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Efficiency of site-specific clicked laccase-carbon nanotubes biocathodes towards O_2 reduction

Solène Gentil,[a,b] Pierre-Rousselot-Pailley,[c] Ferran Sancho, [d] Viviane Robert,[c] Yasmina Mekmouche,[c] Victor Guallar, [d, e] Thierry Tron*[c] and Alan Le Goff*[a]

Abstract: A maximization of a direct electron transfer (DET) between redox enzymes and electrodes can be obtained through the oriented immobilization of enzymes onto an electroactive surface. Here, we present a strategy for obtaining carbon nanotube (CNTs) based electrodes covalently modified with perfectly control-oriented fungal laccases. Modelisations of the laccase-CNT interaction and of electron conduction pathways serve as guide in choosing grafting positions. Homogeneous populations of alkyne-modified laccases are obtained via the reductive amination of a unique surface accessible lysine residue selectively engineered near either one or the other of the two copper centers in enzyme variants. Immobilization of the site-specific alkynated enzymes is achieved via copper click on azido-modified CNTs. A highly efficient reduction of O_2 at low overpotential and catalytic current densities over -3 mA cm^{-2} are obtained by minimizing the distance from the electrode surface to the trinuclear cluster.

Introduction

Multi Copper Oxidases (MCO) are redox enzymes reducing dioxygen via a $4H^+/4e^-$ mechanism at the expense of various substrates oxidation. While oxidation occurs at a surface located mononuclear type 1 (T1) copper center, electrons are transferred from the T1 to an embedded trinuclear Type2/Type3 copper center (TNC) where dioxygen is reduced into water. Immobilized at an electrode surface, high redox potential enzymes (*e.g.* laccases, bilirubin oxidases) are able to achieve the electrocatalytic oxygen reduction reaction (ORR) with a small overpotential (*eg.* 100-300 mV), close to the overpotential of the platinum catalyst traditionally used at the cathode of fuel cells.[1–3] This is the reason why such copper enzymes are envisioned as potential renewable non-precious-metal catalyst for ORR. However, the bulky protein matrix surrounding the copper centers is *de facto* limiting both the catalyst loadings and the electron transfer rates. Transferring electrons between the surface of the electrode up to the TNC is ruled by subtle parameters. In particular, a direct electron transfer (DET), which allows the enzyme to operate at its high potential without any redox partners or mediators, is influenced by the distance between the electron entry point in the enzyme and the surface of the electrode. This distance as well as the presence of insulating medium between the enzyme active site and the electrode has to be minimized. This challenge requires thin-layer modification of the electrode, the use of nanostructured material and a favorable orientation of the enzyme.[3–8] Since the seminal work of Shleev and Gorton,[9–11] numerous articles describe DET reactions of MCOs on electrodes. All together they cover important variations of experimental conditions including enzyme type (laccase, BOD, CueO), electrode type (stationary, or rotating-disk electrode), electrode material (gold, graphite), immobilization method (physi-sorption, supramolecular, covalent), operating conditions (enzyme purity, pH, T°C). Interpretations of results range from conclusions that electrons enter the enzyme via the high-redox-potential T1 copper center just as much that, at least under certain conditions, the TNC might be directly accessible for DET without compromising the ORR.[12–15]

The control of the enzyme orientation on the electrode surface appears as a major challenge in order to avoid random orientation generally resulting in poor electrode performances. To gain control on the immobilization and wiring of MCOs, strategies involving a surface modification have been developed. Most reported attempts rely on a chemical modification of the surface of the electrode. [8,16] For example, Armstrong and colleagues have taken advantage of the hydrophobicity of several amino acids nearby the T1 copper center of a laccase from *Trametes versicolor* to obtain its self-immobilization on a pyrolytic graphite electrode surface modified with polycyclic aromatics as substrate mimics. [17,18] Similar strategies have been successfully exploited to immobilize enzymes at the surface of gold nanoparticles, carbon nanotubes (CNTs) and graphene sheets.[19–26] Nowadays a variety of chemical reactions are available to introduce different functionalities at the surface of these conductive materials. In particular, the chemical or electrochemical reduction of aryldiazonium salts have proven to be a successful mean to modify the surface of the π-extended network of CNT sidewalls.[13,27–29]

