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Analysis of C-Reactive Protein on Amide-Linked *N*-Hydroxysuccinimide−Dextran Arrays with a Spectral Surface Plasmon Resonance Biosensor for Serodiagnosis

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A new label-free array system using amide-linked (AL) NHS−dextran and a spectral SPR biosensor are presented for the high-throughput analysis of C-reactive protein (CRP) in human sera. The AL NHS−dextran layer on the surface of gold arrays was composed of an amide linkage between NHS-modified carboxymethyl−dextran and amine-modified 11-mercaptoundecanoic acid. The topology of the AL NHS−dextran layer was analyzed by atomic force microscopy, and it was found to be superior to the previously used epoxide-linked carboxymethyl−dextran layer in its immobilization of proteins. Specific immunoreactions and a dose-dependent increase of SPR signals were demonstrated on the AL NHS−dextran layer. Then, the label-free array system was successfully applied to the rapid analysis of CRP in 120 human sera. CRP levels in human sera determined by the array-based spectral SPR biosensor showed a good correlation with those determined by the latex-enhanced turbidimetry immunoassay ($n = 120$, $r = 0.945$, $p < 0.0001$). Thus, the array-based spectral SPR biosensor based on the AL NHS−dextran surface is a potential system for rapid and label-free serodiagnosis of human diseases.

Protein arrays have emerged as a key technology in serodiagnosis and proteomics, since the technology allows large-scale and high-throughput analysis of biomolecular interactions.[1,2] A number of techniques have been reported for the analysis of protein interactions on arrays with various methods such as fluorescence labeling,[2,3] mass spectrometry,[4] atomic force microscopy (AFM),[5] and surface plasmon resonance (SPR).[1,6,7] Recently, an accumulating number of reports have demonstrated the potential applications of protein arrays in the serodiagnosis of allergic and infectious diseases by analyzing immunoglobulins in human sera according to the fluorescence labeling method.[8−11] In the serodiagnosis of allergies, protein arrays have been used for monitoring patient-specific antibody profiles to various allergens implicated in allergic diseases.[11] There are reports on the parallel detection of infectious diseases caused by various pathogens such as rubella virus, herpes simplex virus, and hepatitis B virus on peptide or protein arrays.[8,12] Protein arrays have been used to screen specific antigens for the diagnosis of severe acute respiratory syndrome based on fluorescence labeling.[10,13] The in situ SPR method has been used to detect antibodies produced by the infection of virus pathogens in human sera.[14−16] It was reported that anti-adenoviral antibodies were detected and isotyped by the

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SPR method among patients dosed with an adenoviral-based gene therapy vector.[14] However, there is no report on the high-throughput analysis of blood proteins with any array-based SPR biosensors for the serodiagnosis of human diseases such as cardiovascular diseases.

It is important for protein arrays to retain proteins in an active state at high densities and reduce nonspecific interaction since proteins sometimes undergo denaturation while being arrayed on a solid substrate and show nonspecific binding in the analysis of multiple biomolecular interactions.[1,2] One of the most common strategies is to immobilize proteins with activated carboxymethyl-dextran (CM-dextran).[7,17,18] CM-dextran has been widely used to immobilize proteins since Löfås and Johnsson[19] introduced the method to prepare the CM-dextran layer on gold substrate. Dextran is a linear polymer based on 1,6-linked glucose units, and CM-dextran has been used for a wide variety of applications such as biomolecule immobilization, drug delivery devices, and tissue engineering.[20] The CM-dextran layer has been fabricated on gold substrate by the ring-opening reaction of epoxides with dextran and by functionalization of dextran with bromoacetic acid to generate carboxylic groups.[7,19,21,22] One of advantages of the epoxide-linked (EL) CM-dextran layer is that it reduces the potential for denaturation of proteins and minimizes nonspecific binding of ligands to the sensor surface, because it provides an effective barrier between biomolecules and the gold substrate.[19] Another advantage is that the CM-dextran layer can supply more binding capacity than other layers because the CM-dextran matrix has numerous carboxylic group functionalities along its backbone.[23] However, the procedure for the formation of the EL CM-dextran layer is complex, and it requires much time to fabricate the CM-dextran matrix on the gold surface. In addition, the EL CM-dextran layer is inappropriate for a high-throughput analysis of protein interactions on gold arrays because sample solutions can be easily diffused and mixed among spots during incubation.

In this paper, we present a new label-free array system using amide-linked (AL) *N*-hydroxysuccinimide (NHS)−dextran and a spectral SPR biosensor for the high-throughput analysis of C-reactive protein (CRP) in human sera. CRP is used for conventional inflammation diagnosis as well as diagnosis of low-grade inflammation for risk estimation of cardiovascular events.[24] The AL NHS−dextran layer was fabricated on the surface of gold arrays by amide linkage between NHS-modified CM-dextran and amine-modified 11-mercaptoundecanoic acid (MUA), and the surface was analyzed by AFM. A dose-dependent increase of the SPR signal was observed on the AL NHS−dextran surface when monoclonal antibodies were introduced onto the protein arrays prepared with various concentrations of glutathione *S*-transferase (GST) and CRP in buffer and normal human serum. Then, the array-based SPR biosensor was successfully applied to the rapid analysis of CRP in 120 human sera.

EXPERIMENTAL SECTION

Protein and Serum Samples. GST, GST-rhoA, GST-rac1, and GST- p21-binding domain of PAK1 (PBD) were prepared by expressing the genes in *Escherichia coli* (BL21) according to the procedures specified in a previous report.[25] C-Reactive protein was obtained from Scripps Lab (San Diego, CA). Monoclonal antibodies against rhoA and rac1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Human sera were obtained from the Kangwon National University Hospital, divided into aliquots, and stored at −20 °C until use. Experiments using the human samples were performed under the approval by the local Institute Ethics Committee for human subject research.

