Biosensors and Bioelectronics 56 (2014) 286–294

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Development of SPR biosensor for the detection of human hepatitis B virus using plasma-treated parylene-N film

Yong-Hwan Choi [a,1], Ga-Yeon Lee [a,1], Hyuk Ko [a], Young Wook Chang [a], Min-Jung Kang [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] Korea Institute of Science and Technology (KIST), Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 30 September 2013
Received in revised form
3 January 2014
Accepted 19 January 2014
Available online 30 January 2014

Keywords:
Parylene-N
Surface modification
Plasma
Immobilization
SPR biosensor

ABSTRACT

A plasma-treated parylene-N film was presented for the immobilization of proteins through physical adsorption. The changes in surface properties of the parylene-N film after plasma-treatment were analyzed using contact angle microscopy and AFM. To demonstrate the high protein-immobilization efficiency of the plasma-treated parylene-N film, the immobilization efficiencies of differently modified surfaces were compared using model proteins with different surface charges, such as streptavidin (pI=5, negatively charged at pH 7), horseradish peroxidase (pI=6.6, nearly neutral at pH 7), and avidin (pI=10, positively charged at pH 7). The application of the plasma-treated parylene-N film as an SPR biosensor was also tested by immobilizing model proteins. An SPR biosensor based on the plasma-treated parylene-N film was developed for the detection of the human hepatitis virus surface antigen (HBsAg), and the plasma-treated parylene-N film was estimated to improve the sensitivity of SPR biosensor as much as 1000-fold by enhancing immobilization of receptor antibodies.

1. Introduction

Immunoassays are used for the detection of target analytes in complex sample mixtures such as human blood by using highly specific antigen–antibody interactions (Luppa et al., 2001; Panthirana et al., 2000; Chung et al., 2005). Usually, immunoassays are carried out after immobilization of the receptor proteins (antibodies or antigens) on solid supports such as microbeads and microplates. Physical adsorption methods are widely used for the immobilization of these proteins because of their simplicity and high immobilization efficiency (Hornbeck et al., 1991; Jeon et al., 2010a). Immobilization of proteins by physical adsorption is produced by the hydrophobic interactions between the surface of the polystyrene microplate and the hydrophobic parts of the proteins. However, these hydrophobic interactions are known to be restricted in the case of small protein peptides because of their relatively small hydrophobic regions and the variable conformation of their peptides (Schellekens et al., 2000; Goda et al., 2007). Covalent immobilization methods are also used for peptides and small proteins which can be washed out from the solid supports during the repeated washing steps of immunoassays (Pender et al., 2008). In this method, the functional groups of the proteins and solid supports, such as amines and carboxylic acids, are linked by covalent bonding. Parylene is a polymer of *p*-xylene, which is polymerized by pyrolysis of di-*p*-xylene as a precursor. Recently, we applied a modified parylene with primary amino groups ("parylene-A") and formyl groups ("parylene-H") to immobilize peptides and small proteins on conventional microplates (Jeon et al., 2010b, 2011; Ko et al., 2011a,b; Yoo et al., 2011). A microplate with a modified parylene film was used to immobilize short peptides and small proteins through covalent bonding, and the sensitivity of the immunoassay was determined to be far higher than that achieved with the conventionally-prepared microplate using physical adsorption (Jeon et al., 2010b; Ko et al., 2011a).

For commercial immunoassays and biosensor applications, physical adsorption methods are most frequently used because of their simplicity and high immobilization efficiency, but improvement in the immobilization efficiency is required (Hornbeck et al., 1991; Jeon et al., 2010a). In this work, SPR biosensors were used for the detection of a target analyte using a plasma-treated parylene-N film for the immobilization of receptor molecules. Because the SPR biosensor can detect the binding of analytes without any label on the sensor surface, SPRs are used frequently in immunoassays for medical diagnostics such as hepatitis and AIDS by using highly specific antigen–antibody interactions (Chung et al., 2006a, 2006b; Lee et al., 2012a). An SPR biosensor based on a plasma-treated parylene-N film was used for the medical diagnosis of hepatitis B by detection of the human hepatitis virus surface antigen (HBsAg).

* Corresponding author. Tel.: +82 2 2123 5851; fax: +82 2 365 5882.
E-mail address: jcpyun@yonsei.ac.kr (J.-C. Pyun).
[1] Two authors contributed equally to this work.

0956-5663/$ - see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2014.01.035

A plasma-treated parylene-N film (a parylene film without functional groups) is presented for the effective immobilization of proteins through physical adsorption. The changes in the surface properties of the parylene-N film after plasma-treatment were analyzed using contact angle microscopy and AFM. To demonstrate the high protein-immobilization efficiency of the plasma-treated parylene-N film, the immobilization efficiencies of differently-modified surfaces were compared using model proteins with different surface charges. The application of the plasma-treated parylene-N film as an SPR biosensor was also tested by immobilizing model proteins, and an SPR biosensor based on the plasma-treated parylene-N film was developed for the detection of the human hepatitis virus surface antigen (HBsAg).

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), horseradish peroxidase (HRP), streptavidin, avidin and other analytical grade chemicals were purchased from Sigma-Aldrich Korea (Seoul, Korea). Parylene-N dimer was purchased from Femto Science Co. (Korea). Anti-HBsAg antibodies and HBsAg were purchased from AbCam (Cambridge, UK). Chromogenic substrate solutions of 3,3′,5,5′-tetramethyle benzidine (TMB) were purchased from Pierce (Rockford, USA). Polystyrene mircoplates were purchased from SPL Co. (Seoul, Korea).

2.2. SPR chip with plasma-modified parylene-N films

The SPR-chip was prepared by sputtering an adhesive layer of titanium (2 nm), and then a layer of gold (48 nm) to a BK-7 glass disk from KMac (Yousung, Korea). The parylene-N film was layered on the SPR chip using a parylene coater from Femto Science Co. (Seoul, Korea). As previously reported (Jeon et al., 2010b; Ko et al., 2011a), the parylene films were deposited by the following steps: (i) evaporation of monomer at a temperature of 160 °C, (ii) pyrolysis for the production of highly reactive *p*-xylene radicals at a temperature of 650 °C, and (iii) deposition on the microplate under a vacuum of less than 5 Torr at room temperature (Luppa et al., 2001; Panthirana et al., 2000; Chung et al., 2005; Hornbeck et al., 1991; Jeon et al., 2010a). The entire coating procedure was carried out by the microprocessor controller of the parylene coater. The thickness of the parylene-N film was controlled to be 30 nm by adjusting the initial amounts of parylene-N precursors. The plasma-treatment of parylene-N films was carried out by using a plasma generator from Femto Science Co. (Seoul, Korea). The plasma was treated for 1 min with a power of 100 W. The vacuum was controlled to be 0.5–1.0 Torr, and the gas flow was controlled to be 600 cm^3/min. The thickness of the parylene-H coat was measured using an atomic force microscope (XE-100) from Park System Co. (Seoul, Korea).

