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# Fabrication of Protein A-Viologen Hetero Langmuir-Blodgett Film for Fluorescence Immunoassay

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**Abstract** Protein A molecular thin film was fabricated as a platform of antibody-based biosensor. For the immobilization of the protein A thin film, a viologen multilayer was built up using the Langmuir-Blodgett (LB) technique, and then, protein A was adsorbed on the viologen LB film by an electrostatic interaction force, which was formed as a hetero-film structure. For the deposition of viologen, surface pressure area ($\pi$-A) isotherm was investigated. The fabricated protein A-viologen hetero LB film was investigated using atomic force microscopy (AFM). Using the developed molecular film, antibody immobilization and fluorescence measurement was carried out.

*Keywords*: Langmuir-Blodgett film, viologen, protein A, antibody immobilization

## INTRODUCTION

Antibody-based assays, such as immunosensors and protein chip, are the most commonly used type of diagnostic assay and still one of the fastest growing technologies for the analysis of biomolecules [1,2]. In order to enhance the sensitivity of the antibody-based sensor, antibodies such as the immunoglobulin (IgG) molecules, should have the preferential orientation with their Fab fragments exposed to the test solution. One of the well-known methods is the adsorption of IgG molecules on a sublayer of binding receptors that specifically bind the Fc fragment of IgG [3]. Protein A, a cell wall protein of *Staphylococcus aureus*, has been known to be a Fc binding receptor which would mediate the molecular orientation of antibody [4]. The formation of well-ordered protein A sublayer should allow the immobilization of antibodies with a high yield and in functional orientation. When compared with direct deposition of IgG onto a solid substrate, the protein A sublayer is more favorable in that the sublayer serves as a protector for the IgG molecules from the effects of the substrate that might denature the protein molecules and control the direction of the IgG molecules [5].

The Langmuir-Blodgett (LB) technique is a very useful technique for the formation of well-ordered multilayers of organic materials based on various advantages, such as ultra-thin film fabrication, highly ordered molecular arrays, and bio-mimetic membrane fabrication [6-8]. The decrease of detection sensitivity by the interference of other substances in sample, as well as the intrinsic restriction of turbidimetry, can be diminished using the LB technique and fluorescence detection [9]. But, the formation of the well-ordered protein A thin film onto organic LB film and the immobilization of antibody using the hetero-film layer have not been reported.

In this study, the protein A thin film is adopted to be fabricated as the sublayer of Fc binding receptors for IgG immobilization. Due to the existence of protein A thin film, not only can the specific sensitivity be increased, but also the non-specific binding can also be eliminated as well. The hetero-film of protein A/viologen LB film is fabricated, and the surface topographies of fabricated molecular film with respect to the immobilization step are investigated using atomic force microscopy (AFM). Finally, the antibody immobilized onto the fabricated film is investigated by fluorescence measurement.

## MATERIALS AND METHODS

### Materials

Viologen (N-allyl-N′-[3-propylamido-″,N″-di(n-octadecyl)]-4,4′-bipyridinium dibromide) was synthesized using the method suggested by Tundo *et al.* [10]. Chloroform, protein A, and phosphate buffer saline (PBS, 10 mM, pH 7.4) were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA). The glass substrate (Superior, Germany) was used as a solid support for LB film deposition. Human IgG labeled with fluorescein-isothiocyanate (FITC) was also purchased from the Sigma-Aldrich Chemical Company. All solutions were prepared with Millipore (Milli-Q) water followed by distillation.

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### Film Deposition

For the preparation of viologen monolayer, viologen that was dissolved in chloroform to 1 mM was spread onto subphase on the circular-type LB trough (Nima Tech., Covertry, UK), in which deionized distilled water was used as the subphase. Because viologen has good adhesion properties on glass, quartz, gold, silicon wafer, and other supporting materials and glass substrate is more economic than others, glass substrate (Superior, Germany) was chosen as the solid support for this study. The glass substrate was cleaned for 5 min using a piranha solution composed of a mixture of 30% $H_2O_2$ (Sigma-Aldrich, USA) and 70% $H_2SO_4$ (Duksan Chemical Co. Ltd., Korea). After spreading the viologen solution on the subphase, the chloroform started to volatilize in the compartment, and viologen monolayer was prepared for more than 30 min. The LB film was fabricated at a temperature of 20°C and a relative humidity of about 30%. Viologen monolayer was compressed to a surface pressure of 40 mN/m, and the glass was dipped at a speed of 6 mm/min.

Before the immobilization of the protein A molecule, the viologen LB film was dried, and then, it was immersed into the protein A solution dissolved in PBS at a concentration of 0.1 mg/mL for 60 min. By the electrostatic attractive force of the viologen, it was possible to adsorb the protein A on the viologen LB film and build up the viologen-protein A hetero film structure.

