

Quantification of Interleukin-6 in cell culture medium using surface plasmon resonance biosensors

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ABSTRACT

Interleukin (IL)-6, a multifunctional cytokine, is widely used as an index for illnesses such as inflammatory and autoimmune disorders, coronary artery disease, neurological disease, and gestational problems. It is thus very important to be able to precisely quantify the level of IL-6 for disease diagnosis and any subsequent therapy. Surface plasmon resonance (SPR) biosensors are sensitive in detecting the interaction between biomolecules by sensing the changes in the refractive index on the sensor chip. This study investigated the SPR technique to determine the IL-6 secretion of human fibroblast MRC5-CVI cells induced by lipopolysaccharide (LPS). To reduce non-specific binding, a mixed self-assembled monolayer of mercaptoundecanoic acid (MUA) and mercaptohexanol (MCH) was attached to the sensor, and then used for IL-6 determination using a sandwich type immunoassay. In addition, two antibody immobilization methods were applied to the sensor surface—direct immobilization and indirect immobilization via protein G affinity.

The results demonstrated that the direct immobilization method had a better antibody binding capacity on the sensor surface. The level of cellular IL-6 secretion detected by the SPR biosensor showed a consistent correlation with the commercial kit of IL-6 enzyme-linked immunosorbent assay.

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1. Introduction

Interleukin (IL)-6, a 20 kDa molecule, is a multifunctional cytokine which acts on inflammation, acute-phase reaction, growth regulation and differentiation of various cell types [1]. This cytokine is produced by lymphocytes, fibroblasts, endothelial cells, and keratinocytes in response to injury, infection, burns, trauma and a variety of stimulants (e.g. lipopolysaccharide (LPS), IL-1, tumor necrosis factor (TNF) alpha, and platelet-derived growth factor (PDGF)) [2–4]. Aberrant expression of IL-6 causes several diseases including inflammatory and autoimmune disorders, coronary artery disease, neurological disease, and gestational problems [5]. Elevated IL-6 levels have been observed in most types of cancer [6]. It is very important to determine the precise concentration of IL-6 for both disease diagnosis and subsequent therapy.

Most assays used to detect IL-6 are labeled assays such as enzyme-linked immunosorbent assay (ELISA) [7], radioimmunoassay [8] and fluorescence assays [9]. The drawback of these label-based assays is that they are time-consuming, hazardous and require complicated manipulations. ELISA is frequently used to detect the IL-6 level in serum or cell culture medium [7], and possesses high specificity and sensitivity with a detection limit up to

2 pg/mL [10]. However, it is time-consuming, requiring 6–8 h during the analysis process.

Surface plasmon resonance (SPR) biosensors, have been extensively explored during the past two decades, are sensitive enough to detect the small changes in the refractive index (RI) near the sensor chip. With non-specific blocking, the small RI changes can be directly detected presenting the on-going specific interactions [11]. Furthermore, SPR biosensors are useful for sensing the presence and the amount of analytes, as well as for measuring the kinetics of association/dissociation and the affinity constant of ligand–analyte interactions [11,12].

It is crucial that physicians can monitor the level of biological markers in real-time so that they can better understand the progression of an illness. SPR biosensors have the advantage that they are an optical method with the unique features of being non-destructive, highly sensitive and of providing rapid detection without labeling. SPR biosensors perform excellent with respect to accuracy, precision and robustness [13]. However, it remains a challenge when using SPR to assay the analytes of interest in complex biological fluids such as serum, blood plasma, saliva or a cell culture medium, because non-specific binding reduces the detection sensitivity at extreme low concentrations. Non-specific binding of molecules to the sensor chip surface causes false signals to mask the real signal from the analytes. This is a major obstacle for the clinical application of SPR biosensors [14]. In addition, the

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random orientation of the immobilized antibodies affects biological activity and reduces the real signal [15]. To reduce non-specific binding and enhance the sensitivity of the biosensors, two methods are commonly being used in other studies. One method is for the antibody to be covalently bound to a mixed self-assembled monolayer (SAM) using mercaptoundecanoic acid (MUA) and 6-mercapto-1-hexanol (MCH) on a gold surface [16]. Briand et al. [16,17] found a very weak non-specific binding on the mixed SAM modified sensor chip with the 1:3 ratio of MUA and MCH to immobilize antibody. The other method is the sandwich type immunoassay, in which two different antibodies are used. One of the antibodies is first immobilized on the chip, and then antigen and another antibody are flowed through the chip successively to enhance the sensor's sensitivity and specificity.

