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BIOENERGY AND BIOFUELS

Prolongation of electrode lifetime in biofuel cells by periodic enzyme renewal

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Abstract Enzymatically catalyzed biofuel cells show unique specificity and promise high power densities, but suffer from a limited lifetime due to enzyme deactivation. In the present work, we demonstrate a novel concept to extend the lifetime of a laccase-catalyzed oxygen reduction cathode in which we decouple the electrode lifetime from the limited enzyme lifetime by a regular resupply of fresh enzymes. Thereto, the adsorption behavior of laccase from *Trametes versicolor* to buckypaper electrode material, as well as its time-dependent deactivation characteristics, has been investigated. Laccase shows a Langmuir-type adsorption to the carbon nanotube-based buckypaper electrodes, with a mean residence time of 2 days per molecule. In a citrate buffer of pH 5, laccase does not show any deactivation at room temperature for 2 days and exhibits a half-life of 9 days. In a long-term experiment, the laccase electrodes were operated at a constant galvanostatic load. The laccase-containing catholyte was periodically exchanged against a freshly prepared one every second day to provide sufficient active enzymes in the catholyte for the replacement of desorbed inactive enzymes. Compared to a corresponding control experiment without catholyte exchange, this procedure resulted in a 2.5 times longer cathode lifetime of 19±9 days in which the electrode showed a potential above 0.744 V vs. normal hydrogen electrode at 110 $\mu A cm^{-2}$. This clearly indicates the successful exchange of molecules by desorption and re-adsorption and is a first step toward the realization of a self-regenerating enzymatic biofuel cell in which enzyme-producing microorganisms are integrated into the electrode to continuously resupply fresh enzymes.

Keywords Enzymatic biofuel cell · Laccase · Lifetime · Long-term stability · Adsorption · Buckypaper

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Introduction

Redox enzymes are excellent bioelectrocatalysts for biofuel cell applications since they catalyze electron transfer reactions with high turnover rates and reactant specificity. However, their protein shell not only gives them unique selectivity but also limits their lifetime: the vibration of flexible protein regions and chemical modifications of single amino acids can change interactions within the protein chain, thus disturbing the fragile three-dimensional conformation (Rubenwolf et al. 2011). When redox enzymes are applied as biofuel cell catalysts, their lifetime also limits the complete electrode lifetime. At present,

lifetimes reported for enzymatic biofuel cells investigated under operation reach 40 days at best (Cinquin et al. 2010).

To increase the lifetime of enzymatically catalyzed biofuel cell and biosensor electrodes, several strategies and ideas have been published (Rubenwolf et al. 2011). The most common ones aim to stabilize the enzyme itself by immobilization. Laccase has been stabilized for instance by Gellett et al. (2010) using tetrabutylammonium-modified Nafion to encapsulate the enzyme. Another concept is the so-called bioinspired approach that aims to achieve theoretically unlimited lifetime by separating the enzyme lifetime from the electrode lifetime in a similar way how living cells cope with limited enzyme lifetime: deactivated enzymes are simply replaced against fresh and active ones. However, so far, such concepts have only been shown for biosensors with indirect electrochemical conversion and not yet reported for biofuel cells (Atanasov et al. 1997) or mediated enzyme electrodes, where the mediators are a further source of instability (Fishilevich et al. 2009). To our knowledge, the preferable mediatorless approach has not yet been reported. To realize a mediatorless enzyme electrode with a continuous or periodic renewal of the catalyst, the following requirements have to be fulfilled:

- Direct electron transfer between the enzyme and electrode to omit the need for mediators
- Reversible adsorption of the enzyme to the electrode to allow replacement of deactivated enzymes
- Enzyme desorption has to be faster than enzyme deactivation to sustain catalytic activity of the adsorbed enzymes.

Among the commonly used electrode enzymes, laccase from *Trametes versicolor* (EC 1.10.3.2) shows reversible adsorption to carbon (Blanford et al. 2007; Tarasevich et al. 2001) as well as direct electron transfer with various carbon electrodes (Rubenwolf et al. 2010). Previously, we reported on the use of buckypaper, a mechanically stable mat of well-dispersed multi-walled carbon nanotubes, as a highly efficient electrode material for mediatorless laccase-catalyzed oxygen reduction cathodes (Hussein et al. 2011a).

In the present work, we investigate whether the lifetime of this cathode can be prolonged by the periodic exchange of the enzyme-containing catholyte. Thereto, we characterize the adsorption of laccase to buckypaper electrodes by monitoring the decrease of laccase activity in the catholyte compared to laccase deactivation in solution. A further objective was to suggest a catholyte exchange procedure for the long-term operation of buckypaper electrodes.

