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Anthraquinone and *L*-amino Acid Derivatives Schiff Base Cu(II) Complexes as a Mediator between Cathode of Biofuel Cell and Oxygen-reducing Laccase

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Abstract

Background: Cathode materials of biofuel cells sometimes contain mediator (metal complexes) to improve electron transfer efficiency between electrode and laccase. Laccase is an enzyme which catalyzes four-electron reduction of oxygen to water.

Methods: We have synthesized new four anthraquinone and *L*-amino acid (*L*-alanine, *L*-valine, *L*-leucin, and *L*-isoleucin) derivatives Schiff base Cu(II) complexes aiming at low cost materials using abundant Cu metal instead of Os or Ru metals employed commonly.

Conclusion: By means of spectroscopic, electrochemical, and docking simulation studies, the complex of *L*-valine derivative ligand exhibited the best performance as a mediator among these four complexes in view of low reduction potentials, high current density, and appropriate docking features.

Keywords: Biofuel cell; Oxygen reduction; Chiral Schiff base; Copper(II); Laccase

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Introduction

Laccase will not form the intermediate water-soluble peroxide, and can catalyze a four-electron reduction of molecular oxygen to water. This electron reduction, in a peptide polymer, is due to work by four Cu active sites. Cu sites of laccase are divided into three types largely from their features, called type 1 (S-coordinated blue copper), type 2 (normal), and type 3 (O-bridged dinuclear). The type 1 site is found in electron transport chain proteins, with the coordination of cysteine, because they exhibit a blue color due to the strong charge transfer (CT) transitions, also referred to as "blue copper" [1-5].

Type 1 Cu site showing blue color due to strong ligand to metal charge transfer (LMCT) absorption at about 600 nm by coordination of cysteine residues, and they are generally contained in electron transfer metalloproteins. Their electronic spectra exhibit characteristic band from UV region to NIR region besides d-d bands. ESR spectra of type 1 copper exhibit hyperfine coupling, owing to afford a twisting tetrahedral coordination geometry (by His, His (imidazole-N), Met (S), and Cys (S) typically to be Td symmetry or also by additional peptide main chain to be extended C_{3v} symmetry ideally) as well as mixed valence between

Cu(II) and Cu(I) states. Due to the features, redox potential is relatively high value (0.2-0.8 V vs. NHE). Type 2 copper (namely normal copper sites) does not appear such characteristic color and takes a mononuclear coordination environment (by imidazole-N and COO⁻-O) with various geometries. Type 3 copper is dinuclear active centers of phenol-O bridged diamagnetic Cu(I)-Cu(I) or antiferromagnetically coupled Cu(II)-Cu(II) sites (ESR silent), whose magnetic or spectroscopic properties are affected by bond lengths and angles of the dinuclear moieties, in other words, electronic states depend on steric factors. From surface of protein molecule, electrons are provided from (potentially oxidized) substrates and are received by type 1 copper and transferred to the trinuclear cluster of type 2 and type 3 about 1.3 nm distance from the type 1 site, which catalyzes four-electron reduction of oxygen (important process of redox reaction) without forming O₂⁻, H₂O₂, OH⁻ finally. In this way, laccase is called multicopper oxidase (MCO) [2,6,7].

The reaction between the enzyme and the electrode is called direct electron transfer (DET) type catalytic reaction, and in this system, enzyme functions in electrode reaction [8,9]. In the

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case of DET type catalytic reaction, enzyme-interfacial electron transfer rate between the electrodes come directly involved in the performance of the battery. The distance between the redox center of the enzyme and the electrode is short, and easy through the electronic, it reorientation energy on oxidation-reduction is small; it leads to accelerate the electron transfer rate. However, it is very difficult to control these factors in enzyme. On the other hand, a method of improving the electron transfer rate between the enzyme and the electrode has also been proposed [10,11].

