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High-Resolution Multiwavelength Surface Plasmon Resonance Spectroscopy for Probing Conformational and Electronic Changes in Redox Proteins

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To date, surface plasmon resonance (SPR) spectroscopy identifies molecules via specific bindings with their ligands immobilized on a surface. We demonstrate here that a high-resolution multiwavelength SPR technique can measure the electronic states of the molecules and thus allow direct identification of the molecules. Using this new capability, we have studied the electronic and conformational differences between the oxidized and reduced states of cytochrome *c* immobilized on a modified gold electrode. When the wavelength of the incident light is far away from the optical absorption bands of the protein, a ∼0.008° decrease in the resonance angle, due to a conformational change, occurs as the protein is switched from the oxidized to reduced states. When the wavelength is tuned to the absorption bands, the resonance angle oscillates at the wavelengths of the absorption peaks, which provides electronic signatures of the protein.

Surface plasmon resonance (SPR) spectroscopy has emerged as a powerful technique for a variety of chemical and biological sensor applications.[1−8] Its rapid development is evident from a 10-fold increase in the number of research papers using SPR published since 1992.[3] Surface plasmons are collective oscillations of free electrons in a metallic film. Under appropriate conditions, the plasmons can be made to resonate with light, which results in the absorption of light.[9,10] Because the resonance is extremely sensitive to the refractive index of the medium next to the metal film, adsorption of molecules on the metallic film or conformational changes in the adsorbed molecules can be accurately detected. To date, SPR identifies the molecules via specific bindings to their ligands immobilized on the metal film. When applied to study the conformational changes, it measures overall changes in the refractive index and thickness of the adsorbed molecular layer. We present here an SPR technique that has the capability of measuring the electronic states of the molecules. This capability allows one not only to directly identify the molecules but also to determine which parts of the molecules are involved in the conformational changes. As an example, we show here that this capability can be used to study conformational and electronic changes due to an electron-transfer reaction in cytochrome *c* (cyt *c*).

PRINCIPLE

The most widely used SPR methods are based on the attenuated total internal reflection in a prism coated with a thin metal film.[9,10] In these methods, p-polarized light is incident upon the metal film and its reflection is detected (Figure 1). When the angle of the incident light reaches an appropriate value, the reflection decreases sharply to a minimum, corresponding to the resonance of surface plasmons in the film. The resonance angle (θ) shifts proportionately to the changes in the refractive index (Δn) and average thickness (Δd) of an adsorbed molecular layer, as shown by eq 1, where c_1 and c_2 are constants and λ is the

$$\Delta\theta(\lambda) = c_1\,\Delta n(\lambda) + c_2\,\Delta d \qquad (1)$$

wavelength of the incident light. We found that $c_1 = 2.7$ and $c_2 = 0.15$ from a numerical calculation based on Fresnel optics involving four phases: a glass prism ($n = 1.52$), a silver film ($n = 0.073+4.12i$ and thickness $d = 45$ nm), a protein layer ($n = 1.5$ and $d = 3.5$ nm), and a buffer ($n = 1.33$).[11] By measuring $\Delta\theta$ vs λ, both the conformational and the electronic changes in the molecules can be obtained.

When λ is far away from the absorption peaks, Δn is weakly dependent on λ and $\Delta\theta$ measures changes in both the refractive index and the thickness due to conformational changes in the molecules. When λ approaches an absorption peak (Figure 2a), however, an oscillation centered at the wavelength of the peak will occur in a plot of $\Delta\theta$ vs λ (Figure 2b) because Δn oscillates