2.3. Covalent immobilization of proteins on the surface-modified SPR biosensor

The covalent immobilization of proteins (HRP, streptavidin, avidin) on the plasma-treated parylene-N-film-coated microplate was carried out using (a) EDC/NHS (Chung et al., 2005) and (b) glutaraldehyde (Jeon et al., 2010a). In the first method, proteins (HRP, streptavidin, avidin) were covalently coupled to the carboxylic acid groups of the plasma-treated parylene-N film using EDC/NHS (Chung et al., 2005). Coupling reagents of 100 mM EDC/50 mM NHS were prepared in a 10 mM sodium phosphate buffer (pH 5.5). Then, the protein solutions were added and stirred gently for 30 min at room temperature. In the second method, the proteins were immobilized on the amine groups of the plasma-treated parylene-N-film-coated microplate by treating the parylene-N-coated microplate with 150 µl of 5% glutaraldehyde in 50 mM carbonate buffer (pH 9.6) for 2 h at 37 °C (Jeon et al., 2010a). After washing with 50 mM of phosphate-buffered saline (pH 7.0), the microplate was treated with 100 µl of protein solution for 1 h at 37 °C. After the incubation step, the microplate was washed with 1% Tween 20 in PBS using an automated washing machine from Molecular Devices Korea (Seoul, Korea). For the quantification of the immobilized HRP, the TMB solution was treated for 3 min.

2.4. Measurement of SPR signal

An SPR biosensor from KMac Co. (Yousung, Korea) was used for the SPR measurements (Lee et al., 2012a). This SPR biosensor used a home-made SPR chip which was prepared by sputtering an adhesive layer of titanium (2 nm) and then a layer of gold (48 nm) on a BK-7 glass (10×10 mm^2). The SPR biosensor was equipped with a flow cell made of flexi-glass with a capacity of 5 µl. The sample and the washing solution were injected into the flow cell using an injection valve and a peristaltic pump. The pumping rate was set at 1.0 ml/min and the flow of the solution was programmed to stop during the incubation step. For the immobilization of a protein to the surface of SPR biosensor, the protein solution was injected and incubated for 30 min (1800 s). During this incubation step, the protein was physically adsorbed to the sensor surface and it made a corresponding SPR signal. The following "washing" step was repeated three times of (1) PBS injections for 1 min and then (2) incubation for 3 min at the stopped-flow-state as shown in Fig. 5(a). Before the protein adsorption, the baseline of SPR measurement was also established by using the same sequence of the washing step. The SPR signal from the physical adsorption of a protein could be measured from the difference in SPR signals of two washing steps (before and after the protein adsorption step).

3. Results and discussion

3.1. Surface properties of parylene films

Parylene-N is a polymer of *p*-xylene without any functional groups on the benzene ring. After the thermal deposition of parylene-N film, a plasma treatment was performed to modify its surface properties. Usually, modification by plasma treatment produces a change in the hydrophilicity of the polymer surface through introduction of functional groups such as hydroxyl, carboxylic, and amine groups. The change in hydrophilicity is easily detected by contact angle measurements (Trantidou et al., 2012; Chang et al., 2007). As shown in Fig. 1 (a) the contact angle of parylene-N film changed from 84.4° to 13.4° after the plasma treatment. In the case of polystyrene, which is frequently used in microplates for immunoassays, the contact angle changed from 73.9° to 25.2° after the same plasma treatment. These results showed that the contact angle of parylene-N film was lower than that of polystyrene immediately after the plasma-treatment (within 3 min), and implied that parylene-N film had a higher hydrophilicity than polystyrene immediately after the plasma-treatment (within 3 min). Usually, the contact angle decreases (higher hydrophilicity) as the surface energy is increased, and the contact angle is known to increase gradually after the plasma treatment. This change occurs because the modified polymer chain with a higher surface energy tends to have a lower surface energy after hiding the functional groups at the polymer surface (Trantidou et al., 2012; Chang et al., 2007). In the case of polystyrene, the contact angle changed from

