Amplified Immunoassay of Human IgG Using Real-time Biomolecular Interaction Analysis (BIA) Technology

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An automated biomolecular interaction analysis instrument (BIAcore) based on surface plasmon resonance (SPR) has been used to determine human immunoglobulin G (IgG) in real time. Polyclonal anti-human IgG antibody was covalently immobilized to a carboxymethyldextran-modified gold film surface. The samples of human IgG prepared in HBS buffer were poured over the immobilized surface. The signal amplification antibody was applied to amplify the response signal. After each measurement, the surface was regenerated with 0.1 mol/L H_3PO_4. The assay was rapid, requiring only 30 min for antibody immobilization and 20 min for each subsequent process of immune binding, antibody amplification and regeneration. The antibody immobilized surface had good response to human IgG in the range of 0.12—60 nmol/L with a detection limit of 60 pmol/L. The same antibody immobilized surface could be used for more than 110 cycles of binding, amplification and regeneration. The results demonstrate that the sensitivity, specificity and reproducibility of amplified immunoassay using real-time BIA technology are satisfactory.

Keywords amplified immunoassay, biomolecular interaction analysis, surface plasmon resonance, immunoglobulin G

Introduction

The BIAcore instrument, often named as biomolecular interaction analysis (BIA) technology, has been developed into a strong tool for the quantitative and qualitative characterization of reversible interactions between biological macromolecules.[1,2] Its detection principle is based on surface plasmon resonance (SPR), an optical phenomenon arising at interface between a thin gold film and a dielectric medium under the conditions of total internal reflection. Surface plasmon resonance occurs only at a specific angle of incidence named as SPR angle.[3] In real-time BIA, light (wavelength is 760 nm) from a near-infrared light-emitting diode (LED) is focused through the prism on to the sensor chip surface in a wedge-shaped beam, giving a fixed range of incident light angles. The computer interpolation algorithms determine the angle of minimum reflection (the SPR angle) to high accuracy. When protein molecules bind to the sensor chip surface, it will cause the change of refractive index of the surface. Therefore, it will result in the change of the SPR angle. Monitoring the change of SPR angle can provide a real-time measurement of changes in the surface concentration at the sensor surface during the procedure of molecule interaction.[4] In this work, goat anti-hIgG was immobilized on the sensor chip surface, then the hIgG solution was flowed over the surface. Because of the specific interaction of antibody and antigen, the hIgG molecules were captured onto the sensor chip surface and resulted in the change of SPR angle.

Currently, the BIA technology has been widely used in a broad variety of fields. These include receptor-ligand interactions, antigen-antibody interactions and antibody engineering, protein-DNA, protein-carbohydrate and DNA-DNA interactions, signal transduction, cell adhesion molecules, lipid vesicles or planar bilayers *etc*. These applications in different fields have been reviewed

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recently.[1,5-7] The main advantages of this technology include real-time monitoring, label-free detection, rapid analysis, reusable sensor chip, exact sample handling, small sample volume *etc*.[8] However, the major disadvantage of this technology for bioanalytical applications is its difficulties for determination of small molecules or analytes in low concentration.[9-11] The commercial sensor chips, which couple a layer of carboxymethyldextran matrix to a gold surface, could increase reasonably surface loading of biomolecules.[7,8,12-15] Depite the signal amplification attained by dextran matrix, the sensitivity of BIA technology was still finite. In other SPR-based biosensors, some strategies have been proposed to improve the sensitivity by using latex particle[16] colloidal Au,[17] liposome[11] or streptavidin-biotinylated protein complex.[18] These amplifying substrates are about the same as or lager size than the dextran layer of BIA technology and thus can not penetrate into the matrix. Therefore, these enhancing strategies are not suitable for dextran-modified sensor chips. The sandwich method by antibody amplification can be used to enhance the sensitivity of an immune binding level assay. Furthermore, it is very simple, effective and easy to implement. However, there are only a few reports, which show the simple applications of the sandwich method to enhance the response signal.[8,14,16] This paper reports the amplified immunoassay of human immunoglobulin G (hIgG) using real-time BIA technology. The sandwich method by antibody amplification was applied to amplify the response signal. The amplifying effect and sensitivity, specificity and reproducibility of this amplified immunoassay were investigated. As a model system, the determination of hIgG was carried out by this BIA technique.

