8\pm0.5)\times10^{-10}$ mol cm^{-2} found under the same conditions for a SAM of thioctic acid and is consistent with the theoretical value of a close-packed monolayer. Assuming two sulfur atoms are grafted per molecule, the surface concentration of the thiol-NTA linker was finally estimated to be (350 ± 25) pmol cm^{-2}. Metalation of the NTA ligand was achieved by soaking NTA-modified electrodes in a 1 mM solution of $CuCl_2$ (0.1 M phosphate buffer, pH 7.4) for 30 min. The surface coverage of Cu^{II} was estimated as previously reported from its voltammetric response,[25] and a value of (250 ±



Scheme 1. 1,2) Two-step synthesis of the thiol-NTA ligand from thioctic acid, 3) thiol chemisorption on the gold surface, 4) pretreatment of the NTA-modified surface with an aqueous solution of $CuCl_2$, and 5) addition of the histidine-tagged protein to the modified surface.

50) pmol cm^{-2} was obtained, which indicates that more than 70% of the immobilized ligand was coordinated to the Cu^{II} ion. Enzyme immobilization was achieved by immersion of the modified gold electrode in a magnetically stirred 100 nM solution of LAC3 with a 6×His-tag at its C-terminal extremity (Scheme 1). Once loaded with the enzyme, the electrode was washed and transferred to an electrochemical cell containing phosphate buffer at pH 6.0.

The specific binding of His-tagged LAC3 at the electrode is depicted in Figure 1a. A typical catalytic wave was obtained by cyclic voltammetry in the presence of both an excess of O_2 (air-saturated solution) and the one-electron redox mediator $[Os^{III}(bpy)_2pyCl]^{2+}$. The sigmoidal-shaped wave of the voltammogram is characteristic of steady-state conditions. The catalytic response vanished when any of the three components (NTA-thiol, Cu^{II}, or $[Os^{III}(bpy)_2pyCl]^{2+}$) was omitted. Moreover, NTA-modified electrodes loaded with copper were unable to bind laccase that was not His-tagged. These results show that the binding of His-tagged LAC3 is specific and related to the formation of a ternary complex between the anchored NTA ligand, the Cu^{II} ion, and the histidine residues in the recombinant protein. To examine the influence of the orientation of laccase on the catalytic response, the same experiments were repeated with recombinant LAC3 with a 6×His-tag at its N-terminal extremity. In solution, the specific activity of this N-terminal His-tagged LAC3 was found to be of the same magnitude as that of the C-terminal His-tagged LAC3, which indicates that the introduction of the 6×His-tag does not significantly change the catalytic properties of the soluble form of the enzyme. Once immobilized on an electrode under the same conditions as the C-terminal His-tagged laccase, a steady-state catalytic current response of the same intensity was obtained. This result confirms the comparable reactivity of the two recombinant enzymes, even in the immobilized state, and it suggests the mediator has equal access to the primary electron acceptor (type-1 copper) of laccase, whatever the orientation of the protein. In several recent works, it was proposed that by appropriate orientation of laccase at the surface of an electrode, direct electronic communication between the electrode and the type-1 copper of the enzyme could be established, leading to catalytic reduction of O_2 without the mediator.[19,22] It was thus interesting to examine such a possibility. Cyclic voltammograms were thus recorded for both the N- and C-terminal His-tagged laccases immobilized on gold electrodes in the absence of mediator, but no noticeable electrocatalytic reduction of O_2 was recorded (the voltammetric curve was identical to the one recorded in the absence of immobilized laccase). With the N-terminal His-tagged laccase, this result is not really surprising because, on the basis of the crystallographic structure reported for the *Trametes versicolor* laccase,[26] the N-terminal extremity in LAC3 appears localized on the opposite side of the type-1 copper site such that the redox center is too far for efficient electrical communication with the gold surface. In the case of the C-terminal His-tagged laccase, the distance between the redox center and the electrode surface should theoretically be much shorter, but the lack of response in the absence of mediator suggests that this is probably not sufficient. Note that in the absence of dioxygen (i.e., after degassing with argon), the reversible wave of the redox mediator $[Os^{III}(bpy)_2pyCl]^{2+}$ is unaffected by the presence of the chemisorbed ligand and the immobilized protein (the reversible wave can be overlaid on those recorded at an unmodified gold electrode). This clearly indicates that the ligand and enzyme layer do not impede electron transfer.

