

In: Threonine
Editor: Jacob Coleman

ISBN: 978-1-63482-554-2
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Chapter 4

SPECTROSCOPIC AND ELECTROCHEMICAL STUDIES ON METALLOPROTEIN (LACCASE) AND CU(II) COMPLEX MEDIATORS AS MODEL SYSTEMS FOR BIOFUEL CELL CATHODES

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ABSTRACT

Serine, threonine, and alanine are majorly contained amino acid residues in laccase, a metalloprotein which reduces oxygen into water. In which threonine may potentially play a role in intermolecular interaction toward ligands of small molecules and catalytic group. Laccase are employed in typical biofuel cell cathodes with a mediator metal complex such as ferrocene.

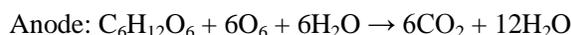
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In this paper, we report on oxygen reduction by laccase with other metal complexes known electron mediators in acetate buffer suspension and in carbon paste electrodes. Furthermore, in order to develop low-cost mediators, we prepared and tested some Cu(II) complexes, namely $[\text{Cu}(\text{phen})_2]^{2+}$, $[\text{Cu}(\text{phen-derivative})_2]^{2+}$, and $[\text{Cu}(\text{Schiff base})_2]$, which were characterized by means of elemental analysis, IR, UV-vis, and CD spectroscopy. Hybrid systems of the complexes and laccase were also prepared for comparison current and potentials of oxygen reduction.

INTRODUCTION OF BIOFUEL CELLS

Generally, the lifetimes of fossil fuels are limited, for example, oil, natural gas, and coal is 40, 60, and 200 years, respectively. Various technologies have been developed as alternative energy especially we focus on biofuel cells in this chapter. Biofuel cells generate electricity (flow of electron to external circuit as energy from negative electrodes) by organic matter oxidized by enzyme or microbial cells. In principle, it is clean devices without increasing CO_2 , because they merely exchange organic matters to electric power using certain catalysts. Among several types of fuel cells, biofuel cells are consisted of enzymes (protein of biocatalysts) and work under mild conditions, namely room temperature and ambient pressure.

Theoretically, biofuel cells react to generate electric power by the following reactions.



Evaluation of oxidation-reduction (redox) potentials of substrates and products is important for performance as battery. As for glucose ($\text{C}_6\text{H}_{12}\text{O}_6$) as substrate, which are oxidized to CO_2 at the anode. On the other hand, O_2 is simultaneously reduced to water at the cathode. These redox potentials are -0.42, +0.82 V respectively, therefore the electric potential difference is +1.24 V [1]. Since 1 mol of glucose generate 24 mol of electron, Faraday's law of electrolysis provides the resulting quantity of electricity from 1 g of glucose for an hour is 3.57 Ah ($24 \times 9.6485 \times 10^4 / (3600 \times 180) = 3.57$), of which electric energy is 1.8 Wh. Compared with the similar electric energy of nickel metal hydride battery (3.9 Wh) indicating high energy density, biofuel cells are suitable for power source of mobile electric appliances.

In addition, the substrate of biofuel cells in the solid state devices may be safer than those of conventional fuel cells using metal catalysts (for example hydrogen or methanol).

In this way, various types of fuel cells (Figure 1) have both merits and weak points. The first biofuel cells containing enzyme was reported in 1964 [2] and fundamental studies on them have mainly been continued so far to improve low electric power generation. Some enzyme biofuel cells generating electric power from abundant biofuel are composed of oxidoreductase [3, 4], whose advantage is simple structure (possibility for easy miniaturization), safety, metal or inorganic catalyst free, and low environmental burden.

On the other hand, there are many serious problems about biofuel cells at present. At present, theoretical conversion efficiency of electron from glucose may be less than 10 %, in which a number of enzymes found in metabolism systems are used without matching each other. Furthermore, stability, especially, durability of enzyme may also be problems, which should be overcome for practical application. For example, durability of enzyme against pH is improved by gene recombination [5, 6]. New immobilization method or immobilization of enzyme in sol-gel matrix maintaining biocompatibility may be expected to improve durability for electric transfer directly from enzyme electrode. As for activity as catalytic systems, gold nano-particles or some carbon nano-materials are expected to improve electron transfer when they bind to enzyme using high surface with porous structures of enzymes. As for cell voltage and output power, new design of biofuel cells (of course maintaining biocompatibility) has been developed from the view points of materials chemistry or chemical engineering.

