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PAPER

Label-free detection of glycoproteins using reflectometric interference spectroscopy-based sensing system with upright episcopic illumination†

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A reflectometric interference spectroscopy (RIfS)-based sensing was demonstrated on the detection of glycoproteins using concanavalin A (Con A)-immobilized silicon nitride sensing chips. When ovalbumin (OVA), a model glycoprotein, was injected, the sensor response increased due to the spectral change in interference reflectance caused by the increase in optical thickness on the sensor chip after the binding of OVA. In contrast, such response was not observed when bovine serum albumin was injected, suggesting that the molecular recognition events such as specific binding of OVA toward the immobilized Con A can be detected by the present sensing system. An apparent dissociation constant was estimated to be 5.4×10^{-6} M from Scatchard analysis, which was a similar order of magnitude to previously reported values. Consequently, the RIfS system appeared to be a tool for the label-free detection of protein recognition events in real time and could provide quantitative information on the binding.

1. Introduction

Glycosylation is a post-translational modification that plays an important role in central biological functions, including immune regulation, inflammation, cell signaling, and expressing glycosylation-related diseases such as cancer, diabetes and rheumatism. Although glycoproteins are the largest group of macromolecules in nature, very little is known in terms of the functional role of their glycan chains. For these reasons, the development of analytical technologies capable of selectively recognizing glycoproteins may have important implications for basic cell biology research as well as in direct clinical applications. There are two types of glycosylation: one is *N*-linked glycosylation and another is *O*-linked glycosylation. Most plasma proteins contain oligosaccharides attached to the polypeptide core. So far, surface plasmon resonance (SPR) methods have been extensively applied to the investigation of oligosaccharides. For example, Mandenius *et al.* reported the interaction between a variety of lectin-based affinity ligands and hemagglutinin from human influenza virus, with the aim of identifying affinity ligands useful for the development of a rapid bioanalytical sensor.[1] Liu *et al.* developed a glycoprotein detection system by using SPR imaging. Chicken ovalbumin (OVA) and immunoglobulin G (IgG), as model glycoproteins, were chemically immobilized by condensation reaction on a gold sensing film having a self-assembled monolayer of 11-mercaptoundecanoic acid, where glycoprotein solutions with different concentrations were orderly dotted using a home-made microspotting device.[2] The glycoprotein-dotted gold films were subjected to concanavalin A (Con A) binding on each dot, and *in situ* SPR images were taken by a CCD video camera. Glycoproteins dotted at either 1.0 mg mL^{-1} or 0.01 mg mL^{-1} were differentiated from non-glycoproteins by reaction with Con A. Although the SPR sensing has gained reputation as a standard method in this field, gold thin layer-coated substrates are commonly utilized to generate evanescent wave on the surface, which is treated with thiol or amine derivatives followed by coupling molecular recognition elements such as antibodies. This routine requirement may sometimes cause the limitation of diverse ranges of surface functionalization and selection of molecular recognition elements.

Reflectometric interference spectroscopy (RIfS) is a potential technique as an inexpensive label-free detection method of the biosensing system,[3–6] since any substrates consisting of a multiple thin layer bearing a different reflective index can be used as a sensing chip, on which molecular recognition elements are immobilized by various chemical modification methods. When incident light is irradiated to the sensing chip, a part of incident light is reflected at each boundary, resulting in observing a reflectometric interference phenomenon. Characteristic reflectometric interference patterns are formed according to the composition of multiple-layer substrates used, and the extent of such reflectometric interference can be dependent on the optical thickness of the outermost surface of the multiple-layer, where the optical thickness is given by multiplying the physical thickness and the reflective index of the outermost layer. When

