Promoting of direct electron transfer of multicopper oxidase by control of enzyme molecule density on multi-walled carbon nanotube

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Abstract
In order to improve the performance of direct electron transfer-type electrode using multicopper oxidase (MCO), it is important to shorten the distance between the redox site of the enzyme and the electrode surface to increase electron transfer efficiency between enzyme and electrode. In this study, we focused on the mobility of the MCO from hyperthermophilic archaeon, Pyrobaculum aerophilum, immobilized onto electrode surface via an affinity tag at the MCO terminus. The mobility of the immobilized enzyme was controlled by changing the density of the immobilized enzyme on the electrode surface by altering the density of the linker for enzyme immobilization. The electrode with low density of MCO immobilized on electrode surface was improved swing ability of the enzyme. It showed 265% higher current density for electrochemical O2 reduction than that with high density of MCO immobilized on electrode surface. Biofuel cell using a cathode with a low density of MCO immobilized on the electrode showed 160% higher power density than a biofuel cell using a cathode with a high density of MCO immobilized on the electrode.

1. Introduction
Enzymatic biofuel cells (EBFCs) are very attractive as clean energy for a sustainable society. EBFCs oxidize biological substances such as organic acids, alcohols, amino acids, and sugars, and biofuel cells (BFCs) convert chemical energy into electrical energy using enzymes as the electrode catalyst [1–4]. However, practical application of EBFCs has not been achieved yet, because of the low power density of EBFCs and the short life of the battery due to low long-term stability of the enzyme.

In order to improve the lifespan of EBFCs, we have investigated biocathodes using enzymes from hyperthermophile as an electrode catalyst, which have high long-term stability compared with enzymes from mesophilic organisms [5–8]. EBFC using multicopper oxidase (MCO) from hyperthermophilic archaeon Pyrobaculum aerophilum (McoP) as the cathode catalyst and pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) from P. aerophilum as the anode catalyst maintained output of 70% after 14 d [5]. Therefore, in this study, McoP was used as a biocatalyst for the biocathode.

In a previous study, we examined a mediated electron transfer-type cathode, in which redox mediator carries electrons between enzyme and electrodes, using 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as a mediator [5]. On the other hand, direct electron transfer (DET)-type electrodes, in which electron transfer occurs directly between electrode and enzyme, has several advantages because it does not use a mediator; has no potential drop; and has no toxicity. Therefore, recently, DET-type electrodes are required for high cell voltage EBFCs and implantable EBFCs. For this reason, we have investigated a DET-type cathode using McoP [9–11]. In this study, the construction of DET-type electrode using McoP was also examined.

MCOs have been known as DET-type enzyme, and the DET-type electrode using MCOs have been the most studied [12–14]. MCOs have four copper atoms classified into Type1 (T1), Type2 (T2) and Type3 (T3)
according to their spectroscopic and magnetic properties. T1Cu receives electrons from the electrode and catalyzes the reduction of dioxygen to water at the trinuclear center consisting of one T2Cu and two T3Cu. Therefore, when constructing DET-type electrode using MCOs, it is important to promote heterogeneous electron transfer by shortening the distance between the electrode and the T1Cu of the MCO immobilized enzyme on the electrode surface [15, 16]. In order to shorten the distance between T1Cu and the electrode, oriented immobilizations of MCO onto the electrode surface by induced-fit using substances similar to the substrate of MCO modified on the electrode surface, or by specific binding via amino acids modified on the enzyme surface by site-directed mutagenesis reported [17, 18]. We have also been studying the oriented immobilization of enzymes using affinity tags introduced to the enzyme to improve the electron transfer between enzyme and electrodes [9–11]. Briefly, a pyrene derivative having an NTA group was modified onto multi-walled carbon nanotube (MWCNT) surface by π–π interaction, which was then coordinated with Ni²⁺. After that, McoP was immobilized in certain orientation by the affinity between the His-tag introduced at McoP terminal and Ni²⁺. The oriented–McoP immobilized electrode showed current density of 190 mA cm⁻² [9]. However, a DET-type cathode using MCO showing a current density of 1 mA cm⁻² or more has been reported [19, 20]. Therefore, further improvement of the DET-type electrode using McoP is required.

