Observation of the Adsorption of Cytochrome *c* and Glucose Oxidase onto an Amphiphilic Monolayer at an Air/Water Interface by the Reflection Spectroscopy of Visible Radiation

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The adsorption of two kinds of chromoproteins, cytochrome *c* and glucose oxidase, was observed at an air/water interface by reflection spectroscopy using optical fibers. The relation between the adsorption of cytochrome *c* onto an amphiphilic monolayer and the deposition of the monolayer with cytochrome *c* by the Langmuir–Blodgett (LB) technique was clarified. It was found out that a good Y-type LB film, with a transfer ratio of 0.9—1.0, was obtained when the adsorbed area of cytochrome *c* was less than about 84% of the maximum average coverage. The reflection spectrum of glucose oxidase, not as such, but stained with coomassie brilliant blue (CBB), could be successfully observed. Since CBB is applicable to almost any protein, the reflection spectroscopy of almost any protein, not only chromoproteins, is possible.

A bioreactor has been developed as an application of protein manipulation. Especially the immobilization of proteins in organic ultrathin films, such as Langmuir–Blodgett (LB) films, realizes ultra-miniature bioreactors.[1] Fromherz has proposed a technique for preparing LB film incorporating protein.[2] In the Fromherz technique, a complex monolayer is prepared by the adsorption of protein in a subphase onto an amphiphilic monolayer; an LB film incorporating protein is obtained by depositing the complex monolayer onto a substrate. Denaturation of the protein at the air/water interface is avoided in this technique. We have realized a multistage bioreactor incorporating glucose oxidase and glucoamylase by this technique.[3,4] It is desirable to use an inexpensive protein in order to determine the conditions of the preparation, because enzymes are generally expensive and unavailable commercially. We thus examined the conditions to prepare LB films incorporating protein by using cytochrome *c*, which matches bovine serum albumin (BSA) in availability. The adsorption of cytochrome *c* to an amphiphilic monolayer has been described.[5—8] However, the building up of LB layers with cytochrome *c* was not thoroughly examined, though it is important for building up LB layers in preparing a bioreactor. We investigated the relation between the adsorption of cytochrome *c* and the building up of LB layers. A defect of the Fromherz technique is the long time required to carry out the operations. It is difficult to examine the adsorbed amount of protein in the LB film. Möbius et al. had both theoretically and experimentally investigated the reflection spectroscopy of visible radiation, and described how the adsorbed amount of dye molecules at an amphiphilic monolayer could be determined.[9—11] We thus studied the adsorption of cytochrome *c* at an air/water interface by reflection spectroscopy.[12]

An LB film incorporating glucose oxidase functions as a sensing device for glucose.[3,13,14] We next studied the adsorption of glucose oxidase.

Experimental

Materials. Cytochrome *c* from horse hearts (95—100%, Type VI, Sigma Chemical) and glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* (Sigma Chemical) were used as protein. Icosanoic acid (Applied Science) and a mixture of trimethyloctadecylammonium chloride (Tokyo Kasei Kogyo) and methyl icosanoate (Applied Science), weight ratio 1 : 4, were used as the materials of an amphiphilic monolayer.[3] The spreading solutions of the materials were prepared in chloroform (special grade, Wako Pure Chemical) at a concentration of 1 $mg\,cm^{-3}$. Ultra-pure water was used as a subphase, and was obtained from an ultra-pure water-production system (Milli Q Organex-Q type, Millipore). The electrical conductivity of the ultra-pure water was more than 17.5 $m\Omega\,cm$. The ionic strength (I) of the subphase was adjusted with sodium chloride (special grade, First Chemical), and the pH was adjusted with hydrochloric acid (special grade, First Chemical) or sodium hydroxide (special grade, First Chemical).[15] A protein solution, of which I and pH were adjusted, was prepared in the subphase. A quartz substrate ($8\times40\times0.5^{t}$ mm^3) treated by trichlorooctadecylsilane (Shin-etsu Chemical) and made hydrophobic was used for the LB technique.[3,4]

Preparation. Fromherz Technique:[2] A Fromherz-type multicompartment trough was used.[3] The trough is shown as the cross-section scheme in Fig. 1; the tech-