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a molecular recognition element is immobilized on the multiple-layer chip surface, and then a target molecule is loaded, the increase in physical thickness and the change in reflective index may occur in the surface layer, resulting in the modulation of reflectometric interference spectra. Consequently, the binding events can be detected and quantified with a label-free manner by continuous monitoring of the reflectometric interference spectral change.[3,7–9] Fujimura *et al.* reported a RIfS-based sensing chip consisting of titanium oxide film (*i.e.* interference layer) on silicon sbustrates.[10] They suggest that the sensitivity of antigen–antibody interaction is in the order of 10 pg mm^{-2}, estimated by both experimental and simulated results. Recently, interaction between ZnO and green fluorescent protein fused with a ZnO-binding peptide was investigated by using a RIfS-based flow system.[11]

In this study, we developed a RIfS-based label-free glycoprotein detection system using a concanavalin A (Con A)-immobilized sensor chip, in which the incident light is perpendicularly irradiated from above the sensor chip, unlike the previously reported systems having an irradiation light point located below the sensor chip. A 66.5 nm silicon nitride coated silicon substrate was employed as a base sensing chip, which enabled the detection of an interference spectrum at visible light region.[10] A transparent micro-flow-cell prepared by polydimethylsiloxane (PDMS) was fixed on the sensor chip to construct the microfluidic system with 4 μL cell volume. To prepare the Con A-immobilized chip, 3-aminopropyltrimethoxysilane was coupled with the surface, followed by the treatment of *N*-hydroxysuccinimide ester of biotin having a tetra(ethylene glycol) spacer. After the addition of avidin, a biotinylated Con A was deposited on the surface. Ovalbumin (OVA), as a model glycoprotein, which has *N*-linked glycan consisting of both high mannose-type and complex-type-structures,[12] was used to demonstrate the recognition of glycosyl moieties of OVA by the RIfS system equipped with the Con A-immobilized sensor chip. Also, immunoglobulin G (IgG) that could serve as a marker for rheumatism[13] was used as another model glycoprotein that is known to bind Con A.[14]

2. Experimental

2.1. Materials and apparatuses

Avidin, ethanol, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), and sodium chloride were purchased from Nacalai Tesque Co. Ltd (Kyoto, Japan). EZ-link®NHS–PEG$_4$–Biotin (*N*-hydroxysuccinimide ester of biotin having tetra-(ethylene glycol) spacer) was purchased from Thermo Scientific Japan (Yokohama, Japan). Biotinylated Con A was purchased from Vector Laboratories Inc. (CA, USA). Ovalbumin (OVA, albumin from chicken egg white, M_w 45 kDa) was purchased from Sigma (St Louis, MO). Rabbit IgG (M_w 146 kDa) was purchased from HyTest Ltd. (Turku, Finland). 3-Aminopropyltriethoxysilane (APTS) was purchased from Shin-Etsu Chemical Co. Ltd (Tokyo, Japan). Manganese(II) chloride tetrahydrate, sodium dihydrogenphosphate dehydrate, disodium hydrogenphosphate 12-water, and bovine serum albumin (BSA, Fraction-V) were purchased from Wako Pure Chemical Co. (Osaka, Japan). Silicon nitride chips (*L* 26 × *W* 18 × *H* 0.725 mm) and the same size of PDMS-based microfluidic cells (flow cell size: *L* 16 × *W* 2.5 × *H* 0.1 mm, total volume: 4 μL) were purchased from Konica Minolta Opto, Inc. (Tokyo, Japan). A halogen lamp was used as a light source, which was irradiated from above, and the reflected light was also detected from above by using an optical fiber bundle. A RIfS-based molecular interaction analyzer (Konica Minolta Opto, Inc., Tokyo, Japan) connected with a pump (PU-980, Jasco, Tokyo, Japan), a degasser (DG-980-50, Jasco, Tokyo, Japan) and an auto-sampler (AS-950-10, Jasco, Tokyo, Japan) was used in experiments except for regeneration and selectivity experiments for monitoring the peak position (550–600 nm) of reflectometric interference spectra measured between 460 and 710 nm ($\Delta\lambda_{peak}$). Regarding regeneration and selectivity experiments, a RIfS-based molecular interaction analyzer, named MI-Affinity LCR-01 (Konica Minolta Opto, Inc., Tokyo, Japan), was used with a syringe pump (Econoflo Syringe pump, Harvard, UK).