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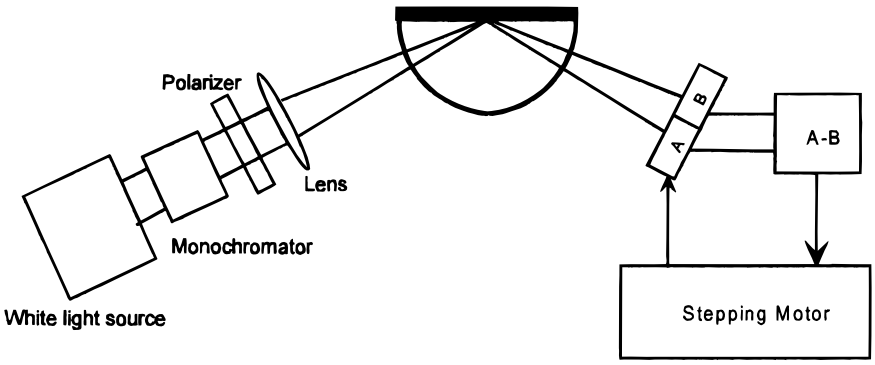

Figure 1. SPR setup. White light from a lamp is sent to a monochromator, and light at a chosen wavelength from the monochromator passes through a polarizer and is then focused onto a prism surface. The total internal reflection is detected with a bicell photodetector whose position is controlled with a stepping motor.

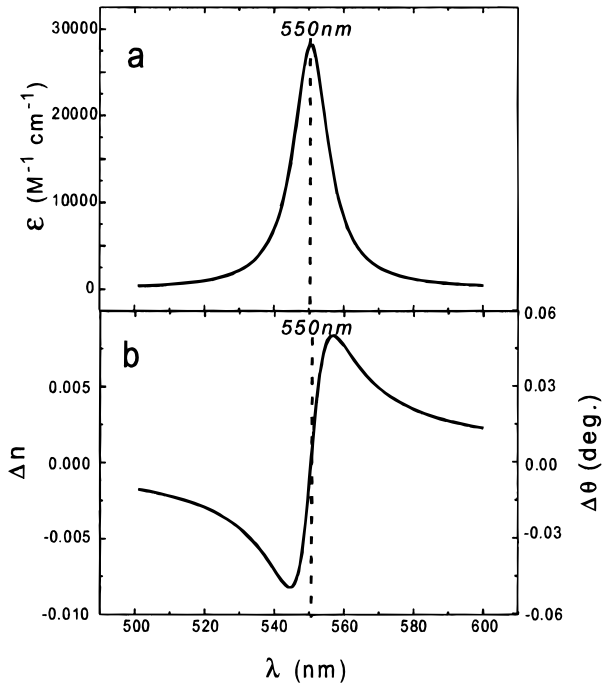

Figure 2. (a) Absorption peak near 550 nm for reduced cyt *c*. (b) Index of refraction variation due to the absorption calculated by using the Kramers−Kronig relation, exhibiting a kink centered at the absorption peak position. Because the shift in the SPR dip angular position is proportional to the change in the index of refraction, the angular position vs wavelength plot also exhibits a similar kink. The proportionality is 6.5°/RIU for a 5 nm thick protein around 550 nm as found by a numerical calculation based on Fresnel optics.

according to the Kramers−Kronig relation[12]

$$\Delta\epsilon(\lambda) = -\frac{2\lambda}{\pi} P \int_0^\infty \frac{\Delta n(\lambda')}{\lambda^2 - \lambda'^2}\, d\lambda' \qquad (2)$$

where ϵ is the molar extinction coefficient and P in front of the integral sign indicates how the infinity at $\lambda' = \lambda$ should be treated. The upper limit of the integral is infinite, but the relation can still provide an accurate result in practice even if data are available over only a limited wavelength range.[13] Therefore, the optical absorption properties of the molecules can be obtained from multiwavelength SPR measurements. This is important because absorption spectroscopy is the most frequently used optical technique in analytical chemistry for purposes ranging from simple concentration determinations to resolutions of complex structural questions.[13] It has, however, been a difficult task to directly measure the absorption spectra of large biological molecules adsorbed on a surface. For example, to detect the absorption spectrum of a monolayer of cyt *c*, a protein that has relatively large molar extinction coefficient (10^4 M^{-1} cm^{-1} at 550 nm; Figure 2a), the intensity measurement must be more accurate than 7×10^{-5}. Using multiwavelength SPR, on the other hand, the absorption peak leads to a 0.04° oscillation in the resonance angle (Figure 2b), which can be easily detected.

