Non-Contact Measurement of Adsorbed Cytochrome *c* with Optical Waveguide Spectrometry; The Effect of Distance between Waveguide and Protein on the Spectral Sensitivity

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Optical waveguide spectrometry with non-contact conditions was performed with the aim of widening the choice of substrate material. The visible absorption spectrum of cytochrome *c* (cyt.*c*) adsorbed on a quartz glass or gold surface was detected with the evanescent wave from a co-faced waveguide. A clear correlation was observed between absorbance of cyt.*c* and the gap between the waveguide surface and the protein.

Nonspecific adsorption of protein occurs when a solution of dissolved proteins contacts most synthetic materials.[1,2] It is known that conformational change of a protein is often induced by the adsorption on a surface, and its activity is partly or completely lost. It is therefore important to analyze and understand such parameters as the shape and the activity of adsorbed protein, and the interaction between adsorbed protein and the substrate surface. The information obtained should be useful in medical, biomaterial, biosensor research and related fields. Recently, optical waveguide (OWG) spectrometry utilizing the evanescent surface wave has emerged as a method for analyzing adsorbed proteins.[1,3-10] OWG spectrometry is very sensitive, since the incident light advances by repeated total reflection in the waveguide substrate, and a sample on the waveguide surface absorbs the evanescent wave at every reflection point. The intensity of the evanescent wave diminishes strongly with distance from the waveguide surface, and only distances similar to the wavelength of the incident light are effective in detecting the spectrum. Accordingly, UV-visible,[6,7] IR,[8] or fluorescence spectra[9,10] of the sample on the waveguide surface are selectively observed. However, all previous reports have been concerned with adsorbed proteins only on a transparent waveguide substrate with a suitable refractive index for total reflection. Absorption of the evanescent wave should be detected when the sample is close enough to the waveguide surface and the medium has an appropriate refractive index. Indeed, the analysis involves not only proteins directly adsorbed on the waveguide surface but also proteins immobilized through a linker such as LB membrane.[5] It is not always necessary that the sample is in direct contact with the waveguide surface. Applications would be greatly expanded if limitations on the kind of substrate could be eliminated.

In the present paper, cytochrome *c* (cyt.*c*) was adsorbed on a quartz glass or gold surface, and these were fixed apart from the waveguide with an appropriate spacer. The absorbance of the cyt.*c* was studied as the function of the distance between the sample and the waveguide surface. This is the first quantitative report on the analysis of adsorbed proteins using OWG spectrometry when the sample is not contact with the waveguide surface.

Horse heart cyt.*c* (Type VI, Sigma) was used, with no further purification. The absorption spectrum of cyt.*c* was studied in the wavelength range 300-700 nm with an OWG spectrophotometer (SIS-50, System Instruments Inc). A quartz plate of thickness 0.2 mm and refractive index 1.46 was used as an optical waveguide. The angle of incidence of the light was 73.5 degrees from the normal to the surface. Gold substrate was prepared by sputter deposition on ITO glass (0.8 x 5.0 cm^2, gold layer thickness 0.2 μm). Cyt.*c* was adsorbed on a quartz plate (1.5 x 2.0 cm^2) or gold substrate by dipping these into 0.1 M phosphate buffer (pH 7.0) solution containing 0.1 mM (1 M = 1 mol dm^{-3}) cyt.*c* for 6 hours. These plates were then thoroughly rinsed with Milli-Q water and fixed above the waveguide surface. Gold leaf of thickness 0.3 μm was used as a spacer. The cyt.*c*-adsorbed quartz glass was tilted with the aid of the gold leaf as a spacer on one side. Latex (polystyrene) beads (for immunoassay, diameter; 0.12 ± 0.003 μm, 0.4 ± 0.01 μm) from Sekisui Chemical were used as a spacer between the waveguide and the cyt.*c*-adsorbed gold substrate.