Theoretical background

A reversible direct contact does not allow covalent binding, but has to depend on physical adsorption with weak interactions, such as van der Waals forces, electrostatic interaction, hydrogen links, and hydrophobic forces between the support and the enzyme. The strength of adsorption depends on the chemical nature of the matrix, the functional groups on the surface of the enzyme, and on the conditions of immobilization (Horozova and Dimcheva 2004). Therefore, the adsorption characteristics have to be determined experimentally for every enzyme–material combination. The amount of adsorbed protein can be detected directly at the electrode via radiolabeling of the protein (Antiohos et al. 2010) or via the electrochemical electrode activity (Lee et al. 1984). Alternatively, it can be calculated from the decrease of protein concentration (Horozova and Dimcheva 2004) or enzyme activity in the electrolyte (Kiiskinen et al. 2004).

The adsorption and the desorption of a monolayer of molecules at a surface with equal adsorption sites have been described by Langmuir (1918): The time t (in seconds), dependent change of the surface coverage Θ (in percent), caused by adsorption depends on the adsorption constant k_{ads} (in milliliters per milligram per second), the concentration of the available adsorbing molecules c (in milligrams per milliliter), and the percentage of free adsorption sites (Langmuir 1918), which itself is described by the difference between the maximum surface coverage Θ_{max} (Chase 1984) and the time-dependent surface coverage $\Theta(t)$.

$$\frac{d\Theta}{dt} = k_{ads} c(\Theta_{max} - \Theta(t)) \tag{1}$$

The time-dependent change of the surface coverage caused by desorption depends on the desorption constant, k_{des} (per second), and the present surface coverage (Langmuir 1918).

$$\frac{d\Theta}{dt} = -k_{des}\Theta(t) \tag{2}$$

At equilibrium, adsorption equals desorption (Langmuir 1918), resulting in the adsorption isotherm where the surface coverage only depends on the concentration of the adsorbing molecules:

$$\Theta(c) = \frac{\Theta_{max} c k_{ads}}{c k_{ads} + k_{des}} \tag{3}$$

With the transformation of Eq. 3 to a linear equation, a measured adsorption isotherm can be used to calculate Θ_{max} and the relation of k_{des} and k_{ads} from its slope and intercept.

$$\frac{1}{\Theta(c)} = \frac{k_{des}}{k_{ads}} \frac{1}{\Theta_{max}} \frac{1}{c} + \frac{1}{\Theta_{max}} \tag{4}$$

Beyond equilibrium, the time-dependent change of the surface coverage (adsorption kinetic) is given by a combination of Eqs. 1 and 2 (Chase 1984).

$$\frac{d\Theta}{dt} = (\Theta_{max} - \Theta(t)) c k_{ads} - \Theta(t) k_{des} \tag{5}$$

When there is no surface coverage in the beginning, a possible solution of integration of Eq. 5 is given in Eq. 6 (Karpovich and Blanchard 1994).

$$\Theta(t) = \frac{\Theta_{\max} c k_{ads}}{c k_{ads} + k_{des}} (1 - \exp(-(c k_{ads} + k_{des})t)) \quad (6)$$

With the help of its transformation to a linear equation given as Eq. 7, k_{ads} can be determined from the time-dependent surface coverage data of the adsorption kinetics when Θ_{max} and the ratio $k_{des}\ k_{ads}^{-1}$ are known from the adsorption isotherm.

$$\ln\left(\frac{\Theta_{\max}}{\Theta_{\max} - D}\right) = k_{ads}\left(c + \frac{k_{des}}{k_{ads}}\right) \quad \text{with} \quad D = \Theta(t)\left(1 - \frac{1}{c}\frac{k_{des}}{k_{ads}}\right) \quad (7)$$

Not all adsorbed molecules, however, remain adsorbed for the same time before they desorb again. Assuming an exponential distribution (Atkins and de Paula 2006), the percentage of the adsorbed molecules that desorb within a given time X (in percent) can be calculated as follows:

$$X = 1 - e^{-t \times k_{des}} \quad (8)$$

Materials and methods

Chemicals and electrode materials

Sodium dihydrogencitrate, sodium hydrogencitrate sesquihydrate, laccase from *T. versicolor* (no. 53739), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and Triton X-100 were purchased from Sigma-Aldrich (Taufkirchen, Germany) and isopropanol from Carl Roth (Karlsruhe, Germany). All chemicals were used as received. If not stated otherwise, deionized water was used as the solvent to chemicals, and 0.1 M citrate buffer (pH 5) was used for the preparation of enzyme and ABTS solutions.