### Analysis of Film Deposition and Surface Topography

The verification of film formation was observed by using the UV/vis. spectrophotometer (V-550, Jasco, Tokyo, Japan). The topography of the protein A-immobilized hetero LB film was observed to verify the protein immobilization and analyze the surface morphology by AFM (Auto Probe CP, Park Scientific Instruments, USA). A contact mode AFM was used for the following conditions: a scan rate of 5 Hz, a set point of 10.4, and 0.6 μm ultralever as cantilever. For the verification of IgG immobilization on the protein A-viologen hetero LB film, a fluorescence spectrophotometer that was equipped with a xenon lamp (SpectraPro 300i, Acton inc., USA) was used with the FITC-labeled human IgG.

## RESULTS AND DISCUSSION

### Deposition of Viologen LB Film and Protein Immobilization

The pressure-area ($\pi$-A) isotherm includes a large amount of information on the phase and stability of the monolayer on the air-water interface, the reorientation of molecules in the two dimensional system, and conformational transformation [4]. Fig. 1 shows the surface $\pi$-A characteristics of the viologen monolayer. The phase regions were not clearly distinguished, but the inflection point indicating the change of the monolayer structure was observed near the surface pressure of 40 mN/m. The collapse of the monolayer was observed around the surface pressure of 60 mN/m. Under the surface pressure of 40 mN/m, 6 layers were fabricated on the glass substrate to prevent the formation of defect in the fabricated thin film due to physical detachment.

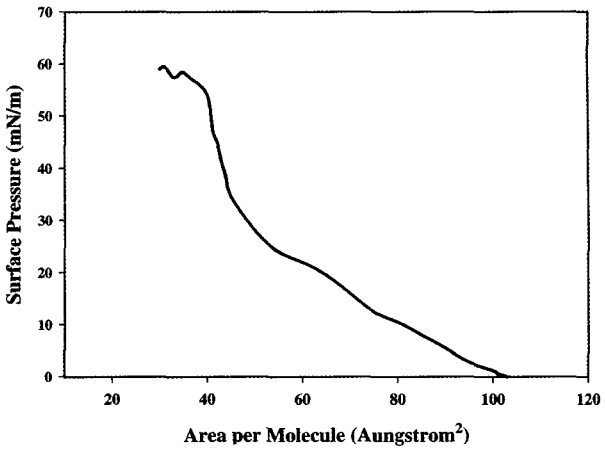


**Fig. 1.** p-A isotherm of the viologen monolayer.

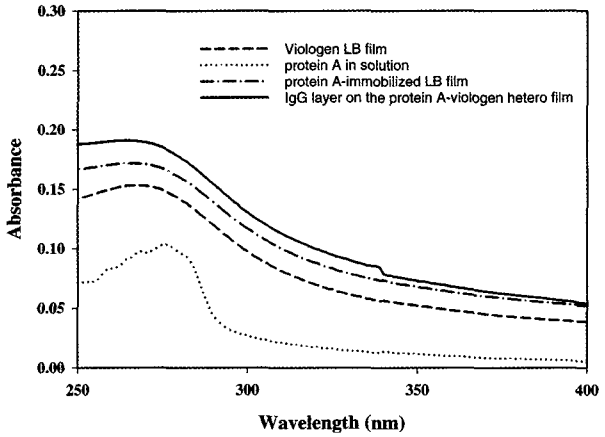


**Fig. 2.** UV/vis. absorption spectra of deposited thin films and the protein A solution. Symbols: protein A solution (dotted line); viologen LB film (dashed line); protein A-immobilized LB film (dashed-dot line); IgG film (solid line).

Because of the isoelectric point of protein A (4.85~5.1), the net charge of protein A at pH 7.4 is negative. Owing to the nitrogen atoms in viologen, the positive charge is always dominant on the viologen LB film. Therefore, adsorption of protein A onto viologen LB film was expected to form in the buffer solution at pH 7.4. The adsorption by electrostatic attractive force would make the protein A molecules immobilized without any activity loss and packed to a high degree.

The thin film formation was investigated by using the UV/vis. spectrophotometer. Measuring the absorbance at 280 nm has been a widely used setting for the determination of protein existence [9]. As shown in Fig. 2, the absorbance peak of about 280 nm was observed in the pro-

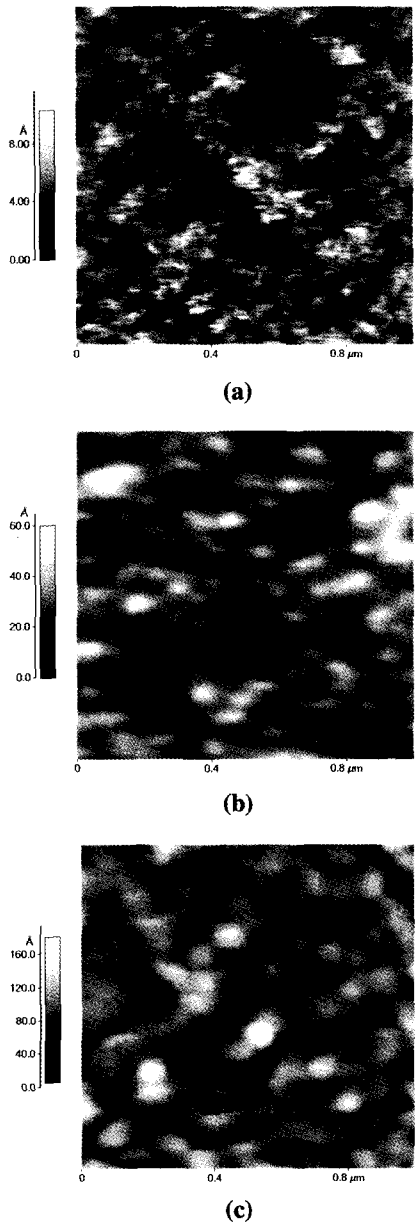


**Fig. 3.** The AFM images of: (a) viologen LB film; (b) protein A absorbed on the viologen LB film; (c) IgG immobilized on the protein A-viologen hetero LB film.