Hydrophobic Modification of Gold Arrays. Gold arrays were fabricated and modified according to the procedures of Jung et al.[25] Briefly, gold arrays with 50 spots each of 2-mm diameter were fabricated by depositing Ti/Au (50/450 Å) films on pyrex glass and were cleaned with a cleaning solution of $NH_4OH/H_2O_2/H_2O$ (1:1:5, v/v) at 70 °C for 10 min. Then, hydrophobic glass surfaces were generated between gold spots according to a previous report[22] to prevent protein solutions from being mixed among the spots during incubation. The gold arrays were incubated with a mixture of hexadecane/tetracarbon chloride/octadecyltrichlorosilane (20:5:0.04, v/v) for 20 min and washed with a mixture of hexadecane/tetracarbon chloride (20:5, v/v), tetracarbon chloride, and ethanol in order.

Surface Modification of Gold Arrays with AL NHS−Dextran and EL CM-Dextran. The surfaces of gold arrays were modified with AL NHS−dextran as described in Figure 1. The gold arrays were incubated with a mixed thiol solution of 0.1 mM MUA and 0.9 mM 6-mercaptohexanol in ethanol for 16 h and then washed with ethanol to remove the excess thiols.[26] The arrays were incubated with a mixture of 50 mM NHS and 200 mM *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) for 10 min, washed with milliQ water, and incubated with 500 mM ethylenediamine for 1 h. To fabricate the AL NHS−dextran layer on gold arrays, the resulting monolayer of *N*-(2-aminoethyl)-11-mercaptoundecanamide and 6-mercaptohexanol was incubated with a NHS−dextran solution, which was freshly prepared by dissolving 0.3 g/mL CM-dextran in 1 mL of 50 mM NHS and 200 mM EDC and incubating at room temperature for 10 min. CM-dextran was prepared according to the procedures of McArthur et al.[27] Six grams of dextran (MW 500 000) was dissolved in 20 mL of 100 mM bromoacetic acid in 2 M NaOH and stirred overnight. Then, the solution was dialyzed against water for 24 h, against 0.1 M HCl for 24 h, and finally against water for 24 h. The dialyzed solution was lyophilized, and the lyophilized CM-dextran was analyzed by FT-IR spectroscopy and stored until use.

The gold arrays were also modified with CM-dextran by epoxide linkage according to a previous report.[21] Briefly, 600 mM

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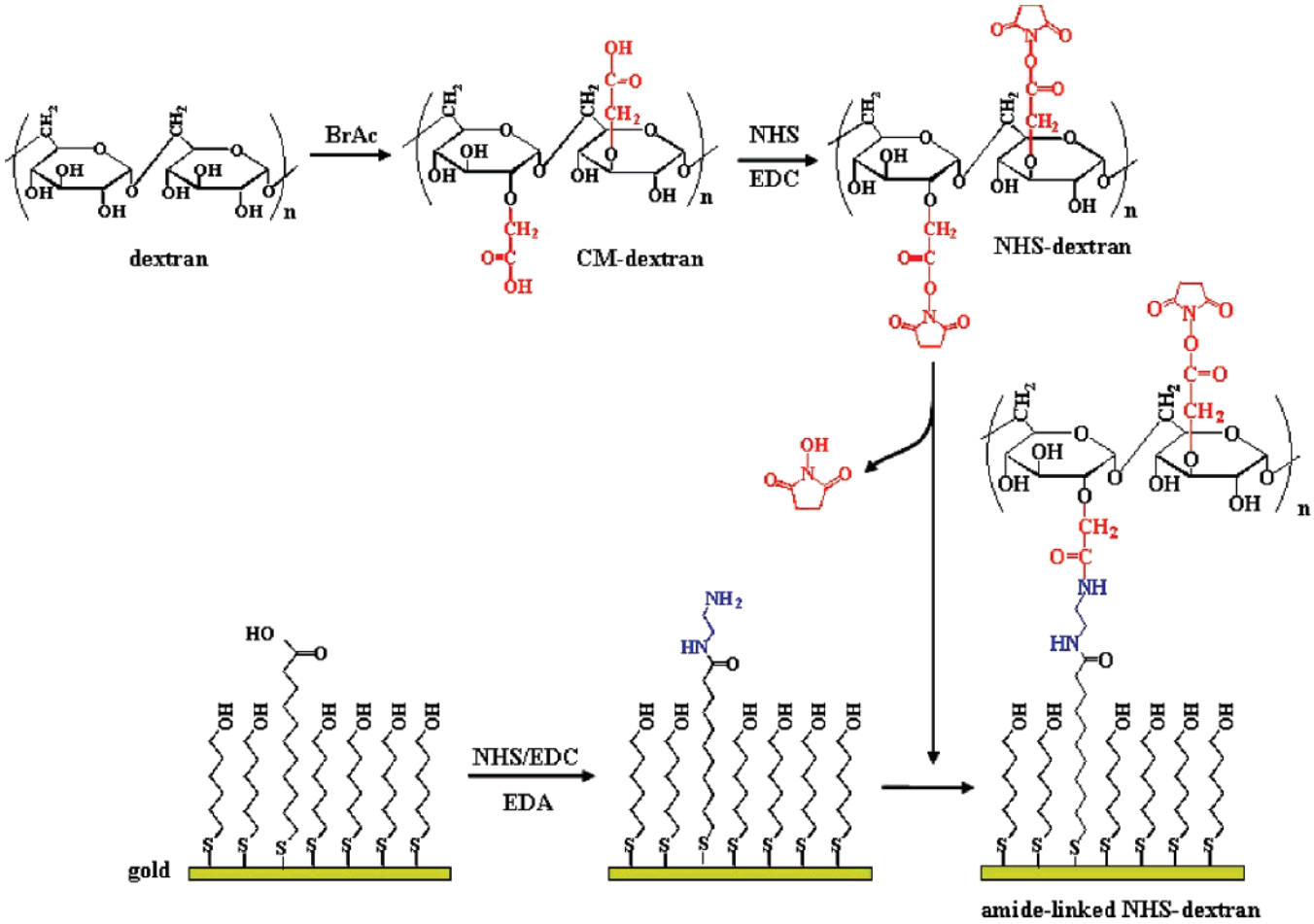

