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Development of Surface Plasmon Resonance Sensor Chip for Multiplex Sample Detection

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Abstract

A method for preparation of a surface plasmon resonance (SPR) sensor chip with 8-stripe pattern for multiplex immunoassay, where several kinds of antibodies can be immobilized, is proposed in this paper. For immobilization of two types of antibody, anti-IgA and anti-IgG antibodies on the sensor chip at 8-stripe pattern alternatively, a flow generated by the centrifugal force was used for introducing the antibody solutions separately into a microchip with 8-channel on the 8-stripe patterned sensor chip. The resulting sensor chip, on which the anti-IgA antibody and anti-IgG antibody were immobilized alternatively, was attached to a flow cell prepared from an acrylic with a single flow channel. Mixed solutions of IgA and IgG at different concentrations were injected into a carrier stream of a pH buffer solution at 7.4, and SPR sensor signals for IgA and IgG were detected separately from the SPR sensor chip with stripes immobilized with corresponding antibodies. As a result, calibration curves for IgA and IgG, which fit to those calculated from the Langmuir adsorption isotherm, were obtained. The detection limit of the SPR sensor was *ca*. 5 ppm.

Keywords multi-channel SPR sensor, centrifugal force, immunoassay

1. Introduction

A sensor based on surface plasmon resonance phenomena, so-called an SRP sensor is one of the highly promising sensors for real-time, no-invasive and sensitive measurements, due to its high sensitivity to a change in the refractive index of a medium at the vicinity surface of a sensor chip [1-3]. The SPR sensor has been widely applied to many kinds of biosensors for tumor maker [4-6], *Escherichia coli* in food [7], some infection virus such as influenza, HIV, and Ebola hemorrhagic fever [8-10] by appropriately modifying the sensor chip with selective receptors for analytes. SPR sensors for the detection of a single analyte have been developed at an initial stage, however, recently, SPR sensors with a multi-detection function have been developed since the needs for multiplex analysis have been increased for environmental and clinical analysis [11-15].

Sensor chips suitable for multi-detection and simultaneous measurements have been reported along with the development of multi-channel SPR sensors. Most of the general sensor chips reported so far were prepared by combing a sample holder, a gasket sheet with microchannels and an Au thin film coated with substrate [16-18]. In these cases, the number of inlets should be the same as that of the sensing channels. Therefore, the same number of syringe pumps and injectors are necessary, which makes the sensing system large and complicated. Another kind of the sensor chip with multi-sensing points was prepared by using a micro spotting printer [19-21]. In this case, more than 20 receptors for analytes were immobilized on the sensor chip with a single channel by using a micro spotting printer, and a mixed sample solution was flowed into the channel. As a result, 20 analytes could be detected simultaneously by the multi-channel SPR sensor. However, since the cost of the micro spotting printer was extremely high, the preparation is not suitable for general use.

In previous paper, we have prepared 30-stripe patterned sensor chip by vapor deposition of Cr onto the bare sensor chip for application to the present SPR sensor with a function of multi-sensing points. In this study, we newly prepared the 8-stripe patterned sensor chip by the Ar ion shower etching method for determination of IgA and IgG instead of the sensor chip prepared by vapor deposition of Cr, taking into account the toxicity of Cr on the sensor chip for immunoassay.

In the previous study, a PDMS microchip with microfluidic network that are divided into two groups (perpendicularly cross each other) was proposed. This design achieved the designated reaction zones and multiplex immunoassay applications [22]. In this study, we proposed a new method for the preparation of an SPR sensor chip with 8 sensing stripes for multiplex sample detection based on the general preparation method. In the proposed method, a flow generated by a centrifugal force instead of pumping system with multiple syringes was utilized for simplicity in immobilizing anti-IgA and anti-IgG antibodies at 8 sensing positons (as shown in Fig. 1).

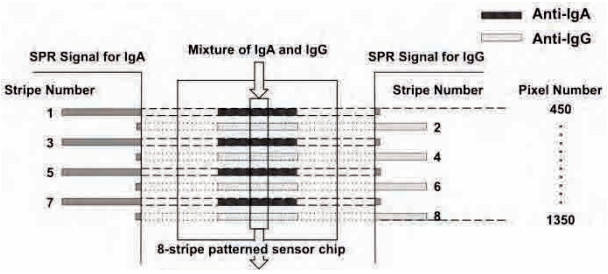

Fig. 1 The image of the anti-IgA and anti-IgG antibodies immobilized sensor chip and the SPR response upon IgA and IgG mixture to the sensor chip.

2. Experimental

2.1 Reagents and solutions

Polydimethylsiloxane (PDMS) prepolymer, Sylgard 184, and its curing agent were obtained from Dow Corning Co. (MI,

USA). The photoresist (SU-8) and its developer were obtained from Nippon Kayaku Co. (Japan). The silicon wafer was obtained from Sumco Co. (Japan). A PBS buffer (pH: 7.4) was obtained from Life Technologies Co. (USA). An Au thin film (Cr: 5 nm/Au: 45 nm) coated cover glass (16 mm x 16 mm x 0.15 mm, simplified as a bare sensor chip) was obtained from Eliotech Co. (Japan). A stainless-steel mask was obtained from Fuji Seimitsu Industries, Japan. A human immunoglobulin G (IgG) and a 1000 ppm solution of its antibody (anti-IgG antibody) from goat, and 1000 ppm solutions of a human immunoglobulin A (IgA) and its antibody (anti-IgA antibody) from goat were obtained from Bethyl Laboratories, Inc. (USA). Bovine serum albumin (BSA) and sucrose were obtained from Wako Pure Chemical Industries, Ltd. (Japan) and Kishida Chemical, (Japan), respectively. Deionized water was purified on a Milli-Q Direct system (Nihon Millipore, Japan). A 100 mM sucrose stock solution was prepared by dissolving 1.7122 g of sucrose into 50 mL pure water, which was provided by the Milli-Q Direct system. The prepared stock solution was then diluted to 15 mM with the Milli-Q water. A 1000 ppm IgG stock solution was prepared by dissolving 3 mg of IgG into 3 mL of the PBS buffer. Mixed solutions of IgA and IgG at the different concentrations from 5 ppm to 100 ppm were prepared from their 1000 ppm stock solutions by using the same buffer. A 50 ppm IgG solution and an IgA solution at the same concentration were prepared from their stock solutions separately. For immobilization of the anti-IgA and anti-IgG antibodies on the SPR sensor chip, 1000 ppm of their original solutions were used as received.

2.2 Fabrication of the SPR sensor chip with 8 sensing stripes used for immunoassay

2.2.1 Fabrication of the 8-stirpe patterned sensor chip

An 8-stripe patterned sensor chip (the width of the stripe and the gap of the neighboring stripes were 500 μm and the length of the stripe was 8 mm) was prepared from a bare sensor chip by using a stainless-steel mask and an Ar ion shower etching apparatus (EI-200ERT, ELIONIX, Japan). The stainless-steel mask shown in Fig. 2 (a) was placed on the bare sensor chip and was then set in the etching chamber. Etching was conducted under the conditions that the applied current of the etching apparatus and the flow rate of an Ar gas in the chamber were 600 mA and 1.4 SCCM, respectively. The resulting 8-stripe patterned sensor chip shown in Fig. 2 (b) was dipped into a piranha solution (H_2SO_4: H_2O_2 = 1:3) at 90 ^oC for 30 min, and then rinsed with the Milli-Q water for 2 min and ethanol for 1 min. The treated sensor chip was kept in ethanol and dried by a N_2 gas just before use.

