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IMAGING ELLIPSOMETRY FOR THE VISUALIZATION OF BIO-MOLECULAR LAYERS

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Abstract -- **Imaging ellipsometry is presented as a visualization technique to study bio-molecular layers. The layers have become more and more attractive in materials science, especially the layers with the thickness similar to the cellular layers and with the physiological activities are very important in molecular biology and medicine. Normally bio-molecular layers are very thin, which thickness is between sub-nanometer and several tens nanometer. They are transparent in visible range of light, so that they are recognized as a phase object in physics. Imaging ellipsometry is non-destructive and exhibits a high sensitivity to phase transitions within thin layers. It is capable of imaging local variations in the optical properties such as thickness due to the presence of different surface concentration of bio-molecule or different deposited molecules.**

Imaging ellipsometry is based on conventional ellipsometry with charge coupled device (CCD) technique. The images are captured with a computer with image processing technique. It has high sensitivity to thickness variation (resolution in the order of angstrom), and high sampling speed (25 pictures with more than 10^5 pixels per second). Although here it had just shown some examples of visualization of bio-molecular layers, it would be possible to show a formation process of bio-molecular layers with a real-time technique.

Index Terms -- **Imaging ellipsometry, bio-molecular layers, visualization, Immunoglobulin G, human serum albumin**

I. INSTRUCTION

Ellipsometry was introduced to study bio-layers in a long history, due to its high sensitivity to thin organic layer (better than 0.1nm). Since 1935, Tronstad measured the thickness of monomolecular films of fatty acids adsorbed on a mercury surface [1]. In 1964, L. Vroman reported his research result on blood coagulation studies with the recording ellipsometry [2]. With the development of ellipsometry technique, a large amount of experimenting went on in many laboratories with all kinds of organic materials. The advantages of ellipsometry show in measurements of thin transparent layer because ellipsometry is very sensitive to the phase variation of light. Normally, bio-molecular adsorption layer is very thin (subnanometer to tens nanometer) and non-absorption. It looks like a phase object in optical measurement and is just suitable for ellipsometry measurement. Here a simple ellipsometric imaging technique to visualize a large field of view, which is Imaging ellipsometry, is performed for the visualization of bio-molecular layers. The initial results to confirm the possibility to image monomolecular layers have been reported [3]. This paper is a continuation further to develop this technique in biochemical applications.

The technique presented here offers unique possibility to analyze bio-molecular layers with a variation of biochemistry reflected to the variation of thickness distribution. Such an approach will be of large value in bio-layer sciences.

II. EXPERIMENTAL

It was concerned with the experimental method and apparatus of imaging ellipsometry. An optical technique was used to visualize an area with different adsorbed bio-molecule patterns on solid substrates. It simultaneously used null and off-null ellipsometry and CCD image technology. It yielded a complete ellipsometric surface analysis in a large field of view ($25\times40mm^2$), thereby obtained the surface distribution of the bio-molecular layer thickness. A thickness variation (in arbitrary unit) was deduced from the intensity of image, and a thickness resolution less than 0.5nm could be detected with a lateral resolution of 5μm. Furthermore, the thickness resolution of 0.1nm and the lateral resolution of 1μm was able to be foreseen by improving optical system.

Ellipsometry principle is based on the analysis of the polarization state of a specularly reflected light to deduce the properties of test surface. The ellipsometric parameter ρ traditionally has been defined with two parts: an amplitude of $\tan\psi$ and a phase Δ [4]. If the sample is an ideal mono-layer on substrate, and it is a function of the incident medium, the layer and the substrate, that is

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$$\rho = \tan\psi\, e^{i\Delta} = \rho(N_0, N_1, d_1, N_2, \phi) \qquad (1)$$

In which N_0, N_1 and N_2 are the refractive indices of the incident medium, the layer and the substrate, d_1 is the layer thickness, and ϕ is the angle of incidence.