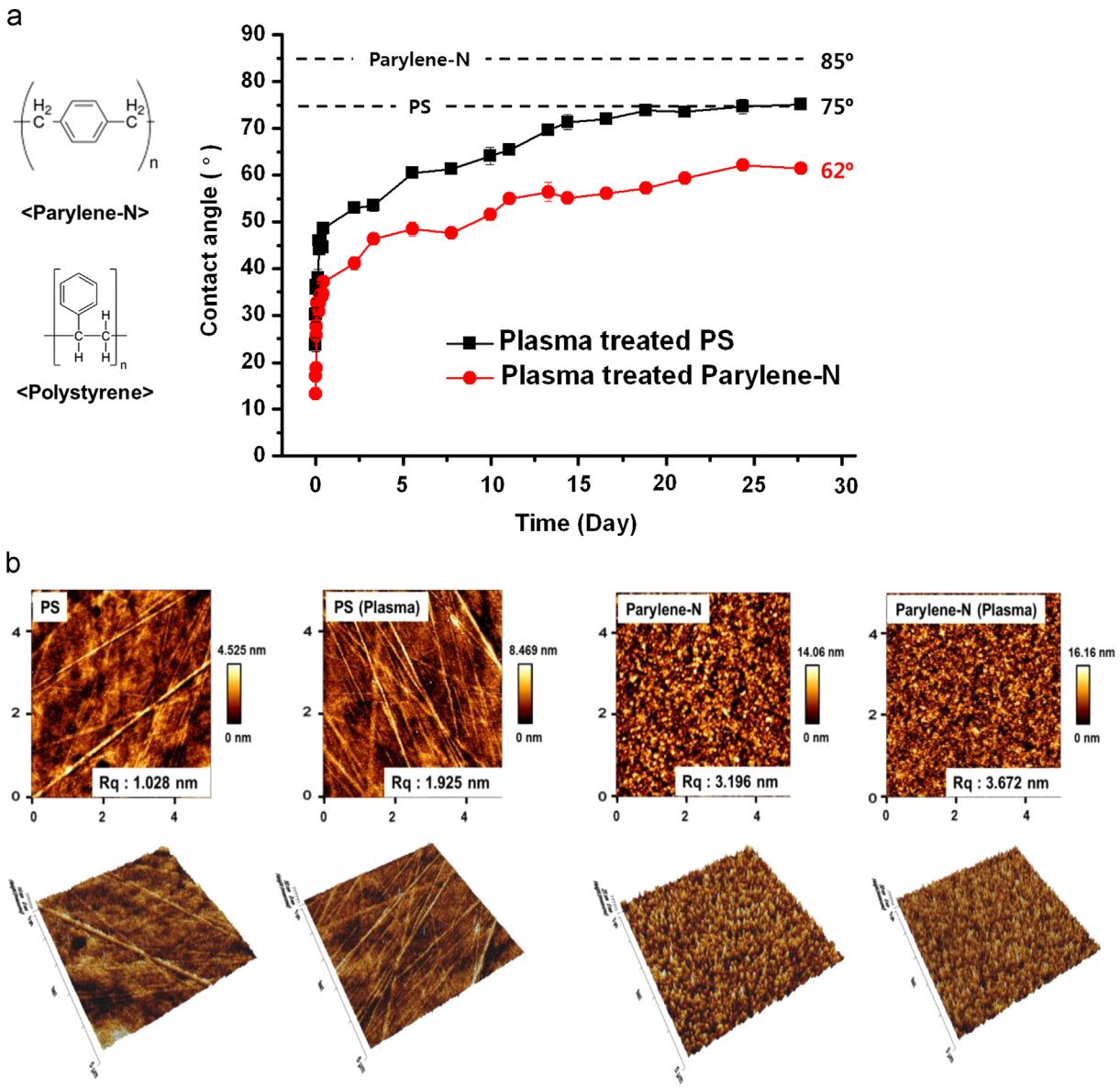

Fig. 1. Surface analysis of parylene-N film after the plasma-treatment: (a) Change in contact angle of parylene-N film after plasma-treatment. (b) AFM analysis of the surface morphology before and after the plasma-treatment. The surface roughness (R_q) was analyzed using AFM images of polystyrene and parylene-N film before and after the plasma-treatment.

25.2° (just after plasma-treatment) to its initial value of 75.0° after 12 h, as shown in Fig. 1(a). This indicates that the effect of the plasma treatment on the hydrophilicity of the polystyrene surface disappeared. However, the contact angle of the parylene-N film changed from 13.4° (just after plasma-treatment) to 62.0°, which is estimated to be 25% lower than the initial contact angle (84.4°). These contact angles were observed to be maintained for 27 days. These results showed that the plasma-treatment of parylene-N film could create a 25% more hydrophilic surface with long-term stability.

Physical damage on the surface could change the surface area and influence the contact angle. To estimate the change in surface roughness caused by the plasma treatment, the surface morphology of the parylene-N film was analyzed before and after the plasma treatment using an atomic force microscope (AFM). As shown in Fig. 1(b), the surface roughness (R_q) of the polystyrene increased from 1.028 nm to 1.925 nm (100%) after the plasma treatment. In the case of parylene-N film, the surface roughness increased from 3.196 nm to 3.672 nm (14.8%). These AFM analysis results showed that the surface roughness of polystyrene was increased 6-fold in comparison with the plasma-treated parylene-N film. Therefore, the change in contact angle of the parylene-N film is thought to result from the hydrophilicity effect rather than the change in surface roughness after the plasma treatment.

3.2. Immobilization of proteins on parylene films

To compare the immobilization efficiencies of the different modified surfaces (parylene-N film, plasma-treated parylene-N film, and polystyrene), three model proteins were physically adsorbed to each surface, and the amounts of immobilized proteins were compared. The model proteins were selected to have different surface charges at pH 7.0, and the three proteins were selected to have different isoelectric points (pI), which is the pH at which the net charge of the protein is zero. If a certain protein has a high composition of basic amino acids, such as lysine and arginine, the pI value of the protein is estimated to be above 7.0 and the protein is expected to be positively charged at pH 7. A protein with a high composition of acidic amino acids, such as

glutamic acid and aspartic acid, is expected to have a pI value below 7.0 and be negatively charged at pH 7. In this work, streptavidin (pI=5, negatively charged at pH 7), horseradish peroxidase (pI=6.6, nearly neutral at pH 7), and avidin (pI=10, positively charged at pH 7) were used as model proteins (Righetti and Caravaggio, 1976; Su et al., 2004; Brett et al., 2002).

As shown in Fig. 2(a), the immobilization efficiencies of HRP on parylene-N film, plasma-treated parylene-N film, and polystyrene were compared after physical adsorption to each surface. From the chromogenic reaction of HRP with a chromogenic substrate of TMB, the surface density of HRP was determined to be more than 2-fold higher on the plasma-treated parylene-N film compared to the other surfaces. Also, the immobilization efficiencies of HRP on both the polystyrene and parylene-N film were determined to be similar, having concentrations in the range of 1–50 μg/ml.

As shown in Fig. 2(b), the biotinylated HRP was physically adsorbed to the parylene-N film, plasma-treated parylene-N film, and polystyrene, then the immobilization efficiency of the biotinylated HRP on each surface was compared. As previously mentioned, the pI value of HRP is 6.6 and its charge is nearly neutral at a pH of 7.0. For the covalent labeling of biotin to HRP, the carboxylic acid group in the biotin was activated by *N*-hydroxysuccinimide, then the activated biotins were reacted with the amine groups in the HRP. Therefore, the biotinylated HRP had a pI value that was more acidic than HRP because the amine groups in the HRP were used for the covalent labeling of biotin. Thus, the biotinylated HRP was more negatively charged at a pH of 7 than the HRP before biotinylation. From the chromogenic reaction of HRP with a TMB substrate, the surface density of HRP was determined to be more than 10-fold higher on the plasma-treated parylene-N film in comparison with the other surfaces. The immobilization efficiency of HRP on both polystyrene and the parylene-N film was found to be similar at concentrations from 1 μg/ml to 50 μg/ml. Therefore, the immobilization results on three different surfaces showed that negatively charged proteins were more favorable to immobilization on the plasma-treated parylene-N than positively charged proteins at pH 7.

The properties of the plasma-treated parylene-N surface were further studied using streptavidin (pI=5, negatively charged at pH 7) and avidin (pI=10, positively charged at pH 7) as model proteins with acidic and basic pIs. As shown in Fig. 2(c) and (d), the surface density of the streptavidin (negatively charged at pH 7) was determined to be more than three times higher on the plasma-treated parylene-N film compared to the other surfaces. The immobilization efficiencies of streptavidin on polystyrene and the parylene-N film were determined to be similar at concentrations from 1 ng/ml to 100 ng/ml. The immobilization efficiency of avidin (positively charged at pH 7) on the plasma-treated parylene-N film was determined to be around 1.5 times higher in comparison with other surfaces. These results also showed that a negatively charged protein could be immobilized with a higher surface density on plasma-treated parylene-N film than a positively charged protein at pH 7.

In summary, the immobilization results of the three model proteins showed that the plasma-treated parylene-N film had a higher immobilization efficiency for the proteins with acidic and basic pIs compared to the parylene-N film and polystyrene. Additionally, the plasma-treated parylene-N film showed a higher immobilization efficiency for the negatively charged proteins than the positively charged proteins at pH 7.