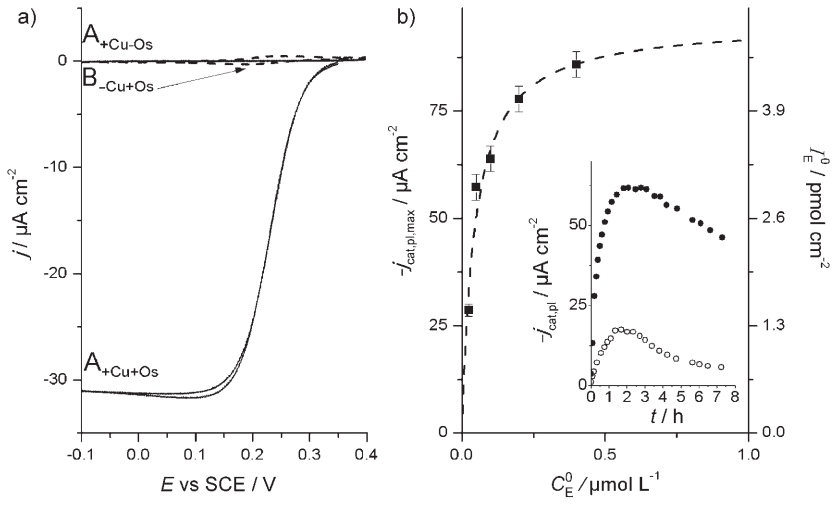


Figure 1. a) Cyclic voltammetry of thiol-NTA-functionalized gold electrodes in phosphate buffer (pH 6.0, T= 25°C, scan rate: 20 mV s^{-1}). Electrode A was loaded with Cu^{II}, whereas electrode B was not. Both were incubated in a 100 nM His-tagged LAC3 solution for 90 min at T=20°C. Electrode A was scanned in the presence (A_{+Cu+Os}, ——) and absence (A_{+Cu-Os}, ——) of 20 µM $[Os^{III}(bpy)_2pyCl]^{2+}$. Electrode B ($B_{-Cu+Os}$, -----) was scanned in the presence of 20 µM $[Os^{III}(bpy)_2pyCl]^{2+}$. b) Maximum current density values obtained for electrodes charged with Cu^{II} and immersed in O_2-saturated solutions with different bulk enzyme concentrations (T= 25°C, 1 mM $[Os^{III}(bpy)_2pyCl]^{2+}$). Contributions of the diffusion current of the mediator and of the residual catalytic activity of the enzyme in solution have been subtracted. The curved line represents the Langmuir isotherm fitting. Inset: Current density as a function of incubation time with 100 nM bulk concentrations of enzyme for electrodes loaded with Ni^{II} (○) or Cu^{II} (●).

The enzyme-binding kinetics were investigated by plotting the catalytic plateau current density, $j_{cat,pl}$, as a function of the immersion time (inset in Figure 1b). The current response at a Cu^{II}/NTA-modified electrode reaches a maximum value after 90 minutes and remains stable for around 2 h before decaying. A similar kinetic profile was obtained at a Ni^{II}/NTA-modified electrode, but the maximum current density was 3.5 times lower (inset

in Figure 1b). The lower catalytic response recorded at the Ni^{II}/NTA-modified electrode suggests poorer affinity binding of the His-tagged enzyme with the Ni^{II}–NTA complex. This result is consistent with the poorer binding of 1,1′-bis(-methylimidazole)ferrocene previously observed at Ni^{II}/NTA-functionalized carbon electrodes compared with Cu^{II}.[25]