Buckypaper electrodes with 60 μm thickness were prepared as described before (Hussein et al. 2011b). In short, 100 mg as-received multi-walled carbon nanotubes (Baytubes C 150-HP, Bayer Material Science, Leverkusen, Germany) were dispersed in 200 ml 1 % Triton X-100 by mechanical stirring for 30 min and ultrasonication for 3 h with a subsequent centrifugation step at 1,107×*g* to remove bigger nanotube agglomerates. Nanotubes of the resulting suspension were deposited on a nylon filter (0.45 μm pore size, Whatman, Maidstone, UK) by vacuum filtration of the supernatant. The filter with the homogeneous nanotube film was washed with excess of deionized water, isopropanol, and acetone to remove the remaining Triton X-100, kept for 30 min at room temperature, and dried in a vacuum oven at 50 °C overnight.

Characterization of protein content and enzyme activity

The protein content of a 0.32 mg ml^{-1} laccase solution was calculated after Warburg and Christian (1941) from the optical absorption at 260 and 280 nm. Therefore, the absorption spectra from 200 to 300 nm against a protein-free citrate buffer as a reference were taken with a spectrophotometer (UV300, Unicam Instruments, Cambridge, UK) using disposable micro-cuvettes (Carl Roth).

The protein content was further investigated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Solutions of 0.32 mg ml^{-1} laccase (lot no. 100891817) in 0.1 M citrate buffer were separated according to Laemmli's procedure (Laemmli 1970) with Bio-Rad (Munich, Germany) gels and cell together with Precision Plus Protein Dual Color Standard (Bio-Rad). Coomassie staining was done with the Serva Blue R Staining Kit (Serva Electrophoresis, Heidelberg, Germany).

Laccase activity was determined spectrophotometrically via its reaction velocity toward the oxidation of ABTS. Triplets of each 100 μl sample were placed in 96-well microplates (Greiner Bio-One, Frickenhausen, Germany). Within the microplate reader Wallac Victor^2 (Perkin Elmer, Rodgau–Jügesheim, Germany) at room temperature, 100 μl of 0.02 M ABTS was injected and the absorption at 405 nm measured every second to monitor its increase. A calibration curve with a freshly prepared laccase solution was included in every run to exclude influences such as differences in room temperature or ABTS aging. Error bars in the graphs indicate twice the standard deviation of the triplets.

Laccase deactivation in solution at room temperature

To investigate laccase deactivation at room temperature, a 0.32 mg ml^{-1} laccase solution was sterile-filtered (0.2 μm, cellulose acetate; Whatman, Dassel, Germany). One-milliliter aliquots of the solution were filled to sterile centrifuge tubes (5 ml volume; Greiner Bio-One) and frozen at −18 °C to stop deactivation. The tubes were sequentially thawed to start the deactivation and stored at room temperature for up to 50 days. This way, the enzyme activity of the samples with different ages could be measured within only one run.

Laccase adsorption kinetics and isotherm

To prepare buckypaper-based electrodes, the filter-supported buckypapers were cut to pieces of 5×5 mm^2 and glued with epoxy (UHU plus sofort fest, Uhu, Bühl, Germany) into a 12-well multi-well plate (BD Falcon, BD Biosciences, Heidelberg, Germany). After hardening of the glue, the electrodes were wetted with isopropanol and thoroughly washed with deionized water to remove the isopropanol. The water was discarded and the microplate clapped upside down to the

bench to remove as much of the adhering water as possible to minimize dilution effects.

Each well was filled with 1 ml catholyte with a laccase concentration of 0.32 mg ml^{-1} (adsorption kinetics) or different concentrations up to 0.92 mg ml^{-1} (adsorption isotherm). Wells without electrodes were used as the negative control. The microplate was incubated at room temperature in a shaker (POS-300, Grant Instruments, Cambridgeshire, UK) at 180 rpm. Ten-microliter samples were taken after incubation for 1 day (to determine the adsorption isotherm) or every hour (to determine the adsorption kinetics). The 10 μl samples were added to 90 μl of citrate buffer for measurement of the enzyme activity.

The adsorption of laccase to carbon electrodes was monitored by the decay of enzyme activity in the catholyte. As enzyme deactivation has no influence within the short observation time, the decay of enzyme activity is a measure of the laccase concentration decay due to adsorbing molecules. To calculate the number of adsorbed molecules, the weight of dry laccase preparation was transformed with the measured protein content and the approximated molecular weight of 70 kDa (Piontek et al. 2002). To calculate the number of adsorption sites, the carbon nanotube load was combined with the specific nanotube surface area taken as 200 m^2 g^{-1} (one-point BET, Sorptomatic 1990, Porotec GmbH, Hofheim/Ts., Germany); the occupied surface area of one laccase molecule was estimated as 3×10^{-17} m^2 from the structural data (Piontek et al. 2002) with the assumption of its random orientation at the surface. Laccase adsorption to walls, glue, or filter was evaluated in reference experiments and is included in the calculations.