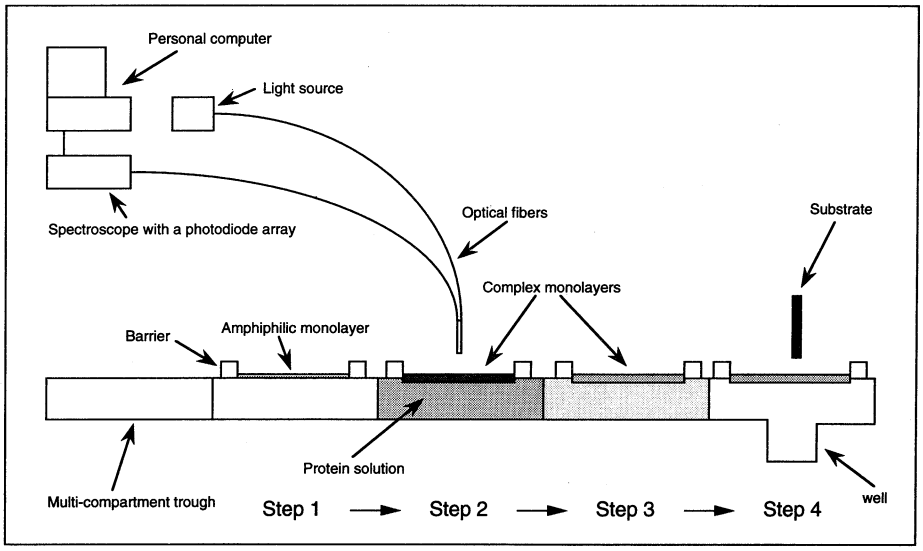

Fig. 1. Scheme of Fromherz trough. A system for an observation of a reflection spectrum of the complex monolayer comprises a spectroscope with photodiode array, a light source, a personal computer, and optical fibers.

nique was carried out in order from Step 1 to Step 4. An amphiphilic monolayer was prepared by compressing amphiphilic molecules spread on a protein-free subphase in a trough compartment (Step 1). The monolayer was moved from the compartment to a following compartment filled with a protein solution; a complex monolayer was organized by adsorbing protein molecules onto the amphiphilic monolayer (Step 2). The complex monolayer was moved to a following compartment with a protein-free subphase, and the desorption of protein molecules from the complex monolayer was carried out (Step 3). Finally, the complex monolayer was moved to a following compartment well filled with a protein-free subphase; the monolayer was transferred to a solid support by means of the LB technique (Step 4). The LB film deposited onto a substrate was measured by UV/vis spectroscopy.[4] The temperature of the trough was kept at 20 °C throughout the operations.

Measurement of the Reflectance of Visible Radiation at an Air/Water Interface: The reflection spectrum of the complex monolayer was measured by an UV/vis spectroscopy system equipped with optical fibers (MCPD-100, Otsuka Electronics).[16] The system comprised a spectroscope with a photodiode-array and a personal computer (PC-9801VX, NEC). The personal computer controlled the spectroscope and processed the measurement data. A 100-W halogen lamp (MC-963, Otsuka Electronics) was used as the light source. The terminals of both the optical fibers from the source and the optical fibers from the spectroscope were put together as a bifunctional probe. The probe was fixed over the trough and the reflection spectrum of the complex monolayer was measured in situ (Fig. 1).

Staining Protein Molecules: Coomassie Brilliant Blue (CBB) G-250 solution (0.5 mg cm^{-3}, Bio-Rad Protein Assay Staining Solution, Bio Rad) was purchased and diluted with ultra-pure water to 0.1 mg cm^{-3} for staining. Stained glucose oxidase molecules were prepared by mixing a glucose oxidase solution with the dye solution, and then used for reflectance measurements.

Results and Discussion

Fromherz prepared two Y-type LB layers with cytochrome *c* by his technique.[7] We also obtained two layers. However, the layers deposited onto the subsequent strokes became similar to Z-type under the same conditions as Fromherz's description, though the substrate with the two initial Y-type layers was thoroughly dehydrated in dry nitrogen gas for 20 min. Y-type layers are normally deposited onto both the down-stroke and the up-stroke, and Z-type layers are deposited onto the up-stroke only.[4,17] It was reported that the complex monolayer was sufficiently saturated with cytochrome *c*.[7,8] It has been described that the ionic strength (I), as well as the pH, affects the adsorption of cytochrome *c* onto amphiphilic molecules.[8,9] We thought that Y-type layers of more than three layers could be prepared by controlling I of the subphase and by decreasing the adsorbed amount of cytochrome *c*.