In a previous study, we reported that a current density of C-terminal his-tagged McoP oriented electrode was 1.3 times higher than that of N-terminal His-tagged McoP oriented electrode [11]. This is because, in McoP, the C-terminal His-tag is closer to T1Cu than the N-terminal His-tag. Therefore, in the C-terminal His-tag McoP oriented electrode, the distance between T1Cu and the electrode surface is closer than that in the N-terminal His-tag McoP oriented electrode. However, due to the use of a linker to modify Ni²⁺ that binds specifically to the His-tag onto the electrode surface, the distance between T1Cu of immobilized McoP and the electrode surface was estimated to be more than 4 nm, which is difficult for electron transfer to occur [10]. Nevertheless, the oriented–McoP immobilized electrode showed good results. From this result, we assumed that McoP immobilized via His-tag was ‘swinging’ and electron transfer occurred when the redox site of McoP and the electrode surface approached. Furthermore, it has been reported that the protein immobilized via affinity tag is swinging [21, 22]. Takatsuji et al reported the relationship between the swing ability and the enzyme activity of an enzyme immobilized on a substrate surface using a fusion protein consisting of HFBI, a class II hydrophobin, tagged with glucose oxidase (GOx–HFBI) with a flexible amino acid linker [21]. The amount of GOx on the substrate was controlled by changing the mixing ratio of GOx–HFBI and HFBI. The swing property of the immobilized GOx was evaluated by the dissipation value (ΔD), which indicates the rate of oscillation decay, obtained from quartz crystal microbalance (QCM) measurements. In addition, it was shown that the enzyme activity of GOx immobilized on substrate was promoted as the ΔD value increased. Thus, in this work, we focused on the ‘swing ability’ of McoP immobilized onto electrode surface via His-tag to construct a high-performance DET-type electrode.

In the present study, as mentioned above, McoP was immobilized on the surface of MWCNT in certain orientation using pyrene derivative (figure 1). Figure 1(a) shows an electrode prepared using only a kind of pyrene derivative (1-pyrenecarboxylic acid N-hydroxysuccinimide ester; PBSE). In figure 1(a), McoP is immobilized at high density. In an electrode on which McoP is immobilized at high density, swing of McoP molecules is hindered by steric hindrance with peripheral McoPs. As a result, it becomes difficult for T1Cu of McoP to approach the electrode surface, and electron transfer between T1Cu and the electrode is limited. Figure 1(b) shows the electrodes prepared in this work. McoP was immobilized via the His-tag using PBSE, NTA and Ni²⁺ as in previous reports. In addition, the amount of McoP on the electrode surface was controlled by changing the mixing ratio of PBSE and 1-pyrenecarboxylic acid (PCA). Since Ni²⁺ is not coordinated to PCA, McoP is not immobilized on PCA and PCA functions as a spacer that expands the distance between McoPs on the electrode surface. The electrode on which McoP is immobilized at low density was decreased steric hindrance between immobilized McoPs and was increased swing ability of McoP on the electrode surface. That is, T1Cu of McoP easily approaches the electrode surface. As a result, the distance between T1Cu and the electrode is shortened and electron transfer is promoted.

The evaluation for electrochemical O₂ reduction of oriented McoP immobilized electrodes was investigated by linear sweep voltammetry (LSV). The QCM measurements were performed by imitating a modified MWCNT surface on a gold electrode using mixed self-assembled monolayer (SAM), and revealed changes in the amount of McoP immobilized onto the electrode surface and swing ability. Finally, the prepared electrode was used as a cathode for EBFC (figure 2). In addition, the bioanodes were prepared using carbon cloth (CC) as the electrode and PQQ-GDH as the catalyst. The EBFC was evaluated by LSV. This study provided a new approach for the construction of high-power DET-type electrodes.
2. Methods

