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Quantitative Assay of Hepatitis B Surface Antigen by Using Surface Plasmon Resonance Biosensor

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Abstract We performed a basic experiment for the rapid, on-line, real-time measurement of hepatitis B surface antigen using a surface plasmon resonance biosensor. We immobilized anti-HBsAg (hepatitis B surface antigen) polyclonal antibody, as a ligand, to the dextran layer on a CM5 chip surface that had previously been activated by *N*-hydroxysuccinimide. A sample solution containing HBsAg was fed through a microfluidic channel, and the reflecting angle change due to the mass increase from the binding was detected. The binding characteristics between HBsAg and its polyclonal antibody followed the typical monolayer adsorption isotherm. When the entire immobilized antibody had interacted, no additional, non-specific binding occurred, suggesting the immunoreaction was very specific. The bound antigen per unit mass of the antibody was independent of the immobilized ligand density. No significant steric hindrance was observed at an immobilization density of approximately 17.6 ng/mm^2. The relationship between the HBsAg concentration in the sample solution and the antigen bound to the ligand was linear up to ca. 40 μg/mL. This linearity was much higher than that of the ELISA method. It appeared the antigen-antibody binding increased as the immobilized ligand density increased. In summary, this study showed the potential of this SPR biosensor-based method as a rapid, simple and multi-sample on-line assay. Once properly validated, it may serve as a more efficient method for HBsAg quantification for replacing the ELISA.

Keywords: SPR, biosensor, HBsAg, binding assay

INTRODUCTION

Hepatitis B, a contagious disease widely spread in Asia, is caused by the infection of HBV (hepatitis B virus). The HBV envelope consists of the S antigen, a membrane protein and two minor proteins, the M and L antigens. The S antigen, isolated from the plasma of contaminated patients as a highly immunogenic polypeptide particle, was expressed as a recombinant protein and has been used commercially since 1986.

Quantitative assays for viruses are traditionally based on an *in vitro* culture assay for measuring the pathogenic effects after infecting test cells with the target virus. Recently, ELISA (enzyme linked immunosorbent assay) has been widely used to quantitatively assay HBsAg (hepatitis B surface antigen).

A SPR (surface plasmon resonance) sensor utilizes the phenomenon that when a thin gold film is exposed to an incident light source, the light will be reflected at different angles depending on the mass density applied. Thus, by measuring the angle change, the mass change on the film surface can be precisely determined. The binding and dissociation between ligand and analyte molecules can be monitored in real-time from a 'sensorgram' [1-5]. The sensorgram is expressed in RU (resonance unit), and 1,000 RU corresponds to 1 ng/mm^2 [6]. The sensor chip consists of a dextran top layer to a very thin (usually 50-nm thick) gold film on a glass slide. Carboxyl groups on the dextran can be derivatized to various functional groups such as aldehyde, thiol, *etc.* Thus, the dextran layer provides a three-dimensional 'nest' for the ligand molecules to give out an amplified binding signal. This chip is connected to an integrated microfluidic cartridge system containing four flow cells. A fluid can independently and continuously flow, *via* microvalve control, through these flow cells. After the ligand molecules are immobilized on the dextran layer by various coupling chemistries, a sample fluid is introduced to the flow cells and the interaction between an analyte and the ligand can be monitored without labeling.

SPR technology has been widely used in biomolecular recognition studies, particularly for screening purposes.