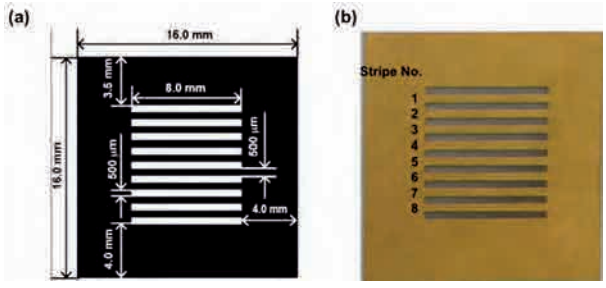

Fig. 2 (a) The design of the stainless-steel mask for preparation of the 8-stripe patterned sensor chip. The gold film of the bare sensor chip at 9 white stripes was removed by Ar ion shower etching. (b) The photo of the 8-stripe patterned sensor chip obtained by Ar ion shower etching. Gray colored stripes are the glass substrate, where the gold film was removed from the bare sensor chip.

2.2.2 Preparation of the PDMS microchip composite with the 8-stripe patterned sensor chip for the immobilization of anti-IgA and anti-IgG antibodies

2.2.2.1 Preparation of the template of the PDMS microchip for composing with the 8-stripe patterned sensor chip

A template of the PDMS microchip for composing with the 8-stripe patterned sensor chip was prepared by a conventional photolithographic method [23]. Fig. 3 (a) is the design of a photo-mask for preparation of the PDMS microchip. The microchip consists of 8 micro-channels and reservoirs for solutions of the anti-IgA and the anti-IgG antibodies, marked by A or G, respectively, and vents for air used for filling the solutions in the reservoirs into the micro-channels at atmospheric pressure. The diameters of the reservoirs and vents were 2 mm and 1 mm, respectively. The width of the micro-channel was 600 μm, the space between the neighboring two micro-channels was 400 μm and the depth of the channel was *ca*. 120 μm. For preparation of the template of the PDMS microchip, firstly, an aliquot of 6 mL of SU-8 was placed on a silicon substrate (120 mmϕ), which was set on a spin coater (K-359S1, Mikasa, Tokyo, Japan), and then the silicon substrate was spun at 500 rpm for 20 sec and at 1200 rpm for 40 sec. The resulting silicon substrate, on which surface was coated with SU-8, was heated on a hot plate (ND-1, AS ONE, Osaka, Japan) at 65 ^oC for 5 min, and then at 95 ^oC for 50 min. After cooled it to the room temperature for 5 min, the photo-mask prepared on an OHP sheet as shown in Fig. 3 (a) was placed on the resulting silicon substrate and UV light at wavelength of 365 nm was irradiated for 90 s in an exposure device (BOX-W9B, Sunhayato, Tokyo, Japan). The photo-mask was detached and the silicon substrate was then

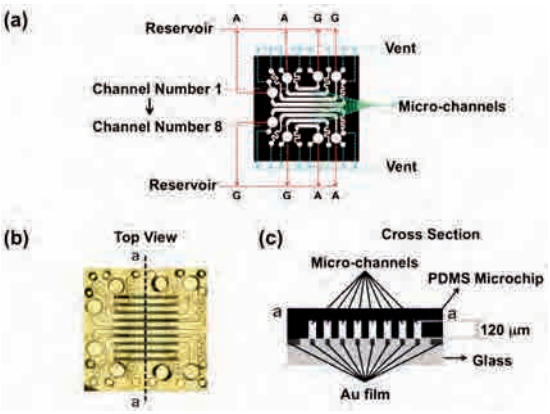

Fig. 3 (a) Design of the photo-mask for preparation of the PDMS microchip with the 8 micro-channels for immobilization of the anti-IgA antibody and the anti-IgG antibody on the sensor chip. Marks of A and G in the figure indicate the reservoirs for the solutions of anti-IgA and anti-IgG antibodies, respectively. Diameter of the reservoirs was 2.0 mm and width of the channels was 600 μm. (b) A photo of the PDMS microchip composite with the 8-stripe patterned sensor chip. (c) The cross section of the composite sensor chip.

heated again on the hot plate at 65 ^{o}C for 1 min for soft baking and then heated at 95 ^{o}C for 5 min for hard baking. After cooled to the room temperature, the resulting silicon substrate was immersed in an SU-8 developer for 10 min. Finally, the template was obtained after heating the developed silicon substrate on the hot plate at 120 ^{o}C for 10 min.

2.2.2.2 Preparation of the PDMS microchip and its composite with the 8-stripe patterned sensor chip

The PDMS prepolymer and its curing agent were mixed thoroughly at a weight ratio of 10:1 and the resulting mixture was then degassed in vacuum for 60 min. The mixture was poured on the template prepared on the silicon substrate and then cured by heating in an oven at 60 ℃ for 4 h to prepare the PDMS microchip with 8 micro-channels. After cooled down to the room temperature, the PDMS microchip was peeled off from the template and the reservoirs (2 mmϕ) and the venting holes (1 mmϕ) were prepared on the PDMS microchip by using punches. The resulting PDMS microchip with 8 micro-channels was placed on the 8-stripe patterned sensor chip under a magnifier scope, where the position of each channel was adjusted to be just on each stripe of the sensor chip. A photo of the PDMS microchip composite with the 8-stripe patterned sensor chip (simplified as a composite sensor chip) is shown in Fig. 3 (b). Nine stripes with gray color in the photo are the naked glass substrate of the bare sensor chip, where Au film was etched by the Ar ion shower. Fig. 3 (c) is the cross section of the composite sensor chip along with the dash line shown in Fig. 3 (b). The depth of the channel was *ca*. 120 μm.

2.2.3 Immobilization of anti-IgA and anti-IgG antibodies using the composite sensor chip and the compact disk (CD)-type PDMS/polycarbonate plate

2.2.3.1 Preparation of the CD-type PDMS/polycarbonate plate for fixing the composite sensor chip

In order to immobilize the anti-IgA and the anti-IgG antibodies on the surface of the 8-stripe patterned sensor chip, the solution of each antibody was filled in the 8 micro-channels by using a centrifugal force. For this purpose, a CD-type PDMS plate supported by a polycarbonate (PC) plate was prepared to fix the composite sensor chip. The preparation method of the CD-type PDMS plate was the same as that of the

