A simplified direct on-chip forward or reverse immune assay for understanding protein-protein interactions in the serum

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Abstract

Background: The identification of protein-protein interactions is of great challenge. Therefore, we conducted this study to fabricate a gold surface biochip with activated sophorolipids in combination with 16-amino-1-hexadecanethiol hydrochloride. Methods: We designed a direct on-chip immunological assay strategy for measuring ligand-receptor interactions in a forward or reverse manner, that is, a ligand was immobilized on the biochip surface and allowed to interact with its specific free receptor in the liquid phase and vice versa. The specificity of molecular interactions on the biochip was evaluated using an immunological blocking assay and a chemiluminescent immunoassay. To test the potential utilization of biochip, we used the serum of hemophagocytic lymphohistiocytosis (HLH) patients as an experimental entity. Results: The receptor CD25-based IL-2 and ligand IL-2-based CD25 assays revealed that the detection limits on the biochip were as low as 156pg/mL and 78pg/mL, respectively. Meanwhile, using the receptor- or ligand-based platforms, we found that the positive rates of free IL-2 and soluble CD25 (sCD25) monomers in the sera of HLH patients were 14.3% and 71.4%, respectively, like our previous specific-antibody-based biochip investigation. Also, the biochip shared a good compatibility with CLIA assay in the measurement of sCD25($r=0.77$, $P<0.01$). Conclusions: The biochip platform can be expanded to protein-specific serological diagnosis as a potential substitute for immunoprecipitation and ELISA to understand the interactions between proteins, ligands and receptors, and enzymes and substrates.

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Running title: Validation of serum protein-protein interactionson biochip

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immobilized on the biochip surface and allowed to interact with its specific free receptor in the liquid phase and vice v*ersa* . The specificity of molecular interactions on the biochip was evaluated using an immunological blocking assay and a chemiluminescent immunoassay. To test the potential utilization of biochip, we used the serum of hemophagocytic lymphohistiocytosis (HLH) patients as an experimental entity. Results: The receptor CD25-based IL-2 and ligand IL-2-based CD25 assays revealed that the detection limits on the biochip were as low as 156pg/mL and 78pg/mL, respectively. Meanwhile, using the receptor- or ligand-based platforms, we found that the positive rates of free IL-2 and soluble CD25 (sCD25) monomers in the sera of HLH patients were 14.3% and 71.4%, respectively, like our previous specific-antibody-based biochip investigation. Also, the biochip shared a good compatibility with CLIA assay in the measurement of sCD25($r=0.77$, $P<0.01$). Conclusions: The biochip platform can be expanded to protein-specific serological diagnosis as a potential substitute for immunoprecipitation and ELISA to understand the interactions between proteins, ligands and receptors, and enzymes and substrates.

Keywords : protein biochip; molecule interaction; ligand; receptor; IL-2; sCD25; sophorolipids; 16-amino-1-hexadecanethiol hydrochloride

1. Introduction

Protein-protein interactions are specifically established events between two or more protein molecules. Developing assays for the detection and identification of protein-protein interactions is a great challenge. Immunoprecipitation (IP), based on the concentrated effects of antibodies and antigens, is a classical method to study protein-protein interactions [1]. IP is an effective way to analyze the cellular and physiological interactions between two kinds of proteins [2-5]. Co-immunoprecipitation and the yeast two-hybrid system are widely used for studying stable and/or high-affinity protein-protein interactions as well as the configuration of molecular complexes[6, 7]. However, in some cases, protein interactions in specific subcellular compartments are transient and unstable[6]. Classical experimental techniques often have intrinsic disadvantages such as the need for more manpower, complicated protocols, expensive materials, and deviated experimental interpretations due to false positives and negatives[8]. Therefore, a new method that can detect protein-protein complexes in the liquid phase needs to be established. Immunoassays are classified into homogeneous and heterogeneous types. Most methodologies for the detection of monomer and/or dimeric molecule interactions belong to heterogeneous assay, such as theenzyme-linked immunosorbent assay (ELISA), which is used for the detection of serum antigens using antibody probes in clinical laboratories[9]. However, ELISA is labor-intensive and time-consuming[10], and most importantly, only one type of protein can be tested at a time. In homogenous enzyme immunoassay, both antigen-antibody reaction and measurement of the degree of immune reaction are performed in solution without separation of the free and antibody-bound components. The assay is based on the conformational change of the active site of the enzyme or the steric hindrance of the substrate by the binding of enzyme-labeled antigen to the antibody. However, regardless of the advantages with rapid and simple operations and utilization for the analysis of small molecules, homogeneous immunoassays are less sensitive than heterogeneous assays [11].All the above techniques are lack of high-throughput parallel comparisons for multiple biomolecules in a single experiment. The biochip technique in combination with a self-assembling monolayer offers a high-throughput platform and enables the detection of multiple proteins in the serum[12-14]

