Assembly of Laccase over Platinum Oxide Surface and Application as an Amperometric Biosensor

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Laccase could be successfully assembled on an amine-derivatized platinum electrode by glutaraldehyde coupling. The enzyme layer formed on the surface does not communicate electron directly with the electrode, but the enzymatic activity of the surface could be followed by electrochemical detection of enzymatically oxidized products. The well-known laccase substrates, ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) and PPD (p-phenylenediamine) were used. ABTS can be detected down to 0.5 μM with linear response up to 15 μM and current sensitivity of 75 nA/μM. PPD showed better response with detection limit of 0.05 μM, linear response up to 20 μM, and current sensitivity of 340 nA/μM with the same electrode. The sensor responses fit well to the Michaelis-Menten equation and apparent K_M values are 0.16 mM for ABTS and 0.055 mM for PPD, which show the enzymatic reaction is the rate-determining step. The laccase electrode we developed is very stable and more than 80% of initial activity was still maintained after 2 months of uses.

Keywords : Laccase, Immobilization, ABTS, PPD, Biosensor.

Introduction

Laccase (p-diphenol:dioxygen oxidoreductase, EC 1.10.3.2) is in multi-copper oxidase family with ascorbate oxidase, ceruloplasmin, etc. Laccase couples catalytic oxidation of phenolic substrates with four electron reduction of dioxygen to water.[1] Various aromatic compounds, such as phenylene-diamines, diphenols, some azo compounds, can be used as substrates for laccase to donate electrons, and oxygen is another specific substrate to accept electrons from reduced substrates through laccase. Laccases can be grouped into two categories, plant and fungal laccases. It is proposed that tree laccase catalyzes the initial polymerization of mono-lignols into oligolignols since most plant laccases are capable of oxidizing monolignols to form dimers and trimers, while fungal laccase works as a depolymerization catalyst to degrade lignin.[2] The laccase activity has been found from a lot of fungal species and laccase is thought to be nearly ubiquitous among fungi. Most of fungal laccases are monomers or homodimers and are glycosylated, but generally to a lesser extent (5-25%) than the plant enzymes (20-50%). Since laccase can use various kinds of aromatic compounds as substrates and relatively stable, it is very useful in lignin degradation, detoxification and decoloration. A lot of efforts can be found in biotechnological applications of laccases in pulp/paper industries, waste water treatment, and textile industries.[3] Recently, several fungal laccases are cloned,[4] and commercial product containing a laccase for decoloring denim is appeared as DeniLite™ from Novo Nordisk Co.

To use laccase in more versatile applications, the immo-bilization is essential for the repetitive uses and easy sepa-ration from products. Immobilization sometimes enhance thermal, or pH stability of the enzyme.[5] There have been a lot of efforts to immobilize laccases onto the surfaces by spontaneous adsorption onto the graphite electrode,[6] glass powder, silica gel, Nylon 66 membranes,[7] chitosan,[8] etc., coupling with activated agarose,[9] and entrapment in polymer films such as gelatin or polyurethane.[10] Our lab demon-strated that *Rhus vernicifera* laccase can be attached to β-mercaptopropionate coated gold electrode by electrostatic interaction.[11] The immobilizations of laccase onto electrode surfaces by polymer films and applications as a sensor to detect phenolic compounds have been reported,[12] but few examples of covalent attachment of laccase onto the electrode surface were found.

Recently we could purify a recombinant fungal laccase from DeniLite™ and we report here that we could success-fully immobilize the laccase onto silanized Pt electrode surface by covalent attachment using glutaraldehyde. The immobilized electrode was tested by two well-known sub-strates for laccase, ABTS (2,2'-azinobis(3-ethylbenzothiazo-line-6-sulfonic acid)) and PPD (p-phenylenediamine) (Figure 1). The substrates were oxidized enzymatically by the immobilized laccase in the presence of oxygen and the oxidized substrates could be immediately detected ampero-metrically when applying suitable reduction potential. This laccase modified electrode can be used as a biosensor to detect various phenolic compounds.