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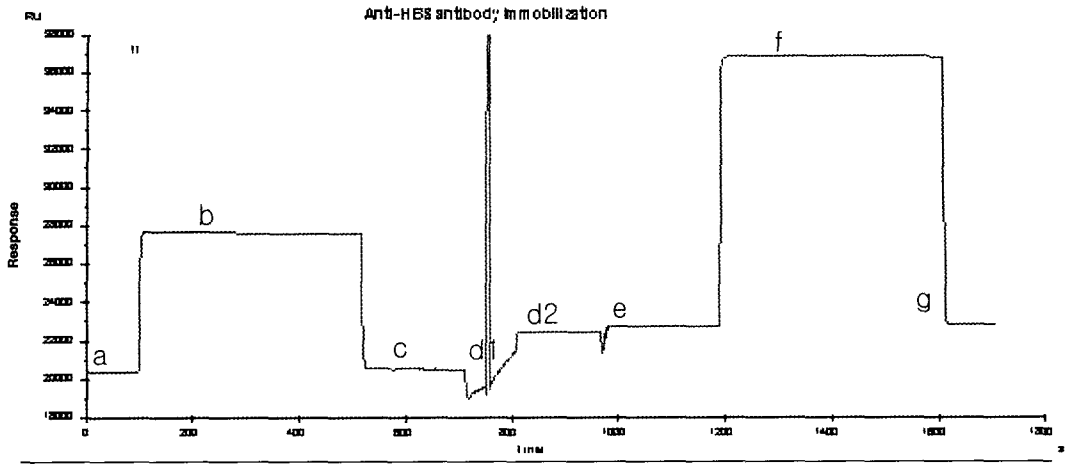
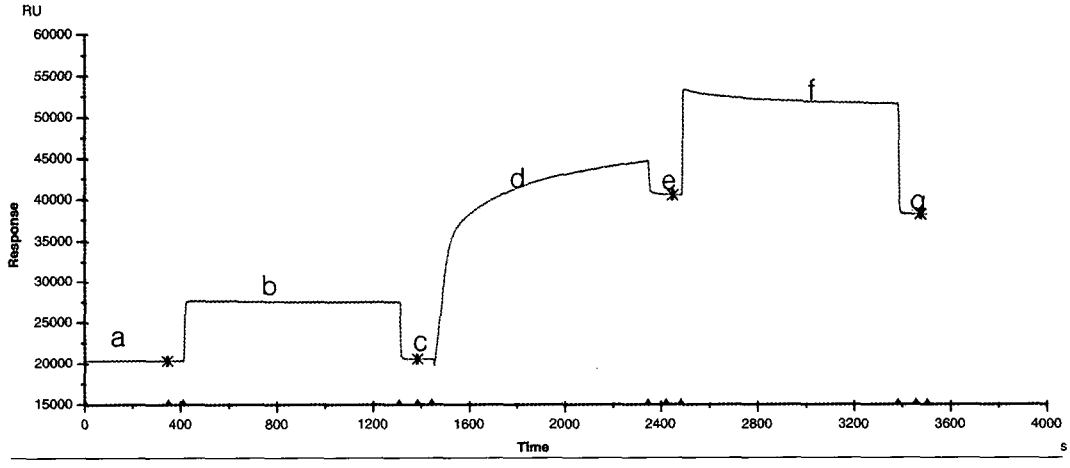
A



Fc	Time (sec)	AbsRe	Baseline	Re	Id
2	38.5	20356.5	Yes	0	Base
2	685.5	20520.2	Yes	163.7	EDC/NHS Activation
2	1152.5	22761.7	No	2241.5	pH 5.0, 50 μg/mL HBsAg Ab
2	1681.5	22824.2	No	2304.2	1 M Ethanolamine

B



Fc	Time (sec)	AbsRe	Baseline	Re	Id
4	344.5	20358.4	Yes	0	Base
4	1383.5	20620.9	Yes	262.5	EDC/NHS Activation
4	2447.5	40576.6	No	19955.7	pH 5.0, 50 μg/mL HBsAg Ab
4	3475.5	38256.8	No	17635.9	1 M Ethanolamine

Fig. 1. The sensorgram of anti-HBsAg polyclonal antibody immobilization. A: low- and B: high-density immobilizations. a: baseline, b: EDC/NHS activation, c: after activation, d: ligand binding, e: after ligand binding, f: deactivation, g: after deactivation.

Generally, larger molecules are immobilized as ligands and smaller molecules, such as peptides, screened for specific interactions. Compared with the ELISA method, a SPR sensor has several potential advantages. It can be used for real-time, on-line monitoring applications, more than 100 assays can be repeatedly performed by a chip, labeling, such as fluorescence or dye tagging, is not necessary, and only a very small sample volume is required (only 20 ng antibodies per flow cell) [2,7-9]. Recently, Tung *et al.* demonstrated that the SPR sensor could be effectively used for epitope mapping of HBsAg in antigen-antibody interactions [7]. They immobilized the antigen and screened the antibodies with different epitopes. In this study, we performed a basic experimental study to apply the SPR sensor to a quantitative assay for recombinant HBsAg. By immobilizing the polyclonal antibody as a ligand, we focused on the feasibility of the method as an alternative rapid assay to ELISA method.