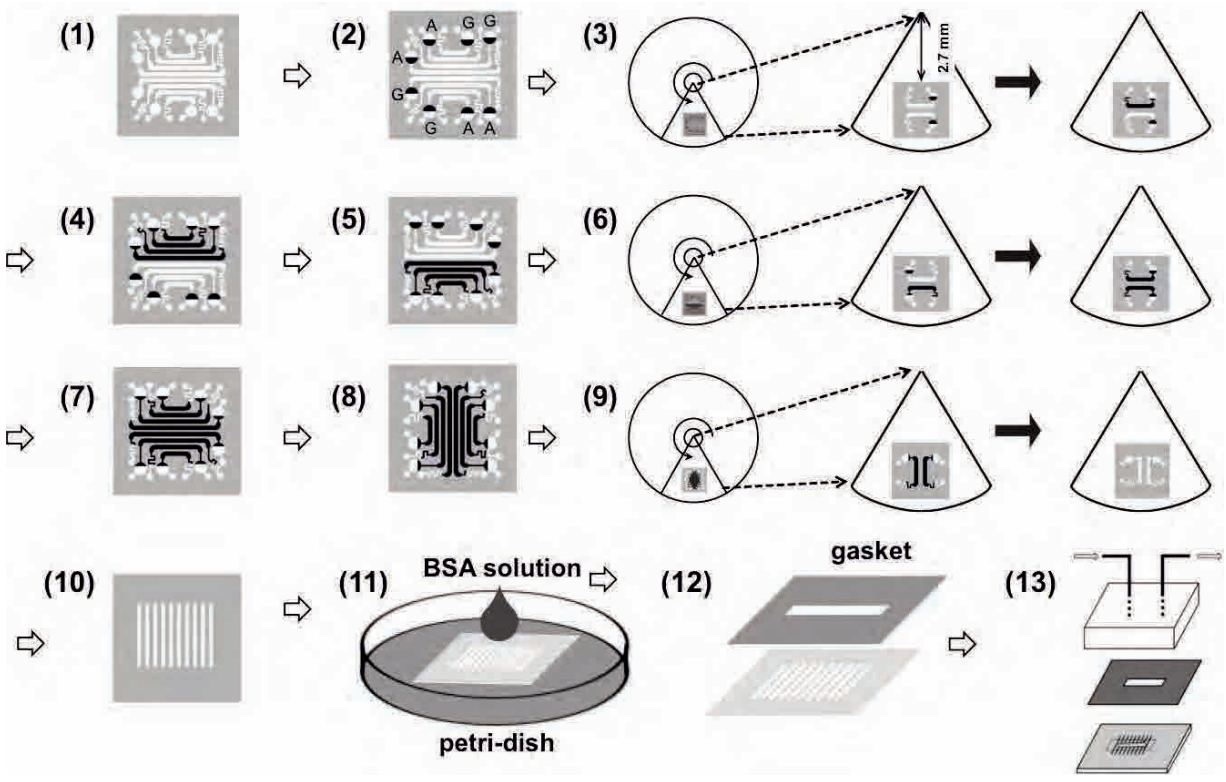

Fig. 4 The process of immobilization of the anti-IgA antibody and the anti-IgG antibody on the surface of the 8-sripte patterned sensor chip. (1) The antibody solutions were injected into the reservoirs of the PDMS microchip composite with 8-stripe patterned sensor chip (sensor chip). (2) A transparent tape was placed on the PDMS microchip to seal the reservoirs and the vents. The vents of the upper 4 reservoirs were opened by a needle. (3) The sensor chip on the CD-type PDMS/PC plate was set on the turn table. (4) The upper 4 channels were filled with the antibody solutions after rotation. (5) The sensor chip was re-set up-side-down, and the vent holes for the channels filled with the antibodies were covered with the transparent adhesive tape. The vents of the 4 upper reservoirs were opened. (6) The sensor chip on the CD-type PDMS/PC plate was set on the turn table. (7) The other 4 channels on the sensor chip were filled with antibody solutions after rotation. (8) The sensor chip was rotated by 90^{o} and the transparent tape was removed. (9) The sensor chip was placed on the CD-type PDMS/PC plate and was set on the turn table and the solutions in the 8 channels were flowed out by the centrifugal force. (10) The PDMS microchip was taken off from the sensor chip. (11) The sensor chip was placed on a petri-dish and 60 μL of a 1000 ppm BSA solution was dropped on the sensor chip. (12) A gasket was put onto the sensor chip after drying the surface of the sensor chip. (13) The sensor chip was assembled with a flow-cell.

PDMS microchip, as described previously. The diameter and the thickness of the CD-type PDMS plate were 120 mm and 2 mm, respectively. A square hole (23 mm x 23 mm) was prepared by cutting out from the PDMS plate, where the upper side of the square was 2.7 cm far from the center of the PDMS plate. The resulting PDMS plate was attached to a circular PC plate (120 mm in diameter and 3 mm in thickness) as a support of the PDMS plate. This composite plate is simplified as the CD-type PDMS/PC plate. The composite sensor chip described in the section 2.2.2.2 was placed in the square hole of the CD-type PDMS/PC plate by using a two-face adhesive tape.

2.2.3.2 Procedures for filling the antibody solutions into the 8 micro-channels of the composite sensor chip

The CD-type PDMS/PC plate, where the composite sensor chip was fixed at the square hole, was set on a turn table. The structure and operation of the turn table were described elsewhere [24]. An aliquot of 5 μL of the 1000 ppm anti-IgA antibody solution and the same volume of the 1000 ppm anti-IgG antibody solution were poured into the eight reservoirs marked as A and G of the composite sensor chip, as shown in Fig. 4 (2). The surface of the composite sensor chip was sealed with a transparent adhesive tape and the vents of the upper 4 reservoirs were opened by a needle. The CD-type PDMS/PC plate was spun at 1000 rpm for 1 min to fill the upper 4 channels of the composite sensor chip with the antibody solutions by the centrifugal force. The image of this process and the composite sensor chip filled with the antibody solutions are shown in Figs. 4 (3) and (4), respectively. The antibody solutions filled in the lower reservoirs were maintained as they were. The opened vents were closed by sealing with the transparent tape. Then, the composite sensor chip in the square hole on the CD-type PDMS/PC plate was replaced up-side-down, and the vents for the upper another 4 reservoirs were newly opened by the needle, as shown in Fig. 4 (5). The CD-type PDMS/PC plate was then spun at 1000 rpm for 1 min again. The other 4 channels were filled with the antibody solutions, as shown in Fig. 4 (7). The composite sensor chip was kept on the turn table for incubation for 15 min to immobilize the antibodies on the surface of the sensor chip. The tapes on the surface of the composite sensor chip were then detached. The composite sensor chip in the square hole of the CD-type PDMS/PC plate was replaced its position by rotating at 90^{o} and then the plate was spun at 1000 rpm for 1 min to discharge the solutions of antibodies from the channels. After these processes, the anti-IgA and anti-IgG antibodies were immobilized on the senor stripes alternatively. The PDMS microchip was detached from the composite sensor chip, and the 8-stripe patterned sensor chip was placed on a petri-dish. Then, 60 μL of the 1000 ppm BSA solution was dropped on the 8-stripe patterned sensor chip and kept in the petri-dish for 15 min after sealing it in order to block the bare sensor surface by BSA (Fig. 4 (11)). The resulting sensor chip was washed with the PBS buffer and then dried by a N_2 gas. Finally, a gasket (16 mm x 16 mm x 0.1 mm^{t}) with a groove (3 mm (W) x 8 mm (L)) was put on the 8-stripe patterned sensor chip (Fig. 4 (12)) perpendicular to the stripe pattern and set up to the flow-cell with the inlet and outlet tubes (Fig. 4 (13)), which was prepared by a polyacrylate resin block. For comparison, a flow-cell with an 8-stripe patterned sensor chip without immobilized antibodies was assembled similarly.

2.3 SPR sensor and flow system

The SPR sensor used in this work is the same one developed by our group for multi-sensing. Details of the SPR sensor are described in the previous paper [25]. Briefly, a linear wedge light was focused on the sensor chip as a line in the length of *ca*. 8 mm and a reflected light was detected with a linear CCD sensor with 2048 pixels. The light intensity of the reflected light was converted by a 12 bit AD converter (4096 digits resolution). The SPR sensor responses as light intensities of the pixels of the CCD sensor were monitored at every 5 seconds and were stored in the memories of the personal computer. The FIA system consisted of a syringe pump (Next-advance, SP300), an injector (Rheodyne 9725, USA) with a 120 μL sample loop and the SPR sensor.

2.4 The measurements of response of the SPR sensor with the 8-stripe patterned sensor chip without antibodies to sucrose solution

The 8-stripe patterned sensor chip without immobilization of antibodies was treated by O_2 plasma for 12 min and was attached to the flow-cell via the gasket. The stripes were oriented perpendicular to the flow direction. The resulting flow-cell was then placed on the prism of the SPR sensor after coating with a matching oil. The flow-cell was connected to the syringe pump and the injector for construction of the FIA system. An aliquot of 120 μL of the 15 mM sucrose solution was injected into a Mill-Q water stream, which was flowed into the flow-cell at the flow rate of 40 μL/min. The SPR sensor responses to the water as a background signals and those to the 15 mM sucrose solution were captured as the function of the pixel number of the linear CCD sensor.

