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Characterization of biotin-avidin recognition system constructed on the solid substrate

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Abstract : The biotin-avidin complex, as a model recognition system, has been constructed through N-hydroxysuccinimide (NHS) reaction on a variety of substrates such as a smooth Au film, electrochemically roughened Au electrode and chemically modified mica. Stepwise self-assembled monolayers (SAMs) of biotin-avidin system were characterized by surface-enhanced resonance Raman scattering (SERRS) spectroscopy, atomic force microscopy (AFM) and surface plasmon resonance (SPR). A strong SERRS signal of rhodamine tags labeled in avidin from the SAMs on a roughened gold electrode indicated the successful complex formation of stepwise biotin-avidin recognition system. AFM images showed the circular shaped avidin aggregates (hexamer) with ca. 60 Å thick on the substrate, corresponding to one layer of avidin. The surface coverage and concentration of avidin molecules were estimated to be 90% and 7.5×10^{-12} mol/cm^2, respectively. SPR technique allowed one to monitor the surface reaction of the specific recognition with high sensitivity and precision.

Key words : Biotin, avidin, stepwise self-assembly, SERRS, AFM, SPR

1. Introduction

Considerable research activities about organic thin films have been performed recently in the field of chemical, biological and material science, due to large number of potential applications. There are conventional methods to construct thin films in molecular-scale, for example, Langmuir-Blodgett (LB) technique and self-assembly (SA).[1] SA method is widely used for studying protein adsorption and molecular recognition system, because it offers the easy construction of organized organic monolayers on various surfaces by the process of spontaneous reaction.[2]

The development of immunoassays during the past decades has revolutionized the determination of drugs and hormones in clinical and pharmaceutical chemistry as well as contaminants in the environmental area.[3] Molecular recognition, namely the specific and selective interaction between two molecules without covalent bonding to each other, has critical importance not only in biology but also in chemistry for the sensor application and for construction of the organized molecular assembly.[4]

Avidin is a well-known protein having four identical subunits, each unit binds to one biotin molecule (which is also called vitamin H). The complex formation of biotin-avidin molecules is nearly irrevers-

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ible due to their high binding constant.[5-6] A number of studies about specific binding of avidin protein with the biotin molecules have been carried out at the air-water interface and on the solid surface.[7-9] Ringsdorf et al showed the two-dimensional crystals of avidin protein with approximately 66% of surface coverage.[10] The other study using biotinylated thiols has indicated that specific interactions can take place between SA monolayers of biotinylated thiols and streptavidin.[11]

Despite many biotin-avidin recognition works performed at the interface or on solid substrate, a detailed characterization of this system in qualitative and quantitative manners is still lacking. Therefore, we tried to obtain comprehensive information for the biotin-avidin recognition systems by employing various analytical tools. Herein, we construct stepwise self-assembled monolayers of the biotin-avidin recognition system on various solid substrates, and characterize their surface structures and binding properties by using surface-enhanced resonance Raman scattering (SERRS) spectroscopy, atomic force microscopy (AFM) and surface plasmon resonance (SPR).

2. Experimental

2.1. Materials

The following chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA): phosphate buffer, 3-aminopropyltrimethoxysilane (APTMS), dihydrochloride cystamine, N-hydroxysuccinimidobiotin (NHS-d-biotin), avidin-rhodamine isothiocyanate labeled (Avidin-R), H_2O_2, H_2SO_4. All chemicals were used as received. Water was purified by double distillation and by passing through UF riverse osmosis purification system from Millipore Co. (NJ, USA).

2.2. Preparation of Substrates and Self-Assembled Monolayers (SAMs)

Three kinds of substrates were used in this work. A smooth gold film was prepared by the electron beam evaporation of 3 nm thick of nickel-chromium as an adhesion promoter, followed by 50 nm of Au, onto the glass slide. This substrate was used for SPR measurements. Electrochemically roughened Au electrode was prepared for SERRS measurements, in which the surface roughness is predominantly associated with electromagnetic enhancement mechanism in SERRS. The electrode was electrochemically roughened by applying an oxidation-reduction cycle in a cell containing 0.1 M KCl solution, previously degassed by purging with N_2 gas for 15 min. A Pt counter electrode and a silver/silver chloride (Ag/AgCl) reference electrode were used.