Comparatively, reports targeting specific modifications of the surface of MCOs – *i.e.* terminal tags, single surface exposed reactive variant or unnatural amino acid – are still few.[30–32]

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Probably seen as complex because a modification of the DNA to encode a site-selective anchor point is a pre-requisite, this strategy has yet no equivalent to generate uniform enzyme/electrode interfaces where almost each enzyme molecule is bound through the same single point. Moreover, in principle, for any given enzyme/electrode couple the interface can be as finely adjusted as moving the anchoring point (*i.e.* the reactive function) from one position to an adjacent surface exposed position. In a recent work, we have precisely introduced a pyrene moiety at the surface of a fungal laccase.[32] This pyrene group has allowed us to immobilize the enzyme at the surface of CNTs (supra-molecular interaction) with a predefined and DET favorable orientation. To the contrary of nonspecific pyrene modifications (through pyrene-N-Hydrosuccinimide), the immobilized enzyme exhibits high direct bioelectrocatalysis of O_2 reduction on both MWCNT and gold nanoparticle-modified electrodes. In order to evaluate the influence of the localization of the anchoring point at the enzyme surface on electrocatalytic properties, we are now exploring the covalent modification of single lysine laccase variants. Here, we functionalized the native LAC3 and two of its variants ($UNIK_{71}$ and $UNIK_{161}$, Fig. 1). We used the well-known alkyne-azide *Huisgen* 1,3-dipolar cycloaddition to efficiently attach these enzymes on MWCNT electrodes covalently modified by 4-azidobenzene diazonium salt [33]. We show that such strategy is highly efficient both for the immobilization and wiring of laccases on nanostructured electrodes. Influence of the grafting conditions and that of the location of the anchoring point at the enzyme's surface on the bioelectrocatalysis of oxygen reduction are discussed.

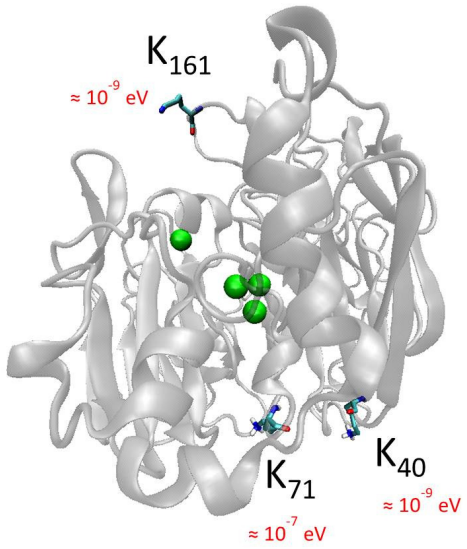


Figure 1. Model of the structure of LAC3 with the three possible locations of lysine in either the native enzyme (K_{71} and K_{40}) or its variants $UNIK_{161}$ and $UNIK_{71}$. View generated with PyMol. *In silico* evaluated electronic coupling values from an electron donating surface to the TNC (in eV) are added in red (see text for details).

Results and Discussion

Electron conduction pathway and laccase engineering

LAC3 from *Trametes* sp. C30 is a typical fungal laccase obtained with high yield as a recombinant enzyme.[34,35] The original LAC3 sequence contains naturally only two lysine residues (K_{40} and K_{71}) located at the surface of the amino-terminus domain (GENEBANK AAR00925.1). These two residues bearing primary amine groups are easily functionalizable with chemical modules.[36] We have recently described a general strategy for obtaining single surface located lysine variants of LAC3 (designed UNIK).[37] From K_0 (*i.e.* variant devoid of lysine) we have already prepared $UNIK_{161}$, a variant which can be functionalized in the vicinity of the T1 Cu^{II} site at a position almost diametrically opposed to positions K_{40} and K_{71} found in LAC3 [37].