In contrast, when the molecular redox materials (electron transfer mediator) sandwiches between the enzyme and the electrode, a method to smooth the electron transfer is ready. This is called the mediator (MET) type enzyme function electrode reaction, can be adapted to most of the oxidation-reduction enzyme system, the selection of the mediator, modification methods must be important [12-16].

In this paper, employing aldehyde precursor of anthraquinone derivatives [17] as the basic backbone, and the amino acid was treated to be synthesized new Schiff base Cu(II) complexes (**Figure 1**). By the use of several kinds of complexes, and suitable method for forming composites of laccase and these Cu(II) complexes, comparison of differences in steric structure of the complexes to fit the hydrophobic region sites at the surface laccase was one of the subject of this research.

Materials and Methods

General procedures

Chemicals of the highest commercial grade available (solvents from Kanto Chemical, organic compounds from Tokyo Chemical Industry and metal sources and MWCNT from Wako) were used as received without further purification. Laccase from *Trametes versicolor* was purchased from Sigma-Aldrich (St. Louis, MO USA). Basically, 2-hydroxy-9,10-dioxo-9,10-dihydroanthracene-1-carbaldehyde was prepared according to the literature method (formylation) [18] with modification of using a Biotage Initiator+microwave synthesis device in trifluoroacetic acid at 403 K for 90 min [19].

Preparations of complexes 1-4

Treatment of 2-hydroxy-9,10-dioxo-9,10-dihydroanthracene-1-carbaldehyde (0.10 g, 0.40 mmol) and NaHCO_3 (0.034 g, 0.40 mmol), and *L*-alanine (0.036 g, 0.40 mmol) in a mixed solution of methanol (50 mL) and water (10 mL) at 333 K for 3 hr, copper(II) acetate monohydrate (0.080 g, 0.400 mmol) were added and stirred for 2 hr to give rise to green precipitates to be filtrated and washed with methanol. Complexes 2-4 were also prepared in similar procedures to **1** using *L*-valine, *L*-leucin, *L*-isoleucin, respectively, instead of *L*-alanine. UV-vis and circular dichroism (CD) spectra and cyclic voltammogram (CV) were depicted in **Supplementary Figures 1** and **2**, respectively.

1: Yield 36.6%. Anal. Found: C, 55.81; H, 3.27; N, 3.96%. Calcd for $\text{C}_{36}\text{H}_{26}\text{CuN}_2\text{O}_{10}$: C, 58.89; H, 3.66; N, 3.62%. IR (KBr): 1628 cm^{-1} (s) (C=N), 1672 cm^{-1} (s) (C=O).

2: Yield 55.5%. Anal. Found: C, 58.49; H, 3.51; N, 3.17%. Calcd for

$\text{C}_{40}\text{H}_{34}\text{CuN}_2\text{O}_{10}$: C, 57.90; H, 4.13; N, 3.38%. IR (KBr): 1624 cm^{-1} (s) (C=N), 1679 cm^{-1} (s) (C=O).

3: Yield 42.2%. Anal. Found: C, 55.81; H, 3.27; N, 3.96%. Calcd for $\text{C}_{42}\text{H}_{38}\text{CuN}_2\text{O}_{10}$: C, 58.80; H, 3.51; N, 3.27%. IR (KBr): 1625 cm^{-1} (s) (C=N), 1677 cm^{-1} (s) (C=O).

4: Yield 44.8%. Anal. Found: C, 58.80; H, 4.02; N, 2.98%. Calcd for $\text{C}_{42}\text{H}_{38}\text{CuN}_2\text{O}_{10}$: C, 58.80; H, 3.51; N, 3.27%. IR (KBr): 1631 cm^{-1} (s) (C=N), 1675 cm^{-1} (s) (C=O).

Docking of complex(1-4)+laccase

For electrochemical or spectral measurements, hybrid materials of complex(**1-4**)+laccase to discuss docking of complex and laccase are prepared as complex solutions (6 μM in 70 mL water:metanol=9:1 solvent) after addition of laccase by 1 mg as solutions of the same solvent.