Amine-functionalized smooth gold film and electrochemically roughened gold electrode were prepared by immersing the substrates in 10 mM aqueous solution of cystamine dichloride for 2 hours, and then rinsed with deionized water several times. The mica was cleaned by immersion in a “piranha solution” (H_2SO_4/30%H_2O_2 = 7/3(v/v)) (**Caution**: Piranha solutions are extremely dangerous and should be handled with extreme caution) at 80°C for 10 min and was subsequently washed several times with deionized water and dried with N_2. Monolayers of 3-aminopropyltrimethoxysilane (APTMS) on mica surfaces were prepared by soaking the substrates in 10 mM 2-propanol solution of APTMS for 10 min, after which the substrates were washed thoroughly with 2-propanol and water, dried under N_2, and baked at 110°C for 10 min.[12]

NHS-d-biotin aqueous solution was prepared in 1 mM in phosphate buffer at pH 8. Avidin-R aqueous solution was prepared in 500 nM in tris buffer including 10 mM NaCl at pH 7.4. Three modified substrates with NH_2 groups were placed in the solution to allow stepwise biotin-avidin SAMs formation. Reaction time was 30 min for all the substrates. After forming each SAMs, the substrates were rinsed thoroughly with distilled water and stored in an inert atmosphere until analysis. All modified substrates were used within 2 hours of preparation.

2.3. Surface-enhanced Resonance Raman Scattering Spectroscopy

Surface enhanced resonance Raman scattering (SERRS) spectra were collected using a triple monochrometer coupled with a blue intensified CCD array detector (Triplemate 1877, Spex Industries, Edison, NJ, USA). The excitation beam was 568.2 nm of a Kr^+ laser (INNOVA 300, Coherent Co., Santa Clara, CA, USA) with 10 mW at the sample. The irradiation angle of the laser excitation source was ca. 45° with respect to the sample plane, and Raman scattered light was collected parallel to the surface normal.

2.4. Atomic Force Microscopy

AFM images of SAMs on APTMS treated mica were obtained by normal contact-mode measurement with a V-shaped silicon nitride cantilevers (spring const.: 0.067 N/m, PSI-LS, Sunnyvale, CA, USA). Before every measurement the instrument was calibrated with standard grid samples and mica.

2.5. Surface Plasmon Resonance Measurements

The measurement of reflective intensity of p-polarized light was performed by the Kretschmann configuration[13] as a function of incident angle. Details of experimental apparatus for measuring SPR angle were described in previously published literature.[14,15] The p-polarized excitation source from laser diode (670 nm) was used as a probe beam. The prism material was BK 7 (n=1.515, SIGMA), and the substrate was prepared by vacuum evaporation of gold (99.999% purity) onto a microscope cover glass after the deposition of adhesion layer of Ni-Cr. Optical contact between the prism and slide glass was achieved by using a refractive index matching fluid (n = 1.515, MERCK). Reflected intensity *via* prism was measured with the photodiode detector (ANDO Electric Co. Ltd., AQ-1135E). The angle was regulated by an automatic motorized rotary stage controller with a resolution of 0.004°.

3. Results and Discussion

3.1. Spectroscopic Studies

Schematic illustration of the stepwise chemical reactions on surface in the present molecular recog-

