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Characterization of Ti/Au Metal Spot Arrays by a Wavelength Interrogation-Based Surface Plasmon Resonance Biosensor

Jong Seol Yuk, Sun-Ju Yi, Seoung-Woo Park[1], Jeong-A Han, Young-Myeong Kim and Kwon-Soo Ha*

Department of Molecular and Cellular Biochemistry and Vascular System Research Center, Kangwon National University School of Medicine, Chunchon, Kangwon-Do 200-701, South Korea
[1]Department of Neurosurgery, Kangwon National University School of Medicine, Chunchon, Kangwon-Do 200-701, South Korea

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We have investigated the characteristics of surface plasmon resonance (SPR) spectra as a function of metal thickness for the application of protein arrays by using a wavelength interrogation-based SPR biosensor (self-developed). Resonance intensity and wavelength were significantly affected by the thickness of Au film. Minimal resonance intensity was observed at a thickness of approximately 450 Å of the Au film and changed slightly according to the incidence angle. A Ti film as an adhesion layer had a significant influence on the half-width of the SPR spectrum and the resonance wavelength, and a 50Å-thick Ti layer under the 450Å-thick Au film yielded a good spectrum in these experiments. In addition, the interaction of anti-tissue transglutaminase and tissue transglutaminase was successfully analyzed on the array of Ti/Au (50 Å /450 Å) metal spots by using the wavelength interrogation-based SPR biosensor. Thus, it is important to consider the optimal thickness of the Au film and Ti adhesion layer when biospecific interactions are analyzed on protein arrays by SPR biosensors.

*Corresponding author, e-mail address: ksha@kangwon.ac.kr

1. Introduction

The application of the surface plasmon resonance (SPR) phenomenon for biosensing purposes was first demonstrated by Liedberg *et al.* in 1983.[1] Since then, the utilization of SPR for the study of biospecific interactions has become widespread because it is possible to perform real-time analysis of biospecific interactions without labeling proteins.[2]

The choice of active metal for surface plasmons is a critical factor in the analysis by SPR biosensors. Even though noble metals such as Au, Ag, Cu and Al are suitable candidates for optical excitation of surface plasmons, most of the experimental works have been performed on Au and Ag.[1–5] In particular, Au is better than Ag from the viewpoint of long-term stability, which means its use in biochemical analysis takes into account the stability consideration. Furthermore, metals such as Ti or Cr have been used for the adhesion layer of noble metals[6–9] because homogeneous Au and Ag are easily detached from the glass substrate during the cleaning process to obtain clean surfaces of metal spots.

There has been a theoretical report on the characterization of the SPR spectrum with respect to Au thickness using Fresnel's equations, which was performed by varying the incidence angle at a fixed wavelength.[10] There is another experimental report on the analysis of SPR spectra obtained using various Ag film thicknesses at a fixed angle of light incidence.[11] In addition, the deposition of a thin metallic film on a thick metal substrate to study the modification of dispersion relations and properties for surface plasmons has been reported.[12,13] However, there are no reports on the properties of SPR spectra with respect to the variation of the adhesion metal layer thickness under a Au substrate for the application of SPR-based biosensors. It is important to apply a SPR measurement system with varying wavelength at a fixed incidence angle for the analysis of protein arrays.[14] Protein arrays consist of several tens to thousands of different proteins immobilized on the solid surface such as metal, glass and plastics. The technology of protein arrays is very important for the analysis of biospecific interactions because it performs highly parallel quantitation of specific proteins in a rapid, low-cost and low sample-volume format.[15–17]

Thus, in this study, we investigated the properties of SPR spectra with respect to the thickness of the Ti adhesion layer by using a wavelength interrogation-based SPR sensor. Ti was selected as an adhesion layer to obtain a sharp SPR spectrum because the dielectric constants of Ti are smaller than those of Cr. Based on the results of SPR measurements, we have discussed the resonance wavelength, depth of reflected intensity and half-width of SPR spectra with respect to the changes of metal thickness. In addition, we have proposed an optimal thickness of the adhesion metal layer and Au film for the use of SPR-based biosensors. The Ti/Au (50 Å/450 Å) metal system successfully analyzed the shift of SPR wavelength caused by immobilizing tissue transglutaminase (tTGase) on the surface of an array of the metal spots modified by dithiobis(succinimidyl propionate) (DTSP).

