Direct Electron Transfer of *Trametes hirsuta* laccase adsorbed at unmodified nanoporous gold electrodes

Urszula Salaj-Kosla[a,b], Sascha Pöller[b], Wolfgang Schuhmann[b], Sergey Shleev[c], Edmond Magner[a,*]

[a] Department of Chemical and Environmental Sciences & Materials and Surface Science Institute, University of Limerick, Limerick, Ireland
[b] Analytische Chemie – Elektroanalytik & Sensorik, Ruhr-Universität Bochum, Universitätsstr. 150, D-44780 Bochum, Germany
[c] Biomedical Science, Faculty of Health and Society, Malmö University, Södra Förstadsgatan 101, SE-20506 Malmö, Sweden

*Corresponding author:
Email address: edmond.magner@ul.ie; tel.: +353 61 202628; fax: +353 61 213529

Keywords: nanoporous gold electrodes, *Trametes hirsuta* laccase, biocathode, bioelectrochemistry

Abstract

The enzyme *Trametes hirsuta* laccase undergoes direct electron transfer at unmodified nanoporous gold electrodes, displaying a current density of 28 $\mu A/cm^2$. The response indicates that *Th*Lc was immobilised at the surface of the nanopores in a manner which promoted direct electron transfer, in contrast to the absence of a response at unmodified polycrystalline gold electrodes. The bioelectrocatalytic activity of *Th*Lc modified nanoporous gold electrodes was strongly dependent on the presence of halide ions. Fluoride completely inhibited the enzymatic response, whereas in the presence of 150 mM Cl^- , the current was reduced to 50% of the response in the absence of Cl^-. The current increased by 40% when the temperature was increased from 20°C to 37°C. The response is limited by enzymatic and/or enzyme electrode kinetics and is 30% of that observed for *Th*Lc co-immobilised with an osmium redox polymer.
Keywords: Laccase, direct electron transfer, nanoporous gold

1. Introduction

Electron transfer (ET) reactions are ubiquitous in nature. Controlling the rate of these reactions can be of significant benefit in developing biochemical systems that utilise redox proteins and enzymes and in particular, in applications that provide power for implanted or portable electronic devices. ET can be achieved directly (direct electron transfer, DET) or by the use of mediators (mediated electron transfer, MET) to shuttle electrons between the redox centre of the enzyme and the electrode. However, mediators are unselective and can also give rise to interfering effects [1-3]. DET-based devices offer high selectivity and sensitivity due to the absence of mediators. In addition, devices based on DET operate at potentials close to the redox potential of the enzyme, maximising the potential difference between the cathode and anode for biofuel cell applications [2]. Moreover, DET can be used to provide detailed information on the kinetics and thermodynamics of the ET process. However, the low stability of the enzyme layer together with the long distances over which ET occurs, represent major obstacles for DET based devices. In addition, the shielding mechanism of the enzymatic redox centre by the protein shell can disrupt DET [2, 4]. One approach in optimizing and enabling rapid DET is to design the electrode surface with an architecture which promotes efficient rates of ET. In this way the electrode morphology ensures the most efficient orientation of the enzymatic redox centre and facilitates communication with the electroactive surface. Porous electrodes can be used to encapsulate the enzyme within a network of cavities, shortening the distance for electron transfer to the redox active site, which will promote more efficient and rapid rates of electron transfer [5]. However, the number of redox enzymes capable of interacting directly with the electrode while catalyzing the enzymatic reaction is limited, with estimates of approximately 5% of all enzymes exhibiting such a response [6].

DET in enzymes was first described in 1978 for laccase (Lc) [7-8]. Lc, together with ceruloplasmin, ascorbate oxidase, and bilirubin oxidase (BOx) belongs to the group of Cu-proteins which are also known as blue multicopper oxidases. More than 60 types of Lc have been isolated from various type of plants, fungi and bacteria sources with different thermodynamic and kinetic properties [9-10]. Lc contains four copper ions classified according to their spectral characteristics into three types, T1, T2 and T3. The T2 and T3 sites form a cluster. Both the T1 and T2 Cu species are EPR-active in contrast to the T3 centre which is EPR silent [11]. The role of each copper redox centre is related to the catalytic properties of the Lc, which catalyze the oxidation of a range of inorganic and organic compounds, mainly phenols with concomitant reduction of O_2 to H_2O. The T1 copper site acts as a primary electron acceptor, which then transfers electrons in an intramolecular ET mechanism (IET) through a His-Cys-His bridge to the T2/T3 sites, where O_2 is reduced to H_2O without the formation of highly reactive oxygen species [12-13].

