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One step immobilization of peptides and proteins by using modified parylene with formyl groups

Hyuk Ko [a], Eun-Hang Lee [a], Ga-Yeon Lee [a], Joil Kim [a], Byung-Jin Jeon [a], Moo-Hwan Kim [b], Jae-Chul Pyun [a,*]

[a] Department of Materials Science and Engineering, Yonsei University, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-749, Republic of Korea
[b] Femto Science Ltd., 323-1, Ban-wol-dong, Hwa-seong-si, Gyeong-gi-do, Republic of Korea

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ABSTRACT

One-step immobilization method for peptides and proteins is developed by using modified parylene film with formyl groups which is suitable for microplate-based immunoassay and SPR biosensor application. The immobilization of peptides and proteins is achieved through the covalent bonding of the formyl group with the primary amine groups of peptides and proteins, which no additional activation step is required. In this work, the immobilization efficiency of parylene-H is estimated in comparison with parylene-A and physical adsorption, using biotinylated-cyclic citrullinated peptide (biotinylated-CCP), human chorionic gonadotropin (hCG) and horseradish peroxidase (HRP) as model proteins. The applicability of parylene-H film to SPR biosensor is demonstrated by estimating the detection range and sensitivity of SPR biosensor at various thicknesses. The immobilization efficiency of parylene-H film for SPR biosensor was compared with physical adsorption by using HRP as a model protein.

1. Introduction

Parylene is a polymer of p-xylene, which is polymerized by pyrolysis of di-p-xylene as a precursor (Mitu et al., 2003; Selvarasah et al., 2008; Rodger et al., 2008). Recently, we applied a modified parylene with primary amino group ("parylene-A") to immobilize short peptides and small proteins to microplates (Jeon et al., 2010). The microplate with modified parylene film was aimed to immobilize short peptides and small proteins through covalent bonding, and the sensitivity of such parylene based immunoassays was determined to be far higher than that of the conventionally prepared microplate by physical adsorption. When the physical adsorption of proteins and peptides are resulted from the hydrophobic interaction between the surface of polystyrene microplate and the hydrophobic part of proteins, the strength of physical adsorption tends to be restricted by the case of small proteins peptides. The covalent immobilization can avoid the elution of small proteins and peptides from the microplate during the repeated washing steps of immunoassays. The parylene-A was also applied to SPR biosensor for immunoassays, and the applicability of parylene as a linker layer to immobilize peptides and proteins to the SPR biosensor was investigated by measuring the SPR signals at different parylene film thicknesses (Jeon et al., 2011). From this work, the parylene film with the thickness of 20 nm was determined to be suitable for the linker layer of SPR biosensor at the required refractive index range for immunoassays.

In the case of parylene-A, the immobilization of peptides and proteins requires the activation of the primary amine group with glutaraldehyde, and then another primary amine group on peptides and proteins reacts to make covalent bonding (Lopez-Gallego et al., 2005). Therefore, the immobilization of peptides and proteins consists of two-step reactions: (1) activation of parylene-A with glutaraldehyde and (2) covalent bonding. In this work, a new parylene modified with formyl group ("parylene-H") is presented for microplate-based immunoassay and SPR biosensor application. Parylene-H can be used for 'one-step immobilization of peptides and proteins' because the formyl group of parylene-H can covalently bond to the primary amine group of peptides and proteins without glutaraldehyde activation step as shown in Fig. 1. In this work, the immobilization efficiency of parylene-H will be estimated in comparison to parylene-A and physical adsorption by using biotinylated-cyclic citrullinated peptide (biotinylated-CCP), human chorionic gonadotropin (hCG) and horseradish peroxidase (HRP) as model biomolecules. The applicability to SPR biosensor will be demonstrated by estimating the influence of parylene-H coating with different thicknesses on the sensitivity and the detection range. The limit of detection (LOD) of SPR biosensor was compared when the parylene-H and physical adsorption were used for protein immobilization.

* Corresponding author. Tel.: +82 2 2123 5851; fax: +82 2 365 5882.
E-mail address: jcpyun@yonsei.ac.kr (J.-C. Pyun).