2. Surface Plasmon Resonance

Surface plasmon resonance (SPR) is an electromagnetic phenomenon in which an evanescent wave excites charge density oscillation along a metal/dielectric interface. Surface plasmons, also called as surface plasmon polaritons (SPP), represent electromag-

netic surface waves that have their intensity maximum in the surface and exponentially decaying fields normal to the surface.[18] Changes in the optical constants close to the metal surface have a significant influence on the resonance condition, which is the basis of the use of SPR for biosensing purposes.[19]

For a smooth surface of a semi-infinite metal with the complex dielectric function $\varepsilon_m(\omega) = \varepsilon_{mr} + i\varepsilon_{mi}$, the wave vector of SPP at the interface between a metal and air is given by

$$k_{sp}^{(0)} = \left(\frac{2\pi}{\lambda}\right)\sqrt{\frac{\varepsilon_m(\lambda)\varepsilon_d}{\varepsilon_m(\lambda)+\varepsilon_d}}, \tag{1}$$

where λ is the free space wavelength of the light and ε_d is the dielectric constant of dielectric on metal.[18] In the case of considering the metal film of finite thickness, the wave vector of SPP is expressed as

$$k_{sp} = k_{sp}^{(0)} + \Delta k_{sp}, \tag{2}$$

where Δk_{sp} characterizes the effect of finite thickness of the metal and decreases as the metal thickness increases. Surface plasmon resonance takes place when the wave vector of SPP matches with the component of the p-polarized incident photon's wave vector, which is parallel to a metal/dielectric interface ($k_{sp} = k_x$). Surface plasmon excitation, SPR, is observed as a characteristic depth in the reflected light intensity. The wave vector of the p-polarized incident photon which is parallel to the metal surface is expressed as

$$k_x = \left(\frac{2\pi}{\lambda}\right) n_p(\lambda)\sin\theta, \tag{3}$$

where n_p is the refractive index of the prism, and θ is an incidence angle of light into the metal surface.

3. Experimental

Metal arrays with eight spots (diameter of each spot, 3 mm) were fabricated by a RF-magnetron sputtering apparatus at a vacuum of 3×10^{-6} Torr (Nuricell, Korea). Three sets of metal spot arrays were prepared by depositing Ti and Au films of various thicknesses on glass substrates. The first set of arrays was prepared by directly depositing Au (99.99%) films of various thicknesses from 300 to 700 Å on the glass substrates. The second set of arrays was made by depositing Au films from 400 to 500 Å thickness on the Ti adhesion layer (50 Å). The last set of arrays was made by depositing a 450Å-thick Au film on Ti layers of various thicknesses.

The metal spot arrays were cleaned by incubation with a cleaning solution of NH_4OH:H_2O_2:H_2O (1:1:5, v/v) at 80°C for 10 min and washing with dH_2O. Then, the

surface of metal spots was modified by DTSP to immobilize proteins by incubation with 5 mM DTSP containing a cleavable disulfide linkage for 3 h at room temperature, resulting in the formation of a self-assembled monolayer of DTSP. For the analysis of the interaction between tTGase and anti-tTGase, tTGase (0.1 mg/ml in PBS, pH 7.4) was immobilized on the DTSP surface for 1 h at room temperature in a water-saturated petridish. After washing with PBS and dH_2O, the surfaces were incubated with anti-tTGase (0.1 mg/ml in PBS, pH 7.4) for 1 h in a water-saturated petridish and washed with PBS. Then, the protein arrays were rinsed with dH_2O, dried under N_2 gas and analyzed in contact with air (*ex situ*) by a SPR wavelength interrogation-based sensor.

