VISUALIZATION OF MOLECULE INTERACTION BETWEEN ANTIGEN AND ANTIBODY
--ONE OF ELLIPSOMETRIC IMAGING APPLICATIONS

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ABSTRACT

It is to investigate molecule interactions between antigen and antibody with ellipsometric imaging technique and demonstrate some features and possibilities offered by applications of the technique.

Molecule interaction is an important interest for molecule biologist and immunologist. They have used some established methods such as immunofluorcence, radioimmunoassay and surface plasma resonance, etc. to study the molecule interaction. At the same time, experimentalists hope to use some updated technique with more direct visual results. Ellipsometric imaging is non-destructive and exhibits a high sensitivity to phase transitions with 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 molecule or different deposited molecules. If a molecular mono-layer (such as antigen) with bio-activity were deposited on a surface to form a sensing surface and then incubated in a solution with other molecules (such as antibody), a variation of the layer thickness when the molecules on the sensing surface reacted with the others in the solution could be observed with ellipsometric imaging. Every point on the surface was measured at the same time with a high sensitivity to distinguish the variation between mono-layer and molecular complexes.

Ellipsometric imaging is based on conventional ellipsometry with charge coupled device (CCD) as detector and images are caught with computer with image processing technique. It has advantages of high sensitivity to thickness variation (resolution in the order of angstrom), big field of view (in square centimeter), high sampling speed (a picture taken within one second), and high lateral resolution (in the order of micrometer). Here it has just shown one application in study of antigen-antibody interaction, and it is possible to observe molecule interaction process with an in-situ technique.

1. INTRODUCTION

Antigen-antibody interaction is the main interest in immunology and medical diagnosis. Here a simple ellipsometric technique to visualize a large field of view, which is imaging ellipsometry, is performed for studies of antigen-antibody binding. The initial results to confirm the applicability to detect the interaction have been reported in a letter [1]. This report is a continuation further to develop this technique in the study of antigen-antibody interaction. In which, the preparation of a simple bio-chip, experimental methods and several results on antigen-antibody interaction have been mentioned. At the same time, it demonstrates some features and possibilities offered by applications of the technique.

2. OPTICAL METHOD

The imaging ellipsometry has been applied in several fields to study super-thin layers since it has high thickness sensitivity. In recent years, Hyuk K. Pak, et al developed a 2D imaging ellipsometric microscope to study the thin-film profile of a spreading liquid drop on a molecular smooth silicon wafer surface [2]. A well-defined multi-layer formation at the air-water and the organization of D, L-a-dipalmitiyl-phosphatidyl-ethanolamine at the oil-water interface were imaged in the Max-Planck Institute [3]. Especially it's worthy to mention that a real time technique of ellipsomicroscopy for surface imaging

was used to image concentration patterns of adsorbed species associated with heterogeneous catalytic reaction by H. H. Rotermund, et al [4]. In our laboratory, a homemade ellipsometric imaging system has been used for the visualization of bio-molecular layers. The basic experimental set-up was a PCSA optical system, that was a polarizer, a compensator, a sample, and an analyser, combined with a charge-coupled device (CCD) camera to realize an imaging ellipsometry with simultaneous null and off-null ellipsometry. It consists of a Xenon arc-lamp and a specific collimating system used as a light source to provide an expanded parallel probe beam with a diameter of about 25 mm. The collimating system consisted of an optical condenser of short focal distance lens and double achromatic lenses with a spatial filter. The beam going out of the collimating system 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 path to increase the ellipsometric contrast of image. The reflected beam passed through an analyzer and an imaging lens with a spatial filter, and then the ellipsometric image of the test surface was captured with a CCD camera. An analog picture converted to a digital one was 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 ellipsometric null condition was set on a bare surface of substrates. The relative intensity distribution in the view field was significant to show the thickness distribution of the adsorbed bio-molecular layer. In our interests for antigen-antibody interaction, the measure of an absolute thickness was not necessary, and the relative variation of the thickness was important. Of course, the imaging technique here is not limited to qualitative measure, but to quantitative one. The thickness calibration may be made by a comparison with reference layers or by an independent null-ellipsometric measurement in one or more selected areas on the sample. The imaging was feasible at different angles of incidence and on any regular reflecting substrate, for example silicon wafers or metallic mirrors, etc., although it exhibited a high sensitivity at the quasi-Brewster angle of the substrate due to the fact that p-polarized light was not reflected at the angle. The thickness accuracy of the imaging system was better than 0.5nm with the lateral resolution of 5μm, and it was enough for our purpose at present, and the thickness sensitivity of 0.1nm and the lateral resolution of 1μm were able to reach with modifying the optical system.