Among such problems and improvement, “mediators” are employed to enhance electron transfer between enzymes and electrodes (bioanodes or biocathodes) regardless of surface area restriction.

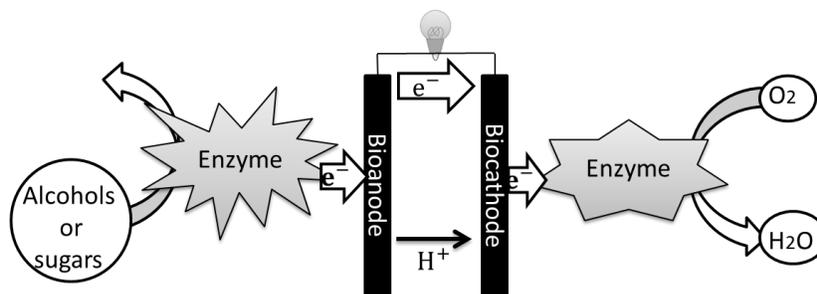


Figure 1. Schematic reaction mechanism of typical biofuel cells.

Kinetics of electron transfer about energy conversion dominates current, because mediators may be key component to improve the efficiency of biofuel cells. In biochemistry, mediator can exchange electrons if metabolic degradation of organic matters proceeds sufficiently. Appropriate mediators indicate similar redox potentials to enzymes (Figure 2, Table 1) [7]. This is molecular design strategy for mediators to avoid low rates of electron transfer as well as proton transfer from anode to cathode or diffusion rates of substrates and oxidant in electrodes, and consequently resulting current density.

In the last section of this paper, our conceptual development of mediators using transition metal complexes without expensive metals will be reported. To adjust proper redox potentials, not only metals but also organic ligands should be designed.

Transition metal complexes are impossible to be included inner space of enzymes, while have advantage to conduct current efficiently. Whereas metal nano-particles are possible to be included inner space of enzyme selectively (better for spatial reasons), while it is hard for them to carry much current.

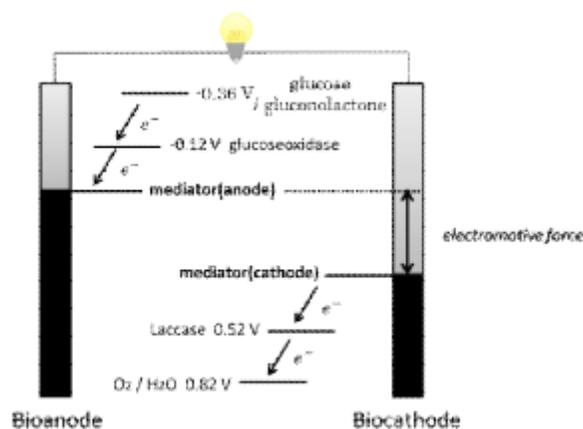


Figure 2. Appropriate redox potentials required for mediators.

Table 1. Redox potentials (vs SCE) for various mediators [8, 9]

mediators	Redox potentials (V)	mediators	Redox potentials (V)
ferrocene	0.2	ABTS	0.65
Fe-porphyrin	0	[Fe(CN) ₆] ³⁻	0.5
[Os(bpy) ₂ Cl ₂] ³⁺	0.1	p-quinone	0.25
[Ru(bpy) ₂ Cl ₂] ³⁺	0.3		

Therefore, we focused on metal complexes as target candidates for suitable mediators.

Moreover, other methodologies have been applied to prepare electrodes. Fixing mediators and enzyme on electrode surface is typical method to enhance electron transfer associated with electrochemical reactions. From this viewpoint, it was reported that fixed polymers binding to osmium complexes and multicopper oxidase (MCO), reducing O₂ to water, exhibit a few mA cm⁻² current density [10]. In this system, not only dissolved oxygen but also oxygen gas can be catalyzed by this cathode of a biofuel cell.

