Zhangfei Su, Maciej Karaskiewicz, Jerzy Rogalski, Renata Bilewicz, Jacek Lipkowski, "Spectroelectrochemical studies of structural changes of laccase adsorbed on modified carbon nanotubes during reduction of oxygen ". *J.Electroanal.Chem*, **2020**, **875**, <http://dx.doi.org/10.1016/j.jelechem.2020.113820>

Spectroelectrochemical studies of structural changes during reduction of oxygen catalyzed by laccase adsorbed on modified carbon nanotubes

Zhangfei Su^d, Maciej Karaskiewicz^{a,b,d}, Jerzy Rogalski^c, Renata Bilewicz^{a*}, Jacek Lipkowski^{d*}

^a Faculty of Chemistry, University of Warsaw, Pasteura 1, 02-093 Warsaw, Poland,

^b College of Inter-Faculty Individual Studies in Mathematics and Natural Sciences, University of Warsaw, Banacha 2, 02-097 Warsaw, Poland

^c Department of Biochemistry and Biotechnology, Maria Curie Skłodowska University, Akademicka 19, 20-031 Lublin, Poland

^d Department of Chemistry, University of Guelph, 50 Stone Road East, Guelph, ON N1G 2W1, Canada

E-mail address: jlipkows@uoguelph.ca (J. Lipkowski), bilewicz@chem.uw.edu.pl (R. Bilewicz)

Zhangfei Su and Maciej Karaskiewicz made equal contribution to this paper

In memory of Professor Andrzej Wieckowski and in recognition of his outstanding contributions to electrochemistry.

Abstract

Reduction of oxygen catalysed by laccase was studied by surface-enhanced infrared absorption spectroscopy (SEIRAS) combined with electrochemical techniques. Laccase molecules were adsorbed on modified carbon nanotubes (CNTs). SEIRAS provided spectra of amide I band of adsorbed laccase. Fourier self-deconvolution and two-dimensional correlation spectroscopies (2DCOS) were employed to reveal the sub-band structure of the amide I band. The analysis demonstrated that laccase adsorbed on CNTs remains its native state both in the oxidized and reduced states. The detailed analysis showed that change of the oxidation state

leads to a small change of the secondary structure of the protein. This change is comparable to the change observed for similar blue-copper oxidases in solution during redox titration. The results of this study demonstrated that CNT's immobilized laccase is an excellent catalyst of oxygen reduction. This study illustrates power of quantitative analysis of IR data to provide information about three-dimensional structure of surface immobilized proteins.

Key-words: arylated carbon nanotubes, SEIRAS, oxygen reduction, laccase, blue copper oxidases.

1. Introduction

Laccase belongs to the blue copper oxidase group and has found many applications in the construction of biosensors and biofuel cells [1-6]. The favorable property of laccase is the ability of transferring electrons in the direct electron transfer (DET) mode, which is rather uncommon among oxidoreductases [7-11]. Even though one can find many publications concerning practical applications of laccase, there is lack of fundamental research on the molecular behaviour of laccase as the catalyst [12, 13]. Figure 1 shows the structure of the laccase from *Cerrena maxima* determined by X-ray crystallography.

Figure 1. A scheme polypeptide chain, forming α -helix and β -sheet (A) and crystallographic structure of laccase molecule from *Cerrena maxima* [14].

Solomon and his group studied extensively the mechanism of ET in laccase. Laccase active site consists of three copper centers: T1, T2 and T3, containing four copper ions [15, 16]. Electrons obtained in the oxidation of aromatic compounds (*e.g.* phenols or aromatic amines), at the T1 site, undergo intramolecular electron transfer (IET) to the T2/T3 cluster located on the opposite side of the molecule.

The DET process between the substrate and the T1 copper ion requires proper orientation of protein molecules. The influence of solid substrate on enzyme orientation and its catalytic activity has been reported for several oxidoreductases [17-21]. Laccase is an example of such dependence as the catalytic reduction of oxygen to water occurs at higher potentials on carbon materials than on metallic substrates [22]. *Cerrena unicolor* laccase molecules adsorbed on carbon electrodes catalyze oxygen reduction at potential very close to formal potential of T1 site, 750 mV *vs.* NHE [23]. The structure of a surface has a great impact as well [23, 24]. The catalytic current on highly ordered pyrolytic graphite (HOPG) has a low value, which is probably caused by relatively flat surface favouring low surface concentration [25]. Protein molecules immobilised on carbon nanomaterials or carbon papers show better electrochemical properties [26, 27]. Stolarczyk *et al.* [28] reported high catalytic activity of laccase adsorbed on pristine carbon nanotubes. Such electrodes had high surface area, which resulted in high catalytic current at the formal potential of the enzyme. Furthermore, the stability of this system was improved in comparison to other substrates [28]. Much better results were obtained by Armstrong and his group when carbon materials were modified with aromatic moieties, enforcing favourable orientation of laccase [22]. As mentioned above, the worst substrates for protein immobilization are bare metals *i.e.* gold [29]. Gupta investigated reduction of oxygen catalyzed by laccase immobilised on gold electrodes and observed higher overpotential of this reaction by 500 mV than on carbon substrates [30]. Such a phenomenon has been reported by many researchers and is well described in a literature [31, 32]. It is assumed that lower potential of oxygen reduction is caused by reversible orientation of molecules and direct electron transfer between electrode and T2/T3 site, which enforces totally different mechanism of reduction [31, 32]. Moreover, even partial denaturation of laccase molecules has been suggested [33]. Increased application of immobilised proteins and characterisation of gold surfaces lead to the use of self-assembled monolayers (SAM), to prevent proteins from denaturation [33, 34]. But even in this case 200 mV overpotential has been observed for several proteins [32].