Preparations of cathode (complex+laccase+MWCNT) [19,20]

For electrochemical measurements as cathode, 1 mg of a complex (**1-4**) was dissolved in 5 mL of methanol and 8 mL of pure water and 50 mg of laccase was added at 293 K for 0.5 hr. After centrifuging at 15000 rpm for 20 min, 1 mL of water and MWCNT (3-20 nm diameter; non-uniform lengths) were added to the precipitates and cast 40 μL of the solution on GC electrode and added 20% methanol solution of glutaraldehyde. After drying

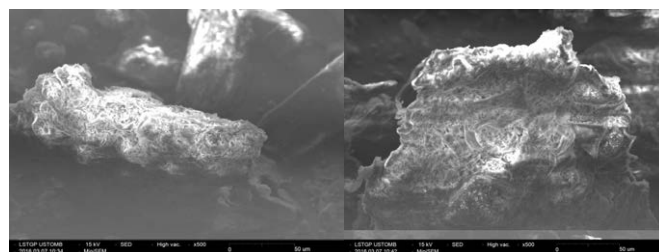


Figure 1 Structures of complexes **1-4** (-R=-CH₃ (**1**), -CH₂(CH₃)₂ (**2**), -CH₂CH(CH₃)₂ (**3**), -CH(CH₂)(C₂H₅) (**4**)).



Figure 2 (a) Spectroelectrochemical changes of **2**. (b) Distribution of HOMO and LUMO of **2** by TD-DFT calculations.

the electrode, 1% aqueous solution of pyrrole was coated. The resulting cathode [19,20] electrode served to electrochemical tests.

Physical measurements

Elemental analyses (C, H, N) were carried out with a Perkin-Elmer 2400II CHNS/O analyzer at Tokyo University of Science. Infrared spectra were recorded as KBr pellets on a JASCO FT-IR 4200 plus spectrophotometer in the range of 4000-400 cm^{-1} at 298 K. Electronic (UV-vis) spectra were measured on a JASCO V-570 UV/VIS/NIR spectrophotometer in the range of 800-200 nm at 298 K. Circular dichroism (CD) spectra were measured as KBr pellets on a JASCO J-820 spectropolarimeter in the range of 800-200 nm at 298 K. (Spectro-)electrochemical (cyclic voltammetry, CV) measurements were carried out on a BAS SEC2000-UV/VIS and ALS2323 system with Ag/AgCl electrodes range of -0.50–0.80 V vs. Ag/Ag⁺.

Computational methods

All calculations were performed using the Gaussian 09W software Revision A.02 (Gaussian, Inc.) [21]. The gas phase geometry optimizations were carried out using TD-DFT with B3LYP functional. The vertical excitation energy was calculated with the LanI2dz for Cu and with the 6-31+G(d) basis set for H, C, N and O method based on the singlet ground state geometry.

Results

Characterization of 1-4

In UV-vis spectra of all **1-4** (Supplementary Figure 1), intense $\pi-\pi^*$ and charge transfer (CT) bands appeared at 260-390 nm and about 560 nm, respectively regardless of carbon numbers of ligands [22-27]. CD spectra of **1-4** (Supplementary Figure 1) are reasonably corresponds to UV-vis spectra of identical complexes. CV recorded with carbon paste working electrodes for **1-4** (Supplementary Figure 2) exhibited similar redox behavior attributed to dinuclear Cu(II)/(I) ions (two peaks around 0.1-0.2 V) and anthraquinone moieties of ligands (additional peaks) [20].