Null ellipsometry principle on a substrate with a high refractive index like silicon is able to obtain a high surface sensitivity to a transparent thin layer on the substrate. A null ellipsometer is mostly operated under the so-called the null condition, which means that the polarizer and analyzer are adjusted for minimum intensity at the detector, when the compensator is fixed. From the azimuthal readings of the polarizer and the analyzer, one then, with an evaluation in an optical model, obtains information about the reflecting surface, e.g. the thickness of a surface layer. For a bio-molecular layer on a solid substrate, the null ellipsometry is operated at an angle of incidence close to the pseudo Brewster angle of the substrate. The layer does not introduce an obvious relative amplitude variation of polarization states of specular reflection. It is most sensitive to the phase variation. The ellipsometric null condition in a field of view can not be obtained on an imaged entire surface with a lateral distribution of different thickness. In this way, a common ellipsometry type of polarizer - compensator - sample - analyzer (PCSA) in null condition and off-null condition is employed simultaneously, so that the null conditions are fulfilled only on somewhere of the surface, such as the bare part of the substrate. At the same time, other where of the surface is in off-null ellipsometric condition, in order to get high ellipsometric contrast and make it easy to deduce the value of thickness [5]. If other parameters, such as N_0, N_1 and N_2 and ϕ for a three-phase sample (ambient – mono-layer - substrate), are known, the detected intensity of specularly reflected light is just a function of thin layer thickness. In fact, this condition is satisfied in a practical measurement of biomolecular layers on a solid substrate, the reflected intensity on the test surface with the layers is then a measure on the layer thickness. An obvious theoretical conclusion is that

$$I = c\, d_1^2 \qquad (2)$$

The detected intensity I in off-null condition is proportional to the square of the thin layer thickness d_1. The coefficient "c" is a proportional constant. Furthermore a relationship between the thickness and the intensity, and even an absolute value of the thickness may be introduced with a calibration or a reference sample whose thickness is known.

The image system was based on a PCSA optical system and a CCD camera to realize an imaging ellipsometry. It consisted of a Xenon lamp and a specific collimating system used as a light source to provide an expanded parallel probe beam with a diameter of about 25mm. The beam passed through a polarizer and a compensator (a quarter wave plate) and finally onto the sample at an incident angle of 75°. An optical filter at 633nm wavelength was placed in the incident optical passage to select wavelength in order to increase the ellipsometric contrast of image. The reflection beam passed through an analyser and an imaging lens with a spatial filter located at its focus plane, and then the ellipsometric image was focused on a sensing area of the CCD camera. A digital image was grabbed by and stored in a computer with a grayscale format (10 bits, 0~1023 grayscale) for further evaluation by an image processing program.

In real operation, the compensator is fixed at 45°. The analyzer and the polarizer are modulated to set null conditions on a bare surface of silicon substrate. The absolute probe intensity and the gain of the CCD camera are not necessary to be calibrated. The relative intensity distribution in the field of view is significant to show the thickness distribution (the intensity distribution in image) of bio-molecular layers. A thickness calibration may be made by a comparison with reference layers or with an independent null-ellipsometric measurement on one or more selected areas on the sample. The thickness accuracy of the imaging system is better than 0.5nm.

Experiments followed the procedure as:

1) Substrate preparation: Small pieces of silicon wafer with a natural silicon dioxide layer (about 1.5nm) on polished flat surface were used as substrates. The wafers were washed in TL1 (a mixture of H_2O, H_2O_2 (30%) and NH_4OH (25%) with volume ratio 5:1:1), rinsed with large amounts of distilled water, and then washed in TL2 (H_2O, H_2O_2 (30%) and HCl (37%) with volume ratio 6:1:1) during the same rinsing process as in TL1 washing. These procedures produce a hydrophilic surface. For our interests, the adsorption of proteins on to hydrophobic surfaces is energetically, due to hydrophobic surface - hydrophobic protein residue interactions, more favorable than on to hydrophilic surfaces. This means that proteins are more firmly bound on hydrophobic surfaces and are therefore not easy to rinse away by buffer or by other proteins. Furthermore, a hydrophobic surface was prepared by silanization with dichlordimethylsilane. The silanization created an additional layer of about 1nm on the surface. Normally, the natural silicon dioxide layer and the silanization layer are considered as a uniform background due to they have almost the same optical characters which keep invariable during the measurement period.

2) Bio-molecular layer preparation: Some bio-molecular products were used, such as: human fibrinogen (Fib), human serum albumin (HSA), human immunoglobulin G (IgG) and bovine serum albumin (BSA) and their antibodies were the products of SIGMA (SIGMA CHEMICAL CO., P.O.BOX 14508 St. Louis, MO 63178, USA). The concentration of 1mg/ml dissolved in Hank's buffer was prepared. The substrates were incubated in solutions during the adsorption time of 30 minutes in order to reach a saturation degree of adsorption on solid surfaces. Then the adsorption surfaces were rinsed to remove all the non-adsorbed molecules. Finally, the surface was dried with nitrogen gas blow.

