***In situ* Observation of Direct Electron Transfer Reaction of Cytochrome *c* Immobilized on ITO Electrode Modified with 11-{2-[2-(2-Methoxyethoxy)ethoxy]undecylphosphonic Acid Self-assembled Monolayer Film by Electrochemical Slab Optical Waveguide Spectroscopy**

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To immobilize cytochrome *c* (cyt.*c*) on an ITO electrode while keeping its direct electron transfer (DET) functionality, the ITO electrode surface was modified with 11-{2-[2-(2-methoxyethoxy)ethoxy]undecylphosphonic acid (CH₃O(CH₂CH₂O)₃C₁₁H₂₂PO(OH)₂, M-EG₃-UPA) self-assembled monolayer (SAM) film. After a 100-times washing process to exchange a phosphate buffer saline solution surrounding cyt.*c* and ITO electrode to a fresh one, an *in situ* observation of visible absorption spectral change with slab optical waveguide (SOWG) spectroscopy showed that 87.7% of the cyt.*c* adsorbed on the M-EG₃-UPA modified ITO electrode remained on the ITO electrode. The SOWG absorption spectra corresponding to oxidized and reduced cyt.*c* were observed with setting the ITO electrode potential at 0.3 and –0.3 V vs. Ag/AgCl, respectively, while probing the DET reaction between cyt.*c* and ITO electrode occurred. The amount of cyt.*c* was evaluated to be about 19.4% of a monolayer coverage based on the coulomb amount in oxidation and reduction peaks on cyclic voltammetry (CV) data. The CV peak current maintained to be 83.4% compared with the initial value for a M-EG₃-UPA modified ITO electrode after 60 min continuous scan with 0.1 V/s between 0.3 and –0.3 V vs. Ag/AgCl.

Keywords Slab optical waveguide spectroscopy, cytochrome *c*, ITO electrode, direct electron transfer, 11-{2-[2-(2-methoxyethoxy)ethoxy]undecylphosphonic acid

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Introduction

In bio-electrochemistry, the protein electron transfer (ET) reaction has been significantly studied. Especially cytochrome *c* (cyt.*c*) is one of the most extensively studied proteins because of its ET reaction activity.^{1–5,8,9} However, still now controlling the protein ET reaction is difficult because of many technical issues. One of them for cyt.*c* electrochemistry adsorbed on ITO electrode is a lack of adsorption ability of cyt.*c* for long-time duration. For example, after 60 min electrode potential scan with 0.1 V/s between 0.3 to –0.3 V vs. Ag/AgCl, about 50% of cyt.*c* adsorbed on a bare ITO electrode was desorbed from the electrode surface in our results. Thus to obtain sufficient data to be compared with different experimental conditions with high-repeatability is almost impossible because of a gradually changing amount of cyt.*c* adsorbed on the ITO electrode.

Slab optical waveguide (SOWG) spectroscopy enabled us to perform *in situ* observations of UV-vis absorption spectra from adsorbed molecules on solid/liquid interfaces under monolayer coverage. Its spectral change can provide information about molecular functionality.^{4,6–9} We have reported *in situ* observations of the DET reaction of cyt.*c* adsorbed on an ITO

electrode from SOWG spectral change due to an electrode potential change.^{4,8,9}

There are considerable numbers of reports, including surface modification techniques to immobilize proteins on solid/liquid interfaces.¹⁰ Recently, phosphonic acid compounds self-assembled monolayer (SAM) film on metal oxide materials have been utilized as surface modifiers, like thiol compounds, for metal surfaces.^{11,12} It is well known that cyt.*c* is positively charged in a neutral condition because its pI is around 10;¹³ thus the authors have reported that 10-CDPA SAM film effected the immobilization and DET functionality continuation of cyt.*c*.⁹

Previous reports showed that polyethylene glycol (PEG) and polyethylene oxide (PEO) modified gold electrodes had the DET reaction activity of cyt.*c*.^{14,15} 11-{2-[2-(2-Methoxyethoxy)ethoxy]undecylphosphonic acid (M-EG₃-UPA) has –OCH₂CH₂ structure, which is a basic unit of PEG and PEO in the molecule. Thus, M-EG₃-UPA SAM modified ITO electrode is expected to offer a positive potential for cyt.*c* immobilization while keeping the DET ability. In the present work, the effectiveness of this modification will be described with compared to 10-CDPA. The main purpose of our research is to prepare a common platform for protein DET experiments and to obtain data with high-repeatability for a long-time duration.