Experimental section

Purification of laccase. DeniLite™, which is a slurry type laccase containing product for decoloration of indigo dye was purchased from Novo Nordisk Co. The product was diluted 5 times with 0.1 M phosphate buffer (pH = 6.0), centrifuged and filtered first. The supernatant was collected
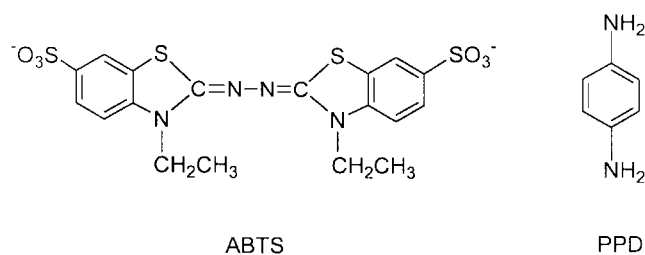
Figure 1. Structures of ABTS and PPD.

and conducted extensive buffer exchange using stirred ultra-filtration cell (Amicon 8200, YM 30 membrane). The solution was loaded onto DEAE sephacel resin (Pharmacia), which is an anion-exchanger, and laccase active fractions were pooled together after step elution by 0.0-0.5 M NaClO$_4$. The SDS-PAGE experiments showed only one band around M.W.=54,000 confirming that the protein was homogeneously purified. The activity of the enzyme was 65 units/mg measured by oxidation of ABTS, which is comparable to those of laccases from other sources.[13]

Reagents and instruments. ABTS and PPD were purchased from Aldrich and stored under inert atmosphere before use. All experiments were performed in 0.1 M potassium phosphate buffer (pH = 6.0) at room temperature. Deionized water from Milli Q water purification system was used for preparing aqueous solutions. Platinum disk working (1 or 4 mm diameter), platinum wire counter, and Ag/AgCl reference electrodes were used for electrochemical measurements. BAS 50W or cDAQ-1604 (Elbio Co., Korea) potentiostat was used to run CVs and measure current-time responses. The solution was continuously stirred by magnetic bar under air during amperometric experiments. The scanning electron microscope (SEM) images of a silanized Pt electrode and laccase-modified electrode were obtained by a FE-SEM (Hitach S-4300) at an acceleration voltage of 10 to 20 kV. On top of the samples platinum/palladium alloy (in the ratio of 8 to 2) was deposited with a thickness of about 15 nm.

Immobilization of laccase. The covalent immobilization was performed by the method to immobilize glucose oxidase in the literature[14,15] with some modifications. A Pt disc electrode (4 mm diameter) was oxidized by 5% solution of potassium dichromate in 15% nitric acid (2 hours, 80 °C) and the electrode was thoroughly rinsed with water, blown dried by N$_2$ gas. The oxidized electrode was incubated in 2 % aqueous solution (pH = 6.8) of 3-aminopropyltriethoxy-silane (APTES) at 37 °C for 1 hour and thoroughly rinsed with water and blown dried by N$_2$ gas. The electrode was further incubated in oven (30 min., 120 °C) to enhance stability of the silane layer. The silanized electrode was further modified with glutaraldehyde by being exposed to vapor of 25% glutaraldehyde solution for 1 hour. The glutaraldehyde activated electrode was rinsed with water, dried by purging N$_2$ gas, and 3 μL of 6 mg/mL laccase solution in 0.1 M phosphate buffer (pH = 6.0) was applied and dispersed uniformly on the electrode using micro-syringe. After drying the enzyme solution for 15 minutes at
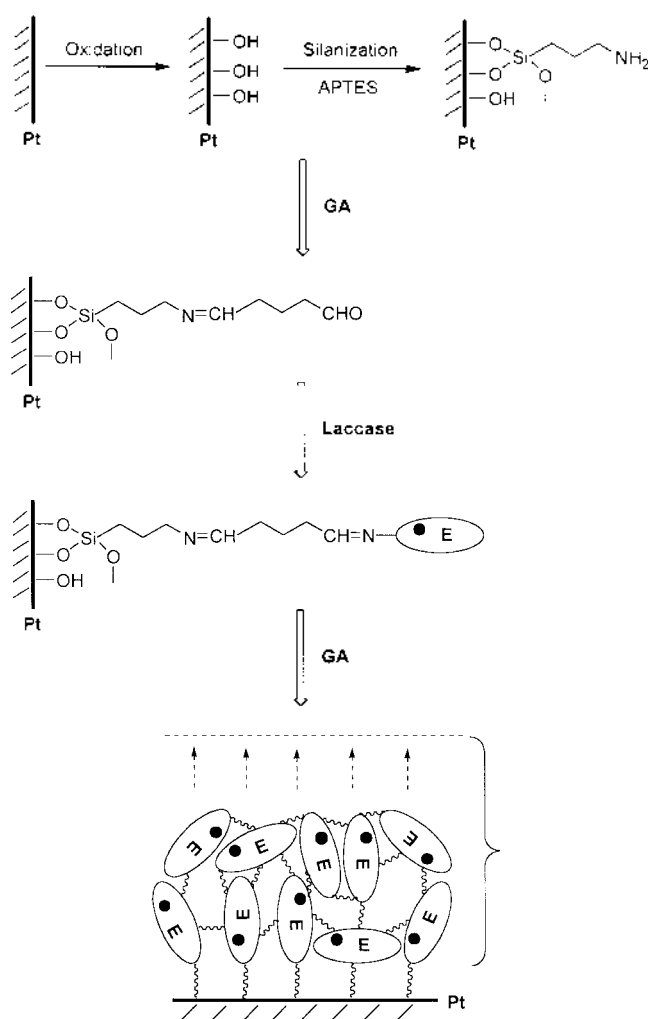
Figure 2. Schematic representation of preparing laccase-immobilized Pt electrode.