Figure 1. Schematic diagram for the surface modification of gold arrays with amide-linked NHS−dextran. BrAc, bromoacetate; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride; EDA, ethylenediamine; NHS, *N*-hydroxysuccinimide.

epichlorohydrin in a mixture (1:1) of 400 mM NaOH and bis-2-methylethyl ether was applied for 4 h to a 11-mercaptoundecanol monolayer of gold arrays. After washing with water and ethanol, the arrays were immersed in 0.3 g/mL dextran in 100 mM NaOH for 20 h, and the dextran surface was functionalized by incubation with 1 M bromoacetic acid in 2 M NaOH for 16 h.

AFM Imaging. The surface topology of three different dextran layers including AL NHS−dextran, EL CM-dextran, and Biacore CM-dextran (CM5 chip) was analyzed by AFM. AFM imaging was performed under air using a Nanoscope IIIa with a J-type scanner (125 × 125 μm) (Digital Instruments) in the contact mode. Cantilevers with Si_3N_4 oxide-sharpened tips (spring constant, 0.12 N/m) were used for the imaging. The applied force was varied from several to tens of nanonewtons. The film thickness of the modified dextran layers was estimated by measuring the depth of an artificial hole that was made by puncturing the layer surface with the contact mode.

Preparation of Protein Arrays on the AL NHS−Dextran Layer of Gold Arrays. Various concentrations of proteins including CRP, rac1, GST, GST-rhoA, GST-rac1, and GST-PBD in 10 mM sodium acetate buffer (pH 4.5) were applied to the AL NHS−dextran surface of gold arrays for 1 h. The arrays were incubated for 30 min with 1% bovine serum albumin (BSA) containing 0.1% Tween 20 in phosphate-buffered saline (PBS) (8.1 mM Na_2HPO_4, 1.2 mM KH_2PO_4, 2.7 mM KCl, 138 mM NaCl, pH 7.4) to reduce nonspecific interactions. The protein arrays were incubated for 1 h with 200 μg/mL monoclonal antibodies against CRP, GST, rhoA, and rac1 in 1% BSA containing 0.1% Tween 20 in PBS. For the analysis of proteins in human sera, the protein arrays were further incubated with anti-mouse IgG or anti-goat IgG to enhance the sensitivity of the SPR biosensor. Then, the arrays were washed twice with 0.1% Tween 20 in PBS for 5 min, rinsed with MilliQ water, flushed with N_2 gas to remove the water, and immediately analyzed with a self-developed spectral SPR biosensor.

Analysis of Protein Arrays by the Line-Scanning Mode of a Spectral SPR Biosensor. The analysis of protein interactions on protein arrays was performed in the line-scanning mode of the spectral SPR biosensor according to a previous report.[28] Briefly, the spectral SPR sensor was configured using the Kretschmann geometry of the attenuated total reflection method. The arrays were coupled with a fused-silica prism via index matching fluid and mounted on an $x-y$ linear stage. Then, the array spots were automatically scanned every 100 μm along the central line by the line-scanning mode.

Analysis of CRP in Human Sera. The level of CRP in human sera was determined by two different methods: the spectral SPR biosensor based on the AL NHS−dextran and the latex-enhanced turbidimetry immunoassay. For analysis by the spectral SPR biosensor, 120 human sera were diluted 10 times with 9.3 mM phosphate buffer (pH 7.4) and immobilized onto the AL NHS−

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dextran layer of arrays. The protein arrays were blocked with 1% BSA containing 0.1% Tween 20 in PBS for 30 min and probed for 1 h with 200 μg/mL monoclonal anti-CRP antibody (2 μL/spot) in 1% BSA containing 0.1% Tween 20 in PBS. After washing with 0.1% Tween in PBS, the arrays were incubated with anti-goat IgG and immediately analyzed with the spectral SPR biosensor. The standard curve was obtained with the Sigmoidal Fit of the Origin program. CRP in human sera was also determined by latex-enhanced turbidimetry immunoassay according to the manufacturer's instructions (Denka Seiken) using an automated chemical analyzer (200 FR, Toshiba). This immunoassay used a monoclonal anti-CRP antibody and a calibrator against the reference preparation CRM 470. Then, correlation between two methods was analyzed with the Linear Fit of the Origin program.