Hemophagocytic lymphohistiocytosis (HLH), a rare but potentially life-threatening illness caused due to pathological activation of the immune system, is characterized by a classical and critical sign of "cytokine storm," which exhibits enhanced levels of IL-10, IL1β, IL-6, IL-8, TNF-α, IL-2, and soluble CD25 (sCD25) in the serum. Previously, we used a cytokine antibody-based biochip strategy, similar to an ELISA assay, to detect specific free or unbound cytokine monomers in the HLH serum[15]. IL-2 specifically recognizes sCD25, one of the three forms of IL-2 receptors. Therefore, in this study, we used HLH as an experimental entity to investigate the potential of an up-to-date biochip platform for on-chip measurements of molecular interactions. We established a novel chemical modification on a gold surface using an activated sophorolipid(SL) in combination with chemical reagent16-amino-1-hexadecanethiol hydrochloride(16-AHDT). We designed a direct on-chip forward or reverse immunological strategy based on the inherent interaction properties of a

ligand and its receptor. We immobilized ligand IL-2 on the biochip and interacted it with free monomer of receptor sCD25 in the serum of HLH patientsand vice versa, enabling the detection of desired monomer proteins on the biochip and their specific binding proteins in the liquid phase, thus, simulating IP. The strategy is shown in Figure 1. Setting up the on-chip IP platform provides a potential technological approach for understanding protein-protein interactions.

2. Materials and Methods

2.1. Reagents and equipment

SL was provided by Soliance (France). 16-AHDTwas purchased from Dojindo (Kumamoto, Japan). 1-(3-dimethylamino) propyl)-N-ethyl carbodiimide hydrochloride (EDC), bovine serum albumin (BSA), phosphate buffer saline (PBS, 0.01 M, pH 7.4), N-hydroxysuccinimide (NHS), sodium chloride (NaCl), sodium hydroxide (NaOH), acetic acid, and hydrochloric acid (HCl) were purchased from Sigma-Aldrich (USA). Goat-anti-human CD25 polyclonal antibody (pAb, BAF223), recombinant human CD25 (223-2A), and recombinant human IL-2 (202-IL) were obtained from R&D Systems (USA). Alexa Fluor@647 labeled anti-human IL-2 monoclonal antibody (mAb,ab199215) was supplied by Abcam (Shanghai, China). Human IgG protein, Cy3-conjugated goat anti-human IgG antibody and Cy3-conjugated donkey anti-goat IgG antibody were purchased from Sangon Biotech (Shanghai, China). PBS with Tween 20 (PBST) buffer (pH7.4)was used as the reaction and washing buffer throughout the experiment. Interactiva Division, ThermoHybaid (Ulm, Germany) supplied the biochips, i.e., standard glass slides (75 mm x 25 mm) covered with a gold film (0.1μm) as well as an initial adhesion layer of Teflon (50 μm). The Teflon film divided the gold surface into a matrix spot with a layout of 8 x 24 frame. Each spot diameter was 1.5 mm. The microarray scanner used was LuxscanTM 10 K-A (CapitalBio Corporation, Beijing, China). An atomic force microscope was provided by Innova (Veeco Co., Ltd., USA), and attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR) was performed using Nicolet 8700 (Thermo Scientific Instrument Co., USA).