2.2. Immobilization of biotinylated Con A *via* avidin–biotin interaction on a silicon nitride chip (Fig. 1)

APTS (100 μL) was added to 10 mL of 95% ethanol, and the solution was stirred for 5 min at room temperature. A silicon nitride chip was cleaned by an UV–O_3 cleaner (Bioforce nanosciences) for 10 min. The silicon nitride chip was soaked in the APTS solution and incubated for 1 h at room temperature. After that the silicon nitride chip was washed with ethanol and distilled water, then dried by N_2 blowing. Finally, the chip was placed on a hot plate for 10 min at 80 °C to obtain an amine-introduced silicon nitride chip.

The amine-introduced silicon nitride chip and the PDMS-based micro-flow-cell were equipped to the RIfS apparatus, and 100 mM sodium borate buffer (pH 8.5) was used as a running buffer at a flow rate of 20 μL min^{-1}. NHS–PEG$_4$–Biotin (0.1 mg mL^{-1}) dissolved in the same buffer (100 μL) was then injected. After 500 s from the injection, the running buffer was changed to 10 mM phosphate buffer (PB, pH 7.4), and 300 s later, 100 μL of BSA (1 mg mL^{-1}) was injected for blocking the free amino groups. Avidin dissolved in 10 mM PB (1 mg mL^{-1}) was then injected (100 μL) after 1400 s from the start, and 100 μL of the biotinylated Con A (0.1 mg mL^{-1}) was injected after 500 s from the injection of avidin. Due to the dead volume of connection tube between the auto-sampler and the flow cell of the RIfS, there was a lag time of 120 s.

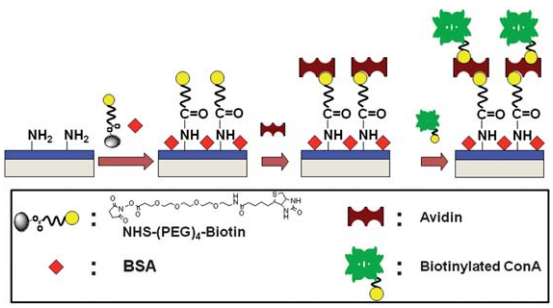

Fig. 1 Schematic illustration of biotinylated Con A immobilization *via* avidin–biotin interaction on a silicon nitride chip.

2.3. RIfS measurements of glycoprotein recognition

Various concentrations (0.05, 0.1, 0.2, 0.4, and 0.5 mg mL^{-1}) of OVA dissolved in 10 mM HEPES (pH 7.4) buffer containing 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$ were prepared. The concentration-dependent change of $\Delta\lambda_{peak}$ against OVA injection was monitored by the RIfS system using the HEPES buffer at a flow rate of 20 μL min^{-1}. Here, each OVA solution (100 μL) was injected into the cell with 200 s interval. As a control, BSA solution (0.1 mg mL^{-1}) was injected. For investigating selectivity of Con A-immobilized surface, OVA solution (0.05 mg mL^{-1}) was injected on an avidin-immobilized silicon nitride chip (without the biotinylated Con A treatment). In a similar manner, the concentration-dependent change of $\Delta\lambda_{peak}$ against rabbit IgG solution (conc. 0.005, 0.01, 0.05, 0.1, 0.2, and 0.4 mg mL^{-1}) was monitored.

2.4. Regeneration experiments

A Con A-immobilized silicon nitride chip was prepared by the same method as described in Section 2.2. After injecting a regeneration reagent (10 mM glycine–HCl, pH 1.5) to wash the sensor chip surface, the concentration-dependent change of $\Delta\lambda_{peak}$ against OVA (0.05, 0.1, and 0.2 mg mL^{-1}) was monitored by the RIfS system at a flow rate of 20 μL min^{-1}. This procedure was repeated 3 times.

