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Protein A thin film was fabricated for antibody immobilization. Cysteine-terminated oligo-peptide composed of polar amino acids was utilized for the promotion of protein A immobilization induced by electrostatic interaction. The formation of the protein A film and the antibody immobilization was investigated with surface plasmon resonance (SPR) and atomic force microscopy, respectively. As the number of layer deposited on gold increased, the angle of plasmon resonance was increased accordingly. The fabricated antibody thin film was applied to the measurement of bovine serum albumin (BSA). The measured SPR angle was increased in proportion to the concentration of the applied BSA, of which the detection limit was 100 pM. These experimental results demonstrate that the developed synthetic peptide played an important role in the antibody immobilization for immunosensors.

Keywords: electrostatic interaction; immunosensor; protein A; self-assembly; synthetic peptide

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1. INTRODUCTION

Antibody-based biosensors which rely upon its specific recognition and binding capacity have been the most commonly used type of diagnostic assay and still one of the fastest growing technologies for the high-throughput analysis [1–4]. Traditional applications have typically utilized an immobilized antibody on a fixed surface, bead, filter or membrane. But this technology has moderate sensitivity and is subject to nonspecific binding error due to the denaturation and the random orientation of the proteins immobilized on the surface [5]. Recently, the roles of surface chemistry and antibody immobilization have been investigated for the enhancement of the attached capture agent [6].

Among the several methods for improving the performance of antibody-based biosensor, the immobilization technique based on protein A or G has been known to be reliable and effective for the enhancement of the fabricated biosensor [7,8]. Protein A, a cell wall protein of *Staphylococcus aureus*, has been used for the immunoglobulin G (IgG) immobilization because of the existence of the Fc binding receptors. There are advantages in IgG deposition onto protein A sublayer if compared with the direct deposition onto a solid substrate: 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 IgG molecules [9,10].

Although the protein A prevents the antibody from denaturation and is helpful to fabricate homogeneous surface, the binding capacity of the immobilized antibody depends on the activity of the deposited protein A on the substrate. The characteristic molecular structure must be maintained on the inorganic surface in order to minimize the activity loss. The engineered IgG binding protein for self-assembly has been reported as a binding mediator between the substrate and antibody [11]. But there existed still activity loss of binding molecule.

Synthetic peptide with specific amino acid sequence has been known to have a binding capacity to a constituent protein of cell wall [12], and its modified peptide fragment was utilized to the immobilization of live cell [13]. Likewise, synthetic oligopeptide with polar charge group and thiol (−SH) group can play a role in the immobilization of the protein A for immunosensors. Due to the weak electrostatic binding between protein A and the polar group of the oligopeptide, the protein A can be immobilized on the fabricated oligopeptide surface with minimized activity loss, which will be ultimately applied to the immunosensors. Even though several groups including the authors have developed the immunosensors utilizing protein A or G [14–16], the

protein A immobilization based on the electrostatic interaction and its application to immunosensor has not been reported yet.

Therefore in this study, protein A thin film is fabricated on the basis of the synthetic oligopeptide with polar charge group. Cysteine-terminated oligopeptide was designed and synthesized for self-assembly and for the promotion of protein A immobilization. The binding interaction on the gold (Au) surface can be quantitatively investigated with surface plasmon resonance (SPR) and the structural analysis of the fabricated thin film is done by atomic force microscopy (AFM). The proposed oligopeptide-mediated protein A thin film is utilized for IgG immobilization for the detection of target analyte. And, finally, the fabricated antibody layer is applied to the detection of bovine serum albumin for feasibility test based on SPR.

2. EXPERIMENTAL DETAILS

Materials

Cysteine-terminated oligopeptide was designed for having positive net charge at neutral pH, of which the sequence of amino acids is CKGRGKGRGKGRGKGRGK. The oligopeptide synthesized by solid-phase synthetic technique was purchased from Peptron Inc. (Korea). Protein A, 2-mercaptoethanol, bovine serum albumin (BSA), and monoclonal antibody (Mab) against BSA were purchased from Sigma (St. Louis, USA). Other chemicals used in this study were obtained commercially as the reagent grade.

Fabrication of Protein A Layer Mediated by Synthetic Oligopeptide

Gold (Au) substrate was fabricated by DC magnetron sputtering on top of cover glass in accordance with the optimized condition for SPR measurement (Cr:2 nm, Au:43 nm) [2,6]. The sputtered Au substrate was cleaned using piranha solution composed of 30 vol.% H_2O_2 (Sigma-Aldrich, MO, USA) and 70 vol.% H_2SO_4 (Duksan Chemical Co. Ltd., Korea) at 60°C for 5 minutes, and then the cleaned substrate was immersed into pure ethanol solution for 30 minutes.

