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Full Length Research Paper

A simple method for quantification of interferon-α2b through surface plasmon resonance technique

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A rapid and efficient immunoassay method for quantification of interferon-α2b using surface plasmon resonance was developed with BIAcore 3000 as a sensor. Two different levels of anti-interferon monoclonal antibody were immobilized onto a CM5 chip using an amine coupling method. Similar binding ratio was observed for both the ligand densities. There was no steric hindrance and loss of antibody activity even at higher ligand density (> 22,000 RU). The sensitivity of the assay was increased up to 45% with the increment in ligand density from 15,400 to 22,360 RU. The binding between interferon-α2b and anti-interferon monoclonal antibody was predominantly controlled by mass transfer rate and the relationship was found linear, ranged from 5 to 400 ng/mL. Total cycle time per analysis was less than 8 min and required only 5 µL of sample injection.

Key words: Immunoassay, surface plasmon resonance (SPR), interferon-α2b (IFN-α2b), BIAcore, biosensor, quantification.

INTRODUCTION

Human interferon-α2b (IFN-α2b) is a physiologically active protein and it is one of the growth factor that comes under the cytokine family. It has excellent antiviral, antimicrobial and antitumor actions. As a result, it is being used as one of the bio-pharmaceutical drugs for diseases like hairy cell leukemia, chronic hepatitis C, chronic hepatitis B (Pestka et al., 2004). Many papers have been published for the production and purification of IFN-α2b (Babu et al., 2000; Lim et al., 2000; Liu et al., 2001; Srivastava et al., 2005; Cao et al., 2006; Valente et al., 2006; Cao et al., 2007; Ayed et al., 2008). In all these papers, the amount of IFN-α2b was quantified either using one or combination of methods like bioactivity assay (Babu et al., 2000; Lim et al., 2000; Liu et al., 2001; Srivastava et al., 2005; Cao et al., 2006; Cao et al., 2007), gel electrophoresis (Lim et al., 2000; Cao et al., 2006; Valente et al., 2006), western blot (Valente et al., 2006) and enzyme linked immunosorbent assay (ELISA) (Srivastava et al., 2005; Cao et al., 2006; Cao et al., 2007; Ayed et al., 2008). These methods are time consuming. Surface plasmon resonance (SPR) can be used to quantify IFN-α2b in a similar manner to ELISA but with faster rates and consumes fewer reagents. It can also be adopted for online monitoring system in a production facility (Chavane et al., 2008).

SPR is used in various fields such as food analysis (Taylor et al., 2006), pharmaceutical (Hwang et al., 2005; Chavane et al., 2008), environmental monitoring (Matsumoto et al., 2005; Shankaran et al., 2006; Habauzit et al., 2008), medical diagnostics (Haes et al., 2005; Yang et al., 2005; Gobi et al., 2007; Masson et al., 2007) and others (Baird et al., 2002; Beseničar et al., 2006; Katsamba et al., 2006). Previously, SPR was used

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to know the binding partners and its relative interaction strength for IFNs (Takacs et al., 1999; Asokan et al., 2006; Schmeisser et al., 2007), immune response against IFN-β (Gibbs and Oger, 2008) and in one particular instance to quantify IFN- γ (Stigter et al., 2005). However, reports on the use of SPR in the quantification of IFN-α2b are not available in the literature.

The working principle of SPR can be found elsewhere (Habauzit et al., 2007; Ravi Shankaran and Miura, 2007; Grieshaber et al., 2008; Homola, 2008). Briefly, the system consists of a plane polarized light which is passed on to a thin metal layer to get the total internal reflection. The angle at which the dip in the intensity of reflection occurred is called a SPR angle. This dip is caused by the excitation of plasmons that occurs on the thin metal layer. The deviation of SPR angle depends on the refractive index of the dense medium (glass) and the medium adjacent to the metal layer, wavelength and the polarization state of the incident light and dielectric constant of the thin metal layer. When other factors are kept constant, it is clear that the angle shift is only due to the properties of the medium adjacent to the metal layer. A shift of 0.1° (1000 Resonance unit (RU)) is equivalent to 1 ng/mm^2 of protein binding (Stenberg et al., 1991). SPR, sensor chip and integrated microfluidic channel (IFC) are essential components for this type of analysis (Löfås et al., 1991; Szabo et al., 1995). While many commercial suppliers provide a robust technology for all those components (Baird and Myszka, 2001), surface of the sensor chip should be modified for one particular use.

The molecules can be immobilized either on the surface of the chip or on the three dimensional layer (free moving layer attached on the surface of the chip). Although immobilization on the surface is more straight forward process, it is often limited by the number of recognition sites (Homola, 2008). A linear chain of hydrophilic carboxymethyl (CM) dextran (hydrogel) is widely used to generate a three dimensional layer on the chip surface and hence providing a very high surface area for immobilization (Shankaran and Miura, 2007). The hydrogel requires only very small amount of protein for immobilization due to the electrostatic attraction between the protein molecules and dextran. Moreover, it suspends the ligand in the solution (Stenberg et al., 1991; Rich and Myszka, 2007). Yet, the use of hydrogel also comes with the limitation of handling more complex crude substances due to non specific adsorption (Shankaran and Miura, 2007) and also due to increase in non specific adsorption with respect to time of usage (Masson et al., 2007). In some cases, non specific adsorption can be reduced by adding CM dextran sodium salt in the samples (Karlsson et al., 1993; Yang et al., 2005). To date, BIAcore and CM5 chip are most widely used equipment and sensor chip (Rich and Myszka, 2005; Rich and Myszka, 2007).