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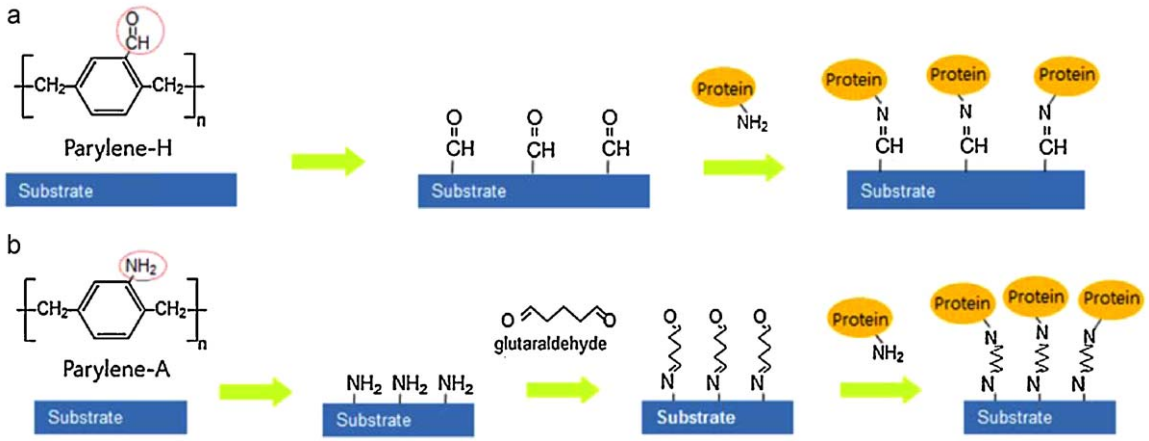

Fig. 1. One-step immobilization of proteins to parylene-H film. (a) One-step immobilization of peptides and proteins by the reaction of formyl groups of parylene-H film and amino groups of peptides and proteins. (b) Two-step immobilization method including the activation step of the amino groups of parylene-A by using glutaraldehyde.

2. Materials and methods

2.1. Materials

Parylene-A and parylene-H were supplied from Femto Science Ltd. (Seoul, Korea). Human choroionic gonadotropin (hCG), horseradish peroxidase (HRP), streptavidin–HRP complex, and albumin from bovine serum (BSA) were purchased from Sigma–Aldrich Korea (Seoul, Korea). Fmoc coupled lysine (Fmoc-lys-OH) was purchased from Nova-Chem (USA). Anti-HRP antibodies (polyclonal) and anti-hCG antibodies (polyclonal) were purchased from ABcam (Cambridge, UK). The chromogenic substrate solution of 3,3′,5,5′-tetramethyle benzidine (TMB) was purchased from Pierce (Rockford, USA). Polystyrene mircoplates were purchased from SPL Co. (Seoul, Korea). The cyclic citrullinated peptide (CCP) with the amino acid sequence HCHQESTXGRSRGCG (X: citrullinated arginine residue, underlined cystein residues: disulfide bond) was synthesized by the standard solid-phase peptide synthesis method by Peptron Co. (Yusung, Korea), and purified to be at least 90% pure by HPLC. The biotinylation to the amino terminal of CCP was carried out by Peptron Co. (Yusung, Korea). The amino acid sequence for the peptide synthesis was selected from the known cDNA sequences of human profilaggrin (Schellekens et al., 2000; Sebbag et al., 1995).

2.2. Preparation of parylene film

The parylene film was coated on a polystyrene microplate by using a parylene coater from KISCO Co. (Tokyo, Japan) and the parylene precursors named parylene-A and parylene-H was synthesized by Femto Science Co. (Seoul, Korea). The parylene coater has a cylindrical coating chamber with a capacitance of 10 l. As previously reported (Jeon et al., 2010, 2011), the parylene films were deposited by polymerization steps: (i) evaporation of monomer at the temperature of 160 °C, (ii) pyrolysis for the production of highly reactive p-xylene radical at the temperature of 650 °C, and (iii) deposition to the microplate under vacuum condition of less than 2 Torr at room temperature. The whole coating procedure was carried out by a microprocessor controller of the parylene coater. In this work, the thickness of the parylene-A film was controlled by adjusting the initial amounts of parylene-A and parylene-H precursors as shown in Fig. 2. The parylene-H films with the thickness of 7.6, 15.4, 23.3, 34.3 nm were obtained by treatment of precursors of 25, 50, 75, 100 mg, respectively. The correlation to deposition thickness was evaluated to be 0.6 nm (parylene-A film)/1 mg (parylene-A precursor) and 0.3 nm (parylene-H film)/1 mg (parylene-H precursor), respectively. The thickness of the parylene-A coating was measured by using an atomic force microscope (XE-100) from Park System Co. (Seoul, Korea).