2.1. Reagents and instruments
A 1-pyrenebutyric acid N-hydroxysuccinimide ester (PBSE) and ABTS were purchased from Sigma-Aldrich (Dorset, UK). N-(5-amino-1-carboxypentyl)iminodiacetic acid (AB-NTA), 11-hydroxy-1-undecanethiol (HUT) and 3,3′-Dithiobis[N-(5-amino-5-carboxypentyl)propionamide-N′,N′-diacetic acid] dihydrochloride (Dithiobis(C2-NTA)) was obtained from Dojindo Laboratories (Kumamoto, Japan). MWCNTs (φ = 10–30 nm, L = 1–2 µm) and PCA was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). PQQ-GDH from microorganism was purchased from TOYOBO (Osaka, Japan). CC (EC-CC1-060) was obtained from TOYO Corporation (Tokyo, Japan). Water used in the present study...
was first deionized and passed through a Milli-Q water purification system (Millipore, Burlington, MA, USA).

2.2. Preparation of His-tagged McoP
Plasmid pET-15b/PAE1888 carrying the pae1888 gene was used as the His-tagged McoP expression vector [9]. The protein was expressed in *Escherichia coli* BL21-CodonPlus (DE3)-RIPL expression host (Agilent Technologies, Palo Alto, CA, USA). In brief, His-tagged McoP was purified by heat treatment at 80 °C for 10 min, anion-exchange chromatography, and Ni-NTA affinity chromatography. ABTS was used as substrate to determine MCO activity of His-tagged McoP in the whole procedure, as described previously [9]. One unit of activity is defined as the amount of enzyme to oxidize 1 µmol of substrates per min at 50 °C.

2.3. Preparation of pyrene derivative-MWCNT complex dispersion
A 1 mg of MWCNT, 0.12 mg of PBSE and 0.66 mg of PCA were added into 1 ml of 50 mM HEPES-NaOH buffer (pH 7.5) (HEPES buffer). The mixture was sonicated using a bath-type sonicator at 28 kHz for 3 h. After sonication, the unreacted reagents were removed by centrifugation. The resulting pellets of pyrene derivative-MWCNT complex were washed three times with HEPES buffer, then resuspended in HEPES buffer.

2.4. Immobilization of His-tagged McoP on the MWCNT
His-tagged McoP was immobilized on the MWCNT surface in accordance with the method proposed by Amano *et al* [9]. Briefly, the MWCNT surface was modified with NTA linkers to confer Ni²⁺ holding capacity on the MWCNTs. A 2.7 mg of AB-NTA was added to 1 ml of the pyrene derivative-MWCNT complex dispersion and incubated at 25 °C for 2 h, and NiCl₂ was added and incubated at 25 °C for 30 min. By centrifugation, the unreacted reagents were removed. The MWCNT pellets were washed twice with HEPES buffer, resuspended in His-tagged McoP solution (2 mg ml⁻¹), and incubated at 25 °C for 5 min (MWCNT–McoP). After incubation in His-tagged McoP solution, the washing step was repeated. The obtained MWCNT–McoP complex was stored at 4 °C until further use. The MCO activity of the MWCNT–McoP was measured using ABTS, as described in section 2.2.

2.5. Preparation of MWCNT–McoP complex modified electrode
The MWCNT–McoP complex dispersion (1 mg ml⁻¹) was dropped onto a glassy carbon electrode (GCE) (No.136002012, geometrical area: 0.07 cm², BAS Inc.) and the electrode was dried at 25 °C for 3 h. After drying, 0.5% Nafion solution (Merck KGaA, Darmstadt, Germany) was dropped onto the electrode and dried at 25 °C for 1 h.

2.6. Electrochemical experiments
LSV was formed using ALS electrochemical analyzer Model 1200B (BAS Inc. Tokyo, Japan). A conventional three-electrode electrochemical cell was assembled using a Pt wire as a counter electrode, Ag/AgCl as a reference electrode and a modified GCE as the working electrode. All the potential values given below are reported with respect to the Ag/AgCl reference electrode. A 100 mM glycine-HCl buffer (pH 3.0) saturated with Ni-NTA and 0.2 mM 11-hydroxy-1-undecanethiol was first deionized and passed through a Milli-Q water purification system (Millipore, Burlington, MA, USA). In brief, His-tagged McoP was purified by heat treatment at 80 °C for 10 min, anion-exchange chromatography, and Ni-NTA affinity chromatography. ABTS was used as substrate to determine MCO activity of His-tagged McoP in the whole procedure, as described previously [9]. One unit of activity is defined as the amount of enzyme to oxidize 1 µmol of substrates per min at 50 °C.