room temperature, the electrode was put into vapor of 25 % glutaraldehyde solution again for 30 minutes at room temperature to make further cross-links among the enzymes. The electrode was thoroughly washed with the buffer and stored in the same solution at 4 °C before use (Figure 2 for the schematic representation of preparing the laccase immobilized electrode).

Results and discussion

SEM image of laccase immobilized electrode. The scanning electron microscopic images of the silanized platinum electrode and the laccase immobilized one are shown in Figure 3. For the silanized platinum surface, some scratches, which were made when the electrode surface was polished with 0.3 μm alumina slurry before the silanization step, are shown. The laccase immobilized surface looks like a few μm thick polymeric structure as expected since the protein solution was dropped onto the electrode, and dried. The covalent linkages between the electrode and proteins, and among proteins should be formed by glutaraldehyde. The SEM images confirm that a stable polymeric structure of

(a)

(b)
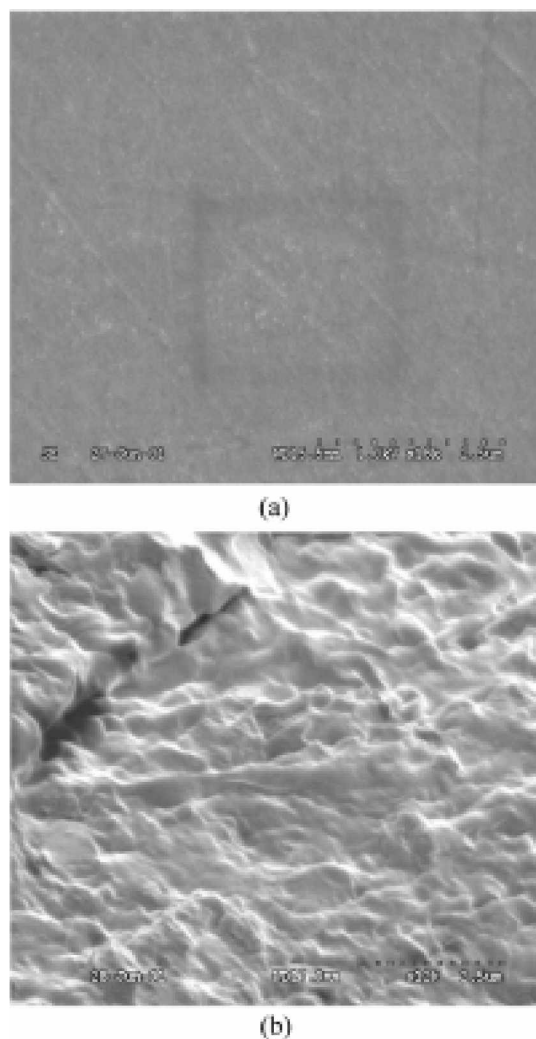
Figure 3. SEM images of silanized Pt electrode (a) and of laccase immobilized Pt electrode (b).