Laccase shows strong dependence of the catalytic behavior on molecular orientation: orientation towards surface or towards substrate material, proved by several spectroscopic investigations [31, 35]. Unfortunately, most of these studies were conducted *ex situ* which do not provide conclusive information on the behaviour of this enzyme during electrocatalysis of oxygen reduction.

The most direct information about native state of the protein provides Amide I band, which is due to the C=O stretching vibrations of amide groups and is observed between 1600 and 1700 cm^{-1} IR region [36]. Due to strong absorption of water in this region, most studies of amide I mode are performed in D_2O solutions. Another difficulty is serious overlap of bands corresponding to secondary structure in the amide I region. Attempts also were made to exploit other vibrational modes, particular amide II and amide III bands [37, 38]. The NH/ND exchange process is limited to outer space of the molecules, and fraction of Amide II band is still retained in D_2O [39, 40]. The Amide II bands are strongly overlapped by bands originating from amino acid side chain vibrations [40-43]. Therefore, even when the intensity of amide II region is strong, the analysis of the protein secondary structure is less straightforward than for the amide I vibration [36]. On the other hand, Amide III bands which is predominantly due to the in-phase combination of N—H in-plane-bending and C—N stretching vibrations, overlaps with the phosphate bands when phosphate buffer is used for *in situ* studies [40].

Palys and her group [31] reported sharp changes of amide I and amide II bands intensity of laccase, registered using *ex-situ* Polarization Modulated Infrared Reflection Absorption Spectroscopy (PMIRRAS), revealing strong relationship between molecule orientation and surface charge. SEIRAS was also used by Vaz-Dominguez and coworkers to monitor orientation of enzyme molecules at SAM of 4-aminothiophenol modified gold electrode [19]. Recently, Climent *et al.* [44] employed SERS and STM to investigate multicopper oxidases found in *Escherichia coli* immobilized at the Au electrode surface modified with self-assembled monolayers of thiols.

In this study electrochemical SEIRAS is employed to study changes in laccase conformation by measurement of intensity of amide I and Amide II bands (Fig. 1A). Protein molecules were adsorbed on Single Walled Carbon Nanotubes (SWCNTs) modified with naphthalene moieties, to enforce the shortest distance between electrode surface and the enzyme T1 site (Fig. 1B). Experiments were conducted *in situ* in oxygen saturated buffer solution to observe differences in the secondary structure for the oxidized and reduced state of the protein. Fourier self-deconvolution (FSD) and two Dimensional Correlation Spectroscopy

(2D-COS) were applied to improve spectral resolution and to deconvolute broad laccase amide I band. Using this approach, this study demonstrated that laccase immobilized at SWCNTs modified with naphthalene moieties retains its native structure in both oxidized and reduced states. Further it showed that the difference in the secondary structure in the two redox states are similar for the surface immobilized protein and the protein in the bulk of solution whose redox state is change by redox titration. Our results explain high electrocatalytic activity of laccase immobilized at SWCNTs. This paper illustrates how molecular structure of redox proteins and hence their active state should be investigated *in situ* in the future.

2. Experimental

2.1 Glassware and chemicals

All glassware was cleaned in hot acid bath (mixture of $\text{HNO}_3/\text{H}_2\text{SO}_4$ in ratio 1:3) for one hour and thoroughly rinsed using Milli-Q ultrapure ($>18.2 \text{ M}\Omega \text{ cm}$) water system (Millipore). The glassware was then soaked in water overnight. The three-electrode glass cell was equipped with platinum foil counter electrode (CE) (Alfa Aesar, 99.99%), which was flame annealed and rinsed with Milli-Q water to remove any contaminations from the surface, Ag/AgCl reference electrode (RE) and thin gold film of gold nanoparticles [45, 46] deposited on a reflecting plane of a hemispherical silicon prism (2.54 cm diameter) as working electrode (WE). Its preparation is described in Ref. [47]. Reagents like: citric acid monohydrate (Sigma-Aldrich), disodium phosphate (Sigma-Aldrich), 48 % hydrofluoric acid (Sigma-Aldrich), sodium tetrachloroaurate (III) dihydrate (Sigma-Aldrich), sodium thiosulfate (Sigma-Aldrich), ammonium chloride (Sigma-Aldrich), sodium sulfite (Sigma-Aldrich) were of analytical grade and their solutions were prepared using Milli-Q water. All spectroscopic and electrochemical measurements were performed in McIlvaine buffer prepared as a mixture of 0.05 M citric acid monohydrate and 0.1 M disodium phosphate to give $\text{pH}=5.3$.

2.2 Spectroscopic Experiments

The details of SEIRAS experiments were described in the literature [45, 46]. The ATR element used in SEIRAS experiments was a non-doped Si hemisphere. All spectra were collected using a Nicolet 8700 FT-IR and MCT-D* detector, 70° angle of incidence and a spectral resolution of 4 cm^{-1} . Spectroelectrochemical cell shown in Figures 9.16 and 9.17 of Ref. [48] was employed to perform these experiments. When spectra are recorded with a

resolution 4 cm^{-1} , the data points are computed every 2 cm^{-1} . Since the bands are plotted by interpolation between these points, the band center position can be determined with uncertainty 0.5 cm^{-1} . The reflecting plane of the Si sphere on which metal is deposited was polished with successively finer grade alumina slurries down to $0.05\text{ }\mu\text{m}$, followed by careful rinsing with water and then sonication in water and next in acetone. Then the surface was exposed to 2% HF for several minutes to remove oxide layer on the Si surface and to terminate it with hydrogen [47]. Deposition of Au was performed at $60\text{ }^{\circ}\text{C}$ by dropping a mixture of a plating solution and 2% HF (2:1 in volume) onto the hydrogen-terminated Si surface. The composition of the plating solution was $0.015\text{ M NaAuCl}_4 \cdot 2\text{H}_2\text{O} + 0.15\text{ M Na}_2\text{SO}_3 + 0.05\text{ M Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O} + 0.05\text{ M NH}_4\text{Cl}$, identical to that reported by Miyake and coworkers [47].