Combined with redox behavior (reduction -0.9 V) and UV-vis spectra, the results of spectroelectrochemical measurements for **1-4** were also performed after 0, 10, 20, 40, 50, 60, 100, 140, and 180 s (Supplementary Figure 3). For example, gradually spectral changes of **2** (Figure 2 (a)) can be explained as follows: Reduction of Cu(II) ion and anthraquinone moieties in the fully oxidized form continued to be the fully reduced form. A peak around 400 nm is MLCT (metal to ligand charge transfer) band due to electron transfer from reduced Cu(I) ion to ligands. Weak intensity at about 410 nm may be ascribed to disappeared $n-\pi^*$ band due to formation hydroquinone from quinone. Red shift of peak around 450 nm is expansion of π -conjugated system by reduction of anthraquinone moieties. Distribution of electron density (Figure 2 (b)) of fully oxidized form also supports this interpretation of MLCT. Excitation from HOMO to LUMO leads to electron transfer from Cu(II) ion to ligand.

Docking of complex and laccase

Figure 3 and Supplementary Figure 4 exhibit changes of CD (and UV-vis) spectra of a constant amount of complex (**1-4**) solutions by gradual addition of laccase under the conditions above [28]. Little changes of CD peak around 230 nm (characteristic to α -helix) suggested keeping activity of laccase without denaturation [29-31]. On the other hand, emerging additional bands suggests interaction between a complex and laccase. Both CD and UV-vis spectra, no additional bands could be observed for **1** and **3**. However, increasing CD intensity at 230 nm and new UV-vis spectral bands at about 250 and 360 nm were observed for **2**. Whereas changes of CD intensity at 288 and 370 nm and new UV-vis spectral bands at 272 nm were observed for **4**, which suggests hydrogen bonds between laccase and a complex. Consequently, experimental results reveal that **2** and **4** bind to laccase well, while **1** and **3** cannot bind to laccase completely.

Figure 4 and Supplementary Figure 5 exhibits docking simulation (CCDC GOLD suits ver. 5.3.0) of laccase (PDB 1GYC) and **1** (left) or **2** (right) optimized by TD-DFT calculation [32]. Mediator complexes, electron transfer between electrode and laccase, are assumed to be included in the hydrophobic pocket around type 1 site of laccase molecules. As summarized in Table 1, the degree of fitness was evaluated by fitness scores,

$$\text{Fitness} = S(\text{hb_ext}) + 1.3750S(\text{vdw_ext}) + S(\text{hb_int}) + S(\text{int})$$

where

$$S(\text{int}) = S(\text{vdw_int}) + S(\text{tors})$$

$S(\text{hb_ext})$ = protein-ligand hydrogen bond energy (external H-bond)

$S(\text{vdw_ext})$ = protein-ligand van der Waals (vdw) energy (external vdw)

$S(\text{vdw_int})$ = ligand internal vdw energy (internal vdw)

$S(\text{tors})$ = ligand torsional strain energy (internal torsion).

The order of docking scores **1** > **2** >> **3** > **4** suggests that both **1** and **2** bind to laccase well theoretically. However binding feature of **1** is different from that of **2** (Figure 4). Both parts of ligands bind to the hydrophobic pocket of laccase for **2**, while half part of ligand manages to bind to laccase and most part of molecule of **1** are out of the pocket for **1**. As experimental results also indicated, only **2** are in the pocket.

Discussion

Electrochemical measurements as a mediator

In cathode of biofuel cell, sole laccase catalyzes four-electron reduction of molecular oxygen showing a reduction peak around

Table 1 Fitness scores (GOLD) of docking of laccase and **1-4**.

	Fitness scores	S(hb_ext)	S(vdw_ext)	S(hb_int)	S(vdw_int)	S(Tor)
1	35.77	2.14	40.10	0.00	-15.62	-5.88
2	32.52	3.21	39.37	0.00	-18.61	-6.21
3	20.99	0.00	37.35	0.00	-21.79	-8.59
4	18.56	1.88	34.98	0.00	-24.58	-6.85