Lc from different sources are often inhibited by halide ions [14] either *via* non-competitive or competitive inhibition mechanisms. The non-competitive inhibitor F^- does not bind to the T1 site, which accepts the electrons from the substrate, but to the T2/T3 trinuclear cluster. As a result, the electron transfer pathway from the T1 to the T2/T3 redox centres is blocked and the activity of the enzyme is inhibited at low concentrations of F^- [15]. In the competitive inhibition mechanism, Cl^- and I^- compete with electron donors for access to the T1 redox centre of the enzyme, reducing the observed activity. In this mechanism, in contrast

to the non-competitive inhibition by F^-, DET is not suppressed by restricted access to the T2/T3 catalytic site [16].

In spite of the large numbers of studies concerning the electrochemical properties of Lc on gold surfaces, the direct mediator-less bioelectrocatalytic reduction of oxygen on bare polycrystalline gold electrodes modified with Lc has not been described thus far. Usually, responses have been reported at low potentials which correspond to the adsorbed enzyme, with no catalytic response observed [17-19]. The low redox potential Faradaic processes (at *ca.* 0.4 V *vs.* NHE) detected for surface confined Lc on gold has been attributed to the redox transformation of copper ion(s) from the T2/T3 Cu cluster [19-23]. An explanation of the absence of bioelectrocatalytic activity has been not clearly identified. Several hypotheses have been proposed including the formation of catalytically inactive but electrochemically active forms of Lc arising from specific enzyme orientation at the electrode surface [19]. Denaturation (protein unfolding) of the enzymes on bare Au surface can also account for the observed effects. The bioelectrocatalytic reduction of O_2 by laccase on modified Au electrodes [24-25] including nanoparticle modified Au electrodes [26] has been described. The catalytic reduction of O_2 by laccase supported on a nanoporous gold film modified electrode has been described, however at low potentials of ca. 0.4 V vs. NHE [27]. Well-pronounced DET-based bioelectrocatalytic reduction of O_2 on bare and modified Au(111) single-crystal electrodes modified with immobilized Lc from different sources has been reported [25]. The voltammetric behaviour of surface-immobilized composite Lc is exceedingly sensitive to the general state of both the electrode surface and the enzymes.

In this study we describe the bioelectrocatalytic reduction of O_2 by a high redox potential fungal Lc from basidiomycete *Trametes hirsuta* (*Th*Lc), which has been confined in unmodified nanoporous gold electrodes. Mediated electron transfer of *Th*Lc with an Os-polymer and the effects of inhibition of the enzyme and its response at different temperatures are also presented.

2. Experimental section

2.1 Reagents

Sulfuric acid, citric acid, disodium phosphate, sodium fluoride, and sodium chloride were obtained from Sigma-Aldrich (Schnelldorf, Germany). All chemicals were of analytical grade and used as received without further purification. Buffers were prepared with ultrapure water (resistivity of 18.2 MΩ cm) from an Elgastat maxima-HPLC (Elga, UK). Aerobic and anaerobic conditions were established by bubbling O_2 and N_2 (BOC Gases, Dublin, Ireland), respectively, through the solutions for 20 min.

2.2 Enzyme

The preparation of homogeneous Lc from the basidiomycete *Trametes hirsuta* (*Th*Lc) was kindly provided by Prof. Alexander I. Yaropolov in the framework of the FP7 project NMP4-SL-2009-229255. The enzyme was produced and purified to homogeneity using published procedures [28-29].

2.3 Synthesis of polymers

2.3.1 Synthesis of copolymer P017-epoxy

Polymerisation was carried out by reacting polyethylene glycol methacrylate (395 mg, 0.75 mM), allyl methacrylate (631 mg, 5.0 mM), and butyl acrylate (545 mg, 4.25 mM) in methanol. Excess monomer was removed by precipitation from water. The final polymer suspension (P017; 4.7 % w/w) was obtained after the solid copolymer was re-dissolved in MeOH. The synthesis of dimethyldioxirane (DMDO) and epoxidation of P017 was performed according to a procedure described previously [30]. The dry polymer obtained was re-dissolved in MeOH (1.0 ml). Water was added drop-wise, until the suspension turned milky, to give the final polymer suspension (P017-epoxy).