LACCASE AS A CATALYTIC ENZYME

Laccase, one of the members of MCO family, has attracted a great deal of attention because of that utility of biofuel cells. The reason is because laccase including four copper active sites can catalyze 4-electron reduction of oxygen except to yield water-soluble peroxide as intermediates. All three types of copper active sites are including into laccase. Depending on spectral properties, they can be classified into so-called type 1, type 2, and type 3.

Type 1 copper (also called blue copper) showing blue color due to strong ligand to metal charge transfer 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 T_d 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 like type 1 and takes a mononuclear coordination environment (by imidazole-*N* and COO⁻-*O*) with various geometries.

Type 3 copper is binuclear 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 binuclear moieties [10, 11], in other words, electronic states depend on steric factors.

As shown in Figure 3, from surface of protein molecules, electrons are provided from (potentially oxidized) substrates 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 4-electron reduction of oxygen (important process of redox reaction) without forming O^{2-} , H_2O_2 , OH^- finally. In this way, laccase is called multicopper oxidase (MCO).

One of the most important factors of these redox reactions may be reaction rate. MCO can react more effectively than copper efflux oxidase (indicating photocatalytic electrode activity) or inorganic Pt catalyst (indicating overpotential) at room temperature, ambient pressure, and neutral pH conditions. Though manifold MCO perform in weak acidity condition, proper pH for manifold enzyme is almost neutral. Neutral condition is necessary for final target of biofuel cells *in vivo*. Not all metalloproteins can be investigated directly by means of electrochemical methods, because active site is far from surface of electrodes and slow reactions of electron transfer.

METAL COMPLEXES PLAYING BIOINORGANIC ROLES

Traditionally, determination of molecular structures is the most important aspects of studies on coordination chemistry.



Figure 3. Structure of laccase with copper active sites.

Structures of chlorophyll are well investigated because of their importance in photosynthesis associated with plants. Besides molecular structures, these molecules form supramolecular assemblies of characteristic arrangements to concentrate light. Their structural studies are in progress at present [12].

Natural chlorophylls contain manganese (Figure 4), while zinc substituted ones are also prepared as attractive model compounds due to their stability [13]. A lot of design of such complexes has reported and applied to various field such as computational chemistry [14], application for dye sensitized solar cells [15].

As for synthetic metal complexes, for example, some binuclear copper complexes (Figure 5) indicate interesting catalytic properties (expected as cooperative reactions) as well as magnetic properties of given spin states [16], which cannot be observed for mononuclear copper complexes [17, 18]. Since they form complicated systems, suitable model compounds should be prepared for detailed studies.

Copper is one of the most common metal elements like iron or zinc. Various valence states, Cu(I), Cu(II), and Cu(III) can be found in (electron transferring) metalloproteins containing copper. As Lewis acids, Cu(I), Cu(II), and Cu(III) are soft, middle, and hard acids, respectively.

According to HSAB theory, Cu(I) tends to coordinate to soft bases such as sulfur ligands. Coordination numbers range from 2 to 12, while 4, 5, 6-coordinated complexes are found widely.

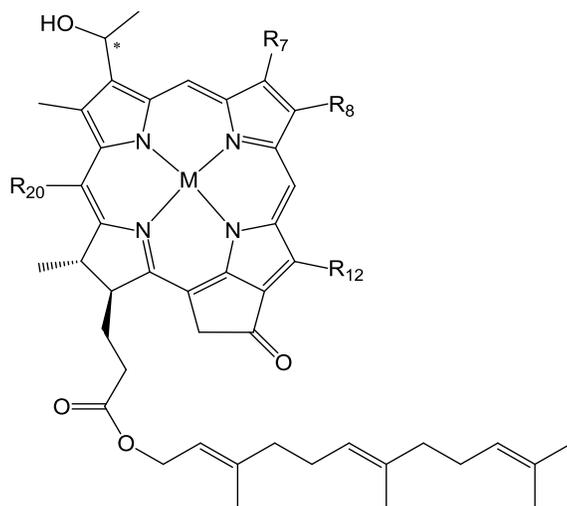
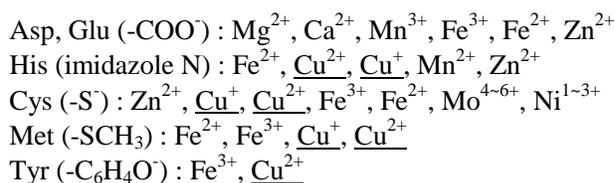


Figure 4. Typical structure of chlorophyll.