Oxidized cytochrome *c* has an absorbance peak at 408 nm. The reflectance change at around 408 nm was measured. An example of the time-dependent change of the spectrum during the adsorption of cytochrome *c* onto a monolayer of icosanoic acid is shown in Fig. 2. It was found that the peak near to 408 nm increased with time. The time dependence of the reflectance change at 408 nm is shown in Fig. 3. The adsorption in any I was equilibrated within 10 min, and the adsorbed amount decreased with increasing I. The results agree with Refs. 5, 7, and 8. Examples of the time-dependent changes of the spectrum during the desorption are shown in Fig. 4. It was found that the peak at approximately 408 nm decreased with time. The desorption was equilibrated within 10 min. The reflectance changes at 408 nm before and after the desorption against I of

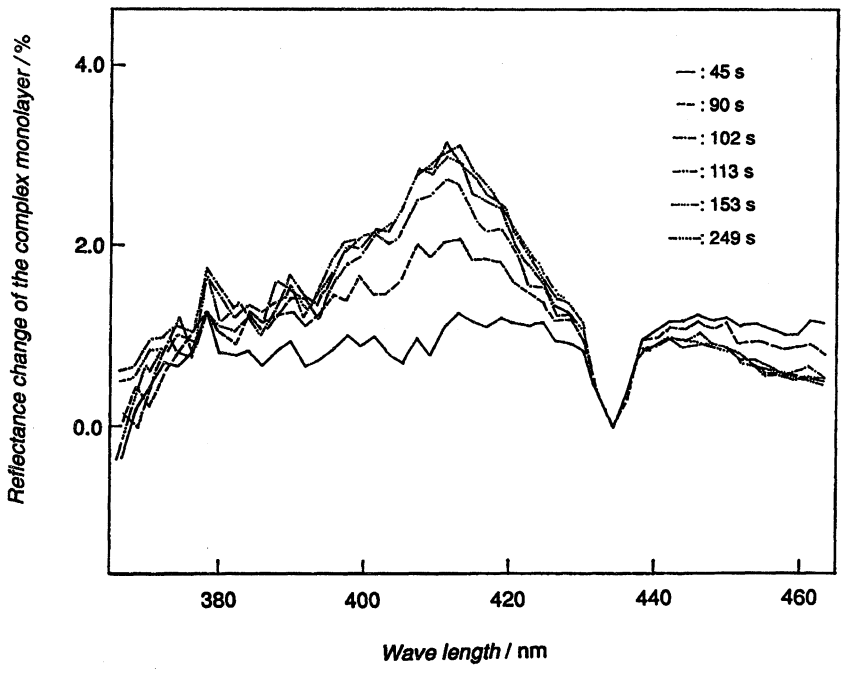

Fig. 2. Spectra of a reflectance change of the complex monolayer during adsorption. The spectrum of the complex monolayer at the time after the beginning of the adsorption of cytochrome *c* under the monolayer of icosanoic acid is shown. pH and ionic strength (I) of the subphase were 7.0 and 0.02, respectively. The concentration of cytochrome *c* was 20 mg dl^{-1}, and the surface pressure of the amphiphilic monolayer was kept at 40 mN m^{-1}. The sampling time for the measurement of the spectrum was 4.8 s.