Following our previous work on the orientation of pyrene-laccase molecules at the MWCNT electrode surface,[32] we initially compared *in silico* the pyrene-$UNIK_{161}$ and $(pyrene)_2$-LAC3 hybrid/MWCNT electrodes (Fig. SI1, see SI for computational details). Modeling of the pyrene grafted K_{40}, K_{71} and K_{161} positions was performed as previously published.[36] Distances from the anchor (pyrene) to the Cu^{II} ions and electron conduction pathways in the protein matrix were evaluated for each hybrid model (see Table 1).

Table 1: electron coupling (in eV) and distances (in Å) calculated between the pyrene moieties and the Cu^{II} ions. Evaluation made with Pathways on the laccase-pyrene/CNT models of Figure SI1.

Cu^{II} center	Anchoring position					
	K_{40}		K_{71}		K_{161}	
	eV	*d* (Å)	eV	*d* (Å)	eV	*d* (Å)
T1	$2\ 10^{-12}$	35	$4\ 10^{-10}$	31	$1\ 10^{-7}$	16
T2	$4\ 10^{-10}$	25	$1\ 10^{-8}$	20	$1\ 10^{-10}$	27
T3α	$1\ 10^{-10}$	29	$1\ 10^{-7}$	24	$4\ 10^{-9}$	23
T3β	$9\ 10^{-10}$	26	$2\ 10^{-8}$	23	$3\ 10^{-9}$	27

Comparison of the electronic coupling values suggests that the TNC of enzymes grafted at K_{40} and K_{71} positions might be directly reducible by the electrode, lysine 71 being predicted to be the position to graft for a potential optimal electron conduction (Fig. 1). The singulation of K_{40} and K_{71} positions was therefore undertaken from LAC3 in order to be able to compare DET performances from singly grafted variants. $UNIK_{71}$ was subsequently constructed and produced as previously described.[37] Positions 71 ($UNIK_{71}$) and 161 ($UNIK_{161}$) are almost mirroring each other relative to the copper centers.

LAC3 and its two variants were independently and covalently grafted to probe the bioelectrocatalytic O_2 reduction activity of the enzyme as a function of a strictly controlled orientation at the electrode surface. A commercial 4-ethynylbenzaldehyde was used for the "alkynylation" (*i.e.* functionalization with an alkyne group) of laccases in a reductive akylation reaction adapted from Mc Farland and Francis [38] (see Supplementary Information). As previously evaluated, this functionalization strategy results in a ≈

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1:1 ratio between the enzyme and the functional group [32,36]. This unique anchor point in the UNIK-alkyne variant is designed to allow only one possible orientation of the laccase molecules after reaction (cycloaddition) at the azido-modified surface of nanostructured electrodes. As previously mentioned, site-specific modifications of the enzyme are a mean to probe a DET between the electrode and the copper centers after enzyme grafting (Fig. 1).

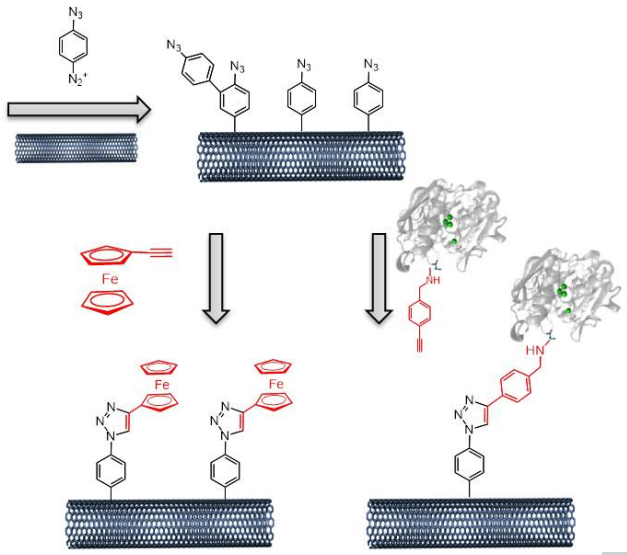


Figure 2. Schematic representation of the covalent modification of CNT by click chemistry of ferrocene-alkyne and alkyne modified LAC3 variants.