The surface preparation is the foremost step to have a proper concentration assay in SPR (Shankaran and Miura, 2007). The choice of appropriate biorecognition elements and immobilization method are of critical importance with direct impact on key performance characteristics of the sensor such as sensitivity, specificity, and limit of detection (Homola, 2008). The difficulties in the determination of low molecular weight analyte (< 20 kDa) at low concentrations using SPR have been reported by Huang et al. (2008). They highlighted different possible strategies that could be applied to enhance the sensitivity and used sandwhich type assay to increase the sensitivity of allergens quantification from *Dermatophagoides farinae* (36-40 kDa). Here we describe a better surface preparation which alone increases the sensitivity up to 45% and it offers very simple, low cost and lesser running time with very much lower volume of reagent than other proposed methods.

MATERIALS AND METHODS

Reagents

Anti-α-IFN Mouse monoclonal antibody (anti-IFN) was purchased from Calbiochem (San Diego, CA, USA). CM5 chip and amine coupling kit [containing 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS) and 1 M ethanolamine hydrochloride (pH 8.5)] were purchased from GE Healthcare (Uppsala, Sweden). IFN-α2b standard was purchased from Affinity Bioreagents (Golden, CO, USA). All other chemicals were purchased either from Sigma-Aldrich (USA) or Merck (USA). HBS-EP buffer (10 mM 4-(2 hydroxyethyl)piperazine-1-ethane-sulfonic acid (HEPES), 150 mM NaCl, 3 mM ethylenediaminetetraacetic acid (EDTA), 0.005% Tween 20, pH 7.4), sodium acetate buffers (10 mM sodium acetate, with pH 4.0 to 5.5 adjusted by adding 1 M HCl) and regeneration buffers were prepared in house, filtered using 0.22 µm filter and degassed prior to use. IFN-α2b expressed in *Escherichia coli* strain Rosetta-gami 2 (DE3) containing pET 26b-IFN vector was used as a crude sample for analysis. The method of preparation of this recombinant strain was already described in details in our previous study (Ramanan et al., 2010).

Instrumentation and software

Biacore 3000 (GE Healthcare) was used in this study. The experiments were conducted either using wizard or customized program. HBS-EP buffer was selected as continuous running buffer at a flow rate of 5 µL/min. All experiments were carried out at 25°C with CM5 chip and the results were further analyzed using BIA evaluation software (Version 4.1).

Optimum conditions for immobilization of anti-IFN

The parameters evaluated for optimum conditions for immobilization of anti-IFN were buffer pH, flow rate and concentration of anti-IFN. The buffer pH was varied from 4.0 to 5.5. The scouting for the preferred pH was conducted in 0.1 mg/mL anti-IFN at two different flow rates, 20 and 5 µL/min. The optimum concentration of anti-IFN was searched from 0.020, 0.05 and 0.1 mg/mL at the preferred pH (pH 5.0). Fifty mM of NaOH was used as a regeneration solution to remove all the electrostatically-adsorbed proteins.

Immobilization of anti-IFN

Anti-IFN immobilization was carried out using amine coupling onto the CM5 chip. Prior to original immobilization, the chip was exposed

to 2 min (20 µL/min) each of 100 mM of HCl, 50 mM of NaOH and 0.5% SDS to swell the gel (Navratilova et al., 2007). The immobilization procedure was conducted at a flow rate of 5 µL/min by activating with equal volumes of 100 mM NHS and 400 mM EDC mixture followed by immobilization with anti-IFN (0.05 mg/mL suspended in 10 mM of acetate buffer pH 5.0) and deactivation of unreacted sites with 1 M ethanolamine (pH 8.5). The contact time of the different solutions was varied to obtain different ligand densities. Immobilization of lower ligand density (LLD) (~15,400 RU) was carried out by flowing the activating, immobilizing and deactivating solutions for 7, 10 and 7 min, respectively. On the other hand, immobilization of higher ligand density (HLD) (~22,360 RU) was carried out by flowing 15 min of activating solution, 30 min of immobilizing solution and 10 min of deactivating solution. The reference flow cell was prepared either with or without immobilizing solution (1 mg/mL of BSA at pH 5.0).

Selection of regeneration solution

EDTA (20 mM), NaOH (50, 10 and 5 mM) and glycine (10 mM, pH 3.0 and 2.5) solutions were used for the regeneration scouting. The capacity of different regenerating solutions was analyzed by passing IFN-α2b solution with an approximate increment of 90 RU and then passing the particular regenerating solution for 1 min at a flow rate of 5 µL/min. In order to check their consistency and its effect on anti-IFN, similar procedure was repeated for few times.

Measurement of standard and sample

Different concentrations of IFN-α2b (0 to 400 ng/mL) prepared from a standard by diluting with HBS-EP buffer were passed to the reference and anti-IFN flow cells for 1 min at a flow rate of 5 µL/min. IFN-α2b expressed in recombinant *E. coli* was extracted through osmotic shock (Ramanan et al., 2009) and glass bead shaking (Ramanan et al., 2008). After separating the cells and the supernatant by centrifugation (rotor model 1189, Universal 22R centrifuge, Hettich AG, Switzerland), the supernatant was mixed with HBS-EP buffer in 1:1 ratio and the resultant solution was passed to the surfaces similar to the standard. After each measurement, the surface was regenerated with one min pulse of 10 mM glycine (pH 2.5) at 5 µL/min.

All the responses were calculated after subtracting the equivalent reference response. The report points were set at 30 s before and 100 s after the measurement. The end point measurement was taken from the difference of two report points. The binding or association rate was taken from the average response rate between 95 and 110 s from the cycle start time. This is considered to be steady-state initial binding rate which is just after the few seconds of injection.