2.2. Study population

Seventy-seven patients with HLH and forty-four healthy negative individuals were enrolled. Mean age of the patients was 21, 56±23,39 years old (range 1 month-73 years) with a median age of 9.5 years old, including 45 men and 32 women. Patients were diagnosed according to the HLH-2004 criteria[16]. Serum sCD25was clinically measured in 65 cases of the HLH patients using a chemiluminescence immunoassay (CLIA). The study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki), and it was approved by the Ethical Committee of Anhui Medical University. Informed consent was obtained from all participants.

2.3. Physicochemical characterization of the biochip surface

For cleaning, the chips were placed in a plastic box containing acetone solution at room temperature (RT) for 1 h, and they were then placed on a horizontal shaker at 60 rpm. Following this, the chips were placed in a mixture of H2O: NH3.H2O:H2O2in a ratio of 5:1:1 (v/v) at 92°C for 4 min. The chips were washed with double-distilled water five times and with ethanol thrice. Finally, the rinsed chips were dried using a nitrogen blow. Two steps were needed to complete the chemical modification of biochips. Step one: 2.4 mg of 16-AHDTwas dissolved in 10 mL of anhydrous ethanol (solution A). The biochips were immersed in solution A, and they were incubated in the dark with shaking at RT overnight. The treated biochips were washed three times with anhydrous ethanol for 2 min, and they were then dried with a nitrogen blow. Step two: 4 g of sophorolipid was dissolved in 10 mL of NaOH (40 g/mol) to obtain solution B (5 M). Solution B was incubated in a water bath with constant stirring at 90°C for 10 min. Following this, the solution was cooled to RT, and 8 mL of HCl (18.5%) was added to solution B with constant stirring to obtain a solution with the SL-COOH group. NHS (50 mM) and EDC (150 mM) were mixed in double distilled water to facilitate the coupling reaction between reagents to obtain solution C. Two milliliters of solution C was added to solution B. The mixed solution was incubated for 1 h to promote the combination of amino and carboxyl groups. The 16-AHDT-modified biochips from the step one were immersed into the mixture solution of B and C and remained incubated for 3 h. The biochips were washed three times with double-distilled water for 2 min, and

they were dried with a nitrogen blow. All incubation steps were performed at RT (24°C) unless otherwise stated. The ready-to-use monolayers were stored at 4°C.

Atomic force microscopy (AFM) and ATR-FTIR were used to characterize the modified biochip surfaces[13, 17]. AFM measurements were performed using a digital atomic force microscope. Scans were rated from 1 Hz to 5 Hz. The size of the cantilever was set to 5μm. The instrument was operated in tapping mode to obtain micrographs. The resulting heights of images were processed using the Nanoscope VII software[14]. The images were flattened to remove the scan lines, and the height scale was set to 20 nm. ATR-FTIR detection parameters were set to 128 scans, and a nominal resolution of 6 cm-1was used after placing liquid nitrogen with a spectrum range of 500-4000 cm-1.Evaluation for binding efficiency of biological molecules on the chemical surface was conducted by a direct on-chip immunoassay for serially diluted human IgG protein and Cy3-conjugated goat anti-human IgG antibody. Of which, 0.01M PBST-0.1%BSA buffer (pH 7.4)was used as a blank control.