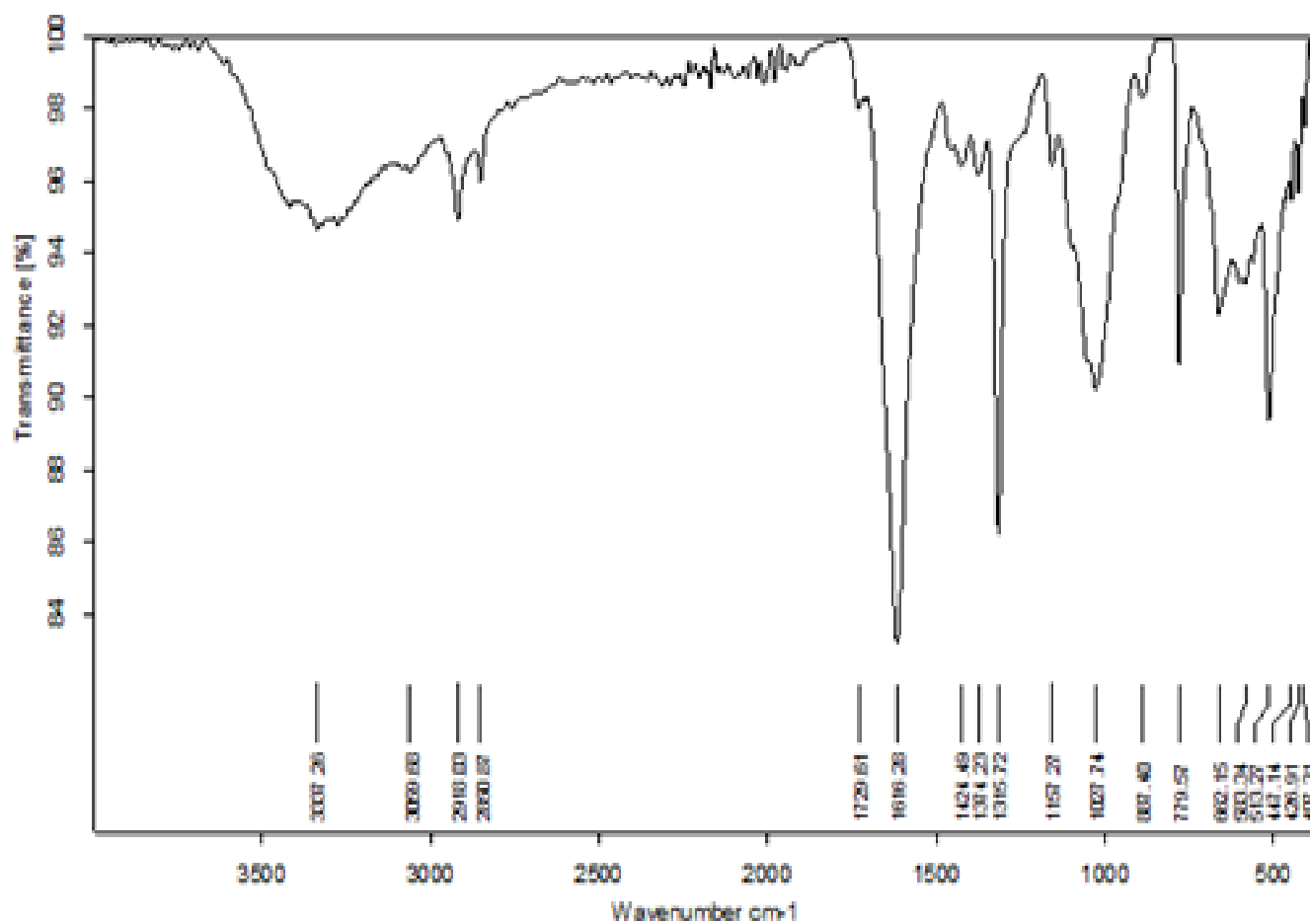


Figure 3 (above) Changes of CD spectra of **1-4** (6 μ M in 70 mL water:metanol=9:1 solvent) after addition of laccase by 1 mg. (below) Comparison of CD spectra for laccase and a complex (**1-4**).