RESULTS

Surface Modification of Gold Arrays with AL NHS−Dextran. In order to modify the surface of gold arrays with AL NHS−dextran, initially, we synthesized CM-dextran by substituting carboxyl groups for hydroxyl groups of dextran and characterized it by FT-IR spectroscopy. The formation of carboxyl groups on dextran was verified by the presence of CO double bond stretching of CM-dextran at 1728 and 1634 cm^{-1} (data not shown). The CM-dextran was activated with EDC and NHS to derive NHS−dextran, as described in Figure 1, and the NHS−dextran solution was applied onto the monolayer of *N*-(2-aminoethyl)-11-mercaptoundecanamide and 6-mercaptohexanol to form the AL NHS−dextran layer on gold arrays. *N*-(2-Aminoethyl)-11-mercaptoundecanamide was formed on the gold arrays by amine modification of 11-mercaptoundecanoic acid with ethylenediamine.

Subsequently, we investigated whether the AL NHS−dextran layer was properly formed on gold arrays by measuring the depth of an artificial hole that was made by puncturing the layer surface in the contact mode of AFM (Figure 2). The surface of AL NHS−dextran layer was uniform, and the thickness of the layer was ~3.2 nm. However, no scratch was made on the mixed thiol layer by AFM (data not shown), indicating that the thickness measured by AFM represented the AL NHS−dextran layer. Similar results were obtained from EL CM-dextran and Biacore CM-dextran layers. The formation of the modified dextran layers on the mixed thiol surface was further investigated with the spectral SPR biosensor. The layer formation of AL NHS−dextran and EL CM-dextran caused the shift of the SPR wavelength by 8.0 and 6.7 nm, respectively, suggesting that the layers were properly formed on the thiol layer. Thus, the AL NHS−dextran layer was appropriately formed on gold arrays.

Analysis of Immunoreactions on AL NHS−Dextran Arrays with the Spectral SPR Biosensor. To test whether the AL NHS−dextran layer was suitable for analysis of serum proteins in an array format, initially, we immobilized six proteins including CRP, rac1, GST, GST-rhoA, GST-rac1, and GST-PBD onto the dextran layer and then analyzed the capacity of the proteins to bind to the AL NHS layer in comparison with EL CM-dextran. As shown in Figure 3, two layers showed a similar binding affinity to CRP and rac1. However, the AL NHS−dextran layer showed a much higher binding affinity to GST or GST-fusion proteins than the EL CM-dextran layer, indicating that the AL NHS−dextran layer might have a strong affinity to GST-fusion proteins for reasons unknown.

We then investigated whether antigen−antibody interactions on the AL NHS−dextran layer were specific, since specific interactions are essential for the analysis of serum proteins. The same six proteins (CRP, rac1, GST, GST-rhoA, GST-rac1, GST-PBD) were immobilized onto the AL NHS−dextran layer to prepare protein arrays, and immunoreactions were analyzed with four monoclonal antibodies against CRP, GST, rhoA, and rac1 in the line-scanning mode of the spectral SPR biosensor. The net shifts of SPR wavelength, which are displayed by color spectra in Figure 4A, represented the amount of four antibodies bound to the protein arrays. As expected, monoclonal antibodies against CRP, rhoA, and rac1 strongly interacted with their antigens, interacting only negligibly with the other proteins. Monoclonal anti-GST antibody showed a strong interaction with GST or GST-fusion proteins. These results indicated specific antigen−antibody interactions on the AL NHS−dextran layer. Thus, AL NHS−dextran was a useful surface to prepare protein arrays and to analyze specific immunoreactions on arrays with the spectral SPR biosensor.

Quantitative Analysis of GST and CRP on AL NHS−Dextran Arrays by the Spectral SPR Biosensor. Since the previous results showed specific antigen−antibody interactions on the AL NHS−dextran layer, we investigated whether quantitative analysis of proteins could be performed on the AL NHS−dextran surface in an array format. To make that determination, first, various concentrations of GST and CRP in acetate buffer (pH 4.5) were applied to AL NHS−dextran arrays, and the resulting arrays were probed with monoclonal antibodies against GST and CRP. Then, the arrays were incubated with anti-mouse or anti-goat IgG to enhance the SPR signal and analyzed in the line-scanning mode of the spectral SPR biosensor. The net shift of the SPR wavelength caused by the secondary antibodies represented the amount of GST or CRP bound to the arrays. As shown in Figure 5A, GST caused a concentration-dependent increase of SPR wavelength shift on being saturated at 50 ng/μL. Similar changes in the SPR signal were obtained by CRP with saturation at 20 ng/μL.

To test whether the AL NHS−dextran arrays were appropriate for the analysis of serum proteins by the spectral SPR biosensor, first, GST and CRP were serially mixed with the normal human serum diluted with acetate buffer (pH 4.5) and applied to the AL NHS surface of arrays. The protein arrays were incubated with anti-GST and anti−CRP antibodies, and the SPR signal was enhanced by incubation with anti-mouse IgG or anti-goat IgG, respectively. As shown in Figure 5B, GST in normal human serum caused a dose-dependent increase of the SPR signal, but the SPR signals were lower than the previous ones obtained by GST in the acetate buffer. And CRP in the normal human serum was detectable only at concentrations over 20 ng/μL (Figure 5B). In addition, the normal serum diluted with the acetate buffer had to be filtered before being used because of its precipitation. These results indicated that the acetate buffer was not appropriate to immobilize CRP in human sera onto the AL NHS−dextran.