3. BIOCHEMICALS

The exterior of proteins is primarily hydrophilic with hydrophobic patches. Protein molecules normally reject one another by coulombic repulsion because they carry a net negative charge at their surfaces. This makes them soluble in water and prevents them from precipitating (sticking to each other). Protein stability is strongly dependent upon their hydrophobic core. Due to these factors, adsorption onto surfaces can involve many different combinations of interactions, which leads to a wide variety of immobilization for each protein. These have different biological implications, such as denaturation degree, bio-activity and also complicated analysis since these different types of interactions have different affinities. Antigen-antibody interactions are very complicated but are governed by two different kinds of interactions: (1) the positioning of antigen binding site to the antibody Fab-portion is induced by electrostatic interactions. (2) The high affinity (tight binding) between the clone (binding site on the antigen) and the antibody is both e-static and chemical (sharing of electrons resulting in a partial covalent binding).

An antibody and its corresponding antigen can form antigen-antibody complexes due to the specific affinity of their interaction [5]. For the antigen-antibody binding studies, the basic idea is that a sensing surface is exposed to a solution containing tested proteins (antibodies or antigens). On this sensing surface there is a pre-adsorbed organic film pattern with known antigens (or antibodies). The types of antigens, their layer thickness and their locations in this pattern are known before it is used as a test chip in the solution. The antibody molecules in the solution interact with its corresponding antigen molecules and form antigen-antibody complexes. The layer in the area where the interaction takes place becomes thicker than before the exposure to the antibody solution. A significant increase of the adsorbed layer thickness in one (or more) of the areas indicates that the solution contained antibodies against the antigen. With a visualization method, the thickness increase is determined and in this way the existence of the antibody molecules in the solution can be verified. This method may be used to test not only one type of antibody in the solution but also a mixture of antibodies in the solution, provided the specific binding of antigen-antibody exists. Furthermore, a sensing surface with multi-protein pattern (a bio-chip) could be prepared for multi-anti-protein test at the same time.

4. EXPERIMENTAL PROCEDURE

The evaluation procedure for qualitative image analysis of antigen-antibody binding studies may be concerned with the following six steps.

4.1 Bio-chip fabrication

A protein pattern on a silicon substrate is generated by adsorption of one or more types of proteins in one or separated areas. Each protein layer has specific affinity with its corresponding antibody molecules. Small pieces of Silicon wafer ($20x30mm^2$ or smaller) with a natural silicon dioxide layer (about 1.5nm) on polished optically 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. 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 bio-molecules. Furthermore, a hydrophobic surface was prepared by silanization to create an additional layer of about 1 nm 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.

The proteins used here are products of SIGMA (SIGMA CHEMICAL CO., P.O.BOX 14508 St. Louis, MO 63178, U.S.A). The buffer contained a normal physiological salt balance (about 1% NaCl) and calcium and magnesium, like that in normal blood. At first, antigen solution with a concentration of 1mg/ml dissolved in Hank's buffer was dropped on the substrate surface, and incubated during the adsorption time of 30 minutes for saturated adsorption. Then the adsorption area was rinsed with Hank's buffer five times and distilled water more than five times, and finally dried with nitrogen blow. This rinse procedure is expected to make only mono-molecular adsorption layer left on the silicon substrate surface as a test surface (a "bio-chip"). It is sensitive to its corresponding antibody.

4.2 Incubation

One of the antigen adsorption layers was incubated in a solution containing poly-clonal antibody which interacted with the antigen on the surface and formed their complexes according to the specific affinity. This interaction lead to a significant thickness increase in the corresponding areas on the chip. After an incubation time, the surface was rinsed and dried.