The purpose of the present study was to determine the IL-6 secretion of human fibroblast MRC5-CVI cells induced by LPS using the SPR technique. The sandwich type immunoassay was adapted in this study since the secondary antibody, which had bound to the primary antibody on the SPR sensor chip, could enhance both the sensor sensitivity and specificity. Two antibody immobilization methods were applied for investigating the detection efficiency of the SPR biosensors in relation to the orientation of the immobilized antibody on the sensor chip: (a) direct antibody immobilization on the mixed SAM and (b) indirect antibody immobilization to the Fc-binding domain of protein G on the mixed SAM. In addition, the detection results of the SPR biosensor were also compared, using a commercial IL-6 ELISA kit.

2. Materials and methods

2.1. Instrument and chemicals

A Biacore 3000 (GE Healthcare, Pittsburgh, PA) was set at 25 °C for all steps during the analytical process, and experimental data were collected at a medium rate. Biacore sensorgrams were analyzed using the BIAevaluation software.

11-Mercaptoundecanoic acid (MUA) and 6-mercapto-1-hexanol (MCH) were purchased from Sigma–Aldrich (St. Louis, MO). Protein G was purchased from BioVision (Mountain View, CA). Affinity purified anti-human IL-6 antibody (primary antibody), biotin anti-human IL-6 antibody (secondary antibody), recombinant human IL-6 (standard protein), and human IL-6 ELISA Ready-SET-GO were purchased from eBioscience (San Diego, CA). Sensor chips (SIA KIT Au) and amine-coupling reagents, ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and ethanolamine HCl, were purchased from GE Healthcare.

2.2. Chip preparation

Sensor chips were pretreated with a piranha solution ($\text{H}_2\text{O}_2:\text{H}_2\text{SO}_4 = 1:3$) and rinsed with absolute ethanol for 15 min before adsorption. The chips were immersed in a binary mixture of 2.5 mM MUA and 7.5 mM MCH in absolute ethanol for 3 h to ensure an optimal homogeneity of the mixed adsorbed layer. The mixed SAM chips were subsequently rinsed in ethanol and dried under a flow of clean air. Except for the above step, other immobilization procedures were completed using the SPR instrument.

2.3. Buffer preparation

Phosphate buffered saline (PBS) with pH 7.4 (2.7 mM KCl, 1.76 mM KH_2PO_4 , 137 mM NaCl, 10 mM Na_2HPO_4 , 0.005% Tween 20) was prepared in Millipore water. PBS was used as the running buffer for IL-6 antibody immobilization with protein G. PBS buffer

with 0.02% BSA was used as a blank buffer, as a running buffer during the binding steps, and for the preparation of IL-6 samples and the secondary antibody.

2.4. Cell culture

Human fibroblast (MRC5-CVI) cells used in this study were obtained from Chang Gung Memorial Hospital. Cells grew in DMEM supplemented with 10% fetal bovine serum (FBS), 2.5% HEPES, 3.7 g/L NaHCO_3 and antibiotics (all from Invitrogen, Carlsbad, CA). A confluent cell number (5×10^4 /well) was seeded in a 6-well plate, and stimulated with 10 $\mu\text{g}/\text{mL}$ LPS under 37 °C and 5% CO_2 for 2 days. The supernatant of culture medium was respectively, collected at 6, 12, 24 and 48 h after LPS stimulation, and reserved at 4 °C for further SPR and ELISA analysis.