To confirm the introduction of different functional groups by the plasma-treatment, parylene-N film was treated with plasmas of two different gases: oxygen and nitrogen. The changes in chemical composition on the surface were analyzed by using X-ray photoelectron spectroscopy (XPS). And, then the amount of functional groups on the plasma-treated parylene-N film was estimated using the chemical reagents of EDC/NHS for carboxylic acid groups and glutaraldehyde reactions for amine groups to allow covalent coupling in HRP. As shown in Fig. 3(a), the chemical composition of the parylene-N film was analyzed to be 100% C_{1s}. After the plasma-treatment of the paryelen-N film, the chemical composition was changed to be 77.54%, 21.48%, 0.98% for C_{1s}, O_{1s}, N_{1s}, respectively. These results represented the introduction of functional groups with oxygen to the parylene-N film with 100%

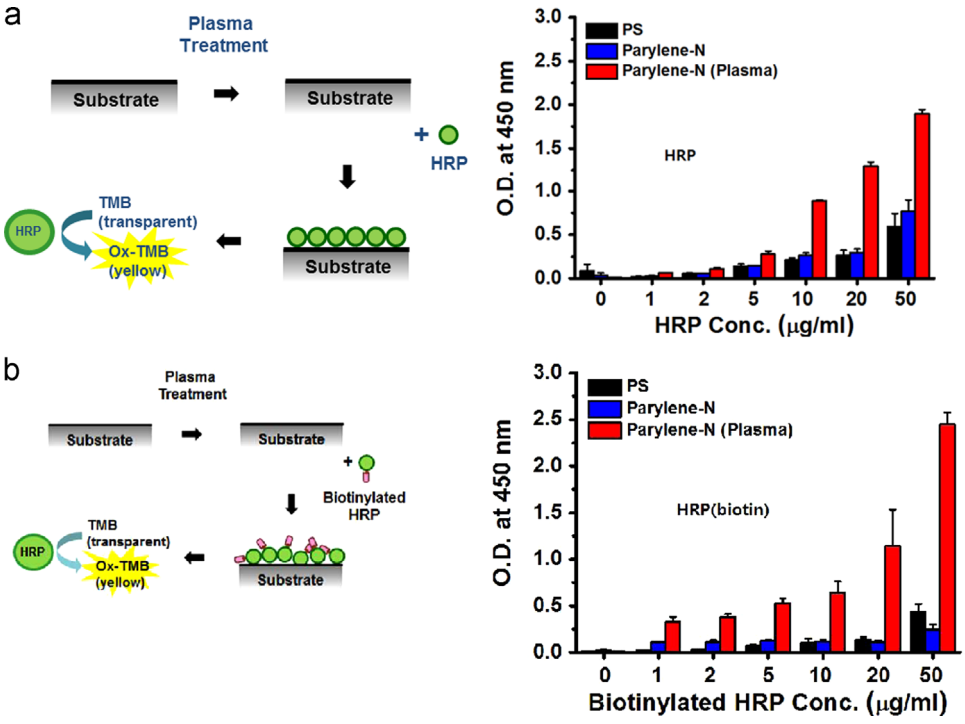

Fig. 2. Comparison of protein adsorption on polystylene and parylene-N film before and after plasma-treatment: (a) HRP (pI=6.6, nearly neutral at pH 7), (b) biotinylated HRP, (c) streptavidin (pI=5, negatively charged at pH 7), (d) avidin (pI=10, negatively charged at pH 7).

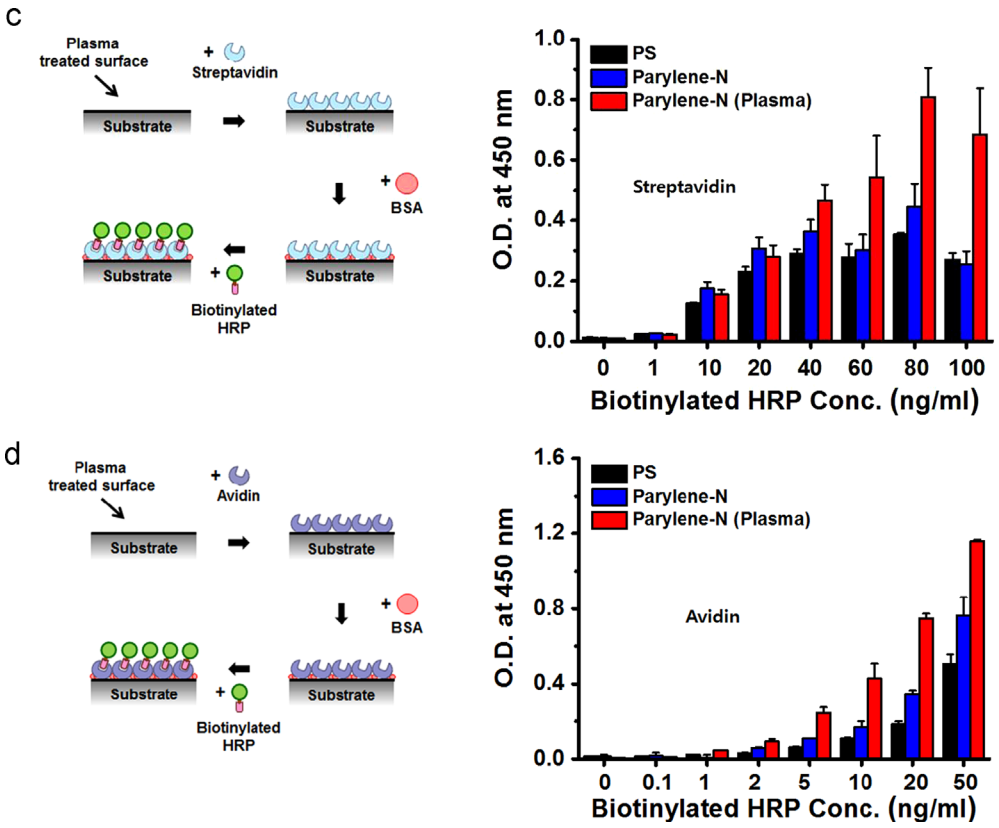

Fig. 2. (*continued*)