Covalent functionalization of MWCNT electrodes and electrochemistry in non-turnover conditions

MWCNT electrodes were prepared as previously-described.[21,23] Figure 2 illustrates the functionalization technique for the immobilization of an alkyne-modified ferrocene used here as an electrochemical probe and alkyne-modified LAC3 variants.

Electrografting of 4-azidobenzene diazonium tetrafluoroborate was performed either by spontaneous or electrochemical grafting of the 4-azidobenzene diazonium salt. Spontaneous grafting corresponds to a simple soaking step in a solution of 4-azidobenzene diazonium tetrafluoroborate while the electrografting corresponds to CV scans performed below the irreversible reduction of the 4-azidobenzene diazonium salt. Spontaneous grafting corresponds to the ability of diazonium salts to react with electrode surface, especially graphitized ones, by spontaneous electron transfer from the surface to the diazonium salt.[39,40] This technique was compared to electrografting as it allows to avoid undesirable thick polyphenylene layer formation inevitably formed during the electrografting process. Typical CV scans for the electrografting of a 2 mM solution of 4-azidobenzene diazonium tetrafluoroborate in MeCN are given in Supplementary Information (Fig. SI2). An irreversible reduction is observed at E_{red} = +0.05 V vs. Ag/AgNO3. This reduction corresponds to the generation of aryl radicals followed by their reaction with the surface of MWCNTs and the subsequent formation of a polyphenylene layer. The “click reaction” was then performed on azido-modified MWCNT electrodes by soaking each of them in a deaerated solution of the proper alkyne-modified derivative in the presence of $CuSO_4$, sodium ascorbate and tris-hydroxypropyltriazolylmethylamine (THTPA) for 60 min at room temperature. The modification of MWCNT electrodes was also monitored by QCM-D experiments. The functionalization of MWCNT-coated quartz crystal was monitored for both spontaneous grafting of 4-azidobenzene diazonium and immobilization of alkyne-modified laccases (Fig. S3-S5). A low Sauerbrey mass uptakes was observed for the immobilization of nonmodified LAC3 compared to alkyne-modified LAC3. This underlines that weak interactions between the enzyme and nonmodified crystals exist and corroborates the covalent attachment of enzymes on azido-modified quartz crystals. Considering a 50% degree of hydration for the protein layer,[41] final Sauerbrey mass uptakes would correspond to a surface coverage of 16 10^{-12} mol.cm^{-2} for LAC3-modified MWCNT-coated crystal. It is noteworthy that QCM-D performed on the laccase variants did not show any striking difference in terms of immobilization rates or mass uptakes. These QCM-D experiments unambiguously demonstrate that the spontaneous modification of electrodes with the 4-azidobenzene diazonium results in the grafting of a thin polyphenylene layer at the surface of MWCNTs. In turn, this active layer allows a rapid, efficient and stable immobilization of clickable laccases.

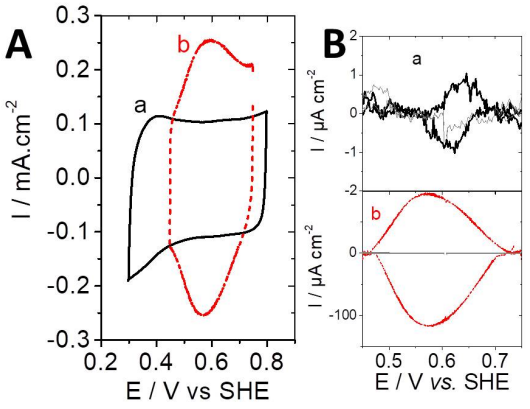


Figure 3. (A) CVs and (B) background-subtracted CVs of (a) alkyne-UNIK$_{161}$ and (b)- ethynylferrocene functionalized MWCNT electrodes under argon at pH = 5. The gray line corresponds to a non-modified MWCNT electrode (0.05 mol L^{-1} in acetate buffer solution, v = 10 mV s^{-1}).