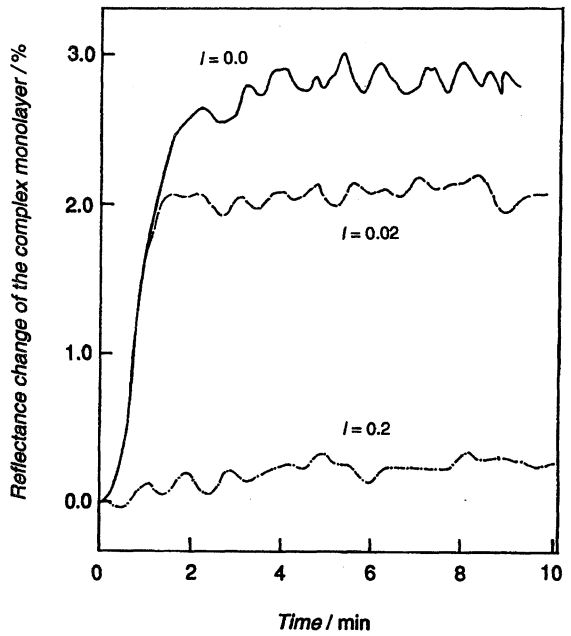

Fig. 3. Time dependence of a reflectance change of the complex monolayer at 408 nm in adsorption. pH was 7.0, the concentration of cytochrome *c* was 20 mg dl^{-1}, and the surface pressure of the monolayer of icosanoic acid was kept at 40 mN m^{-1}. The sampling time for the measurement of the spectrum was 4.8 s.

the subphase are plotted in Fig. 5. The values of the reflectance change before the desorption are the values of the reflectance change just after the adsorption process, and decreased with increasing I. The values of the reflectance change after the desorption were constant at up to 0.08 of I, and decreased at down to 0.08 of I. The difference between the values of reflectance change before and after the desorption was larger for less than 0.08 of I. The cytochrome *c* molecules desorbed at up to 0.08 of I may be weakly adsorbed onto the icosanoic acid monolayer.

The LB deposition of the complex monolayer on six down- and up-strokes was carried out. The adsorbed amount of cytochrome *c* in the LB film was determined by UV/vis spectroscopy. Twenty-four LB layers were obtained when Y-type LB film is deposited. Good LB films (transfer ratio 0.9—1.0) were deposited at down to 0.1 of I. However, layers deposited onto the two initial Y-type layers became Z-type-like layers at up to 0.1 of I. The transfer ratios were 0.8 and approximately 0.5 at 0.04 of I and down to 0.02 of I, respectively. The hydrophobicity of the surface of the two initial Y-type layers was studied after dehydration of the layers. It was found that the surfaces of the two initial Y-type layers were sufficiently hydrophobic at down to 0.1 of I of the subphase, and that the surfaces were not sufficiently hydrophobic at up to 0.1 of I. It was predicted that the next layers deposited onto the layers with a sufficiently hydrophobic surface became Y-type layers, while the next layers deposited onto the layers with an insufficiently hydrophobic surface became Z-type-like layers. The adsorbed amounts of cytochrome *c* at the amphiphilic monolayer at up to 0.1 of I were larger than

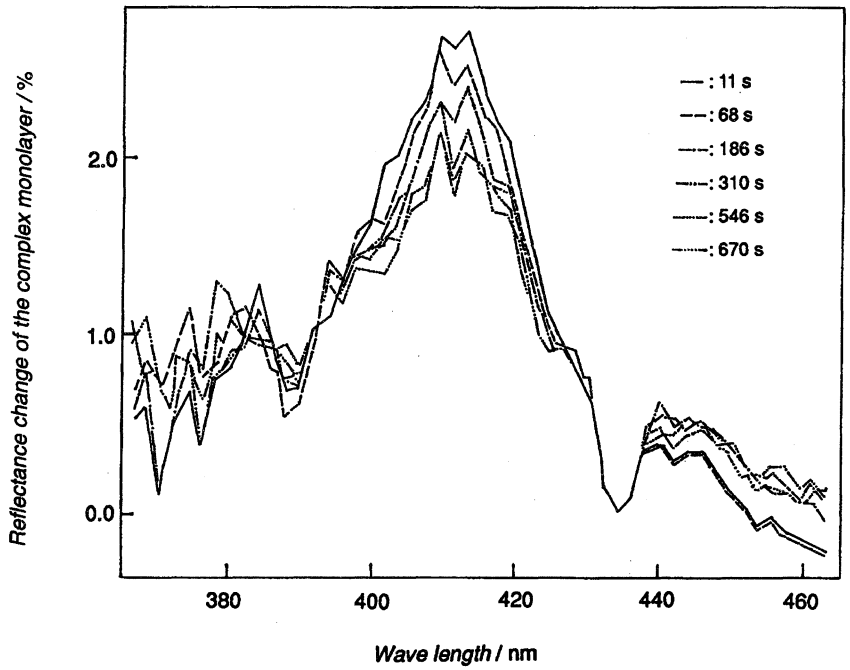

Fig. 4. Spectra of a reflectance change of the complex monolayer during desorption. The spectrum of the complex monolayer at the time after the beginning of the desorption of cytochrome c under the monolayer of icosanoic acid is shown. pH and ionic strength (I) of the subphase were 7.0 and 0.002, respectively. The concentration of cytochrome c was 20 mg dl^{-1}, and the surface pressure of the amphiphilic monolayer was kept at 40 mN m^{-1}. The sampling time for the measurement of the spectrum was 4.8 s.

