Development of a Two-Dimensional Evaluation Method for Thin Layers Using Surface Plasmon Resonance

Soichi Otsuki,* Kensuke Murai,† and Susumu Yoshikawa††
Human Stress Signal Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Midorigaoka, Ikeda, Osaka 563-8577
†*Photonics Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Midorigaoka, Ikeda, Osaka 563-8577*
††*Institute of Advanced Energy, Kyoto University, Gokanosho, Uji, Kyoto 611-0011*

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A two-dimensional evaluation method for intermolecular interactions based on surface plasmon resonance (SPR) was developed. The measurement was conducted at varying wavelengths. The wavelength affording the minimum brightness (SPR wavelength) was determined at each pixels of the image. The spatial distribution of SPR wavelength was converted to a thickness distribution in nm scale, which well presented structures of thin materials attached on silver film.

Recently, two-dimensional surface plasmon resonance (2DSPR) technique has begun to be explored to observe variations in thickness or refractive index of thin films with high sensitivity.[1–7] The 2DSPR technique has successfully applied for label-free characterization or monitoring of laterally structured organic layers,[8] adsorption of antigens, proteins, or biopolymers on patterned surfaces,[9–11] DNA and RNA hybridization in an array format.[7,11,12]

Originally in the surface plasmon resonance (SPR) technique, a p-polarized light irradiates the interface between metal and dielectric thin film in an attenuated total reflection configuration. Two typical configurations have been proposed. One employs a focused light for illumination and measures the intensity of the reflected light by a linear photodiode array to determine refractive index at each point, and the point of incidence is mechanically scanned.[3] However, the mechanical scanning takes very long time for characterization of the full surface. The other configuration illuminates a specimen with a plane light at a fixed angle and detects reflected light with a CCD camera.[1] The image consists of brighter or darker regions corresponding to spatially localized difference in the specimen. However, it is difficult to predict in advance for the measurement if a variation in material gives rise to increasing or decreasing the brightness. Different thickness or refractive index can often give the same brightness as the incidence angle goes out of the resonance point and the reflected intensity does not change any more.

Measurement of 2DSPR at multiple angles has been explored to characterize the lateral thickness heterogeneities of a polymer film.[2] Lines of lowest reflected intensity were drawn for each picture taken at varying angles. The thickness contour of a polymer film was obtained by Fresnel calculations to convert the angular data to thickness and synthesizing the lines for all angles. However, the thickness profile was discontinuous and small variations in the distribution were neglected. White light has been used as an exciting light to conduct a 2DSPR experiment.[4] The results were color images of reflected light with areas of different color corresponding to those of different thickness, but were as far from qualitative as conventional 2DSPR methods. A 2DSPR measurement at multiple wavelengths has also attempted to visualize continuously varying refractive indices of solutions in micro wells.[6] However, no attempt obtaining spatial distributions of the refractive index has been made using data given by the multiple-wavelength measurement.

In this study, images corresponding to spatial distributions of the refractive index or thickness of samples were obtained for the first time by analyzing images of reflected light measured at varying wavelengths and determining local minima of the reflected intensity based on SPR.

The experimental set-up is shown in Figure 1. A thin silver layer, 50 nm, was vacuum-deposited on BK7-glass substrates of 1 mm thickness. A sample layer was prepared on the silver layer as noted below. The bare glass side of the substrate was attached on one surface of the equilateral prism using a matching oil. The light from a halogen lamp-monochrometer combination passed through a double convergent lens collimator with a pinhole and changed to an expanded parallel beam which irradiated the sample through the prism. The reflected beam passed through a polarizer and a focusing lens was projected on the sensor area of a CCD camera. All measurements were carried out in air at room temperature (about 25 °C). The camera was connected to a video digitizing system for capturing frames with a computer. The images were 640 × 512 pixels in size and the brightness of each pixel was stored in grayscale format (8 bit).

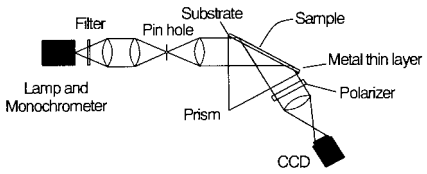

Figure 1. Optical configuration for 2DSPR measurements.

Firstly, to compensate the wavelength dependency of the system, images of reflected light were recorded at varying wavelengths at an incident angle where the SPR did not occur. The values of brightness at all pixels over the image were averaged at each wavelength. The averaged brightness was normalized to the highest value to obtain the relative brightness of image (background). Then, the measurement was made at an incident angle desired for detection of SPR, as the wavelength was scanned at intervals. The images obtained were corrected in brightness by the background. The wavelength affording the minimum brightness (SPR wavelength) was determined at each pixel of the image by fitting the brightness vs wavelength data to a secondary polynomial equation.