Multiwavelength SPR is more than just a high-sensitivity absorption spectroscopic technique. First, the oscillations at the wavelengths of the absorption peaks provide detailed information about the electronic states of the molecules which are specific for each molecule, so multiwavelength SPR will allow direct identification of the molecules. Second, because SPR probes molecules near surfaces, it is particularly suitable for studying interactions of molecules with their counterparts immobilized on the surface. In contrast, conventional absorption spectroscopy cannot easily distinguish molecules on a surface from those in solution. Finally, SPR measurements provide information about the overall conformational changes in molecules at wavelengths far away from the absorption bands, where conventional absorption spectroscopy provides no information.

To accurately measure the oscillations in the resonance angle, it is essential to have a high-resolution SPR technique. We recently developed a high-resolution technique that can achieve an angular resolution of 10^{-5} deg.[14] The method uses a convergent beam focused onto a metal film, but the reflection is collected by a bicell photodetector instead of a CCD or LDA (Figure 1). The components of the reflected light falling onto the two cells of the photodetector are first balanced such that the SPR dip is located near the center of the photodetector. A small shift in the dip position causes a large imbalance in the two cells, which is

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detected as a change in the differential signal of the bicell photodetector. Because the differential signal is linearly proportional to the shift in the resonance angle and can be easily amplified without saturation problems, it provides an accurate detection of SPR.

EXPERIMENTAL SECTION

In the SPR setup (Figure 1), a BK7 plano-cylindrical lens (Melles Griot) was used as prism. On the prism, a BK7 glass slide, coated with a 45 nm thick silver film by a sputtering coater, was placed with an index-matching fluid. White light from a 150 W xenon lamp (Oriel) was sent to a monochromator. Monochromatic light with a bandwidth of ~0.5 nm from the monochromator was collimated and then focused by a 14 mm local length lens through the prism onto the silver film. The intensity of the incident light was adjusted to ~30 μW to avoid possible photoinduced degradation in the protein. Light reflected from the silver film was detected with a bicell photodiode detector (Hamamatsu Corp., model S2721-02) which was mounted on a precision translation/rotation stage. Both the differential, $A - B$, and sum, $A + B$, signals were measured. Before each measurement, the prism was rotated such that a dark line, corresponding to the SPR dip, was located at the center of the laser beam. The components of the reflected light falling onto the two cells of the photodetector were then balanced by adjusting the photodetector position with the translation stage until $A - B$ approached zero.

The silver film was modified with a self-assembled 3-mercaptopropionic acid (MPA) monolayer by exposing the film in 1 mM MPA aqueous solution overnight. A Teflon sample cell was mounted on the silver film to hold the sample solutions. The cell has two ports for the entrance and exit of sample solutions and a port for purging O_2 from the solutions with N_2. For controlling the electrochemical potential of the silver film, Pt wire and Ag/AgCl electrodes were used as counter and reference electrodes, respectively. The electrochemical potential of the silver film was controlled with a potentiostat (Pine Instruments). Horse heart cyt *c*, obtained from Fluka Chemie AG, was used without further purification. The protein was dissolved in 50 mM phosphate buffer (pH 7.0) to a concentration of 300 μM. The solutions were prepared with Nanopure water from a bioresearch grade purification system (Barnstead Co.). The protein was immobilized onto the MPA-modified surface by exposing the surface to the protein solution. The process required 10−20 min according to the real-time measurements of the SPR angle vs time.[14]

RESULTS AND DISCUSSION

We have applied high-resolution multiwavelength SPR spectroscopy to the electron-transfer reaction in cyt *c*, a redox protein whose main function is to shuttle electrons between enzymes.[15] A basic question directly related to the electron-transport function is whether there is a large conformational difference between its oxidized and reduced states.[16−20] Experiments such as hydrodynamic[18] and small-angle X-ray scattering measurements[20] detected large differences between the two states which were attributed to a large conformational difference. However, X-ray crystallography[21] and NMR spectroscopy[22] observed only small structural differences between the two states.