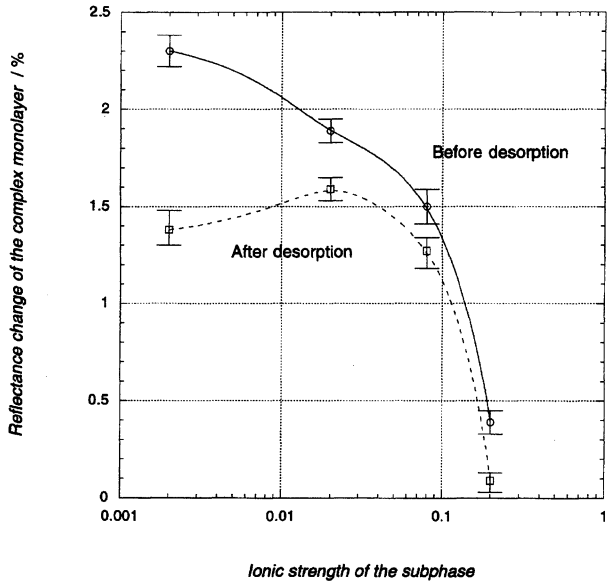

Fig. 5. Reflectance change of the complex monolayer vs. ionic strength of a subphase. The value (average±standard deviation) was measured at 408 nm. pH of the subphase was 7.0, the concentration of cytochrome c was 20 mg dl^{-1}, and the surface pressure of the monolayer was kept at 40 mN m^{-1}. The sampling time for the measurement of the spectrum was 4.8 s.

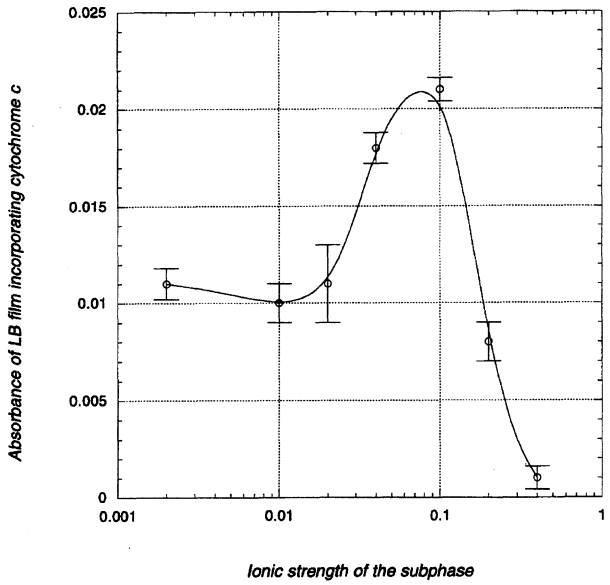

Fig. 6. Absorbance of LB film incorporating cytochrome c vs. ionic strength of the subphase. The value (average±standard deviation) was measured at 408 nm. pH of the subphase was 7.0, the concentration of cytochrome c was 20 mg dl^{-1}, and the surface pressure of the monolayer was kept at 40 mN m^{-1}. LB deposition was carried out on six down- and upstrokes.