Galvanostatic cathode characterization

Galvanostatic polarization curves and the long-term stability of buckypaper-based laccase cathodes were tested in half-cell configuration at room temperature. The electrodes were prepared as described previously (Hussein et al. 2011a). In short, the electrodes were cut into pieces of 0.6×1.5 cm^2, connected to platinum wires (0.1 mm diameter, 99.9 %; Chempur, Karlsruhe, Germany) with a conducting carbon cement (Leit-C, Plano, Wetzlar, Germany), cured for half an hour at 70 °C, wetted with isopropanol, and washed thoroughly with deionized water. The electrochemical reactor has been described in detail recently (Kloke et al. 2010). It consists of polycarbonate frames alternately stacked with silicone gaskets; channels and vents are incorporated as bore holes or cavities. The reference electrode compartment is separated by a cation exchange membrane (Fumapem F-950®, FuMA-Tech, St. Ingbert, Germany). Sterile conditions can be maintained in long-term experiments by protecting the gassing channels with syringe filters (FP 30/0.2 CA-S, Whatman, Dassel, Germany) and the medium exchange port with a septum closure (Greiner Bio-One).

The reactors were equipped with buckypaper cathodes and platinum mesh (Goodfellow, Huntington, UK) counter electrodes, filled with 4 ml laccase-free citrate buffer, and autoclaved. Afterwards, the buffer was exchanged against sterile filtered citrate buffer with 0.32 mg ml^{-1} laccase and purging with humidified air was started. The electrode potential was measured against saturated calomel electrodes (SCE; 0.244 V vs. normal hydrogen electrode (NHE) at 25 °C) as the reference electrodes, while load currents were applied by an electronic load as described before (Kerzenmacher et al. 2009). For all experiments, the enzyme was allowed to adsorb overnight at open-circuit conditions. To record the galvanostatic electrode polarization, current was increased in hourly steps of 50 μA in the range of 0–300 μA followed by steps of 8 μA in the range of 300–800 μA. To record the cathode long-term performance, a constant current of 110 μA cm^{-2} was applied. In these experiments, the catholyte was exchanged against freshly prepared catholyte every second day. In reference experiments, only the evaporated water was replaced through the sterile filter. Evaluation of electrode performance was done at 0.744 V vs. NHE (0.5 V vs. SCE) as described before (Rubenwolf et al. 2010). Electrode lifetime is defined as the time where the electrode potential drops below 0.744 V for the first time.

Results

Deactivation of dissolved laccase at room temperature

Deactivation of various enzymes follows first-order kinetics with a constant deactivation rate, assuming that a single structural change results in a total loss of activity. But the enormous complexity of enzyme conformation also allows deactivation via one or more intermediates with altered activity, and deactivated enzymes may also influence active enzymes. This can result in the deactivation rates continuously decreasing or increasing as well as in deactivation-free grace periods (Sadana 1988).

Figure 1 shows the time-dependent relative activity of laccase solution at pH 5 when stored at room temperature. The enzyme exhibits a deactivation-free grace period of about 2 days before it undergoes a first-order deactivation with a half-life of 7 days. The occurrence of a grace period may be attributed to a two-state independent unfolding as observed at the homologous enzyme laccase 1 from the basidomycete PM1 (Coll et al. 1994).

Characterization of Langmuir adsorption and desorption

The protein content of the laccase preparation was determined by the optical absorptions at 260 and 280 nm of a 0.32 mg ml^{-1} solution after Warburg and Christian (1941).

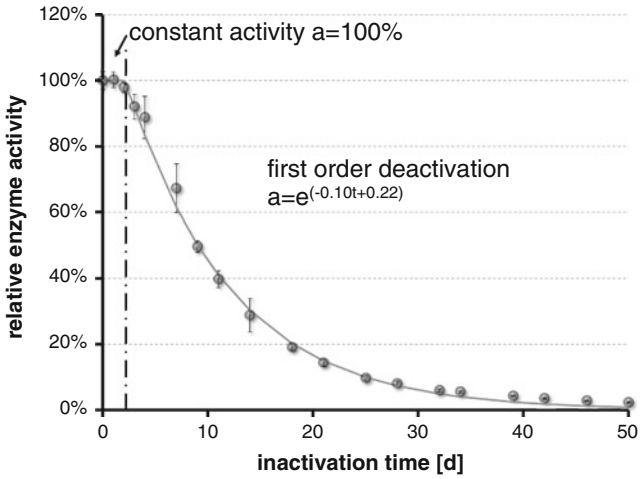


Fig. 1 Deactivation of dissolved laccase at room temperature. Experimental data are given as *circles* and the calculated fit as a *solid line*. For the fit, constant activity was supposed for the first 2.2 days followed by fist-order deactivation according to Eq. 2 (with a modification due to the deactivation-free grace period)

