Electrochimica Acta 110 (2013) 105–111

Contents lists available at ScienceDirect

Electrochimica Acta

journal homepage: www.elsevier.com/locate/electacta

# Application of Polarization Modulated Infrared Reflection Absorption Spectroscopy for electrocatalytic activity studies of laccase adsorbed on modified gold electrodes

Piotr Olejnik, Aleksandra Pawłowska [1], Barbara Pałys [*,2]

*University of Warsaw, Pasteur Street 1, 02-093 Warsaw, Poland*

ARTICLE INFO

*Article history:*
Received 30 November 2012
Received in revised form 7 March 2013
Accepted 10 March 2013
Available online 21 March 2013

*Keywords:*
Enzyme
Characterization
Adsorption
Oxygen electro-reduction
Biofuel cells

ABSTRACT

Orientation of the enzyme macromolecule on the electrode surface is crucially important for the efficiency of the electron transport between the active site and electrode surface. The orientation can be controlled by affecting the surface charge and the pH of the buffer solution. In this contribution we study laccase physically adsorbed on gold surface modified by mercapto-ethanol, lipid and variously charged diazonium salts.

Polarization Modulated Infrared Reflection Absorption Spectroscopy (PMIRRAS) enables the molecular orientation study of the protein molecule by comparison of the amide I to amide II band intensity ratios assuming that the protein secondary structure does not change. We observe significant differences in the intensity ratios depending on the kind of support and the enzyme deposition. The comparison of infrared spectra and cyclic voltammetry responses of variously prepared laccase layers reveals that the parallel orientation of beta-sheet moieties results in high enzyme activity.



## 1. Introduction

Enzymes immobilized on solid supports are basic elements of electrochemical sensors [1,2] and biofuel cell electrodes [3,4]. Laccase is applied for both oxygen sensing [5–7] and biofuel cell applications [8,9]. Laccase belongs to multicopper oxidases [10–12]. It catalyze reduction of molecular oxygen directly to water without formation of reactive oxygen intermediates. The active site of laccase contains four copper atoms classified in accordance with their spectroscopic characteristics as T1 (responsible for the blue color of laccase), T2 and T3 sites. The T1 site of the enzyme is responsible for binding and oxidation of the substrate. Typical laccase substrates are aromatic phenols or amines being the electron source for the oxygen reduction. The electrode may serve as the electron donor as well. The T2 and T3 sites are involved in the transport of the electron and binding molecular oxygen causing its reduction to two molecules of water.

The influences of the support on the enzyme activity have been reported for several enzymes. Laccase is an example of such influence, since the enzyme adsorbed on graphite and boron doped diamond electrodes catalyses direct reduction of molecular oxygen to water at high potential values [13–15]. Adsorption of laccase on the highly ordered pyrolytic graphite (HOPG) leads to diminished activity due to the enzyme aggregation [16]. The laccase electroactivity on the bare gold surface is worse than on carbon electrodes. The partial denaturation of the enzyme has been even suggested [17,18]. The disadvantageous influence of the enzyme–gold interaction might change the mechanism of the electrocatalytic reaction, for example $H_2O_2$ was detected as the by-product of the electrocatalytic reduction of molecular oxygen [17,18]. Modification of the gold surface with a thiol monolayer prevented the $H_2O_2$ formation. Laccase adsorbed on p-aminothiophenol modified gold surface revealed the direct electrocatalysis at higher potentials comparing to bare gold, although the catalytic performance was diminished comparing to carbon supports [17,19]. Alternative for thiols are layers formed by the electrochemical reduction of phenyldiazonium salts [20–22]. The reduction of diazonium salts gives aromatic moieties bond directly to the electrode surface without the sulfur bridge. Conductivity of such layers is presumably higher comparing to thiol-linked layers. Pita et al. presented recently mixed amino-phenyl–mercapto-hexanol films as support for laccase covalent binding [23]. Laccase revealed direct electrocatalytic oxygen currents at potential values close to the T1 redox potential with moderate current values. Similar potential values with slightly higher electrocatalytic currents were observed for laccase bond to amino-ethyl-phenyl (AEP) layers and