The length of the cyt.*c*-adsorbed quartz glass was 2.0 cm on the light path, from which the number of reflections is estimated to be 15. The depth of penetration of the evanescent wave, d_p, is calculated to be 0.17 μm at 407 nm, which is the maximum absorption wavelength of oxidized cyt.*c*. Generally the evanescent wave penetrates significantly $3d_p$ from the waveguide surface. It is expected theoretically that the absorption spectrum of the sample can be detected within 0.5 μm of the waveguide surface. The cyt.*c*-adsorbed quartz glass was set on the waveguide substrate at a slant with gold leaf as a spacer. Milli-Q water (of refractive index 1.33) was gently soaked into the gap. The OWG spectra were measured at intervals corresponding to each 0.01 μm distance between the cyt.*c* and the waveguide surface. The inset to Figure 1 shows a typi-

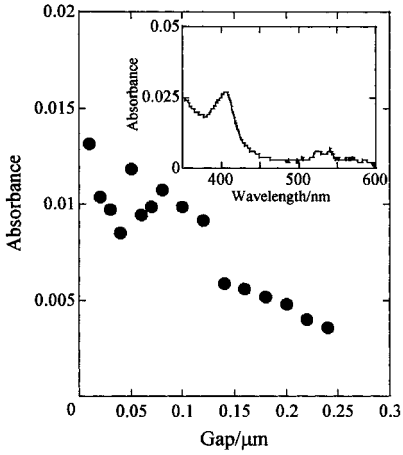

Figure 1. Effect of gap between quartz glass plate and waveguide on the absorbance (407 nm) of cyt.*c* on the substrate. Inset: Typical OWG spectrum for adsorbed cyt.*c*, gap = 0.01μm.

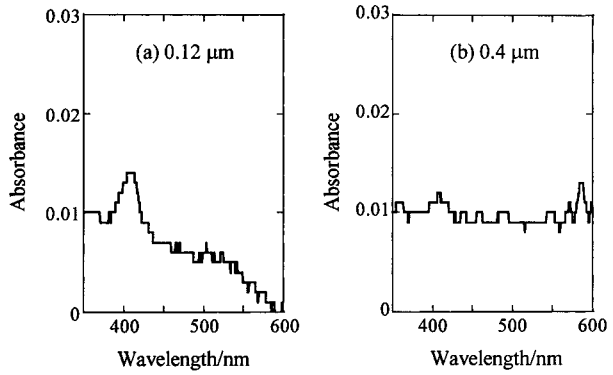

Figure 2. OWG spectra for adsorbed cyt.*c* on the gold substrate. Gap was 0.12 (a) and 0.4 μm (b), respectively. Gap was filled with Milli-Q water containing 0.01 weight % latex bead.

cal cyt.*c* OWG spectrum at a gap of 0.01 μm. Figure 1 shows the relation between the absolute value of the absorbance based on cyt.*c* at 407 nm, and the distance between cyt.*c* and the waveguide surface. The absorbance at 407 nm was 0.013 at a gap of 0.01 μm. This value was about one-sixth times that of cyt.*c* directly adsorbed on the waveguide surface (0.083). The signal intensity was lowered by making the gap because the intensity of the evanescent wave decreased exponentially with distance. This result shows that there is a clear relationship between absorbance and the distance between cyt.*c* and the waveguide surface. The OWG spectrum of the adsorbed protein, which was set apart from the waveguide surface, could be detected when the distance between the sample and the waveguide surface was 0.01-0.24 μm.

Gold leaf of thickness 0.3 μm was used as a spacer in this experiment. However, handling of this gold film was troublesome and latex bead was also used as a spacer. This contained no emulsifying agent, dispersed stably in solution, and did not aggregate over a wide pH range in the buffer. Gold was selected as a substrate for cyt.*c* adsorption since it has often been used as a substrate to immobilize enzymes or proteins. The length of the cyt.*c*-adsorbed gold substrate was 5 cm on the light path, so that the number of reflections was 37. The two types of latex bead were mixed with Milli-Q water at a concentration of 0.01 weight %, and these mixed solutions were injected into the gap between the cyt.*c*-adsorbed gold substrate and the waveguide. Figure 2 shows the OWG spectra for cyt.*c* adsorbed on a gold electrode when each type of latex bead was used as a spacer. The absorption peak based on cyt.*c* at 408 nm was observed when 0.12 μm latex beads were used. The absolute value of the absorbance was 0.0065, which is less than that of cyt.*c* adsorbed on quartz glass (0.0095) at the same gap distance, although the light path length was 2.5 times greater than for quartz glass. This small absorbance value was due principally to roughness of the gold substrate. However, the absorption peak was not observed when the 0.4 μm latex beads were used instead. This result is reasonable because the distance $3d_p$ was estimated to be up to 0.5 μm. These results reveal that latex beads can control the gap between the waveguide and a sample.

From these results it is concluded that the OWG spectrum of the adsorbed protein, which is not in direct contact with the waveguide substrate, can be detected when the gap between the protein and the waveguide surface is properly controlled.

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