at down to 0.1 of I. It is thought that the LB layers with larger cytochrome c become insufficiently hydrophobic, becase cytochrome c is a hydrophilic protein. The absorbance of the LB film at 408 nm is plotted against I of the subphase in Fig. 6. The largest value for the absorbance is found at 0.1 of I. It was thought

that the decrease in the absorbance at down to 0.1 of I was caused by a decrease in the adsorbed amount of cytochrome c at the amphiphilic monolayer shown in Fig. 5, and that the decrease of the absorbance at up to 0.1 of I was caused by a decrease in the transfer ratio of the LB layers. The values for the absorbance at up to 0.02 of I were constant, and about half of the largest value for the absorbance in Fig. 6. The reason is that the layers deposited onto the two initial Y-type layers were deposited as Z-type layers. The shape of a cytochrome c molecule is approximately spherical and the molecular weight, density, and molar extinction coefficient at 408 nm (ε^{408}) are 12500, 1.33 g cm^{-3}, and 47250, respectively.[18] Therefore, the projected area of the molecule in the monolayer was estimated to be 7.55×10^{-14} cm^2, and the absorbance of 24 layers of the complex monolayer at 408 nm was calculated to be 0.025 for an average coverage of 100%. The calculated values agreed with the reported values.[8] The average coverage at 0.1 of I was calculated to be 84% (Fig. 6) and the reflectance change at 0.1 of I was 1.1 (Fig. 5). Therefore, 100% average coverage may be equivalent to a reflectance change of about 1.3. The constant values of the reflectance change shown after desorption are about 1.3 in Fig. 5 and correspond to 100% average coverage.

The reflectance change is plotted against the pH of the subphase in Fig. 7. The adsorption peak was at neutral pH and adsorption was not observed at pH 3.0. The next two Y-type layers could be deposited onto the two initial Y-type layers at pH 3, 5, 9, and 11, but not at pH 7. In summary, Y-type films incorporating cytochrome c were obtained with less than about 84% average coverage of cytochrome c molecules under the icosanoic acid monolayer.

Glucose oxidase is a chromoprotein with peak absorbance at 450 nm; the molar extinction coefficient (ε^{450}) is 37860. However, a reflection spectrum could not be observed during the adsorption of glucose oxidase under the same condition as that for cytochrome c. The reason is that the average coverage is 30% and may be smaller.[4] It was described that the amount adsorbed onto the monolayer of a mixture of trimethyloctadecylammonium chloride and methyl icosanoate is larger than that onto that of icosanoic acid.[14] However, the optical sensitivity of the spectroscope was also inadequate for measuring the mixed monolayer. We thus used glucose oxidase molecules modified with dyes having a larger molar extinction coefficient. A dye-staining method using coomassie brilliant blue (CBB) is used in protein assays.[19] If we can use our reflectivity method for CBB-stained glucose oxidase it would be possible to extend the applicability of this method, because CBB molecules are adsorbed on basic aromatic amino acid residues of protein and can stain almost any protein.[19] CBB solutions for protein assays are commercially available. A commercial solution (CBB G-250) was used.

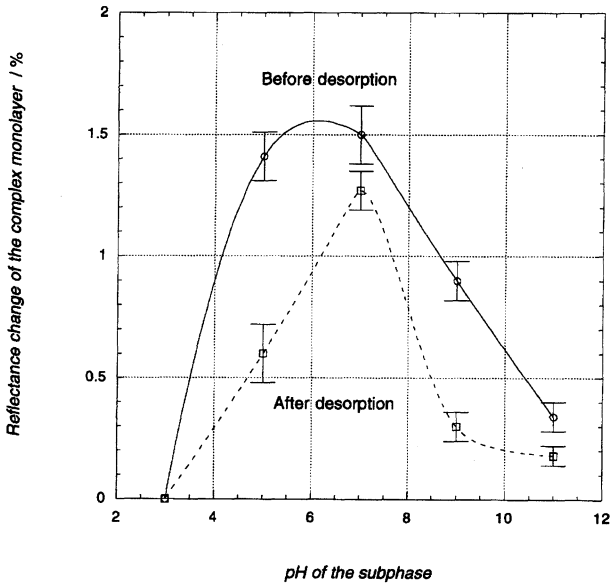

Fig. 7. Reflectance change of the complex monolayer vs. pH of the subphase.The value (average±standard deviation) was measured at 408 nm. Ionic strength (I) of the subphase was 0.08. The concentration of cytochrome c was 20 mg dl^{-1}, and the surface pressure of the monolayer was kept at 40 mN m^{-1}. The sampling time for the measurement of the spectrum was 4.8 s.