laccase is covered on the electrode surface. If the protein layer is constructed in a similar way on a bare Pt surface or on a silanized Pt electrode instead of a silanized and glutaraldeyde-treated one, the stability between the protein layer and the electrode is very poor and the protein layer is easily peeled off upon electrochemical oxidation/reduction steps. This shows that the covalent linkages through glutaraldehyde between the electrode and the proteins are essential in stabilizing the immobilized laccase layer.

CV of immobilized laccase. The cyclic voltammogram of the immobilized laccase in a plain buffer was taken, and no voltammetric waves were shown between 0.0 and +0.7 V vs. Ag/AgCl range where redox reactions of blue copper proteins or multicopper oxidases are reported to be occurred (Figure 4).[1] This means that a direct electron transfer is not feasible between the electrode and the immobilized laccase. and shows that the polymeric laccase structure as shown in the SEM image is non-conductive. The nearest distance between the electrode and the enzyme surface is not too far (~10 Å), therefore the redox center might be deeply buried in the enzyme and outer part of the enzyme is not a good
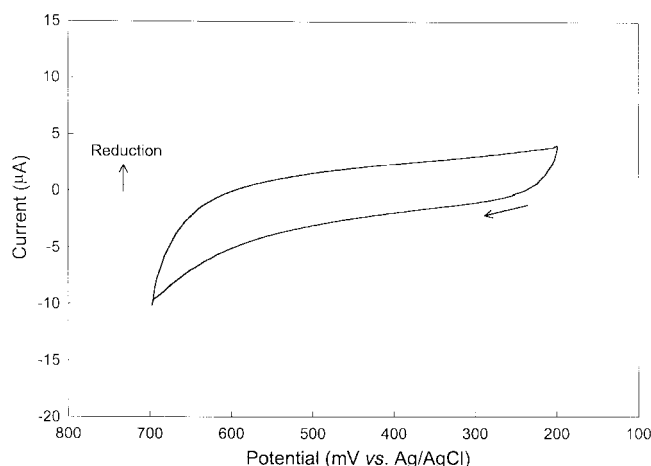

Figure 4. Cyclic voltammogram of a laccase immobilized Pt electrode (ϕ 4 mm) in 0.1 M phosphate buffer (pH 6.0) under Ar. Scan rate: 50 mV/s.

conductor to communicate electrons with the electrode surface.

Although direct electron transfer was not possible, we tried electrochemical detection of enzymatically transformed product in order to confirm the enzyme is successfully immobilized with retaining activity. The well-known substrates for laccase. ABTS and PPD were chosen, and their electrochemical responses at Pt electrode were measured first to decide the applying potential values in amperometric measurements.