MATERIALS AND METHODS

HBsAg and Anti-HBsAg Antibody

The antigen was a recombinant HBsAg expressed from *Hansenula polymorpha*, and the antibody was a polyclonal anti-HBsAg IgG from goat, obtained from Green-Cross Vaccine Corp (Yongin, Korea). A reference ligand with no affinity to HBsAg was necessary to identify the baseline, and the rabbit anti-interferon-α2 antibody, a polyclonal IgG purchased from Sigma (St. Louis, MO, USA), was used for this purpose.

SPR Sensor and Ligand Immobilization Process

The BIACORE 3000 (Biacore AB, Uppsala, Sweden) equipped with CM5 (carboxymethyl) chip was used. For better conjugation with the amine groups on the antibody, the carboxyl groups on the dextran layer were treated for 10 min with 0.2 M EDC (*N*-ethyl-*N'* dimethylaminopropyl carbodiimide) and 0.05 M NHS (*N*-hydroxysuccinimide) for their conversion to more reactive NHS esters. As a running buffer, 10 mM phosphate buffer saline (pH 7.4) was used at a flow rate of 30 μL/min. The ligand and reference ligand were added to 10 mM sodium acetate buffer (pH 5.5) to a concentration of 50 μg/mL, and each solution fed to the activated flow cells. The reference ligand (anti-interferon-α2 antibody) was immobilized to flow cells # 1 and 3 (Fc 1 and Fc 3), and to the ligand of interest (anti-HBsAg antibody) to Fc 2 and Fc 4. By varying the surface activation time, we induced 'low-density' and 'high-density' immobilization of the ligand, with the objective of observing any effect of the ligand density on the antigen-antibody interactions and to look for a possible steric hindrance effect in the high-density case. To prevent unspecific binding and remove the unconjugated ligand, 1 M ethanolamine was fed for 8 min to deactivate or block the unreacted NHS esters.

Analysis of Antigen-antibody Interactions

HBsAg solutions of various concentrations were fed through the flow cells, and the mass of the bound HBsAg measured in RU. The RU values obtained from Fc 2 and 4 were converted to mass values after excluding the non-specific binding effect by subtracting the RU values from Fc 1 and 3. The bound HBsAg masses were plotted against the concentrations of HBsAg in the sample. The linearity range was determined as the concentration where the correlation coefficient was maintained higher than 0.95 [10,11]. By applying the double-reciprocal method, we calculated the maximum binding capacity, and then determined the molar ratio of the antigen-antibody interaction [11,12].

Regeneration of Sensor Chip

For the repeated use of the sensor chip, the regeneration step should completely remove the bound HBsAg, regardless of the bound mass. To identify the optimum regeneration conditions, buffers of various compositions were evaluated (10 mM glycine, pH 2.2 or 1.7; 50 or 100 mM HCl with 1 M NaCl; 1, 5, 10, 25 and 50 mM NaOH with 1 M NaCl) [13].