Calculation of binding ratio and increase in response

Binding ratio was calculated according to Löfås et al. (1993) using equation 1. The molecular weight of anti-IFN and IFN-α2b used in the equation was 150 and 19 kDa. The response of anti-IFN used in the equation for LLD and HLD was 14,618 and 20,963 RU which was taken just before the start of experiment. Increase in response was calculated using equation 2.

$$Binding\ ratio = \frac{\mathrm{Re}\,sponse\ of\ IFN-\alpha 2b \times MW\ of\ anti-IFN}{Amount\ of\ anti-IFN \times MW\ of\ IFN-\alpha 2b} \quad (1)$$

$$Increase\ in\ response\ (\%) = \frac{100 \times (HLD\ response - LLD\ response)}{LLD\ response} \quad (2)$$

RESULTS AND DISCUSSION

Optimum conditions for immobilization of anti-IFN

The main conditions that affect the density of immobilized ligand are the concentration of ligand, ionic strength, pH, flow rate, activation time and immobilization time. As most of these conditions (except the activation time and immobilization time) were used to transfer the protein from the liquid surface to CM dextran layer, these conditions can be scouted before immobilization by passing on to the chip. Low ionic strength was preferred for this type of electrostatic attraction (Johnsson et al., 1991; Löfås et al., 1993) and it was fixed as 10 mM through out the experiment. The pH of the buffer was varied between 4 and 5.5 to identify the preferred pH for immobilization. Generally, this would be assessed for a period of two min. At the end of two min run with 20 µL/min, most of the curves were still in the increasing trend (Figure 1). Higher flow through time (> 2 min) was required for some specific proteins to identify its maximum response (Johnsson et al., 1991). In order to identify the maximum response, twelve min run was carried out at a flow rate of 5 µL/min. Although the maximum RU obtained for two min run and twelve min run were at similar pH (pH 5.0), the trend was different for other pH.

The difference in charges for dextran and the protein at different pH has made the difference in the attraction rate and the maximum attraction. For example, the rate of attraction was higher at pH 4.0 and 4.5, but the maximum attraction was higher at pH 5.0 and 5.5. At lower pH, the proteins were more positive (pI ~ 5.0 to 7.0), thus the rate of transfer was higher. At similar condition, the total negative charge of dextran was lower, which in turn, reduced the maximum attraction. This is in contrary for the higher pH.

Three different ligand concentrations (0.02, 0.05 and 0.1 mg/mL) with two different flow rates were run at the preferred pH (5.0). Even though the higher attraction rate was observed in higher flow rate and at higher concentration, all the maximum attraction response was more than 50,000 RU, which was well above the RU that could attain after immobilization. Considering the attraction rate and cost of antibody, the antibody concentration for immobilization experiment was fixed at 0.05 mg/mL with a flow rate of 5 µL/min.

Variation of immobilization level

Two different immobilization levels were attained by varying the activation and immobilization times. Figure 2 shows the comparison of maximum protein adsorption during scouting and actual immobilization. The maximum protein adsorption was reduced during immobilization and it was noticed that the reduction was proportional to the activation time. The reduction of negative site in CM layer by the formation of NHS ester would be the possible reason for this. Yet, the maximum immobilization amount