SWCNTs were functionalized with naphthyl residues, as described in [49]. The stable SWCNTs suspension (1 ml), prepared from 1mg of nanotubes in 10 ml of absolute ethanol, was dropped on bare gold surface and dried in Ar stream. Immobilization of laccase on CNTs surface was done by dissolving a vial of the protein in 2 ml of 0.15 M McIlvaine buffer solution, pH 5.3. Half of this volume was used to cover one electrode surface. Experiments were performed after incubation in such solution overnight. Next, the enzyme solution was replaced with pure McIlvaine or phosphate buffer, used for most spectroelectrochemical experiments.

2.3 Laccase preparation

Laccase *Cerrena unicolor C-139* was obtained from the culture collection of the Regensburg University and deposited in the fungal collection of the Department of Biochemistry (Maria Curie-Sklodowska University, Poland) under the strain number 139. Laccase from the fermenter scale cultivation was obtained according to already reported procedure after ion exchange chromatography on DEAE-Sepharose (fast flow) and lyophilized on Labconco (Kansas City, USA, FreeZone 12 Lyophiliser) in clear stoppering chambers [50]. Laccase activity was measured spectrophotometrically with syringaldazine as the substrate. The protein content was determined according to Bradford with bovine albumin as the standard [51]. The concentration of isolated and frozen ($-18\text{ }^{\circ}\text{C}$) enzyme was $C_{\text{lacc}} = 178\text{ }\mu\text{g}/\text{cm}^3$. After lyophilizing, laccase activity in each vial containing 1 mg of protein after dilution in 1 ml of water was 256 U ml^{-1} .

3. Results and discussion

For consistency with previous electrochemical studies of laccase immobilized at naphthylated SWCNTs [32], preliminary experiments were performed in aqueous solutions of McIlvaine buffer. The activity of laccase immobilized on the SWCNTs deposited onto the Au film deposited at Si ATR window were characterized with the help of cyclic voltammetry, before every IR experiment. Figure 2 shows the CVs recorded in solutions in the absence and presence of oxygen.

Figure 2. Cyclic voltammograms recorded in (black) deoxygenated and (red) oxygenated McIlvaine buffer, pH = 5.3 using Si with thin gold film covered with naphthylated SWCNTs and laccase; scan rate: 1 mV s^{-1} .

In the presence of oxygen the cyclic voltammetry curve has a wave shape corresponding to the catalytic process of dioxygen reduction and appears at a potential close to that of the T1 site as discussed in a details in our earlier paper [32]. Here we focus on the IR characteristics of the process.

Figure 3 shows IR spectra recorded during adsorption of laccase molecules on the CNTs functionalized with aminoethyl residues and deposited onto a film of Au nanoparticles covering the ATR window. The spectra are presented as $\log(I_{\text{ref}}/I_{\text{sample}})$ where I_{ref} and I_{sample} refer to reflectivities measured before and after addition of laccase and a waiting time ranging from 1h to 14 h during which the spectra were collected at the interval of one hour at the open circuit potential. For comparison, dashed black line plots ATR spectrum of laccase recorded

using silicon widow without Au film and without CNTs. The I_{ref} for this spectrum was the reflectivity of the silicon widow covered by laccase free buffer solution. Since the penetration depth of the evanescence wave was several hundred nm, this spectrum plots the spectrum of laccase in solution.

Figure 3. SEIRA spectra of laccase collected during the adsorption of enzyme molecules on modified CNTs *in situ*. Each spectrum was registered after one hour from last acquisition in McIlvaine buffer solution, 0.15 M, pH = 5.3. The dashed line represents the ATR spectrum of laccase in McIlvaine buffer in H₂O.

The spectra recorded for laccase in the bulk of solution display strong amide I and Amide II bands at 1640 and 1547 cm⁻¹ respectively. They are in excellent agreement with transmission spectra reported by Ragusa *et al* at pH=7.2 (1641 cm⁻¹ for Amide I and 1548 cm⁻¹ for Amide II band) [52]. The SEIRAS spectrum of laccase recorded 1h after addition of the protein to the electrode covered by SWCNTs is compared to the ATR spectrum of laccase in solution in Figure S1 of the Supporting Information (SI). The two spectra of laccase are similar (the SEIRAS spectrum is slightly red shifted due to environmental effects), indicating that laccase immobilized at the naphthylated SWCNTs retains its native form. This is a very significant result because it explains strong laccase activity for oxygen reduction described in [35].

The intensities of Amide I and Amide II bands were increasing, with time indicating a progressive adsorption lasting up to 16 h. The position of the amide II band was almost

constant and equal to $1539 \pm 1 \text{ cm}^{-1}$. In contrast the position of amide I band shifted from 1636 to 1632 cm^{-1} during the 16 h of laccase adsorption (see Figure 2S of the SI). The ratio of the intensity of the amide I to amide II bands also increased with time. This behavior suggests that spectral interferences may be present in the spectra. To address these issues, several complementary experiments were performed and are presented in the Supporting Information. Figure S3 of the SI shows the ATR spectrum of the McIlvaine buffer using H_2O as the reference. The spectrum shows band at $\sim 1650 \text{ cm}^{-1}$ corresponding to water bending vibration and bands at ~ 1710 and $\sim 1580 \text{ cm}^{-1}$ corresponding to $\text{C}=\text{O}$ and carboxylic group vibrations of citrate present in the buffer. These bands disappear if the spectrum is recorded in the phosphate buffer without citrate. The presence of water bending mode in the two spectra indicates that the cancelation of this vibration is not complete. Figure S4 of SI compares ATR spectra of laccase in the bulk of McIlvaine and phosphate buffers. The spectra are quite similar indicating that phosphate buffer may be used in further research to avoid spectral interferences due to the presence of citrate. Finally, Figure S5 of SI shows spectra of McIlvaine and phosphate buffer recorded in D_2O . The water bending band at $\sim 1650 \text{ cm}^{-1}$ disappears, however a new broad band at $\sim 1450 \text{ cm}^{-1}$ corresponding to HOD bending mode is present in these spectra. The spectrum of the phosphate buffer in D_2O has a flat background in the 1800 to 1500 cm^{-1} region and is free of citrate and water bending bands. Therefore, further studies were conducted in D_2O and in the phosphate buffer in the presence of oxygen.