Experimental

Instrument and reagents

The BIAcore 1000 system, sensor chip CM5 (a carboxymethyldextran hydrogel coupled to a gold-coated glass surface according to the method described elsewhere[7]), HBS buffer (pH 7.4, consisting of 10 mmol/L 4-[2-hydroxyethyl] piperazine-1-ethane-sulfonic acid, sodium chloride (150 mmol/L), EDTA (3.4 mmol/L), 0.005% (V/V) surfactant P-20), and amine coupling kit containing N-hydroxysuccinimide (NHS), N-ethyl-N'-(3-diethylaminopropyl) carbodiimide (EDC), and ethanolamine hydrochloride were obtained from Pharmacia Biosensor AB (Uppsala, Sweden).

hIgG was obtained from the Institute of Microbiology, Chinese Academy of Military Medicine Science (Beijing, China). Purified goat anti-hIgG antibody was purchased from Sina-Am Biotech. Co. (Beijing, China). Bovine serum albumin (BSA) and mouse immunoglobulin G (mIgG) were purchased from Sigma (St Louis, USA). All other reagents and solvents were analytical reagent grade and MilliQ-grade water was used. All solutions prepared were filtered (0.22 μm) and thoroughly degassed prior to use.

Antibody immobilization

A constant flow rate of 5 μL/min and a temperature of 25 ℃ were chosen for immobilization procedure and other operations subsequently. Antibody immobilization was performed according to the general procedure described elsewhere.[15]

Amplified immunoassay

The immobilized antibody surface was used to determine the hIgG concentration. The sample (40 μL) was diluted with HBS and poured over the immobilized surface by autosampler injection of 40 μL. Goat anti-hIgG antibody (40 μL, 0.5 mg/mL) was subsequently injected to amplify the response signal. Response value was read 40 s after the end of the sample injection. After each measurement, the surface was regenerated by injection of H_3PO_4 (5—15 μL, 0.1 mol/L). The amplified response (ΔR) was measured as the change of the SPR signal before and after each injection of the amplification antibody.

Results and discussion

Antibody immobilization on sensor chip surface by amine coupling method

Amine coupling is used for antibody immobilization. N-hydroxysuccinimide esters were introduced into the surface matrix by modification of the carboxymethyl groups with a mixture of NHS and EDC by amine coupling. These esters then reacted spontaneously with amines of the antibody protein which was concentrated into the matrix by electrostatic adsorption. Subsequence to the coupling reaction of antibody to activated surface, 1 mol/L

ethanolamine hydrochloride was injected to deactivate the unreacted esters, and then, H_3PO_4 (0.1 mol/L) was injected to desorb noncovalently bound protein from the sensor surface.[19]

Several parameters such as protein concentration, protein solution ionic strength, pH, reaction time, and reagent concentration can influence the immobilized amount of protein ligand. Among them, solution ionic strength and pH are the main effect factors which can affect the electrostatic attraction force between the negatively charged hydrogel matrix and positively charged proteins, which can concentrate the protein ligand into the matrix. Therefore, a low ionic strength and proper pH value below the isoelectric point of the protein can enhance the immobilization amount of the protein ligand.[20,21] Here we use acetate buffer (10 mmol/L) at pH 5.6 with antibody protein solution (0.1 mg/mL) for the immobilization after optimization to these parameters.

The SPR response of such an activation-concentration-coupling-deactivation-desorption sequence of goat anti-hIgG antibody immobilization is shown in Fig. 1. Antibody was immobilized in a solution of acetate buffer (10 mmol/L) at pH 5.6 with goat anti-hIgG antibody (0.1 mg/mL). A merit of the BIA technique for immobilization is that the response of the whole immobilization procedure can be followed in real time. The immobilized amount of anti-hIgG antibody (ΔR) is about 9160 RU after complete washing. The estimated surface concentration of the immobilized antibody on the sensor chip surface was calculated to be 5.73×10^{-12} mol/cm^2 (according to the correlation of SPR response with surface protein concentration,[4] 1 kRU = 1 ng/mm^2, and the molecular weight of the anti-hIgG antibody, about 160,000 Da). The theoretical maximum coupling amount of IgG on a flat surface to form a dense stack monolayer is about 2.37×10^{-12} mol/cm^2 (with an end-on orientation as reported[22,23]) according to X-ray crystallographic data of IgG (10 nm × 14 nm × 5 nm).[24] The results show that the dextran matrix on sensor chip surface enhances the immobilization capacity of biomolecules. Antibody immobilization is highly reproducible under the same conditions with an RSD of 3.5% ($n = 4$).