Thus, we prepared various concentrations of CRP in the normal human serum diluted with phosphate buffer (pH 7.4) instead of the acetate buffer (pH 4.5), and the samples were analyzed on the AL NHS−dextran arrays by the spectral SPR biosensor. As shown in Figure 6A, the sensitivity was significantly enhanced,

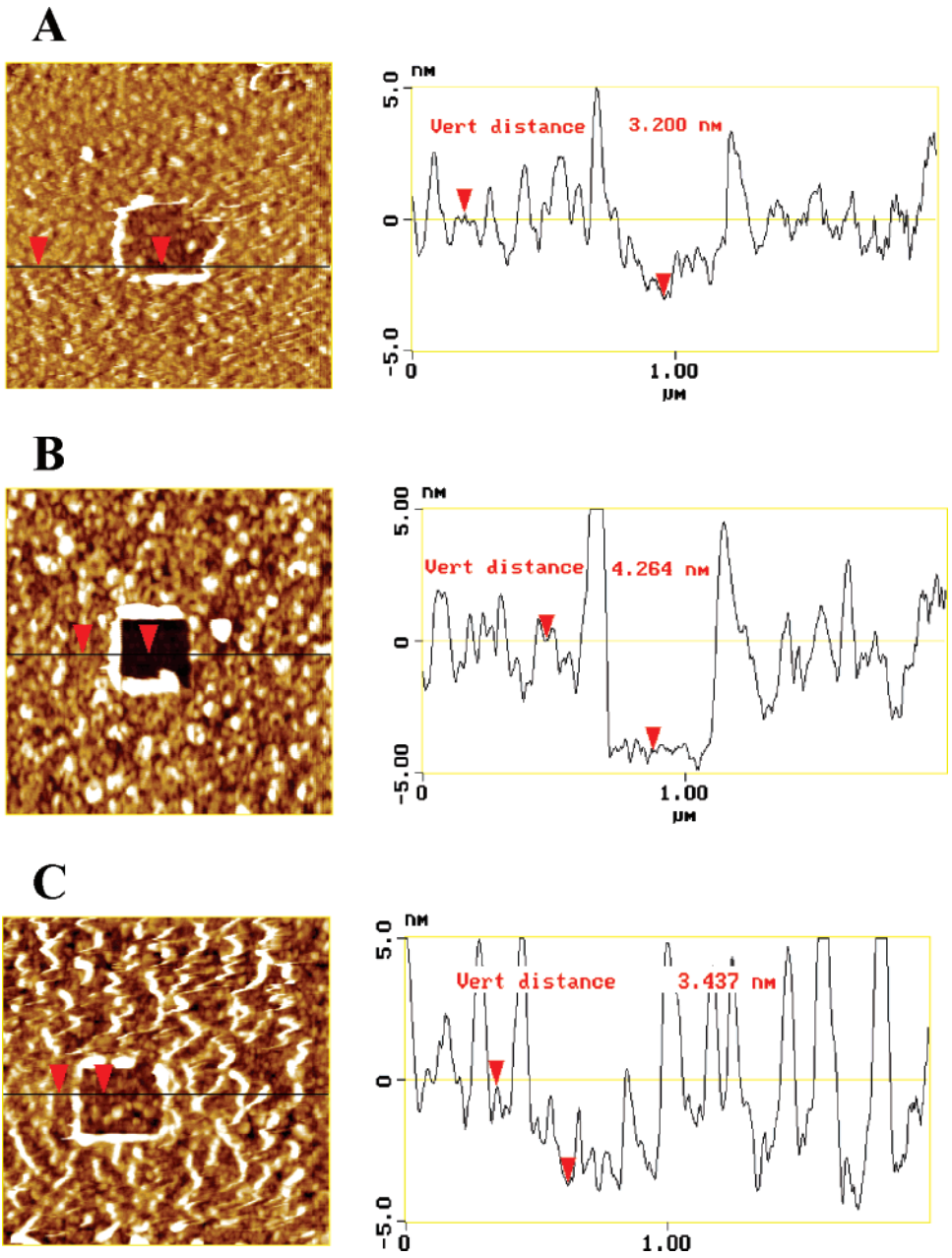

Figure 2. Topology analysis of three dextran layers by AFM. AFM imaging was performed in the contact mode of Nanoscope IIIa as described in the Experimental Section. (A) Amide-linked NHS−dextran, (B) Epoxide-linked CM-dextran, (C) BIAcore CM-dextran (CM5 chip).

being detectable at 0.1 ng/μl. Thus, proteins in human serum can be analyzed on AL NHS−dextran arrays by the spectral SPR biosensor, even though the sensitivity is dependent on the pH environment of serum samples.

High-Throughput Analysis of CRP in Human Sera. Because CRP spiked in normal human serum was successfully analyzed on the AL NHS−dextran, we applied the array system to the rapid analysis of CRP in 120 human sera by the spectral SPR biosensor. First, the level of CRP in human sera was determined by a commercialized method, the latex-enhanced turbidimetry immunoassay using an automated chemical analyzer. The serum samples were also used in the determination of the CRP level by the spectral SPR biosensor. In this method, SPR signals obtained from the human sera were used to calculate the amount of CRP with the standard curve of Figure 6A. Then, the relationship between the results obtained by two methods was investigated with the Fit Linear of the Origin program. As shown in Figure 6B, the CRP levels of human sera determined by the spectral SPR biosensor using the AL NHS−dextran surface showed a good correlation with those determined by the latex-enhanced turbidimetry immunoassay ($n = 120$, $r = 0.945$, $p < 0.0001$). Thus, the spectral SPR biosensor based on the AL NHS−dextran surface is a potential system for the rapid and label-free serodiagnosis of human diseases in an array format.