2.3.2 Synthesis of P002-P91 polymer

Allyl methacrylate (15.0 mM, 1.89 g, 1.00 eq), 2-(dimethylamino)ethyl 2-methylacrylate (30.0 mM, 4.72 g, 2.00 eq) butyl acrylate (55.0 mM, 7.05 g, 3.66 eq), polymer P002 (100 mg) and Os-complex P91 (5.7 mg) were used to carry out the polymerisation of the P002-P91 polymer according to the procedure previously described [30].

2.4 Preparation of nanoporous gold electrodes

Nanoporous gold electrodes with a non-uniform Ag distribution were fabricated by co-sputtering of a silver: gold alloy. The silver component of the alloy was removed by heating in the presence of HNO_3 as described previously [31]. The electrodes were cleaned using the following procedure. The potential was scanned over the range 0 – 1.5 V at a scan rate of 200 mV/s in 0.5 M H_2SO_4 until well-defined voltammograms were obtained. The potential was then scanned between 0 and 0.8 V at a scan rate of 50 mV/s in 0.1 M citrate-phosphate buffer (pH 4.0) for 15 min. The nanoporous gold electrodes were then dried in a vacuum chamber for 10 min.

2.5 Modification of nanoporous gold electrodes

2.5.1 *Th*Lc based nanoporous gold for DET analysis

15 µl of 40 µg/ml *Th*Lc solution was placed on a clean, dry nanoporous gold electrode. The electrode was left in the vacuum chamber under pressure for *ca.* 3 min to allow the enzyme to adsorb. The *Th*Lc-modified nanoporous gold electrode was then removed from the chamber, covered with 10 µl of P017-epoxy solution and allowed to dry in air at room temperature for 20 min. A control electrode was prepared by placing a 15 µl aliquot of *Th*Lc solution (40 µg/ml) on the surface for 2 h at 4° C in the absence of a vacuum step.

2.5.2 *Th*Lc based nanoporous gold for MET analysis

30 µl of the mixture containing 40 µg/ml *Th*Lc and 230 µg/ml P002-P91 polymer in 1:1 *v*/*v* ratio was divided in two equal portions. The first portion was placed on a clean, dry nanoporous gold electrode. The electrode was then left in the vacuum chamber under reduced pressure for *ca.* 3 min to allow the enzyme to adsorb. The *Th*Lc-modified nanoporous gold was then removed from the chamber, covered with the second aliquot of the enzyme and polymer solution and allowed to dry in air at room temperature for 2 h.

2.6 Electrochemical measurements

Electrochemical measurements were performed with an CHI832 bipotentiostat (CHI Instruments, Austin, Texas, USA) using a three-electrode cell with Pt wire, Ag/AgCl (3M

KCl) and nanoporous gold as the counter, reference and working electrodes, respectively. The supporting electrolyte consisted of 0.1 M citrate-phosphate buffer solution at pH 4.0. The measurements were performed at 20° C and 37° C. Current densities were estimated using the geometric surface area of the working electrode. All potentials are reported *vs.* Ag/AgCl (3 M KCl).

3. Results and discussion

Bioelectrocatalytic currents of *ca.* 0.5 µA were obtained at *Th*Lc-modified electrodes prepared by placing a drop of enzyme solution onto the surface of vacuum dried nanoporous electrodes (**Fig 1A**). On changing the immobilization conditions to vacuum deposition of the *Th*Lc solution on a bare, vacuum dried nanoporous gold electrode, followed by covering the electrode with a P017-epoxy cap, significantly higher currents of 7 µA (**Fig 1B**) were obtained. As reported previously, the linkages formed by chemisorption of the NH_2 groups of the lysine residues of *Th*Lc on nanoporous gold are as strong as those for –SH groups on gold [32]. However, a P017-epoxy cap was also applied [33] to prevent any possible leaching of the enzyme from the gold surface. The P017-epoxy stabilises the adsorbed enzyme by creating covalent bonds with amine, thiol, or hydroxyl nucleophilic groups on the surface of the enzyme and enables the enzyme to be retained in the pores [34]. Using a similar immobilisation method for the adsorption of BOx resulted in an O_2-sensitive BOx biocathode with a biocatalytic current density of *ca.* 200 µA (on a 0.246 cm^2 electrode) [33]. The response of this electrode was limited by diffusion of O_2. On replacing BOx with Lc, the faradaic response was reduced by a factor of *ca.* 30, indicating that diffusion of substrate can not be a limiting factor. Note that no catalytic response was observed at a laccase modified planar gold electrode, indicating that the orientation of the enzyme on the electrode was unsuitable for efficient rates of DET and/or the surface loading of enzyme was too low to provide a measurable faradaic response.