Characterization of the UNIK$_{161}$ electrode

Electrode functionalization with the alkyne-UNIK$_{161}$ was evaluated by comparison with an electrode modified with ethynylferrocene (electrochemical probe). Figure 3 displays CV performed under argon for MWCNT electrodes covalently-modified by ethynylferrocene and alkyne-modified UNIK$_{161}$ after spontaneous grafting of the 4-azidobenzene diazonium salt.

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As expected for the ferrocene-modified electrode, a reversible system characteristic of ferrocene moieties grafted at the surface of MWCNTs [33] is observed at $E_{1/2}$ = +0.57 (+/-0.01) V (ΔE = 0 mV) *vs.* SHE. For $UNIK_{161}$-modified MWCNT electrodes, a tiny reversible system is observed at $E_{1/2}$ = +0.63 (+/-0.01) V (ΔE = 20 mV, pH 5) *vs.* SHE. This reversible system is likely attributable to the electroactivity of the enzyme. It is noteworthy that the redox potential of the T1 center of LAC3 has been measured at + 0.68 V *vs.* NHE at pH 6.[40] Control experiments with non-modified MWCNTs do not show any redox system in this region, confirming the immobilization of both ferrocene and $UNIK_{161}$ by click chemistry (Figure SI6). Unfortunately, redox peaks associated to $UNIK_{161}$ rapidly vanished when the electrode was tested at different pHs or at different scan rates. This is mostly caused by the high background capacitive current generated by MWCNTs.[13,43] By integration of the charge under the symmetrical peaks surface, coverages of 0.5 10^{-12} and 1.0 10^{-9} mol cm^{-2} were respectively estimated for $UNIK_{161}$ and ferrocene. If a large difference of surface coverages is expected given the size difference between ferrocene and the enzyme, note that the surface covered by the enzyme may be here underestimated as electroactivity of copper active sites of laccases under non-turnover conditions has been always highly difficult to distinguish by CV. [3] This is corroborated by the higher surface coverage measured by QCM-D (Fig. S5).

Electrochemistry under O_2 and influence of the grafting conditions

CV of $UNIK_{161}$-modified MWCNT electrodes were performed under constant O_2 bubbling in the supporting electrolyte. We studied the influence of the mode of electrode surface functionalization with 4-azidobenzene diazonium on the electrocatalytic reduction of O_2 by comparing MWCNT electrodes modified either by spontaneous or electrografting during 5 consecutive scans (Fig. 4).

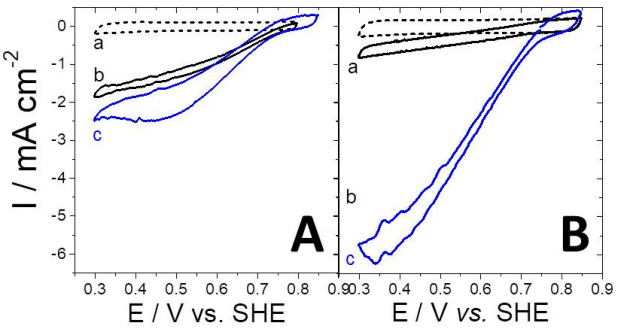
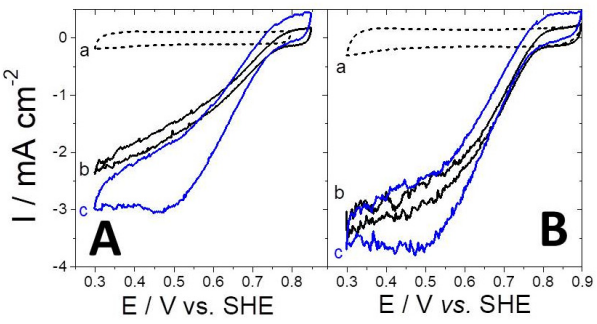


Figure 4. CV of alkyne-$UNIK_{161}$ MWCNT electrodes after the spontaneous (A) or the electro grafting (5 CV scans) of 4-azidobenzene diazonium tetrafluoroborate (B). CVs obtained (a) under Ar, (b) under O_2 and (c) after the addition of 10 mM ABTS (0.05 mol L^{-1} acetate buffer solution pH 5, v = 10 mV s^{-1}).