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Experimental

Materials

Cyt.*c* (equine heart) was purchased from Sigma Chemical Co. (95%, St. Louis, MO, USA), and its solution (20 μ M) was prepared with a 10 mM phosphate buffered saline (PBS) solution (pH 7.4, Kanto Chemical Co., Inc., Japan). Cyt.*c* solution was prepared just before each experiment. M-EG₃-UPA ($\text{CH}_3\text{O}(\text{CH}_2\text{CH}_2\text{O})_3\text{C}_{11}\text{H}_{22}\text{-PO}(\text{OH})_2$ (Dojindo Molecular Technologies, Japan) and tetrahydrofuran (THF, super dehydrated, stabilizer free, Wako Pure Chemical Ind. Ltd., Japan) were used as received. All the solutions used for the electrochemical experiment were deaerated by nitrogen-gas bubbling for at least 30 min.

Electrochemical measurements

ITO electrodes, which were formed on a finely polished quartz plate with optical grade ($50 \times 20 \times 0.05$ mm) by vapor deposition, has a 20-nm thickness and 20×20 mm surface area (Atoc Co. Ltd., Japan). The surface of the ITO electrode was cleaned with acetone and ethanol, and then washed with Milli-Q pure water. The electrode potential was controlled with a potentiostat (ALS/CHI electrochemical analyzer Model 720C, BAS Inc., Japan). A platinum wire and Ag/AgCl saturated KCl electrode were used as a counter and reference electrodes, respectively. An electrochemical cell made of glass was set on the ITO electrode. The surface area of the ITO electrode touching the sample solution and solution volume were 1.54 cm² and 1.0 mL, respectively. In this cyt.*c* adsorption experiment, the ITO electrode potential was set at the rest potential, and cyt.*c* was oxidized form in the sample water solution as prepared.

Slab optical waveguide systems

In the current system, the direct insertion of an optical fiber into a glycerol drop put on SOWG has been utilized for the light coupling forward core layer. The white light from the Xe lamp transmitted through optical fiber was guided to SOWG by a glycerol drop laid on the SOWG surface. SOWG transmittance spectra were measured by a charge-coupled device (CCD) detector, which included a monochromator (PMA-11, Hamamatsu Photonics K. K., Japan).

To evaluate the adsorption ability of cyt.*c* on the ITO electrode surface, the PBS solution in the SOWG cell was continuously exchanged by using a pipette (PIETMAN, M&S Instruments Inc., Japan) by hand. This process is described as washing process in this manuscript. In this process, 0.8 mL of PBS solution in the cell attaching to the ITO electrode surface was extracted, and the same amount of the fresh PBS solution was supplied to the cell by using a micropipette so as to never expose the electrode surface to the air, and repeated 100 times or more in one experiment.

Surface modification process of ITO electrode

M-EG₃-UPA was dissolved into THF, and the concentration was adjusted to be 0.2 wt%. Firstly, the ITO electrode was dipped in this solution in a beaker under a N₂ gas atmosphere for 3 min. Secondly the ITO electrodes were moved to another beaker with fresh THF, and placed there as a washing procedure to make SAM film. As the third step, ITO electrodes were moved to another beaker with fresh THF again, and placed in a supersonic cleaning machine for about 1 min. Finally, the ITO electrodes were dried in the air atmosphere under room temperature.

Fig. 1 SOWG absorption spectra of cytochrome *c* immobilized on the ITO electrode modified with 11-[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]undecylphosphonic acid (M-EG₃-UPA) self-assembled monolayer film. The ITO electrode potential was (a) 0.3 and (b) -0.30 V, respectively. The electrolyte solution was 0.01 M PBS solution (pH 7.4).

Fig. 2 The absorbance change in the Soret band peak at 408 nm in SOWG spectra of cytochrome *c*, depending on washing process numbers by 0.01 M PBS solution (pH 7.4). (a) ITO electrode modified with 10-calboxydecylphosphonic acid (10-CDPA) self-assembled monolayer film; (b) ITO electrode modified with 11-[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]undecylphosphonic acid (M-EG₃-UPA) self-assembled monolayer film; (c) bare ITO electrode.