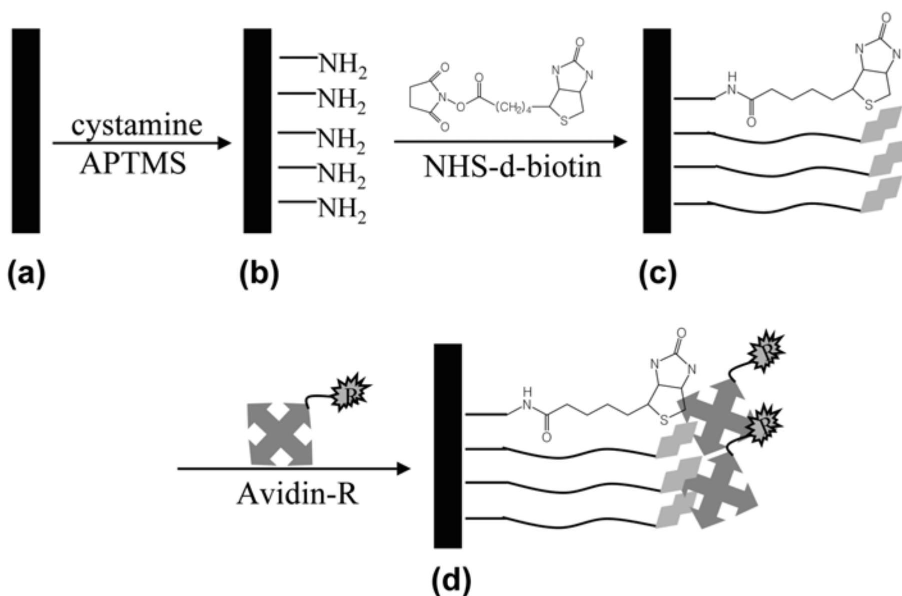

Fig. 1. Schematic illustration of the stepwise chemical reactions on the substrate.

nition system is shown in *Fig.* 1. Coupling of NHS-activated biotin to -NH_2 functionalized surface is an important step for the recognition efficiency in the system. The surface modified with the primary amine group is allowed to form covalent amide bonds between the coupling agent (Cystamine and APTMS, which are well-known molecules for forming well-ordered self-assembled monolayers) and immunoreagent (NHS-d-biotin) through NHS reaction.

Because the rhodamine molecules labeled in avidin proteins used herein are SERRS active, we could measure SERRS signal of the SAMs on the surface to conform that the avidin proteins have indeed formed the complex with biotin molecules by specific interactions. The electrochemically roughened gold electrode was used as a SERRS-active substrate, and 568.2 nm wavelength of Kr^+ laser was used as an excitation source in order to induce optical resonance with rhodamine dye tags (The rhodamine dye is orange-red color and have an absorption band maximum centered at 555 nm). *Fig.* 2 shows SERRS spectra of avidin-R SAMs on the various surfaces. Dried drops of avidin-R solution on roughened bare gold electrode revealed a strong SERRS signal of rhodamine (*Fig.* 2(a)). Main SERRS bands observed at 1645, 1527, 1506, 1356, 1276 (cm^{-1}), and other weak bands are all originated from the C-C, C-N and C-O-C aromatic stretching modes in rhodamine molecules.[16]

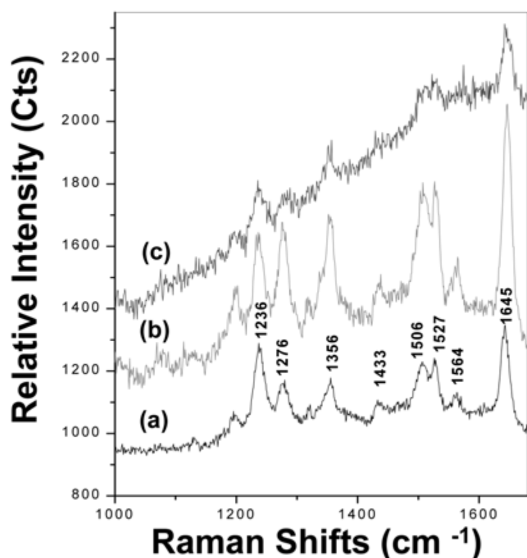

Fig. 2. SERRS spectra of avidin-R SAMs on gold roughened electrode. (a) Au electrode//avidin-R (dried drop), (b) Au electrode//cystamine SAMs/NHS-d-biotin/avidin-R, (c) Au electrode//cystamine/avidin-R.