Redox reactions of ABTS. It is known that ABTS undergoes two consecutive one electron oxidation around +500 and +900 mV vs. Ag/AgCl region to produce ABTS$^+$ and ABTS^{2+}.[16] The stabilities of ABTS$^+$ and ABTS^{2-} are very much dependent on solution composition, pH, electrode material, etc., therefore, we examined the voltammetric response of ABTS in our condition focused only on the first oxidation. ABTS shows quasi-reversible oxidation at Pt electrode in pH 6.0. 0.1 M phosphate buffer ($\Delta E_p = E_{pa} - E_{pc}$
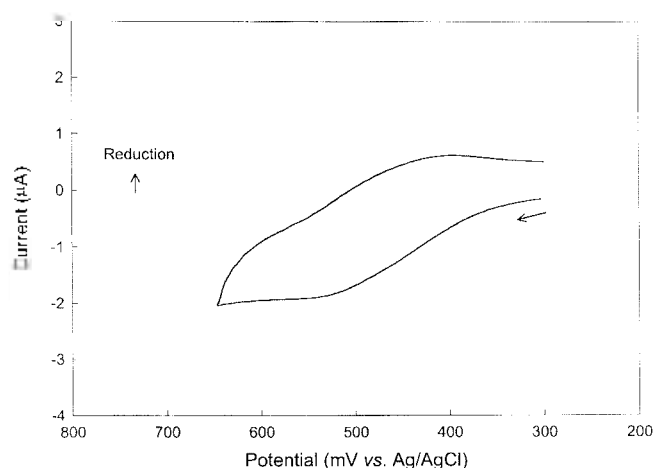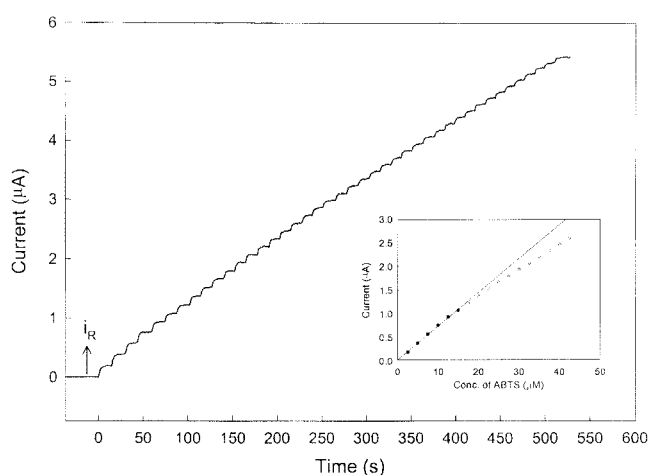

Figure 5. Cyclic voltammogram of 1 mM ABTS at Pt electrode (ϕ 1 mm) in 0.1 M phosphate buffer, pH 6.0 under Ar. Scan rate: 50 mV/s.

= +530 - 396 = 134 mV at 50 mV/sec) (Figure 5). The oxidized ABTS⁻ radical is stable in our voltammetric time scale and can be reduced back to ABTS again in +400 ~ +300 mV range. Based on the above result, we chose +350 mV as the applied potential for the amperometric measurement of ABTS on the laccase-immobilized electrode since ABTS⁻ can be generated by laccase catalyzed oxidation in the presence of oxygen. [17] In other words, amperometric detection of ABTS was conducted by electrochemical reduction of the enzymatically oxidized product.

Detection of ABTS by the immobilized laccase electrode. The laccase immobilized electrode was tested as a biosensor to detect ABTS. 2.5 μL of 5 mM ABTS solution was added consecutively to 5 mL of phosphate buffer solution (pH = 6.0) upon continuous stirring under air while holding the electrode potential at +350 mV to monitor the electrochemical reduction current (Figure 6). The electrochemical response is quite fast and a stable current response was reached in 5 seconds. As increasing the substrate concentration by successive injections, the current response increases linearly (r^2 = 0.999) up to 15 μM with a sensitivity of 75 nA/μM. For a bare Pt electrode, no current response was found in a similar experiment. This means that the enzyme is successfully immobilized with retaining activity. The immobilized enzyme catalyzes ABTS oxidation and the enzymatically oxidized product, ABTS⁺ radical can diffuse fast through the immobilized enzyme network to the electrode surface and re-reduced. The laccase-immobilized electrode can be used as a biosensor to detect ABTS and the calibration curve is shown in the inset of Figure 6. The detection limit can be estimated down to 0.5 μM. If non-linear fitting is used, up to a few hundreds μM of ABTS can be measured by this sensor. The electrochemical responses fit well to Lineweaver-Burk type plot of Michaelis-Menten equation for the kinetic analysis of the biosensor showing apparent K_M = 0.16 mM. It shows that the reaction is kinetically controlled and the enzymatic reaction is a rate-limiting process. [18] We found that the response time became faster after running several experiments, which implies that the electrode was possibly conditioned upon electrochemical activations. It will be further discussed later in experiments with PPD. The electrochemical response drops down gradually upon repetitive uses, which might be attributed to the degradation of laccase by ABTS⁺ radical. It is well known that ABTS⁻ damages laccase[19] although it is a good mediator for the enzyme. In our case, we could observe that the immobilized enzyme layer changed its color to purplish red ABTS⁺ radical after several uses, and the colored material could not be removed by washing or electrochemical redox reactions. It seems that the colored material originated from ABTS⁻ radical was incorporated into the enzyme layer strongly by electrostatic or covalent interaction. If PPD was used instead of ABTS, no color change was observed for the immobilized layer and more stable and sensitive responses were obtained.