Figure 4 ATR spectra of laccase in phosphate buffer in D_2O (red solid line) and phosphate buffer in H_2O (black dashed line).

Figure 4 compares ATR spectrum of laccase recorded in the phosphate buffer in D₂O to the spectrum recorded in H₂O. The Amide I band corresponds chiefly to C=O stretching of the amide bond and hence is not affected by the isotopic exchange. In contrast, the Amide II band has a strong component of N-H vibration and is strongly shifted towards lower wavenumbers when D₂O is used as the solvent. It almost disappears in the spectrum recorded in the D₂O solution.

Figure 5 shows the SEIRAS spectra recorded in the phosphate buffer at several electrode potentials. In addition, Figure S6 of the SI (equivalent to Figure 2) shows how the spectrum changes as a function of laccase deposition time. In comparison to the ATR spectrum of laccase in the bulk of the buffer (Figure 4), the SEIRAS spectrum of the enzyme immobilized at CNTs show residual amied II band indicating incomplete isotopic substitution of the N-H bond. The Fourier self-deconvolution (FSD) procedure was applied to reveal the sub-bands in the broad spectral envelope of the two amide bands region. The FSD narrows bands in the spectrum revealing the fine structure of a broad spectral feature [53]. Figure 6 shows the Fourier self-deconvoluted spectrum. It reveals many bands hidden in the broad envelope of the 1750-1500 cm⁻¹ region. Table 1 lists the strongest bands and their assignment.

Figure 5. Potential dependence of SEIRA spectra recorded for laccase molecules on the CNTs functionalized with aminoethyl residues and deposited onto Au film covered Si window. The spectra were recorded after 8 h deposition time (see Figure S6 of the SI) Spectra were recorded *in situ* at open circuit potential in the 0.1 M phosphate buffer, pH = 5.4 and D₂O as the solvent. The potentials were measured vs. Ag/AgCl in sat. KCl solution. The spectra were plotted as $A=\log(I_{\text{ref}}/I_{\text{sample}})$ where I_{ref} is the spectrum before adding laccase to the electrolyte and I_{sample} is the spectra of laccase at different electrode potentials.

Figure 6 Difference between SEIRAS spectra of laccase in phosphate buffer in D₂O with oxygen recorded at sample at $E=0.8$ V vs Ag/AgCl SEIRA and more negative potentials indicated in the figure.

Crystallographic structure of laccase from *Cerrena maxima* [14], which is very similar that of *Cerrena unicolor*, shows that laccase has three cupredoxin domains. Domain I comprises of eight β -strands and two α -helices. The second domain has eleven β -strands and the third domain has ten β -strands and four α -helices [14]. A few α -helices can be found in the vicinity of T1 and T2/3 active sites. A typical IR spectrum of the protein with such secondary structure displays amide I and amide II bands at *ca.* 1630cm^{-1} and 1530cm^{-1} , respectively [52, 54, 55], indicating that the bands are dominated by absorption by the β -sheets. In some reports these bands are shifted slightly to higher values suggesting that absorptions by α -helices also contribute to the spectrum [31, 56]. Indeed, in Figure 5 absorptions at ~ 1630 (amide I) and 1530 cm^{-1} (amide II) dominate in the two spectral regions. Small bands above 1700 cm^{-1} correspond to vibrations of amino acid residues in side chains [54].

Table 1 Positions of sub bands in the envelope of the amide I band in the ATR spectrum of laccase and comparison with results from [52].

2D-COS / cm^{-1}	FSD / cm^{-1}	Data from Ragusa <i>et al.</i> [52] / cm^{-1}	band assignment [52, 54, 55]
---------------------------	------------------------	--	------------------------------

1510	1512	1515	Amino acid in side chains (tyrosine)
1523	1530		Amide II
1546	1545	1545	Amide II
1566	1566	1558	Amino acid in side chain (glutamate)
1588	1582	1584	Amino acid in side chains (aspartate)
	1601	1611	Amino acid in side chains (tyrosine)
1616	1623		β -strand
1634	1639	1636	β -strand
1658	1657	1659	α -helix
1690	1690	1678	Aminoacids in side chains

Figure 5 shows that the spectrum changes little with potential indicating small differences between the secondary structure of the enzyme in the reduced and oxidized states. These changes are small but are measurable. Figure 6 plots the difference spectra that correspond to difference between absorbance of the oxidized and the reduced form. The changes at $E_s=0.7$ V vs. Ag/AgCl are small and hence this spectrum is plotted as the background. The potential difference spectra are bipolar where the positive lobes represent oxidized state and negative lobes reduced state of the enzyme. In Figure 7 the difference between SEIRAS spectra recorded at 0.8V and 0V vs Ag/AgCl is compared to the FSD of the SEIRAS spectrum recorded at 0.0V vs Ag/AgCl.