3. Results and discussion

It is well known that biotin–avidin interaction can be utilized to immobilize various biotinylated proteins, antibody, or biomarkers on biosensor chips. In the present study, Con A was immobilized on the surface of sensor chips *via* biotin–avidin interaction. Since silanol groups may exist on the sensor chip surface, which are originated from the silicon base substrate, silane coupling reagents such as APTS can be applied in order to introduce amino groups. After the APTS treatment, biotinylation was performed by using NHS–PEG_4–Biotin.

When NHS–PEG_4–Biotin solution was injected (Fig. 2a), the $\Delta\lambda_{peak}$ value increased up to around 0.15. After changing the buffer from 100 mM sodium borate buffer to 10 mM PB (Fig. 2b), $\Delta\lambda_{peak}$ value did not decrease, suggesting that PEG_4–Biotin was successfully immobilized onto the amine-introduced silicon nitride chip. For blocking the remaining unreacted free amino groups, the BSA solution was injected (Fig. 2c), which increased the $\Delta\lambda_{peak}$ value to around 0.8. When avidin was injected (Fig. 2d), further increase was observed to 1.8, confirming that the avidin surface could be formed on the sensor chip. Finally, the biotinylated Con A was introduced (Fig. 2e), and the $\Delta\lambda_{peak}$ values reached to 2.5. As can be seen, the immobilization was confirmed by observing the elevated baseline of the sensing system, the Con A immobilization process could be monitored by changing the $\Delta\lambda_{peak}$ value due to the successive increase in optical thickness in conjunction with each treatment.

In order to confirm the binding between Con A and a glycan part of OVA, OVA was injected into the RIfS systems as compared with BSA, which has no oligosaccharide. When the OVA solution (0.1 mg mL^{-1}) was injected, the $\Delta\lambda_{peak}$ value increased to 0.14, as shown in Fig. 3. In contrast, only the increase of 0.03 was observed for the injection of BSA, suggesting that the RIfS system may not respond to serum proteins bearing no sugar moiety even in real biological fluids. Also to investigate selective binding of the Con A surface toward OVA, OVA solution (0.05 mg mL^{-1}) was injected into the avidin-immobilized sensor chip, which had no Con A on the chip. When the Con A-immobilized chip surface was used, the $\Delta\lambda_{peak}$ value increased to 0.6, while the $\Delta\lambda_{peak}$ value increased to only 0.2 after injecting onto the avidin-immobilized chip interface (see ESI, Fig. S1†). These results indicate the surface-immobilized Con A was bound to the glycan part of OVA selectively.

Fujimura *et al.* proposed the calculation theory of the amount of protein adsorption by computational simulation for RIfS.[10] According to them, 0.1 nm change in the $\Delta\lambda_{peak}$ value is attributed to the analyte binding of 0.1 ng mm^{-2}. Since the area of the binding surface in the flow cell was 40 mm^2, the bound OVA and BSA were calculated to be 0.123 and 0.032 pg, respectively.

In order to quantitatively evaluate the OVA binding activity of the Con A-immobilized silicon nitride surface, OVA was injected at a range from 50 to 500 μg mL^{-1} (Fig. 4A). The cumulative increase in $\Delta\lambda_{peak}$ values was observed after the injection of each OVA solution. From the binding isotherm observed by the

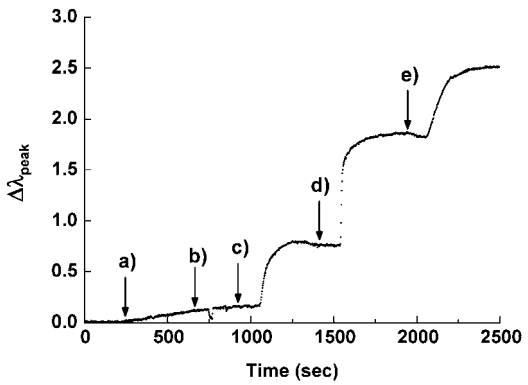

Fig. 2 *In situ* monitoring of the surface modification by the change in $\Delta\lambda_{peak}$ values after injections of (a) NHS–PEG_4–Biotin, (b) changing buffer from 100 mM sodium borate buffer (pH 8.5) to 10 mM PB (pH 7.4), (c) BSA blocking, (d) avidin binding, and (e) immobilization of biotinylated Con A.