The solution of synthetic oligopeptide (0.1 mg/mL) was applied to the prepared Au surface for self-assembly and then incubated at least overnight. In order to prevent the nonspecific binding, 200 mM of 2-mercaptoethanol was applied on the surface and incubated for 2 hr subsequently. The modified surface with the oligopeptide was immersed in the protein A solution dissolved in PBS, and then

incubated overnight. Each step was accompanied by the washing procedure with phosphate buffered saline (PBS, 10 mM, pH 7.4, Sigma-Aldrich) and deionized distilled water. By the electrostatic attractive force of the immobilized oligopeptide at neutral pH, it is possible to adsorb the protein A on the oligopeptide thin film and construct the oligopeptide-protein A hetero film. To deposit the monoclonal anti-bovine serum albumin (anti-BSA, IgG, Sigma-Aldrich, MO, USA) onto the protein A hetero film, the fabricated surface was immersed into an anti-BSA solution (120 nM) for 6 hours. Because of the specific binding ability of protein A with respect to IgG, anti-BSA can be well-organized onto the self-assembled protein A layer.

Measurement of Binding Interaction and Topographic Analysis of the Fabricated Films

Molecular binding interaction was investigated by surface plasmon resonance spectroscopy (Multiskop^{TM}, Optrel GmbH, Germany) at a wavelength of 632.8 nm and at incidence angles from 38° to 50°, which was used to monitor interactions at or above the gold-coated glass sensor surface [17,18]. All samples were monitored at a constant temperature of 20°C. The formation of protein A hetero film, the anti-BSA (IgG) layer, and the BSA bound to anti-BSA layer were monitored by SPR spectroscopy.

The topography of protein A-immobilized hetero film was observed to verify the protein immobilization and analyze the surface topography by AFM (Auto Probe CP, Park Scientific Instruments, USA). AFM was operated in contact mode and the topographic image was acquired with a scan size of 1 μm × 1 μm under the optimized feedback control.

3. RESULTS AND DISCUSSION

Protein A Immobilization Mediated by Oligopeptide

Figure 1 shows the SPR spectroscopies of the thin films fabricated by each step. On the basis of gold surface, the formation of oligopeptide layer, the immobilization of protein A, and binding event of antibody was observed through the significant shift of the SPR angle. Because the isoelectronic point of protein A is between 4.85 and 5.1, the net charge of protein A in the PBS solution (pH 7.4) is negative [19]. Owing to the arginine and lysine in oligopeptide chain, the positive net charge is always dominant on the fabricated thin film. Therefore, adsorption of protein A onto the oligopeptide surface was expected to form in the buffer solution of pH 7.4. The adsorption by electrostatic

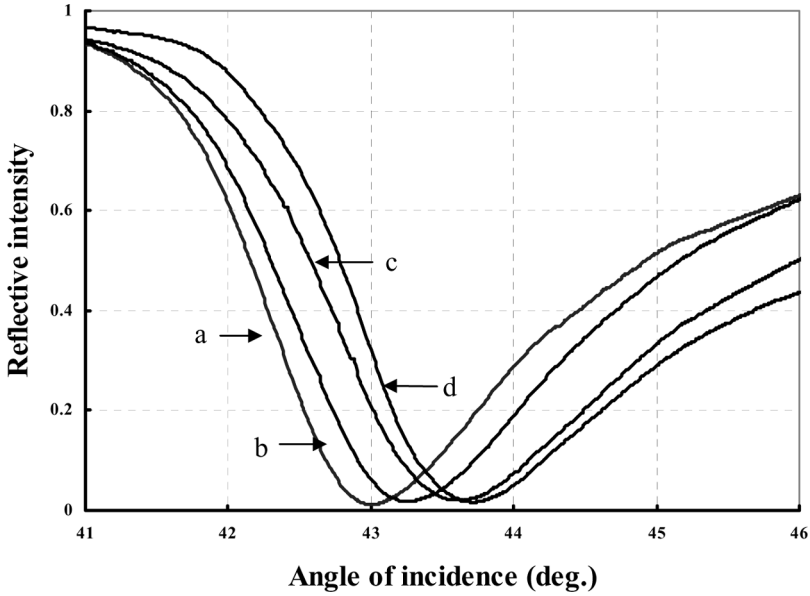


FIGURE 1 SPR spectroscopies of the fabricated films: (a) gold substrate; (b) oligopeptide layer immobilized by self-assembly technique; (c) protein A bound on the fabricated oligopeptide layer; and (d) antibody layer immobilized on the fabricated protein A film.

attractive force will make the protein A molecules immobilized without any activity loss and packed with high degree.