Directly adsorbing proteins onto bare metal surfaces often results in denaturation. So an MPA monolayer adsorbed on the silver film was used to protect cyt *c* from denaturation.[23−25] The negatively charged groups of the MPA monolayer attract the positively charged lysine groups of cyt *c*, which immobilizes cyt *c* molecules on the surface. The electrostatic attraction is also expected to allow the protein to adopt an appropriate orientation for the fastest electron transfer between the heme group and the electrode.

The electron-transfer current (Figure 3a) and the resonance angle (Figure 3b) were simultaneously recorded in 50 mM phosphate buffer (pH 7.0) as a function of potential for various wavelengths, from 670 to 520 nm. The current vs potential plot exhibits two peaks corresponding to the well-known reduction and oxidation of cyt *c*. From the peak areas, the coverage of cyt *c* was estimated to be $\sim 7 \times 10^{-12}$ mol/cm^2, which is close to the coverage estimated from the tapping-mode AFM images.[26,27] The resonance angle showed a reversible sigmoidal change of ~0.008° as the protein was switched between the oxidized and reduced states by the potential. To ensure that the sigmoidal change was due to the change in the oxidation state of the protein, we performed the measurement without cyt *c* on the electrode and found only a small linear change in the resonance angle. The linear change is due to a change in the electron density of the electrode induced by the potential.[28] The time derivatives of the SPR angle vs potential have been calculated for both the protein and the buffer, which can be more directly compared with the cyclic voltammograms.

In contrast to the current vs potential measurement, the sigmoidal change in the SPR angle is strongly wavelength dependent (Figure 4). We have plotted the resonance angle shift as a function of the wavelength (Figure 5b). The plot exhibits two interesting oscillations centered at 520 and 550 nm, which coincide with the wavelengths of the two absorption peaks of the reduced cyt *c* (Figure 5a). Note that the absorption spectrum of oxidized cyt *c* is much flatter in the wavelength window. The oscillations in the resonance angle are due to the changes in the refractive index of the protein near the absorption bands as expected from the Kramers−Kronig relation discussed earlier. For comparison, the resonance angle without cyt *c* on the surface

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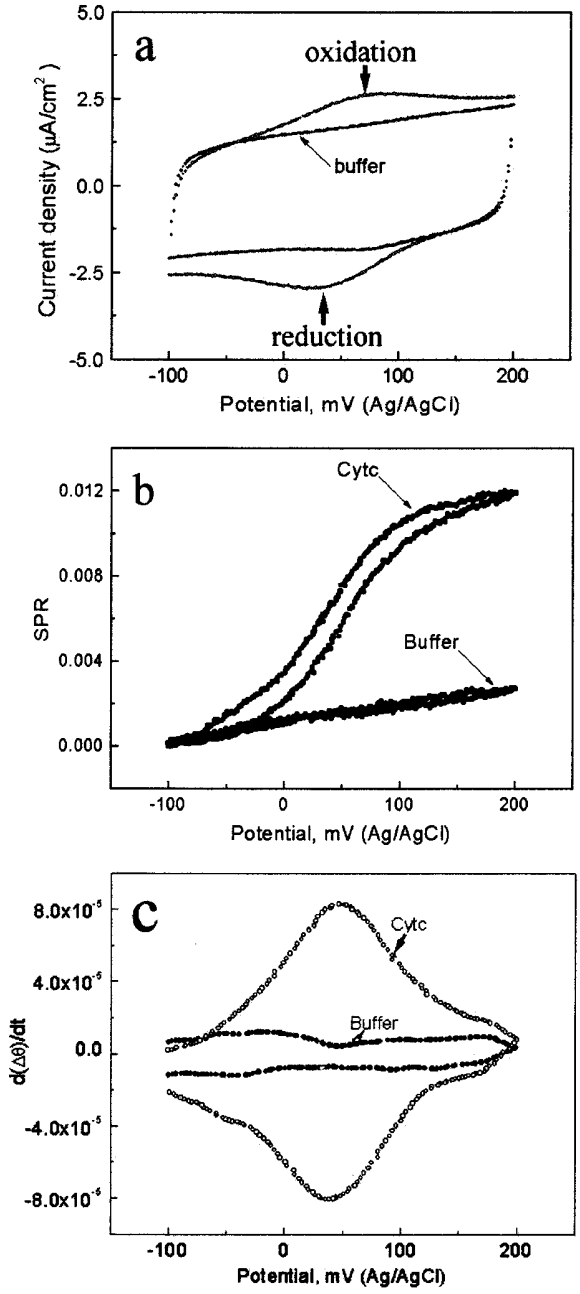