Here Figure 1

In an O_2 saturated solution, the onset potential of *ca.* 650 mV for the biocatalytic reduction of O_2 at *Th*Lc-modified nanoporous gold electrode (**Fig. 1B**) is in good agreement with the redox potential of the T1 site of the enzyme [22]. Such a well-defined biocatalytic response for *Th*Lc is in contrast to the complete absence of this process on Lc-modified planar polycrystalline Au electrodes [35]. Preferential orientation of Lc in the nanocavities of the electrode appears to enable efficient electron transfer *via* the T1 redox site of the enzyme [36]. The lower catalytic current observed in air arises from the lower concentrations of O_2 in solution (**Fig. 1B**), while *Th*Lc biocathodes did not demonstrate a catalytic response in N_2 saturated solution (**Fig. 1A** and **B**).

Here Figure 2

Although the mechanism of inhibition is different, halide ions influence both DET and MET reactions in the biocatalytic response of Lc. Fluoride acts as a non-competitive inhibitor

by binding to the T2/T3 copper cluster, blocking the ET pathway from the T1 site to the T2/T3 cluster [15-16, 37-38]. This is confirmed by the complete removal of the biocatalytic activity of *Th*Lc-modified nanoporous gold electrodes at a low concentration (2 mM) of F^- (**Fig. 2A**). Contrary to the inhibition by F^-, Cl^- and I^- act as competitive inhibitors interacting with the T1 Cu site of the enzyme [39]. Usually, in the presence of the bulkier Cl^-, the access of redox mediators to the T1 site is blocked and bioelectrocatalytic responses from Lc-modified electrodes are suppressed [16]. However, as previously reported, Cl^- does not suppress the bioelectrocatalytic reduction of O_2 in certain cases, *e.g.*, for DET-based biodevices with appropriately oriented Lc on the electrode surface [26] and for MET-based bioelectrodes where the enzyme is incorporated into redox hydrogels [40]. In this study, the addition of Cl^- causes a reduction in the bioelectrocatalytic current. Although DET between *Th*Lc and the surface of the unmodified nanoporous gold electrode takes place, the biocatalytic response of *Th*Lc biocathode decreased by *ca.* 50% in the presence of 150 mM NaCl (**Fig. 2B**).

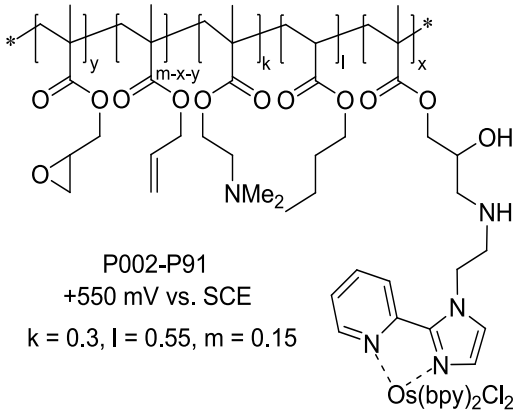
Here Figure 3

The temperature dependence of the catalytic reduction of O_2 at 20°C and 37°C at *Th*Lc-modified nanoporous gold electrode was investigated (**Fig. 3**). In both cases a bioelectrocatalytic response was observed, however, a higher (*ca.* 40%) current at 0.2 V was obtained at 37°C. These results are in agreement with previous reports where the maximal bioelectrocatalytic activity of Lc from different sources was observed in the temperature range 35 – 50°C [41-44]. While the solubility of O_2 decreases with increasing temperature, the increase in the diffusion coefficient offsets this decrease, resulting in an increased response [37, 45-46] which is in agreement with the results reported for homogeneous *Th*Lc catalysis which has a temperature optimum of 45°C [47]. A small peak was observed at 0.7 V. The origin of the peak is unclear, it is not related to temperature effects at 45°C as it has been observed previously at room temperature [22, 35].