In order to confirm electrostatic interaction between protein and oligopeptide for protein A immobilization, the proteins that have other isoelectric point were applied to the oligopeptide surface. Figure 2 shows the changes of the SPR angle with respect to the applied proteins for immobilization. When the cytochrome c (pI: 10.1) which has a strong positive net charge at pH 7.4 was applied to the surface for immobilization, the angle of plasmon resonance was observed not to be significantly changed on the basis of oligopeptide layer. In contrast to the case of cytochrome c, the angle of plasmon resonance was significantly increased when the bacteriorhodopsin (pI: 6.0), which has a little negative net charge at pH 7.4, was applied to the oligopeptide surface. Moreover, when the protein A was immobilized on the oligopeptide layer, the change of SPR angle increased much compared with that of bacteriorhodopsin. Therefore, this experimental result demonstrates that the negative net charge promoted the amount of protein A immobilization on the fabricated oligopeptide surface.

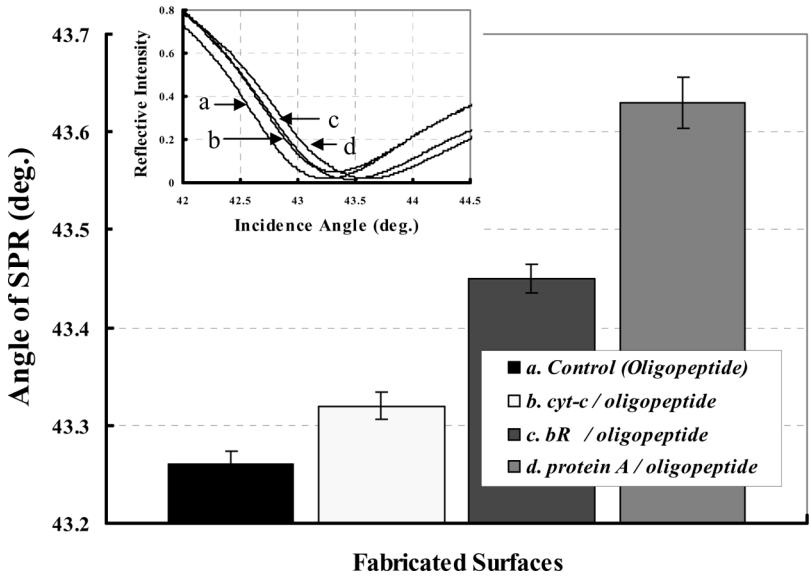
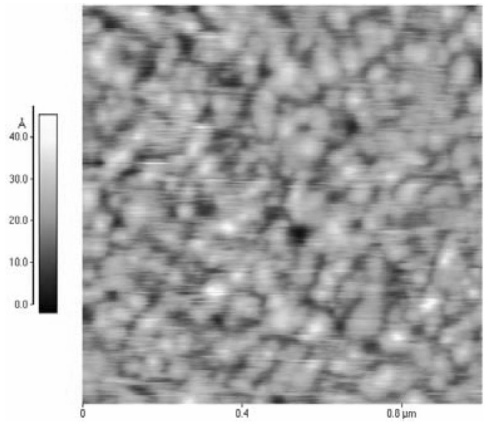


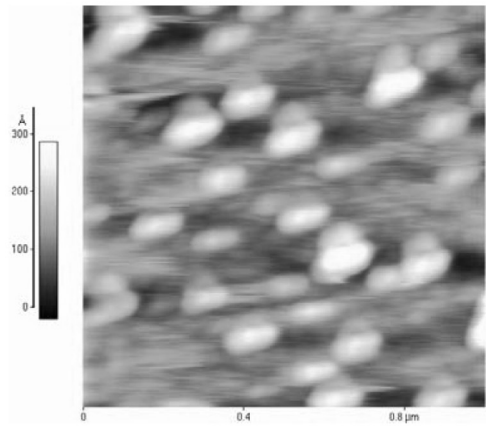
FIGURE 2 Angles of plasmon resonance with spectroscopic view with respect to the applied proteins for immobilization: (a) control (self-assembled oligopeptide only); (b) cytochrome-c immobilized on the oligopeptide; (c) bacteriorhodopsin immobilized on the oligopeptide; and (d) protein A immobilized on the oligopeptide.