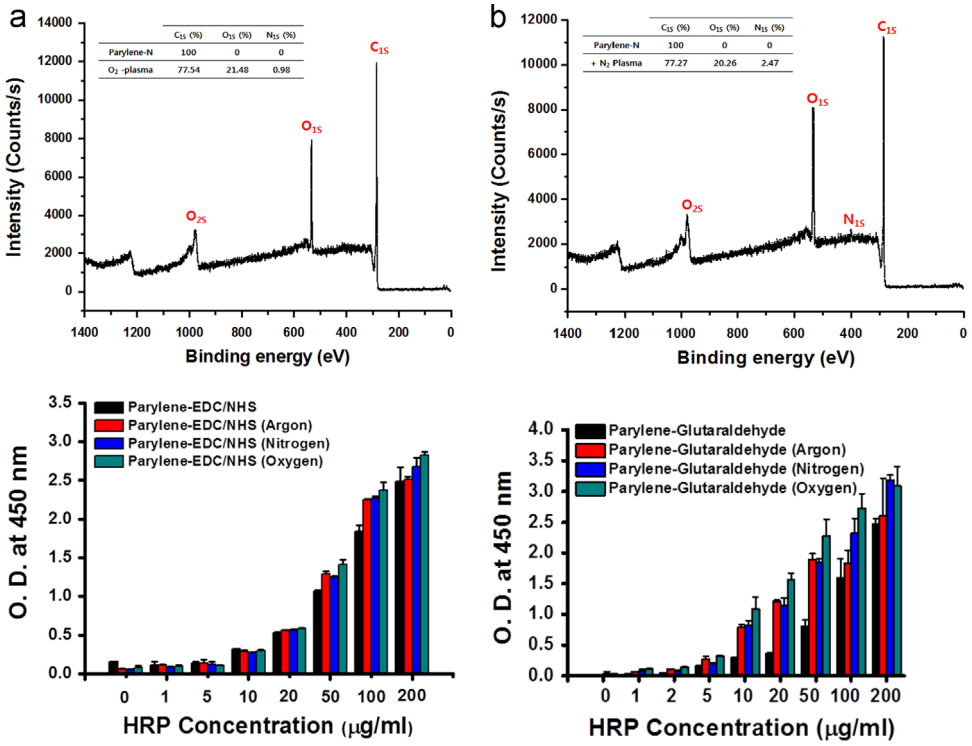

Fig. 3. XPS analysis and covalent immobilization of HRP on the parylene-N film after treatment of oxygen and nitrogen plasmas: (a) XPS spectrum after oxygen plasma-treatment and the amount of covalent bonding through carboxylic acids on the surface with the reaction of EDC/NHS. (b) XPS spectrum after nitrogen plasma-treatment and the amount of covalent bonding through amines on the surface with the reaction of glutaraldehyde.

C_{1s}, such as carboxylic acids, hydroxyl groups and so on. And then, the plasma-treated parylene-N film was reacted with the reagent EDC/NHS to create covalent bonding between the carboxylic acid groups on the film and the amine groups in the HRP. In the case of the oxygen-plasma-treated parylene-N film, the amount of immobilized HRP was estimated to be higher than on the parylene-N

film treated with argon and nitrogen plasmas. As shown in Fig. 3 (b), the chemical composition of the parylene-N film after the plasma-treatment was analyzed to be 77.27%, 20.26%, 2.47% for C_{1s}, O_{1s}, N_{1s}, respectively. These results represented the introduction of functional groups with oxygen as well as nitrogen to the parylene-N film, such as carboxylic acids, hydroxyl and amino groups and so on. And then, the plasma-treated parylene-N film was reacted with the reagent glutaraldehyde to create covalent bonding between the amine groups on the plasma-treated parylene-N film and the amine groups in the HRP. In the case of the nitrogen-plasma-treated parylene-N film, the amount of immobilized HRP was estimated to be higher than in the parylene-N film treated with argon and oxygen plasmas. These results showed that the oxygen and nitrogen plasma-treatments created a higher surface concentration of carboxylic acid and amine groups in comparison with the other plasma-treatments. However, the amount of covalent immobilization was not significantly higher than with the plasmas of the other gases. For example, the EDC/NHS reagent induced more covalent immobilization of HRP with the oxygen plasma-treatment, and the other plasmas also induced comparable covalent immobilization of HRP in the oxygen-plasma-treated parylene-N film. These results showed that the immobilization efficiencies of the plasma-treated parylene-N film were similar regardless of the plasma gas used, and the immobilization of HRP was mainly carried out by physical adsorption. As shown in the immobilization results for different pI values, the plasma-treated parylene-N film had a positive net charge at pH 7, however, the results from the different plasmas showed that the surface was composed of both positively charged and negatively charged functional groups, such as amines and carboxylic acids.

3.3. Application to SPR biosensor

It has been published that parylene-A film with amine groups can be used in SPR biosensors by the covalent immobilization of antibodies and antigens. To use a parylene-A film as a SPR biosensor, the thickness of the parylene-A film on the gold surface should be controlled to less than 50 nm thick. In this work, the plasma-treated parylene-N film was applied to the SPR biosensor by protein immobilization through physical adsorption. As shown in Fig. 4(a), the SPR response curve according to the thickness of the parylene-N film was recorded. The SPR angle increased to 14,950, 19,986, 23,750, and 27,938 as the thickness of the parylene-N film was increased from 0 (bare gold), to 10 nm, 30 nm, and 50 nm. The sharpness of the SPR curves was indexed by the full width at half maximum (FWHM) which was calculated to be 1.00, 1.03, 1.12, and 1.21 (in an arbitrary unit, relative index to the gold surface) for a parylene-N film with thicknesses of 0 nm, 10 nm, 30 nm, and 50 nm. These results showed that the sharpness of the SPR curve decreased as the parylene-N film became thicker.

To estimate the influence of the thickness of the parylene-N film on the sensitivity of the SPR measurement, standard sucrose samples at the known refractive index (RI) were prepared and injected onto the SPR chip fabricated with a parylene-N film of known thickness. In this work, the standard sucrose samples were prepared in the RI range between 1.332 and 1.337. By using these standard samples, the change in sensitivity of SPR sensor according to the thickness on the sensor surface was evaluated from the change in SPR signal against the RI change on the sensor surface. As shown in Fig. 4(b), the slope of the response curve (SPR signal per RI change) was calculated to be 0.98 (r=0.982), 1.11 (r=0.977), 0.99 (r=0.989), and 0.99 (r=0.993) ($\times 10^6$, r=linearity factor) for the SPR chip with parylene-N films with thicknesses of 0 nm, 10 nm, 30 nm, and 50 nm, respectively. These results showed that the sensitivities of the SPR biosensor would be similar for SPR biochips using parylene-N films with thicknesses from 0 nm to 50 nm. For the parylene-N film with a thickness of 80 nm, an SPR peak was not observed and the FWHM steeply increased. To have a suitable sensitivity, the SPR biosensor was prepared using a parylene-N film with a thickness of 30 nm.

