In situ Observation of Desorption Reaction of Cytochrome c from Solid/Liquid Interfaces with Slab Optical Waveguide Spectroscopy

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An automated solution exchange (SE) mechanism has been introduced to slab optical waveguide spectroscopy to estimate the protein immobilizing ability of a slab optical waveguide (SOWG) surface. In each SE process, SOWG spectral change in absorbance at peak position of cytochrome c (cyt.c) Soret band at 409 nm was observed to analyze the desorption ratio of cyt.c adsorbed on SOWG surface. Continuous SE processes for 100 times have successfully brought us a kind of master desorption curve of cyt.c, which was well fitted by a double exponential equation, indicating the existence of three kinds of adsorbed states, including weakly adsorbed, strongly adsorbed, and immobilized cyt.c. The present results showed that around 30 times SE processes were enough to anticipate the ratio of desorbed and immobilized amounts of cyt.c adsorbed on SOWG surface.

Keywords In situ observation, desorption process, slab optical spectroscopy, automated mechanism, visible absorption spectra

(Received February 14, 2017; Accepted February 25, 2017; Published April 10, 2017)

Introduction

Numerous results concerning direct electron transfer (DET) reaction of cytochrome c (cyt.c) have been reported since 1970s.1–7 The main purpose of these studies is to build novel bio-electronics devices through bottom-up technology with using its DET functionality. One of the largest technical issues in developing bio-electronics device utilizing DET reaction of cyt.c adsorbed on interfaces is a decrease of cyt.c due to rather easy desorption even during experiments in laboratory. For example, the coulometric amount of redox wave peaks in DET reaction of cyt.c adsorbed on a bare ITO electrode decreased by about 40% of the initial value after 60 min cycling as reported in our previous papers.8,9 Surface modification of substrates is one method to solve this, though it is not easy to estimate its effect on protein immobilization, because analysis of the change in adsorbed amount is very difficult.

For this purpose we have reported in situ observation results with slab optical waveguide (SOWG) spectroscopy of desorption reaction against a 100-times washing process with a pipette by hand; however, the change in absorbance obtained from a series of SOWG absorption spectra is not smooth enough to fit any kinetic curve.8,9

To improve the experimental accuracy of our SOWG spectroscopy, in this research an automation solution exchange (SE) mechanism was introduced to SOWG spectroscopic instrument. Then, the phosphate-buffered solution (PBS) in SOWG cell was automatically exchanged at precisely same velocity and volume while continuously carrying out in situ observation of SOWG absorption spectra. The desorption process of cyt.c from glass surface was selected as a model case. The detailed experimental procedure and the results are described in this paper.

Experimental

Materials

Cyt.c (equine heart) was purchased from Sigma Chemical Co. (95%, St. Louis, MO, USA), and used as received without further purification. The cyt.c solution (20 μM) was prepared with 10 mM PBS (pH 7.4, Kanto Chemical Co., Inc., Japan). The cyt.c solution was prepared just before the experiment. A 50 μ-thick glass plate (50 × 20 × 0.050 mm, Matsunami Glass, Japan) was used as a SOWG.

SOWG spectroscopy instruments

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

**Automated solution exchange mechanism**

An automated solution exchange (SE) system was composed of an orthogonal tri-axial robot (ICSA3, IAI Co. Ltd., Japan), and an auto-cylinder (RCA2, IAI Co. Ltd., Japan) as shown in Fig. 1. The computer was utilized to control the movement. A pipette (PIPETMAN, M&S Instruments Inc., Japan) was fixed to auto-cylinder for the solution-exchange (SE) process.

To evaluate the adsorption ability of cyt.c on the glass surface, the PBS in the jacketed SOWG cell was continuously exchanged to analyze the desorption ratio at 30°C by circulating temperature-controlled water. In this process, 0.8 mL of PBS in the cell attached to the glass surface was extracted, and the same amount of fresh PBS was supplied to the cell with the use of a micropipette so as never to expose the glass surface to the air, and repeated 100 times in one experiment. It took 12.4 s to carry out one cycle of such a SE process.

**Procedure of SE process**

For this operation, two beakers were set close to the SOWG cell. One was for a container used to discard PBS from SOWG cell, the other was to replenish fresh PBS to SOWG cell. The former and the latter are named container A and B, respectively.

To observe the desorption process of cyt.c on glass surfaces, cyt.c was adsorbed on SOWG surface as the first stage with 20 μM of cyt.c in PBS for 10 min. As the initial state, SOWG cell was filled with a 1.0-mL sample solution containing 20 μM cyt.c. After adsorption, 0.8 mL of this sample solution was extracted from SOWG cell and moved to container A. Then, 0.8 mL of the fresh PBS was imported to SOWG cell from container B. Again 0.8 mL of the PBS with diluted cyt.c was extracted from SOWG cell to container A, and the same amount of the fresh PBS was imported from container B to SOWG cell. This SE process was repeated 100 times.