The SPR wavelength interrogation-based sensor was self-developed using a 20 W quartz tungsten halogen lamp as the light source due to its stable output (Oriel, Inc. USA).[20] Optical excitation of surface plasmons by the ATR method, Kretschmann-Raether configuration, was used in our SPR sensor. A Glan-Tayler polarizer was positioned at the input light path to obtain transverse magnetic polarized light. An optical prism coupler made of fused silica was mounted on an x-y linear stage and the incidence angle was adjusted by a beam steering device to obtain resonance wavelength. The incidence angle was fixed at 49° for the measurement of the SPR spectrum and the reflected light from the prism was collected by an optical fiber whose diameter was 200 μm. In order to collimate nonsymmetrical beams, a plano-cylindrical glass lens was positioned in front of the optical fiber. An AVS-S2000 spectrometer, which provides 0.4 nm resolution in the range of 500–700 nm (Avantes, Inc. Netherlands), was used to find the resonance wavelength of reflected light. Data acquisition, processing, and display were performed by self-developed programs using LabVIEW software.

4. Results and Discussion

Initially, we investigated the characteristics of SPR spectra as a function of the Au thickness on a glass substrate using a wavelength interrogation-based SPR biosensor, because the thickness of the surface plasmon active metal is a key parameter in determining the resolution of the SPR sensor. First, a series of SPR spectra were obtained according to Au thickness at the incidence angle of 47° (Fig. 1). A characteristic SPR spectrum, which is produced by excitation of surface plasmons at the interface of Au/air, was observed for a Au film thickness of 400 Å. It has been reported that minimal resonance intensity takes place when the internal damping of $\mathrm{Im}\left\{k_{sp}^{(\bullet)}\right\}$ and radiation damping of $\mathrm{Im}\left\{\Delta k_{sp}\right\}$ are equal and it depends on the dielectric function of metal.[18] At 450 Å, we obtained a sharper SPR spectrum with the minimal normalized intensity of the resonance, donated by resonance intensity. However, from 500 to 700 Å, the resonance intensity of SPR spectra significantly elevated with the decrease of resonance wavelength. It was impossible to observe a normal SPR spectrum at a 300 Å thickness of Au film. These results suggest that a suitable thickness of Au film for protein arrays is approximately 450 Å at the incidence angle of 47°.

However, it was necessary to lower the resonance wavelength by the increase of incidence angle, since macromolecular interactions on protein arrays cause a large shift of resonance wavelength. Thus, we investigated SPR spectra by the same metal arrays with

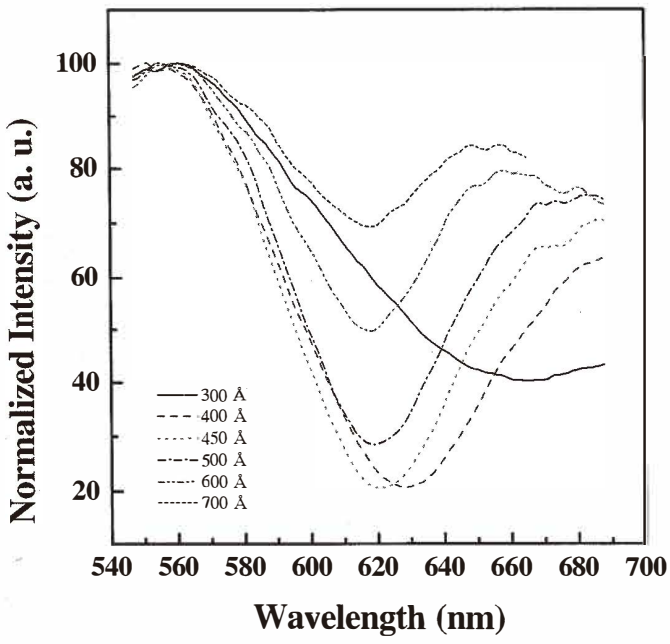

Fig. 1. SPR spectra obtained from Au films of various thicknesses on glass substrates as a function of the wavelength at a fixed incidence angle of 47°. SPR spectrum of 300 Å (solid line), 400 Å (dash line), 450 Å (dot line), 500 Å (dash-dot line), 600 Å (dash-dot-dot line), and 700 Å (short dash line) thick Au films.

