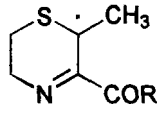
tablished by the various spectral data, and elemental analyses.[6] Similar results (whole mixture yield: 59%) were obtained with the 2-bromide **5** and 2-aminoethanethiol hydrochloride in the presence of potassium hydroxide in methanol at room temperature.

Surprisingly, a suspension of dihydro-1,4-thiazine hydrochloride **8** in methylene chloride was stable at room temperature in a nitrogen atmosphere for a few hours in the presence of water while the above mixture when subjected to air or oxygen afforded a mixture of acetylthio **6** and 1,3-thiazolidine **7** under the same conditions. Similar evidence was found in various aqueous solutions. As a result, we concluded that oxygen was involved in the rearrangement of dihydro-1,4-thiazine hydrochloride **8** in the presence of water.

Concerning the reaction mechanism, a free radical such as **11** could be considered as the reactive intermediate which is prone to autoxidation.[7] The stability of such a hybrid radical would arise from odd-electron delocalization into α/d-orbitals of sulfur as well as the possibility of an electron releasing effect, characteristic of the sulfur atom. In-



11

dependent syntheses of **6** and **7** as well as mechanistic study related to the mechanism are in progress.

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4. mp 106-109 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.85 (d, 3H, *J*=7.0 Hz, CH_3), 5.59 (q, 1H, *J*=7.0 Hz, CHBr), 7.18-7.68 (m, 5H, ArH), 8.71 (br. s, 1H, NH); ms: m/z 256 (M^+).
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6. For **6**: mp 155-157 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.36 (s, 3H, $COCH_3$), 3.10 (t, 2H, *J*=9.6 Hz, SCH_2), 3.56-3.59 (m, 2H, NCH_2), 7.31-7.67 (m, 5H ArH), 8.15 (br. s, 1H, NH), 9.48 (br. s, 1H, NHC_6H_5); ^{13}C NMR (78.5 MHz, $CDCl_3$) δ 29.09, 31.15, 40.30, 120.68, 125.99, 129.80, 137.17, 157.99, 159.66, 161.03; Anal. Calcd. for $C_{12}H_{14}N_2O_3S$: C, 54.13, H, 5.26, N, 10.53, S, 12.03. Found: C, 53.9, H, 5.25, N, 10.3.
For **7**: mp 141.5-143 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.57 (s, 2H, 2-$COCH_3$), 2.73 (br. s, 1H, 3-NH), 3.00-3.10 (m, 2H, 5-CH_2), 3.74-3.80 (m, 2H, 4-CH_2), 7.09-7.61 (m, 5H, ArH), 9.50 (br. s, 1H, NHPh); ^{13}C NMR (78.5 MHz, $CDCl_3$) δ 26.06, 37.41, 53.77, 86.55, 119.60, 124.78, 129.08, 137.24, 167.20 167.20, 200.23; Anal. Calcd. for $C_{12}H_{14}N_2O_2S$: C, 57.57, H, 5.64, N, 11.19, S, 12.81, Found: C, 57.86, H, 5.47, N, 11.27.
7. Similar autoxidations of benzothiazines and cyclic pyrindene were reported. See Carelli, V.; Moracci, M. M.; Liberatore, F.; Cardellini, M.; Lucarelli, M. G.; Marchini, P.; Liso, G.; Reho, A. *Int. J. Sulfur Chem.* **1973**, *3*, 267. and Cohen, L. A.; Witkop, B. *J. Am. Chem. Soc.* **1955**, *77*, 6595.

Immobilization of Laccase onto the Gold Electrode Using β-Mercaptopropionate

Kyung Hee Hyung, Kyu Yeon Jun, Hun-Gi Hong[†], Hyun Soo Kim[†], and Woonsup Shin*

Department of Chemistry, Sogang University, Seoul 121-742, Korea
[†]Department of Chemistry, Sejong University, Seoul 133-747, Korea
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Immobilization of enzymes onto the electrode surfaces is very important in developing biosensors and enzyme reactors for industrial applications.[1] The immobilization is essential to reuse expensive enzymes and to facilitate the separation of reaction products without sacrificing the enzyme. In this communication, we report the immobilization of *Rhus vernicifera* laccase from Chinese lacquer tree onto the β-mercaptopropionate coated gold electrode. There have been several reports dealing with the immobilization of fungal laccases, and their uses for some catalytic reactions.[2] They were immobilized onto the graphite electrode by adsorption or in polymer films such as gelatin or polyurethane. Our effort in this paper is the first example of the immobilization of tree laccase onto the gold electrode using a self-assembly technique.[3]

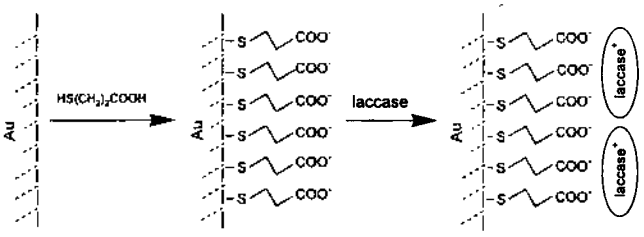
Laccase (*p*-diphenol : O_2 oxidoreductase, EC 1.10.3.2) is a copper containing oxidase which is widely distributed in higher plants and fungi.[4] It belongs to multicopper oxidases