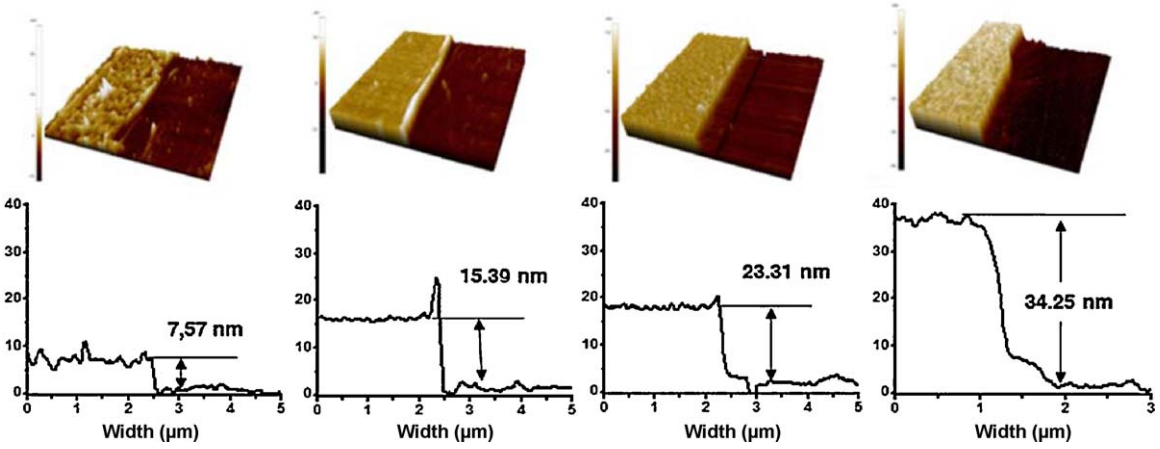

Fig. 2. AFM image of parylene-H film. The parylene-H films with the thickness of 7.6, 15.4, 23.3, 34.3 nm were obtained by treatment of precursors of 25, 50, 75, 100 mg, respectively. After parylene-H film was deposited on the gold surface, the parylene-H film was partly removed by scratching with a scalpel.

2.3. Immobilization of proteins to parylene films

The immobilization of proteins and peptides to the parylene film was demonstrated by using a short peptide called biotinylated-CCP, and proteins hCG and HRP. For the immobilization of the proteins and the peptide to the microplate with parylene-A film, the microplate was first treated with 2.5% glutaraldehyde (150 μl) in 50 mM carbonate buffer (pH 9.6) for 2 h at 37 °C and then washed with 50 mM phosphate buffered saline (PBS, pH 7.0). For the immobilization of the proteins and the peptide to the microplate with parylene-H film, glutaraldehyde treatment was not required. The proteins (hCG and HRP) and the peptide (biotinylated CCP) were immobilized to the parylene-H coated microplate by incubation at each microplate for 1 h at 37 °C. Then, the microplate was washed with 1% Tween 20 in PBS by using an automated washing machine from Molecular Devices Korea (Seoul, Korea). For the quantification of the immobilized HRP, TMB solution was treated for 3 min. To quantify the immobilized hCG, HRP conjugated anti-hCG antibodies (1:5000 dilution) were treated for 30 min at 37 °C. For the quantification of immobilized biotinylated-CCP, streptavidin–HRP complex at the concentration of 10 μg/ml was treated for 30 min at 37 °C, and then TMB solution was treated for 3 min. In all cases, TMB reaction was quenched with 2 M sulfuric acid and the optical density was measured at the wavelength of 450 nm with a reference wavelength of 650 nm by using an ELISA reader (VESAmax from Molecular Devices, Korea). For the comparison of immobilization efficiencies, the control plates were prepared by physical adsorption of proteins and peptide to the conventional polystyrene microplates. For the control plates, the same concentrations of proteins and peptide as the covalent immobilization to parylene-A coated microplates were used.