2.5 Immunoassay for IgA and IgG by using the SPR sensor with the 8-stripe patterned sensor chip immobilized with the anti-IgA and anti-IgG antibodies

The performance of the SPR sensor equipped with the 8-stripe patterned sensor chip, on which surface the anti-IgA antibody and the anti-IgG antibody were immobilized alternatively, was evaluated by using the FIA system. The 8-stripe patterned sensor chip immobilized with the anti-IgA and anti-IgG antibodies was attached to the flow cell via the gasket. The flow cell was set on the prism of the SPR sensor in the similar manner described previously. A carrier solution of the PBS buffer at pH 7.4 was flowed into the flow cell at the flow rate of 20 μL/min. In order to confirm that the anti-IgA and anti-IgG antibodies were immobilized on the 8-stripe patterned sensor chip, as expected from the immobilization protocols described in section 2.2.3.2, a 50 ppm IgA solution and a 50 ppm IgG solution were injected into the carrier stream separately, and snap shots of the SPR sensor responses to IgA and IgG were obtained as the light intensity as a function of the pixel number of the linear CCD sensor. For simultaneous immunoassay for IgA and IgG, mixed solutions of IgA and IgG containing at the same concentrations from 5 ppm to 100 ppm were injected into the carrier stream, which was led to the 8-stripe patterned sensor chip, on which surface was immobilized with the anti-IgA and anti-IgG antibodies. After the measurements of the sensor responses to IgA or IgG, a dissociation solution (a buffer solution of glycine-HCl at pH 2.0) was injected into the carrier stream each time, in order to dissociate the immuno-complex of IgA and IgG bound to their

antibodies on the sensor surface. The sensor surface was each time regenerated to the same initial state as the immobilization of the anti-IgA and anti-IgG antibodies by the dissociation solution. The SPR sensor responses were obtained as a function of the pixel numbers every 5 sec. The sensor signals from the 8-stripe patterned sensor chip were obtained by averaging the light intensities of 40 pixels for each sensor stripe using data stored in the memories of the PC.

2.6 Evaluation of cross reactivity of anti-IgG antibody on the sensor chip to IgA and IgG

The cross reactivity of anti-IgG antibody on the sensor chip to IgA against IgG was evaluated by using the bare sensor chip, where the anti-IgG antibody was immobilized by physical adsorption. A 50 μL of the 1000 ppm anti-IgG antibody solution was placed on the bare sensor chip for 30 min. After washing the sensor chip, 50 μL of the 1000 ppm BSA solution was placed on the sensor chip for 15 min. The sensor chip was washed with a 100 μL of the PBS buffer 5 times in order to remove the extra anti-IgG antibody and BSA from the sensor surface. The resulting sensor chip was then attached to the flow-cell via the gasket, and placed on the prism of the SPR sensor. IgA and IgG solutions at various concentrations from 5 ppm to 100 ppm was injected separately into the PBS buffer stream, which was flowed at the flow rate of 20 μL/min under the same condition as described in the section of 2.5.

3. Results and Discussion

3.1 The responses of SPR sensor with 8-stripe patterned sensor chip to sucrose solution

The SPR sensor used in this work has a function of multi-point sensing, where the refractive index change on the sensor chip with 7.5 mm in width can be detected by *ca*. 750 pixels (from pixel number 450 to 1350) of the linear CCD sensor as the reflected light intensity from the sensor chip.

Fig. 5 shows the snap shots of the light intensities obtained by the linear CCD sensor of the SPR sensor as a function of pixel numbers, when water and the 15 mM sucrose solution were introduced into the flow-cell with the 8-stripe patterned sensor chip without antibodies on the sensor surface. As can be seen from Fig. 5, the changes in light intensities between water and the sucrose solution are observed at 8 parts, numbered in the figure. These signals come from the 8-stripe patterned sensor chip due to the SPR phenomena occurred on the gold film. The light intensities at the other parts are more than 4000 digits, which means that the incident light completely reflected by the sensor chip without gold film. This is due to the fact that the SPR phenomena do not occur on the glass substrate without the gold film.

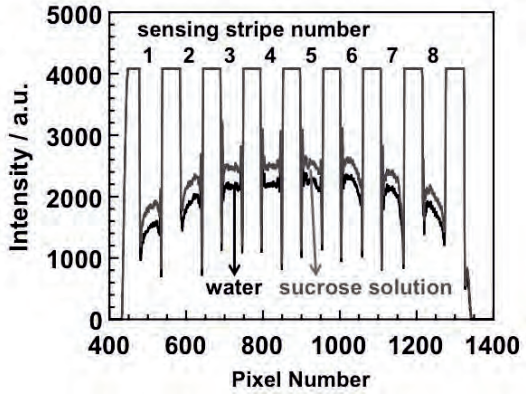

Fig. 5 The responses of SPR sensor with 8-stripe patterned sensor chip to water and a 15 mM sucrose solution observed as light intensities of the pixels of the linear CCD sensor as the sensor signals. The numbers in the figure are the sensing stripe numbers corresponding to those shown in Fig. 2 (b).

The light intensities along with the pixel number are convex for both water and the 15 mM sucrose solution, as can be seen from Fig. 5. This tendency was also observed when the bare sensor chip was used for the present SPR sensor. We have confirmed that the convex-shaped light intensity was due to the distribution of the intensity of the incident light from the light source of the present SPR sensor [25]. However, the difference of the light intensity between water and the 15 mM sucrose solution was almost the same for the 8 sensing stripes. This indicates that the change in refractive index on the 8 sensing stripes is able to be detected equally, if the light intensities for sample solutions are appropriately subtracted by those at each pixel for water (background signals). In addition, we confirmed that the noise level of the light intensities is reduced by averaging the light intensities among pixels [25].

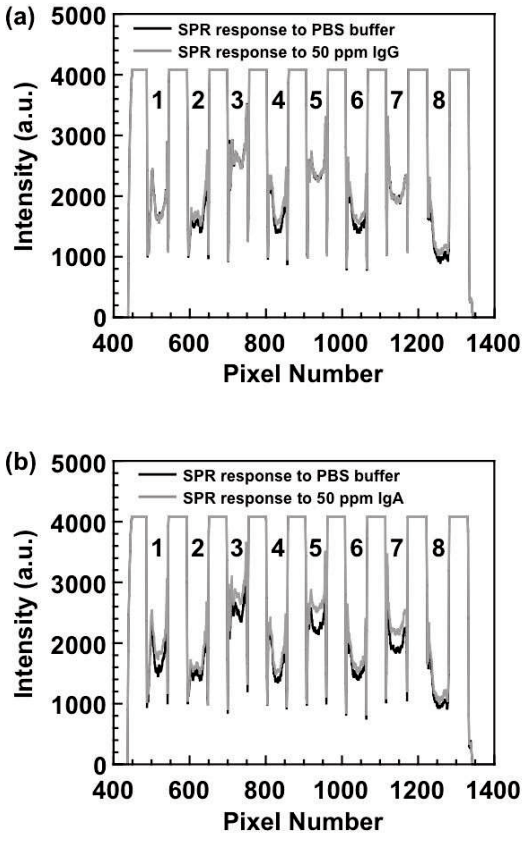

Fig. 6 The SPR response of the 8-stripe patterned sensor chip immobilized with the anti-IgA and anti-IgG antibodies to (a) 50 ppm IgG and (b) 50 ppm IgA. The anti-IgA antibody was immobilized on the sensing stripes of 1, 3, 5, and 7, and the anti-IgG antibody was immobilized on the sensing stripes of 2, 4, 6, and 8.

3.2 Confirmation of immobilization of anti-IgA and anti-IgG antibodies on 8-stripe patterned sensor chip

The purpose of the present study is to demonstrate the capability of the SPR sensor for simultaneous determination of multi-analyte by using the 8-stripe patterned sensor chip immobilized with two different kinds of antibodies. Figs. 6 (a) and (b) show the snap shots of the SPR sensor response (light intensity as a function of the pixel number of the linear CCD sensor) of the 8-stripe patterned sensor chip, where the anti-IgA and the anti-IgG antibodies were immobilized alternatively, when the PBS buffer and the 50 ppm IgG solution and the 50 ppm IgA solution were introduced into the flow cell separately, as the same as shown in Fig. 5. The numbers 1 – 8 in the figures mean the sensing stripes of the SPR sensor chip.