The measured optical absorptions A_{260}=0.22±0.01 and A_{280}=0.16±0.01 correspond with a nucleic acid content of 5 % and a protein content of 23±1 % as the amino acid composition of laccase is comparable to that of the enolase enzyme used by Warburg and Christian (Fahraeus and Reinhammar 1967; Malmstrom et al. 1959). In addition to the protein, every laccase molecule contains four copper molecules and a glycosylation content of 10–45 % of the total enzyme weight (Yaropolov et al. 1994). Further contents of the preparation may be salts and other culture medium components as well as enzyme-stabilizing agents.

The protein content was further investigated using SDS-PAGE. The stained gel is shown in Fig. 2. Due to the different glycosylation contents, laccase molecules from different microorganisms have different molecular weights around 70 kDa (Piontek et al. 2002). The only visible band corresponds to a molecular weight of around 65 kDa, matching the molecular weight of laccase. Minor contents of impurities not visible in the gel are tolerable as we use the highly specific enzyme activity as a measure of concentration.

To obtain values for the maximum surface coverage, Θ_{max}, and the ratio of adsorption and desorption constant, $k_{ads}\,k_{des}^{-1}$, the adsorption isotherm was recorded. We used the enzyme activity test to monitor the laccase decrease in the catholyte as this test is very sensitive and does not interfere with citrate buffer. Buckypaper electrodes were incubated for 1 day in catholytes containing different concentrations of laccase to measure the adsorption at equilibrium conditions. Figure 3 shows the resulting adsorption isotherm, which clearly shows a Langmuir-type behavior with monolayer adsorption. The insert of Fig. 3 shows the transformation of the adsorption isotherm according to Eq. 4, giving the parameters Θ_{max}= 11 % and $k_{ads}k_{des}^{-1}=6\times10^{-3}$ mg ml^{-1}.

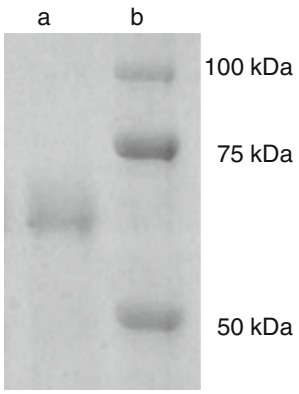


Fig. 2 Polyacrylamide gel of the laccase preparation (**a**) and the protein standard (**b**)

The low surface coverage may mainly depend on the surface structure. Buckypaper is a highly porous material with a wide distribution of pore sizes, predominantly in the mesopore range of 2–50 nm (Hussein et al. 2011b), where the smaller pores reduce the surface availability for enzyme adsorption. For example, they can be blocked by one or few adsorbed laccase molecules (dimension, 6.5×5.5×4.5 nm according to Piontek et al. 2002) and thereby hinder laccase diffusion to underlying pores. Furthermore, the hydrated enzyme may occupy a larger surface area than estimated, and impurities may also affect laccase adsorption.

Using the calculated parameters, the adsorption and desorption constants can be estimated from the adsorption kinetics. For this experiment, electrodes were incubated with laccase solution and the time-dependent adsorption was monitored, as shown in Fig. 4. The insert shows the transformation according to Eq. 7 giving $k_{ads}=1\times10^{-3}$ ml mg^{-1} s^{-1} and $k_{des}=6\times10^{-6}$ s^{-1}. Therewith, the mean residence time of every

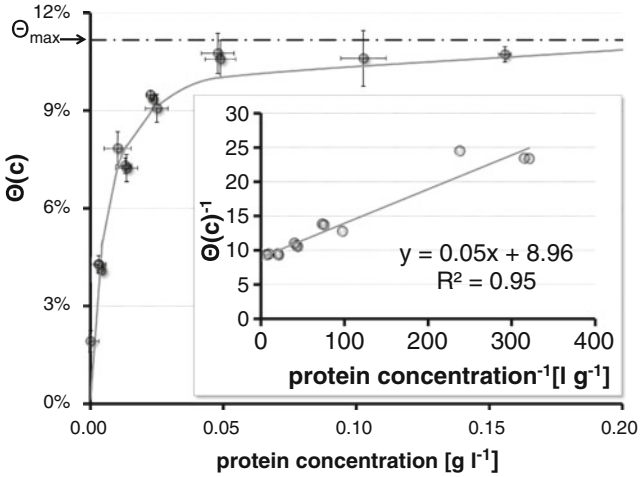


Fig. 3 Adsorption isotherm of laccase at buckypaper electrodes at equilibrium conditions. Experimental data are given as *symbols* and the calculated curve according to Eq. 3 as a *gray solid line*; the *black dashed line* indicates maximum surface coverage. The *insert* shows the transformation according to Eq. 4 to obtain the parameters for calculation of the isotherm