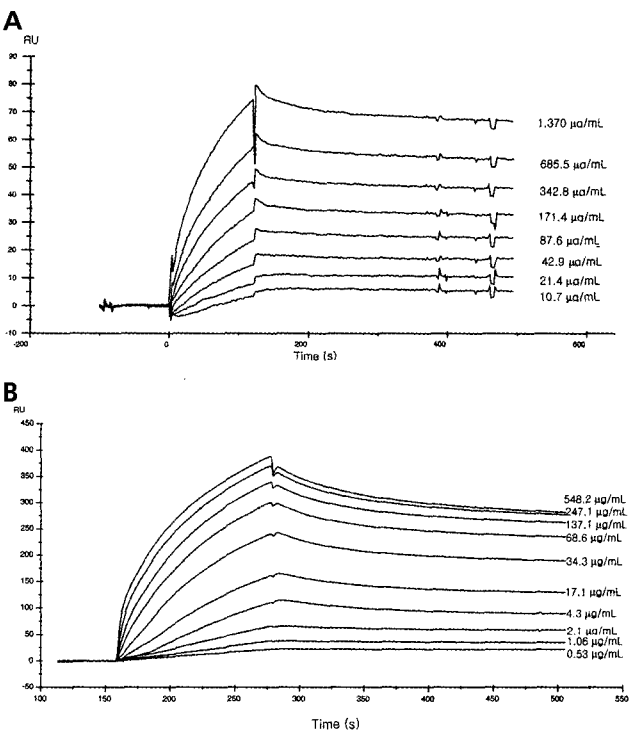


Fig. 2. Time-course profiles of HBsAg and anti-HBsAg antibody interactions at various HBsAg concentrations. A: low-density sensor chip (Fc 2~Fc 1) for 10.7, 21.4, 42.9, 87.6, 171, 343, 686, and 1,370 μg/mL (from the bottom plot upward), and B: high-density sensor chip (Fc 4~Fc 3) for 0.53, 1.06, 2.1, 4.3, 17.1, 34.3, 68.6, 137, 274, and 548 μg/mL (from the bottom plot upward).

RESULTS AND DISCUSSION

Ligand Antibody Immobilization

Since the dextran layer was negatively charged, sodium acetate buffer at an acidic pH was used as a positively charged coupling buffer. When various pHs (4.0, 4.5, 5.0 and 5.5) of 10 mM sodium acetate were evaluated, pH 5.5 turned out to be optimum for the ligand immobilization. Fig. 1 shows the sensorgrams of the ligand immobilization procedure. As soon as the surface had been treated with EDC and NHS for activation, the RU increased (interval "B"), and when re-exposed to the running buffer, it decreased (interval "C"). After the "C" interval, there was a mass increase from the original baseline in the "A" interval, due to the conversion of the carboxyl groups to NHS esters. As the ligand antibody was immobilized, the sensorgram increased (interval "D"), and when the running buffer was re-introduced it de-

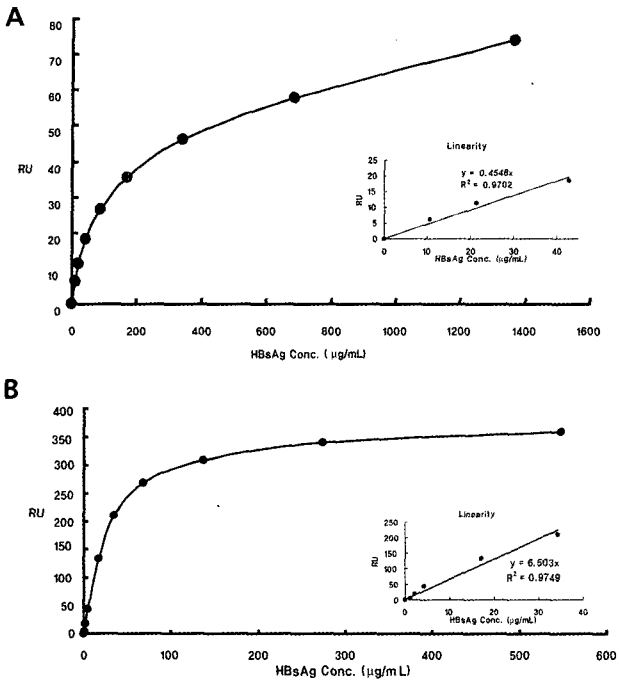


Fig. 3. Relationship between HBsAg concentrations in the analyte solution and the HBsAg mass bound to the immobilized antibody. A: Low-density and B: High-density immobilizations.