As can be seen from Fig. 6 (a), an increase in the light intensities for the 50 ppm IgG solution from the light intensities for the PBS buffer is observed on the sensing stripes No. 2, 4, 6, and 8, where the anti-IgG antibody was immobilized, but no response is observed on the other stripes, where the anti-IgA antibody was immobilized. Fig. 6 (b) shows the SPR responses of the 8-stripe patterned sensor chip to the PBS buffer and the 50 ppm IgA solution. Increases in the light intensities from those for the PBS buffer to those for the IgA solution are observed on the sensing stripes No. 1, 3, 5 and 7, where the anti-IgA antibody was immobilized. This is due to the fact that the anti-IgA antibody immobilized on the surface of the sensor chip binds with IgA in the solution. While the responses on the other stripes, where the anti-IgG antibody is immobilized, are also observed due to the cross reaction of the anti-IgG antibody with IgA. This comes from the low selectivity of the anti-IgG antibody used in this work. The cross reactivity of the anti-IgG antibody with IgA was examined and discussed in the section 3.5. The results shown in Fig. 6 indicate that the anti-IgA antibody and the anti-IgG antibody were confirmed to be correctly immobilized on the expected sensing stripes. Namely, immobilization of the anti-IgA and anti-IgG antibodies on the alternative sensor chip was successfully carried out by using the centrifugal force. It is worthy to note that, the light intensities on the sensing stripes No. 2, 4, 6, 8 are lower than those on the sensing stripes No. 1, 3, 5, 7, as can be seen in Figs. 6 (a) and (b). This fact means that the amount of the anti-IgA antibody immobilized on the sensor stripes No. 1, 3, 5, 7 was larger than that of the anti-IgG antibody immobilized on the sensing stripes No. 2, 4, 6, 8, taking into account that the higher light intensity corresponds to the higher refractive index on the sensor chip for the present SPR sensor.

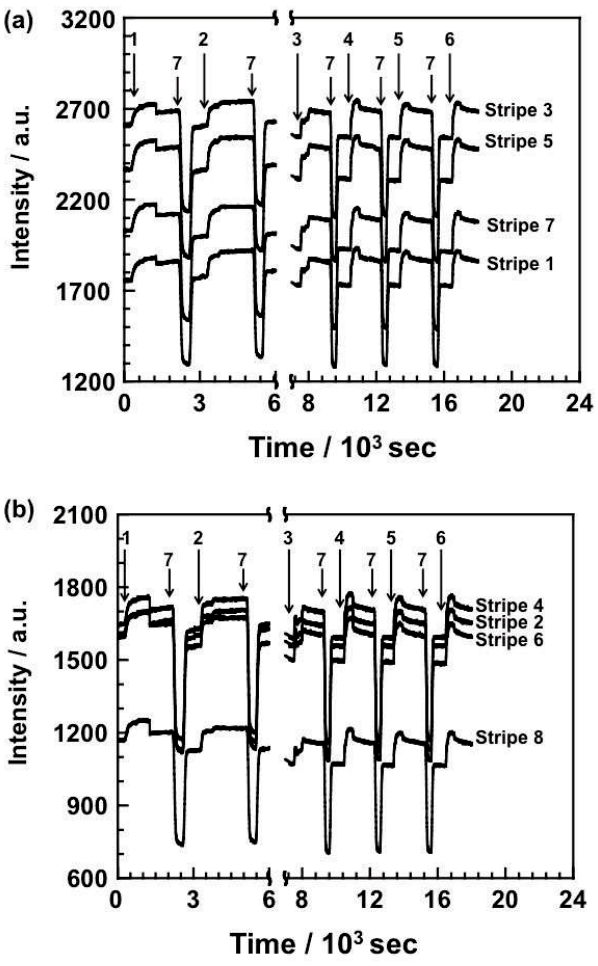

Fig. 7 (a) The sensorgrams for simultaneous immunoassay for IgA obtained on the sensing stripes No. 1, 3, 5, 7 and (b) those for IgG obtained on the stripes No. 2, 4, 6, 8. Concentrations of IgA and IgG in the mixed solution injected are indicated by the numbers in the figure, 1: 5 ppm, 2: 10 ppm, 3: 20 ppm, 4: 40 ppm, 5: 50 ppm, 6: 100 ppm. Number 7 means the injection of the dissociation solution of glycine-HCl buffer (pH=2.0).

3.3 Immunoassay of IgA and IgG on the 8-stripe patterned sensor chip

When the mixed solutions of IgA and IgG at the concentration from 5 ppm to 100 ppm were introduced to the sensor chip, the antibody-antigen reactions between the IgA and IgG in the solution and the anti-IgA and anti-IgG antibodies immobilized on the corresponding sensing stripes were respectively observed as the changes in the light intensity. The sensorgrams for the mixed solutions of IgA and IgG observed on the sensing stripes immobilized with the anti-IgA antibody and those immobilized with the anti-IgG antibody are shown in Fig. 7(a) and (b), respectively. The light intensity determined by the PBS buffer as a carrier is different among the sensing stripes, due to the different points of the sensing stripes on the sensor chip. The arrows numbered 1 ~ 6 and numbered 7 in Fig. 7 indicated the injection of the mixed solutions of IgA and IgG and the injection of the dissociation solution, respectively. A part of the sensorgram in Fig. 7(a), which was observed on the sensing stripe 1 is shown in Fig. 8, as an example, when the mixed solution of IgA and IgG at 50 ppm and the dissociation solution were injected at the time indicated by the arrows 5 and 7. When the mixed solution of IgA and IgG was introduced at a time indicated as arrow 5, the light intensity steeply increased with time and reached the maximum value. This indicates that the anti-IgA antibody immobilized on the sensing stripe 1 binds with IgA. The light intensity gradually decreased with time after reached the maximum, due to dissociation of IgA at a small extent from the anti-IgA antibody because the PBS buffer was continuously flowed on the sensor chip after passing the IgA solution through the sensor chip. The difference in the light intensity between the baseline determined by the carrier and that the mixed solution has passed though the sensor chip, indicated as ΔI in the figure, is taken as the sensor signal for the IgA solution. When the dissociation solution was injected at a time indicated by arrow 7, the light intensity was abruptly decreased and was returned to the baseline, the level of which was the same as that before the injection. This indicates that the immune-complex the anti-IgA antibody with IgA was completely dissociated. The changes in the intensities ΔI,

observed for the mixed solutions of IgA and IgG at the different concentrations in Fig. 7(a) were plotted against the concentrations of IgA in the mixed solutions. The results obtained for the sensing stripes No. 1, 3, 5, and 7 are shown in Fig. 9. The curves in the figure are calculated ones by using the binding constant of the anti-IgA antibody and ΔI_{max}, which are described in the section 3.5.

The sensorgrams observed on the sensing stripes No. 2, 4, 6 and 8, the surfaces of which were immobilized with the anti-IgG antibody, are shown in Fig. 7(b). Since the anti-IgG antibody showed cross reactivity to IgA, the changes in the light intensities include the binding with IgA and IgG. This response is reasonably explained by taking into account the binding constants of the anti-IgG antibody with IgA and IgG, which is discussed in the section 3.6.

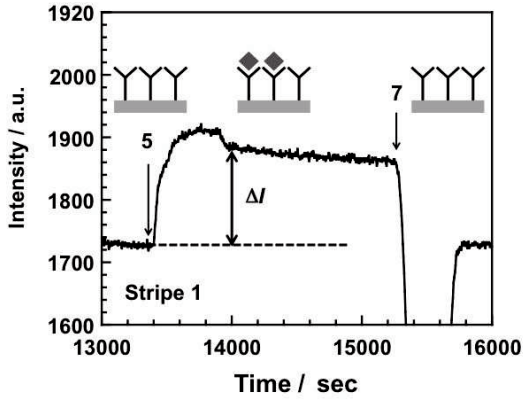

Fig. 8 The sensorgram observed on sensing stripe No. 1 for the 50 ppm IgA solution and the dissociation solution. The arrows 5 and 7 indicate the injections of the 50 ppm IgA solution and the dissociation solution, respectively. Estimated scheme on the sensor chip are shown in the figure. ΔI is the change in the light intensity due to binding IgA with the anti-IgA antibody on the sensor chip. .