The SPR signal from the adsorption of proteins was first compared using SPR chips with a gold surface, parylene-N film, and plasma-treated parylene-N film. The SPR measurement was carried out using BSA as a model analyte, and the SPR signal was measured by the repeated process of (1) baseline establishment by PBS injections repeated three times, (2) sample injection and incubation for 30 min, (3) washing and signal measurement. To compare the efficiency of protein immobilization on differently modified SPR chips, the SPR measurement was carried out using SPR chips with a gold surface, a self-assembled monolayer (SAM) with an 11-mercapto-1-undecanol, parylene-N film, and a plasma-treated parylene-N film. As shown in Fig. 5(a), the SPR signal was measured using the gold surface, and was observed to decrease with repeated injections of the sample (BSA). The same SPR

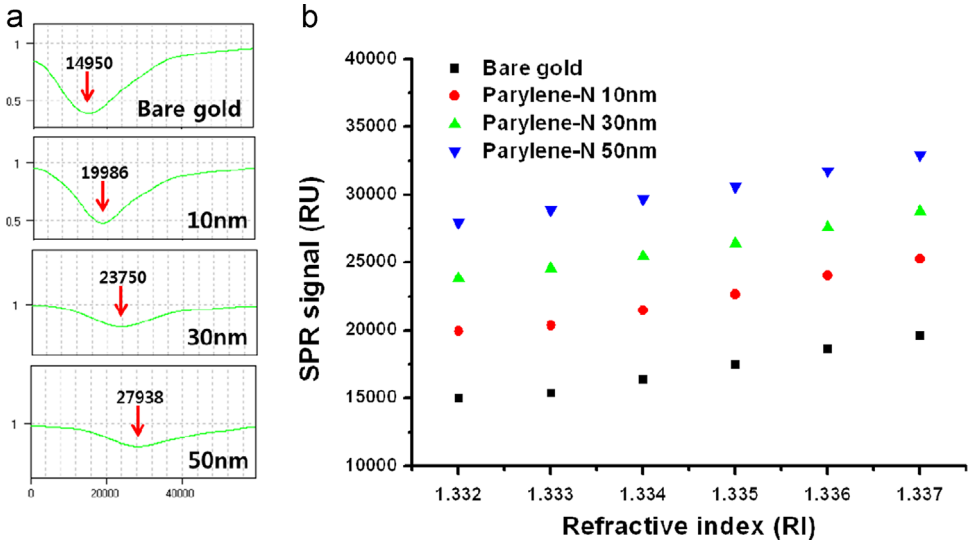

Fig. 4. SPR response according to the thickness of parylene-N film on the gold surface of an SPR chip: (a) SPR curve according to the thickness of parylene-N film. (b) SPR signal according to the refractive index of sucrose samples.

measurement was carried out using the SPR chip with a parylene-N film as shown in Fig. 5(b), and the SPR signal from the adsorption of protein (BSA) was observed to decrease significantly compared to the previous SPR measurements on the gold surface. As shown in Fig. 5(c), the SPR signal from the plasma-treated parylene-N film was observed to be significantly larger than from both the gold surface and the parylene-N film. These SPR signals from protein adsorption on the different SPR chips are compared in Fig. 5(d). The protein adsorption to the SAM and parylene-N films was estimated to be the lowest by repeated injection of the sample (BSA). The plasma-treated payrlene-N film was estimated to have the highest protein adsorption capacity of the modified surfaces. In particular, the immobilization efficiency of the plasma-treated parylene-N film was estimated to be 185% higher than the conventional gold surface at the concentration of SPR signal saturation. These results showed that the plasma-treated parylene-N film could be effectively used for protein immobilization on SPR biochips through physical adsorption.

3.4. SPR biosensor for the detection of HBsAg

A plasma-treated parylene-N film was applied to a SPR biosensor for the detection of HBsAg. Usually, the sensitive detection of a target analyte requires a high surface density of receptor