DISCUSSION

In this report, we present a new approach to the high-throughput analysis of CRP in human sera by the array-based spectral SPR biosensor with AL NHS−dextran. The AL NHS−dextran layer was prepared on the surface of gold arrays by the amide linkage between amine-modified MUA and NHS-modified CM-dextran. The AL NHS−dextran surface was superior to the

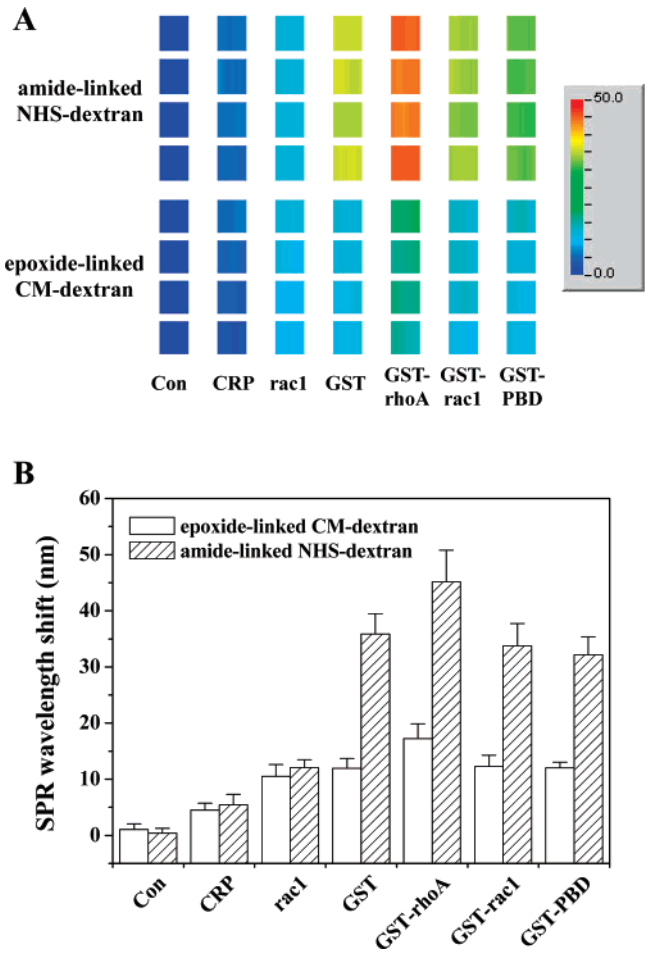

Figure 3. Comparison between amide-linked NHS−dextran and epoxide-linked CM-dextran in the binding capacity of various proteins. Various proteins including CRP, rac1, GST, GST-rhoA, GST-rac1, and GST-PBD (100 μg/mL in 10 mM sodium acetate buffer, pH 4.5) were immobilized onto the surfaces of amide-linked NHS−dextran and epoxide-linked CM-dextran, and then the arrays were analyzed in the line-scanning mode of the spectral SPR biosensor as described in the Experimental Section (A). The results are expressed as means ± SD from three separate experiments (B).

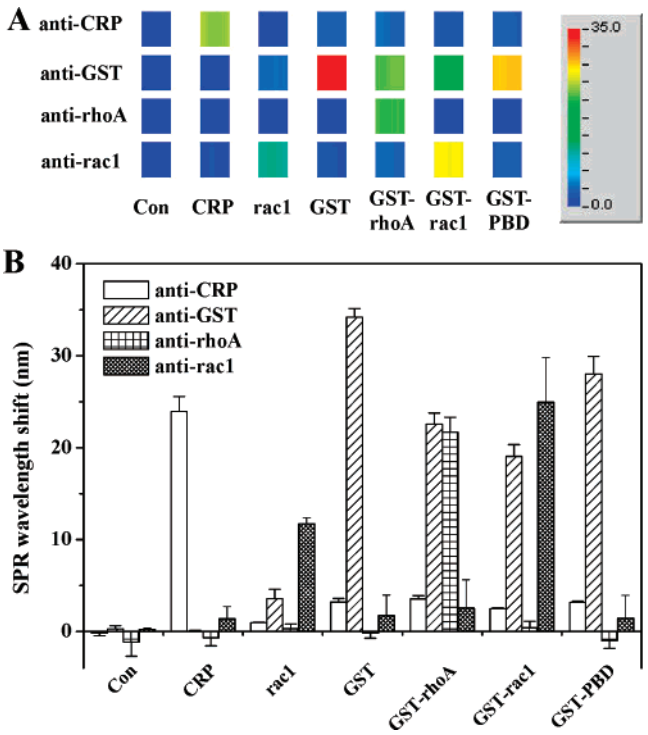

Figure 4. Analysis of antigen−antibody interactions on amide-linked NHS−dextran arrays by the line-scanning mode of the spectral SPR biosensor. Protein arrays were prepared by immobilizing various antigens including CRP, rac1, GST, GST-rhoA, GST-rac1, and GST-PBD (100 μg/mL in 10 mM sodium acetate buffer, pH 4.5) onto the amide-linked NHS−dextran layer, and the arrays were incubated with four monoclonal antibodies against CRP, GST, rhoA, and rac1. Then, the arrays were analyzed in the line-scanning mode of the spectral SPR biosensor as described in the Experimental Section (A). The results are expressed as means ± SD from three separate experiments (B).

previously used EL CM-dextran surface in the binding of proteins. Specific interactions between antigens and antibodies were observed on the AL NHS−dextran surface of arrays in the line-scanning mode of the spectral SPR biosensor. The array-based spectral SPR biosensor was successfully applied to the rapid analysis of CRP in 120 human sera. The CPR levels determined with the SPR biosensor showed a good correlation with those determined with the commercialized latex-enhanced turbidimetry immunoassay. Thus, the AL NHS−dextran layer combined with the spectral SPR biosensor has a strong potential for the rapid serodiagnosis of human diseases in a high-throughput format.