*To whom correspondence should be addressed.

including ascorbate oxidase and ceruloplasmin which are capable of 4-electron reduction of dioxygen to water. Laccase has a rather low specificity with regard to the reducing substrates which are the source of electrons to oxygen through the enzyme. In other words, substrates which are hardly oxidized directly by oxygen can be oxidized easily in the presence of laccase. The substrates are various kinds of mono-, di-, polyphenols (hydroquinone, 4-methylcatechol) and amines (p-phenylenediamine, 2,7-diaminofluorene, ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)).[5]

The electrode modification was performed as follows. First, a gold electrode was thoroughly cleaned according to the published procedure[6] and immersed into 3 mM β-mercaptopropionate solution in ethanol for 1 day. The negatively charged electrode surface could be produced by the carboxylic group of β-mercaptopropionate after making an self-assembly on the gold surface. Second, the electrode was thoroughly washed with water and immersed in 0.16 mM laccase containing solution for 1 day at 4 °C. The isoelectric point (pI) of the tree laccase is around 10 and the enzyme has a net positive charge in neutral solution. The enzyme could be attached to the negatively charged β-mercaptopropionate-modified electrode successfully by the electrostatic interaction and a schematic diagram for the preparation of the laccase-immobilized gold electrode can be depicted as Scheme 1.

The laccase-immobilized electrode was thoroughly cleaned with water and transferred to 0.1 M phosphate buffer solution (pH 7.0). Cyclic voltammograms were taken at 50-500 mV/sec. Typical voltammograms at 100 mV/sec and 500 mV/sec are shown in Figure 1. The cathodic peak has a typical bell-shape of a surface-bound redox reaction. The cathodic peak potential is +300 mV *vs.* NHE and invariant to scan rate up to 200 mV/sec. It shifts about 10 mV cathodically at 500 mV/sec. The cathodic peak current increases linearly as the scan rate increases up to 500 mV/sec. The anodic peak is broader than the corresponding cathodic peak and broadens further as increasing the scan rate. The anodic peak current relative to the corresponding cathodic peak current is about unity at low scan rate but decreases as increasing scan rate. The anodic peak potential is +360 mV *vs.* NHE and invariant to scan rate up to 200 mV/sec. The anodic peak potential shifts more anodically at higher scan rate, but the accurate determination is difficult since the peak broadens too much. The peak potential difference (ΔE_p) is around 60 mV and is not much changed upon increasing scan rate up to 200 mV/sec. The formal potential ($E^{0'}$) of this redox couple is +330 mV *vs.* NHE taken as the average of the cathodic and anodic peak potentials.



Scheme 1. Schematic diagram for the preparation of the laccase-immobilized gold electrode using β-mercaptopropionate.

These results are consistent with the surface-confined redox reaction and verify that the enzyme is successfully immobilized. The peak potential difference of 60 mV may come from the quasi-reversibility of the electron transfer in the immobilized enzyme. The broadening and decreasing of anodic peak as increasing the scan rate can be explained as follows. The reduction of copper site can cause a conformational change of the protein structure since the preferred geometry of Cu(II) and Cu(I) sites are quite different

