Detection of lysozyme adsorption on titania using surface plasmon resonance on Au layer coated with thin titania film

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Titanium metal, which is used as artificial bone, has a titania surface layer. In this study, titania was investigated using surface plasmon resonance (SPR) in terms of protein adsorption, which occurs during the initial process of cellular attachment by employing a multilayer device consisting of a titania layer on Au. The titania film was deposited on the Au layer in the multilayer device by atmospheric chemical vapor deposition. A flow cell was used to evaluate protein adsorption on the titania top layer of the multilayer device. Phosphor buffer solutions with and without the protein lysozyme (Lyz) were injected into the flow cell. From the attenuation of reflected light, the SPR angle was determined as the angle with minimum reflection intensity. The observed behavior of the SPR angle indicated that Lyz was adsorbed on the titania film surface, and the obtained SPR angles increased with the Lyz concentration on the titania surface. These results indicate that the titania/Au multilayer SPR device can easily be used to detect the adsorption of protein.

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1. Introduction

Titanium metal is a relatively bioinert metal with good biocompatibility that exhibits sufficient mechanical strength to be used as artificial human bone.1) Titanium metal has recently been used in dental implants,2) artificial joints,3) and artificial bones.4) Its surface is coated with a natural oxide layer of titania. Thus, the body usually comes in contact with titania rather than titanium. Titania or titania is generally placed in a culture medium in general biocompatibility testing. Then the sample are completely dried and examined by fluorescence microscopy.5) In these tests, the methods used to evaluate biocompatibility require a long time for each process. Hence a simple method of evaluating biocompatibility in a short time is required.

It is well known that the first step of cell adsorption a material surface is protein adsorption.6)† Namely, the initial protein adsorption determines the behavior of the subsequent cell adsorption. Direct detection methods for protein adsorption using surface plasmon resonance (SPR),7)–10) ellipsometry,11) optical waveguide light mode spectroscopy,12)–15) a quartz crystal microbalance,12)–14) and surface-acoustic-wave devices have been reported.16) In particular, the SPR sensing technique has high sensitivity and enables real-time analysis. SPR has been applied to the detection of biomolecules adsorbed on a metal surface under a liquid.15)–18)

SPR was first reported by Otto, who found that when a glass prism is brought near a metal-vacuum interface, surface plasma waves can be optically excited by the evanescent waves that undergo total reflection.17) A system with a Kretschmann configuration that employs the phenomenon of total internal reflection was then developed for detecting SPR.15)–18) When a metal film is placed on a glass prism, the light reflected from the glass generates an evanescent wave that penetrates through the metal film. The evanescent field $K_{ev}$ is given by

$$K_{ev} = n_p \omega/c \sin \theta,$$

where $n_p$ is the refractive index of the prism, $\omega$ is the frequency of the incident light, $c$ is constant as the speed of light in vacuum, and $\theta$ is the incidence angle of the light. Plasmon waves are also excited on the external surface of the film, and the wave vector of a surface plasmon ($K_{sp}$) can be approximated

$$K_{sp} \approx \omega/e_{in} \cdot n_s^2/(e_{in} + n_s^2)^{1/2},$$

where $e_{in}$ is the dielectric constant of the metal film and $n_s$ is the refractive index of the dielectric medium. The surface plasmon is resonantly excited under the condition of $K_{sp} = K_{ev}$ which causes the reflection intensity of the laser from the back side of the metal film to be rapidly reduced. The laser incidence angle under this condition is defined as the SPR angle $K_{sp}$ or $\theta_{sp}$. From Eq. (1), $K_{sp}$ is dependent on the refractive index of a water/air medium on a metal film, which can be monitored up to approximately 200 nm above the metal surface (within the detection limit of SPR). Therefore, when the refractive index above the metal surface changes owing to the adsorption of a protein, $\theta_{sp}$ is shifted. By monitoring $\theta_{sp}$ during the adsorption process, the adsorption behavior can be observed by the shift in $\theta_{sp}$. SPR has been used to detect the interaction between inorganic films and proteins. From the consideration of the detection limit of SPR, detection using SPR is possible when the inorganic thin film is deposited within 200 nm from the metal film surface. Thus, protein adsorption on a film can be detected when a film with a thickness of nanometers is deposited on a metal. Kishimoto et al. reported that lysozyme adsorption on a silica surface could be detected using a multilayer structure of silicate glass/metal by employing SPR.20) To investigate protein adsorption on the surface of titania which is a commonly used biomaterial, we used multilayered SPR device to detect protein

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adsorption on a titania surface. Hence, a structure consisting of a thin titania film deposited on a metal may be used to detect protein adsorption on a titania film.

In this study, we attempted to detect protein adsorption on a titania surface through the detection of SPR on a titania/metal multilayer structure. Furthermore, the structural dependence of the efficiency of protein adsorption on the titania film was studied using such a multilayer SPR device.

2. Experimental methods

The device with a titania/metal multilayer structure used for SPR detection is shown in Fig. 1. Gold (Au) was used as the metal. An optical glass substrate with dimensions of 25 × 25 × 1 mm³ and a refractive index of 1.778 was chosen. A 1.5-nm-thick chrome (Cr) layer was used as an adhesion layer between the Au film and the substrate. The Cr layer was deposited by magnetron sputtering using a Cr target (99.9%) in Ar gas. Furthermore, a 50-nm-thick Au film was deposited on Cr/glass by sputtering; a pure Au target (99.95%) was used for the deposition. For SPR detection, titania obtained from titanium tetraisopropoxide [Ti(O-CH(CH₃)₂)₄] was deposited on Au/Cr/glass by atmospheric chemical vapor deposition (CVD). The titanium tetraisopropoxide was vaporized at 80°C then transported to the nozzle of the CVD apparatus using a carrier gas (N₂) with a flow of 31/min, and the titania film was deposited on the gold layer on the substrate, which was maintained at 400°C. A titania film was also synthesized on a silicon substrate to investigate the crystalline structure by X-ray diffractometry (XRD). Finally, the SPR device consisting of titania/Au/Cr/glass multilayers was integrated with a glass triangular prism using index-matching oil (n = 1.778).