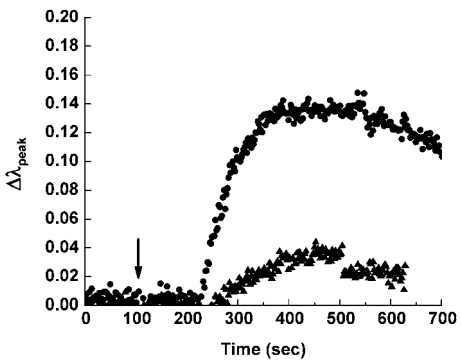

Fig. 3 Real time monitoring of $\Delta\lambda_{peak}$ values after the injection (100 μL) of OVA (0.1 mg mL^{-1}) (circles) and BSA (0.1 mg mL^{-1}) (triangles). Running buffer: 10 mM HEPES (pH 7.4) buffer containing 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$. Flow rate: 20 μL min^{-1}.

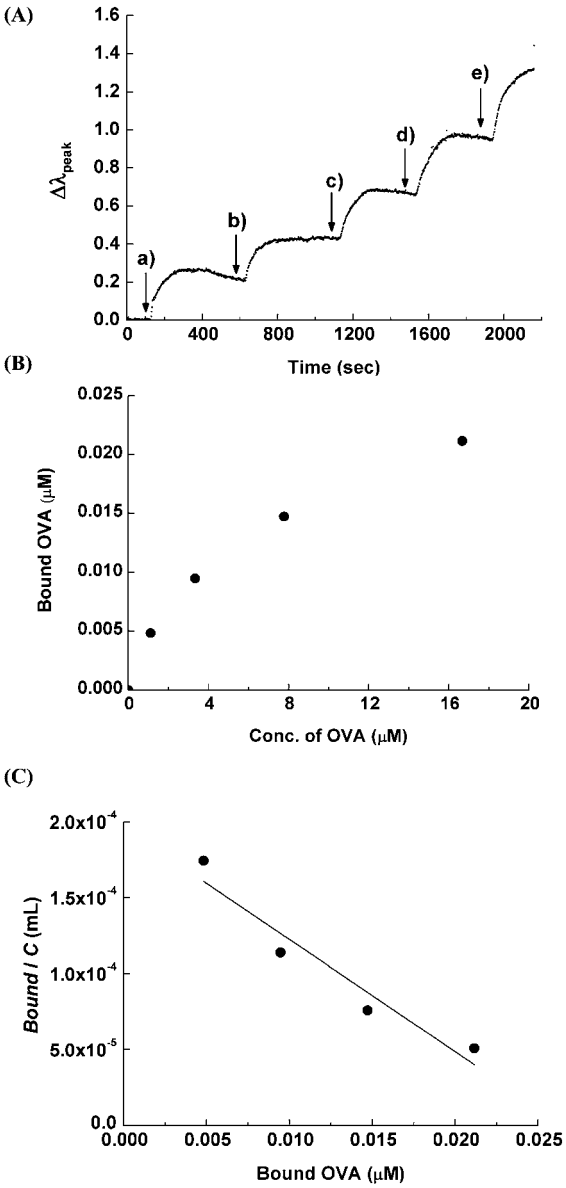

Fig. 4 (A) Cumulative change in $\Delta\lambda_{peak}$ values after the consecutive OVA injections: (a) 0.05, (b) 0.1, (c) 0.2, (d) 0.4 and (e) 0.5 mg mL^{-1}. (B) Binding isotherm of OVA of the Con A-immobilized silicon nitride chip based on the change in optical thickness calculated from the $\Delta\lambda_{peak}$ values.[10] (C) Scatchard plot from the binding data of OVA.