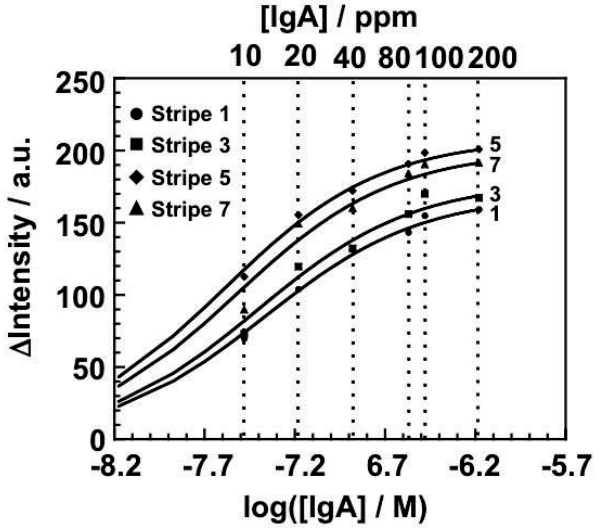

Fig. 9 The calibration curves for IgA obtained from sensing stripes No. 1, 3, 5, and 7. The curves are simulated ones from the binding constant, K and ΔI_{max} calculated from Eq. (6).

3.4 Estimation of the binding constants of the anti-IgA antibody with IgA

The binding constants of an immuno-complex of an antibody immobilized on the sensor chip with its antigen, can be evaluated from the SPR sensor response by assuming the Langmuir adsorption [26]. An immuno-reaction of the antibody immobilized on the sensor chip, Ab, with the antigen, Ag, and the binding constant, K, are expressed by Eqs. (1) and (2), respectively.

$$\underline{\text{Ab}} + \text{Ag} \rightarrow \underline{\text{Ab-Ag}} \quad (1)$$

$$K = [\underline{\text{Ab-Ag}}] / [\underline{\text{Ab}}][\text{Ag}] \quad (2)$$

where the under bar indicates that the species is on the sensor chip. [Ag] is the molar concentration of the antigens in the solution and [Ab] and [Ab-Ag] are the surface concentration of the antibody and the immuno-complex of the antibody with the antigen on the sensor chip, respectively. From the mass balance of the surface concentration of the antibody immobilized on the sensor chip, the following equation holds;

$$[\underline{\text{Ab}}]^{\text{T}} = [\underline{\text{Ab}}] + [\underline{\text{Ab-Ag}}] \quad (3)$$

where $[\underline{\text{Ab}}]^{\text{T}}$ is the total surface concentration of the antibody on the sensor chip. From Eqs. (1) and (3), the following equation can be derived;

$$[\underline{\text{Ab-Ag}}] / [\underline{\text{Ab}}]^{\text{T}} = K \cdot C_{\text{Ag}} / (1+K \cdot C_{\text{Ag}}) \quad (4)$$

where [Ag] can be replaced by the initial concentration of the antigen in the sample solution, C_{Ag}, because the concentration of the antigen in the sample solution is much higher than the surface concentration of the antibody on the sensor chip, then the change in the concentration of the antigen is assumed to be negligibly small.

In the present SPR sensor, since the surface concentration of the immuno-complex of the antibody with the antigen, Ab-Ag, is related to the change in the light intensity of the SPR sensor, ΔI, the following equation holds;

$$[\underline{\text{Ab-Ag}}] / [\underline{\text{Ab}}]^{\text{T}} = \Delta I / \Delta I_{max} \quad (5)$$

where ΔI_{max} is the change in the SPR sensor signal, when the antibody immobilized on the surface of the sensor chip is completely bound with the antigen. From Eqs. (4) and (5), the

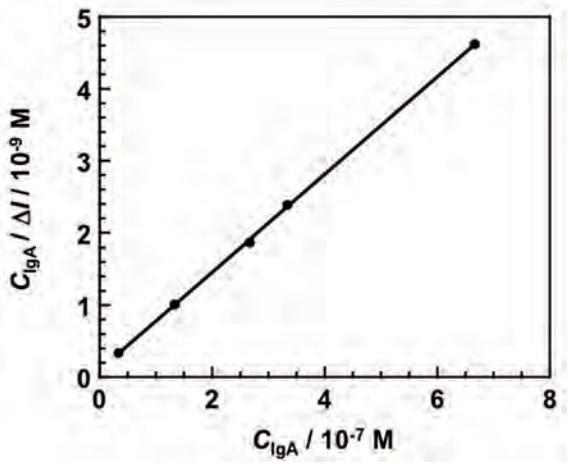

Fig. 10 The Langmuir plot based on Eq. (6) for estimation of binding constant of IgA of the immuno-complex of the anti-IgA antibody with IgA for sensing stripe No. 1.

following equation is derived;

$$C_{Ag}/\Delta I = C_{Ag}/\Delta I_{max} + 1/(\Delta I_{max} \cdot K) \quad (6)$$

From Eq. (6), the ΔI_{max} and K are respectively calculated from the slope and intercept calculated of the $C_{Ag}/\Delta I$ vs.C_{Ag} plot [25]. Fig. 10 shows an example of the Langmuir plot for binding IgA obtained from the sensorgram of the sensing stripe No. 1, showing in Fig. 7 (a). The value of ΔI_{max} and K were calculated to be 172 a.u. and 1.9 x 10^7 M^{-1} from the slop and intercept, respectively. By using the same method, a series of binding constant and ΔI_{max} for IgA measured on sensing stripes No. 3, 5, 7 was calculated, and the values are shown in Table 1.

3.5 Estimation of cross reactivity of anti-IgG antibody with IgA and IgG

As described in the sections 3.3 and 3.4, the anti-IgG antibody used in this work showed cross reactivity with IgA. In order to evaluate the SPR response of the sensor chip immobilized with the anti-IgG antibody to the mixture of IgA and IgG, which was shown in Fig.7 (b), the cross reactivity of the anti-IgG antibody was evaluated, according to the procedures described in the section 2.6. Figs. 11(a) and (b) show the sensorgrams for IgA and IgG solutions at the different concentrations observed at pixels No. 815 – 851, which are corresponded to the sensing stripe No. 4.

The change in the light intensity due to the immuno-reaction of IgA and IgG with the anti-IgG antibody was determined in the same way as that described in the previous section. The light intensities are plotted against the concentrations of IgA and IgG in Fig. 12. The solid curves in Fig. 12 are the simulated ones from the assumption of Langmuir adsorption by using the binding constants of the anti-IgG antibody with IgA and IgG, and ΔI_{max}, which were obtained from Fig. 13. From the slopes and intercepts of the Langmuir plots for IgA and IgG in Fig. 13, the K and ΔI_{max} were calculated to be 1.4 x 10^7 M^{-1}, 139 a.u. for IgA, and 1.8 x 10^7 M^{-1}, 156 a.u. for IgG, respectively. Such data taken from the SPR response corresponding sensing stripes No. 6 and 8 and were listed in Table 1.