Influence of hIgG concentration on the immune reaction time of primary response

The influence of the concentration of hIgG on the immune reaction time between hIgG and immobilized antibody is illustrated in Fig. 2. The reaction procedure of hIgG in different concentrations was monitored in real time based on the advantage of BIA technique. From the sensorgrams of immune reaction, the influence of hIgG concentration on the initial binding rate of immune reaction and reaction equilibrium time can be followed by direct visual observation of binding events at the immobilized surface. As shown in Fig. 2, the initial binding rate of analyte is more rapid at higher concentration and a stable equilibrium binding level would be reached during a shorter time.

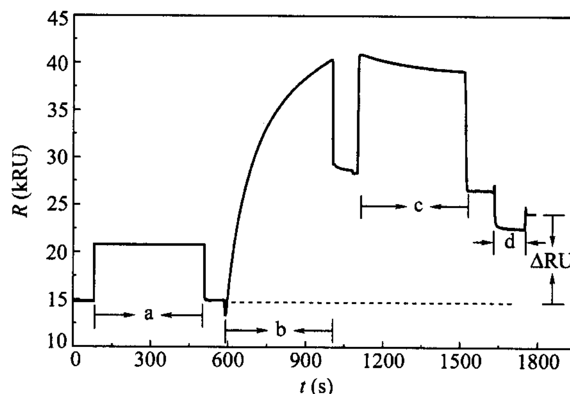

Fig. 1 Sensorgram showing the procedure and response of goat anti-hIgG antibody immobilization on sensor surface. The captions indicate injections, (a) 35 μL of NHS (0.05 mol/L)/EDC (0.2 mol/L), (b) 35 μL of 0.1 mg/mL goat anti-hIgG antibody (pH 5.6, 10 mmol/L acetate buffer), (c) 35 μL of ethanolamine (pH 8.5, 1 mol/L), (d) 10 μL of H_3PO_4 (0.1 mol/L).

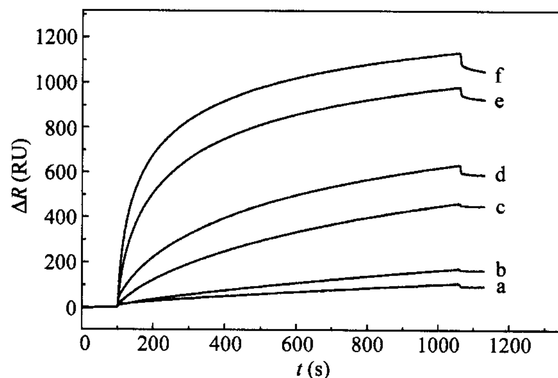

Fig. 2 Overlay of six sensorgrams showing the procedure of real time binding of hIgG in different concentrations ((a) 3 nmol/L, (b) 6 nmol/L, (c) 30 nmol/L, (d) 60 nmol/L, (e) 300 nmol/L, (f) 600 nmol/L as indicated) to the antibody immobilized surface.

Amplified effect by the amplification antibody

The amplified effect of the amplification antibody with different concentrations was investigated in real time. Different concentrations (0.05, 0.1, 0.2 and 0.5 mg/mL) of goat anti-hIgG antibody were separately poured over the sensor surface after each immune reaction of 30 nmol/L hIgG. As shown in Fig. 3, the real time sensorgram gives clear impression on the amplifying effect at different concentrations of the amplification antibody (goat anti-hIgG antibody). Fig. 4 shows the amplified response signals as a function of the primary response signals of hIgG at different concentrations. The slope of each line represents the amplifying effect of different concentrations of the amplification antibody respectively. The enhancement times appeared to depend on the concentration of signal amplification antibody used. With concentration of 0.5 mg/mL of this antibody, the highest enhancement could be obtained.