2.7. Swing ability evaluation of immobilized His-tagged McoP by QCM
The QCM electrode (geometrical area: 4.909 × 10⁻⁴ cm²) was washed using Piranha solution (30% H₂O₂/H₂SO₄ (1:3, v/v)). To construct a His-tagged McoP-modified QCM electrode, an Au electrode was immersed in mixed SAM solution contained 8 × 10⁻⁵ mM C₆-NTA and 0.2 mM 11-hydroxy-1-undecanethiol at 25 °C for 3 h. Then the electrode was immersed in 100 mM NiSO₄ at 25 °C for 30 min. Finally, His-tagged McoP solution (2 mg ml⁻¹) was dropped on the electrode surface and kept at 25 °C for 30 min. The QCM
measurements were performed in HEPES buffer at 25 °C. The mobility of immobilized enzyme was estimated from the value of dissipation ($\Delta D$) using AFFINIX QN Pro Viewer (ULVAC Inc. Kanagawa, Japan).

2.8. EBFC

2.8.1. Preparation of electrode

The cathode electrode was prepared by dropping the MWCNT–McoP complex (1 mg ml$^{-1}$) onto CC (10 mm × 10 mm) treated with oxygen plasma, and then drying the electrode at room temperature for 2 h. For the anode electrode, the PQO-GDH solution was dropped onto CC (30 mm × 30 mm) treated with oxygen plasma and dried at room temperature for 2 h.

2.8.2. Evaluation of EBFC

At the anode cell, 50 mM potassium phosphate buffer (pH 7.0) was used as an electrolyte, containing 1 mM 1,1$'$-ferrocenedicarboxylic acid as a mediator, along with 5 mM D-glucose. At the cathode cell, 50 mM glycine-HCl buffer (pH 3.0) was used as the electrolyte with O$_2$ as a substrate. These cells were separated by Nafion membrane (Merck KGaA, Darmstadt, Germany). LSV was performed from open circuit potential to 0 V with scanning of 0.001 V s$^{-1}$ at 50 °C. Polarization curves were calculated from LSV results.

3. Results and discussion

3.1. MCO activity of MWCNT–McoP complex

To control the swing ability of immobilized His-tagged McoP on MWCNT surface, PBES modified MWCNT and two types of pyrene derivative (PBSE and PCA) modified MWCNT were prepared. The specific activities of MWCNT-(PBSE)-McoP and MWCNT-(PBSE/PCA)-McoP were 0.17 units mg solid$^{-1}$ and 0.08 units mg solid$^{-1}$. It was suggested that the difference in surface modification of MWCNT affected the amount and/or activity of the McoP immobilized on MWCNT surface.