The next stage of analysis is transformation of the distribution of SPR wavelength to that of thickness. The SPR wavelength could be estimated by means of Fresnel calculations for varying sample thickness at an interval. The following conditions were assumed for

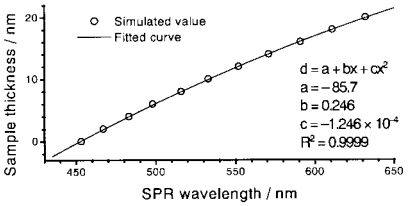

Figure 2. Sample thickness vs estimated SPR wavelength relationship. In the inset equation, d: thickness, x: SPR wavelength, a, b, and c: coefficients, R^2: coefficient of determination.

simulation; the refractive index of the sample was fixed at 1.45, the refractive index of the prism (BK7 glass) and the metal film (silver, 50 nm) was thought wavelength-dependent and was taken from the literature,[13,14] and the incident angle was fixed at 45.0°. These were the same as usually used in actual measurements including one giving results mentioned below in this paper. The sample thickness was plotted against the estimated SPR wavelength in Figure 2. The relationship of thickness vs SPR wavelength could be well fitted to a secondary polynomial noted in the figure ($R^2 = 0.9999$).

For a preliminary experiment, aqueous solutions of several proteins were spotted on a silver-deposited substrate at a constant volume (0.5 μL) with varying protein concentrations as 0.1%, 0.02%, 0.004%, 0.0008% and 0.00016% by weight. Evaporation of water in air left round spots of residue with diameter of about 1 mm which consisted of dried protein. It was expected that the thickness of the spot could be controlled by the protein concentration.

Figure 3(a) shows an image of protein spots (albumin) represented as an intensity distribution of the reflected light which was measured at 470 nm and an incident angle of 45.0° according to the conventional 2DSPR procedure. The image is multiplied by two in brightness for ease to be seen. Spots were arranged so that one formed from the solution with the higher concentration is at the more right side at a 2-mm spacing. As can be seen, the two spots from the higher concentrations are brighter than the basement whereas the three spots from the lower concentrations are darker. It can be said that the SPR occurs apart from the basement, but near the dark three spots and far from the bright two spots, and that the brighter the spot, the thicker it is. However, the result is annoying to be interpreted and gives only qualitative and limited information, as it is very difficult to estimate thickness of the spots.

Figure 3(b) shows the thickness distribution of the same spots calculated from the SPR wavelength distribution obtained by multiple-wavelength measurements from 400 nm to 670 nm at a 10-nm step. The incident angle was set at 45.0°. Since the measurement condition used was the same as in the aforementioned simulation, the thickness-wavelength relationship obtained above could be applied to the calculation. The image is represented so that the thicker the spot, the higher the brightness. Figure 3(c) displays a line profile of the spots along the cutting line indicated in Figure 3(b). Figure 3(d) presents a surface profile. The basement of these profiles is almost flat and free from apparent noises, though it is slightly shifted from zero estimated by the simulation. The slight deviation of the basement from zero may mostly arise from inaccurate setting of the incident angle with the primitive version of 2DSPR instrument used in this experiment, since a slight change in incident angle causes large shift in SPR wavelength.

The thickness of the sample was separately measured by mechanical scanning as 25–30 nm for the concentration of 0.1% and 4–10 nm for 0.02%. These values mostly coincide with the results obtained by the 2DSPR method. However, for the spots

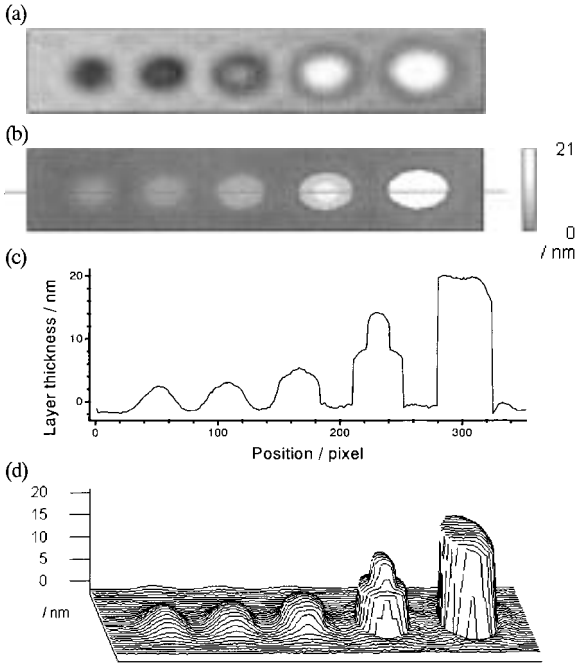

Figure 3. Images and profiles of protein thin spots with different thickness. Distribution of the intensity of reflected light (a), an image as thickness distribution (b), a line profile (c), and a surface profile (d).

from further dilute solutions, the thickness could not be measured by the mechanical scanning. In addition, there was fair scattering in the thickness values for the two high concentrations. The line profile of the sample using the mechanical scanning showed undulations of about ± 10 nm height per 0.5 mm length and irregulars of about ± 2 nm height, which reflect the real surface of the glass plate. This may explain the difficulties with the mechanical scanning. In contrast, the thickness of the spots could be measured with a good reproducibility even among several kinds of protein using the 2DSPR method (data not shown). It seems that the 2DSPR measurement is not much influenced by such surface irregulars.

In conclusion, the newly developed 2DSPR technique afforded a good quantitativity to the conventional one, and will make it a label-free optical tool for characterization of ultra thin layers, high-throughput analysis for microarrays of DNA, proteins, other chemicals, etc.

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