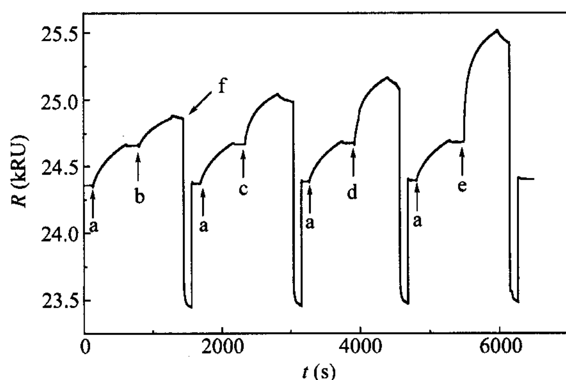

Fig. 3 Sensorgram showing the immune reaction, antibody amplification and regeneration by signal amplification antibody at different concentrations. First, 40 μL of hIgG (30 nmol/L) (a) was injected across antibody immobilized surface, then 40 μL of signal amplification antibody at different concentrations of 0.05 mg/mL (b), 0.1 mg/mL (c), 0.2 mg/mL (d), 0.5 mg/mL (e), finally followed by 10 μL of H_3PO_4 (0.1 mol/L) (f), respectively.

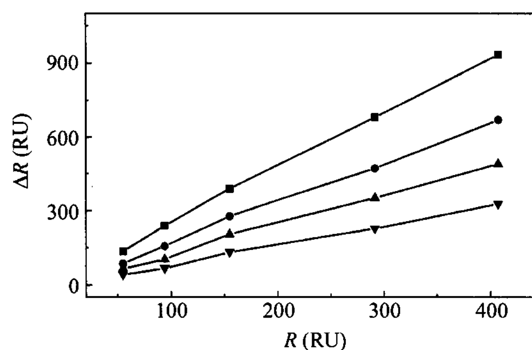

Fig. 4 Amplified response of hIgG by the signal amplification antibody in different concentrations versus primary response of hIgG. The concentrations of the enhanced antibody are (▼) 0.05 mg/mL, (▲) 0.1 mg/mL, (●) 0.2 mg/mL, (■) 0.5 mg/mL, respectively. The values of primary response are measured to hIgG samples with concentrations of 3, 6, 12, 30 and 60 nmol/L.

Amplified immunoassay of hIgG

After the immobilization, the target analyte (hIgG) and the amplification antibody (goat anti-hIgG, 0.5 mg/mL) are poured over the surface subsequently. The sensorgrams of SPR response due to antibody-antigen reaction are recorded in real time (Fig. 5). The response signal is amplified based on the sandwich method by introducing the analyt-specific antibody for better sensitivity and specificity of the analyte concentration assay.[14] After completing one assay, the sensor surface is regenerated by H_3PO_4 (0.1 mol/L) for the following assays. Often, a fraction of analyte bound remained on the surface after each regeneration step due to incomplete desorption. This may be too small to affect the primary response signal, but causes interference for the amplified assay. Therefore the amplified response signal was corrected by the bulk response in our experiments. Fig. 6 shows the primary response and the amplified response of the analyte as a function of protein concentration. According to the primary response without amplifying operation, the immobilized antibody surface shows a response to hIgG in the range of 0.6—60 nmol/L. The detection limit is determined to be less than 0.3 nmol/L. However, when the signal amplification antibody was applied to enhance the response signal, hIgG protein could be detected in a range of 0.12—60 nmol/L with a detection limit of 60 pmol/L. We know that the change of the SPR signal normally depends on the change of surface mass concentration of protein on the sensor chip surface. Therefore, small molecules and low concentrations can not be detected directly, because binding to the surface hardly changes the mass concentration. The binding of amplification antibody (goat anti-hIgG) to the target analyte can enhance the mass concentration of the sensor chip surface. Therefore, it can enhance the sensitivity of the detection.

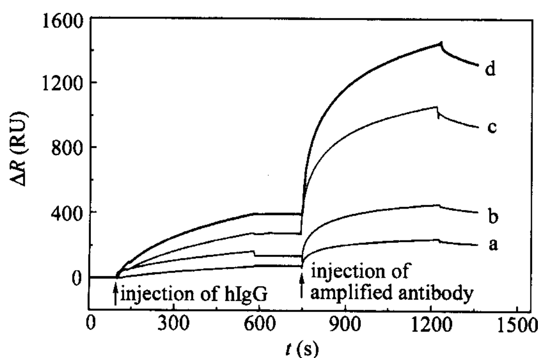

Fig. 5 Overlay of four sensorgrams showing the procedure of immune reaction and antibody amplification. 40 μL of hIgG protein at different concentrations of 6 nmol/L (a), 12 nmol/L (b), 30 nmol/L (c), 60 nmol/L (d) (as indicated) was injected across the antibody immobilized surface, then 40 μL of signal amplification antibody of 0.5 mg/mL was injected, respectively.