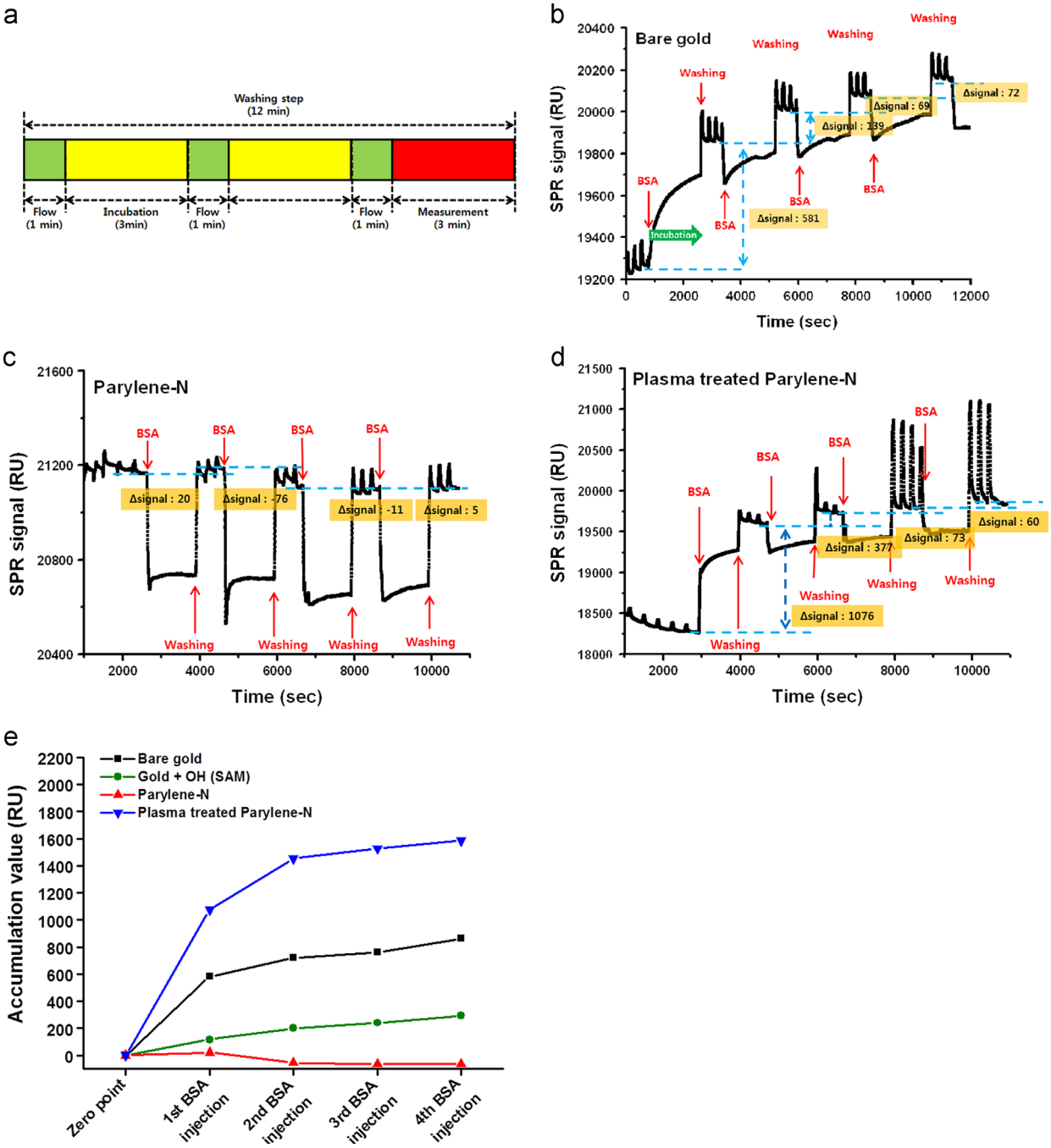

Fig. 5. Response of SPR biosensor according to the adsorption of bovine serum albumin (BSA) to differently modified sensor surfaces: (a) Flow control during the washing step of SPR measurement. (b) Bare gold. (c) Plasma-treated parylene-N film. (d) Comparison of protein adsorption to differently modified sensor surfaces. (e) Comparison of SPR signal of the adsorbed BSA by repeated treatment to differently prepared surfaces.

molecules on a solid support of immunoassays. In this work, a plasma-treated parylene-N film was used for the immobilization of anti-HBsAg antibodies through physical adsorption. First, the efficiency of physical adsorption of anti-HBsAg to the plasma-treated parylene-N was compared on differently prepared surfaces. As shown in Fig. 6(a), anti-HBsAg antibodies were physically adsorbed to a parylene-N film, plasma-treated parylene-N film, and polystyrene, then the immobilization efficiency of the anti-HBsAg antibodies on each surface was compared after treatment with HRP labeled secondary antibodies. As previously mentioned, the plasma-treated parylene-N film showed a higher immobilization efficiency for the negatively charged proteins than the positively charged proteins at a pH of 7. As the antibodies (IgG) were known to have a positive charge at pH 7 (Lee et al., 2012b), the immobilization efficiencies of both the parylene-N film and the plasma-treated parylene-N film were not significantly different. However, the surface density of anti-HBsAg antibodies was determined to be more than two times higher on the plasma-treated parylene-N film compared to the conventional polystyrene surface in the concentration range of 10–500 ng/ml.

For the detection of HBsAg with an SPR biosensor, the immobilization efficiencies of proteins on the modified SPR biochips were tested using several proteins. SPR chips were prepared with a bare gold surface, parylene-N film, and plasma-treated parylene-N film. Then, the immobilization efficiency was compared for BSA, streptavidin, and anti-HBsAg using differently modified SPR chips. As shown in Fig. 6(b), the SPR chip with the plasma-treated parylene-N film was estimated to have the highest immobilization efficiency compared to the bare gold surface and the untreated parylene-N film. In addition, the anti-HBsAg antibodies were estimated to be most effectively immobilized compared to the BSA and streptavidin. In this work, the detection of HBsAg was carried out using an SPR biochip with a plasma-treated parylene-N film, and a freshly-prepared SPR biochip with a plasma-treated parylene-N film was used for each measurement.

As shown in Fig. 6(c), the baseline drift of SPR biosensor with the plasma-treated parylene-N film was estimated to be 9.8 (RU) when PBS was injected as a sample ($n=3$). Based on these results, the limit of detection was estimated to be less than 10 pg/ml with a significantly higher SPR signal of 22.0 (RU) in comparison with the baseline drift, and the detection range for HBsAg was estimated to be 10 pg/ml–1 μg/ml. For the SPR biosensors with the bare gold surface and the parylene-N film, the limits of detection were estimated to be 10 ng/ml and 100 ng/ml, respectively.

The feasibility of the SPR biosensors for medical diagnosis was tested by comparing the sensitivity with a commercial ELISA kit from BioRad Laboratories (CA, USA). The cut-off value for the determination of HBsAg-positiveness was established to be an optical density (OD) of 1.5 at the wavelength of 450 nm by using standard HBsAg-positive and HBsAg-negative sera. Usually, the sample was determined to be HBsAg-positive if the immunoassay were higher than the cut-off value. Otherwise the sample was determined to be negative. From this ELISA test, the cut-off concentration for HBsAg-positiveness was estimated to be 30 ng/ml (arrow) in Fig. 6(c). In the case of the SPR biosensor with the plasma-treated parylene-N film, the limit of detection as well as the detection range was suitable for the medical diagnosis of HBsAg. From the comparison of standard curves, the SPR biosensors could detect the HBsAg in the concentration of 10 pg/ml–40 ng/ml, which could not be detected from the conventional ELISA kit. These results showed that the SPR biosensor with the plasma-treated parylene-N film could achieve more than 1000-fold improved sensitivity in comparison with the conventional ELISA kit, and such an improved sensitivity was considered to be resulted from the enhanced immobilization of receptor antibodies against HBsAg by using the plasma-treated parylene-N film.