The crystalline structure and surface roughness of the deposited titania film were evaluated by XRD (M03XHF22, MAC Science Co., Ltd.) with Cu Kα radiation and atomic force microscopy (AFM), respectively. The surface morphology of the films was observed by AFM (Seiko Instruments: SPA300) at a scan speed of 0.170 Hz.

Lysosome (Lyz) was used as the protein with a phosphor buffer (PB) as the buffer solution. The PB was a mixture of Na₂HPO₄, 12H₂O and NaH₂PO₄·2H₂O with a phosphate concentration of 1.8 mM and the pH adjusted to 7. Lyz-containing PB solutions were prepared with Lyz concentrations of 7, 21, 35, and 70 μM.

The SPR sensor operated in the Kretschmann configuration, which employs the phenomenon of total internal reflection to achieve the resonant condition. The incident light was a beam from a laser diode with a wavelength of 635 nm (Sigma Koki LDU-33-635-3). The intensity of the reflected light was detected using a photodetector (Advantest Q82324-ADCE8250A). The laser diode and photodetector were mounted on separate rotating stages, which were moved using a stepping motor with a maximum resolution of 0.0025° (Sigma Koki KST-120YAW). An iris diaphragm mounted between the laser diode and the prism was used to reduce the beam diameter to approximately 0.8 mm. A flow cell with a volume of approximately 0.5 ml was attached to the multilayer SPR device to investigate Lyz adsorption. The experimental procedure for investigating the adsorption of Lyz on the titania film surface was as follows. First, the initial resonance angle was measured for only the PB solution injected into the flow cell. Next, the PB solution in the flow cell was replaced with a Lyz-containing PB solution, and the resonance angle was measured. Then, the protein solution was again replaced with PB solution to remove the nonadsorbed Lyz and the resonance angle was measured.

3. Results

Figure 2 shows the XRD profile of the titania film on the Si substrate. The peaks at 32.95 and 69.15° were assigned to the Si substrate. The peaks at 25.30, 37.00, 37.75, 38.55, 48.05, 53.90, 55.10, 62.70, and 75.05° were assigned to the (101), (103), (004), (112), (200), (105), (211), (204), and (215) planes of anatase titania, respectively. These peaks indicated that the deposited titania film was oriented in the [101] direction. An AFM image of the titania surface of the device is shown in Fig. 3. The film surface had a grainlike structure and the surface roughness was 3.59 nm.

The dependence of reflectivity on the laser incident angle used to evaluate Lyz adsorption on the titania layer on a Au layer, is shown in Fig. 4. By considering the attenuation of reflected light, the laser incident angle resulting in the weakest reflectivity was defined as θ₁λ, which was determined to be 74.46° for PB solution. After this initial measurement, a Lyz-containing solution was injected onto the titania surface, and θ₁λ was measured. θ₁λ increased from 74.46 to 74.90° upon the injection of PB solution containing 7μM Lyz. To remove the nonadsorbed Lyz from the titania surface, PB solution was again injected onto the titania surface, which caused θ₁λ to decrease from 74.90 to
After that, PB solution was repeatedly injected onto the surface, and after each injection we investigated the dependence of reflectivity on the laser incident angle. However, $\theta_{q}\text{er}$ remained in the range of $74.83 \pm 0.1^\circ$. This uniform $\theta_{q}\text{er}$ suggests that the adsorbed Lyz was immobilized on the titania surface. The shift of $\theta_{q}\text{er}$ indicated a change in the dielectric constant caused by the change in Lyz concentration on the titania surface. Thus, the shift of $\theta_{q}\text{er}$ showed that SPR measurement using the titania/Au multilayer device can be used to detect protein adsorption on a titania surface.

To investigate the concentration dependence of the adsorption of Lyz on titania, we repeated the above procedure for PB solution containing other concentrations of Lyz. The dependence of reflectivity on the laser incident angle for each concentration of Lyz after the Lyz-containing PB solution was replaced with PB solution is shown in Fig. 5. $\theta_{q}\text{er}$ increased with the concentration of injected Lyz. $\theta_{q}\text{er}$ for each concentration of

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**Fig. 3.** AFM image of titania surface of titania/Au. The observation area is $1000 \text{ nm}$.  

**Fig. 4.** Dependence of reflectivity on laser incident angle of titania/Au multilayer device after injection of (1) PB solution, (2) 7 $\mu$M Lyz containing PB solution, and (3) PB solution to replace Lyz containing PB solution.  

**Fig. 5.** Dependence of reflectivity on laser incident angle after replacing with the PB from each Lyz-containing PB solution. (1) After PB injection for initial, After replacing with the PB solution from (2) 7 $\mu$M, (3) 36 $\mu$M, and (4) 70 $\mu$M.
that Lyz was adsorbed on the titania film surface, and that most of the available adsorption sites on the titania surface were absorbed by Lyz.

4. Conclusions

The detection of protein adsorption using a multilayer SPR device consisting of a titania layer on Au was investigated. The top layer of the SPR device was titania deposited by atmospheric plasma CVD. By considering the attenuation of reflected light, \( \theta_{opt} \) was defined as the angle with minimum reflection intensity. Upon evaluating the adsorption of Lyz as the protein, the observed behavior of \( \theta_{opt} \) indicated that Lyz was adsorbed on the titania film. Our results indicate that the titania/Au multilayer SPR device can easily be used to detect protein adsorption on a titania surface.

References