various thicknesses at the incidence angle of 49° (Fig. 2). The minimal resonance intensity was observed at 400 Å, but the half-width of the resonance curve decreased by the increase of Au film thickness from 400 to 500 Å. In addition, the resonance wavelength at 49° was much lower than that at 47° (compare Figs. 1 and 2), indicating that a 49° incidence angle has a larger available spectral range than a 47° one for the analysis of protein arrays by the wavelength interrogation-based SPR biosensor. Considering that accurate analysis of protein arrays by SPR biosensors is dependent on resonance intensity and sharpness of the SPR spectrum, it is likely that the optimal Au thickness is approximately 450 Å at the incidence angle of 49°.

Next, in order to investigate the influence of the adhesion layer under the Au film on the SPR spectrum, we obtained SPR spectra from metal spot arrays coated with Ti layers of three different thicknesses (50, 100 and 150 Å) and a 450Å-thick Au film, and analyzed the characteristics of the SPR spectra. As shown in Fig. 3, the resonance wavelength and intensity elevated as the thickness of the Ti adhesion layer increased from 50 to 150 Å. The increase of Ti thickness also caused the half-width increment of the spectra by broadening of the spectra, indicating that 50 Å was the optimal thickness in these experiments. The

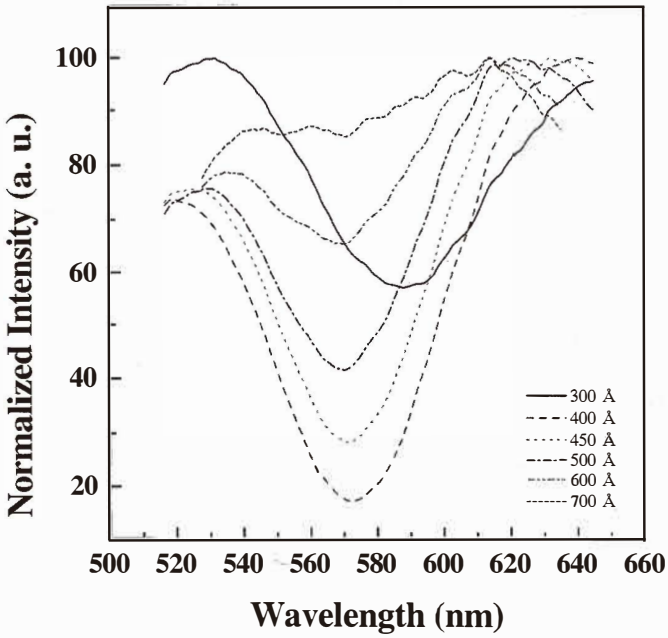

Fig. 2. SPR spectra obtained from Au films of various thicknesses on glass substrates as a function of the wavelength at a fixed incidence angle of 49°. SPR spectrum of 300 Å (solid line), 400 Å (dash line), 450 Å (dot line), 500 Å (dash-dot line), 600 Å (dash-dot-dot line), and 700 Å(short dash line) thick Au films.