2.5. Protein G and IL-6 antibody immobilization

Immobilization and quantification procedures were carried using a Biacore 3000 instrument. Protein G was diluted to 25 $\mu\text{g}/\text{mL}$ with acetic acid buffer at pH 5.5. Protein G and IL-6 antibody were individually immobilized on three flow cell surfaces at different densities (i.e. different densities were generated by applying a different flow-time of immobilization). A low-density surface was generated in flow cell 2, a medium-density surface in flow cell 3, and a high-density surface in flow cell 4. BSA (50 $\mu\text{g}/\text{mL}$) was immobilized on the surface of flow cell 1 to serve as a reference flow cell for the subtraction of the systematic noise and drift of the instrument [18].

The immobilization on the mixed SAM surface was obtained by using amine-coupling chemistry. At a flow rate of 10 $\mu\text{L}/\text{min}$, carboxyl groups were activated using 1:1 ratio of 0.1 M NHS and 0.4 M EDC for 7 min. Then the diluted antibody was injected to couple with the activated surface until the appropriate immobilization level was achieved. Excess activated carboxyl groups were blocked by 1 M ethanolamine (pH 8.5) for 7 min.

2.6. Preparation of IL-6 and secondary antibody

Solutions of IL-6 standard protein and secondary antibody were prepared in PBS/0.02% BSA buffer. The stock concentration of IL-6 standard protein was 100 ng/mL, and diluted into the serial concentrations (0.78–12.5 ng/mL) by culture medium. A secondary antibody was prepared at a concentration of 10 $\mu\text{g}/\text{mL}$. PBS/BSA buffer was also used to equilibrate the injection signal at the beginning of the SPR assay, and served as a blank injection throughout the experiments.

2.7. SPR assay of IL-6 concentration

The IL-6 level of the standard protein solution and cell culture supernatants were sensed by two antibody immobilization methods: direct and indirect (i.e. via protein G). In the direct method, the IL-6 antibody was directly immobilized on the mixed SAM surface. IL-6 solution was injected consecutively into four flow cells for 3 min at a flow rate of 20 $\mu\text{L}/\text{min}$, and then a volume of 60 μL 10 $\mu\text{g}/\text{mL}$ secondary antibody was eluted to enhance the response signal. The used SAM surface was regenerated by two short pulses of glycine–HCl solution (pH 2.5). In the indirect method, the protein G was immobilized on the mixed SAM surface, and then 2.5 $\mu\text{g}/\text{mL}$ IL-6 antibody was flowed over the surface for 3 min at a flow rate of 20 $\mu\text{L}/\text{min}$. The SPR signal can be reduced by IL-6 antibody dissociation as a result of non-covalent binding. To minimize this possibility, a 10 min PBS/BSA buffer elution was carried out before the injection of the IL-6 solution. The following steps were the same as the direct method mentioned above.

The first 5 binding cycles of the buffer solution were used to equilibrate the instrument and minimize the drift as a result of temperature fluctuation. The IL-6 solutions were injected according to their respective concentration. Each experiment was performed in triplicate to confirm the reproducibility of the assay. The limit of detection was defined as three times the standard deviation of the average value of the blank injection. The IL-6 standard curve from the serial concentration of the standard protein solution was used to calculate the IL-6 concentration of the cell culture supernatant.

2.8. ELISA determination of supernatant IL-6

A commercial ELISA was used to evaluate the accuracy of the IL-6 level determined by the Biacore SPR sensor. The supernatant from the cultured cells was collected and centrifuged at 1500 rpm (10 min, 4 °C). The concentration of IL-6 was determined using the corresponding ELISA Ready-SET-GO kit (eBioscience). The optical density in each kit well was detected using a VERSAmix microplate reader (Molecular Devices, Sunnyvale, CA) and the IL-6 level which was deduced from the absorbance value by extrapolation from a standard curve generated in parallel.