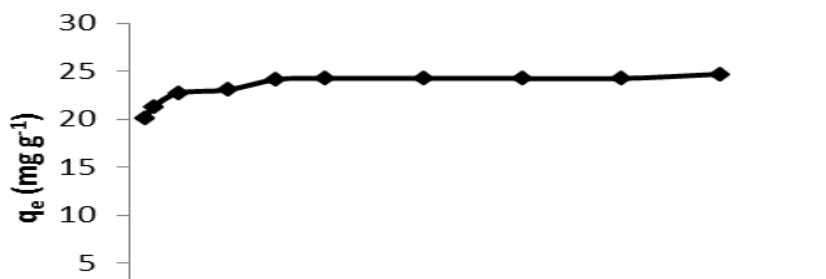


Figure 4 Docking simulation (GOLD suite ver. 5.3.0) of laccase (PDB 1GYC) and a **1** (left) and **2** (right) optimized by TD-DFT.

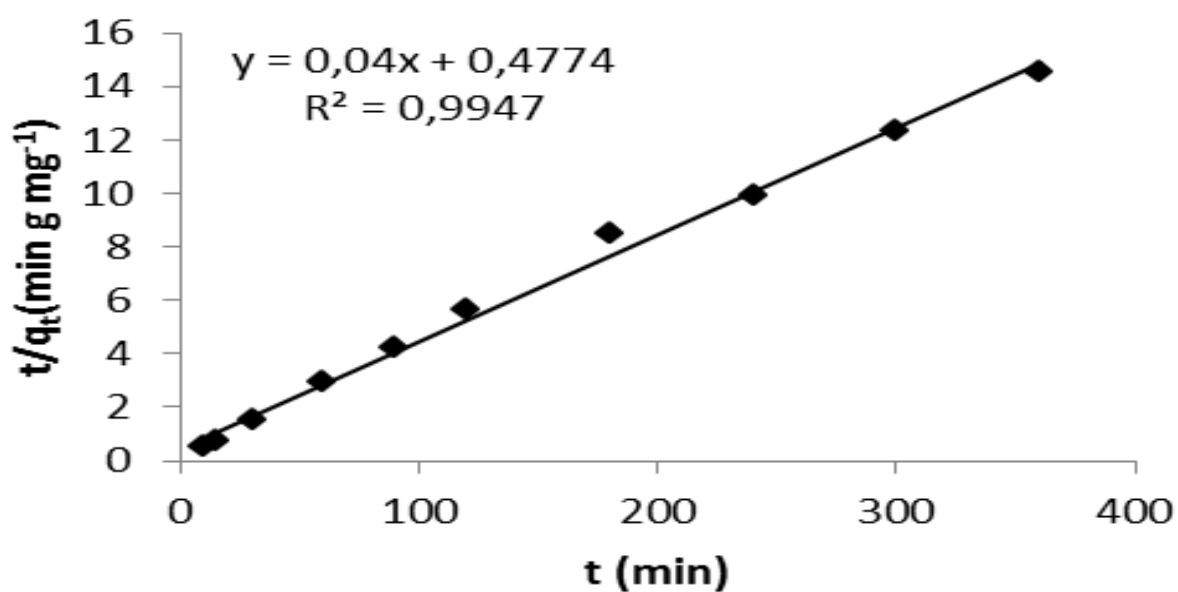


Figure 5 The CV (vs Ag/AgCl, scan rate 0.05 V/s, in acetate buffer pH 5.0, 200 mM) of oxygen reduction reactions with cathodes containing complex(1-4)+laccase+MWCNT.