Redox reactions of PPD and response to the laccase immobilized electrode. PPD was oxidized reversibly on Pt
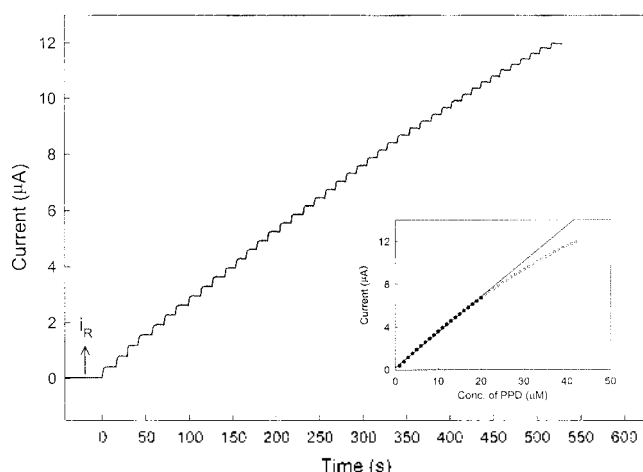

Figure 6. Current-time response of the laccase immobilized Pt electrode with increasing concentration of ABTS. 2.5 μL of 5 mM ABTS was injected into 5 mL of 0.1 M phosphate buffer (pH 6.0) at each step under air. The applied potential was +350 mV vs. Ag/AgCl. Inset: calibration curve.
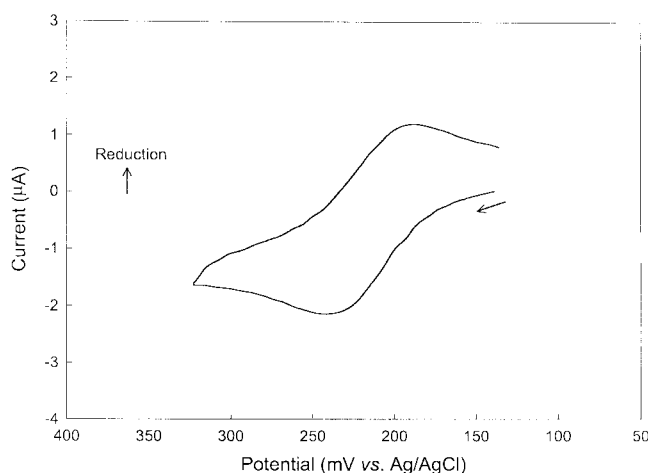
Figure 7. Cyclic voltammogram of 1 mM PPD at Pt electrode (ϕ 1 mm) in 0.1 M phosphate buffer, pH 6.0 under Ar. Scan rate: 50 mV/s.

electrode (E_{pa} = 243 and E_{pc} = 186 mV vs. Ag/AgCl. $\Delta E p$ = 57 mV. i_{pc}/i_{pa} = 0.98. at 50 mV/sec, Figure 7). PPD is known to undergo one or two electron oxidation and be further oligomerized or polymerized depending on kind of solvent and method of oxidation. [20] In our case, CV of PPD shows reversible two electron oxidation reaction in the experimental time scale, which can be judged by the voltammetric parameters. The enzymatically oxidized product from PPD, could be detected at +150 mV with a linear range (r^2 = 0.998) up to 20 μM (Figure 8) and detection limit can be estimated down to 0.05 μM. The current response was 340 nA/μM, which is 4.5 times higher than ABTS. The response time is less than 3 seconds, which is even faster than that of ABTS. If non-linear fitting is used, about a hundred μM of PPD can be detected. The responses to PPD also well fit to Lineweaver-Burk type plot and shows apparent K_M = 0.055 mM. The faster and better response for PPD might be attri-

Figure 8. Current-time response of the laccase immobilized Pt electrode with increasing concentration of PPD. 5 μL of 1 mM PPD was injected into 5 mL of 0.1 M phosphate buffer (pH 6.0) at each step under air. The applied potential was +150 mV vs. Ag/AgCl. Inset: calibration curve.

buted to the weaker interaction of the enzymatically oxidized PPD⁻ radical with the immobilized enzyme layer resulting in an easier transport through the layer to the electrode surface, although K_M for PPD is much smaller than that of ABTS. The long-term stability of the immobilized enzyme is much better for PPD than for ABTS as we'll discuss further in the following section.