Here Figure 4

Efficient ET between redox enzymes and a range of electrode surfaces can be achieved by co-immobilisation of the enzymes with Os-complex modified polymers [4, 48]. The response of *Th*Lcs on nanoporous gold electrodes was significantly improved (3-fold) when the enzyme was co-immobilised with the Os-complex modified polymer (P002-P91) (**Scheme 1**) displaying currents of *ca.* 20 μA in O_2 saturated electrolyte (**Fig. 4**). Co-immobilization of the redox enzymes with the Os polymer results in an increase in the capacitive current, which arises from a change in the magnitude of the capacitance, not from a change in the surface area. In addition to promoting more efficient ET between the enzyme and the electrode, the three-dimensional network of the polymer can increase the amount of electroactive *Th*Lc molecules, improving the bioelectrocatalytic response in comparison to the Lc modified nanoporous gold electrode. The magnitude of the current indicates that the response is limited by a combination of enzyme kinetics and/or the kinetics of ET between the enzyme and the

electrode. As expected, no bioelectrocatalytic response was observed with the polymer modified enzyme electrodes in N_2 saturated electrolyte, and lower catalytic currents were observed in air-saturated buffer (**Fig. 4**).



Scheme 1. Structural formula of P002-P91 Os-complex.

4. Conclusions

Well-pronounced bioelectrocatalytic responses were obtained from *Th*Lc-modified nanoporous gold electrodes. The responses observed indicate that *Th*Lc was immobilised at the surface of the nanopores in a manner which promoted DET, in contrast to results obtained previously with high redox potential Lcs at unmodified polycrystalline gold electrodes. The activity of *Th*Lc immobilised on nanoporous gold was strongly dependent on the presence of halide ions. Fluroide completely inhibited the enzymatic response, whereas the addition of Cl^- reduced the bioelectrocatalytic activity by 50%. Higher responses were obtained when the temperature was increased from 20°C to 37°C. The current obtained was 30% of that observed for *Th*Lc co-immobilised with an osmium redox polymer, indicating that higher amounts of enzyme are electrochemically addressable in the presence of the redox polymer. In both systems, the response was substantially lower than the diffusion limited current, indicating that the current was limited by enzyme kinetics and/or the rate of electron transfer between the enzyme and the electrode. The main advantage of nanoporous electrodes is that the use of DET enables devices such as fuel cells to operate at higher potentials than can be obtained with MET. Higher loadings of electroactive enzyme are required to provide devices that are limited by diffusion of the substrate. Different electrode architectures are under examination to maximise the surface loading of electroactive enzyme. Such electrodes coupled with alternative enzymes that display more rapid rates of DET and increased stability are currently under investigation.

Acknowledgements

This work was supported by the European Union FP7 project, 3D-nanobiodevices (NMP4-SL-2009-229255) and the HEA PRTLI4 programme, INSPIRE. The authors thank Prof. Alexander I. Yaropolov for the provision of a homogeneous preparation of *Trametes hirsuta* laccase.

References

[1] A. Heller, Electrical connection of enzyme redox centers to electrodes, J. Phys. Chem. 96 (1992) 3579-3587.

[2] L. Gorton, A. Lindgren, T. Larsson, F.D. Munteanu, T. Ruzgas, I. Gazaryan, Direct electron transfer between heme-containing enzymes and electrodes as basis for third generation biosensors, Anal. Chim. Acta 400 (1999) 91-108.

[3] J.D. Newman, A.P.F. Turner, Home blood glucose biosensors: a commercial perspective, Biosens. Bioelectron. 20 (2005) 2435-2453.

[4] R.S. Freire, C.A. Pessoa, L.D. Mello, L.T. Kubota, Direct electron transfer: an approach for electrochemical biosensors with higher selectivity and sensitivity, J. Brazil. Chem. Soc. 14 (2003) 230-243.