Figure 7 Difference between SEIRAS spectra recorded at $E=0.8$ V and $E=0.0$ V vs Ag/AgCl (red line) and the bottom black line is the FSD of SEIRA spectrum of laccase in phosphate buffer in D_2O with oxygen at $E=0.0$ V vs Ag/AgCl.

The comparison shows that the most prominent changes are observed at $\sim 1725\text{ cm}^{-1}$ (side chains), $\sim 1655\text{ cm}^{-1}$ (α -helices), $\sim 1630\text{ cm}^{-1}$ and $\sim 1530\text{ cm}^{-1}$ (β -strands). The amplitudes of the potential difference spectra are about an order of magnitude smaller than the amplitude of the SEIRAS spectra (Figure 5). However, Figure 8 shows that their amplitude is comparable to the amplitude of the difference spectra (oxidized – reduced form) measured for related proteins such as plastocyanine [55] and halocyanine [57]. The spectrum for plastocyanine was scanned from Figure 5 of Ref. [55] and the spectrum for halocyanine was scanned from Figure 4 of Ref. [57]. All three spectra display similar changes of the amide I, amide II and side chains bands. These changes reflect small differences between secondary structure of the enzyme in the oxidized and reduced states. The positive bands in differential spectra indicate a shift towards higher wave numbers in the oxidation state of the protein. Such small shift may have several origins; (i) elongation of the Cu-oxygen distance in the oxidation state [57], (ii) changes in local pH (a flux of OH^- -product of oxygen reduction may increase local pH at negative potentials), (iii) environmental effects due to change in protein hydration and the nature of surrounding ions [55]. In Refs. [55] and [57], the proteins were in a free state in the bulk of solution and their redox state was changed using redox mediators. In the present work the protein was surface immobilized and the redox state was changed by direct electron transfer to the electrode. The similarity of the differential spectra reported in this work and in Refs. [55] and [57] despite different environment is significant. It confirms that the protein immobilized at the electrode surface retains its native state.

Figure 8. Oxidized-minus-reduced FTIR difference spectra of halocyanin (ii, blue) [57] and plastocyanin (iii, black) [55] in D₂O. The red line is the average potential difference spectra of laccase in phosphate buffer in D₂O (i). The positive going band is indicated as black, and the negative going band is marked as purple color.

The potential difference spectra in Figure 6 could be used to calculate two-dimensional correlation spectra (2D COS). They were constructed to analyze changes in the Amide I and Amide II spectral regions. The 2D COS analysis calculates correlation function from the potential difference spectra. It gives synchronous and asynchronous correlations of the spectral changes caused by changing electric field. In addition, it provides information about sequential changes of the bands [58, 59]. Figure 9 plots the 2D COS spectra calculated from the potential difference spectra in Figure 6. Figure 9A shows synchronous and Figure 9B asynchronous spectra. Red color marks positive and blue color negative bands. In synchronous spectrum the autocorrelation bands are located on the diagonal of the plot. They are always positive. The cross correlation bands are located off diagonal. Positive sign (red color) of these bands indicates that the bands change in the same direction. Negative sign (blue color) indicates that they change in the opposite direction. The synchronous spectra could be used as an additional tool to identify bands hidden in a broad spectral envelope of overlapping vibrations. The bands positions identified using 2D COS are listed in Table 2.

Figure 9 2D-COS of laccase in phosphate buffer in D₂O at pH=5.4 (the potential difference spectra (Figure6) were used as the input file). (A) synchronous spectrum, and (B) asynchronous spectrum.

The asynchronous spectra have no autocorrelation bands. They are weaker than synchronous spectra indicating that the potential induced changes are to a large degree correlated. The (ν_1, ν_2) bands are positive (red color) if a change at wavenumber ν_1 takes place before the change at wavenumber ν_2 . The bands are negative (blue color) if the change at ν_2

precedes the change at ν_1 . Table 2 lists signs of the bands in the synchronous and asynchronous 2D COS.

Table 2. Signs of bands in the synchronous and asynchronous 2D COS analysis.

ν_1	1659		1633		1616		1566	
ν_2	asyn	asyn	syn	asyn	syn	asyn	syn	asyn
1659			+	-	-	+	-	+
1633	+	+			-	+	-	+
1616	-	-	-	-			+	-
1566	-	-	-	-	+	+		

The 2D COS spectra show poor correlation of the $\sim 1690\text{ cm}^{-1}$ band (side chain) with α -helix (1650 cm^{-1}) and β -strand bands (1634 and 1616 cm^{-1}) bands. In contrast it shows a strong correlation between 1510 and 1566 cm^{-1} bands corresponding to the side chains vibrations, confirming that the assignment was correct. The most interesting result of this analysis is the positive cross correlation between α -helix and β -strand bands ($1656, 1616$) and ($1656, 1634$) in the asynchronous spectrum. It indicates that the potential induced changes of the α -helix take place before changes of the β -strand. Indeed, crystallographic data show that α -helices are located close to the T1 and T2/3 active sites. Consequently a change of the oxidation state of Cu is expected to affect α -helices first.

4. Conclusions

The conformational changes of laccase immobilised on SWCNTs modified with naphthalene moieties were investigated using Surface-Enhanced Infrared Absorption Spectroscopy coupled with electrochemical techniques. It has been shown that gold electrodes covered with modified carbon nanotubes have completely different properties than for enzymes deposited on SAMs reported in the literature. The present study has proven that carbon nanotubes provide favorable environment for the immobilization of enzymes. SEIRAS spectra of the Amide I band demonstrated that laccase retains its native state both in oxidized and reduced states. The structural differences between the two redox states are small and for

the CNT immobilized protein are similar to changes observed for related blue-copper proteins in the bulk of solution. The significant conclusion of this study is that immobilization of laccase on SWCNTs modified with naphthalene moieties does not affect its three dimensional structure and hence its electrocatalytic activity

Acknowledgement

This work was supported by a grant from Natural Sciences and Engineering Research Council of Canada (NSERC) to JL (RG-03958).