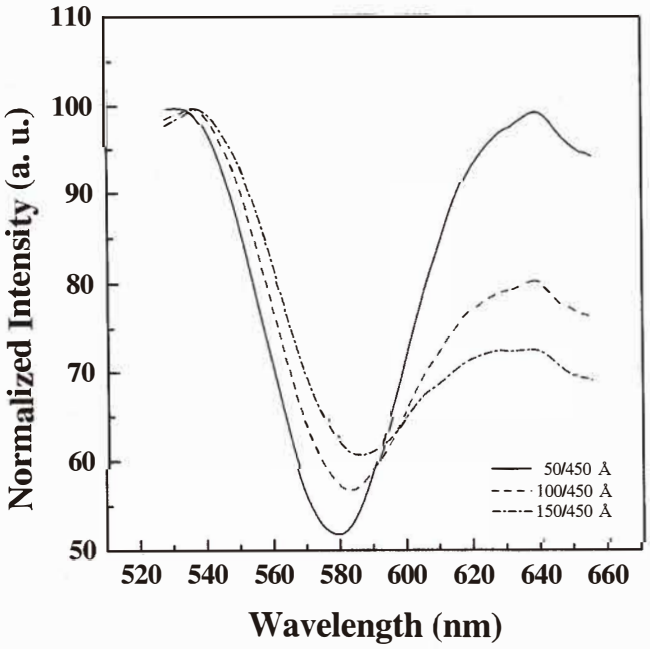

Fig. 3. SPR spectra obtained from Ti films of various thicknesses under Au substrates (450 Å) at the fixed incidence angle of 49°. SPR spectrum of 50 Å (solid line), 100 Å (dash line), and 150 Å (dash-dot line) thick Ti films.

changes of resonance intensity and wavelength could be interpreted as being due to damping, which is related to $\sim\varepsilon_{mi}d$ of Au.[13] These results suggested that the thickness of the adhesion layer strongly influenced the intensity and half-width of the SPR spectrum. In our experiments, a 50Å-thick Ti layer was chosen for metal spot arrays since a thinner Ti layer did not yield a superior Au surface after incubating the surface with a cleaning solution of NH_4OH:H_2O_2:H_2O (1:1:5, v/v).

Our previous results showed that the SPR wavelength decreased with increasing the thickness of the Au film without a Ti adhesion layer (Figs. 1 and 2). Thus, we have tested whether a Ti layer (50 Å) under the Au film affects the changing pattern of the SPR spectrum by variation of Au film thickness. When we increased the thickness of the Au film from 400 to 500 Å on the Ti layer, the SPR wavelength decreased as it did without a Ti adhesion layer (data not shown). The characteristic responses of resonance wavelength to the thickness of the Au film on the Ti layer were opposite to those to the thickness of the Ti adhesion layer under a Au film. The difference in the response of the SPR wavelength could be caused by different dielectric constants of Au and Ti because these dielectric constants are involved in damping.[13] SPR intensity also increased with respect to the thickness of the Au film. These results suggested that the adhesion layer of metal spot arrays did not influence the resonance wavelength and intensity. Thus, the Ti/Au system is a good surface for analyzing protein interactions on protein arrays by the wavelength interrogation-based SPR sensor.

To test whether it is possible to regenerate the Ti/Au metal spots without lifting off the Au layer from the glass substrate, the surface of Ti/Au metal spots was repeatedly modified by DTSP, immobilized with proteins and cleaned with cleaning solution of NH_4OH:H_2O_2:H_2O (1:1:5, v/v). Then the SPR wavelength was determined by the SPR wavelength interrogation-based sensor (Fig. 4). When the surface was cleaned, the resonance wavelength returned exactly to the basal level, implying that all the layers on the Au surface were clearly removed. The same resonance wavelength was obtained each time after 7 successive modifications with DTSP. Thus, the arrays of Ti/Au metal spots can be efficiently regenerated by the cleaning solution.

Then, we investigated whether the Ti/Au (50/450 Å) system is able to monitor protein interactions on protein arrays. We have obtained SPR spectra following the incubation of anti-tTGase and tTGase on the arrays of metal spots. tTGase is a Ca^+-dependent cross-linking enzyme which has various biological functions such as the induction of apoptosis, cell adhesion and morphology, neurodegenerative diseases and cell growth.[21,22] As shown in Fig. 5, there was a small increase of SPR wavelength by modification with DTSP, as expected from the low molecular weight of DTSP. The interaction of anti-tTGase and tTGase on the DTSP surface was clearly analyzed by the wavelength interrogation-based SPR biosensor. It was predicted that the shift of SPR wavelength was caused by the increase of refractive index by protein interactions and thickness increment on the surface of a Ti/Au metal spot array. Therefore, it was concluded that Ti/Au was a good active metal system for monitoring biospecific interactions on protein arrays by a wavelength interrogation-based SPR biosensor, and the optimal thicknesses of Ti and Au were approximately 50 and 450 Å, respectively.