3) Molecule interaction: If necessary, the prepared surface would be incubated in another solution with some certain molecules. When the molecules in the solution interacted with the molecules on the surface and formed combined molecules or complexes. This interaction would form a combined layer, which leads to a significant thickness increase.

4) Visualization of bio-molecular layers: The reflection intensity distributions of the surface were measured with imaging ellipsometry and an analog video picture was observed on a monitor. The null condition was set on the bare surface of silicon substrate to form a black background and the image of the layer thickness pattern was obtained in an intensity distribution.

5) Picture capture and digitalization: The image of the surface with bio-molecular layer were captured by a CCD camera to obtain an analog video picture (signal-frame and video sequences) which was converted (10 bits, 1024 gray scales) to a digital one in grayscale with a Matrox digitizing system and Compaq-compatible PC.

6) Image processing: A thickness distribution of the surface structure was deduced with a theoretical model, such as: the thickness was proportional to the square root of the intensity in image (eq.2), and an image processing program. Here the thickness were given in arbitrary units as 16 times the square root of the intensity from each pixel, with the intensity still processed in 8-bit digital grayscale format. A three-dimension image of thickness distribution was transformed with converting the grayscale to the thickness.

III. RESULTS AND DISCUSSION

The use of the imaging ellipsometry for the visualization of bio-molecular layers here is demonstrated with several examples.

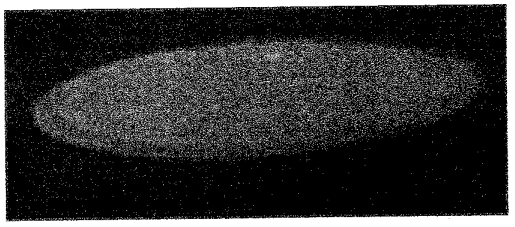

Fig. 1. Image of a IgG adsorption layer on the hydrophobic surface of a silicon substrate.

Fig.1 shows the image of a round IgG adsorption layer with a diameter of about 5mm on the hydrophobic surface of a silicon substrate. It looks like a bright ellipse plate in dark background since the probe light falls on the surface at the incident angle of 75°. The dark background is the silicon surface in the null condition of ellipsometry. The intensity in the image is corresponding to the thickness. That is the lighter, and the thicker the layer is. Its three-dimensional image of thickness distribution is shown in Fig.2. The adsorption layer is expected as a mono-molecule adsorption layer. The roughness on the layer surface reflects the molecule surface density in the layer. On our previous calibration with conventional ellipsometry, the average thickness of a saturated adsorption layer of IgG molecules is about 3.5nm.

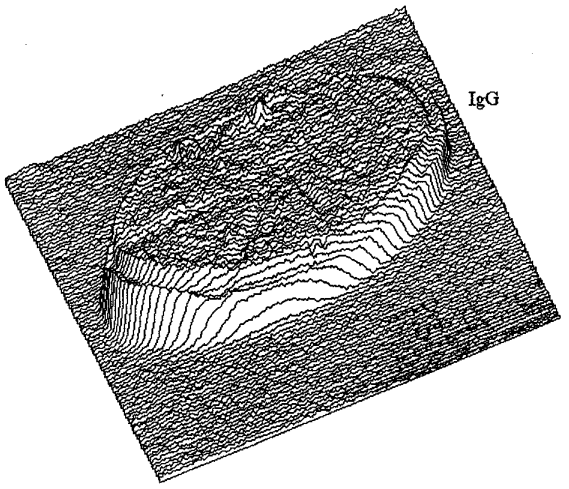

Fig. 2. Thickness distribution of a IgG adsorption layer on the hydrophobic surface of a silicon substrate.