$\Delta\lambda_{peak}$ of each cumulative concentration (Fig. 4B), a dissociation constant (K_d) was calculated from a Scatchard plot based on the following equation:

$$\text{Bound}/C = -(\text{Bound}/K_d) + (B_{max}/K_d)$$

where Bound is the bound amount of OVA, and C is the free concentration of OVA in the buffer. From the slope of the plot (Fig. 4C), K_d was estimated to be 5.4×10^{-6} M. The obtained value was a similar order of magnitude as the value calculated by isothermal titration calorimetry.[15]

In a similar manner, rabbit immunoglobulin G (IgG) was injected on Con A-immobilized silicon nitride surface from 0.005 to 0.4 mg mL^{-1} (Fig. 5A). Each injection of IgG induced the increase in the $\Delta\lambda_{peak}$ values stepwise. From the sensorgram, the binding isotherm and the corresponding Scatchard plot could be drawn as shown in Fig. 5B and C. Unlike the case of OVA, the Scatchard plot obtained was not linear, where at least two kinds of K_d values can be observed, *e.g.* 1.1×10^{-5} M and 2.0×10^{-6} M. This is reasonably acceptable, because IgG bears multiple-type oligosaccharides,[16] the apparent binding activity may differ according to the affinity of glycan part of IgG toward the binding site of Con A. Consequently, the sensor system can

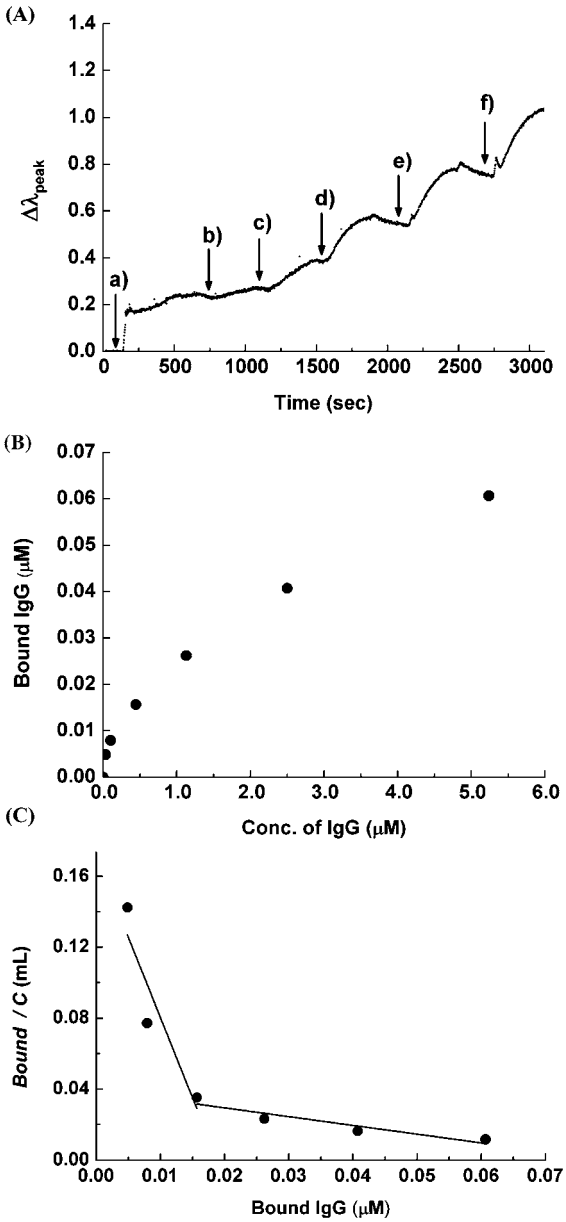

Fig. 5 (A) Cumulative change in $\Delta\lambda_{peak}$ values after the consecutive OVA injections: (a) 0.005, (b) 0.01, (c) 0.05, (d) 0.1, (e) 0.2 and (f) 0.4 mg mL^{-1}. (B) Binding isotherm of rabbit IgG on the Con A-immobilized silicon nitride chip based on the change in optical thickness calculated from the $\Delta\lambda_{peak}$ values.[10] (C) Scatchard plot from the binding data of rabbit IgG.