Fig. 2(b) shows SERRS spectrum of the recognized avidin-R on the stepwise SAMs (Au roughened electrode//cystamine SAMs/NHS-d-biotin SAMs /avidin-R). Of particular interest here are the peak positions from rhodamine dye and their comparison with spectrum (a). There is no noticeable difference except for the signal-to-noise ratio. All the major peaks from physisorbed avidin-R (*Fig.* 2(a)) match with ones from avidin-R which is captured by specific molecular recognition (binding affinity: $Ka = 10^{15}\ mol^{-1}$) between biotin and avidin-R molecules[6] (*Fig.* 2(b)). The vibrational features, however, were not observed in the case of the roughened gold electrode//cystamine SAMs/avidin-R in the same condition. There is no specific affinity between cystamine SAMs and avidin-R molecules. As shown in *Fig.* 2(c), the SERRS spectrum for this case revealed a high background and poor SERRS signal. This result indicates that the molecular recognition system prepared by the stepwise SAMs is successful at least in a qualitative way.

3.2. AFM Images

Fig. 3(a) shows AFM image of a biotin monolayer immobilized on APTMS treated mica surface through the covalent bonding between the NHS-d-biotin molecules and primary amines of APTMS on the surface. In spite of their inhomogeneity (there exist some holes and different regions in height), the monolayer seems to be stable and relatively well organized (no aggregated domains). Root mean square (rms) surface roughness is measured to be 3.8 Å. From the line profile along AB shown in *Fig.* 3(b), the high regional area exhibits a depth of approximately 20-22 Å from the top to the bottom of the

hole, and the low regions are ca. 10-12 Å. Assuming that the NHS-d-biotin molecules are successfully reacted with the primary amine group on the surface and has a nearly perpendicular orientation to the surface (see *Fig*. 1(c)), the thickness of the monolayer can be calculated to be ca. 20 Å by the CPK model. This is in good accordance with the value measured from the AFM image. Schematic illustration for the biotin monolayer can be drawn on the basis of the line profile (b) as shown in *Fig*. 3(c).

Fig. 4 shows AFM images of avidin proteins adsorbed on the biotin-functionalized SAMs by specific recognition. Rms roughness was increased up