3. Results and discussion

3.1. Immobilization of IL-6 antibody ligand

In both the direct and indirect antibody immobilization methods, flow cell 1 with immobilized BSA on the surface served as the reference flow cell. IL-6 antibody and protein G were immobilized at three different densities, “high”, “medium”, and “low” relative to the RU value, on the other three flow cells in order to provide the information relevant to the sensitivity of the ligand density. The immobilization density was accomplished right after a serial pulse of ligand was flowed over the activated surface until the expected immobilization level was achieved. For example, in the indirect antibody immobilization method, the –COOH group on the chip surface was reacted with NHS and EDC, and protein G was injected into the flow cell to activate the chip surface for immobilization. After three injections, the signal was saturated, and the protein G injection into the flow cell was stopped. The un-reacted esters were then deactivated by 1 M ethanolamine HCl (pH 8.0). The injection time of BSA was controlled by checking the signal, and injection was stopped when the signal was saturated. The low immobilization density of IL-6 antibody and protein G in flow cell 2 was 1331 RU and 574 RU, respectively. The medium immobilization density of antibody and protein G in flow cell 3 was 2268 RU and 1002 RU, respectively. The high immobilization density of antibody and protein G in flow cell 4 was 2768 RU and 1300 RU, respectively. The immobilization density of BSA in flow cell 1 was 1908 RU and 1651 RU, respectively.

3.2. Detection of IL-6 standard protein by direct antibody immobilization

The IL-6 standard protein solution was diluted by culture medium and detected at a concentration range from 0.78 to 12.5 ng/mL. Fig. 1 shows the real-time sensorgram of 12.5 ng/mL IL-6 detection and enhancement assay (10 µg/mL secondary antibody in running buffer) in flow cell 4. A sudden increase and decrease of the signal at the beginning and end of each injection was due to the time difference in each flow cell and the refractive index change caused by the culture medium and the running buffer. For saving the overall detection time and the amount of sample being used, we set a shorter waiting time (i.e. 180 s) for the sample injection. After finishing the secondary antibody injection, the surface was flowed with buffer for 3 min to wash out the unspecific

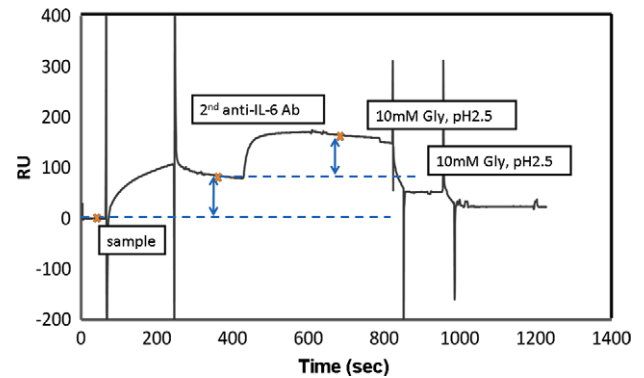


Fig. 1. Sensorgram of IL-6 detection by direct antibody immobilization. The result of flow cell 1 was subtracted from that of flow cell 4.

binding. In the last regeneration step, we chose the 10 mM glycine (pH 2.5) as the regeneration solution due to its good performance.

In this direct antibody immobilization method, the non-specific binding in response to the culture medium was 57 RU. This response was higher than the signal of a buffer injection (5 RU, data not shown). Although the mixed SAM chip was coated with BSA, the non-specific binding remained. Fig. 2 shows that the SPR binding signal depended upon the IL-6 concentration and that it was enhanced by secondary antibody. Fig. 2 shows the positive linear correlation between the IL-6 level and the enhanced signal ($R^2 = 0.956$). Unfortunately, the primary signal (i.e. without 2nd antibody) shows a nonlinear and negative response to the IL-6 level. This suggests that the culture medium containing 10% FBS will lead to competitive binding and interfere with the binding of the primary antibody with the IL-6 protein. Because the IL-6 has smaller molecular weight than FBS, the competition between IL-6 and FBS will decrease the signal in the low level of IL-6. The sandwich type method for immobilizing antibody not only amplifies the detection signal but also increases the specificity. The results reflect the combined specificities showing that the primary antibody was specifically bound to the analyte and that the secondary antibody was bound to the primary antibody. The SPR signals corresponding to the secondary antibody binding to IL-6 at various antibody immobilization levels (i.e. High, medium, and low) were also investigated (not shown in the figure). At the same IL-6 concentration, different levels of immobilization did not provide a significant difference in sensitivity.