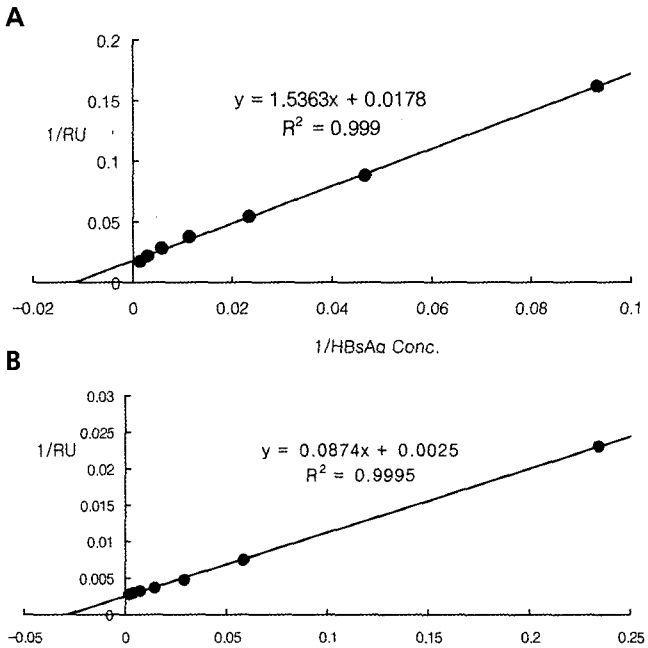


Fig. 4. Double-reciprocal plots to determine the maximum mass of HBsAg bound to the immobilized antibody. A: Low-density and B: High-density immobilizations.

creased slightly because the physically entrapped antibodies were washed off the chip surface (interval "E"). The difference between "C" and "E" corresponded to the immobilized antibody. When the chip was treated with the deactivation or blocking buffer (interval "F"), the RU increased, but decreased when the running buffer was fed (interval "G"), with the difference between E and G being due to the washing off of non-specifically bound antibodies. The masses of ligand antibody immobilized were 2.3 and 17.6 ng/mm^2 for the low- and high-density-immobilization, respectively. They were calculated from the RU values, taking 1,000 RU corresponding to 1 ng/mm^2 [6]. The main difference between them was the time period of the surface activation. As seen in Fig. 1, the longer the activation time, the more antibodies were immobilized, suggesting the degree of activation directly impacted on the immobilization density.

Binding Characteristics and Determination of Linearity Range

Various concentrations of HBsAg solution (10.7, 21.4, 42.9, 87.6, 171, 343, 686, and 1,370 μg/mL) and (0.53, 1.06, 2.1, 4.3, 17.1, 34.3, 68.6, 137, 274 and 548 μg/mL) were fed through the low- and high-density chips, respectively. Fig. 2 shows the sensorgrams from each interaction and Fig. 3 the plots of the mass of bound HBsAg against the HBsAg concentration in the solution. The linearity range, defined as the concentration range in which the correlation coefficient of higher than 0.95 could be applied, was estimated at 30~40 μg/mL in both cases (see the inset Fig. 3). It was reported that the linearity range of the ELISA method on HBsAg was 100 ng/mL [14]. Therefore, the SPR biosensor method yielded a much wider range of linearity.

The slopes within the linearity ranges depended on the immobilized ligand density. They were 0.46 and 6.50 RU $\mu g^{-1}mL^{-1}$ for the low-density (2.3 ng/mm^2) and high-density chips (17.6 ng/mm^2), respectively. At a given antibody concentration, the antigen binding mass difference was approximately 14.1-fold for a 7.6-fold difference in the immobilized antibody density. This seemed to indicate the binding was stronger at a higher immobilized ligand density, especially when the analyte concentration was low. Since the binding stoichiometry was the same between the ligand densities, and there was no steric hindrance effect in the binding (see the next section of "Maximum binding capacity and molar ratio of the antigen-antibody interaction"), this deviation might be explained by the physical phenomenon of the analyte molecules having a higher probability and/or affinity for binding when the ligand was more densely populated. It will be interesting to systematically look for the effect of the ligand density on the degree of binding.

Maximum Binding Capacity and Molar Ratio of the Antigen-antibody Interaction

The binding curve in Fig. 3 demonstrates the typical monolayer adsorption behavior and suggested the Langmuir equation could be applied.