3.6 Simulation of SPR response of the sensor chip immobilized with the anti-IgG antibody to the mixture of IgA and IgG

Here, we consider the case that the antibody, Ab, shows a cross reaction with different antigens, Ag_1 and Ag_2. In the present case, the anti-IgG antibody showed the cross reaction with IgA. When a sample solution of the mixture of IgA and IgG at [Ag_1] and [Ag_2] is introduced to the sensor chip, where the anti-IgG antibody is immobilized, the following equations are derived. An immuno-reaction of the antibody immobilized on the sensor chip, Ab, with the antigen, Ag_1 (IgA), Ag_2 (IgG) and the binding constant for IgA, K_1, and the binding constant for IgG, K_2 are expressed by Eqs. (9) and (10), respectively.

$$\underline{Ab} + Ag_1 \rightarrow \underline{Ab\text{-}Ag_1} \quad (7)$$

$$\underline{Ab} + Ag_2 \rightarrow \underline{Ab\text{-}Ag_2} \quad (8)$$

$$K_1 = [\underline{Ab\text{-}Ag_1}] / [\underline{Ab}][Ag_1] \quad (9)$$

$$K_2 = [\underline{Ab\text{-}Ag_2}] / [\underline{Ab}][Ag_2] \quad (10)$$

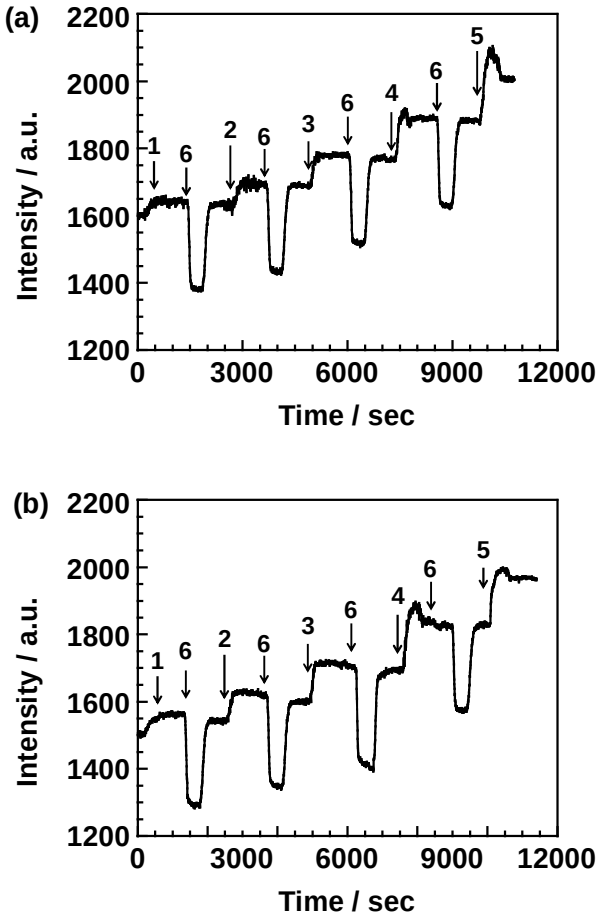

Fig.11 (a) The sensorgram for immunoassay of IgA obtained at pixels No. 815-851 on the CCD sensor, and (b) IgG obtained at the same pixels of the CCD sensor. Concentrations of IgA and IgG in the separate solution injected are indicated by the numbers in the figure, 1: 5 ppm, 2: 10 ppm, 3: 20 ppm, 4: 50 ppm, 5: 100 ppm. Number 6 means the injection of the dissociation solution of glycine-HCl buffer.

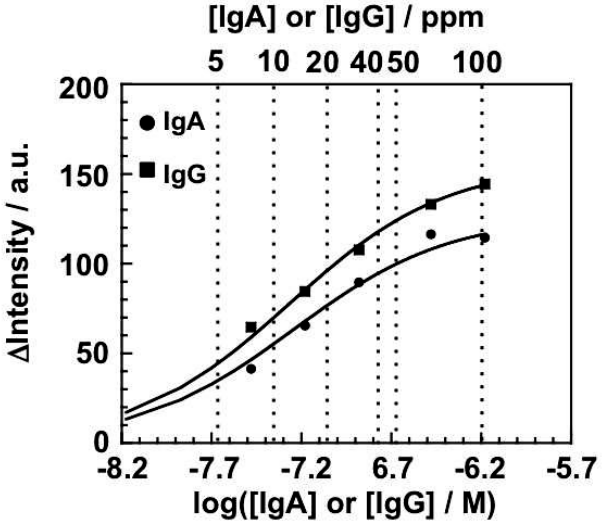

Fig. 12 The calibration curve of IgA and IgG

From the mass balance of the surface concentration of the

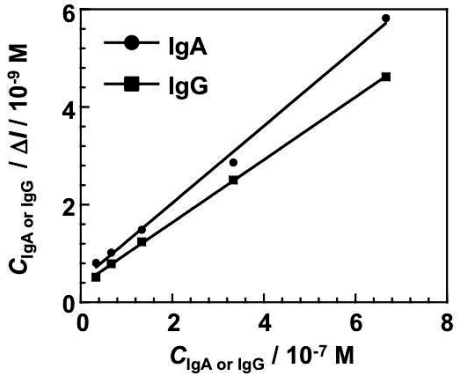

Fig. 13 The Langmuir plot for estimation of binding constant of the anti-IgG antibody with IgA and IgG for pixels No. 815 – 851 of the CCD sensor.

antibody immobilized on the sensor chip, the following equation holds;

$$[\underline{Ab}]^T = [\underline{Ab}] + [\underline{Ab\text{-}Ag_1}] + [\underline{Ab\text{-}Ag_2}] \quad (11)$$

From Eqs. (8) to (11), the following equation can be derived;

$$([\underline{Ab\text{-}Ag_1}] + [\underline{Ab\text{-}Ag_2}]) / [\underline{Ab}]^T = (K_1 \cdot C_{Ag,1} + K_2 \cdot C_{Ag,2}) / (1 + K_1 \cdot C_{Ag,1} + K_2 \cdot C_{Ag,2}) \quad (12)$$

where $[Ag_1]$ and $[Ag_2]$ can be replaced by the initial concentration of the antigen in the sample solution ($C_{Ag,1}$ and $C_{Ag,2}$, respectively) from the similar reason to that described in Section 3.4.

Similarly to the Eq. (5), the following equation holds;

$$([\underline{Ab\text{-}Ag_1}] + [\underline{Ab\text{-}Ag_2}]) / [\underline{Ab}]^T = \Delta I / \Delta I_{max} \quad (13)$$

From Eqs. (11) and (12), the following equation is derived;

$$(K_1 \cdot C_{Ag,1} + K_2 \cdot C_{Ag,2}) / \Delta I = (1 + K_1 \cdot C_{Ag,1} + K_2 \cdot C_{Ag,2}) / \Delta I_{max} \quad (14)$$

When the sample solution contains IgA and IgG at the same concentration, as C_{Ag}, *i. e.* $C_{Ag,1} = C_{Ag,2} = C_{Ag}$, the Eq. (14) can be expressed as following;

$$C_{Ag} / \Delta I = C_{Ag} / \Delta I_{max} + 1/(\Delta I_{max} \cdot (K_1 + K_2)) \quad (15)$$

From Eq. (15), the ΔI_{max} is calculated from the slope of the C_{Ag} vs. $C_{Ag}/\Delta I$ plot. The K_1 and K_2 is the value that is calculated from the measurement of IgA and IgG on anti-IgG antibody immobilized sensor chip, which are shown in the previous section 3.4. The calculated I_{max} and $K_1 + K_2$ were used to simulate the calibration curve from 1 ppm to 100 ppm (6.67×10^{-9} M to 6.67×10^{-7} M). The calibration curves from experiment and those simulated by using value shown in Table 1 at sensing stripes 2, 4, 6, and 8 are shown in Fig. 14. As can be seen in Fig. 14, K_1 and K_2 fitted the experiment results well.

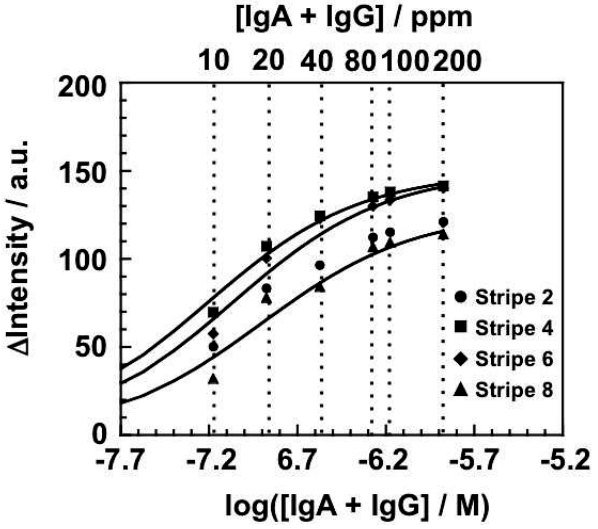

Fig. 14 The calibration curve of IgA and IgG mixture solution obtained from sensing stripe 2, 4, 6, and 8.