For all electrodes, an irreversible electrocatalytic wave for O_2 reduction is observed at high potentials. This confirms that a DET is obtained for the clicked $UNIK_{161}$ achieving the bioelectrocatalytic reduction of O_2. Maximum current densities of -1.76 (+/-0.3) and -0.75(+/-0.2) mA cm^{-2} were obtained after spontaneous and electrografting respectively (fig. 4, black curves). It is noteworthy that a simple adsorption of the enzyme on non-modified MWCNTs led to a sluggish irreversible reduction with a maximum current density of -0.52(+/-0.2) mA cm^{-2} (Fig. SI7). Reduction of O_2 was also investigated after addition of ABTS. This well-known laccase redox mediator unravels the enzymatic activity of immobilized $UNIK_{161}$ which might not be in direct contact with the electrode. In this case, maximum current densities of -2.39 (+/-0.5) mA cm^{-2} and -5.91 (+/-0.8) mA cm^{-2} were respectively observed after spontaneous and electrografting (Fig. 4, blue curves). Ratio of mediated/unmediated electrocatalytic current values suggests that spontaneous grafting induces the most efficient DET. Therefore, although the electrografting mode allows to reach a high density of azide groups at the surface of the electrode eventually leading to a high amount of immobilized enzymes, these enzymes appear poorly connected (Fig. 4B, ≈ 15% DET). To the contrary, with a thinner polyphenylene layer induced by the spontaneous grafting of the diazonium salt, this electrode offers less azido groups to click the enzyme but these enzymes are fairly well connected to the electrode (Fig. 4A, ≈75% DET). These experiments underline the fact that the grafting of the functional layer has to be optimized in order to prevent inhibition of electron tunneling while maximizing enzyme surface coverage. This being said, the CV of the alkyne-$UNIK_{161}$ modified electrode under O_2 is very similar to that previously obtained with the pyrene-$UNIK_{161}$ modified electrode in similar conditions.[32] Eventually, the stability of the electrodes was evaluated performing one-hour chronoamperometry at +0.4 V *vs.* SHE under O_2 bubbling (Figure SI8). After one hour, the catalytic current decreased from 1.7 (+/-0.2) to 1.1 (+/-0.2) mA cm^{-2}. This catalytic current stabilized around 0.5 mA cm^{-2} after 3 days. These experiments did not reveal obvious differences linked to the use of different immobilization techniques or enzyme variants.[32]

Influence of the orientation of the enzyme

In order to unveil an influence of the location of the grafting point on the DET obtained with laccase variants, both LAC3 and $UNIK_{71}$ were further modified with alkyne groups and immobilized at azide-modified MWCNT electrodes, as described above for $UNIK_{161}$. Figure 5 displays CVs performed under O_2 in the absence or presence of ABTS.



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Figure 5. CV of (A) $(alkyne)_2$-LAC3 MWCNT electrodes and (B) alkyne-$UNIK_{71}$ MWCNT electrodes; (a) under Ar, (b) under O_2 and (c) after the addition of 10 mM ABTS (0.05 mol L^{-1} acetate buffer solution pH 5, v = 10 mV s^{-1}).

The general behavior of the two electrodes both possessing an alkyne functionalized K_{71} as grafting point is globally similar. Both electrodes perform O_2 reduction at a similar onset potential (nearly + 0.85 V *vs.* SHE), that is itself comparable to that already obtained for the alkyne-$UNIK_{161}$ (Fig. 4A). The $UNIK_{71}$-modified electrode (Fig. 5B) exhibits a high current density under O_2, accompanied with a negligible increase after addition of ABTS. This indicates that all immobilized $UNIK_{71}$ enzymes establish a direct electronic contact with the electrode. The second anchoring point present in the $(alkyne)_2$-LAC3 (Fig. 5A) does not seem to improve neither the grafting (ABTS trace) nor the DET. Note that in this case the signal may be composite, potentially including contributions of enzymes resulting from two possible single point (K_{40} or K_{71}) and a double point (K_{40} and K_{71}) functionalization of the nanotube surface. It is therefore difficult to infer which situation modulates the signal from the raw CV.