The affinity binding (K_b) of His-tagged LAC3 to the NTA-modified electrode loaded with Cu^{II} was determined from the plot of a Langmuir isotherm. The maximum catalytic plateau current density, $j_{cat,pl,max}$, which was assumed to occur at equilibrium binding, was recorded at several enzyme concentrations, C_E^0, in solution (Figure 1b). The experimental data were fitted to the Langmuir equation [Eq. (1), in which $j^s_{cat,pl,max}$ is the maximum catalytic plateau current density for a saturated protein surface concentration]. From the fitting we obtained $j^s_{cat,pl,max} = 100\ \mu A\,cm^{-2}$ and $K_b = 2.2\times10^7\ M^{-1}$. The value of the binding constant is comparable to that reported for the binding of a His-tagged protein to a Ni–NTA lipid bilayer at pH 7.4[27] or to the affinity binding measured for a histidine-modified peroxidase at a Cu^{II}/NTA-functionalized carbon electrode.[25] The decay of the catalytic current in Figure 1b was attributed to a slow dissociation of the enzyme from the surface and not to a deactivation of the immobilized enzyme. This assertion is supported by the fact that a similar decay of the catalytic response upon prolonged immersion was previously observed in the kinetics binding curve of histidine-modified peroxidase at Cu^{II}/NTA-modified carbon electrodes,[25] which suggests that the loss of activity at enzyme–NTA-modified electrodes is a general phenomenon. This effect might be attributed to the intrinsic reversibility of the binding of the metal ion; the metal ion can slowly dissociate from the NTA-modified electrode by competitive binding with the 6×His-tag of the enzyme, as previously reported for other NTA-modified surfaces.[10,28] This hypothesis is corroborated by an acceleration of the decay at low pH values. Note also that the recombinant His-tagged laccases were not especially unstable because after a few weeks of storage at 4 °C there was no significant loss of their activity.

$$j_{cat,pl,max} = \frac{j^s_{cat,pl,max} K_b C_E^0}{1 + K_b C_E^0} \qquad (1)$$

The relative stability of the enzyme layer after 90 min of enzymatic binding (Figure 1b) allowed us to analyze at the same modified electrode the catalytic kinetics of immobilized LAC3. The catalytic current response of the immobilized His-tagged LAC3 was analyzed on the basis of the laccase mechanism proposed in Figure 2a. The catalytic cycle of laccase was assumed to proceed through a reaction scheme analogous to a ping-pong mechanism in which four electrons are sequentially taken up from reducing substrates by the accessible blue type-1 copper site (T1), which transfers them to a buried trinuclear copper center (T2 and T3 sites) about 13 Å away.[26] Once fully reduced, the T2/T3 copper redox center binds O_2 and then plays a key role in the reduction of O_2 to H_2O. This accepted mechanism suggests that the main functional role of the T1 center is to facilitate the first three long-range intramolecular electron transfers from the substrate to the three copper centers in the T2/T3 trinuclear cluster (the fourth electron reduces the T1 site).[29,30] The reductive half-reaction of the enzyme with the electrochemically reduced form of the mediator was therefore considered to occur through four sequential one-electron transfers from $[Os^{II}(bpy)_2pyCl]^+$ to the Cu^{II} T1 site, which includes after each of the first three bimolecular electron transfers an intramolecular electron-transfer step. It was also postulated that there is no precursor complex between the reduced osmium(II) complex and the copper T1 site of laccase and that, at high substrate concentration, the rate-determining step of the reductive half-reaction is the intramolecular electron transfer from the T1 to the T2/T3 cluster. This hypothesis is reasonable because with a high driving force between the mediator and the copper T1 center, a rapid bimolecular outer-sphere electron transfer should occur. To verify whether a large driving force exists between the $[Os^{II}(bpy)_2pyCl]^+$ mediator and the copper T1 center of LAC3, the standard potential of the copper T1 center of LAC3 was determined from a spectroelectrochem-