The CM-dextran surface has been widely used in the analysis of biomolecular interactions since the first report[19] on CM-dextran in the immobilization of biomolecules including proteins. The CM-dextran surface was used to immobilize various biomolecules such as proteins,[19,29] oligonucleotides,[21] and viruses.[14] The EL CM-dextran surface has advantages such as less denaturation of proteins and minimized nonspecific protein interactions.[19] However, the EL CM-dextran layer required a significant amount of time (~2 days) to fabricate on the prepared mixed thiol surface of gold arrays. And, sample solutions were easily mixed by diffusion among spots of gold arrays during incubation since the glass surface of the gold arrays became less hydrophobic by the treatment with epoxides (data not shown). It was assumed that the partial loss of hydrophobicity of the glass surface was caused by mechanisms including the formation of diols by the ring-opening reaction of epoxides with silanol groups on the glass surface. Thus, in this paper, we presented a new approach to modify the surface of gold arrays with the NHS−dextran layer, which was formed by the amide linkage of NHS-modified CM-dextran and *N*-(2-aminoethyl)-11-mercaptoundecamide. In contrast to EL CM-dextran, the AL NHS−dextran layer was fabricated on the monolayer of mixed thiols within 2 h. In addition, protein samples were not mixed among the spots of the arrays during incubation, demonstrating that the problems caused by EL CM-dextran could be overcome with AL NHS−dextran. Thus, the AL NHS−dextran surface is appropriate for the high-throughput analysis of protein interactions by the array-based spectral SPR biosensor.

Antibody arrays have been reported to analyze the conformational change of Bax protein, protein expression profiling, and low

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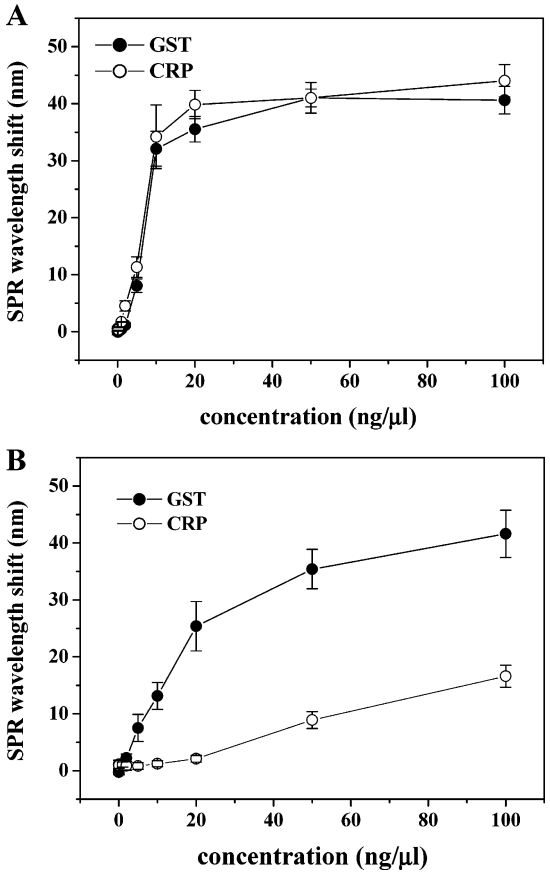

Figure 5. Quantitative analysis of GST and CRP in buffer (A) or normal human serum (B) on amide-linked NHS−dextran arrays with the spectral SPR biosensor. The indicated concentrations of GST or CRP were prepared in 10 mM acetate buffer (pH 4.5) (A) or normal human serum (10 times diluted with the acetate buffer) (B) and immobilized onto the AL NHS−dextran layer of gold arrays. The protein arrays were probed with monoclonal antibodies against GST and CRP and incubated with secondary antibodies. Then, the arrays were analyzed with the spectral SPR biosensor as described in the Experimental Section. The results are expressed as means ± SD from three separate experiments.

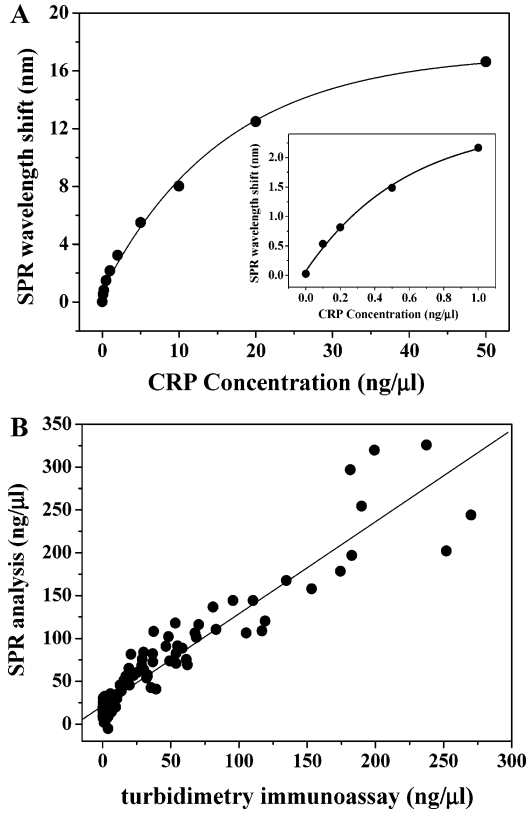

Figure 6. Analysis of CRP in human sera. (A) Standard curve of CRP. The indicated concentrations of CRP were prepared in normal human serum (10 times diluted with 9.3 mM phosphate buffer, pH 7.4) and immobilized onto the dextran layer. The protein arrays were incubated with anti-CRP antibody and anti-goat IgG and analyzed with the spectral SPR biosensor as described in the Experimental Section. (B) Analysis of CRP in human sera. The level of CRP in 120 human sera was determined by the latex-enhanced turbidimetry immunoassay and the spectral SPR biosensor as described in the Experimental Section. Then, correlation between the CRP levels determined by the latex-enhanced turbidimetry immunoassay and those determined by the spectral SPR biosensor was analyzed by the Fit Linear of the Origin program ($n = 120$, $r = 0.945$, $p < 0.0001$).