References

- [1] L.C. Almeida, R.D. Correia, G. Squillaci, A. Morana, F. La Cara, J.P. Correia, A.S. Viana, Electrochemical deposition of bio-inspired laccase-polydopamine films for phenolic sensors, *Electrochim. Acta* 319 (2019) 462-471.
- [2] L. Zhang, H. Cui, Z. Zou, T.M. Garakani, C. Novoa-Henriquez, B. Jooyeh, U. Schwaneberg, Directed Evolution of a Bacterial Laccase (CueO) for Enzymatic Biofuel Cells, *Angew. Chem. Int. Ed.* 58(14) (2019) 4562-4565.
- [3] Y. Liang, R. Cai, D.P. Hickey, J.P. Kitt, J.M. Harris, S.D. Minter, C. Korzeniewski, Infrared Microscopy as a Probe of Composition within a Model Biofuel Cell Electrode Prepared from *Trametes versicolor* Laccase, *ChemElectroChem* 6(3) (2019) 818-826.
- [4] Q. Fu, A. Sutherland, E. Gustafsson, M.M. Ali, L. Soleymani, R. Pelton, Relating Redox Properties of Polyvinylamine-g-TEMPO/Laccase Hydrogel Complexes to Cellulose Oxidation, *Langmuir* 33(32) (2017) 7854-7861.
- [5] L. Liu, S. Anwar, H. Ding, M. Xu, Q. Yin, Y. Xiao, X. Yang, M. Yan, H. Bi, Electrochemical sensor based on F,N-doped carbon dots decorated laccase for detection of catechol, *J. Electroanal. Chem.* 840 (2019) 84-92.
- [6] S. Mukerjee, J. Ziegelbauer, T.M. Arruda, K. Artyushkova, P. Atanassov, In Situ X - Ray Spectroscopy of Enzymatic Catalysis: Laccase - Catalyzed Oxygen Reduction, in: H.R. Luckarift, P. Atanassov, G.R. Johnson (Eds.), *Enzymatic Fuel Cells: From Fundamentals to Applications*, John Wiley & Sons 2014, pp. 304-336.
- [7] J. Liu, Y. Xie, C. Peng, G. Yu, J. Zhou, Molecular Understanding of Laccase Adsorption on Charged Self-Assembled Monolayers, *J. Phys. Chem. B* 121(47) (2017) 10610-10617.
- [8] O. Aleksejeva, I. Mateljak, R. Ludwig, M. Alcalde, S. Shleev, Electrochemistry of a high redox potential laccase obtained by computer-guided mutagenesis combined with directed evolution, *Electrochem. Commun.* 106 (2019).
- [9] Z. Han, L. Zhao, P. Yu, J. Chen, F. Wu, L. Mao, Comparative investigation of small laccase immobilized on carbon nanomaterials for direct bioelectrocatalysis of oxygen reduction, *Electrochem. Commun.* 101 (2019) 82-87.
- [10] G. Hong, D.M. Ivnitski, G.R. Johnson, P. Atanassov, R. Pachter, Design parameters for tuning the type 1 Cu multicopper oxidase redox potential: insight from a combination of first principles and empirical molecular dynamics simulations, *J. Am. Chem. Soc.* 133(13) (2011) 4802-4809.
- [11] N.S. Parimi, Y. Umasankar, P. Atanassov, R.P. Ramasamy, Kinetic and mechanistic parameters of laccase catalyzed direct electrochemical oxygen reduction reaction, *ACS Catal.* 2(1) (2011) 38-44.
- [12] F.A. Armstrong, N.L. Barlow, P.L. Burn, K.R. Hoke, L.J. Jeuken, C. Shenton, G.R. Webster, Fast, long-range electron-transfer reactions of a 'blue' copper protein coupled non-covalently to an electrode through a stilbenyl thiolate monolayer, *Chem. Commun.* (3) (2004) 316-317.