Results and Discussion

Clarifying if the M-EG₃-UPA SAM film was effective on immobilizing cyt.*c* on the ITO electrode surface or not, an *in situ* SOWG spectroscopic observation of the cyt.*c* adsorption reaction on ITO electrode surface was performed. The SOWG absorption spectrum after reaching to the adsorption equilibrium state looked similarly, as shown in Fig. 1(a), and the peak position of the Soret band of cyt.*c* was observed at 408 nm, corresponding to that of the oxidized form. The steep increase of absorbance at a peak position of the Soret band was observed in the SOWG spectra, and the initial adsorption process finished in about 5 to 10 s; the adsorption process was maintained so as to assure reaching at the equilibrium state for 10 min.

The desorption process of cyt.*c* on the ITO electrode was performed with the washing process and *in situ* observation of SOWG spectroscopy. As a comparison, the same experiments

Table 1 Summarized data of the change in the absorbance at the peak position of the Soret band of cyt.*c* adsorbed on bare, 10-CDPA modified, and M-EG3-UPA modified ITO electrodes with respect to the numbers of washing process with a PBS solution

Numbers of washing process	Bare	10-CDPA	M-EG ₃ -UPA
0	0.356	0.396	0.310
3	0.305	0.373	0.309
10	0.253	0.356	0.297
20	0.234	0.340	0.285
30	0.223	0.335	0.281
40	0.215	0.327	0.270
50	0.213	0.315	0.270
60	0.209	0.318	0.269
70	0.208	0.322	0.271
80	0.206	0.314	0.258
90	0.203	0.315	0.267
100	0.199	0.316	0.272

with bare and 10-CDPA modified ITO electrodes were carried out. The change in the absorbance at the peak position of the Soret band of the SOWG absorption spectra at 408 nm of cyt.*c* adsorbed on three types of surfaces; bare, 10-CDPA modified, and M-EG₃-UPA modified ITO electrodes with respect to the numbers of washing process, are summarized in Table 1. Figures 2(a), 2(b) and 2(c) show these absorbance change data at the Soret band peak position with the 10-CDPA modified, M-EG₃-UPA modified and bare ITO electrodes shown in Table 1, respectively. Concerning all data in Fig. 2, the first dots mean that the 0th time of the washing process was the result before the washing process; thus, there was sample solution in the SOWG cell, and the origins of absorbance were from cyt.*c* both in the bulk near the ITO/solution interfaces and adsorbed on ITO electrode surface.

There are some points to be noted concerning the data given in Table 1 and Fig. 2. Firstly, the absorbance decrease in the final 50-times washing process was very small for both the 10-CDPA and E-MG₃-UPA modified ITO electrodes.

Secondly, comparing the absorbance of the 10-CDPA modified and M-EG₃-UPA modified ITO electrodes at the 0th washing process, they were 0.396 and 0.310. If the values of the absorbance are proportional to the amount of cyt.*c* adsorbed on the ITO electrode, that on the M-EG₃-UPA modified ITO electrode was less about 22% compared to that on the 10-CDPA modified one.

Finally, the decreased ration after 100-times washing process of the 10-CDPA modified and M-EG₃-UPA modified ITO electrodes were 20.2 and 12.3%, respectively. Considering the decreased ration for the bare ITO of 44.1%, in both cases the surface modification was clearly effective. It appeared that the M-EG₃-UPA modified ITO electrode was more effective than the 10-CDPA modified one. Quantitative analysis with SOWG spectroscopy is not easy, and thus any comparison of the effectiveness with these two surface modification methods would be confirmed after several times of same experiments.

In situ SOWG absorption spectroscopy was employed to observe the DET reaction of cyt.*c* immobilized on an ITO electrode modified with M-EG₃-UPA SAM film. Figures 1(a) and 1(b) were *in situ* SOWG absorption spectra of cyt.*c* immobilized on an ITO electrode modified with M-EG₃-UPA SAM film at 0.3 and -0.3 V, respectively. These coincided with those of the oxidized and reduced forms of cyt.*c*, which were

Fig. 3 Cyclic voltammogram of cytochrome *c* immobilized on ITO electrode modified with 11-{2-[2-(2-methoxyethoxy)ethoxy]ethoxy}-undecylphosphonic acid (M-EG₃-UPA) self-assembled monolayer film in 10 mM PBS solution. (a) Background, and (b) after cytochrome *c* immobilization. The scan rate was 0.1 V/s.