3.3. Detection of IL-6 standard protein by indirect antibody immobilization

Fig. 3 shows the real-time sensorgram of IL-6 detection by indirect antibody immobilization. Following the primary antibody

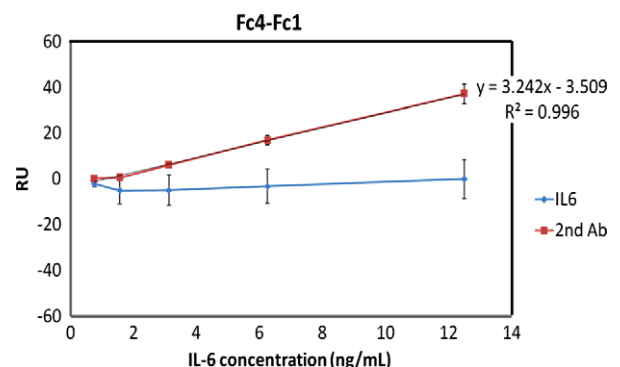


Fig. 2. The IL-6 response and the enhancement by secondary antibody for the direct immobilization of the primary antibody on the surface of the chip.

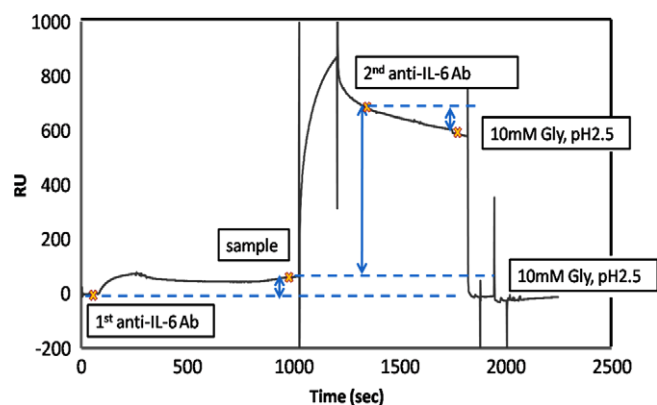


Fig. 3. Sensorgram of IL-6 detection by indirect antibody immobilization. The result of flow cell 1 was subtracted from the result of flow cell 4.

injection, the association time for the primary antibody binding to protein G was set at 10 min, in order to reach the steady state. In this indirect antibody immobilization, the response of non-specific binding was around 860 RU higher than for direct antibody immobilization. The obvious non-specific binding signal might be as a result from FBS which contains IgG bound to protein G which was immobilized on the SAM chip. Protein G has a non-specific conjugation potency, and thus protein G in this study may bind with primary antibody, secondary antibody and with other IgG in a culture medium. It is interesting to note that a linear relationship remained between the SPR signal and the IL-6 concentration following the enhancement by secondary antibody (Fig. 5, $R^2 = 0.996$). This suggests that the sandwich type immunoassay may have enhanced the detection specificity.

Regardless of direct (Fig. 2) or indirect (Fig. 4) immobilization of primary antibody, the linear relationships were only presented for the enhancement of secondary antibody response. The detection limit was calculated as 1.3 and 5.7 ng/mL, respectively, for direct and indirect immobilization. The indirect immobilization had a worse detection limit which might be due to the non-specific binding of FBS to the sensor surface, and because part of the primary antibody binding to protein G. The detection signal of SPR is proportional to the mass on the sensor chip, implying that the amount of protein binding to the sensor chip could be estimated accordingly. The immobilization level of protein G was about 1300 RU. When all of the primary antibodies (160 kDa) were bound to protein G (34 kDa) (1:1), the corresponding SPR signal reached about 6118 RU ($(160/34) \times 1300$). However, the signal only increased by 452 RU after the injection of the primary antibody, which meant that 7.4% protein G on the sensor chip was bound with primary antibody. This low binding ratio corresponds to the higher detection limit of indirect immobilized antibody. The level of secondary antibody used in this study for enhancing the SPR response was 10 μ g/mL, which is a much lower value than in previous studies [19,20]. Consequently, an increased concentration of secondary antibody should be applied to increase the detection limit of indirect immobilized antibody.