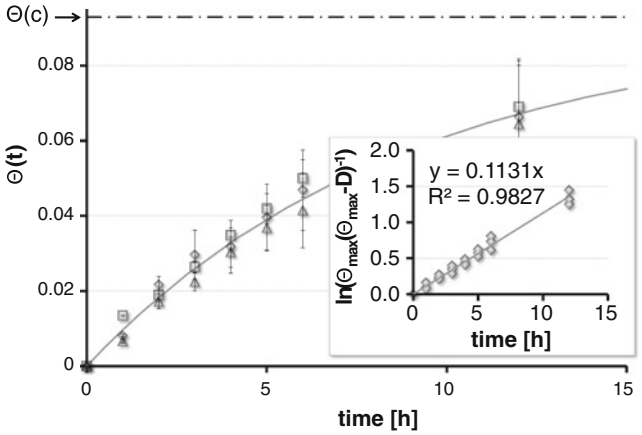


Fig. 4 Adsorption kinetics of laccase at buckypaper electrodes. Experimental data are given as *symbols* and the calculated curve according to Eq. 6 as a *gray solid line*; the *black dashed line* indicates equilibrium surface coverage at 0.32 mg ml^{-1}. The *insert* shows the transformation according to Eq. 7 to get the parameters for calculation of the kinetics with $D = \Theta(t)\left(1 - \frac{1}{c}\frac{k_{des}}{k_{ads}}\right)$

adsorbed molecule at the electrode before desorbing again can be calculated as $t_{ads}=k_{des}^{-1}=2$ days.

Adsorption, desorption, and deactivation within an electrode with periodic exchange of catholyte solution

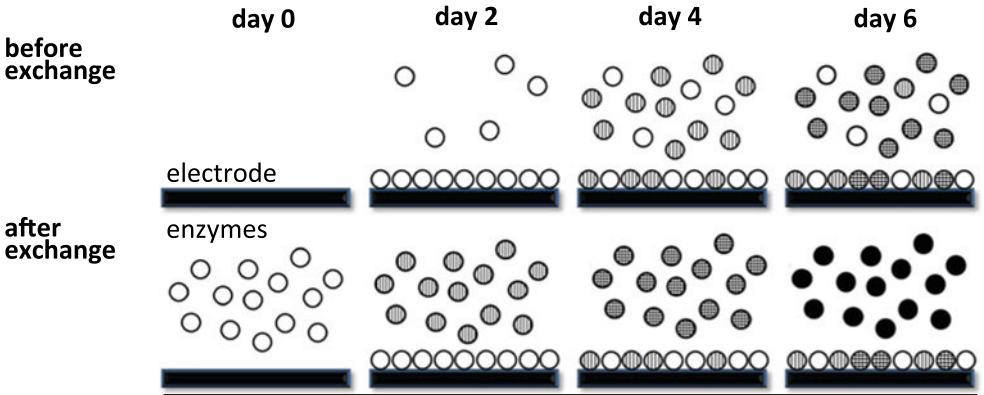
With the data from laccase deactivation and desorption, a rough quantitative estimation of the processes within the electrode compartment can be done: Whenever the catholyte solution is exchanged by a fresh solution, within the following 2 days, 64 % (corresponding to the fraction e^{-1}) of the adsorbed laccase molecules will be replaced by molecules from the fresh solution. As the laccase concentration of the used buffer is lowered compared to the fresh one because of enzyme adsorption, there is an increase of total enzyme (adsorbed to the electrode and in solution) within the first exchange cycles, reaching a plateau after the second exchange. Figure 5 illustrates the adsorption and desorption of enzymes within the first four exchange cycles and the corresponding distribution of enzyme age. At day 0, there is no laccase adsorbed to the electrode and the catholyte is a laccase-free citrate buffer. The exchange of the solution introduces laccase molecules to the catholyte. At day 2, the concentration-dependent equilibrium surface coverage $\Theta(c)$ is reached and the age of all laccase molecules either adsorbed or in the solution is 2 days. With the help of the adsorption isotherm, we can calculate that 67 % of the enzymes are adsorbed while 33 % are still in the solution. The exchange of the catholyte removes the old enzymes from the solution and introduces fresh ones. At day 4, 64 % of the adsorbed enzymes have desorbed while the same number of enzymes have adsorbed from the solution, as defined for equilibrium in Eq. 3. To simplify the calculations, we assume them to be all exchanged against fresh laccase molecules supplied during the last 2 days so that 64 % of the adsorbed enzymes are 2 days old while the rest is 4 days old. According to the adsorption isotherm, now 46 % of the enzyme is adsorbed. The exchange of the catholyte at day 4 again leads to freshly dissolved enzymes only. At day 6, analogously 64 % of the enzymes at the electrode got exchanged and are 2 days old, while from the other adsorbed enzyme molecules, 64 % are 4 days old and 36 % are 6 days old. This results in the following age distribution of adsorbed enzymes: 64 % of all adsorbed molecules are 2 days old, 23 % are 4 days old, and 13 % are 6 days old.