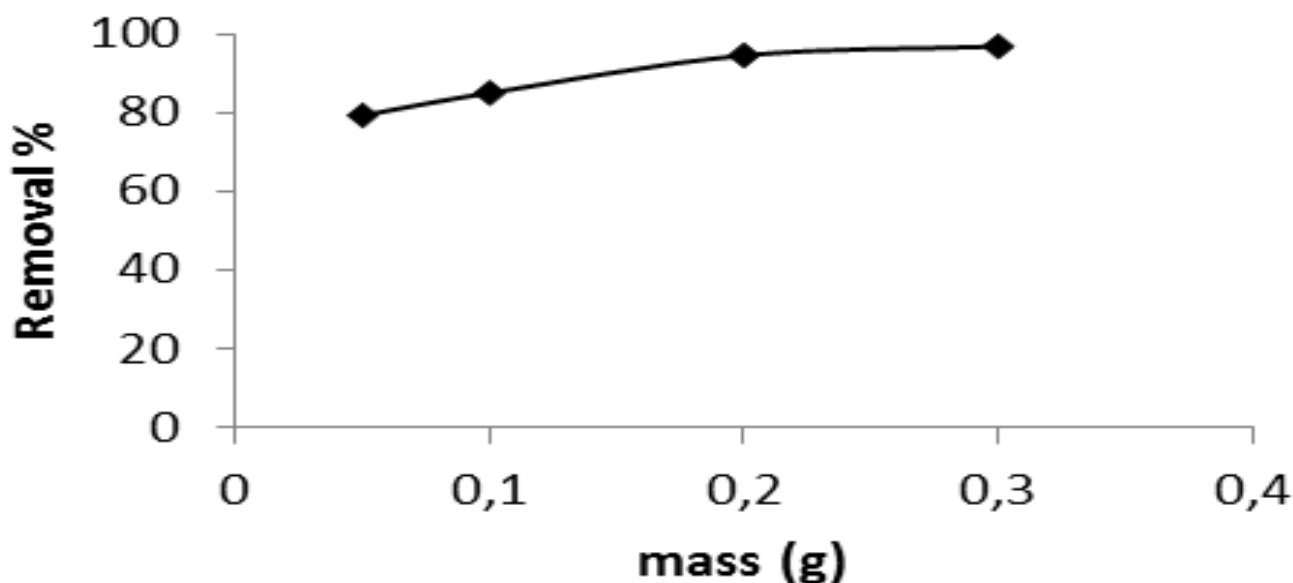


Figure 6 (left) OCP of laccase and laccase + **1-4**. (right) schematic summary of potentials of oxygen reduction.

0.6 V (vs. Ag/AgCl) and reduction of the type 2 site showing a peak around -0.01 V (**Supplementary Figure 5**), which was confirmed by enhancing current density from nitrogen purged system to oxygen purged one (**Supplementary Figure 6**) [33].

The performance of cathodes containing **1-4** prepared by the procedure in the previous section was compared (**Figure 5**). In all CVs a peak of oxygen reduction by laccase was appeared. The current density per area of **2** exhibited the largest value among four CV, which also indicated the highest amount of oxygen reduced. The highest performance as a mediator for **2** is in agreement with well docking with laccase supported by both spectral change features and docking scores.

After CV tests, in order to confirm potentials of oxygen reduction by laccase, OCP measurements were carried out under oxygen purged conditions (**Figure 6**). Reaction of oxygen reduction occurs at 0.93, 0.59, and 0.51 V for natural oxygen, catalyzing by laccase, and catalyzing by **2**+laccase, respectively. This decreasing of potentials indicated that catalytic performance of laccase was improved by the best mediator (**2**) effectively.

Conclusion

In conclusion, valine-derivative **2** exhibited the best performance as a mediator between cathode and laccase among four Cu(II) complexes showing similar redox behavior of both dinuclear Cu(II)/(I) ions and anthraquinone moieties. Spectral changes and docking simulations suggested appropriately docking of **2** towards the hydrophobic pocket of the molecular surface of laccase near type 1 sites (for oxidation of substrate naturally [34]), which is advantage for transferring electrons. Improperly docking features of **1** and low docking scores of **3** or **4** ascribed to disadvantage for the function as a mediator. Actually, electrochemical results also supported the highest performance of **2**. From different views, other methods have been reported recently, [35] indeed, and we are also proposing good way to improve oxygen reduction electrodes.

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