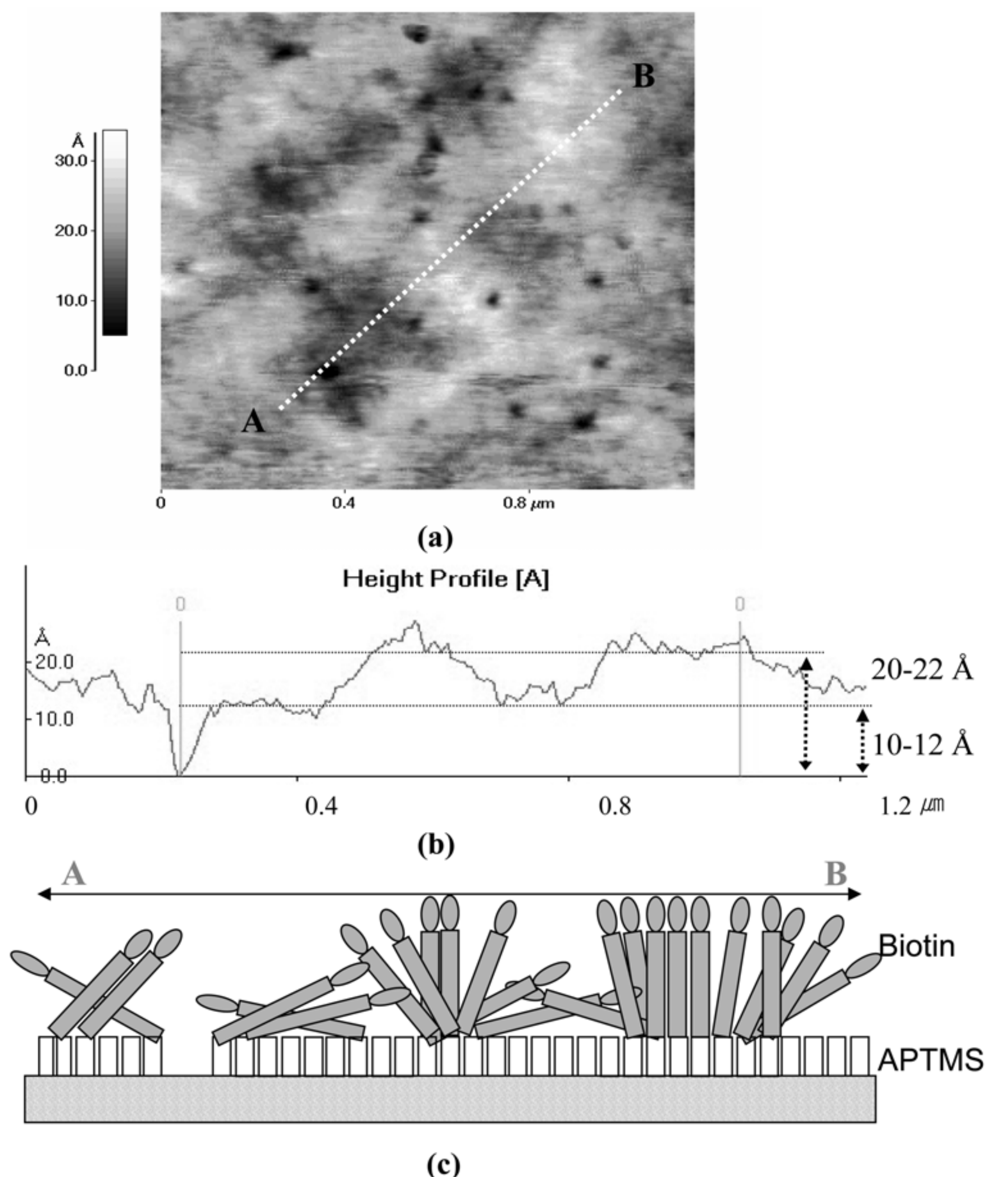

Fig. 3. AFM image of biotin monolayer on modified mica surface. (a) AFM topographic image, (b) Line profile of AB on the AFM image, (c) Schematic illustration of the biotin monolayer.

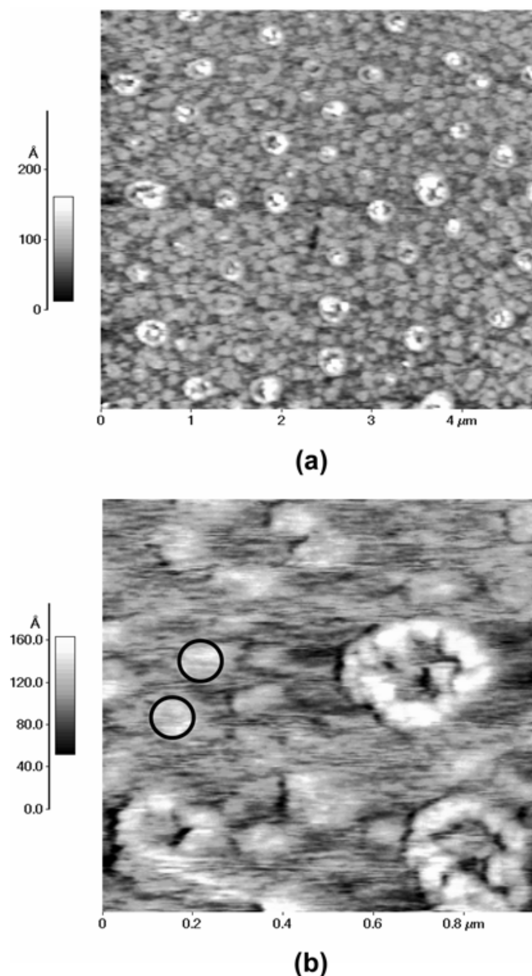

Fig. 4. AFM images ((a): large scanning area, (b): small scanning area) of avidin proteins adsorbed on biotin-functionalized SAMs.