After a few exchange cycles, the distribution of enzyme age is already quite stable. Right before exchange, 64 % of the enzyme is 2 days old, 87 % of the enzyme is not older than 4 days, and 98 % of the enzyme is not older than 8 days. Assuming that the adsorbed enzyme is not deactivated faster than the dissolved enzyme, we can calculate from the distribution of enzyme age that there are more than 90 % active enzymes at the electrode. These calculations do not take into account that enzymes may also be exchanged against itself or other desorbed older molecules. Nevertheless, the example demonstrates that the process of supplying fresh enzymes every second day leads to a stable situation of electrode coverage with the active enzyme. This should significantly prolong electrode lifetime.

Comparison of electrode long-term stability at constant galvanostatic load

A detailed characterization of buckypaper electrodes with adsorbed laccase has already been reported elsewhere (Hussein et al. 2011a). To choose the appropriate current density for long-term experiments, the galvanostatically controlled polarization curves of buckypaper-based laccase cathodes were recorded. As can be seen in Fig. 6, they start at open-circuit potentials of 0.879±0.023 V. At current densities up to at least 150 μA cm^{-2} at 0.744 V (0.5 V vs. SCE), they show a stable potential with only few losses due to activation losses and ohmic resistances. Afterwards, the potentials drop rapidly due to mass transport limitations. Stabilization of the electrode potential below −0.6 V vs. NHE instead of around −0.2 V indicates oxygen transport to be the limiting factor (Hussein et al. 2011a). The high variations between the electrodes may derive from the slightly different oxygen intakes caused by the stacked setup of the electrochemical reactors and the inhomogeneities of the electrode material. For the long-term experiments, a current density of 110 μA cm^{-2} was chosen to exclude oxygen transport limitations.

To evaluate electrode lifetime extension by enzyme renewal, the electrode potential was monitored at a constant galvanostatic load of 110 μA cm^{-2}, while every second day the catholyte was exchanged against a freshly prepared catholyte. In reference experiments, the catholyte was not exchanged,



enzyme age [d]	electrode age [d]						
	2	4	6	8	10	12	14
2	100%	64%	64%	64%	64%	64%	64%
4		36%	23%	23%	23%	23%	23%
6			13%	8%	8%	8%	8%
8				5%	3%	3%	3%
10					2%	1%	1%
12						1%	0%
14							0%

Fig. 5 Illustration of the enzyme renewal process by periodic exchange of catholyte solution. The adsorbed and dissolved enzyme in the electrode compartment is shown before and after exchange of the catholyte at days 0, 2, 4, and 6 with the corresponding distribution of enzyme age. Enzymes added on days 0, 2, 4, and 6 are depicted by *empty*, *lined*, *checkered*, and *filled circles*, respectively

but evaporated water was replaced. As can be seen from Fig. 7, conventional electrodes sustain potentials of at least 0.744 V vs. NHE for 8±3 days. The curves show small potential increases whenever evaporated water is refilled, indicating the influence of the ionic strength of the catholyte. We found out that this does not alter enzyme activity (experiments not shown), but it may influence enzyme adsorption. The regular supply of fresh enzyme by exchanging the catholyte periodically causes much higher potential increases and thereby prolongs the time the electrodes sustain potentials of at least 0.744 V vs. NHE to 19±9 days. This means that the higher percentage of active laccase molecules in the catholyte also leads to a higher bioelectrocatalytic activity of the electrodes, indicating the successful exchange of molecules by desorption and re-adsorption.

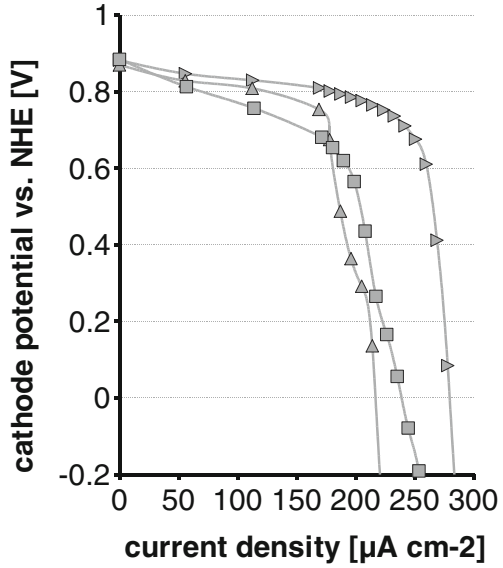


Fig. 6 Galvanostatic polarization curves of a triplet of buckypaper-based laccase cathodes. The *dashed line* indicates the current density of 110 $\mu A\,cm^{-2}$, which was chosen for the long-term experiment