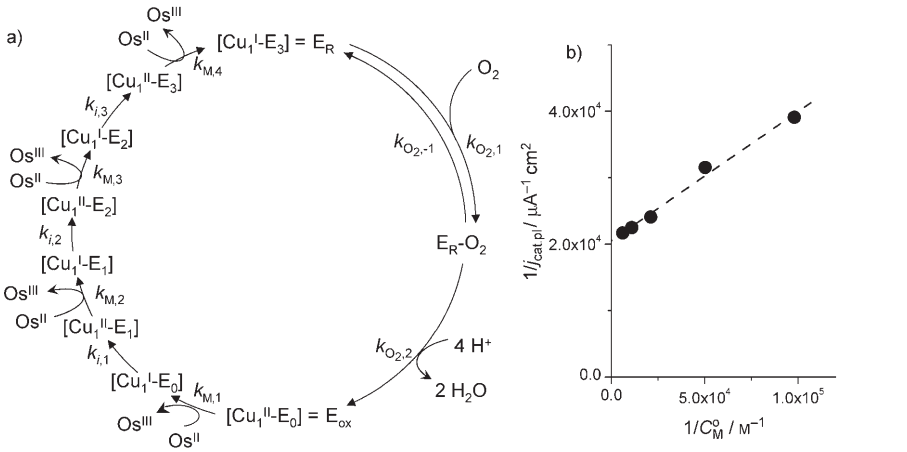


Figure 2. a) Reaction scheme used for the kinetic analysis of laccase under steady-state conditions. The mechanism assumed a classic Michaelis–Menten-type reaction towards O_2 followed by sequential four rapid electron transfers from the osmium(II) complex $[Os^{II}(bpy)_2pyCl]^+$ to the oxidized copper T1 of laccase, the first three steps were followed by rate-determining intramolecular electron transfer to the trinuclear cluster. b) Reciprocal plot of the catalytic plateau current density versus mediator concentration. The data were obtained at a NTA-electrode loaded with Cu^{II} and incubated in a 100 nM His-tagged LAC3 solution for 90 min, and finally scanned by cyclic voltammetry in an O_2-saturated phosphate buffer (pH 6.0) containing 20 μM $[Os^{III}(bpy)_2pyCl]^{2+}$ (scan rate: 20 mV s^{-1}, T = 25 °C).

ical titration (Figure 3). The perfect fitting of the redox titration curve of LAC3 with a one-electron Nernst equation and the full reversibility of the titration process (Figure 3)

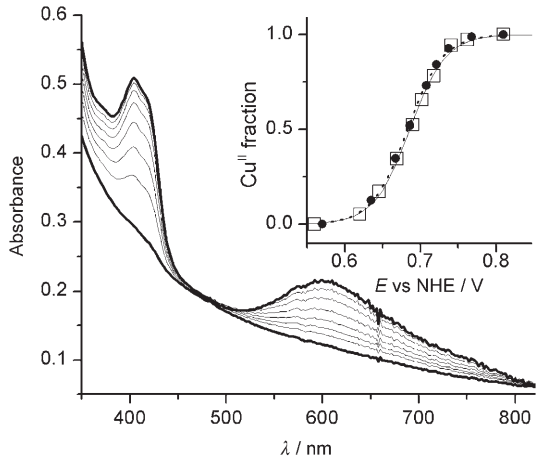


Figure 3. UV/Vis spectra of mediated spectroelectrochemical titration of His-tagged laccase (20 μM LAC3) in the presence of 180 μM $[Os(CN_6)]K_4$ (pH 6.0, $T=20$°C), recorded at different equilibrated applied potentials. Inset: Fraction of the oxidized T1 site of laccase (measured at $\lambda=$ 605 nm) as a function of the applied potential. Starting from the fully reduced laccase, the enzyme was first (□) progressively oxidized by forwarding the potential in the anodic direction and then (●) reduced by reversing the potential in the cathodic direction. Solid and dashed lines represent the fitting of the Nernst equation to the experimental data obtained during the application of the forward and reverse potential, respectively.

are in quite good agreement with the reversible one-electron transformation of blue type-1 copper. From the fitting, a formal potential value of $E^{0'}=0.68$ V versus NHE ($n=1$) at pH 6.0 was obtained, which is 230 mV more positive than the standard potential of the redox mediator involved in our cyclic voltammetric experiments ($E^0=0.45$ V versus NHE for the $[Os^{III}(bpy)_2pyCl]^{2+}/[Os^{II}(bpy)_2pyCl]^{+}$ couple). The half-oxidizing reaction was considered to follow a Michaelis–Menten-type reaction because the reaction of fully reduced native laccase with O_2 has been shown to produce a transient species, also called a peroxy intermediate, that is further rapidly oxidized ($k_{O_2,2}>1000\ s^{-1}$) to a native intermediate.[30]