2.4. SPR measurement

A SPR biosensor from K-Mac Co. (Yusung, Korea) was used for SPR measurements. SPR chip was made by sputtering titanium layer with the thickness of 2 nm and by additional sputtering of gold layer with the thickness of 48 nm. The sample and washing solution were injected to the flow-cell by using an integrated injection valve and a peristaltic pump. The pumping rate was set to be 1.0 ml/min, and the flow of the solution was programmed to stop during the incubation step as described in previous works (Jeon and Pyun, 2008; Jose et al., 2010). To determine the correlation of the resonance angle shift for the sensitivity change of SPR biosensor, a series of sucrose solutions with known refractive index (RI) values were injected and then, the SPR signal was measured. The RI value was measured by using a standard RI meter from Leica microsystems (Wetzler, Germany).

2.5. Estimation of surface concentration of formyl groups

In order to quantify the surface density of formyl groups on the parylene-H film, Fmoc-lys-OH was reacted with the formyl groups to make immines, and then the Fmoc-group was eluted for the quantification of reacted formyl groups (Zhang et al., 1998). Sample preparation was done by coating the glass vials (inner diameter = 14.316 mm) with the parylene-H film. The glass vials were treated with 1 ml of Fmoc-Lys-OH solution in aqueous hydrochloric acid (pH 5.4) at the concentration of 1 mg/ml for 30 min. After three times of washing with distilled water, the Fmoc group was removed from the immobilized Fmoc-lys on the parylene film by treating 20% piperidine in dimethylformamide (DMF, Sigma–Aldrich, Korea) solution for 30 min with gentle shaking. The optical density of the eluted 20% piperidine solution was measured at the wavelength of 268.5 nm and the amount of Fmoc-group was quantified by using a standard curve. The surface concentration of formyl group was estimated to be 0.93 ± 0.43 pmol/mm^2 ($n = 3$) by the reaction with Fmoc-lysine (Zhang et al., 1998).

3. Results and discussion

3.1. Efficiency of protein immobilization to parylene film

Various methods have been developed for the immobilization of antibodies to improve a sensitivity of immunoaffinity biosensors, which can be classified into (1) increase the surface density of antibodies and (2) orientation control of antibodies. This work is focused on the first issue 'Increase the surface density of antibodies'. The immobilization efficiency of parylene film was estimated by using a short peptide (biotinylated-CCP), and small proteins such as hCG and HRP. Usually such peptides and small proteins show low immobilization efficiency through the physical adsorption by rinsing out during washing steps (Jeon et al., 2010, 2011). The peptide and proteins were separately immobilized on the microplates coated with parylene-H and parylene-A, then the amount of immobilized peptide and proteins were quantified as described in Section 2. For the negative control, the peptide and protein were immobilized to the conventional polystyrene microplates by physical adsorption. The peptide called biotinylated-CCP (molecular weight of 1896 Da) has two arginine residues of primary amine which can react with the fomyl group of parylene-H film and with the glutaraldehyde of parylene-A film. As shown in Fig. 3(a), the peptide shows significantly higher immobilization efficiency compared to physical adsorption as well as parylene-A film coated microplates. In the case of hCG (molecular weight of 36 kDa) (Cole, 2010), the immobilization efficiency is observed to have a similar trend to the biotinylated-CCP. As shown in Fig. 3(b), the parylene-H film coated microplate shows far higher immobilization efficiency of hCG (molecular weight of 36 kDa) in comparison to physical adsorption as well as parylene-A coated microplates. For the immobilization of HRP (molecular weight of 66 kDa) (Bartonek-Roxå et al., 1991), the difference of immobilization efficiency among three differently prepared microplates was not so significantly large as the case of small biomolecules such as biotinylated-CCP and hCG as shown in Fig. 3(c). These results indicate that a high immobilization efficiency can be expected for the immobilization of short peptides or small proteins by using the parylene-H film. Furthermore, the parylene-H film showed the higher immobilization efficiency than the parylene-A based on two step reaction. These results show that the parylene-H film can be effectively used for the one-step immobilization of short peptides and small proteins.