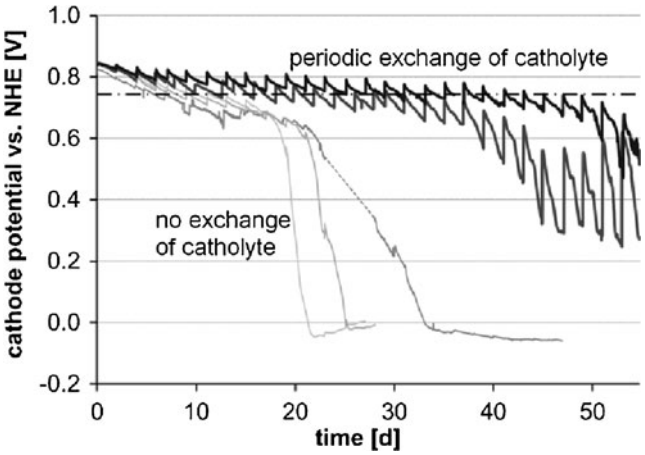


Fig. 7 Long-term stability of electrodes. The *two bold lines* refer to electrodes with periodic exchange of catholyte every second day. Every potential increase is caused by the exchange of the enzyme-containing catholyte. *Thin lines* correspond to identically prepared electrodes without exchanging the catholyte. Electrode lifetime is defined as the time where the electrode potential drops below 0.744 V vs. NHE (indicated by the *dashed line*) for the first time

Discussion

In the present work, we demonstrate for the first time that the lifetime of a mediatorless laccase-catalyzed oxygen reduction cathode can be extended by a regular resupply of fresh enzymes. We clearly show the feasibility of decoupling the electrode lifetime from the enzyme lifetime. This is a prerequisite for the realization of a self-regenerating enzymatic biofuel cell in which enzyme-producing microorganisms are integrated into the electrode to continuously resupply fresh enzymes.

In previous works, lifetime extension by regular enzyme resupply has only been shown for an indirect glucose biosensor anode not compatible with the self-regenerative approach because of the chemical immobilization of glucose oxidase to carbon particles (Yang et al. 1997), and lifetime extension by continuous enzyme resupply has only been suggested for an indirect glucose oxidase biosensor electrode (Yamaguchi et al. 2008). In comparison to the recently presented biofuel cell in which genetically modified yeast cells exhibit surface display of enzymes (Szczupak et al. 2012), our approach is advantageous since it does not require mediators, which themselves exhibit limited lifetimes (Binjamin et al. 2001). Furthermore, our concept of secreting soluble enzymes instead of using surface display would allow using organisms that secrete enzymes naturally (e.g., the wood-degrading fungus *T. versicolor* for laccase; Yaropolov et al. 1994) and, thus, enables the realization of self-regenerating biofuel cells without genetically modified organisms, suitable for environmental applications.

Compared to a control experiment, the periodic exchange of the enzyme-containing catholyte in the present work resulted in a significant 2.5-fold longer cathode lifetime of 19±9 days, defined as the time in which the electrode showed a potential above 0.744 V vs. NHE at 110 μA cm^{-2}. This lifetime under continuous operation already exceeds the 350 working hours (~15 days) shown for a state-of-the-art methanol fuel cell using laccase stabilized by immobilization at the cathode (Gellett et al. 2010). However, a direct comparison to this work is difficult since their fuel cell was operated discontinuously and a different laccase from *Rhus vernificera* was employed.

Nevertheless, even with regular exchange, our electrodes show a progressing loss of activity. A reason for this behavior may be the accumulation of poisoning substances from the laccase preparation used in our work. In separate experiments (data not shown, publication in preparation), we observed that components from typical culture media can strongly decrease the electrocatalytic activity of laccase electrodes. Furthermore, it has to be considered that not every desorption of a laccase molecule leads to the adsorption of a fresh one and that denatured enzymes can exhibit increased surface hydrophobicity or aggregate (Gianazza et al. 2007). Both mechanisms may potentially lead to an accumulation of inactive enzymes on the electrode surface.

Important topics for future work will thus be the clarification of the poisoning mechanism, the development of a fermentation medium for microbial laccase production without poisoning components, and the integration of the laccase-producing microorganisms in the electrode, resulting in a self-renewing biofuel cell and eliminating the need for regular maintenance.

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