Surface Structure of the Fabricated Antibody Layer

For the investigation of the protein A layer which was immobilized by cysteine-terminated oligopeptide, AFM analysis was carried out as shown in Figure 3. The topographic image can be obtained in contact AFM mode [20]. The surface topographies of gold and protein A hetero layer were shown in Figure 3(a) and Figure 3(b), respectively. In Figure 3(a) it could be observed that the height difference of gold substrate was about 40 Å. There were not severe height differences in the overall region of the Au substrate. In Figure 3(b), the height of protein A hetero film was about 300 Å, in which the increment of height was attributed to the formation of protein A film. Because the size of protein A molecule is larger than oligopeptide, the height difference was much more than that of gold. Besides, the surface roughness observed in the Figure 3(b) was significantly increased, which was owing to the locally distributed electrostatic force generated by the oligopeptide.



(a)



(b)

FIGURE 3 The AFM topographic images of: (a) gold and (b) protein A immobilized on the oligopeptide film. Scan size was $1\,\mu m \times 1\,\mu m$.

Measurement of Target Analyte with Respect to Concentration

The variation in the SPR angle induced by the binding with BSA at various concentrations was shown in Figure 4. The shift in the minimum SPR angle increases almost linearly with the increase in the antigen concentration in spite of several deviations from the average.

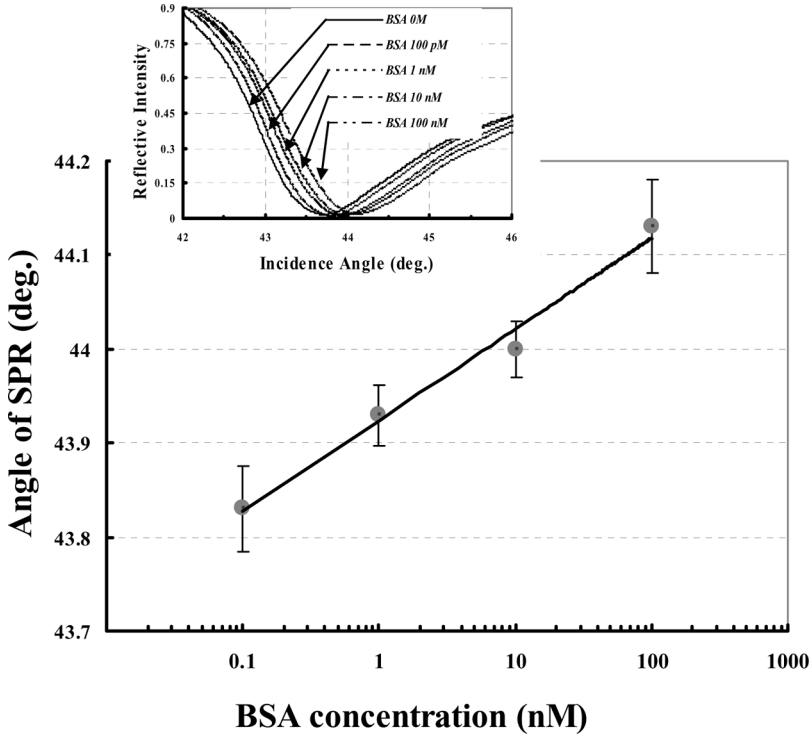


FIGURE 4 Change of SPR angle position as a function of antigen (BSA) concentration including linear calibration line.

When the 100 pM of BSA was applied to the fabricated antibody layer, the angle shift was observed in a degree of 0.12 degree. At the concentration of BSA less than 100 pM, the angle of plasmon resonance was included in the error region of the control (data are not shown). Therefore, when BSA was measured by the proposed SPR immunosensor, lower detection limit and correlation coefficient were 100 pM and 0.987, respectively. Considering the overall experimental results, it was confirmed that the fabricated protein A-oligopeptide hetero film played an important role in the construction for the immunosensor.

4. CONCLUSIONS

Protein A was immobilized for the construction of antibody-based biosensor by cysteine-terminated oligopeptide, which was adsorbed on

the Au surface by means of self-assembly. The binding event between the oligopeptide and protein was owing to the opposite charge status of each molecule, which played a role in the attractive force. The immobilization of protein A and the formation of antibody layer were confirmed by SPR spectroscopy. Due to the localized electrostatic force of the oligopeptide, the surface roughness of the immobilized antibody layer was significantly increased, which was confirmed by AFM. When the fabricated immunosensor was applied to the measurement of BSA based on SPR, the angle of plasmon resonance was linearly increased in accordance with the BSA concentration, of which the detection limit was 100 pM. These experimental results suggest a direction of peptide-based immobilization technique that is able to control the amount and the activity of the immobilized proteins.

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