Fig. 4 Peak current of reduction peak in the cyclic voltammogram of cytochrome *c* immobilized on ITO electrode in 10 mM PBS solution. (a) ITO electrode modified with 10-calboxydecylphosphonic acid (10-CDPA) self-assembled monolayer film; (b) ITO electrode modified with 11-{2-[2-(2-methoxyethoxy)ethoxy]ethoxy}-undecylphosphonic acid (M-EG₃-UPA) self-assembled monolayer film; (c) bare ITO electrode.

reported previously; and the Soret band peak positions were 408 and 416 nm, respectively.⁹ These results apparently proved DET activity preservation of cyt.*c* immobilized on the M-EG₃-UPA SAM modified ITO electrode.

Figure 3 shows cyclic voltammograms (CVs) of cyt.*c* immobilized on the ITO electrode modified with M-EG₃-UPA SAM film. The scan rate was 0.1 V/s. The electrode potentials of the oxidized and reduced peaks were 0.044 and 0.034 V, respectively. The peak positions of both peaks and the peak separation were almost the same as that for the 10-CDPA modified ITO electrode.

From the reduction and oxidation wave area, the coulomb amount was observed to be about 3.11×10^{-7} C/cm². The amount of cyt.*c* adsorbed on the ITO electrode surface was calculated to be 3.22 pmol/cm². Assuming that the occupied area by a cyt.*c* molecule would be 10 nm², the ITO electrode surface coverage by immobilized cyt.*c* was nearly 19.4% of a monolayer.

For the purpose to estimate the ability for cyt.c DET reaction activity preservation by M-EG₃-UPA SAM film modified ITO electrode, CVs were continuously measured for 60 min. The electrode potential was scanned between 0.3 and -0.3 V with a scan rate of 0.1 V/s, so 5 cycles were conducted for each minute. Figure 4 shows the rational change in the CV current for the cathodic peaks with respect to the CV scan time duration. The obtained peak current ratios for the 10-CDPA modified and M-EG₃-UPA modified ITO electrodes almost corresponded with each other. For example, after 60 min, the peak current decreased in about 83.4% of the initial one in the case of the M-EG₃-UPA modified ITO electrode. The peak current for the bare and the 10-CDPA modified ITO electrodes were about 41.4 and 84.3% after 60 min, respectively. It was clearly shown that M-EG₃-UPA SAM film is significantly effective for preserving the DET reaction activity of cyt.c on an ITO electrode.

In the electrochemistry of cyt.c on PEG and PEO modified gold electrodes, well-defined redox waves in CVs were observed. Especially, Kurusu *et al.* have reported on the redox response of cyt.c fixed on a CHO-PEO₄₀₀-SH modified gold electrode.¹⁵ An aldehyde group was expected to give a selective binding site between an amino group of protein. These results indicated that the effect of PEG, which resists nonspecific adsorption for cyt.c, would be less than that for the other biomolecules. One of the candidates to interpret the results obtained here is the existence of a phosphoric group of M-EG₃-UPA, which has two -OH groups after the binding surface as a modifier. It is then reasonable to obtain almost the same results from 10-CDPA and M-EG₃-UPA modified ITO electrodes.

Such results shown here would introduce us the next step to test DET reaction of cyt.c for a long time duration with utilizing some significant parameters, which is requested for practical use in electronics device. Then it is reasonable that the excellent platform for protein DET reaction experiments was prepared with phosphonic acid compounds modified ITO electrodes. Further experimental results will be described in near future.

Conclusions

We have demonstrated that the M-EG₃-UPA SAM film modified SOWG has the ability and potential for immobilizing protein

while maintaining the DET reaction activity on the electrode surfaces. As a perspective view, searching proper experimental condition for preparing SAM film as surface modification techniques are being required for the future practical applications of the protein DET reaction. Moreover, the combination of SOWG spectroscopy and other *in situ* surface observation techniques would be strongly required for obtaining much more data to comprehend the phenomena.

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