A Cu(I) complex commonly affords a tetrahedral coordination geometry, while a Cu(II) complex flexibly affords square planar, trigonal bipyramidal, or distorted octahedral coordination geometries. In actual copper proteins exhibiting redox reactions, structural features can be controlled by valence states as well as steric restriction by peptide chains.

The affinity of complexes to residue of amino acid is classified as follow roughly.



Bioinorganic chemistry deals with Werner type complexes, while bioorganometallics deals with non-Werner type ones having not lone-pair donating coordination M-L bonds between Lewis acid (M) of metal and Lewis base (L) of ligands but covalent M-C(carbon) bonds. Various structures of them have been also determined by means of X-ray crystallography.

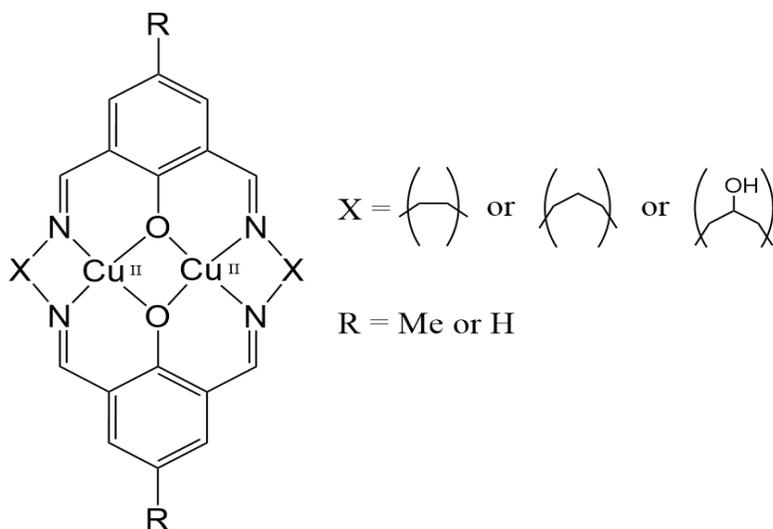


Figure 5. Functional binuclear copper complexes.

Structures of complexes lead to expect their properties, hence structural determination is important. Indeed, ferrocene was studied by Wilkinson and Fischer since early 1952 [19].

DOCKING OF METAL COMPLEXES AND PROTEINS

Metal complexes, which have various structures, show multifarious biochemical behavior [20]. Metal complexes can also be useful as a homogeneous catalysis similar to biocatalysts not only sole species but also hybrid catalysts with biopolymers (proteins or DNA), which is called artificial metalloenzymes or 'Artzymes'. Artificial metalloenzymes indicate high selectivity for substrate by designing ligands or combination with components, though they are weak against heat. Their functions can be applied organic reactions such as hydrogenation, benzannulation and Diels-Alder [21-23].

To date, docking of metal complexes into protein has been studied, though accurate binding features have not been elucidated clearly.

However, development of protein-ligand docking simulation software, for example GOLD or Protein DF, enables docking simulation, and becomes clear about accurate bind formation to discuss catalytic activity [24] based on information proteins-ligands or proteins-proteins [25].

PLELIMINARY STUDY ON NEW COPPER MEDIATORS

In this section, we report on testing some complexes as mediators and preliminary results of electrochemical and spectroscopic properties. Commonly, ferrocene or osmium complexes (Figure 6) binding to polymers are used for this purpose. The source of osmium metal is expensive relatively.