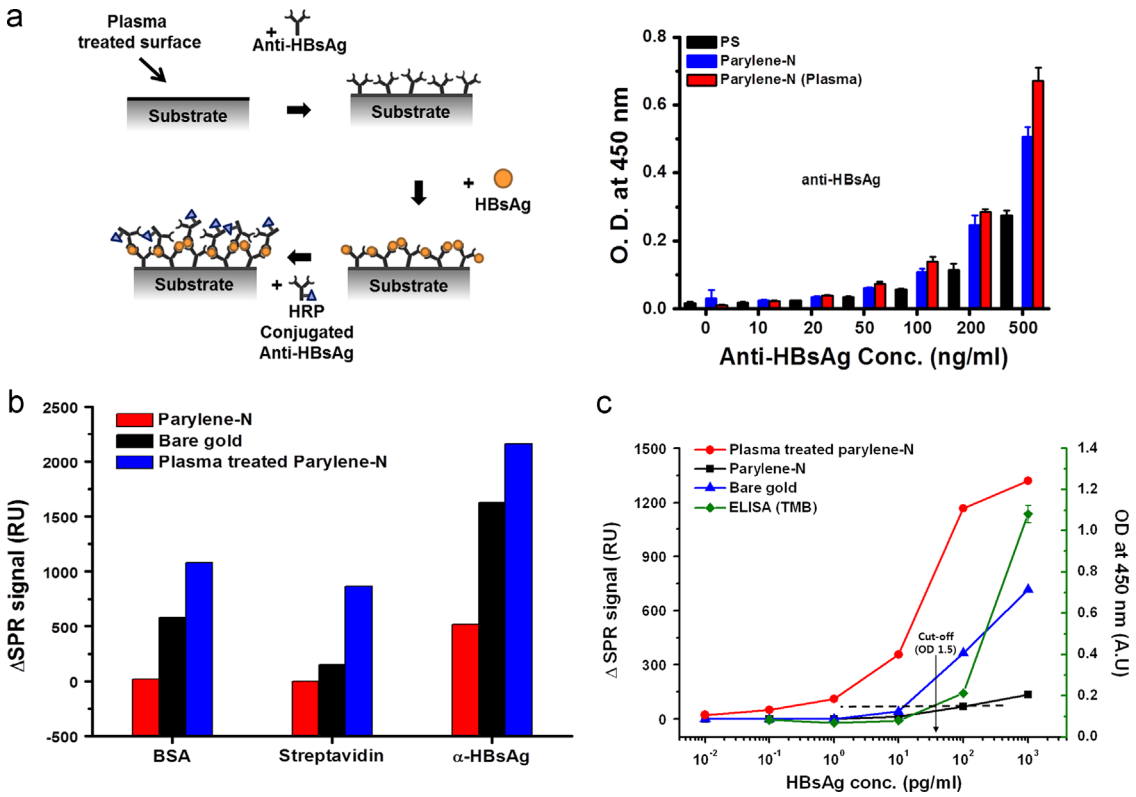

Fig. 6. Detection of HBsAg with SPR biosensor: (a) Comparison of the immobilization efficiency of anti-HBsAg to differently modified surfaces. (b) Comparison of SPR signals by immobilization of proteins to differently modified surfaces. (c) Comparison of SPR signals according to the concentration of standard samples of HBsAg. The cut-off value (dash, OD=1.5) for the determination of HBsAg-positiveness was established by using a commercial ELISA kit for HBsAg.

4. Conclusions

A parylene-N film was modified with a plasma-treatment for immobilization of proteins through physical adsorption. The changes in the surface properties of the parylene-N film after plasma-treatment were analyzed using contact angle microscopy and AFM. The plasma-treatment of the parylene-N film created a 25% more hydrophilic surface with long-term stability, and the change in contact angle of the parylene-N film was thought to result from the hydrophilicity effect rather than the change in surface roughness after the plasma-treatment. To apply plasma-treated parylene-N films to immunoassays, the efficiency of protein immobilization was compared with that of conventional polystyrene and parylene-N film using three model proteins with different surface charges (isoelectric point): streptavidin (pI=5, negatively charged at pH 7), horseradish peroxidase (pI=6.6, nearly neutral at pH 7), and avidin (pI=10, positively charged at pH 7). The model proteins with acidic and basic pIs showed higher immobilization efficiencies on the plasma-treated parylene-N film compared to the parylene-N film and polystyrene. To use the plasma-treated parylene-N film in an SPR biosensor, the sensitivity of the biosensor was estimated according to the thickness of the parylene-N film. The plasma-treated parylene-N film was shown to effectively immobilize protein on the SPR biochips through physical adsorption using BSA, streptavidin, and anti-HBsAg antibodies. For the detection of HBsAg, an SPR biosensor was developed using the plasma-treated parylene-N film. In this work, the detection of HBsAg was possible in the concentration range from 10 pg/ml to 1 μg/ml, and the limit of detection was estimated to be less than 10 pg/ml. In comparison with the commercial ELISA kit with the limit of detection of 40 ng/ml, the SPR biosensor with the plasma-treated parylene-N film could achieve more than 1000-fold improved sensitivity in comparison with the conventional ELISA kit, and such an improved sensitivity was considered to be resulted from the enhanced immobilization of receptor antibodies against HBsAg by using the plasma-treated parylene-N film.

Acknowledgements

This research was supported by the National Research Foundation of Korea (2013-056331, and 2013-056267).

References

Brett, P.J., Tiwana, H., Feavers, I.M., Charalambous, B.M., 2002. J. Biol. Chem. 277, 20468–20476.
Chang, T.Y., Yadav, V.G., Leo, S.D., Mohedas, A., Rajalingam, B., Chen, C.L., Selvarasah, S., Dokmeci, M.R., Khademhosseini, A., 2007. Langmuir 23, 11718–11725.
Chung, J.W., Kim, S.D., Bernhardt, R., Pyun, J.C., 2005. Sens. Actuators, B 111–112, 416–422.
Chung, J.W., Kim, S.D., Bernhardt, R., Pyun, J.C., 2006a. Sens. Actuators, B 118, 28–32.
Chung, J.W., Kim, S.D., Bernhardt, R., Pyun, J.C., 2006b. Sens. Actuators, B 114, 1007–1012.
Goda, T., Konno, T., Takai, M., Ishigara, K., 2007. Colloids Surf., B 54, 67–73.
Hornbeck, P., Winston, S.E., Fuller, S.A., 1991. Current Protocols in Molecular Biology. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (Eds.), Wiley and Sons, New York, p. 11.2.8
Jeon, B.J., Kim, M.H., Pyun, J.C., 2010a. J. Immunol. Methods 353, 44–48.
Jeon, B.J., Kim, M.H., Pyun, J.C., 2010b. J. Immunol. Methods 353, 44–48.
Jeon, B.J., Kim, M.H., Pyun, J.C., 2011. Sens. Actuators, B 154, 89–95.
Ko, H., Lee, G.Y., Jeon, B.J., Pyun, J.C., 2011a. BioChip J. 5, 242–245.
Ko, H., Lee, E.H., Lee, G.Y., Jeon, B.J., Kim, M.H., Pyun, J.C., 2011b. Biosens. Bioelectron. 30, 56–60.
Lee, E.H., Yoo, G., Park, M., Jose, J., Pyun, J.C., 2012a. BioChip J. 6, 231–238.
Lee, E.H., Lee, J.K., Kang, M.J., Pyun, J.C., 2012b. BioChip J. 6, 319–324.
Luppa, P.B., Sokoll, L.J., Chan, D.W., 2001. Clin. Chim. Acta 314, 1–26.
Panthirana, S.T., Barbaree, J., Chin, B.A., Hartell, M.G., Neely, W.C., Vodyanoy, V., 2000. Biosens. Bioelectron. 15, 135–141.
Pender, A.B., Prager, A., Elsner, C., Buchmeiser, M.R., 2008. J. Appl. Polym. Sci. 112, 2701–2709.
Righetti, P.G., Caravaggio, T., 1976. J. Chromatogr. A 127, 1–28.
Schellekens, G.A., Visser, H., de Jong, B.A., van den Hoogen, F.H., Hazes, J.M., Breedveld, F.C., van Venrooij, W.J., 2000. Arthritis Rheum. 43, 155–163.
Su, S.V., Hong, P., Baik, S., Negrete, O.A., Gurney, K.B., Lee, B., 2004. J. Biol. Chem. 279, 19122–19132.
Trantidou, T., Prodromakis, T., Toumazou, C., 2012. Appl. Surf. Sci. 261, 43–51.
Yoo, G., Park, M., Lee, E.H., Jose, J., Pyun, J.C., 2011. Anal. Chim. Acta 707, 142–147.