Stability of the laccase immobilized electrode. The long-term stability of the electrode was monitored for two months. Figure 9 shows the relative current response of the enzyme electrode during this period by running the same experiment as shown in Figure 8. The electrode was used 3 times per day in first two weeks and once a day in the second one week. Afterwards, the frequency of using the sensor is decreased to every second days for 10 days and every 5th days for next 30 days. The electrode was stored in the buffer at 4 °C while not in use. More than 80% of the initial
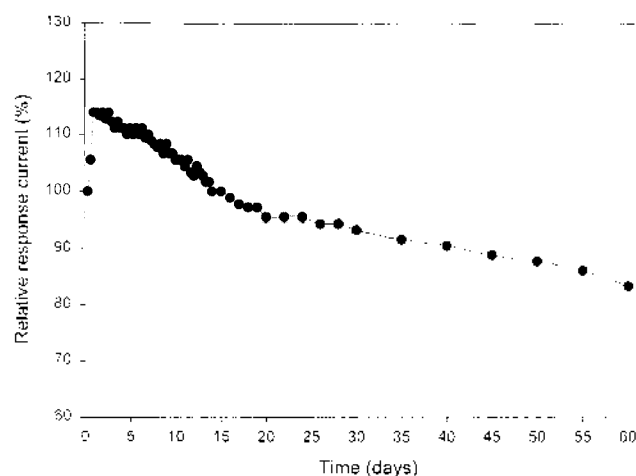
Figure 9. Long-term stability of laccase immobilized Pt electrode. The measurements for PPD were made at +150 mV vs. Ag/AgCl in 0.1 M phosphate buffer (pH 6.0) at room temperature.

response was retained after two months of uses. Therefore, the laccase-immobilized sensor we developed can be used to detect PPD more than two months without significant decrease in sensitivity. Further lifetime test for PPD and other substrates are being conducted in our lab now. In case of ABTS, the sensor response drops down to 50% after 2 weeks upon 3 times uses per day. This also confirms that ABTS⁻ is harmful to laccase.

We tested and found that the laccase solution in 0.1 M phosphate. pH 6.0 lost 30% of its activity in 5 days. On the other hand, the immobilized laccase lost less than 3% of its highest activity. It is clear that the immobilization method we developed enhances the stability of the laccase greatly, if it is compared to that in solution. The glutaraldehyde coupling is known to be able to enhance the stability of enzymes in some cases.[15]

An initial rise in sensor response and following steady state response after several uses as shown in Figure 9 is typical for the most immobilized enzyme systems.[21] This phenomenon was explained by the creation of diffusional channels for substrate transport in the enzyme layer.

We cannot compare our data with other published ones since no systematic electrochemical responses for ABTS and PPD were reported using laccase-immobilized electrode. We are investigating the responses of other well-known substrates such as catechols, diphenols. catecholamines with the laccase-immobilized electrode to make better comparison with published results and to find more versatile uses.

Conclusions

We could prepare an enzyme electrode from newly isolated laccase from a cheap commercial product. Although the glutaraldehyde coupling method is very common to link enzymes and proteins, our approach here is the first application to immobilize a laccase onto the platinum electrode. The enzyme layer could be successfully constructed on the surface of platinum electrode and it is demonstrated that ABTS and PPD can be detected down to less than a μM level. The enzyme layer showed more than two months of stable response for detecting PPD.

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17. The voltammetric behavior of ABTS on the laccase-immobilized electrode is similar to that on a bare Pt electrode except decrease in peak current. The peak potential values are not much changed. These phenomena are similar for PPD, too.

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