4.3 Visualization of the film thickness pattern

The reflection intensity distribution of the surface was measured with imaging ellipsometry and an analog video picture was obtained. The compensator was fixed at the azimuth of 45°, then the azimuths of the polarizer and the analyzer were adjusted to set the null condition on the bare surface of silicon substrate. In this case, the bare surface means the real hydrophobic surface of silicon wafer. That is the silicon surface with a natural silicon dioxide and silanization layer on it.

4.4 Picture capture and digitalization

The image of the bio-chip surface is captured with a CCD camera to obtain an analog video picture, which is shown on a TV monitor. For further picture processing with computer, the analog picture is converted to a digital one with an A/D convertor. The Metrox digitizing system (consisting of hardware and software) allows capture signal-frames and video sequences with Compaq-compatible PC. The analog video picture from the CCD camera is converted to an intensity distribution in digital format in a grayscale (10 bits, 0-1023 gray scales) and stored in the computer for further picture processing.

4.5 Image processing

A thickness distribution of the surface structure is deduced with a theoretical model, such as: the thickness is proportional to the square root of the intensity in image [6], and an image-processed program. However, here the thickness are given in

arbitrary units as 16 times the square root of the intensity from each pixel, with the intensity is still recorded in 8-bit digital grayscale format (0-255).

4.6 Evaluation

A comparison between both thickness distributions of the sample surface before and after the incubation in a solution containing the antibodies is finally done. If the difference is significant, conclusions can be drawn about the existence of the antibody.

5. EXPERIMENTAL RESULTS

A serial of experiments on the molecule interaction between human immunoglobulin G (IgG) and its antibody (anti-IgG) from goat (SIGMA products) has been demonstrated here.

Fig.1. shows a three dimension image with three regions like steps corresponding to the thickness distribution of two kinds of bio-molecular layers on a silicon substrate. The lowest is the hydrophobic surface of silicon substrate. The middle one is a IgG molecule mono-layer in saturated state which means no molecule could adsorb in the layer anymore. The highest is a specific binding layer of IgG and anti-IgG, which is made with an incubation of the IgG layer in a solution of anti-IgG (concentration about 100μg/ml) in Hank' buffer for more than 30 minutes. Owing to the specific affinity between antigen and antibody, a saturated interaction of IgG and anti-IgG was expected during the incubation time and then with completely rinsing. In fact, there is no more thickness increase when the incubation time in the solution of anti-IgG is prolonged. The measurement shows that the step height of anti-IgG binding to IgG is about 1.1 time of the IgG' s.

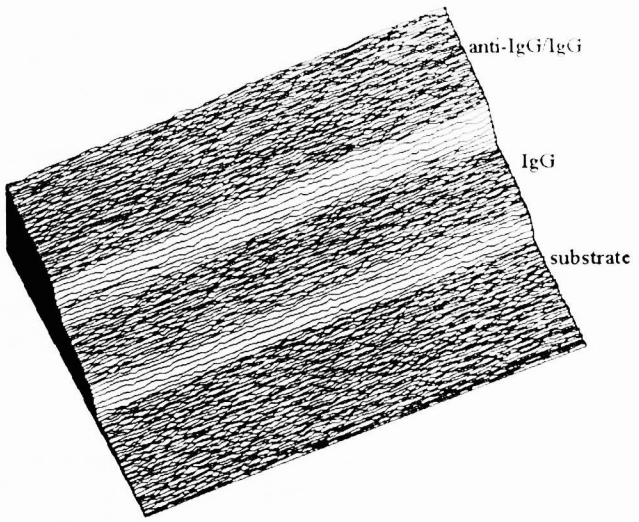

Fig.1 Three dimension image of the thickness distribution of anti-IgG and IgG complexes , and IgG adsorption monolayer on a hydrophobic surface of silicon

For present antigen-antibody binding studies, the absolute value of the thickness is not needed, only the relative variation of the thickness before and after the incubation of antibody solution is significant. For optimizing experimental conditions, such as the concentration of antibody solution and the incubation time, etc., we defined a relative thickness variation (RTV) as the ratio of the thickness increase of the anti-IgG binding to IgG layer to the thickness of the IgG layer. That is:

$$RTV = (\text{thickness of anti-IgG / IgG} - \text{thickness of IgG}) / 1.1\ (\text{thickness of IgG})$$

which reflects the level of the interaction between IgG and its antibody and the full level is 1 reflecting the saturated binding. Here the thickness is averaged over a layer.