* Corresponding author. Tel.: +48 22 8220211; fax: +48 22 8225996.
*E-mail addresses:* polejnik@chem.uw.edu.pl (P. Olejnik), alekspawlows@gmail.com (A. Pawłowska), bpalys@chem.uw.edu.pl, bjpalys@gmail.com (B. Pałys).
[1] Present address: Institute of Physical Chemistry, Polish Academy of Sciences, Kasprzaka 44/52, 01-224 Warsaw, Poland. Tel.: +48 22 8220211.
[2] ISE member.

0013-4686/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.electacta.2013.03.089

carboxyl-ethyl-phenyl (CEP) on gold – without addition of a thiol [24].

The diazonium chemistry has been used recently for modification of the gold nanoparticles with aminophenyl functional groups [25]. The gold nanoparticles were immobilized on the low density graphite electrodes. Such nanogold–graphite electrodes were used as a support for covalent binding of the *Trametes hirstuta* laccase. Very high electrocatalytic currents were obtained at the potential range close to the redox potential of the laccase T1 site. Furthermore, the bioelectrocatalytic properties of the immobilized enzyme were affected by size of the gold nanoparlices.

Although, the protein denaturation in contact with gold surface is often suggested, the knowledge about the structural changes upon adsorption and the mechanism of the possible denaturation remains unexplained. Studies of Bovine Albumin Serum – the model protein, reveal that the protein–gold interaction has weaker impact on the protein structure than the adsorption on a polymer surface [26]. Adsorption of another model compound lysozyme on gold nanorods leads to highly stable system – even under denaturing conditions [27], proving that the gold–protein interactions still requires thorough studies.

Besides the interaction with the surface the orientation of the enzyme macromolecule on the electrode surfaces influences the electrocatalytic performance. Orientation of the laccase molecule with the T1 center toward the electrode enables the direct electron transfer (DET) between the enzyme and the electrode and the electrocatalytic oxygen reduction at high potential values corresponding the T1 redox potential. The orientation with the T2 center toward the electrode surface enables the DET between the T2 center and the electrode. In such case the direct electrocatalysis occurs at the potential value related to the T2 center, which is approximately 0.2 V more negative than the T1 potential. The direct electrocatalysis involving the T2 site might also lead to the $H_2O_2$ formation [17,18]. The effect of the orientation on DET has been also reported for hydrogenase [28].

The enzyme structure can be stabilized by the interaction with phospholipid films, as it was demonstrated for catalase and horseradish peroxidase incorporated in DPPG films. The activity of both enzymes in DPPG films was higher by up to 23% comparing to the enzyme solution [29,30]. Lipid mono- or bilayers deposited on the electrode surface mimic the cell membrane [31,32]. Studies of proteins in such model membrane systems visualize the influence of the protein–lipid interactions on the structure of lipid bilayers [31,33,34]. The lipid–protein interactions might influence the activity of enzymes incorporated in lipid layers. Laccase and tyrosinase were immobilized in amphiphilic polymer layers for the construction of the colorimetric sensor of phenol compounds [35,36].

Infrared spectroscopy is valuable probe of the structure and orientation of proteins at interfaces. The peptide bond motions – amide I and amide II bands are sensitive to the secondary and the tertiary structure of a protein [26–37]. The deconvolution of the amide I bond is carried to investigate the protein structure and stability [38,39]. The shift or re-shaping of the amide I band suggests the changes in the secondary structure of the protein backbone. The amide bands of the fungal laccase from *Rigidoporus lignosus* have been studied for the native and the metal depleted enzyme [40]. The infrared spectra revealed small content of α-helices and the prevailing amount of β-sheets, staying in agreement with crystallographic data available for other laccases [41,42].