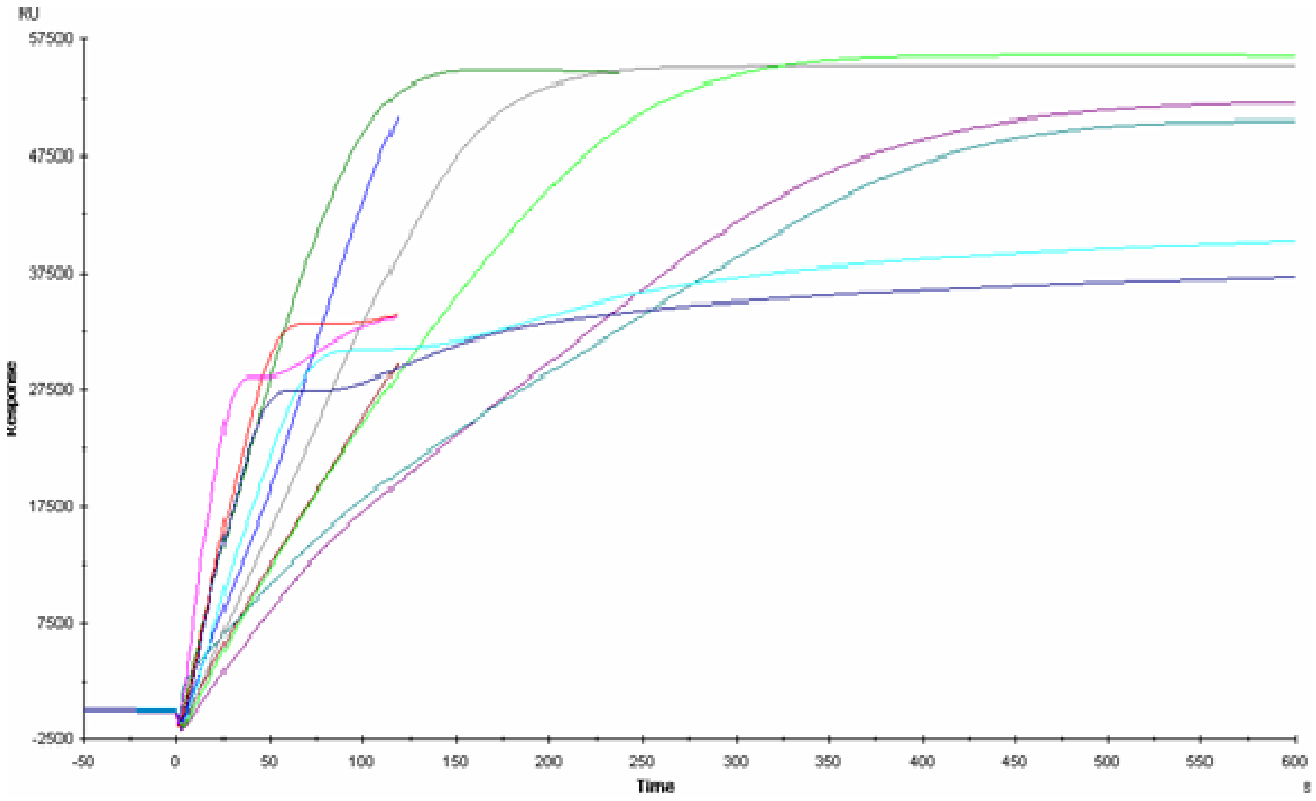


Figure 1. Scouting for the preferred pH at different flow rates and concentrations of anti-IFN performed on CM5 chip using BIAcore 3000. Two chips were used for these experiments. All experiments were run on the same chip, except the experiment conducted at pH 5.0, 50 µg/mL, 5 µL/min was run using different chip. Scouting was performed at 2, 4 and 12 min. The variations in this scouting experiment are as shown in the symbols. Symbols: pH 4.0,100 µg/mL, 5 µL/min; pH 4.5, 100 µg/mL, 5 µL/min; pH 5.0, 100 µg/mL, 5 µL/min; pH 5.5, 100 µg/mL, 5 µL/min; pH 5.0, 50 µg/mL, 5 µL/min; pH 5.0, 20 µg/mL, 5 µL/min; pH 5.0, 100 µg/mL, 5 µL/min; pH 4.0, 100 µg/mL, 20 µL/min; pH 4.5, 100 µg/mL, 20 µL/min; pH 5.0, 100 µg/mL, 20 µL/min; pH 5.5, 100 µg/mL, 20 µL/min.

was increased by increasing the activation time.

Figures 3a and 3b show the total cycle of immobilization for LLD and HLD, respectively. At 7 min of EDC/NHS activation time (Figure 3a), ~15,400 RU was obtained which was around 29% of total protein adsorbed during scouting. Huang et al. (2008) reported that in amine coupling, 60 to 80% of adsorbed protein would be immobilized and they had achieved around 62.5%. In their study, the optimum conditions were scouted for two min interval and information on maximum adsorbed protein was not available. In fact, the immobilization amount depends on the amount of activated site in the chip. Typically, 7 min run of activation solution may activate 30 to 40% of CM dextran (Johnsson et al., 1991) and the immobilized amount would attain the similar percentage.

Higher immobilization (~22,360 RU) (Figure 3b) was achieved by increasing the activation time to 15 min and immobilization time to the maximum of 30 min. It should be noted that the half life period of activated NHS is 15 min (Johnsson et al., 1991). In this study, no changes in the immobilized amount were observed when immobilization was extended more than 30 min.

Selection of better regeneration condition

The ideal regeneration condition should be in such a way that it should remove all the analyte bound during the injection step and it should not affect the binding capacity of the ligand. It was observed that the use of chelating solution (20 mM EDTA) did not significantly remove the analyte. On the other hand, the use of high pH caused the removal of the ligand. These two conditions affect the successive analysis. The use of low pH gave the satisfactory result. Subsequently pH 2.5 was chosen after showing the consistent response for 5 successive cycles (Figure 4). Though this test was performed at the increment of 90 RU, 5 µL of regeneration solution was found to be effective until 600 RU. The incomplete regeneration for higher response was observed, even after several injections of regeneration solution (glycine, pH 2.0) (Gobi et al., 2007). Similar observation was also