[5] R.A. Marcus, N. Sutin, Electron transfer in chemistry and biology, Biochim. Biophys. Acta (BBA) 811 (1985) 265 - 322.

[6] A. Christenson, N. Dimcheva, E.E. Ferapontova, L. Gorton, T. Ruzgas, L. Stoica, S. Shleev, A.I. Yaropolov, D. Haltrich, R.N.F. Thorneley, S.D. Aust, Direct Electron Transfer Between Ligninolytic Redox Enzymes and Electrodes, Electroanal. 16 (2004) 1074-1092.

[7] I.V. Berezin, V.A. Bogdanovskaya, S.D. Varfolomeev, M.R. Tarasevich, A.I. Yaropolov, Bioelectrocatalysis. Equilibrium oxygen potential in the presence of laccase, Dokl. Akad. Nauk SSSR 240 (1978) 615- 618.

[8] M.R. Tarasevich, A.I. Yaropolov, V.A. Bogdanovskaya, S.D. Varfolomeev, Electrocatalysis of a cathodic oxygen reduction by laccase, Bioelectrochem. Bioener. 6 (1979) 393-403.

[9] C.F. Thurston, The structure and function of fungal Laccases, Microbiol. 140 (1994) 19 - 26.

[10] A.I. Yaropolov, O.V. Skorobogatki, S.S. Vartanov, S.D. Varfolomeyev, Laccase: properties, catalytic mechanism, and application, Appl. Biochem. Biotechnol. 49 (1994) 257 - 280.

[11] D.L. Johnson, J.L. Thompson, S.M. Brinkmann, K.A. Schuller, L.L. Martin, Electrochemical Characterization of Purified Rhus vernicifera Laccase: Voltammetric Evidence for a Sequential Four-Electron Transfer†, Biochem. 42 (2003) 10229-10237.

[12] T. Sakurai, K. Kataoka, Basic and applied features of multicopper oxidases, CueO, bilirubin oxidase, and laccase, Chem. Record. 7 (2007) 220-229.

[13] E.I. Solomon, U.M. Sundaram, T.E. Machonkin, Multicopper oxidases and oxygenases, Chem. Rev. 96 (1996) 2563-2605.

[14] P. Kavanagh, P. Jenkins, D. Leech, Electroreduction of O2 at a mediated Melanocarpus albomyces laccase cathode in a physiological buffer, Electrochem. Commun. 10 (2008) 970-972.

[15] F. Xu, R.M. Berka, J.A. Wahleithner, B.A. Nalson, J.R. Shuster, S.H. Brown, A.E. Palmer, E.I. Solomon, Site-directed mutations in fungal laccase: effect on redox potential, activity and pH profile, Biochem. J. 334 (1998) 63-70.

[16] C. Vaz-Dominguez, S. Campuzano, O. Rüdiger, M. Pita, M. Gorbacheva, S. Shleev, V.M. Fernandez, A.L. De Lacey, Laccase electrode for direct electrocatalytic reduction of O2 to H2O with high-operational stability and resistance to chloride inhibition, Biosens. Bioelectron. 24 (2008) 531-537.

[17] M. Gelo-Pujic, H.H. Kim, N.G. Butlin, G.T. Palmore, Electrochemical studies of a truncated laccase produced in *Pichia pastoris*, Appl. Environ. Microbiol. 65 (1999) 5515-5521.

[18] K.H. Hyung, K.Y. Jun, H.-G. Hong, H.S. Kim, W. Shin, Immobilization of laccase onto the gold electrode using b-mercaptopropionate, Bull. Korean Chem. Soc. 18 (1997) 564-566.

[19] S. Shleev, A. Christenson, V. Serezhenkov, D. Burbaev, A. Yaropolov, L. Gorton, T. Ruzgas, Electrochemical redox transformations of T1 and T2 copper sites in native *Trametes hirsuta* laccase at gold electrode, Biochem. J. 385 (2005) 745-754.

[20] S. Shleev, J. Tkac, A. Christenson, T. Ruzgas, A.I. Yaropolov, J.W. Whittaker, L. Gorton, Direct electron transfer between copper-containing proteins and electrodes, Biosens. Bioelectron. 20 (2005) 2517-2554.