Among infrared experimental techniques the attenuated total reflectance (ATR) [43,44] or PMIRRAS are most commonly used to investigate the structure of bio-molecules immobilized on the electrode surface [31,45,46]. PMIRRAS enables also studies of the molecules on the air–water interface [29,38,47]. Due to selection rules for specular infrared reflectance only motions perpendicular to the gold surface contribute to the spectrum [48]. For this reason

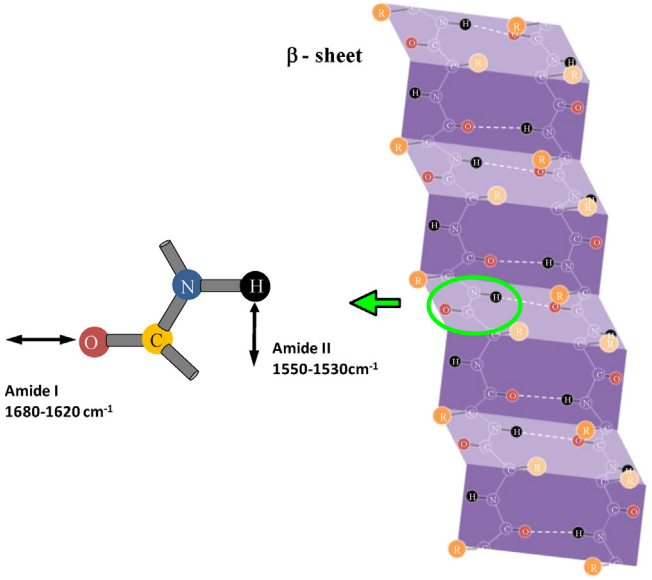


**Fig. 1.** Directions of amide I and amide II motions and the position of the peptide bond within the β sheet.

infrared reflectance spectra are a straightforward tool to evaluate the orientation of molecules deposited on gold surfaces. The PMIRRAS technique is the improvement of the specular infrared reflectance, where the polarization modulation is applied to maximize the signal to noise ratio [31]. The selection rules for PMIRRAS and the specular reflectance are identical.

As the peptide bond motions, the amide I and amide II bands are strong signals in infrared spectra of all peptides and proteins. Amide I corresponds to the carbonyl stretching motion, while the amide II mode involves mainly the bending motion of the N—H bond. Since the two motions are perpendicular to each other (Fig. 1), the intensity ratio between the amide I and amide II bands depends on the orientation of the peptide bond with respect to the surface. Assuming that the secondary protein structure is not affected by the interaction with the surface, the amide I to amide II intensity ratio is sensitive to the orientation of the peptide chain on the surface.

For single peptides the tilt angle with respect to a surface normal can be evaluated by comparison of the intensity ratio of the amide I to amide II band in the reflectance spectrum of the monolayer with the amide I to amide II intensities ratios for the randomly oriented sample [49–51]. The spectra of randomly oriented molecules can be measured in KBr pellet. The tilt angle values obtained from infrared spectra stay in agreement with those evaluated using AFM based thickness measurements [52]. For larger biological molecules analysis of orientation is more complicated though even qualitative results might be helpful for elucidating complex mechanisms of bioelectrocatalytic reactions.

The ATR redox difference spectroscopy probes the protein spectrum at various redox states, when the protein is not directly bond to the electrode surface [53,54]. The redox state of the protein is modulated by the redox mediator oxidized or reduced at the electrode surface. In consequence, the thick protein film (few micrometers range) might be investigated. The ATR redox difference studies have been carried out for CueO and bilirubin oxidase [54]. These enzymes belong to the multi-copper oxidases having the four copper active site structure similar to laccase. The studies revealed that the oxidation of the enzymes influence positions of the amide I band indicating structural changes of the protein backbone. The oxidation of the enzyme was accompanied by the deprotonation of the glutamine residue (Glu506 of CueO