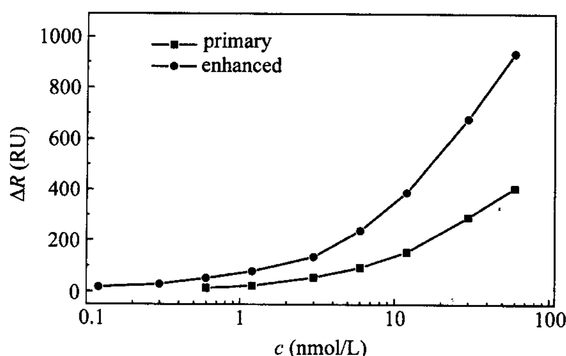

Fig. 6 Primary response and amplified response of hIgG versus hIgG concentration.

Specificity of amplified immunoassay

Apart from enhancing the response signal, the amplification antibody may increase the specificity of immunoassay, especially, when an analyte of low concentration is measured in crude samples with a high background noise.[14,25] The goat anti-hIgG antibody immobilized surface was tested for the cross-reactivity with sheep serum and other proteins such as bovine serum albumin (BSA) and mouse IgG (mIgG). After the non-specific reaction, the signal amplification antibody was injected to flow over the sensor surface. The primary response was about 13 RU for BSA (1 mg/mL), compared to 58 RU for mIgG (0.5 mg/mL). After the injection of the signal amplification antibody, the enhanced response is less than 10 RU for both BSA and mIgG. The primary response is about 127 RU for sheep serum, however, the amplified response is only 23 RU. These results show that the specificity of immunoassay has also been increased greatly by using the amplification antibody.

Reproducibility of amplified immunoassay

The same immobilized surface was used for a series of measurements after H_3PO_4 (0.1 mol/L) regeneration.[19] The amplifying immunoassay of hIgG (30 nmol/L) was carried out for 110 cycles on the same immobilized surface. As shown in Fig. 7 (A), an upward drift of the baseline of about 1300 RU can be observed for 110 cycles. This is mainly due to the incomplete elution of molecules bound that leads to a non-specific accumulation in the matrix. In Fig. 7 (B), a decrease of the response signal of about 22.8% is observed over 110 cycles. The average loss of reactivity per regeneration is 0.21%. The relative standard deviation of assays is 6.9% for 110 assays. The recovery of hIgG standard sample added to human serum (1 : 1000 dilution) is determined to be 96.4%—102.3%.

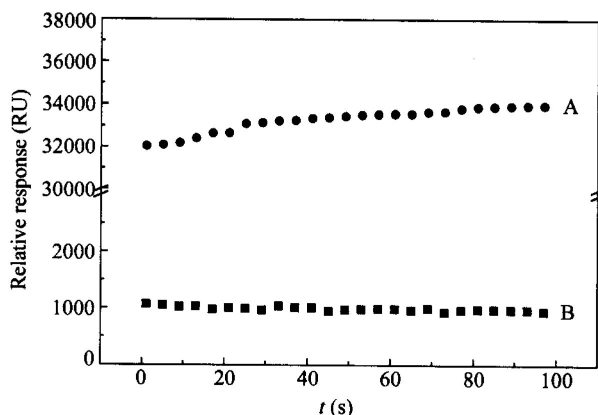

Fig. 7 Regeneration effect of the antibody immobilized surface by 10 μL of H_3PO_4 (0.1 mol/L) elution reagent. (A) represents the baseline data obtained after each acid elution cycle as a function of cycle number. (B) represents the response data on the binding of hIgG (30 nmol/L) solution to the same antibody immobilized sensing surface as a function of cycle number.

Conclusion

Real-time BIA technique has been used to the determination of hIgG protein. The sandwich method by antibody amplification was applied to enhance the response

signal. This amplifying strategy is very simple, effective and easy to implement. Apart from enhancing the response signal, the amplified antibody could increase the specificity of immunoassay. Human IgG protein could be detected in the range of 0.12—60 nmol/L with a detection limit of 60 pmol/L.

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