2.4. Limit of detection (LOD) of IL-2 and CD25 on the biochip

The LOD values of IL-2 and CD25 proteins were measured to validate the mobilization efficacy of the biochip modified with 16-AHDT and activated SL (16-AHDT-SL). Briefly, recombinant human IL-2 protein was dissolved in 0.1 M acetic acid-0.1%BSA buffer (pH 2.27) and recombinant human CD25 protein was dissolved in 0.01M PBST-BSA buffer (pH 7.4), respectively. After immobilizing 6.25μg/mL of the protein solutions onto the chemically modified biochips, we incubated the biochips in a humid chamber at RT for 2 h. We rinsed the biochips with double distilled water and then thrice with0.01M PBST buffer (pH 7.4) for 2 min, and subsequently, we dried them with a nitrogen blow. Meanwhile, we prepared additional recombinant human IL-2protein solution with 0.1M acetic acid-0.1%BSA (pH 2.27) and recombinant human CD25 protein solution with 0.01M PBST-0.1%BSA (pH 7.4), respectively, to get two-fold serial dilutions ranging from 50 μg/mL to 0.049 μg/mL. We then supplied the serially diluted recombinant human IL-2 protein solution individually onto the reaction spots (1 μL/spot) onaCD25-coated biochip prepared as described above, and we incubated the biochip at RT for 1 h. After washing and drying the biochip, we incubated it with 12.5 μg/mL of Alexa Fluor@647-conjugated anti-human IL-2 mAb in a dark environment at RT for 30 min. Similarly, we added two-fold serial dilutions of recombinant human CD25 protein onto one IL-2-coated biochip (1 μL/spot) and incubated the biochip at RT for 1 h. Following this, we then incubated the biochip with 25 μg/mL of goat anti-human CD25pAb at RT for 1 h. After washing and drying, we incubated the biochip with 2.5 μg/mLof Cy3-conjugated donkey anti-goat IgG antibody in the dark at RT for 30 min. After washing and dry procedure, the fluorescence intensity of each reaction spot on the biochips was visualized using a microarray scanner (LuxscanTM 10 K-A, CapitalBio Corporation) equipped with two color channels, 532 (green, PMT 350, Power 90) and 635 (red, PMT 600, Power 90), respectively. The images were then exported as TIFF files for analysis using image processing software in the scanner. Acetic acid-BSA buffer (pH 2.27) or PBST-BSA buffer (pH 7.4) was used as the blank controls.

2.5. Immunological blocking assays for evaluating the binding specificities of IL-2 and sCD25 interaction

We immobilized 6.25 μg/mL of commercially recombinant human CD25 or IL-2 protein onto two individual biochips. We selected four known IL-2- or sCD25-positive sera from HLH patients as positive controls and four known IL-2- or sCD25 free serum samples from healthy individuals as negative controls. Before incubating the biochips with the serum samples, an immunological blocking assay was performed in the vials to verify the interaction specificities of ligand IL-2 and receptor sCD25 in serum. Briefly, fourIL-2-positive sera samples were pretreated with a commercially specific recombinant human CD25 protein in 0.01M PBST-0.1%BSA buffer (pH 7.4)at concentrations of 0 μg/mL, 0.1 μg/mL, 1 μg/mL, 10 μg/mL, and 100 μg/mL in five individual vials at RT for 1 h. We added the CD25-pretreated sera onto one recombinant human CD25-coated biochip, incubated the biochip at RT for 1 h. We rinsed the biochip with PBST buffer (pH 7.4) thrice and dried with a flow of nitrogen and then treated it with 12.5μg/mL of Alexa Fluor@647 conjugated anti-human IL-2 mAb in a dark environment at RT for 30 min. In this case, free IL-2 in the serum was neutralized by gradually increasing concentration of recombinant human CD25 molecule in the vials, thus, the reaction between free IL-2 in serum and CD25 on the biochip became impossible due to the saturation

of binding sites, and fluorescence signals from the known positive sera turned negative. Similarly, before biochip assay, four known receptor sCD25 positive sera were individually neutralized with commercially specific recombinant ligand IL-2 protein at logically diluted concentrations of 0, 0.1, 1, 10, and 100 μg/mL in 0.1M acetic acid-0.1%BSA buffer (pH2.27)in additional five vials at RT for 1 h. Following this, we supplied the IL-2-pretreated sera onto one recombinant human IL-2-coated biochip, and we incubated the biochip at RT for 1 h. After washing and drying procedure, we incubated the biochip with 25 μg/mL of goat anti-humanCD25pAb at RT and then with 2.5 μg/mL of Cy3-conjugated donkey anti-goat IgG antibody in the dark at RT for 1 h and30 min, respectively. After washed and dried, fluorescence intensities of the spots on biochips were analyzed using a microarray scanner.