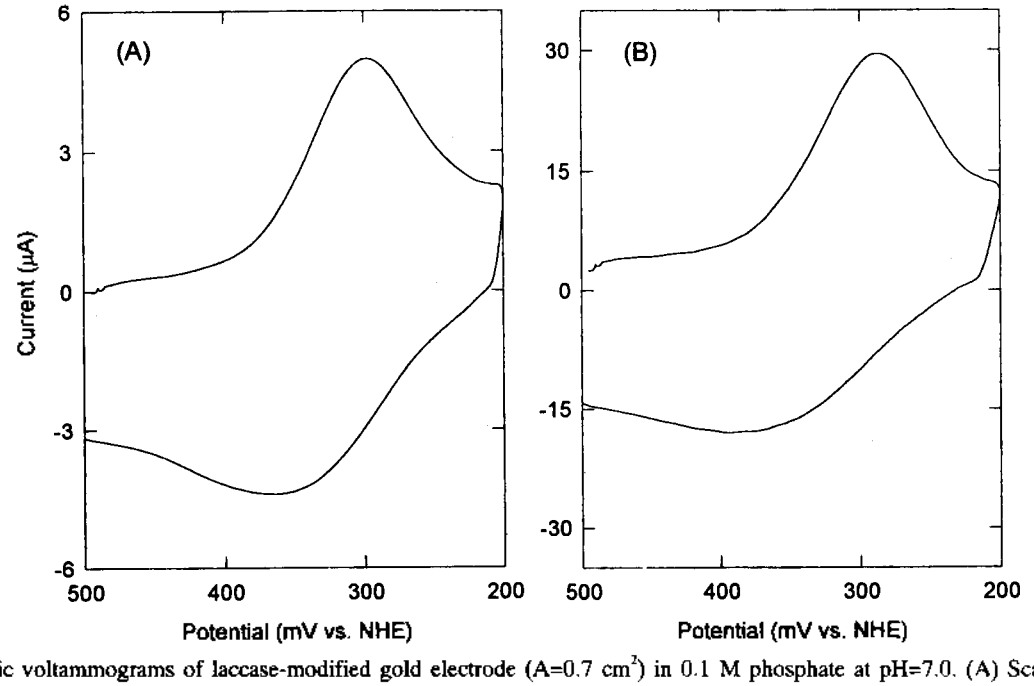


Figure 1. Cyclic voltammograms of laccase-modified gold electrode (A=0.7 cm^2) in 0.1 M phosphate at pH=7.0. (A) Scan rate: 100 mV/sec, (B) Scan rate: 500 mV/sec.

in normal copper complexes. Redox dependent conformational changes of metalloproteins are quite common.[7] It takes time for the reduced protein to change its conformation back to the shape that can be oxidized easily, and its consequence is shown in the anodic shape of the voltammogram. The conformation change could be seen in our experimental time scale which can be estimated as 0.1-1 seconds.

The redox potential of the metal site of the tree laccase has been previously determined potentiometrically (+394 mV at pH 7.5, +430 mV at pH 7.0. Different mediators are used).[8] The redox potential of +330 mV (at pH 7.0) measured in our experiments for the surface-bound laccase is a little different from the values measured for the free-laccase in solution. This implies that some structural change of protein occurred upon binding to the surface. The structural change can modify the geometry of the metal site and results in the shift of the redox potential of the site. Bare Pt, Au, glassy carbon electrodes were unsuccessful to obtain cyclic voltammogram of laccase since the size of the enzyme is quite big (M.W.=110,000) and the direct electron transfer between the electrode and the enzyme is not feasible. However, the β-mercaptopropionate coated gold electrode could transfer electrons to laccase and cyclic voltammogram could be obtained. The peak current is initially big and decreases as time goes on. The shape of the voltammogram changes to bell shape and the redox potential shifts toward more negative direction to +330 mV from initial +380 mV. The shape of the voltammogram becomes the same as the immobilized one finally. This implies that β-mercaptopropionate coated gold electrode can communicate with laccase in solution initially and the surface-confined redox process remains only after laccase is immobilized onto the electrode. This also supports the idea of the redox potential shift upon immobilization. The details of these phenomena are currently being investigated.

ABTS oxidation capability was used to determine the activity of the immobilized enzyme.[8] The laccase-immobilized-electrode was immersed in 1 mM ABTS containing solution and the absorption spectra of the solution were taken with time without applying potential. The green color of $ABTS^+$ developed as the electrode was immersed. The absorbance at 405 nm (ε_{405}=35,000 $M^{-1}cm^{-1}$) increases with time and flattens after ~70 hours at 4 °C (Figure 2). This means the activity of the immobilized enzyme is sustained around 70 hours. To assure the activity was not from the free laccase which might be around in solution, the enzyme-bound electrode was transferred to a fresh ABTS solution at each measurement and the rate of absorbance change was used to assess the activity of the enzyme. The rate of the absorbance change was not altered upon transfer and we could conclude that the enzyme was still active in bound state.

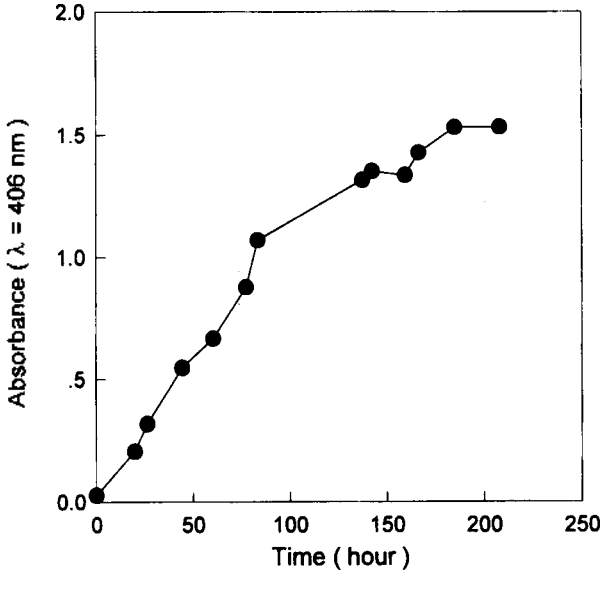


Figure 2. ABTS oxdation by the immobilized laccase: 1 mM ABTS in 8 mM MES (4-Morpholineethanesulfonic acid) buffer; pH=5.3, under air, T=4 °C.

We are now trying to characterize the enzyme-bound surface in more detail using other spectroscopic techniques. Also, we are investigating the possibility to use the laccase-modified electrode as an electrocatalyst for oxygen reduction. Other immobilization techniques are currently being used to compare the results in this paper.

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