The absorbance peak of the CBB was altered from 465 to 595 nm upon adsorption to the protein. The absorbance spectrum of glucose oxidase stained with CBB is shown in Fig. 8. A coomassie brilliant blue solution (0.1 mg cm^{-3}) was injected at volumes of 0.2 ml into 1.0 ml of a glucose oxidase solution (0.1 mg cm^{-3}). At higher concentrations of CBB the peak of free CBB can be observed at 465 nm. Therefore, the volume ratio between the CBB solution and the glucose oxidase solution for staining was chosen to be 4 : 5, and the molar extinction coefficient (ε^{595}) of the glucose oxidase stained with CBB was determined to be 2.0×10^8. The spectrum of the reflectance change of the complex monolayer of glucose oxidase stained with CBB and the mixed monolayer at a surface pressure of 20 mN m^{-1} is shown in Fig. 9. The peak near to 640 nm was larger than that near 600 nm. It has been reported that the surface of a glucose oxidase molecule holds both positive and negative groups, and that the molecules aggregate on the amphiphilic monolayer.[4,20] Therefore, the interactions among the CBB molecules on the surfaces of the glucose oxidase molecules may cause the peak to shift 600 to 640 nm. The surface pressure of the complex monolayer was altered from 20 to 10 mN m^{-1}, kept at 10 mN m^{-1} for 5 min, and returned to 20 mN m^{-1} by altering the complex monolayer area. The spectrum after this process is shown in Fig. 10. Both of the peaks near to 600 and 640 nm increased, and the peak near to 600 nm became remarkably larger. It was thought that the interaction among CBB molecules on the glucose oxidase molecules

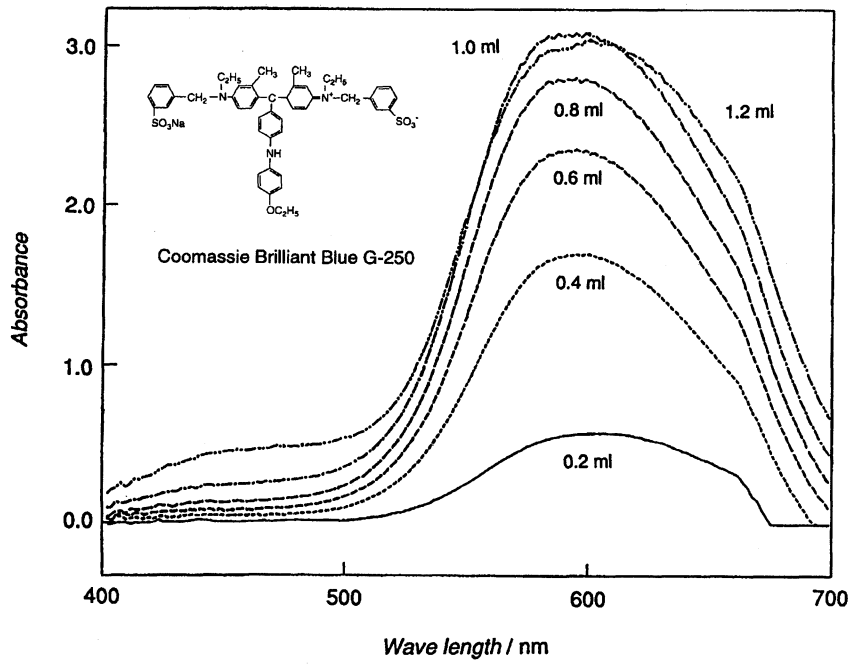

Fig. 8. Absorbance of glucose oxidase with coomassie brilliant blue G-250. A coomassie brilliant blue solution (0.1 mg cm^{-3}) was injected at volumes of 0.2 ml into 1.0 ml of a glucose oxidase solution (0.1 mg cm^{-3}).