Figure 3. (a) Cyclic voltammograms with and without cyt *c* immobilized on the surface in 50 mM phosphate solution. (b) Corresponding SPR dip shifts (wavelength = 670 nm). (c) Derivatives of the SPR shifts shown in (b).

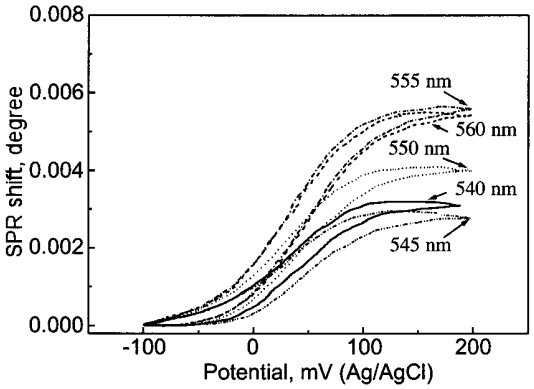

Figure 4. Electron-transfer-induced shift in the resonance angle for a monolayer of cyt *c* immobilized on an MPA-coated silver surface at various wavelengths.

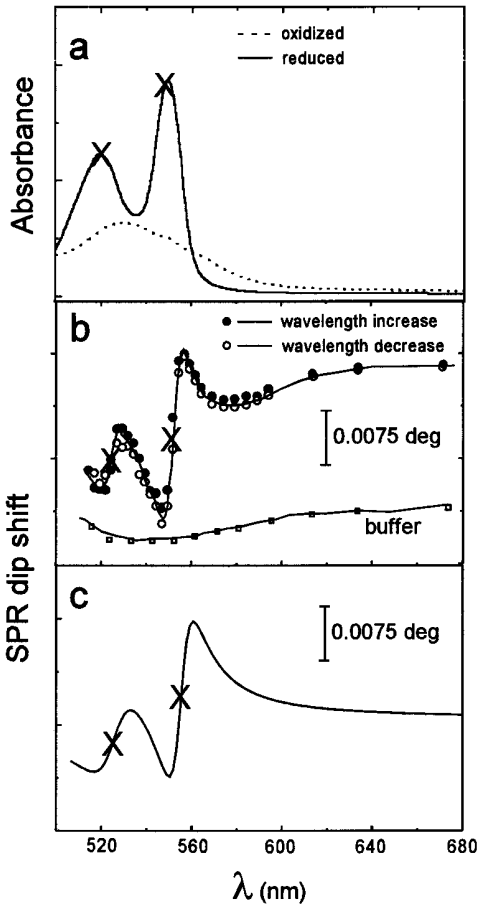

Figure 5. (a) Absorption spectra of reduced and oxidized cyt *c*. (b) Experimental SPR shift of cyt *c* (open and filled circles) as it switches from oxidized to reduced states (the kinks occurring at the absorption peaks 550 and 520 nm) and the shift in pure phosphate buffer (squares). (c) Theoretical SPR shift based on the absorption peaks and the Kramers−Kronig relation.

was also measured over the wavelength range, which shows little wavelength dependence.