2.6. Direct forward or reverse on-chip detection for free IL-2 or sCD25 monomer in serum

Protein-protein interactions were detected on the biochips in a forward or reverse manner. To capture free IL-2 monomer in the serum, 6.25μg/mL of the recombinant human CD25was immobilized on a biochip at RT for 2 h. This biochip was rinsed with PBST buffer (pH 7.4) thrice and then dried with a flow of nitrogen. Sera from 77 patients with HLH and 44 known negative controls were diluted to 1:4 in 0.01M PBST- 0.1%BSA buffer (pH 7.4). One microliter of the diluted sera was individually added onto each spot of the biochip. The biochip was incubated at RT for 1 h, and then rinsed with 0.01M PBST buffer (pH 7.4) thrice and then dried with a flow of nitrogen. Subsequently, the biochip was reacted with 12.5 μg/mL of Alexa Fluor@647 conjugated anti-human IL-2 mAb in a dark environment at RT for 30 min. Similarly, to capture free sCD25 monomer in the serum, sera from patients at a dilution concentration of 1:20 in 0.01M PBST-0.1%BSA buffer (pH 7.4) were incubated with a recombinant IL-2-coated biochip at RT for 1 h. After washed and dried, the biochip was reacted with 25 μg/mL of goat anti-human CD25pAb at RT for 1 h. Finally, the specific binding was verified using 2.5 μg/mL of Cy3-conjugated donkey anti-goat IgG antibody at RT in the dark environment for 30 min. After washing and drying procedure, the fluorescent signals on biochips were detected using the microarray scanner. The fluorescence intensity was analyzed thrice over the average value of the known negative sera as the cut-off value.

2.7. Statistical analysis

The statistical analyses were performed by SPSS Statistics (version 23.0, IBM). Data were presented as the mean±SD unless indicated otherwise. Correlation efficiency analysis was performed using Pearson's test. The P values reported in this study were two-sided and $P<0.05$ was significant.

3. Results

3.1. AFM and ATR-FTIR characterization of 16-AHDT-SL-modified biochip

In this study, we developed a novel self-assembled molecular monolayer on a gold surface using 16-AHDT-SL. Figure 2 revealed various immunological reaction efficacies in binding and detecting human IgG protein via a direct on-chip immunoassay for serial dilutions of human IgG protein and Cy3-conjugated goat anti-human IgG antibody. The immobilization efficacy of the biochip with a combined modification of 16-AHDT-SL was much better than that of the biochip modified with 16-AHDT alone. A decreased fluorescence signal was visible on the biochip as surface loading of human IgG protein was serially diluted.

Physicochemical characterization of 16-AHDT-SL was performed using ATR-FTIR and AFM. Figure 3a shows the main functional groups in the absorbance of 16-AHDT-SL using ATR-FTIR. Amide bonds were observed at 1410 cm-1 (C-N), 1715 cm-1 (C=O), and 3270 cm-1 (NH), indicating that amidation reactions formed covalent bonds between the outermost NH2and COOH groups of 16-AHDTand SL, respectively. Other peaks were observed at 3329 cm-1 (OH), 2930 cm-1 (CH2), 2870 cm-1 (CH3), 1410 cm-1 (C-N), and 1056 cm-1 (C-C) n. The main carbon chain of 16-AHDT was located at 1056 cm-1, and functional groups were observed at 3329 cm-1in different planes of several free hydroxyl groups at the end of SL. A wide peak at 3329 cm-1 resulted in several free hydroxyl groups, forming a polymolecular association oscillation at the outer end of SL. Three-dimensional images of the chemically modified biochip and unmodified clean chip were observed using AFM. The modified biochip surface (Figure 3b) was much rougher than that the unmodified