For a monolayer of laccase immobilized on the electrode, the current flowing through the electrode according to the reaction scheme shown in Figure 2a may be expressed by Equation (2). This expression is formally the same as that for a classical ping-pong mechanism[31,32] except that the rate constants k_{cat} and k_M are a combination of several elementary rate constants.

$$-i=-FSD_M\left(\frac{\partial[M]}{\partial x}\right)_{x=0}+\frac{4FS\Gamma_E^0}{\frac{1}{k_{cat}}+\frac{1}{k_{O_2}[O_2]_{x=0}}+\frac{1}{k_M[M]_{x=0}}} \quad (2)$$

in which $\frac{1}{k_{cat}}$, $\frac{1}{k_M}$, and k_{O_2} are defined as follows:

$$\frac{1}{k_{cat}}=\frac{1}{k_{i,1}}+\frac{1}{k_{i,2}}+\frac{1}{k_{i,3}}+\frac{1}{k_{O_2,2}}$$

$$\frac{1}{k_M}=\sum_{n=1}^{4}\frac{1}{k_{M,n}}$$

$$k_{O_2}=\frac{k_{O_2,1}k_{O_2,2}}{k_{O_2,2}+k_{O_2,-1}}$$

D_M is the diffusion coefficient of the mediator, F is the Faraday constant, S is the electrode area, $[O_2]_{x=0}$ and $[M]_{x=0}$ are the dioxygen and mediator concentrations at the electrode surface ($x=0$), Γ_E^0 is the protein surface concentration, k_{cat} is the turnover number (in s^{-1}), and k_{O_2} and k_M are the global bimolecular rate constants (in $M^{-1}s^{-1}$) for the oxidation of the reduced laccase (E_R) by O_2 and the one-electron reduction steps of the oxidized copper T1 by the osmium(II) mediator, respectively. The factor four in Equation (2) is related to the four equivalents of the mediator required in the catalytic cycle to reduce O_2 to H_2O. The pure catalytic contribution of the current can be obtained by subtracting the diffusion contribution [the first term in Eq. (2)] from the total current. Taking into account the fact that at the catalytic plateau current, $[M]_{x=0}=C_M^0$ (in which C_M^0 is the con centration of the mediator in solution), and that the catalytic current does not significantly change when passing from an air-saturated to a dioxygen-saturated solution (i.e., $k_{O_2}[O_2]_{x=0}\gg k_{cat}$), the catalytic plateau current density, $j_{cat,pl}$, may be simplified to Equation (3).

$$-j_{cat,pl}=\frac{-i_{cat,pl}}{S}=\frac{4F\Gamma_E^0}{\frac{1}{k_{cat}}+\frac{1}{k_M C_M^0}} \quad (3)$$

The catalytic plateau current was recorded under an O_2-saturated atmosphere and at different mediator concentrations, ranging from 0.01–1 mM. The resulting values are plotted in Figure 2b in the form of the reciprocal catalytic current as a function of the reciprocal mediator concentration. From linear regression analysis, one obtains from the intercept $k_{cat}\Gamma_E^0=(1.3\pm0.2)\times10^{-10}$ mol cm^{-2} s^{-1} and the slope $k_M\Gamma_E^0=(1.3\pm0.2)\times10^{-5}$ cm s^{-1}. To determine the absolute values of k_{cat} and k_M, it was necessary to know precisely the concentration of the enzyme at the surface. This was achieved by desorbing the His-tagged LAC3 immobilized on the electrode into a small volume (80 μL) of a 0.1 M acidic phosphate buffer (pH 3.6) for 60 min and then spectrophotometrically measuring the residual enzyme activity contained in the slightly acidic solution (see the Experimental Section). Complete release of the enzyme from the electrode surface was confirmed by the total disappearance of the electrocatalytic response. From the activity of the recovered laccase, an enzyme surface coverage of $\Gamma_E^0=(2.7\pm0.1)$ pmol cm^{-2} was estimated for a series of electrodes obtained under the same conditions as those described in Figure 2b. Values of $k_{cat}=(50\pm5)\ s^{-1}$ and $k_M=(4.8\pm0.5)\times10^6\ M^{-1}s^{-1}$ were calculated. The value of k_{cat} is close to the