Organic/inorganic hybrid materials composed of metal complexes and polymers have potential to emerge various functions, for example, exhibiting photochromism by containing azo-groups [26, 27], emission [28], or chirality [29]. Herein we expand their application to the cathode of biofuel cells of mediators and laccase to improve electron transfer between electrode and enzyme. Although osmium or ruthenium complexes are usually employed as mediator, they are commonly expensive. Then, we prepared some copper(II) complexes, **1**, **2**, **3**, and **4** according to the literatures [30-33], and ferrocene **5** (Figure 7) expected as low costs and abundant resources and tested them.

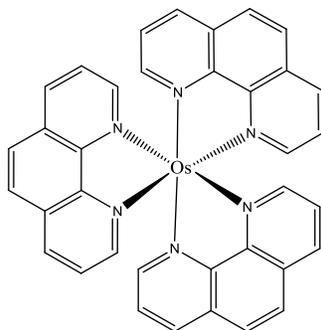


Figure 6. Example of an osmium complex as a mediator. Valence states are omitted for clarity.

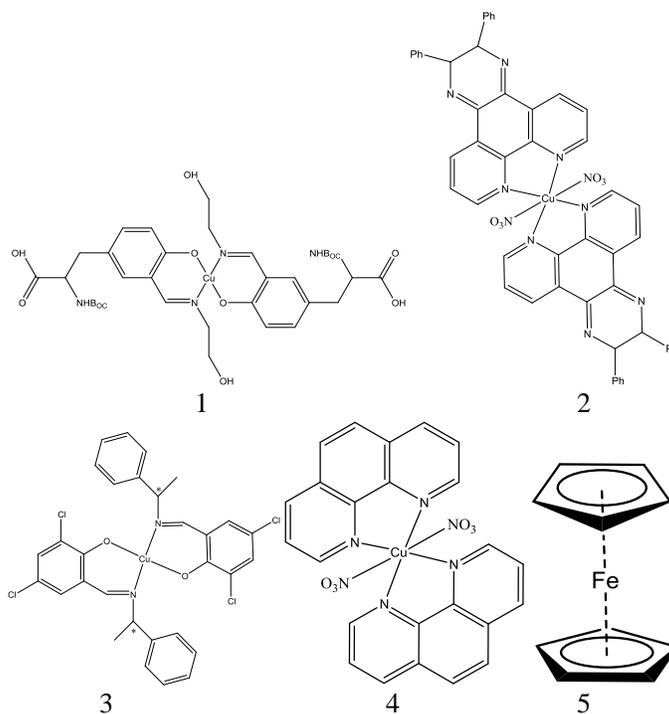


Figure 7. Complexes **1-5** tested as mediators.

Complexes **1-5** have been investigated as the mediators to laccase as hybrid systems [34]. Complex **4** is bis(1,10-phenanthroline)copper(II) cation, which is typical example of copper(II) complexes. Besides copper(II) complexes, only complex **5** is ferrocene (iron (II/III) complex) which is used a

reference substance for redox reactions commonly. In acetate buffer (pH 4.5, 50 mM), UV-vis spectra appeared predominant peaks at 220, 340, and 360 nm for **1**, 212, 252, 285, 365 nm for **2**, 211, 240, 280, 304, and 380 nm for **3**. As for chiral complexes, CD spectra appeared predominant peaks at 220, 340, and 360 nm for **1**, 233, 256, 300, 360, and 400 nm for **3**.

CV (cyclic voltammetry) (in DMSO with 0.1 M TBAP, at 100 mV/s) exhibited oxidation/reduction potentials of Cu(II)/Cu(I) at 0.42/0.18 V for **1**, -0.16/-0.26 V for **2**, -0.12, -0.36, -0.54/ -0.16, -0.44, -0.68 V for **3**, -0.14/0.24 V for **4** (and for comparison, 0.25/0.15 V for **5**) (Figure 8). All redox reactions are reversible, and only **3** exhibited multi-step reaction accompanying with organic ligands including halogen.