tein A solution. On the deposited thin films, the absorbance intensity at the wavelength of 280 nm was increased as the viologen LB film was fabricated, protein A was adsorbed on the viologen layer, and IgG was immobilized on the fabricated protein A/viologen hetero-film, which means the amount increase of the protein in the constant area. Therefore, the protein A-immobilized LB film was fabricated by electrostatic attractive force.

## Surface Topographies and Fluorescence Detection

AFM is a high-resolution instrument for measuring

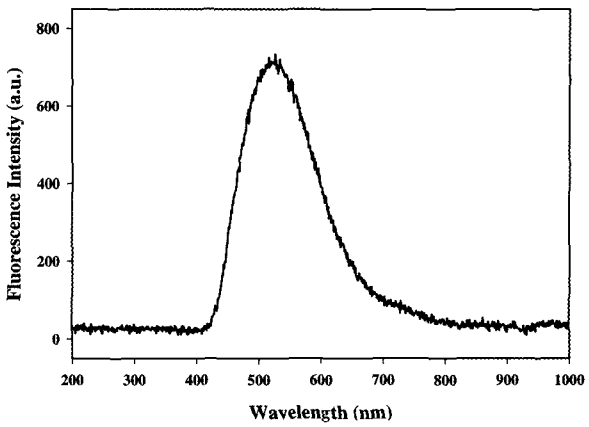


**Fig. 4.** Fluorescence spectrum of the FITC-labeled IgG layer (Ed: Figures – All four figure legends should be more detailed. They should be detailed enough to be comprehensive when observed separately).

and visualizing objects on the scale of nanometers. An important property of AFM is its ability to measure the exact height of observed rigid objects [12]. The surface topographies of the viologen LB film, protein A-immobilized LB film, and IgG-immobilized layer were shown from Fig. 3(a) to Fig. 3(c), respectively. The images of the surfaces coated with proteins can be obtained in contact AFM mode [13]. In Fig. 3(a), the height of the viologen LB film was about 8 Å. There were not severe height differences in the overall region of the viologen LB film on the glass substrate. In Fig. 3(b), the height of protein A-viologen hetero LB film was about 60 Å, in which the increment of height was due to the formation of the protein A film. Considering the mean size of protein A to be 3 nm [14], the protein A-immobilized LB film has a monolayer configuration. Spatially vacant places were locally observed in the overall region. Compared with the Fig. 3(a), there were several tightly immobilized regions (topographically high) and several loosely immobilized regions (topographically low). Because molecular density in the local region is proportional to the charge density in the viologen LB film, the electrostatic interaction force in the tightly immobilized regions would be much more than that of the loosely immobilized region. Fig. 3(c) shows the topographic image of the antibody-immobilized biosurface on the protein A-viologen hetero LB film. As ever, relative height deviation was observed in the span of 160 Å. Because the size of the IgG molecule is larger than protein A and viologen, the height difference was much more than the other topographies. The vacant site in the topographic image resulted from the absence of protein A on the viologen LB film.

Detection sensitivity may be reduced by the interference of other substances in a sample as well as by the intrinsic restriction of turbidimetry. In order to verify fluorescence immunoassay on the protein A-viologen hetero LB film, fluorescence spectrophotometry was carried out using the FITC-labeled IgG. Fig. 4 shows the fluorescence emission peak when the excitation light at the

wavelength of 495 nm was irradiated into the bound IgG layer on the protein A-viologen hetero LB film. The emission peak was observed near the wavelength of 525 nm. From the above results, it can be concluded that IgG molecules were specifically immobilized on the deposited protein A-viologen hetero LB film, and the protein A-viologen hetero LB film was fabricated for the fluorescence immunoassay.

## CONCLUSION

In this study, the protein A-viologen hetero LB film was fabricated for antibody immobilization. Protein immobilization was verified using UV/vis spectroscopy. On the fabricated protein A-viologen hetero LB film, various kinds of IgG can be immobilized, which gives a possibility of producing hetero LB films of Fc binding receptors. Since IgG could be immobilized stably onto the protein A thin film, the proposed technique could be used for the investigation of immunoassays and also for the fabrication of biosensors. AFM topography showed the monolayer formation of protein A. Protein A was electrostatically immobilized following the surface charge density of the viologen LB film. Using the fabricated protein A-viologen hetero LB film, fluorescence measurement was successfully carried out. The proposed immobilization technique of biomolecules can pave an effective way for the production of antibody-based protein chips in biomedical research and clinical chemistry.

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