Under the room temperature about 22°C, the optimization of the concentration of anti-IgG in Hank's buffer between 5 μg/ml and 100 μg/ml is shown in the Fig.2 with the incubation time of 30 minutes. It 's a relation of the RTV in percentage and the concentration of anti-IgG, which shows that the RTV is lower than 50% when the concentration is lower than 10 μg/ml and the RTV reaches to the full level when the concentration is higher than 100 μg/ml. In real measurements, the phenomenon of IgG and anti-IgG interaction is obviously observed when the RTV is over 10%, which means that the concentration of anti-IgG being higher than 5 μg/ml can be tested in 30 minutes. Then the concentration of 5 μg /ml is chosen to optimize the interaction time. The Fig.3 shows a normalized relation of the RTV and the incubation time for IgG and anti-IgG specific binding, which indicates that the RTV over 50% needs the incubation time of 4 hours or longer and the full level needs an incubation time longer than 12 hours. The scattered experimental results when the incubation time is lower than 2 hours are

induced by the instability of the thickness variation of IgG reacted with anti-IgG to form complexes. In the ellipsometric images, the uniformity of layers can be seen clearly with a full field of view in an intensity distribution. For the lower concentration, it's observed that the concentration of 1μg/ml anti-IgG with an incubation time of 18 hours would result in the RTV over 50%.

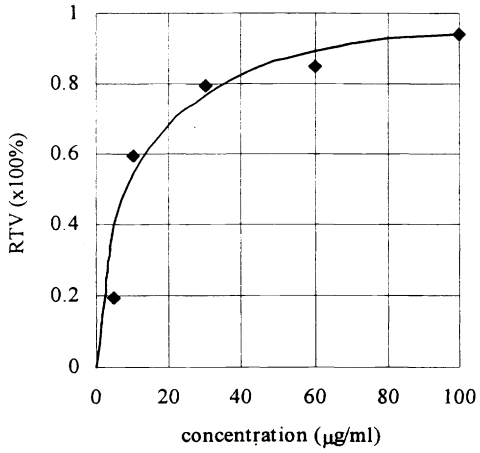

Fig.2. A normalized relation between the RTV with an interaction time of IgG & anti-IgG and the concentration of anti-IgG in Hank's buffer.

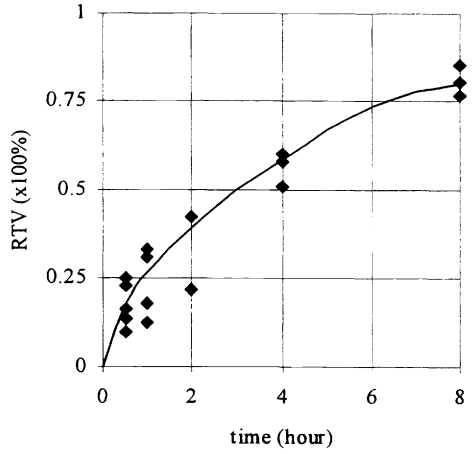

Fig.3. A normalized relation of the RTV with a 5μg/ml concentration of anti-IgG and the IgG & anti-IgG interaction time.

It is possible to study the antigen-antibody interaction process with in-situ imaging ellipsometry. Applications are not limited to antigen-antibody interaction but to all systems, such as ligand-receptor, and protein-protein, etc. where specificity is found. One obvious possibility is clinical use for routine analysis. This would give a sensing surface to detect some antibody (or antigen) in serum. A practical analysis instrument for clinic or biochemical laboratory could be previewed on present results. Surface structure inspection is also a big potential area, such as bio-molecular layers, and surfaces of micro-electronic elements.

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