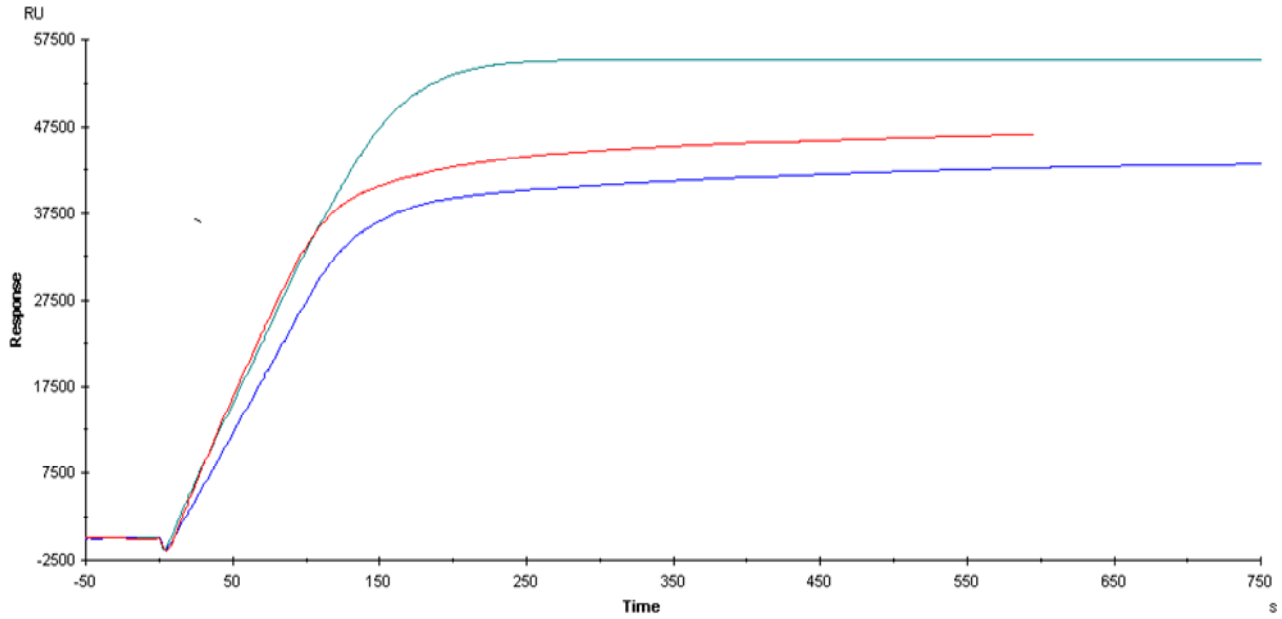


Figure 2. Comparison of immobilization and pH scouting of anti-IFN performed on CM5 chip using BIAcore 3000. Symbols: Response of protein adsorption during pH scouting; Response of protein adsorption during immobilization with 7 min of activation; Response of protein adsorption during immobilization with 15 min of activation.

noted in this study. For higher response, even though incomplete regeneration was observed, 8 µL of regeneration solution gave consistent result for the subsequent analysis.

Activity of immobilized ligand

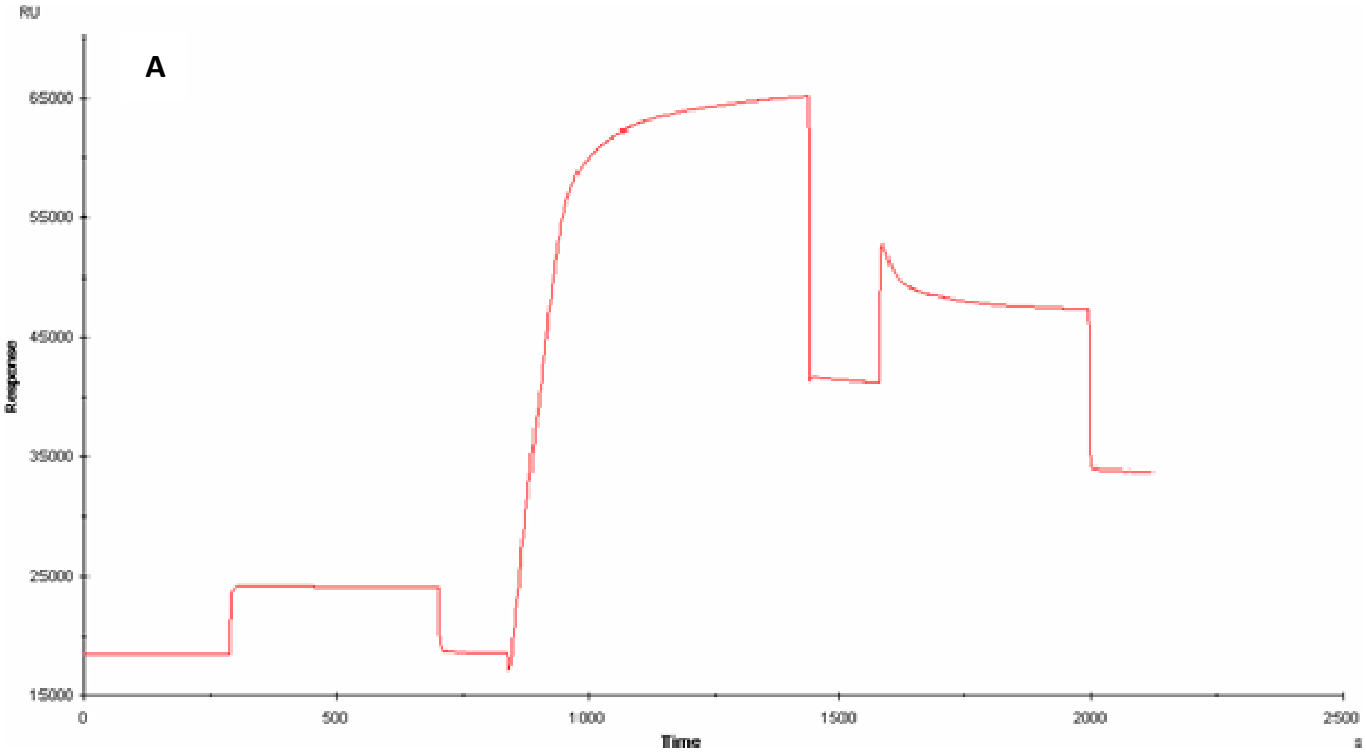
The ligand activity is generally affected by the activation time and the amount of immobilized ligand. Since the amine coupling immobilize the ligand randomly, the reduction of activation time would reduce the cross-linking of ligand and increase the activity but reduce the amount of immobilized ligand (Löfås et al., 1995). On the other hand, higher immobilized ligand would shift the response from kinetics limited to mass transfer limited assay which would be good for concentration assay (Karlsson et al., 1993). Higher immobilized ligand would also increase the sensitivity of assay (Löfås et al., 1993) but in some cases the sensitivity would be reduced due to the steric hindrance of the ligand itself (Löfås et al., 1993; Johnsson et al., 1995; Yarmush et al., 1996).

The binding ratio between the analyte and the immobilized ligand was found to be similar for both the ligand density, though the total amount of analyte bound was increased at a particular concentration (Figure 5). The average sensitivity was increased to 40% (Table 1) by simply immobilizing higher ligand density. Karlsson et al. (1993) reported that the increment of ligand density would lead to mass transfer limited reaction only and the binding rate observed would be independent of the amount of ligand density. Results from this study showed that this was not achieved for this interaction even up to 22,360 RU (Table 1).