- [13] A.J. Augustine, M.E. Kragh, R. Sarangi, S. Fujii, B.D. Liboiron, C.S. Stoj, D.J. Kosman, K.O. Hodgson, B. Hedman, E.I. Solomon, Spectroscopic studies of perturbed T1 Cu sites in the multicopper oxidases *Saccharomyces cerevisiae* Fet3p and *Rhus vernicifera* laccase: allosteric coupling between the T1 and trinuclear Cu sites, *Biochemistry* 47(7) (2008) 2036-2045.
- [14] A.V. Lyashenko, I. Bento, V.N. Zaitsev, N.E. Zhukhlistova, Y.N. Zhukova, A.G. Gabdoulkhakov, E.Y. Morgunova, W. Voelter, G.S. Kachalova, E.V. Stepanova, X-ray structural studies of the fungal laccase from *Cerrena maxima*, *J. Biol. Inorg. Chem.* 11(8) (2006) 963-973.
- [15] D.W. Randall, S.D. George, B. Hedman, K.O. Hodgson, K. Fujisawa, E.I. Solomon, Spectroscopic and Electronic Structural Studies of Blue Copper Model Complexes. 1. Perturbation of the Thiolate-Cu Bond, *J. Am. Chem. Soc.* 122(47) (2000) 11620-11631.
- [16] D.W. Randall, S.D. George, P.L. Holland, B. Hedman, K.O. Hodgson, W.B. Tolman, E.I. Solomon, Spectroscopic and Electronic Structural Studies of Blue Copper Model Complexes. 2. Comparison of Three- and Four-Coordinate Cu (II)-Thiolate Complexes and Fungal Laccase, *J. Am. Chem. Soc.* 122(47) (2000) 11632-11648.
- [17] M. Klis, E. Maicka, A. Michota, J. Bukowska, S. Sek, J. Rogalski, R. Bilewicz, Electroreduction of laccase covalently bound to organothiol monolayers on gold electrodes, *Electrochim. Acta* 52(18) (2007) 5591-5598.
- [18] C. Nowak, D. Schach, J. Gebert, M. Grosserueschkamp, R.B. Gennis, S. Ferguson-Miller, W. Knoll, D. Walz, R.L. Naumann, Oriented immobilization and electron transfer to the cytochrome c oxidase, *J. Solid State Chem.* 15(1) (2011) 105-114.
- [19] C. Vaz-Domínguez, M. Pita, A.L. de Lacey, S. Shleev, A. Cuesta, Combined ATR-SEIRAS and EC-STM study of the immobilization of laccase on chemically modified Au electrodes, *J. Phys. Chem. C* 116(31) (2012) 16532-16540.
- [20] A. Ciaccafava, P. Infossi, M. Ilbert, M. Guiral, S. Lecomte, M.T. Giudici - Orticoni, E. Lojou, Electrochemistry, AFM, and PM - IRRA spectroscopy of immobilized hydrogenase: role of a hydrophobic helix in enzyme orientation for efficient H₂ oxidation, *Angew. Chem. Int. Ed.* 51(4) (2012) 953-956.
- [21] Y. Ulyanova, S. Babanova, E. Pinchon, I. Matanovic, S. Singhal, P. Atanassov, Effect of enzymatic orientation through the use of syringaldazine molecules on multiple multi-copper oxidase enzymes, *Phys. Chem. Chem. Phys.* 16(26) (2014) 13367-13375.
- [22] C.F. Blanford, R.S. Heath, F.A. Armstrong, A stable electrode for high-potential, electrocatalytic O₂ reduction based on rational attachment of a blue copper oxidase to a graphite surface, *Chem. Commun.* (17) (2007) 1710-1712.
- [23] C. Lau, E.R. Adkins, R.P. Ramasamy, H.R. Luckarift, G.R. Johnson, P. Atanassov, Design of carbon nanotube - based gas - diffusion cathode for O₂ reduction by multicopper oxidases, *Adv. Energy Mater.* 2(1) (2012) 162-168.
- [24] S.O. Garcia, C.N. Villarrubia, A. Falase, P. Atanassov, Gas-diffusion cathodes integrating carbon nanotube modified-Toray paper and bilirubin oxidase, *J. Electrochem. Soc.* 161(9) (2014) H523-H528.
- [25] K.G. Arzola, Y. Gimeno, M. Arevalo, M. Falcon, A.H. Creus, Electrochemical and AFM characterization on gold and carbon electrodes of a high redox potential laccase from *Fusarium proliferatum*, *Bioelectrochemistry* 79(1) (2010) 17-24.
- [26] C.F. Blanford, C.E. Foster, R.S. Heath, F.A. Armstrong, Efficient electrocatalytic oxygen reduction by the 'blue' copper oxidase, laccase, directly attached to chemically modified carbons, *Faraday Discuss.* 140 (2008) 319-335.
- [27] J. Biener, M. Stadermann, M. Suss, M.A. Worsley, M.M. Biener, K.A. Rose, T.F. Baumann, Advanced carbon aerogels for energy applications, *Energy Environ. Sci.* 4(3) (2011) 656-667.
- [28] K. Stolarczyk, E. Nazaruk, J. Rogalski, R. Bilewicz, Nanostructured carbon electrodes for laccase-catalyzed oxygen reduction without added mediators, *Electrochim. Acta* 53(11) (2008) 3983-3990.
- [29] G. Gupta, V. Rajendran, P. Atanassov, Laccase biosensor on monolayer - modified gold electrode, *Electroanal.* 15(20) (2003) 1577-1583.
- [30] G. Gupta, V. Rajendran, P. Atanassov, Bioelectrocatalysis of oxygen reduction reaction by laccase

on gold electrodes, *Electroanal.* 16(13 - 14) (2004) 1182-1185.

[31] P. Olejnik, A. Pawłowska, B. Pałys, Application of Polarization Modulated Infrared Reflection Absorption Spectroscopy for electrocatalytic activity studies of laccase adsorbed on modified gold electrodes, *Electrochim. Acta* 110 (2013) 105-111.

[32] M. Karaskiewicz, D. Majdecka, A. Wieckowska, J.F. Biernat, J. Rogalski, R. Bilewicz, Induced-fit binding of laccase to gold and carbon electrodes for the biological fuel cell applications, *Electrochim. Acta* 126 (2014) 132-138.

[33] M. Pita, S. Shleev, T. Ruzgas, V.M. Fernández, A.I. Yaropolov, L. Gorton, Direct heterogeneous electron transfer reactions of fungal laccases at bare and thiol-modified gold electrodes, *Electrochem. Commun.* 8(5) (2006) 747-753.

[34] M. Pita, C. Gutierrez-Sanchez, D. Olea, M. Velez, C. Garcia-Diego, S. Shleev, V.M. Fernandez, A.L. De Lacey, High redox potential cathode based on laccase covalently attached to gold electrode, *J. Phys. Chem. C* 115(27) (2011) 13420-13428.

[35] P. Olejnik, B. Pałys, A. Kowalczyk, A.M. Nowicka, Orientation of laccase on charged surfaces. Mediatorless oxygen reduction on amino-and carboxyl-ended ethylphenyl groups, *J. Phys. Chem. C* 116(49) (2012) 25911-25918.

[36] A. Barth, Infrared spectroscopy of proteins, *Biochim. Biophys. Acta* 1767(9) (2007) 1073-1101.