molecular weight protein biomarkers by SPR imaging.[30−32] In this paper, however, an alternative method, protein arrays, was used to analyze CRP in human sera for two reasons. First, antibody arrays prepared with anti-CRP on the AL NHS−dextran surface were not so sensitive to analyze CRP at low concentrations (data not shown). Second, it might be necessary to check whether the SPR signals obtained by the binding of CRP to antibody arrays were specific. Thus, in this work, the method based on protein arrays rather than antibody arrays was chosen to analyze CRP in sera with secondary antibodies to amplify SPR signals. Someone might ask how the protein arrays worked in the analysis of CRP in sera, since CRP might compete with other blood proteins while being immobilized onto the AL NHS−dextran surface. That was true in part when we analyzed CRP in the normal human serum diluted with the acetate buffer (pH 4.5). The sensitivity was much lower when CRP samples were prepared in normal human serum rather than in the acetate buffer (Figure 5). However, the sensitivity significantly increased up to 0.1 ng/μL when CRP was spiked with the normal human serum diluted with a phosphate buffer (pH 7.4) (Figure 6A). The difference between two buffers in the sensitivity for CRP might be explained by differential binding of blood proteins onto the NHS−dextran surface in different pH environments because of their pI values. For example, the net shift of SPR wavelength caused by application of haptoglobin (100 μg/mL) onto the AL NHS−dextran surface, one of the major blood proteins, was 16.4 nm in the acetate buffer (pH 4.5), but the haptoglobin binding tremendously decreased to 0.2 nm in the phosphate buffer (pH 7.4). Thus, protein arrays can be used to analyze blood proteins under an appropriate pH environment.

It was interesting that the AL NHS−dextran layer showed a higher binding affinity to GST or GST-fusion proteins than the EL CM-dextran. GST-fusion proteins have been widely used to study biological functions of proteins, since the proteins can be easily prepared via bacterial expression systems.[25] Recently, GST-fusion proteins have been used to prepare protein arrays by immobilizing the proteins onto the GSH surface, the arrays being

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used to investigate the functions of small G-proteins.[25] In this report, a higher SPR signal was obtained by GST-fusion proteins such as GST-rac1 than normal proteins including CRP and rac1 on the AL NHS−dextran layer. These results suggested that GST-fusion proteins have a strong affinity to the AL NHS−dextran layer, and thus, the AL NHS−dextran layer has the potential for the preparation of GST-fusion protein arrays. To investigate the mechanism of the GST-fusion proteins binding to the AL NHS−dextran surface, GST-rac1 was immobilized onto the AL NHS−dextran arrays and treated with thrombin to digest GST-rac1 into GST and rac1. Then, the arrays were probed with anti-GST and anti-rac1 antibodies and analyzed with the spectral SPR biosensor. The results showed no difference in the SPR signal caused by the antibodies between control spots and thrombin-treated spots, indicating that thrombin treatment did not release rac1 from GST-rac1 on the AL NHS−dextran surface (data not shown). Thus, it is likely that both GST and rac1 were immobilized onto the AL NHS−dextran layer, even though the detailed mechanism remains to be elucidated.

There have been reports showing the potential use of protein arrays in the serodiagnosis of infectious and allergic diseases based on the fluorescence labeling method.[8−12] Recently, in situ SPR biosensors have been used to analyze antibodies produced in human sera by the infection of pathogens.[14,15] The SPR biosensor has also been used to analyze the antibody produced against human respiratory syncytial virus in 26 infants.[15] There is a recent report on serodiagnostic comparison between ELISA and SPR methods for the detection of antibody titers of *Mycoplasma hyopneumoniae* in pig sera.[33] However, there is no report on the high-throughput analysis of antibodies produced in human sera or serum proteins by SPR biosensors in an array format. In this paper, we presented a new approach to analyzing CRP in human sera on arrays. To our understanding, this is the first report on the high-throughput analysis of serum proteins by SPR biosensors in an array format.

In summary, we coupled a label-free array system based on a spectral SPR biosensor and AL NHS−dextran to the analysis of CRP in human sera in a high-throughput manner. This novel approach is simple, label-free, and rapid and thus has the great potential for the rapid serodiagnosis of human diseases.

CONCLUSIONS

In this paper, we demonstrated a new array-based spectral SPR biosensor based on the AL NHS−dextran surface for the high-throughput analysis of CRP in human sera. The AL NHS−dextran surface was superior to the previously used EL CM-dextran surface in the immobilization of proteins. Specific interactions of antigens and antibodies were analyzed on the AL NHS−dextran surface of gold arrays. Then, the label-free SPR system was successfully applied to the rapid analysis of CRP in 120 human sera, and the results obtained by the SPR sensor showed a good correlation with those determined with the commercialized latex-enhanced turbidimetry immunoassay. Thus, the AL NHS−dextran array combined with the spectral SPR biosensor has a strong potential for the rapid serodiagnosis of human diseases in a high-throughput format.

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