chip surface (Figure 3c). Uneven peaks and valleys were observed on the modified biochip surfaces. The peak height of the modified surface varied between 6 nm and 9 nm, indicating that the reagents were fixed on the surface of the biochip. A self-assembled molecular monolayer was efficiently formed in combination with 16-AHDT-SL at RT, which remained stable at 4 °C for months.

3.2. Qualification controls for CD25 and IL-2 detection using the biochip

To determine the optimal detection limit of molecules on the biochip, we analyzed serially diluted concentration curves for both ligand IL-2-based and receptor CD25- based assays on the biochip. A buffer containing 0.01 MPBST- 0.1%BSA (pH 7.4) was used as the blank control. Figure 4 shows that the fluorescence intensities tended to attenuate as the concentration of ligand IL-2 or receptor CD25 proteins on the sampling points of biochips gradually decreased. We chose a fluorescence value three times the mean value of the blank control as the cut-off value. In this study, the visualized detection limits inCD25-based ligand IL-2 assay and ligand IL-2-based receptor CD25 assay were156 pg/mL (Figure 4a) and 78 pg/mL (Figure 4b), respectively.

To determine the optimal serum dilution, three sera with known positive immune reactions of ligand IL-2 or receptor sCD25 and three sera with known negative immune reactions for both the molecules were selected. The average fluorescence ratio values between the positive and negative sera were outlined in the ordinate, and the layout of dilution ratios was assigned in the abscissa (data not shown). Among the ratio spectra of > 3, we chose 1:20 as the optimal serum dilution concentration for the detection of serum receptor sCD25. Similarly, a 1:4 dilution ratio was optimal for the detection of serum IL-2.

3.3. Serum immunological blocking experiment on the protein biochip

Serum immunological blocking assay is an important approach for evaluating the binding specificity of a capture molecule that combines a serum biomarker on the detection platform. As shown in Figure 5, we selected additional four known biomarker-positive sera and four known biomarker-negative sera. Our data showed that fluorescent signals of either CD25-based IL-2 assay or IL-2-based sCD25assay gradually attenuated as recombinant ligand IL-2 (Figure 5a)or recombinant receptor CD25 (Figure 5b)was added to the sera in vials at concentrations of 0.1, 1, 10, and 100 μg/mL. The histogram indicated that such a protein-protein interaction between the ligand IL-2 and receptor sCD25 monomer in sera were completely blocked after adding 0.1μg/mL of recombinant ligand IL-2 protein or recombinant receptor CD25protein to the vials (Figure 5c). Data from the immunological blocking assays demonstrated the binding specificities of IL-2 and sCD25 on-chip assays.

3.4. Direct on-chip immunological assays for the detection of serum IL-2 or sCD25 monomer

In this study, we evaluated an on-chip measurement strategy for capturing free receptors or ligand monomers in HLH sera in a forward or reverse manner by means of its intrinsic intermolecular recognition property. We performed a direct immunological binding assay using either IL-2-or CD25-coated biochips (Figure 6). We chose a cut-off value three times the mean value of negative controls. The positive rates of free ligand IL-2 and receptor sCD25 monomers in 77 HLH sera detected using the ligand/receptor-based biochip assays were 14.3% and 71.4%, respectively. We compared ratios of IL-2 and sCD25 values in the HLH patients in the biochip assay (Supplementary Table 1) and found that vast majority of sCD25/IL-2 ratios in the patients were over one. Again, to validate the accuracy of the biochip assay in detecting immune responses to ligand IL-2 and receptor sCD25, we evaluated the biochip-based methodology in comparison with a conventional CLIA for sCD25 in identical HLH patients. We found that the biochip assay had a satisfying correlation with CLIA assay in the measurement of sCD25 ($r=0.77$, $P<0.01$, Pearson test, Supplementary Table 2), indicating that the effectiveness of the biochip platform and CLIA were in close accordance with each other. Thus, the biochip with its high-throughput advantage reduced the shortcomings of IP and performed multiple measurements of a variety of associated proteins.