to ca. 19 Å, and there were many circular-shaped aggregated domains. The size of unit features of the aggregate domains (circles in *Fig*. 4 (b)) was 100 nm in diameter 60 Å high. The height of the aggregate domain obtained from the AFM image is in good agreement with the length of long axis of the 3-D dimension.[17] The results suggest that the avidin molecules form two-dimentional aggregate domains, indicating lateral order of the proteins on the biotin-functionalized surface when they are adsorbed on the surface by specific molecular recognition. Haussling L. *et al*.[18] had reported the direct observation of streptavidin proteins adsorbed on biotin-functionalized SAMs and LB monolayer using scanning tunneling microscope. They showed that the individual avidin proteins with large regions free of proteins were visible by the STM technique only on the mixed monolayer of the biotin derivative and the inactive mercaptoundecanol in the ratio 1:50. They also showed that proteins were not detectable on the pure biotin disulfide SAMs in the case of the non-dilute SAMs of the biotin because the biotin molecule was completely engulfed by the protein.[19] AFM results obtained in the study, in contrast to their work, are very different and remarkable, since the recognized avidin proteins on the packed biotin SAMs form relatively well organized monolayer. One aggregate domain sized ca. 100 nm is composed of the 300 avidin molecules with lateral arrangement. This result is reasonable, because unlike Langmuir monolayer, chemisorbed monolayers on solid surface exhibit only limited lateral and no vertical mobility.[20] In order to calculate the surface concentration of the avidin molecules in AFM image, the surface coverage was estimated to be ca. 90%. Assuming the avidin molecule as a simple rectangular shape (4.5×4.5 nm), the area/molecule of avidin on surface becomes 20 nm^2. The surface concentration can be calculated to be 7.5×10^{-12} mol/cm^2.

3.3. SPR Measurements

Fig. 5 shows *ex-situ* SPR curves of the stepwise SAMs on Au evaporated film. All *ex-situ* SPR measurements are performed in air condition. Each SPR curve of A, B, C and D corresponds to the schematic description (a), (b), (c) and (d) shown in *Fig*. 1. SPR angle on bare gold was measured as 43.75°, and the following stepwise SAMs showed gradual shifts in resonance angle. SPR angle changes and the calculated thickness by the fitting according to the angle shifts are summarized in *Table* 1. For cystamine SAMs on Au film the thickness was estimated to 6 Å, and it was increased upto 19 Å when the NHS-d-biotin molecules immobilized on the

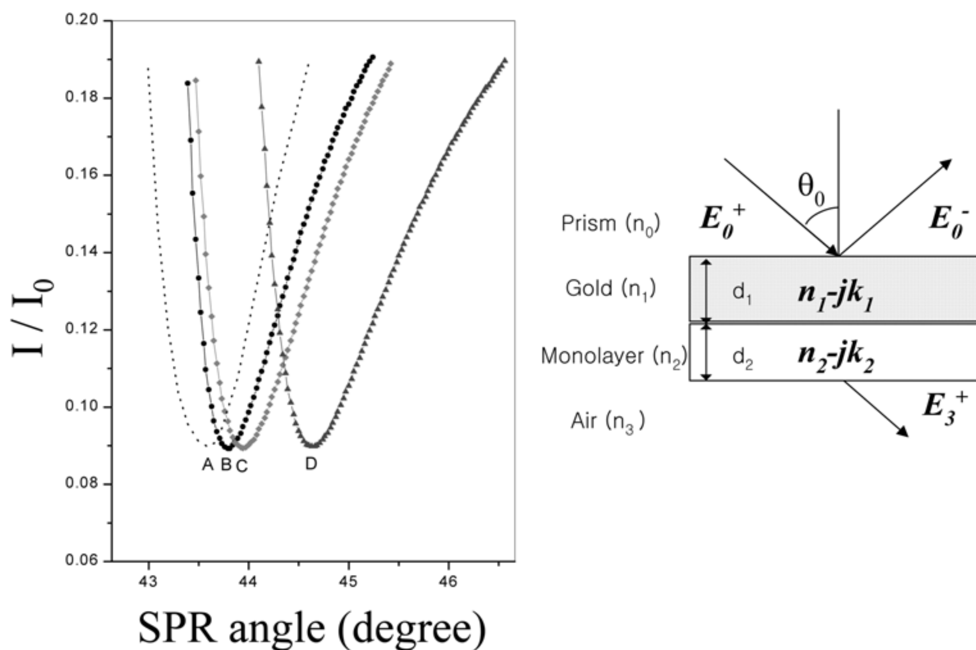

Fig. 5. SPR curves of the stepwise SAMs on Au evaporated film. A, B, C, and D are corresponding to Fig. 1 (a), (b), (c), and (d), respectively.