$$A = A_{max} * c / (K + c)$$

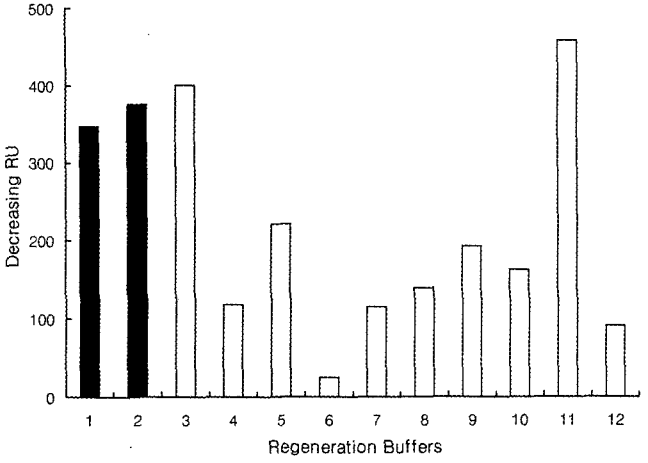


Fig 5. Effect of desorption buffer compositions on removal of the bound HBsAg. 1 to 12: 10 mM glycine (pH 2.2), 10 mM glycine (pH 1.7), 50 mM HCl, 50 mM HCl + 1 M NaCl, 100 mM HCl, 1 mM NaOH, 5 mM NaOH, 10 mM NaOH, 25 mM NaOH, 25 mM NaOH + 1 M NaCl, 50 mM NaOH, 50 mM NaOH + 1 M NaCl.

where A and A_{max} were the amount and the maximum amount of the adsorbed HBsAg, respectively, c the HBsAg concentration in the analyte solution, and K a constant. To determine the saturation value of the HBsAg binding to the antibody (A_{max}), the above equation was double-reciprocally plotted and the results are shown in Fig. 4. Both plots were linearized with very high accuracy (both correlation coefficients were higher than 0.999), which indicated the interaction indeed followed a monolayer adsorption, without inhibition or competition. From the y-intercept values, we calculated A_{max} values of 0.056 and 0.400 ng/mm^2, respectively, for the low- and high-density immobilization chips, based on the fact that 1,000 RU corresponds to 1 ng/mm^2 [6]. The difference appeared to be proportional to the antibody immobilization density. For confirmation, we calculated the mass of the HBsAg saturated per unit mass of the antibody immobilized, and found that it was independent of the immobilization density (0.024 and 0.023 ng-HBsAg/ng-antibody for the low- and high-density). This clearly indicated that the stoichiometry of the immunobinding was the same. On a molar basis, approximately six antibody molecules were used for the binding of one antigen molecule (the molecular weights of HBsAg and the antibody were 22 and 150 kD, respectively, for the calculation). Furthermore, it could be concluded that no steric hindrance effect was observed up to a ligand immobilization density of ca. 17.6 ng/mm^2.

Evaluation of Desorption Buffers

To use the biosensor for repetitive assays, the bound HBsAg must be fully and effectively removed from the chip surface by a desorption buffer. We evaluated various concentrations of glycine, HCl, NaOH and NaCl at various pHs to see how well the sensorgram decreased back to the original baseline value [13]. The results are shown in Fig. 5. The addition of NaCl to the buffer had no effect at all. This was understandable and expected, since the antigen-antibody interaction was independent of the ionic strength. Glycine, at low pH, and HCl itself were very effective. Tung *et al.* used 20 mM HCl to regenerate the HBsAg-immobilized surface [7], and Takacs *et al.* also used 10 or 20 mM HCl to successfully remove the bound anti-IFN antibodies [15]. Increasing the NaOH concentration up to 50 mM proportionally decreased the RU value. However, when a fixed concentration of NaOH was used, the decrease in the RU value was not reproducible, which could pose an operational problem in reusing the chip. If the desorption buffer was too harsh buffer the ligand antibody would be denatured in such a way that the bioactivity could not be regained. When the buffer was too mild, the dissociation would not be complete. Therefore, to reuse the chip on a routine basis it was imperative to identify a condition where the chip surface could be effectively and reproducibly regenerated, regardless of the antigen mass bound.

CONCLUSION

A SPR sensor was used to study the binding behavior between recombinant HBsAg and its polyclonal antibody. The interaction followed the Langmuir's adsorption isotherm, which was very specific. On a molar basis, approximately six antibody molecules interacted with one antigen molecule at saturation. The upper limit of the linearity range (ca. 40 μg/mL) was much higher than that of the ELISA (ca. 100 ng/mL). The saturation values per unit mass of the immobilized antibody were independent of the immobilized ligand density, suggesting the same binding stoichiometry, with no steric hindrance effect. This study has indicated the potential of using the biosensor for the quantitative and non-labeling assay of the antigen, during various steps of the HBsAg production process. However, the reproducible regeneration conditions need to be identified for repeated use of the sensor chip.

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