3.2. SPR sensor response change by parylene-H film

In this work, the parylene-H film was deposited on a SPR biosensor and the influence of parylene-H film on SPR signal was observed by using different thicknesses of parylene-H films. The parylene films with functional groups have been made to be as thin as possible on the various substrates (Jeon et al., 2010, 2011). The surface plasmon waves are known to be generated between metal and dielectric layers. The surface plasmon wave propagates along the interface, and the intensity of evanescence field decays exponentially in a direction perpendicular to the direction of propagation (Homola et al., 2008). In order to improve the sensitivity of SPR biosensor, the antigen–antibody interaction should be occurred at the strong intensity range of the evanescence field. Therefore, the parylene layer deposited on the metal layer of the SPR biosensor should be deposited as thin as possible so that the antigen–antibody interaction can be occurred at the closest region of the gold surface.

As shown in Fig. 4(a), the resonance angle shift of SPR biosensor was observed by using parylene-H films at the

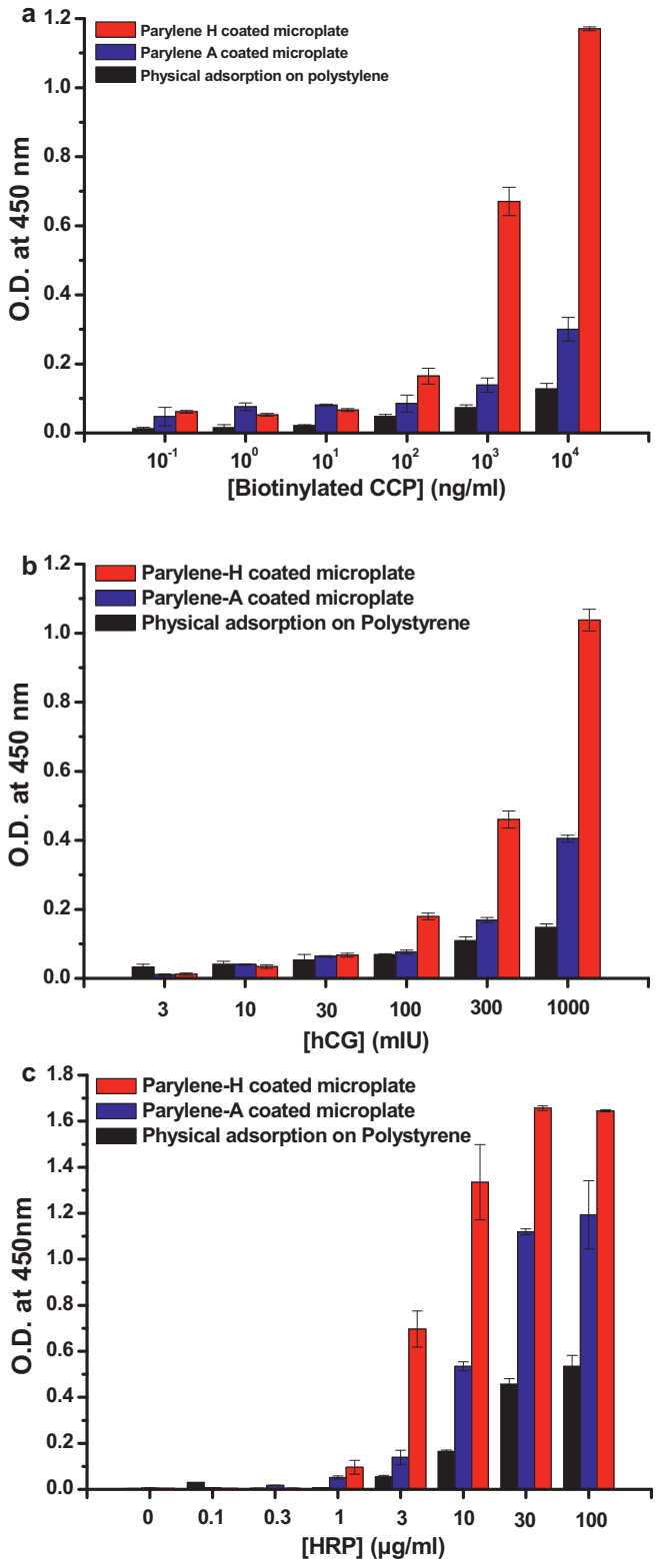

Fig. 3. Comparison of immobilization efficiency of parylene-H film with conventional physical adsorption on polystyrene and parylene-A film. A peptide called biotinylated-CCP (molecular weight of 1 kDa) and proteins called hCG (molecular weight of 36 kDa) and HRP (molecular weight of 66 kDa) were used for comparison.