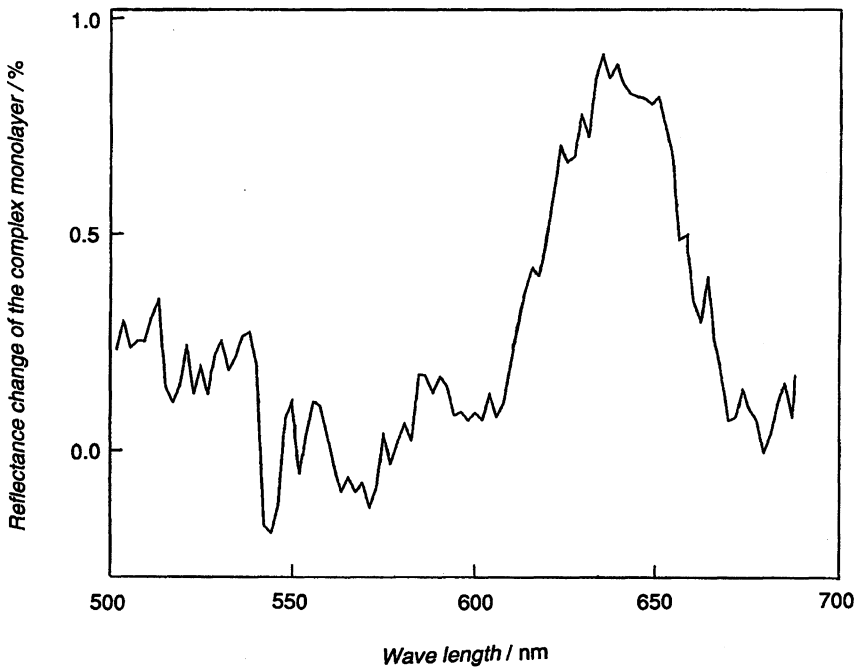

Fig. 9. Spectrum of the reflectance change of the complex monolayer in adsorption. The spectrum of the complex monolayer of the amphiphilic monolayer of the mixture with glucose oxidase stained with coomassie brilliant blue is shown. The surface pressure of the monolayer was kept at 20 mN m^{-1}. The sampling time for the measurement of the spectrum was 4.8 s.

decreased, because the glucose oxidase molecules were moved into the amphiphilic monolayer and covered by the amphiphilic molecules. The reflectance change at nearly 600 nm could be adequately measured in spite of the appearance of a peak shift. It was clarified that the CBB staining method is useful in the reflection spectroscopy of visible radiation at the air/water interface.

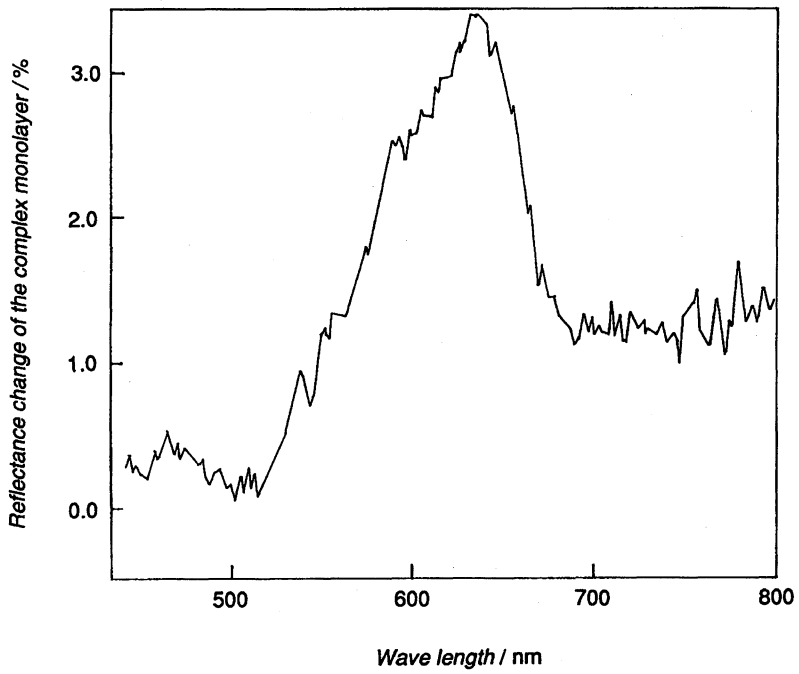

Fig. 10. Spectrum of the reflectance change after expansion and compression of the complex monolayer. The spectrum of the complex monolayer of the amphiphilic monolayer of the mixture with glucose oxidase stained with coomassie brilliant blue is shown. The surface pressure of the monolayer was decreased from 20 $mN\,m^{-1}$ to 10 $mN\,m^{-1}$, and returned to 20 $mN\,m^{-1}$ by altering the complex monolayer area. The sampling time for the measurement of the spectrum was 4.8 s.

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