Modelization

The electrocatalytic response of the different electrodes was simulated using the model developed by Armstrong and Léger.[44–46] In particular, this model takes into account a distribution of orientations of catalytically active immobilized enzymes. Background-subtracted experimental curves and simulated curves obtained for $(alkyne)_2$-LAC3, alkyne-$UNIK_{161}$ and alkyne-$UNIK_{71}$ are displayed on Figure 6.

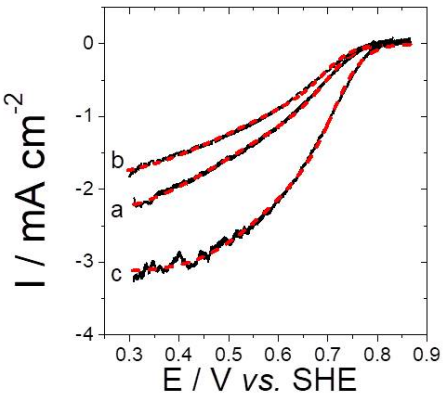


Figure 6. Background-subtracted CV of $(alkyne)_2$-LAC3 (a), alkyne-$UNIK_{161}$ (b) and alkyne-$UNIK_{71}$ (c) functionalized MWCNTelectrodes under O_2 and respective simulated CV scan (red trace) (0.05 mol L^{-1} acetate buffer solution pH 5 , v = 10 mV s^{-1}. Background-subtracted CVs take into account the average between the backward and forward CV scans.

Despite a potential inaccuracy linked to diffusion limitations into the MWCNT film that are not specifically taken into account by this model, CV curves were all well-fitted (R^2 > 0.99) using equations developed in ref [44–46] (see supplementary materials for details). The simulation allowed us to estimate several constant parameters: the catalysis redox potential (E_{cat}), the ratio between the catalytic rate constant (k_{cat}) and the interfacial ET rate constant at minimal distance d_{min} (k_0^{max}) and the ET tunneling factor βd_0 which is the product of the medium exponential decay constant β and d_0, the interval of distances between the electrochemical relay center and the electrode allowing ET (Table 2).

Table 2. Fitting parameters of simulated curves from Figure 6 for alkyne-modified native LAC3, $UNIK_{161}$ and $UNIK_{71}$.

	LAC3	$UNIK_{161}$	$UNIK_{71}$
E_{cat}	0.706	0.701	0.720
k_{cat}/k_0^{max}	0.047	0.034	0.029
j_{lim}	2.33	1.82	3.13
βd_0	10.3	10.6	8.4
R^2	0.998	0.997	0.998
%ABTS[a]	27%	34%	4%

[a] 100*(1-(j_{lim} (-ABTS)/ j_{lim} (+ABTS))

The location chosen for the grafting positions at the surface of the enzymes and distances estimated from the graft to the copper centers point the Cu^{II} T1 and the TNC as electrochemical relay centers respectively for $UNIK_{161}$ and for both $UNIK_{71}$ and LAC (Fig. 1, Table 1). Our initial *in silico* evaluation of the systems suggests that energies coupling the electron donating surface to their closest electrochemical relay centers are similar for $UNIK_{161}$ and $UNIK_{71}$ (Table 1). The slightly higher performance of the $UNIK_{71}$ based electrode is therefore directly associated to a lower distribution of tunneling distances. This is observed with the lowest βd_0 product corresponding to a decreasing effect of the dispersion of tunneling distances which might arise from a shorter electronic coupling distance between the electrochemical redox center and the electrode.[44,46] Interestingly, the βd_0 value for the $Unik_{71}$ electrode is also significantly lower than that of the LAC3 electrode whereas these enzymes are having both K_{71} as grafting point. Beyond the simple ascertainment that grafting the enzyme through the two surface positions (K_{40}, K_{71}) located ca. 14 Å away (Cα-Cα distance) might not reduce heterogeneity this could alternatively reveal a significant proportion of the LAC3 enzyme actually connected through the position K_{40} only.