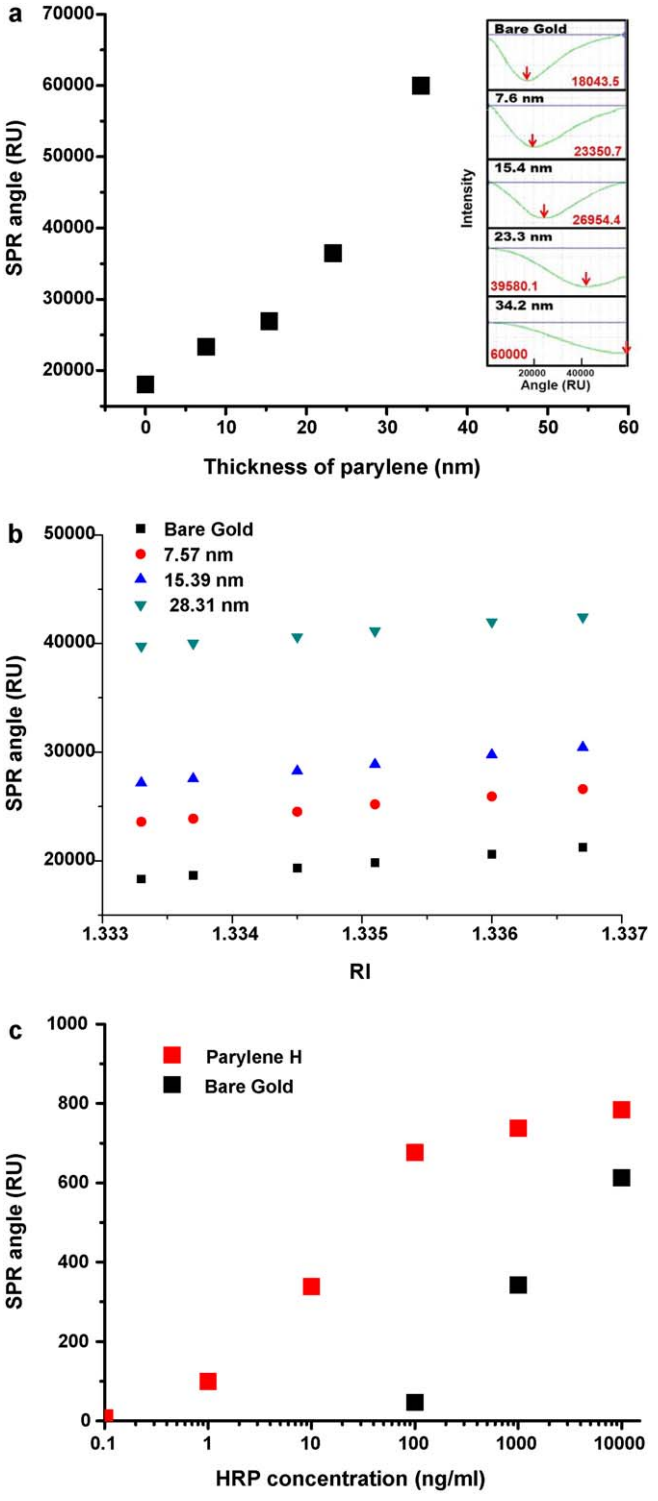

Fig. 4. SPR signal with parylene-H film. (a) SPR shift according to the thickness of parylene-H film. (b) SPR signal at different RI samples. For the SPR biosensor with a parylene layer at a thickness of 35 nm showed no sensor response at the RI range. (c) Comparison of immobilization efficiency of parylene-H film with conventional physical adsorption on the gold surface of SPR biosensor.