At the same time, SOWG spectra were continuously recorded, 1250 times in every 1 s. Each spectrum was accumulated for 0.20 s (20 ms × 10 times) with an interval of 0.80 s. Then, in situ observation of the desorption reaction of cyt.c from glass surface was carried out about 21 min to follow 100 times SE process.

Results and Discussion

Figure 2 shows a series of SOWG absorption spectral changes due to SE process which was repeated 100 times. In the first absorption spectrum, SOWG was attached by the sample solution containing 20 μM cyt.c.

Figure 3(a) shows a normalized absorbance decrease curve at 409 nm, obtained from Fig. 2. Figure 3(b) is normalized absorbance change that resulted in the first 10 cycles shown in Fig. 3(a). At each SE process, the decay curve of SOWG absorbance reached the equilibrium value in about 4 s. This result showed that in each SE process the replenishment of fresh PBS induced desorption of a part of cyt.c was adsorbed on the glass surface, and that the desorption reaction reached the equilibrium between cyt.c adsorbed on glass surface and existing in PBS.

A simple kinetic equation was utilized to fit the decay curve in Fig. 2(a), as described below:

\[
A = A_0 + A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2),
\]

where \(A\) is normalized absorbance at 409 nm; \(A_0, A_1, A_2\) are normalized absorbance of each component; \(t\) is the number of solution exchange cycles; \(\tau_1, \tau_2\) are constants to specify each desorption component, respectively.

In particular, normalized absorbance decrease curves including 100, 90, ..., 20, and 10 times of SE process were fitted independently. The calculated results are summarized in Table 1. Several significant results were obtained from these fittings.

Firstly, the regression value (\(R^2\)) given in Table 1, obtained by fitting Eq. (1) for 10 to 100 SE cycles, was almost unity, which strongly suggested that the decay curves in Fig. 2(a) were well expressed by this simple equation. In addition, fitting parameters obtained for 30 to 100 SE cycles coincided within experimental errors. These results showed that around 30 cycles of SE process was sufficient to estimate the adsorption ability of cyt.c molecules on glass surfaces.

Secondly well-fitted results by two decaying components strongly suggested that three kinds of adsorbed states of cyt.c existed on the glass surface. They can be called as weakly adsorbed, strongly adsorbed, and immobilized cyt.c, respectively. The \(\tau_1\) value corresponded to the inflection point of the decay curve, and shows the characteristic desorption cycle number of weakly adsorbed cyt.c. Thus, before \(\tau_1\), the desorption process was dominated by weakly adsorbed states of cyt.c. After \(\tau_1\), the desorption was caused by mainly strongly adsorbed states of cyt.c.
obtained from silanol sites having pK_a based on the existence of surface silanol groups. Detailed studies at different pH values and with several substrates indicated that about 45% of cyt. c was desorbed by SE process if this model works in our case. However, quantitative discussions and assignments of observed three states of cyt. c are difficult until detailed studies at different pH values and with several substrates for cyt. c adsorption/desorption measurements will be made. As one big advantage of the present result, this process provides simple methodology to obtain the immobilized fraction of adsorbed cyt. c from only 30 cycles of SE process. It is very difficult to obtain absolute amount of adsorbed molecules with other in situ observation tools. The fraction of immobilized and desorbed amounts of molecules evaluated from the present method will be useful to estimate the effects of experimental parameters on immobilization.

Efforts are being made to improve the present method and to elucidate the desorption mechanism of cyt. c by the use of surface modification with self-assembled monolayer films of phosphoric acid derivatives in addition to different pH conditions.

Acknowledgements

The authors would like to acknowledge Ms. Keiko Shimizu for her assistance in data analysis.

References


Finally, the most important result was that this decay curve, or normalized absorbance decrease curve, easily and immediately brought us the fraction of immobilized cyt. c. After calculating the fitting curve, \( A_0, A_1, \) and \( A_2 \) values, which were the absorbance contributed based on the immobilized, weakly adsorbed, and strongly adsorbed states, were evaluated from the experimental results. The fraction of immobilized states was obtained from \( A_i/(A_0 + A_1 + A_i) \). It was estimated to be about 55% from Table 1 for 30 - 100 times washing. Then, this indicated that about 45% of cyt. c was desorbed by SE process if this process was infinitely continued.

One possible explanation for these findings will be made based on the existence of surface silanol groups with different structure and acidity (pK_a). Ong et al. reported two types of silanol sites having pK_a of 4.5 and 8.5 and fractions of 19% and 81%, respectively, from surface second-harmonic generation studies.\(^\text{10}\) The acid-base behavior of surface silanols was later studied in detail from first principles.\(^\text{11,12}\) Their results were almost consistent with that proposed by Ong et al., except for some details. Under the present experimental condition with pH 7.4, silanol site with pK_a 4.5 is completely deprotonated and that with pK_a 8.5 will be deprotonated by about 10%. Thus about 30% of deprotonated surface silanol sites is expected to make considerably different interactions with cyt. c, if this model works in our case. However, quantitative discussions and assignments of observed three states of cyt. c are difficult until detailed studies at different pH values and with several substrates.