3.2. Electrochemical O$_2$ reduction of MWCNT-(PBSE/PCA)-McoP complex

The enzymatic electrochemical O$_2$ reduction was evaluated by LSV measurements. Figure 3 shows the linear sweep voltammograms (LSVgrams) of MWCNT-(PBSE)-McoP complex and MWCNT-(PBSE/PCA)-McoP complex. Under nitrogen saturated conditions, the MWCNT-(PBSE)-McoP complex modified electrode showed a very small reduction current. Therefore, the reduction current at the MWCNT-(PBSE)-McoP complex or MWCNT-(PBSE/PCA)-McoP complex modified electrode is based on the reduction of O$_2$ by McoP. The O$_2$ reduction current densities of MWCNT-(PBSE/PCA)-McoP complex and MWCNT-(PBSE)-McoP complex were 378 $\mu$A cm$^{-2}$ and 193 $\mu$A cm$^{-2}$ at 0 V, respectively. The onset potentials for O$_2$ reduction of MWCNT-(PBSE/PCA)-McoP complex and modified MWCNT-(PBSE)-McoP complex were 0.45 V and 0.41 V, respectively. Although the specific activity for ABTS of MWCNT-(PBSE/PCA)-McoP complex was 47% compared with that of MWCNT-(PBSE)-McoP complex, the current density for electrochemical O$_2$ reduction of MWCNT-(PBSE/PCA)-McoP complex showed was 265% higher than that of MWCNT-(PBSE)-McoP complex. The increase of the O$_2$ reduction current density in the electrode using MWCNT-(PBSE/PCA)-McoP complex indicates the increase of the amount of electrochemically active enzyme on the electrode or the improvement of the electrochemical activity of McoP immobilized on the electrode. In addition, the positive shift of onset potential for O$_2$ reduction exhibits a decrease in overvoltage due to shortening the distance between T1Cu of McoP and the MWCNT surface. From these facts, it was suggested that the PCA modified on the MWCNT surface contributed to the swing ability and amounts of enzyme on the electrode surface. Therefore, it is important to use both PBSE and PCA for modification of MWCNT. Other groups reported higher current densities of MCO modified electrode than that of the MWCNT-(PBSE/PCA)-McoP modified electrodes [19, 23]. The low current density of McoP modified electrode is due to low activity of McoP. In order to construct an McoP-modified electrode with higher output, it is necessary to obtain highly active McoP by genetic engineering. Furthermore, the current density of C-terminal his-tagged McoP oriented electrode was 1.3 times higher than that of N-terminal His-tagged McoP oriented electrode [11]. Therefore, a high current density can be obtained by applying the C-terminal His-tag McoP to the method in the present study.

3.3. Swing ability evaluation of immobilized McoP

The surface of Au electrode for QCM measurements was modified with a mixed SAM using HUT having a hydroxy group at the end and Dithiobis (C$_2$-NTA) having an NTA group at the end, to mimic the amount of McoP immobilized on MWCNT. McoP was immobilized on the Au electrode via a coordination bond between His-tag and Ni$^{2+}$
coordinated to NTA group, and HUT functions as a spacer that expands the gap between McoPs on Au electrode surface.

Table 1 shows dissipation (\(\Delta D\)) and the amount of enzyme on the C\(_2\)-NTA modified QCM electrode and mixed SAM modified QCM electrode. The amount of enzyme molecules on the electrode surface of C\(_2\)-NTA modified QCM electrode was 159% higher than that of mixed SAM modified QCM electrode. The control of the amount of enzyme on the electrode was successfully achieved using mixed SAM (HUT:C\(_2\)-NTA = 2500:1). From the results of QCM measurement, it can be explained that the difference in the MCO activity of each MWCNT–McoP complex was mainly caused by the difference in the amount of enzyme on MWCNT surface.

To investigate the difference of immobilized enzyme swing ability, \(\Delta D\) values were compared. As mentioned above, the \(\Delta D\) value indicates the rate of oscillation decay, with a large \(\Delta D\) value indicative of a protein that can efficiently adsorb vibrational energy \([21]\). Therefore, we consider that the increase in \(\Delta D\) value is the increase in swing ability of the enzyme immobilized on electrode surface via His-tag. The \(\Delta D\) value on electrode surface of mixed SAM modified QCM electrode was higher than that of C\(_2\)-NTA modified QCM electrode. This is because the swing inhibition of enzyme molecules was decreased by increasing the distance between enzyme molecules. McoP immobilized via His-tag, which has less swing inhibition, and was easily accessible to the MWCNT surface. As a result, efficient electron transfer was allowed at the position where the distance between T1Cu of McoP and MWCNT surface was close. McoP immobilized via His-tag, which has less swing inhibition, was easily accessible to the MWCNT surface. As a result, the amount of electrochemically active McoP was increased and the overvoltage for O\(_2\) reduction was decreased. In this study, the enzyme density on the electrode was not optimized. However, for the swing of the enzyme molecule on the electrode, it is considered preferable that the distance between the enzyme molecules on the electrode is equivalent to that of an enzyme molecule. When the enzyme is immobilized at a distance greater than or equal to the size of one molecule of the enzyme, the current value is considered to decrease as the amount of the enzyme on the electrode decreases.