thickness of 7.5–35 nm. From ellipsometric measurement, the refractive indexes of parylene without modification, parylene-A and parylene-H were measured to be 1.658 ± 0.005 ($n = 3$), 1.637 ± 0.013 ($n = 3$), 1.590 ± 0.002 ($n = 3$),respectively (Fortin and Lu, 2001). Generally, the surface plasmon resonance is generated when the momentum of the surface plasmon matches that of the incident light. It is found that this wave-matching condition is very easily disrupted by even very tiny changes in the interface conditions, such as the changes in the refractive index or thickness of the medium adjacent to the metal film (Chien and Chen, 2004). For

thin dielectric films (thickness < 100 nm) such as the parylene film, the surface plasmon resonance angle shift is reported to be roughly proportional to the changes in refractive index and thickness of the film (Steiner, 2004). Therefore, the shift of surface plasmon resonance angle of Fig. 4(a) can be explained to have resulted from the changes in the refractive index and the parylene film thickness. To determine the correlation of the resonance angle shift to the sensitivity change of SPR biosensor, a series of sucrose solutions with known refractive index (RI) values were injected and the SPR signal was measured. As shown in Fig. 4(b), the SPR sensors with parylene layers at different thicknesses showed a quantitative sensor response at the RI range of sucrose solutions. The sensitivity of each SPR biosensor was calculated from the slope of each curve to be 8.4×10^5, 8.7×10^5, 9.4×10^5, 12.3×10^5 for the thickness of parylene film of 0 (bare gold surface), 7.6, 15.4, 28.3 nm, respectively. However, the SPR biosensor with the parylene film at the thickness of 35 nm showed no sensor response when the same sucrose solutions were injected. These results show that the SPR biosensors with the parylene-H layer at the thickness less than 30 nm can be applied at the RI range of 1.333–1.337, which is wide enough to measure the antigen–antibody interactions. In this work, the parylene linker layer with the thickness of 20 nm was selected for immunoaffinity biosensor application which required the detection range of 1.333–1.340 (Melendez et al., 1997; Elkind et al., 1999). The efficiency of one-step protein immobilization by parylene-H film was demonstrated by using HRP as the model protein. The SPR signals from the covalent immobilization to the parylene films and that from physical adsorption method were compared according to the HRP concentrations used for the immobilization as shown in Fig. 4(c). In the case of the covalent immobilization to the parylene-H, the SPR signal could be measured at the HRP concentration of lower than 0.1 ng/ml. These results show that the limit of detection (LOD) of SPR biosensor can be improved more than 1000-fold by covalent immobilization with parylene-H film compared to the conventional physical adsorption method. These results indicate, not only can the parylene-H film be used for one-step immobilization of peptides and proteins, but also improve the LOD and the sensitivity of SPR measurements by increasing the surface density of proteins.

4. Conclusions

One-step immobilization method for peptides and proteins was developed by using a modified parylene film with formyl groups. The formyl groups immobilize the peptides and proteins by covalently bonding to the primary amine groups of peptides and proteins without additional activation step. In this work, the immobilization efficiency of parylene-H was estimated by using biotinylated-cyclic citrullinated peptide (biotinylated-CCP), human chorionic gonadotropin (hCG) and horseradish peroxidase (HRP) as model proteins, where the parylene-H film coated microplate shows far higher immobilization efficiency in comparison with physical adsorption as well as parylene-A coated microplates. The applicability of parylene-H film to SPR biosensor is demonstrated by estimating the detection range and sensitivity of SPR biosensor with different thicknesses. The SPR biosensors with the parylene-H layer at the thickness less than 30 nm showed linear sensor response for the samples at the RI range of 1.333–1.337 which was considered to be wide enough to measure the antigen–antibody interactions. The immobilization efficiency of parylene-H film for SPR biosensor was compared with physical adsorption by using HRP as the model protein, and the limit of detection (LOD) of SPR biosensor was determined to be improved more than 1000-fold by the covalent immobilization with parylene-H film in comparison with the conventional physical adsorption method. From these results, parylene-H film was determined to be effective for one-step immobilization of peptides and proteins as well as for the improvement of the LOD and the sensitivity of SPR measurements by increasing the surface density of proteins.

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