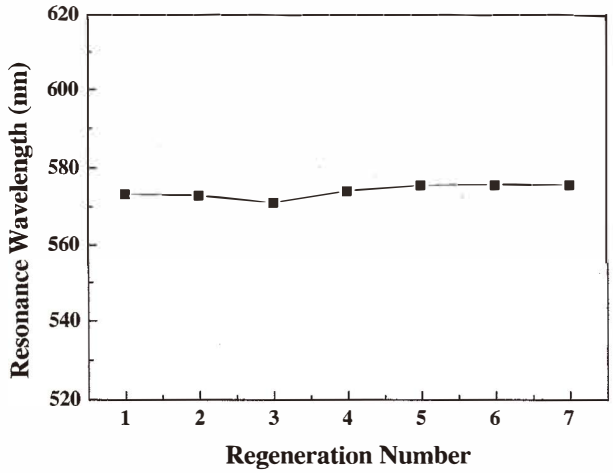

Fig. 4. Regeneration of protein arrays. A protein array was repeatedly regenerated by incubation with NH_4OH:H_2O_2:H_2O (1:1:5, v/v), and then resonance wavelength was determined.

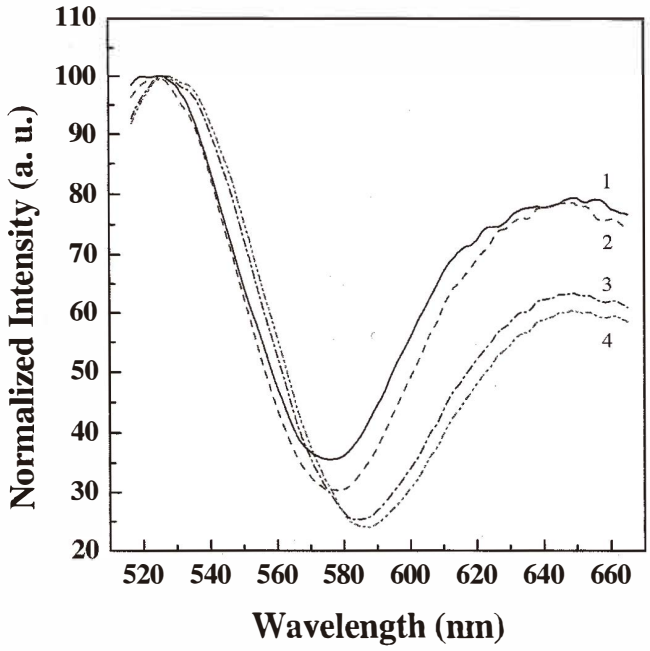

Fig. 5. Changes of SPR spectrum by successive incubation with DTSP, tTGase and anti-tTGase on the Ti/Au (50/450 Å) system of protein arrays. SPR spectra were measured at the incidence angle of 49°. SPR spectrum of Au (1), Au/DTSP (2), Au/DTSP/tTGase (3), and Au/DTSP/tTGase/anti-tTGase (4).

5. Conclusions

We have investigated the characteristics of SPR spectra with respect to the thickness of the Au film and Ti adhesion layer by using a wavelength interrogation-based SPR biosensor. Pronounced shifts of resonance intensity and wavelength with respect to the thickness of Au and Ti have been observed. Particularly, Ti as an adhesion layer had a significant influence on the half-width of the resonance curve and resonance wavelength, and we found that the optimal thicknesses of the Ti/Au metal system were approximately 50 and 450 Å, respectively. Also, we successfully analyzed the interaction of anti-tTGase and tTGase on arrays of Ti/Au metal spots.

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