[21] M. Pita, S. Shleev, T. Ruzgas, V.M. Fernandez, A.I. Yaropolov, L. Gorton, Direct heterogeneous electron transfer reactions of fungal laccases at bare and thiol-modified gold electrodes, Electrochem. Commun. 8 (2006) 747-753.
[22] S. Shleev, M. Pita, A.I. Yaropolov, T. Ruzgas, L. Gorton, Direct Heterogeneous Electron Transfer Reactions of Trametes hirsuta Laccase at Bare and Thiol-Modified Gold Electrodes, Electroanal. 18 (2006) 1901-1908.
[23] D. Ivnitski, P. Atanassov, Electrochemical studies of intramolecular electron transfer in laccase from *Trametes versicolor*, Electroanal. 19 (2007) 2307-2313.
[24] 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 (2011) 13420-13428.
[25] V. Climent, J. Zhang, E.P. Friis, L.H. Oestergaard, J. Ulstrup, Voltammetry and Single-Molecule in Situ Scanning Tunneling Microscopy of Laccases and Bilirubin Oxidase in Electrocatalytic Dioxygen Reduction on Au(111) Single-Crystal Electrodes, J. Phys. Chem. C 116 (2012) 1232-1243.
[26] M. Dagys, K. Haberska, S. Shleev, T. Arnebrant, J. Kulys, T. Ruzgas, Laccase-gold nanoparticle assisted bioelectrocatalytic reduction of oxygen, Electrochem. Commun. 12 (2010) 933-935.
[27] H. Qiu, C. Xu, X. Huang, Y. Ding, Y. Qu, P. Gao, Adsorption of Laccase on the Surface of Nanoporous Gold and the Direct Electron Transfer between Them, J. Phys. Chem. C 112 (2008) 14781-14785.
[28] S.V. Shleev, O.V. Morozova, O.V. Nikitina, E.S. Gorshina, T.V. Rusinova, V.A. Serezhenkov, D.S. Burbaev, I.G. Gazaryan, A.I. Yaropolov, Comparison of physico-chemical characteristics of four laccases from different basidiomycetes, Biochimie 86 (2004) 693-703.
[29] E. Gorshina, T. Rusinova, V. Biryukov, O. Morozova, S. Shleev, A. Yaropolov, The dynamics of oxidase activity during cultivation of basidiomycetes from the genus Trametes Fr, App. Biochem. Microbiol. 42 (2006) 558-563.
[30] S. Pöller, Y. Beyl, J. Vivekananthan, D.A. Guschin, W. Schuhmann, A new synthesis route for Os-complex modified redox polymers for potential biofuel cell applications, Bioelectrochem. 87 (2012) 178-184.
[31] M.D. Scanlon, U. Salaj-Kosla, S. Belochapkine, D. MacAodha, D. Leech, Y. Ding, E. Magner, Characterization of Nanoporous Gold Electrodes for Bioelectrochemical Applications, Langmuir 28 (2011) 2251-2261.
[32] H. Qiu, C. Xu, X. Huang, Y. Ding, Y. Qu, P. Gao, Immobilization of Laccase on Nanoporous Gold: Comparative Studies on the Immobilization Strategies and the Particle Size Effects, J. Phys. Chem. C 113 (2009) 2521-2525.
[33] U. Salaj-Kosla, S. Pöller, Y. Beyl, M.D. Scanlon, S. Beloshapkin, S. Shleev, W. Schuhmann, E. Magner, Direct electron transfer of bilirubin oxidase (Myrothecium verrucaria) at an unmodified nanoporous gold biocathode, Electrochem. Commun. 16 (2012) 92-95.
[34] C. Mateo, R. Torres, G. Fernández-Lorente, C. Ortiz, M. Fuentes, A. Hidalgo, F. López-Gallego, O. Abian, J.M. Palomo, L. Betancor, B.C.C. Pessela, J.M. Guisan, R. Fernández-Lafuente, Epoxy-Amino Groups: A New Tool for Improved Immobilization of Proteins by the Epoxy Method, Biomacromolecules 4 (2003) 772-777.
[35] 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 (2006) 747-753.
[36] H.-X. Zhou, K.A. Dill, Stabilization of Proteins in Confined Spaces, Biochem. 40 (2001) 11289-11293.
[37] T. Beneyton , Y. Beyl , D.A. Guschin, A.D. Griffiths, V. Taly, W. Schuhmann The Thermophilic CotA Laccase from Bacillus subtilis: Bioelectrocatalytic Evaluation of O2 Reduction in the Direct and Mediated Electron Transfer Regime, Electroanal. 23 (2011) 1781-1789.