one obtained from the spectrophotometric steady-state kinetics study of LAC3 in homogeneous solution ($k_{cat}=60\ s^{-1}$ at pH 6.0). These results clearly illustrate that the immobilized laccase remains virtually as active as in homogeneous solution despite the use of a short thiol-NTA linker that brings the enzyme very close to the gold metal surface. They also demonstrate that the as-proposed immobilization strategy prevents deactivation of the enzyme by the metallic surface. Moreover, the value of k_M/k_{cat} was found to be identical for electrodes charged with Ni^{II} or Cu^{II}, which shows that the metal ion used to anchor LAC3 does not interfere with the enzyme reactivity.

From the enzyme kinetic parameter k_{cat} and the maximal current density of the Langmuir isotherm established at high mediator concentrations ($C^0_M=1$ mM), it was simple to estimate the maximal enzyme coverage because $k_{cat} \ll k_M C^0_M$, and so $-j_{cat,pl}=4k_{cat}\Gamma^0_E$. A maximal enzyme surface concentration of $\Gamma^0_{E,max}=(5.1\pm0.5)\ pmol\,cm^{-2}$ was deduced. This value agrees with the coverage that can be estimated from the size of laccase and for a saturated monolayer of His-tagged LAC3 on a perfectly flat surface. A theoretical saturated surface concentration of 5–7.6 $pmol\,cm^{-2}$ can be calculated for a compact monolayer of *Trametes versicolor* laccase ($65\times55\times45$ $Å^3$),[26] depending on the orientation of the protein upon immobilization. The fact that the maximal enzyme surface concentration derived from electrochemical experiments is consistent with the theoretical value calculated for a densely packed monolayer of laccase is further evidence that the proposed immobilization strategy leads to a fully active monolayer of laccase.

Conclusion

We have shown that the surface of a gold electrode can be easily modified by a short NTA-terminated alkanethiol in a single step and that it can serve as an efficient platform to affinity immobilize a packed and well-ordered monolayer of a histidine-tagged laccase. We have also shown that the catalytic activity of the immobilized enzyme can be investigated by cyclic voltammetry in the presence of a diffusive one-electron redox mediator. From a detailed kinetic analysis of the catalytic reduction of dioxygen and indirect quantification of the enzyme coverage, we concluded that the immobilized laccase is as active as in homogeneous solution, without noticeable loss of its intrinsic activity. This means that despite the use of a short thiol-NTA linker, which brings the enzyme very close to the gold surface, the enzyme activity is not affected by the close proximity of the metallic surface. It was, however, not possible to induce direct electron transfer between the copper T1 site of laccase and the gold surface, even with C-terminal His-tagged recombinant laccase, probably because of a too long-range electron transfer. We expect that shortening the thiol-NTA linker will favor direct electrical communication between the gold electrode and the redox center of laccase. Work is in progress in this direction.

Experimental Section

Synthesis: *N*-[5-(1,2-Dithiolan-3-ylpentanoylamino)-1-carboxypentyl]iminodiacetic acid was synthesized as follows: Step 1 was performed according to ref. [33]. In step 2, freshly distilled triethylamine (1.8 mL) was added to a solution containing N^{α},N^{α}-bis(carboxymethyl)-L-lysine hydrate (0.5 g; Fluka) dissolved in dried DMF (15 mL). The solution was stirred and heated for 90 min to allow complete dissolution of the solid. The activated ester (0.55 g) was then added to the mixture and the solution heated at reflux for 4 h. The solution was then filtered and the solvent removed under reduced pressure. The resulting orange oil was recuperated and dried in a desiccator. The oil was redissolved in H_2O/EtOH (1:1) and left for the night at 4 °C; a white powder precipitated from solution. After filtration, the powder was partially redissolved in hot acetone and filtered. The filtrate was finally evaporated under reduced pressure to give the thiol-NTA product as a pale-yellow solid (0.505 g, 62 %). ^{1}H NMR (250 MHz, CD_3OD): $\delta=3.62$ (d, 4H; 2×NCH_2COOH), 3.55 (1H; m, SCH), 3.46 (t, 1H; C*H*COOH), 3.18 (m, 2H; SCH_2), 3.32 (m, 2H; CH_2NH), 2.46 (m, 1H; SCH_2CH_2CHS), 2.19 (t, 2H; CH_2CO), 1.89 (m, 1H; SCH_2CH_2CHS), 1.80–1.20 ppm (m, 12H; CH_2); ^{13}C NMR (250 MHz, CD_3OD): $\delta=177.0$ (COOH), 67.7 (*C*HCOOH), 58.6 (SCH), 56.5 (*C*H_2COOH), 42.3, 41.1, 40.4, 38.0, 36.8, 31.8, 31.0, 27.8, 25.8 ppm; elemental analysis calcd (%) for $C_{18}H_{30}N_2O_7S_2$ (450.6): C 48.1, H 6.7, N 6.2 %; found: C 48.1, H 6.7, N 5.8. MS (ESI$^+$): *m*/*z*: 451 $[M+H]^+$, 473 $[M+Na]^+$.