Hwang et al. (2005) observed no reduction in ligand activity for the quantification of hepatitis B surface antigen (~22 kDa) up to 17,600 RU. In this study, similar result was also observed up to 22,360 RU. In fact, the results from Löfås et al. (1993) delineated that the maximum immobilized ligand activity depends on the mass of analyte for antibody and antigen interaction.

Inclusion of reference surface

The subtraction of response of the actual surface from the reference surface is useful to remove the non-specific adsorption on the chip and it is very important while analyzing the crude samples. The reference surfaces used by some of the previous studies were blank surface (Huang et al., 2007), deactivated surface (Yang et al., 2005; Chavane et al., 2008) and also other antibodies (Gonzales et al., 2002; Hwang et al., 2005). The usage of other antibodies would mimic their specific surface but the cost would be high for long run. In this study, deactivated surface and bovine serum albumin (BSA) have been used as the reference surface in two different chips and observed that the increment of response after regeneration in the reference surface in long run. This was observed only when low pH was used during regeneration and it was less pronounced with the addition of 1 M NaCl in the regeneration buffer. At low pH, some proteins were denatured and would be bound back in the



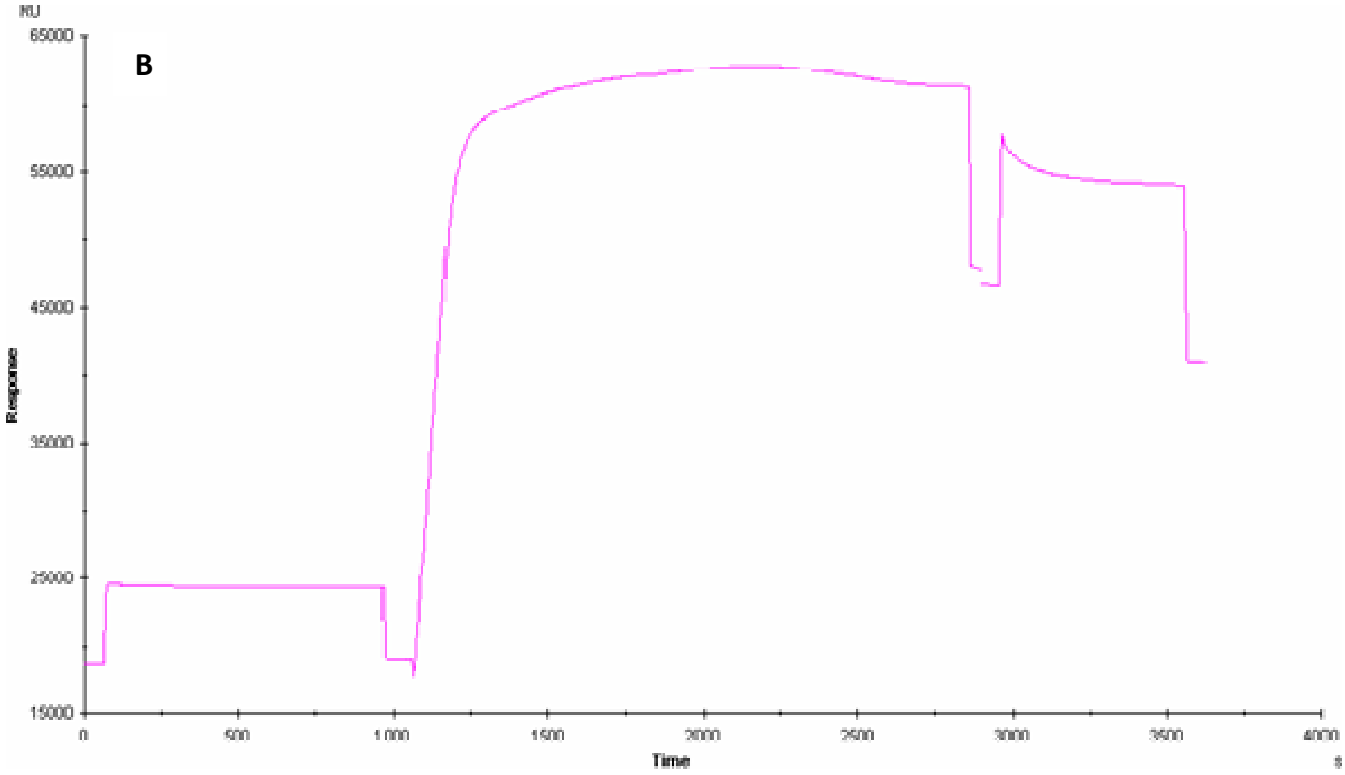


Figure 3(a and b). Total cycle of immobilization for LLD (a) and HLD (b) performed on CM5 chip using BIAcore 3000. 1- Response during the flow of activation solution (NHS/EDC). 2- Response during the flow of immobilizing solution (Anti-IFN). 3- Response during the flow of deactivating solution (Ethanol amine).