[38] A. Naqui, S.D. Varfolomeev, Inhibition mechanism of polyporus Laccase by fluoride ions, FEBS Letters 113 (1980) 157 - 160.
[39] F. Xu, Catalysis of novel enzymatic iodide oxidation by fungal laccase, App. Biochem. Biotech. 59 (1996) 221-230.
[40] Y. Beyl, D.A. Guschin, S. Shleev, W. Schuhmann, A chloride resistant high potential oxygen reducing biocathode based on a fungal laccase incorporated into an optimized Os-complex modified redox hydrogel, Electrochem. Commun. 13 (2011) 474-476.
[41] I. Zawisza, J. Rogalski, M. Opallo, Electrocatalytic reduction of dioxygen by redox mediator and laccase immobilized in silicate thin film, J. Electroanal. Chem. 588 (2006) 244-252.
[42] C. Fernández-Sánchez, T. Tzanov, G.M. Gübitz, A. Cavaco-Paulo, Voltammetric monitoring of laccase-catalysed mediated reactions, Bioelectrochem. 58 (2002) 149-156.
[43] W.E. Farneth, B.A. Diner, T.D. Gierke, M.B. D'Amore, Current densities from electrocatalytic oxygen reduction in laccase/ABTS solutions, J. Electroan. Chem. 581 (2005) 190-196.
[44] B. Chefetz, Y. Chen, Y. Hadar, Purification and characterization of laccase from Chaetomium thermophilium and its role in humification, Appl. Environ. Microbiol. 64 (1998) 3175 - 3179.
[45] A. Majcherczyk, C. Johannes, A. Huttermann, Oxidation of aromatic alcohols by laccase from *Trametes versicolor* mediated by the 2,2'- aziono-bis-(3-ethylbenzothiazoline-6-sulphonic acid) cation radical and dication, Appl. Microbiol. Biotechnol. 51 (1999) 267 - 276.
[46] R.C. Walsh, M.J. Astle, W.H. Beyer, CRC Handbook of Chemistry and Physics, in, CRC Press, Inc., Boca Raton, Florida, 1985, pp. F-44.
[47] O. Morozova, G. Shumakovich, M. Gorbacheva, S. Shleev, A. Yaropolov, "Blue" laccases, Biochem. (Moscow) 72 (2007) 1136-1150.
[48] K. Habermüller, M. Mosbach, W. Schuhmann, Electron-transfer mechanisms in amperometric biosensors, Fresenius' J. .Anal. Chem. 366 (2000) 560-568.

List of figures

Figure 1 Cyclic voltammogram of (A) *Th*Lc/nanoporous gold electrode in the presence of O_2 (full line) and of N_2 (dotted line); (B) ThLc/nanoporous gold electrode with P017-epoxy as

stabilizer in the presence of O_2 (full line), air (dashed line) and N_2 (dotted line). Electrodes were tested in 100 mM phosphate-citrate buffer, pH 4.0; scan rate: 5 mV/s.

Figure 2 Effect of (A) F^- and (B) Cl^- inhibition on the biocatalytic current corresponding to the reduction of oxygen at *Th*Lc modified nanoporous gold electrodes at 0.2 V (vs. Ag/AgCl) in 0.1 M citrate-phosphate buffer, pH 4.0.

Figure 3 Cyclic voltammogram of *Th*Lc adsorbed on nanoporous gold electrode recorded at 20 °C (full line) and 37 °C (dotted line) in oxygen saturated, 100 mM citrate-phosphate buffer, pH 4.0; scan rate: 5 mV/s.

Figure 4 Cyclic voltammograms of a *Th*Lc and P002-P91 Os polymer modified nanoporous gold electrode in the presence of O_2 (full line), air (dashed line) and N_2 (dotted line). Electrodes were tested in 100 mM phosphate-citrate buffer, pH 4.0; scan rate: 5 mV/s.