Conclusion

A new method of immobilizing the anti-IgA and anti-IgG antibodies on the 8-stripe patterned sensor chip alternatively by the centrifugal force using the microchip prepared by PDMS is described. Due to the low price of PDMS kit, SU-8 and its

Table 1 The values of K and ΔI_{max} calculated from Eq. (6) by using SPR response obtained for the 8 sensing stripes

	Anti-IgA antibody*		Anti-IgG antibody**			
*Stripe Number	K_{IgA} / M^{-1}	$\Delta I_{max\ IgA}$ / a.u.	K_{IgA} / M^{-1}	$\Delta I_{max\ IgA}$ /a.u	K_{IgG} / M^{-1}	$\Delta I_{max\ IgG}$ /a.u
1	1.9×10^7	172	-	-	-	-
2	-	-	-	-	-	-
3	2.8×10^7	179	-	-	-	-
4	-	-	1.4×10^7	139	1.8×10^7	156
5	4.6×10^7	208	-	-	-	-
6	-	-	1.1×10^7	134	1.3×10^7	166
7	2.5×10^7	200	-	-	-	-
8	-	-	0.7×10^7	122	0.9×10^7	139
Average	3.0×10^7	190	1.0×10^7	132	1.3×10^7	154

* The anti-IgA antibody was immobilized on the sensor stripes No. 1, 3, 5, and 7.

** The anti-IgG antibody was immobilized on the bare sensor chip and values were obtained from the sensor response to IgA and IgG observed at the pixels corresponding to each sensing stripes No. 4, 6, and 8.

developer, this method is simple and cost-effective to immobilize a sensor chip with multiplex receptors such as antibodies compared to other methods such as a material spotting ink-jet printer (around or more than 1 million Japanese Yen). By measuring the single solution of IgA and IgG with the concentration of 50 ppm, the fact that the anti-IgA antibody and anti-IgG antibody were correctly immobilized on the expect stripes can be proved. Furthermore, from the results of the detection of IgA and IgG, 8 calibration curves that are similar to the Langmuir adsorption curve are obtained. From the calibration curves, a series of binding constant and ΔI_{max} of IgA measured on sensing stripe 1, 3, 5, 7 and those of IgG measured on sensing stripe 2, 4, 6, 8 are obtained. However, due to the anti-IgG antibody used in this study included both the light and heavy chain, cross reactions resulted from the IgA and the heavy chain of the anti-IgG antibody were observed on sensing stripe 2, 4, 6, and 8 when 50 ppm IgA solution flowed from the sensor chip. Because of the cross reaction between IgA and anti-IgG antibody observed on sensing stripe 2, 4, 6, and 8, on which the anti-IgG antibody was immobilized, the measurement of the single solution of IgA and IgG with various concentrations on the anti-IgG antibody immobilized sensor was carried out, and the binding constant for IgA and IgG were obtained. These results proved that the multiplex sample detection can be carried out on this sensor chip correctly. The proposed method of preparation of the sensor chip can be expected to be useful in the clinical examination, such as the detection of the several kinds of cancer markers at the same time.

References

[1] O. H. Mei, X. R. Ding, Y. Y. Chen, J. Ding, K. Lee, H. X. Chen, Y. M. Yin, *Microchim. Acta*, **178**, 83 (2012).

[2] H. X. Chen, F. Liu, K. Koh, J. Lee, Z. H. Ye, T. T. Yin, L. Z. Sun, *Microchim. Acta*, **180**, 431 (2013).

[3] H. X. Chen, O. H. Mei, Y. F. Hou, J. Lee, B. Chen, L. Fang, X. L. Zhao, *Sens. Actuators B: Chem.*, **181**, 38 (2013).

[4] T. Špringer, J. Homola, *Anal. Bioanal. Chem.*, **404**, 2869 (2012).

[5] X. Chen, X. Jia, J. Han, J. Ma, Z. Ma, *Biosens. Bioelectron.*, **50**, 356 (2013).

[6] Y. Uludag, I. E. Tothill, *Anal. Chem*., **84**, 5898 (2012).

[7] A. D. Taylor, J. Ladd, Q. Yu, S. Chen, J. Homola, S. Jiang, *Biosens. Bioelectron.*, **22**, 752 (2006).

[8] H. Bai, R. Wang, B. Hargis, H. Lu, and Y. Li, *Sensors*, **12**, 12506 (2012).

[9] C. Esseghaier, A. Ng, and M. Zourob, *Biosens. Bioelectron.*, **41**, 335 (2013).

[10] J. Luczkowiak, S. Sattin, I. Sutkevičiūtė, J. J. Reina, M. Sánchez-Navarro, M. Thépaut, L. Martínez-Prats, A. Daghetti, F. Fieschi, R. Delgado, A. Bernardi, and J. Rojo, *Bionconjugate Chem.*, **22**, 1354 (2011).

[11] M. Piliarik, M. Vala, I. Tichý, and J. Homola, *Biosens. Bioelectron.*, **24**, 3430 (2009).

[12] M. Vala, K. Chadt, M. Piliatik, and J, Homola, *Sens. Actuators B: Chem.*, **148**, 544 (2010).

[13] J. Dostálek, J. Homola, and M. Miler, *Sens. Actuators B: Chem.*, **107**, 154 (2005).

[14] M. Piliarik, L. Párová, and J. Homola, *Biosens. Bioelectron.*, **24**, 1399, (2009).

[14] F. Bardin, A. Bellemain, G. Roger, and M. Canva, *Biosens. Bioelectron.*, **24**, 2100 (2009).

[15] A. Hemmi, R. Mizumura, R. Kawanisi, H. Nakajima, H. Zeng, K. Uchiyama, N. Kaneki, and T. Imato, *Sensors*, **13**, 801 (2013).

[16] H. Kawazumi, K. V. Gobi, K. Ogino, H. Maeda, and N. Miura, *Sens. Actuators, B*, **108**, 791 (2005).

[17] S. J. Kim, K. V. Gobi, H. Iwasaka, H. Tanaka, and N. Miura, *Biosens. Bioelectron*., **23**, 701 (2007).

[18] M. Vala, K. Chadt, M. Piliarik, and J. Homola, *Sens. Actuators, B*, 148, 544 (2010**)**.

[19] F. Bardin, A. Bellemain, G. Roger, and M. Canva, *Biosens. Bioelectron*., **24**, 2100 (2009).

[20] J. Dostálek, J. Homola, and M. Miler, *Sens. Actuators, B*, **107**, 154 (2005).

[21] P. Y. Li, B. Lin, J. Gerstenmaier, and B. T. Cunningham, *Sens. Actuators, B*, **99**, 6 (2004).

[22] G. Shao, J. Wang, Z. Li, L. Saraf, W. Wang, Y. Lin, *Sens. Actuators, B*, **159**, 44 (2011).

[23] http://www.kayakumicrochem.jp/

[24] S. Guo, R. Ishimatsu, K. Nakano, T. Imato, **133**, *Talanta*, 100, (2015).

[25] J. Zhan, K. Furui, H. Nakajima, N. Kaneki, R. Ishimatsu, K. Nakano, T. Imato, A. Hemmi, *Anal. Sci*. (in press).

[26] M. Tanaka, Y. Li, H. Nakajima, N. Soh, K. Nakano, K. Sakamoto, T. Imato, *J. Flow Injection Anal*., **25**, 172, (2008).

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