3.4. Supernatant IL-6 of cell culture

The supernatant of MRC5-CVI cells was collected for IL-6 analysis after LPS stimulation for 6, 12, 24 and 48 h. These supernatant media were analyzed, respectively by ELISA and SPR assay. Surprisingly, the indirect antibody immobilization method could not correctly quantify the concentration of IL-6 (data not shown) due to the influence of non-specific binding on the sensor surface. As shown in Fig. 5, LPS can stimulate MRC5-CVI cells to secrete IL-6, and the IL-6 level was positively correlated to the time involved in the LPS treatment. The detection patterns based on IL-6 concen-

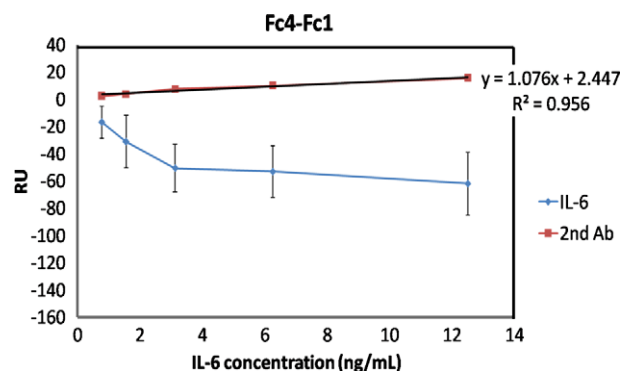


Fig. 4. The IL-6 response and the enhancement by secondary antibody for the indirect immobilization of the primary antibody on the chip surface.

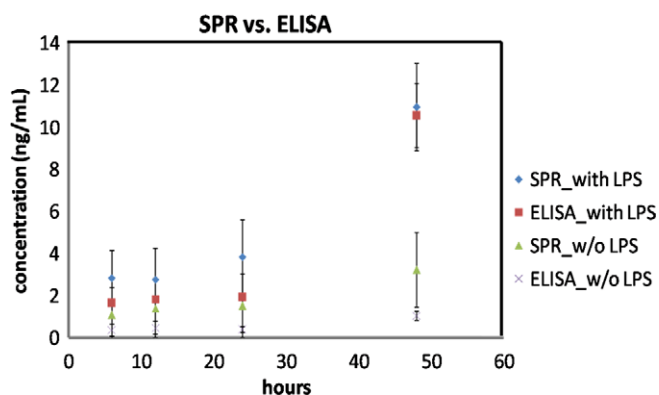


Fig. 5. Quantification of supernatant IL-6 level of MRC5-CVI cells with/without LPS stimulation by ELISA and SPR biosensor.

tration in ELISA and SPR assays were similar. The discrepancy between them was lower than 2 ng/mL, which was within the error bar, and at the IL-6 level it was below 4 ng/mL. At the higher level of IL-6 (above 4 ng/mL), the analytic results of these two detect assays were almost the same. Previous studies [14] demonstrated using SPR to determine the exogenous IL-6 analyst added into biological fluid. To the best of our knowledge, this is the first study to detect the spontaneous IL-6 secretion in cells stimulated by LPS.

4. Conclusion

This study demonstrated that the SPR biosensor successfully quantified the IL-6 level secreted by cells. The cell culture medium was analyzed directly without any prior procedures such as centrifugation or dilution. The sensor surface was modified by mixed SAM to reduce non-specific binding, and the sensitivity was improved by sandwich type immunoassay using secondary antibody as a signal-amplifying agent. The SPR sensor surface with direct antibody immobilization provided better detection results of IL-6 than did the indirect antibody immobilization via protein G. This suggests that protein G tends to be non-specific binding with IgG of FBS and secondary antibody. The detection of IL-6 in cell culture medium elucidated that SPR biosensors offer a tool to quantify analyte in complex biological fluid, and with potential applications in medical screening and diagnosis.

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