On the other hand, CV data were also measured using carbon paste electrode in order to apply for metal complexes-laccase hybrid systems, in which laccase was supplied commercially. In acetate buffer (pH 4, 200 mM, at 50 mV/sec), CV (Figure 9) exhibited oxidation/reduction potentials at 0.3/-0.4 V (5 μ A) for laccase (as control), 0.1, 0.3, 0.5/-0.3, -0.1 V for **1**, 0.3/-0.1 for **2**, 0.1/-0.3 for **3**, 0.3, 0.0/0.1, -0.1 for **4**, and 0.4/0.2 for **5**. Only **1** and **4** exhibited multi-step reactions in the conditions. As for metal complexes-laccase hybrid systems whose potential indicated negative shifts, observed current values (potentials) were 27 μ A (-0.3 V) for **1**+laccase, 10 μ A (-0.3 V) for **2**+laccase, 100 μ A (-0.3 V) for **5**+laccase. No current was observed for **4**+laccase.

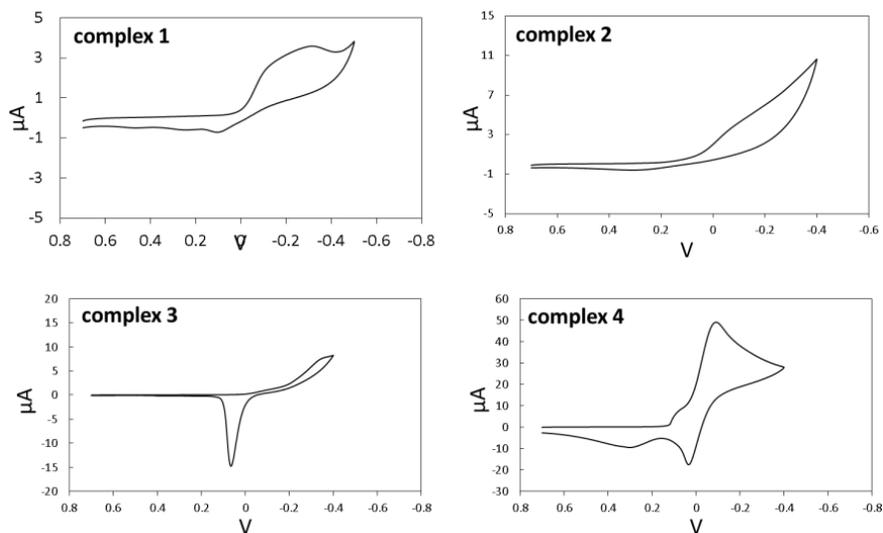


Figure 8. CV data for complexes **1-4**.

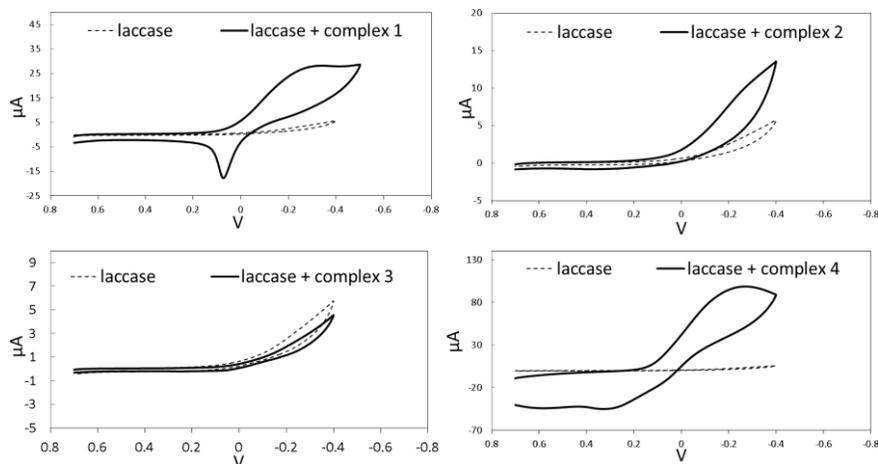


Figure 9. CV data for hybrid systems of **1-4**+laccase.

Consequently, compared with control value of 115 μA (0.1 V) for **5**+laccase, **4**+laccase may be the best mediator systems among the present copper(II) complexes (Figure 9). Correlation between geometrical or electronic structures (binding to proper sites or residues of proteins by molecular recognition) and electrochemical functions (especially large current for a good mediator) will be elucidated in future.

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