[37] F.-N. Fu, D.B. Deoliveira, W.R. Trumble, H.K. Sarkar, B.R. Singh, Secondary structure estimation of proteins using the amide III region of Fourier transform infrared spectroscopy: application to analyze calcium-binding-induced structural changes in calsequestrin, *Appl. Spectrosc.* 48(11) (1994) 1432-1441.

[38] K.A. Oberg, J.M. Ruysschaert, E. Goormaghtigh, The optimization of protein secondary structure determination with infrared and circular dichroism spectra, *Eur. J Biochem.* 271(14) (2004) 2937-2948.

[39] S. Englander, N. Downer, H. Teitelbaum, Hydrogen exchange, *Annu. Rev. Biochem.* 41(1) (1972) 903-924.

[40] S. Cai, B.R. Singh, Identification of β -turn and random coil amide III infrared bands for secondary structure estimation of proteins, *Biophys. Chem.* 80(1) (1999) 7-20.

[41] J. Kong, S. Yu, Fourier transform infrared spectroscopic analysis of protein secondary structures, *Acta Biochim. Biophys. Sin.* 39(8) (2007) 549-559.

[42] Z. Ganim, H.S. Chung, A.W. Smith, L.P. DeFlores, K.C. Jones, A. Tokmakoff, Amide I two-dimensional infrared spectroscopy of proteins, *Acc. Chem. Res.* 41(3) (2008) 432-441.

[43] J.J. Leitch, C.L. Brosseau, S.G. Roscoe, K. Bessonov, J.R. Dutcher, J. Lipkowski, Electrochemical and PM-IRRAS Characterization of Cholera Toxin Binding at a Model Biological Membrane, *Langmuir* 29(3) (2013) 965-976.

[44] V. Climent, Y. Fu, S. Chumillas, B. Maestro, J.-F. Li, A. Kuzume, S. Keller, T. Wandlowski, Probing the electrocatalytic oxygen reduction reaction reactivity of immobilized multicopper oxidase cueO, *J. Phys. Chem. C* 118(29) (2014) 15754-15765.

[45] M. Osawa, Dynamic processes in electrochemical reactions studied by surface-enhanced infrared absorption spectroscopy (SEIRAS), *Bull. Chem. Soc. Jpn.* 70(12) (1997) 2861-2880.

[46] M. Grossutti, J.J. Leitch, R. Seenath, M. Karaskiewicz, J. Lipkowski, SEIRAS studies of water structure in a sodium dodecyl sulfate film adsorbed at a gold electrode surface, *Langmuir* 31(15) (2015) 4411-4418.

[47] H. Miyake, S. Ye, M. Osawa, Electroless deposition of gold thin films on silicon for surface-enhanced infrared spectroelectrochemistry, *Electrochem. Commun.* 4(12) (2002) 973-977.

[48] V. Zamlynny, J. Lipkowski, Quantitative SNIFTIRS and PM IRRAS of organic molecules at electrode surfaces, in: R. Alkire, D.M. Kolb, L. J. (Eds.), *Advances in Electrochemical Science and Engineering: Diffraction and Spectroscopic Methods in Electrochemistry*, Wiley-VCH, Weinheim, 2006, pp. 315-376.

[49] E. Nazaruk, M. Karaskiewicz, K. Żelechowska, J.F. Biernat, J. Rogalski, R. Bilewicz, Powerful connection of laccase and carbon nanotubes: Material for mediator-free electron transport on the enzymatic cathode of the biobattery, *Electrochem. Commun.* 14(1) (2012) 67-70.

- [50] J. Rogalski, G. Janusz, Purification of extracellular laccase from *Cerrena unicolor*, *Prep. Biochem. Biotech.* 40(4) (2010) 242-255.
- [51] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72(1-2) (1976) 248-254.
- [52] S. Ragusa, M.T. Cambria, F. Pierfederici, A. Scirè, E. Bertoli, F. Tanfani, A. Cambria, Structure–activity relationship on fungal laccase from *Rigidoporus lignosus*: a Fourier-transform infrared spectroscopic study, *Biochim. Biophys. Acta* 1601(2) (2002) 155-162.
- [53] W.K. Surewicz, H.H. Mantsch, New insight into protein secondary structure from resolution-enhanced infrared spectra, *Biochim. Biophys. Acta* 952 (1988) 115-130.
- [54] Y.N. Chirgadze, O. Fedorov, N. Trushina, Estimation of amino acid residue side - chain absorption in the infrared spectra of protein solutions in heavy water, *Biopolymers* 14(4) (1975) 679-694.
- [55] S.G. Taneva, U. Kaiser, A.A. Donchev, M.I. Dimitrov, W. Mäntele, A. Muga, Redox-induced conformational changes in plastocyanin: an infrared study, *Biochemistry* 38(30) (1999) 9640-9647.
- [56] B. Endrodi, A. Kormanyos, C. Janaky, O. Berkesi, C. Visy, Fixation of laccase enzyme into polypyrrole, assisted by chemical interaction with modified magnetite nanoparticles: A facile route to synthesize stable electroactive bionanocomposite catalysts, *Electrochim. Acta* 122 (2014) 282-288.
- [57] M. Brischwein, B. Scharf, M. Engelhard, W. Maentele, Analysis of the redox reaction of an archaeobacterial copper protein, halocyanin, by electrochemistry and FTIR difference spectroscopy, *Biochemistry* 32(49) (1993) 13710-13717.
- [58] I. Noda, A. Dowrey, C. Marcott, G. Story, Y. Ozaki, Generalized two-dimensional correlation spectroscopy, *Appl. Spectrosc.* 54(7) (2000) 236A-248A.
- [59] I. Noda, Two-dimensional codistribution spectroscopy to determine the sequential order of distributed presence of species, *J. Mol. Struct.* 1069 (2014) 50-59.

Graphical Abstract