Beyond the above noted differences it appears that the three electrodes all perform the electrocatalytic reduction of O_2 with a relative efficiency (current densities > 1.8 mA cm^{-2} and a DET efficiency from 70% to nearly 100%) and that whatever the orientation chosen for the enzyme at the CNTs surface. Turnover frequencies (TOF) values are in the order of 5.0 10^3 s^{-1} according to the enzyme surface coverage measured by QCM-D; these are in good agreement with TOF values measured for BOD[43] or for laccases.[47] The $UNIK_{161}$ enzyme

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offers a "classical" orientation with the Cu^{II} T1 as electrochemical relay to the dioxygen reduction center (TNC).[5, 8] The $UNIK_{71}$ enzyme (as well as the LAC3 enzyme) offers an opposite orientation with no relay to the TNC, a TNC that however can be apparently directly reached by electron tunneling though the protein matrix. Orientation dependent electron tunneling from the surface to the TNC has been proposed for several MCO for which a marked difference of redox potential of the copper centers has been measured.[46, 47] This is the case for MCOs for which experimental study conditions are apparently favoring the Alternative Resting (AR) state of the enzyme, a form of TNC partly-reduced and fully reducible only at low potential. [12–15]. This is also the case for low potential Cu^{II} T1 enzymes like CueO. [50] In LAC3 and in its variants (i.e. $UNIK_{71}$ and $UNIK_{161}$) the copper centers are almost iso potential. [51] Apparently, none of our experimental conditions was favoring an AR state of the TNC. This shows up in the CVs of the three different electrodes we tested as they perform O_2 reduction at the same onset whatever the orientation of the enzyme at the CNTs surface. Moreover, the similar Ecat obtained for all enzymes is probably related to the fact that TNC and T1 redox potentials are close.[51] Overall, our results establish that both the Cu^{II}T1 and the TNC centers of a high potential laccase can be efficiently targeted from discrete surface areas selected through the choice of single grafting points.

Conclusions

We achieved an efficient DET based electrocatalytic reduction of dioxygen using diazonium modified MWCNTs easily functionalized through a simple alkyne-azide *Huisgen* 1,3-dipolar cycloaddition of alkynylated enzymes. Targeting specific modifications of the surface of MCOs through single surface exposed reactive variants, uniform enzyme/electrode interfaces were generated. Hence, each enzyme molecule is bound through the same single point. With this strategy, we established that each of the two catalytic centers of a high potential laccase, namely the Cu^{II}T1 and the TNC centers, can be efficiently reached from discrete surface areas selected through the choice of single grafting points. The next step towards improvements on the immobilization of our laccase variants at the surface of MWCNTs requires to get more insights into the structural characterization of the grafted electrodes. In particular, an *in situ* evaluation of both active-site-electrode distances and of the structural integrity (*i.e.* identifying a partial deformation or unfolding of the enzyme upon immobilization) is prioritized in order to better understand which parameters influence the electrochemical behavior of a copper enzyme to another.

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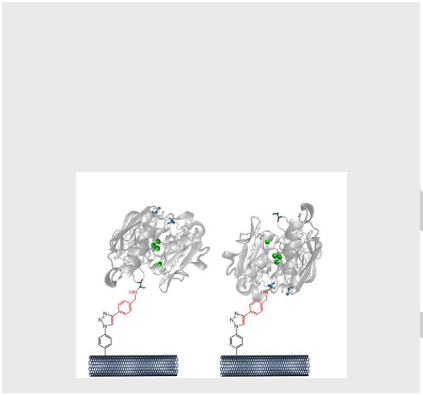
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