The $[Os^{II}(bpy)_2pyCl]PF_6$ complex was synthesized as previously described.[34] The corresponding osmium(III) (Os^{III}) complex was obtained through its oxidation by LAC3 and purified using a 10 kDa cutoff ultrafiltration cartridge (Vivaspin).

Protein expression/purification: The LAC3 encoding sequence from the plasmid pAKY145[12] was modified by site-directed insertion using the Quick-Change protocol from Promega with the following mutagenic primers: Y145_NH top (bottom = reverse and complement) 5′CCC AAA TAT CTG CAG CAA TAC ACC ATC ACC ACC ACC ATG GAC CTG TCA CGG ACT TGA CC 3′ and Y145_CH top (bottom = reverse and complement) 5′CCC GAC GGT CTC GGG CGC CAC CAT CAC CAC CAT CAT TGA GAG GCG AAG CAG CTT C 3′. The resulting six histidine coding extensions in pACY1 (C-terminal) and pACY2 (N-terminal) were controlled by sequencing (Genome express). For each construct, transformants were obtained from the yeast *S. cerevisiae* W303-1A (*MAT*α ade2-1, *his3-11*, *15*, *leu2-3*, 112, *trp1-1*, *ura3-1, can1-100*). For the recombinant protein production, cells were grown as previously described.[12] All purification steps were carried out at 4 °C. The mycelium was removed by centrifugation for 30 min at 10000 rpm. The supernatant was filtered through glass microfiber filters (Whatman, final porosity 2.5 μm) and concentrated to 100 mL using a 10 kDa cutoff ultrafiltration cartridge (prep/scale-TFF 2.5 ft^2). The fluid obtained was further concentrated (10 times) and dialyzed against 20 mM phosphate buffer containing 0.5 M NaCl and 25 mM imidazole at pH 7.4 (buffer A) in an Amicon ultrafiltration stirred cell by using type YM 10 membranes. The sample was centrifuged for 30 min at 10000 rpm again and then applied to a pre-equilibrated Ni-NTA column (GE Healthcare, HisTrap FF, 1 mL). The enzyme was eluted from the column with 20 mM phosphate buffer containing 0.5 M NaCl and 500 mM imidazole at pH 7.4 (buffer B) at a flow rate of 1 $mL\,min^{-1}$. The active fractions were collected, pooled, and dialyzed against 20 mM phosphate buffer at pH 6.0 and then finally concentrated on a Microcon YM-10 column at 4 °C. Tagged LAC3 (either N- or C-tagged) represented the unique proteinaceous component of fractions that were eluted from the Ni-NTA column (as controlled by PAGE-SDS).