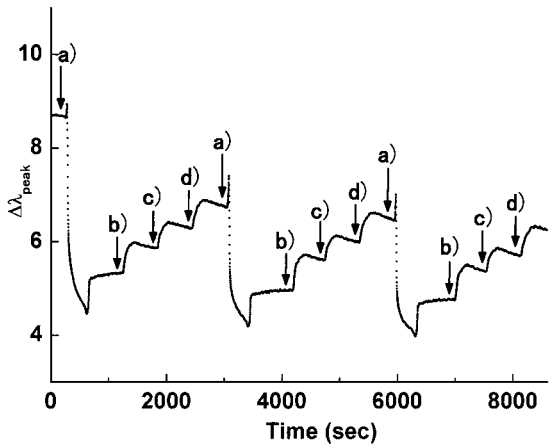

Fig. 6 Monitoring of the regeneration process by the concentration-dependent change in $\Delta\lambda_{peak}$ against the injections of (a) 10 mM glycine–HCl (pH 1.5), (b) 0.05 mg mL^{-1}, (c) 0.1 mg mL^{-1}, and (d) 0.2 mg mL^{-1} of OVA solutions.

provide the structure information on biomacromolecules having glycan structures.

Reproducibility of the response to the binding events on the Con A-immobilized silicon nitride surface was examined by repetitively injecting a set of various concentrations of OVA, where 10 mM glycine–HCl (pH 1.5) was used as a regeneration reagent (100 μL). As shown in Fig. 6, the injection of glycine–HCl allowed the sensor chip to be regenerated after about 1000 s from the injection, and the cumulative binding of OVA was confirmed to be reproducible. The $\Delta\lambda_{peak}$ decrease by the first injection of glycine–HCl may be due to the removal of weakly or improperly immobilized Con A on the surface.

The main advantages of RIfS against SPR are the non-dependency of the surface plasmon and evanescent field generation. In the case of SPR, the decay of the evanescent field (2–300 nm) perpendicular to the surface causes a decrease in sensitivity. In addition, the sensitivity decreases with changes in bulk refractive index and temperature. On the contrary, RIfS is less temperature dependent due to the measurement of the optical thickness, involving mainly the change in physical thickness caused by the analyte binding. In addition, unlike SPR and QCM measurements, the RIfS response reflects the change in optical thickness that is in proportion to the refractive index and the physical thickness on the sensor chip. Thus, RIfS may possess a unique detection property toward not only the binding amount but also the shape and/or size of the analyte. The incident white light in the RIfS system can partially be reflected on the sensor chip surface, at which the physical thickness can be measurable up to ~1.4 μm, depending upon the coherence length at each wavelength.[17] Therefore, the RIfS system can be applied not only for the detection of molecular-level interaction but also for μm order binding such as cell adsorption.

4. Conclusions

The RIfS system was applied for detection of glycoproteins OVA and IgG as model glycoproteins. The silicon nitride coated silicon-based sensor chip was biotinylated to immobilize biotinylated Con A *via* avidin. Those surface modifications could be monitored by increased $\Delta\lambda_{peak}$ values in real time. From the results of cumulative increase in $\Delta\lambda_{peak}$ values after the injection of each OVA and IgG, the dissociation constants could be estimated by Scatchard analysis. Furthermore, the proposed Con A-immobilized sensor chip-based RIfS system could provide not only quantitative information on the binding events of glycoproteins, but also on structural differences in glycan substructures of glycoproteins.

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