### 3.4. PQQ-GDH modified CC

For the construction of EBFC, PQQ-GDH modified CC electrodes were prepared and evaluated by CV. Figure 4 shows the cyclic voltammograms (CVgrams) of the PQQ-GDH modified CC electrode. The CV was performed from \(-0.1\) V to 0.7 V. When 5 mM D-glucose existed in the electrolyte solution, the catalytic current arising from the oxidation reaction catalyzed by PQQ-GDH was observed from the vicinity of 0.2 V. The oxidation current was 176 \(\mu\)A cm\(^{-2}\) at 0.6 V. In this study, commercial PQQ-GDH was modified onto CC by physical adsorption. Therefore, the PQQ-GDH modified CC electrode showed sufficient current density for EBFC using with MWCNT-(PBSE/PCA)-McoP complex modified electrode.
3.5. Evaluation of EBFC

To evaluate performance as a biocathode for EBFC, the McoP modified electrode controlled the amount of McoP on MWCNT was combined with bioanode using PQQ-GDH from microorganism. A highly active PQQ-GDH from microorganism was used as anode catalyst so that the cell output was not limited by the anode performance. The EBFC was evaluated at 50 °C considering the optimum temperature of McoP. Figure 5 shows polarization curves of EBFCs using each MWCNT–McoP modified electrode as cathode. The maximum power densities of EBFC using MWCNT-(PBSE/PCA)-McoP complex modified electrode and EBFC using MWCNT-(PBSE)-McoP complex modified electrode were 1.76 $\mu$W cm$^{-2}$ (at 0.17 V) and 1.10 $\mu$W cm$^{-2}$ (at 0.14 V), respectively. The open circuit voltage (OCV) of EBFC using MWCNT-(PBSE/PCA)-McoP complex modified electrode increased by 40 mV compared with EBFC using MWCNT-(PBSE)-McoP complex modified electrode. This result was similar to the increasing tendency of onset potential for O$_2$ reduction in cathode evaluation. However, the power density and OCV of the EBFC were lower compared with that in a previous study. In previous work, we used expensive PQQ as mediator for anode. In this study, we used 1,1'$'$-ferrocenedicarboxylic acid, which is cheaper and has higher redox potential than PQQ. Therefore, as a result of the difference between the anode potential and the cathode potential being reduced, the OCV was reduced. In the future, in order to improve the cell voltage, we are aiming to construct a DET-type anode as well as the cathode. Furthermore, in the present study, PQQ-GDH from microorganisms was used as the anode catalyst. In addition, LSV measurement takes longer than CV measurement. Therefore, it is possible that PQQ-GDH is partially inactivated during the measurement at 50 °C. It is considered that stability problems are solved by using PQQ-GDH from hyperthermophilic archaea which was used in previous studies.
4. Conclusion

In the present study, we indicated the promoting effect of increasing swing ability of an immobilized enzyme on DET. The swing ability of McoP immobilized onto MWCNT via His-tag was increased by controlling the amount of McoP on MWCNT surface using spacer (PCA). By the increased swing ability, the accessibility of T1Cu of McoP to electrode surface was promoted and thereby electron transfer efficiency was improved due to decreasing the distance between the T1 site and the electrode surface. As a result, the current density for electrochemical O$_2$ reduction of McoP modified electrode using spacer introduced MWCNT showed 256% higher than that of McoP modified electrode using non-spacer modified MWCNT. The EBFC using McoP modified electrode using spacer introduced MWCNT as a cathode showed 1.76 $\mu$W cm$^{-2}$ (at 0.17 V) of maximum power density and 0.25 V of OCV. Further research is needed to construct a biocathode by optimizing the density of enzyme molecules on the electrode surface to obtain high power biofuel cells.

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Conflict of interest

The authors declare that they have no competing interests.

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