Enzyme activity measurement: Homogeneous oxidation of the $[Os^{II}(bpy)_2pyCl]^+$ (100 μM) complex by laccase (0.2–5 nM) in an air-saturated phosphate buffer (0.1 M, pH 6.0 or 3.6, $T=25$ °C) was followed by monitoring the initial rates (during the first 60 s) of the disappearance of osmium(II) at 500 nm by UV/Vis absorption spectroscopy (diode-array spectrophotometer Hewlett-Packard 8452A). At this wavelength the molar extinction coefficients of the reduced and oxidized forms of the osmium complex are 8900 and 500 $M^{-1}\,cm^{-1}$, respectively. Under these ex-

perimental conditions, $C_M^0 \gg k_{cat}/k_M$ and so the measured activity is only a function of the k_{cat} value, in accordance with Equation (4).

$$\frac{-d[Os^{II}]}{dt} = 4k_{cat}C_E^0 \quad (4)$$

k_{cat} values of 60 and 190 s^{-1} were reproducibly obtained for the C-terminal His-tagged LAC3 at pH 6.0 or 3.6, respectively. The k_{cat} value obtained at pH 3.6 was then used to determine the amount of active laccase desorbed from the electrode in the small volume (80 μL) of acidic solution at pH 3.6.

Electrochemical measurements: Prior to thiol immobilization, the polycrystalline gold electrodes ($S = 0.0123\ cm^2$) were polished with 3, 1, and 0.1 μm alumina, and analyzed in 0.5 M H_2SO_4 between 0.2 and 1.8 V versus SCE. Evaluation of the effective area of the gold electrode was carried out as previously reported.[35] We reproducibly found a rugosity factor value of around two. Cyclic voltammetry was carried out with a PST20 Autolab potentiostat (Eco-Chemie) interfaced with a PC computer. Voltammetric experiments were performed in a water-jacketed electrochemical cell maintained at a controlled temperature with a circulating water bath. For the cyclic voltammetric experiments with laccase, solutions (0.1 M phosphate buffer, pH 6.0) were saturated with dioxygen by flushing the cell with dioxygen gas for a few minutes.

Spectroelectrochemistry: The UV/Vis-mediated spectroelectrochemical titration of LAC3 was performed in solution by using a homemade one-compartment bulk electrolysis cell adapted from that of Tsujimura et al.[36] The working electrode was a fine wire mesh gold minigrid (Goodfellow). A platinum wire (1 mm diameter), separated from the bulk of the solution by a vycor frit and Teflon heat-shrink tubing (Princeton Applied Research) filled with the buffer solution, was used as an auxiliary electrode. A DRIREF-2 Ag/AgCl/KCl 3 M (World Precision Instruments) was used as the reference electrode ($E^0 = 0.21$ V vs. NHE, $T = 20\,°C$). The three electrodes were inserted into a 3 mL quartz cell (1 cm path length) through a silicon cap that hermetically closes the cell. An additional tygon tube for degassing was introduced. The spectroelectrochemical cell was bubbled with argon throughout the entire experiment. 20 μM laccase in a total volume of 1.4 mL of 0.05 M phosphate buffer (pH 6.0) was used. The titration was performed in the presence of 180 μM $[Os(CN_6)]K_4$ ($E^0 = 0.64$ V vs. NHE). Electrolysis at a controlled potential was carried out under magnetic stirring with a homemade potentiostat. The optical absorbance spectra of the electrolyzed solution were monitored with a 8452A diode-array spectrophotometer (Hewlett-Packard). After each applied potential step, the solution was left to equilibrate until two identical UV/Vis spectra were recorded. The absorption spectrum at 0.8 V versus NHE was identical to that of fully oxidized LAC3, exhibiting a characteristic LMCT band with a maximum at $\lambda = 605$ nm. A potential of 0.4 V versus NHE was applied for 20 min to remove any traces of dioxygen. The anaerobicity of the cell was verified by checking the stability of the reduced enzyme in the absence of any applied potential over a few minutes. Oxidative titration from 0.4 to 0.8 V was then performed. After oxidative titration, the potential was swept back to re-reduce the protein. The data were analyzed by using the Nernst equation [Eq. 5], in which n is the number of electrons (in the present case, $n = 1$), E_{appl} is the applied potential, and $E^{0'}$ is the formal potential of interest ($F = 96500\ C\,mol^{-1}$, $R = 8.31\ J\,mol^{-1}K^{-1}$). An average $E^{0'}$ value of 0.68 V versus NHE (pH 6.0) was found with n varying between 0.95 and 1.05.

$$\text{fraction of } Cu^{II} = \{\exp[(E^{0'} - E_{appl})nF/RT] + 1\}^{-1} \quad (5)$$

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