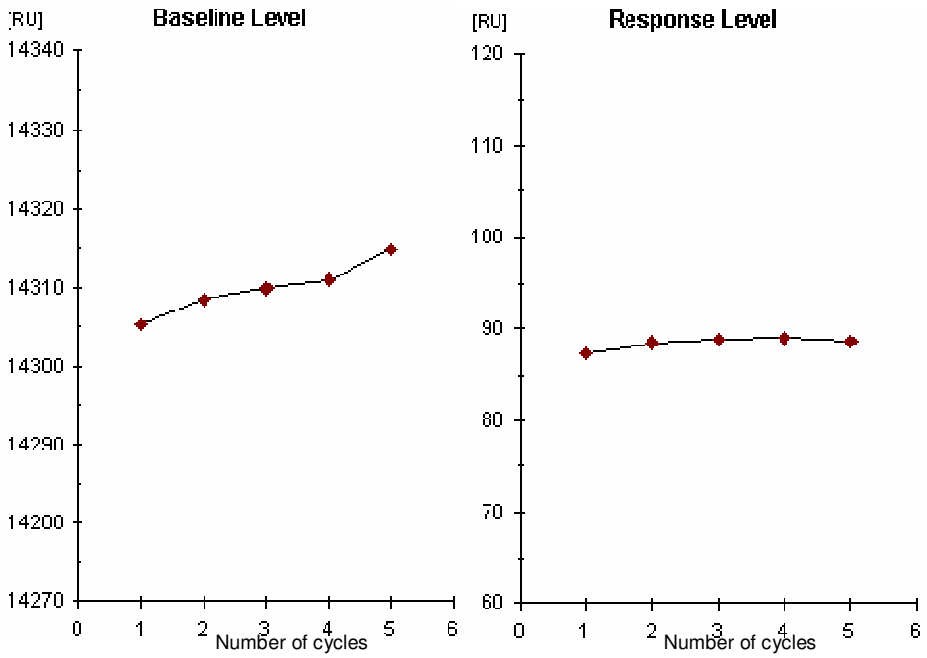


Figure 4. Baseline and response levels (Y axis) during 5 consecutive cycles (X axis) of analyte addition (5 µL/min, 1 min) followed by regeneration solution (10 mM glycine, pH 2.5, 5 µL/min, 1 min) performed on LLD of CM5 chip using BIAcore 3000. The baseline and response levels shown in the figure have been reference subtracted.

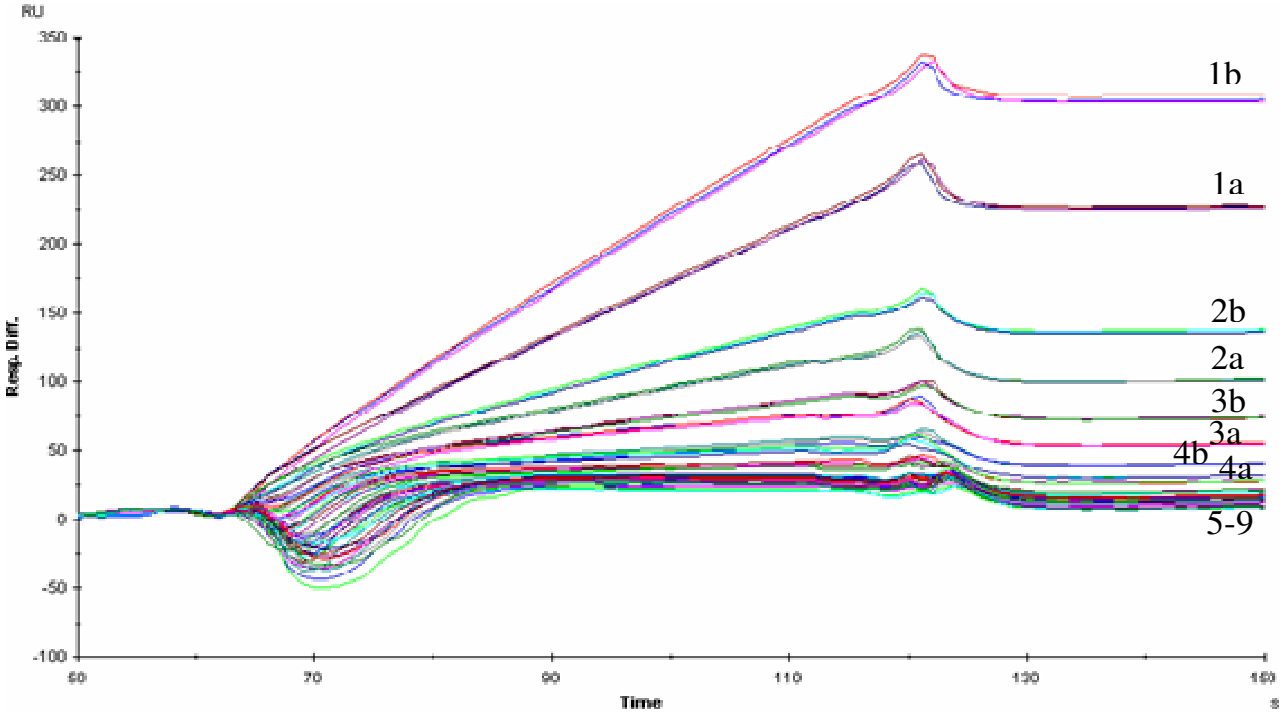


Figure 5. Response of different IFN-α2b concentrations passed on LLD and HLD of CM5 chip using BIAcore 3000. The responses 1 to 9 (reference subtracted) were generated by passing the standards (in triplicates) from 150 to 0 ng/mL to both LLD and HLD. The standards were diluted serially from 150 to 1.17 ng/mL and running buffer was used for 0 ng/mL. Symbols a and b refer to the response of LLD and HLD for the particular IFN-α2b concentration. The concentrations with respect to the points 1 to 9 are 150, 75, 37.5, 18.75, 9.38, 4.69, 2.34, 1.17 and 0 ng/mL.

Table 1. Calculation of binding rate, end point response and binding ratio of both ligand densities for different IFN-α2b concentrations.