To compare the experimental data with theory quantitatively, we have calculated the electron-transfer-induced SPR shift as a function of the wavelength using the experimental absorption spectra shown in Figure 5a as inputs. We first calculated the refractive index of the protein in both the reduced and oxidized states with the Kramers−Kronig relation. Using the refractive index, we then determined the resonance angles of the reduced and oxidized states on the basis of the four-phase Fresnel calculation. The calculated difference between the resonance angles of the two states is in an excellent agreement with the experimental data near the absorption bands (Figure 5c).

Far away from the absorption peaks (>630 nm), the SPR angle shift vs potential is still sigmoidal and much greater than that of the pure buffer (see Figure 3b), but the shift in the SPR angle between the two states becomes wavelength independent (Figure 5b). This means that the shift at large wavelengths is not due to

the electronic change associated with the optical absorption, and therefore it must originate from a structural change in the protein layer. Because the structural change will change both the refractive index and the average thickness of the protein (see eq 1), the SPR shift alone is not adequate to directly determine both the refractive index and the thickness. However, the refractive index is related to the average thickness by the Lorentz−Lorenz relation.[12] The relation, together with a simplied model given below, can be used to extract the change in the average thickness in the protein layer upon oxidation or reduction.

According to the Lorentz−Lorenz relation

$$\frac{n^2 - 1}{n^2 + 2} = \frac{4\pi}{3V}(N_p \sum_i \alpha_i + N_w \alpha_w) \qquad (3)$$

where N_p and N_w are the numbers of cyt *c* and water molecules in the protein layer and α_i and α_w are the polarizabilities of the *i*th atom in the protein and a water molecule, respectively. V in eq 3 is the volume of the protein layer and is given by $V = V_p + V_w$, where V_p and V_w are the volumes of the protein and water molecules in the layer, respectively. If the conformational change causes a change in the protein thickness by Δd, the Lorentz−Lorenz relation predicts a refractive index change, Δn, given by

$$\Delta n = -\frac{1}{6n}(n^2 + 2)^2 \left(\frac{n^2 - 1}{n^2 + 2} - \frac{{n_w}^2 - 1}{{n_w}^2 + 2} \frac{V_p}{V} \right) \frac{\Delta d}{d} \qquad (4)$$

where n_w is the refractive index of water. From the surface coverage of cyt *c* determined from the current peaks, $V_p/V_w = 0.52$, together with that $n = 1.5$, $n_w = 1.33$, and $d = 3.5$ nm, we have $\Delta n = -0.11\Delta d$. Combining this result and eq 1, we have $\Delta\theta \sim -0.15\ \Delta d$. So a ∼0.008° decrease in the resonance angle corresponds to an increase of ∼0.05 nm in the protein thickness. This change is rather small, which is consistent with the X-crystallography data.[21]

Finally, we note that the angular resolution of the multiwavelength setup is 10^{-3}−10^{-4} deg, which is not nearly as good as the resolution of a differential SPR setup that we have achieved using a diode laser.[14] The primary reason is the use of the bulky xenon lamp and monochromator, which make the setup more susceptible to thermal and mechanical noise than the compact diode laser setup.

CONCLUSIONS

By accurately measuring the SPR angle as a function of the incident light wavelength with a differential detection method, we have demonstrated that both conformational and electronic information regarding molecules adsorbed on a surface can be determined. The electronic information provides not only direct identification of the molecules but also new insights into changes in the electronic states due to various processes. Using SPR, we have studied the electron-transfer reaction of cyt *c*. Near the absorption bands at 520 and 550 nm, oscillations centered at the two wavelengths were observed in the resonance angle vs wavelength plot, which agrees quantitatively with the Kramers−Kronig relation. Far away from the absorption bands, a ∼0.008° decrease in the resonance angle accompanies the reduction of cyt *c*. This decrease depends little on the wavelength and is due to a conformational change in the protein associated with the electron-transfer reaction.

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