Table 1. Properties of SAMs formed by the stepwise adsorption on glod film. The thickness for each samples were calculated using a refractive index of 1.45.

Sample	SPR angle	Thickness(Å)
Au//	43.75	-
Au//cystamine	43.80	6
Au//cystamine/biotin	43.94	19
Au//cystamine/biotin.avidin-R	44.65	82

cystamine SAMs through the chemical reaction. This value is consistent with the result from AFM study. Recognized avidin protein-layer by the biotin-functionalized SAMs on gold surface, Au//cystamine/biotin/avidin-R, was also investigated by SPR method. The resulting angle was 44.65°, corresponding to the thickness of 82 Å. The thickness of the only avidin layer except the cystamine/biotin double layers becomes 63 Å, which is almost the same with the previous AFM result.

It is very useful to perform *in-situ* SPR measurements for elucidating the sensor application and demonstrating the interfacial phenomenon. After measuring SPR angle of Au bare film at the gold-water interface in the cell, the cystamine, NHS-d-biotin and avidin-R solutions were injected into cell step by step. Changing solution was carried out after confirming the saturation of SPR angle. *Fig*. 6(a) shows SPR angle changes *vs* contacting time of the Au film with the following solutions. SPR angle changes can be seen with the time, and it is also observed that SPR angle is jumped up to the initial state as soon as each solutions is injected. This is due to the fact that the SPR angle change is affected by the refractive index of the solution. From the SPR data over the total recognition system, assuming that the refractive index of solution has the same value as the one of water, the calculated SPR curves can be obtained as shown in *Fig.* 6(b). Estimated thickness of the avidin protein layer was in the range of 62-70 Å. From the *in-situ* SPR measurements, the sensing application

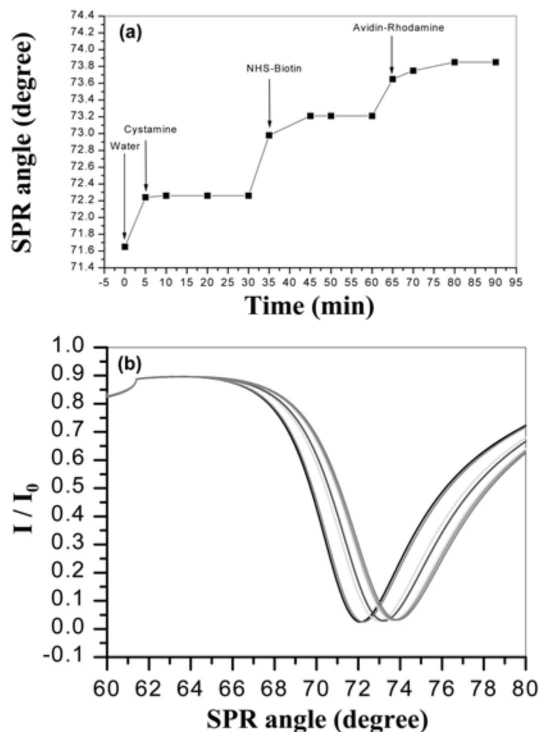

Fig. 6. *In-situ* SPR angle measurements. (a) SPR angle with the time, (b) Simulated SPR angle according to the results in (a).

was successfully tested. The result showed that formation of stepwise SAMs was finished within 30 min, and it was clearly observed that the avidin proteins were recognized on the biotin-functionalized SAMs.

4. Conclusion

In the present paper, we demonstrated the biotin-avidin recognition system constructed through NHS chemical reaction on a variety of substrates. SERRS spectroscopic results indicated that the successful complex between biotin layer and avidin proteins was formed. From AFM images, we observed that the avidin molecules were adsorbed on the biotin-activated surface as a circular shaped aggregate with 60 Å thick, corresponding to monolayer of avidin molecules, and the results of which are consistent with the SPR analysis. The surface coverage and concentration of the avidin proteins were estimated to be 90% and 7.5×10^{-12} mol/cm^2, respectively.

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