Fig.3 shows an image with three regions in different grayscales. It is a sample with two kinds of bio-molecular layers on a silicon substrate. The dark region is the hydrophobic surface of silicon substrate. The middle region is

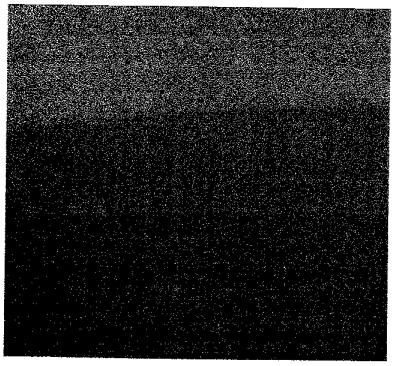

Fig. 3. Image of a HSA monolayer, and a HSA and anti-HSA complexes layer on the hydrophobic surface of a silicon substrate.

a HSA molecule monolayer in saturated state, which means no molecule could adsorb in the layer anymore. The bright region is a specific affinity layer of HSA and anti-HSA [6], which is made with an incubation of the HSA layer in a solution of anti-HSA (concentration about 100 μg/ml) for more than 30 minutes and then with completely rinsing. The

layer is thicker than the HSA monolayer so that a stepped thickness distribution is formed. It is obviously seen a three dimension image of thickness distribution in Fig.4.

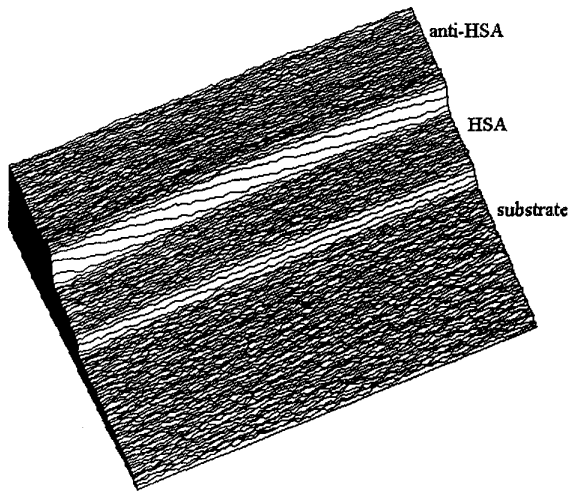

Fig.4. Thickness distribution of a HSA mono-layer, and a HSA and anti-HSA binding layer on the hydrophobic surface of a silicon substrate.

Besides antigen and antigen-antibody complexes layers, such as IgG, HSA, HSA and anti-HSA as above, antibody saturated adsorption layers on the hydrophobic surface of a silicon substrates are shown in Fig.5. They are three round antibody adsorption layers of anti-IgG, anti-HSA, and anti-Fib, respectively with a diameter of about 3mm. We may see the different thickness of these three antibodies. The layer of anti-Fib is thicker than that of anti-IgG and anti-HSA is thinner than it.

With present system, the mono-layer of dichlordimethylsilane even with a molecule weight 129 could be visualized.

Bio-molecular layers will be of large value in future materials science, and a rapid and accurate analysis is crucial for their applicability. The technique is not limited to visualize bio-molecular layers, but to study molecule interactions, such as antigen-antibody interaction, ligand-receptor and protein-protein, etc. Surface analysis is also a big potential area, such as adsorption, chemical attachment, and surfaces non-uniformity studies. Another area is the possibility of characterizing films at the liquid-solid interface or air-liquid interface, the beginning step for in-situ analysis has been taken with the technique.

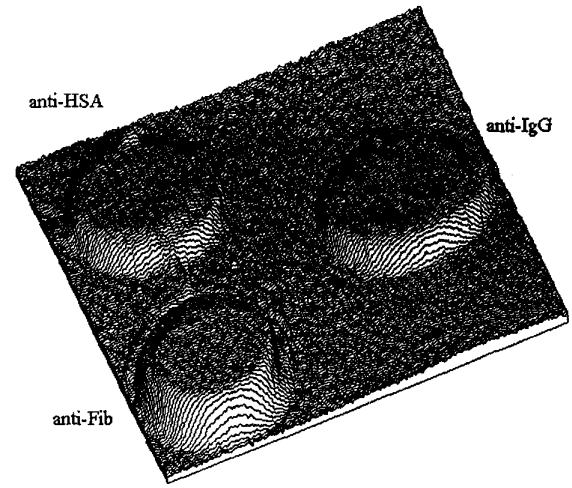

Fig.5. Thickness distribution of anti-IgG, anti-HSA and anti-Fib adsorption layers on the hydrophobic surface of a silicon substrate.

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