IFN-α2b concentration ng/mL	Binding rate RU/S		End point response RU		Binding ratio		Increase in response (%)
	LLD	HLD	LLD	HLD	LLD	HLD	
150	3.92	5.18	224.31	303.46	0.121	0.114	35
75	1.62	2.20	100.14	137.64	0.054	0.052	37
37.5	0.75	1.07	53.45	73.61	0.029	0.028	38
18.75	0.32	0.51	30.21	42.57	0.016	0.016	41
9.38	0.17	0.29	20.46	29.33	0.011	0.011	43
4.69	0.02	0.09	13.92	20.16	0.008	0.008	45
2.34	0.01	0.09	12.91	18.04	0.007	0.007	40
1.17	0.01	0.06	12.80	17.62	0.007	0.007	38
0.00	-0.10	-0.04	8.99	10.95			22

The binding rate and end point response were taken from the Figure 5. Binding rate was calculated from the average response rate between 95 and 110 s. End point response was calculated from the difference between after 100 s and before 30 s of injection. Binding ratio and increase in response was calculated using equation 1 and 2. Percentage Coefficient of Variance (%CV) was found to be less than 6% for all the standards using end point response where as in the binding rate less than 8% CV obtained for standards more than 9.38 ng/mL.

Table 2. Quantification of IFN-α2b in the samples using binding rate and end point response standard curves from HLD (Figure 6).

Sample no	Binding rate (RU/s)	Calculated value (ng/mL)	End point response (RU)	Calculated value (ng/mL)
1	1.73	54.24	93.24	45.14
2	4.01	119.97	222.53	111.97
3	5.59	165.81	309.98	157.17
4	4.36	130.26	237.01	119.45
5	1.95	60.61	105.51	51.48
6	5.79	171.47	317.26	160.93
7	2.51	76.73	132.10	65.23
8	2.45	74.92	144.35	71.56
9	2.66	81.09	149.78	74.37
10	3.38	101.92	188.57	94.42
11	2.67	81.33	146.34	72.59
12	5.12	152.11	265.86	134.36
13	2.58	78.86	149.88	74.41
14	1.50	47.70	89.73	43.33
15	4.03	120.56	227.98	114.79

Samples were run in duplicates and the average was reported here. The %CV of all the samples was less than 10%.

reference cell. Similar problem was also reported by Gonzales et al. (2002). However, this situation did not affect the actual assay.

Standard curve and sample determination

A standard curve was constructed using IFN-α2b standard at concentration ranging from 0 to 150 ng/mL where a linear relationship was observed with respect to its RU. In fact, linearity was found up to the maximum concentration analyzed (400 ng/mL). Both the initial binding rate (slope) and the end point measurement could be taken in constructing the standard curve (Figure 6). As shown in Table 2 both measurements gave comparable results. While additional steps were needed to calculate the binding rate, there was no further step to generate the end point measurement. Moreover, the slope end point measurement was high with respect to the binding rate. Hence the calibration and quantification using end point

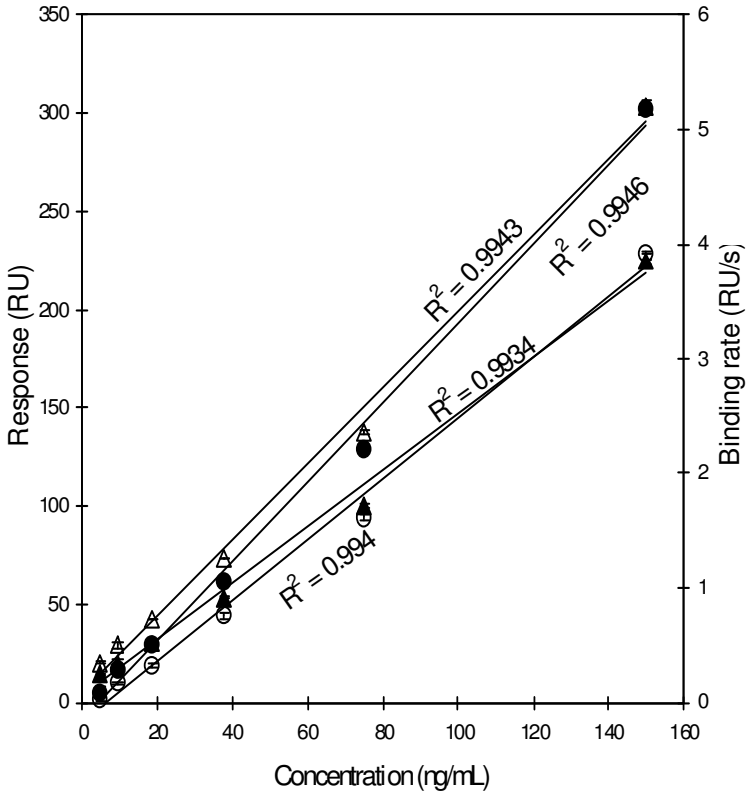


Figure 6. Standard curves using end point response and binding rate for both ligand densities. The values from Table 1 were used to construct the standard curve. The error bar shows the standard deviation among the samples, run in triplicates. Symbols: (▲) End point response for LLD, (∆) End point response for HLD, (○) Binding rate for LLD, (●) Binding rate for HLD.

measurement is suggested to be used for the routine analysis.

Conclusion

Results from this study have demonstrated that the surface Preparation is a key step in SPR analysis. With the proper design, the sensitivity could be increased and thereby reducing the cost of analysis. The sensitivity of the analysis could be increased by immobilizing higher ligand density. The quantification of IFN-α2b in the samples could be analyzed by the determination of end point measurement. This is mainly due to the interaction between the IFN-α2b and anti-IFN is predominantly controlled by mass transfer rate. Further work will be carried out to validate the assay as per the ICH guidelines.

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