4. Discussion

In this study, we developed a new biochip strategy to understand potential protein-protein interactions in solutions. First, we developed a novel self-assembled molecular monolayer on a gold surface using an activated

sophorolipid in combination with 16-amino-1-hexadecanethiol hydrochloride. Chemical characterization of the surface was performed using ATR-FTIR and AFM techniques, as shown in Figure 2. Crosslinking of 16-AHDT and SL enhanced the protein binding capacity of the biochip by means of free hydroxyl groups at the end of SL. A previous study demonstrated that SL has a strong wettability due to both the large exposure of C-OH groups of glucose and prominent surface roughness while glucose moieties are parallel to the surface (low roughness) and more C-OH groups are involved in hydrogen bonding with their close neighbors [17]. SL molecules with double bonds can present strong combined functions due to the preferential exposure of C-OH groups[18]. Additionally, our direct immunological reaction to human IgG molecules on the modified biochip provided a valuable demonstration for the efficient binding of molecules.

Second, using the newly modified biochip surface, we simulated a direct on-chip IP assay, revealing immunological interactions between the two molecules, ligand IL-2 and its receptor CD25. Protein-protein interactions were detected on the biochips in either a forward or reverse manner. Detection of human receptor CD25 in a liquid phase, which was combined to a ligand IL-2-based biochip, was achieved by adding a specific anti-human CD25 antibody and fluorescence-labeled secondary antibody, respectively. Alternatively, after we immobilized recombinant receptor CD25onto the biochip surface, integration of ligand IL-2 in the liquid phase with the receptor CD25-based biochip was revealed by means of a specific fluorescence-labeled anti-human IL-2 IgG antibody. In this study, the immunological detection efficacy on the biochips indicated that ligand IL-2-based CD25 assay revealed more sensitive than receptor CD25-based IL-2 assay (78 pg/mL vs.156 pg/mL). The LOD values were similar to those reported previously [12, 15]. We supposed that molecular size of proteins as linkers on the biological surface would be attributable to the potentials. IL-2 and CD25 show molecular weights of 15kDa and 55 kDa, respectively. Small molecule IL-2 as a linker on the biochip would enable less efficient steric hindrance in molecular interaction reaction. Data from immunological blocking assays showed the specificity of on-chip IL-2 and sCD25 interaction assays.

Third, the practicability of biochip platform was further evaluated using the sera from 77 HLH patients. HLH is rich in IL-2 and its receptor, sCD25. The ligand IL-2 molecule on the biochip specifically captures its receptor CD25, if any, in HLH serum. In this study, using the ligand/receptor-based biochip assays, on the one hand, we found that the positive rates of ligand IL-2 and receptor sCD25 monomers in the sera were 14.3% and 71.4%, respectively, which were in accordance with our previous α-IL-2 or α-sCD25antibody-based biochip investigation, in which ligand IL-2 and receptor sCD25 monomer in the HLH sera have been detected using their relevant specific antibodies[15]. Our data indicated that the biochip shared an impressive compatibility with CLIA assay in the measurement of sCD25. This further provided evidence that the biochip could detect the interaction specificity between ligand IL-2 and receptor sCD25 monomers in the HLH sera. On the other hand, we observed that almost all sCD25-to-IL-2 ratios in individual patients were over one, indicating that there would b emore abundant sCD25 than IL-2 in the serum of the HLH serum. Moreover, the data also supported the evidence that elevated sCD25levels in HLH are inversely associated with reduced IL-2 levels [15, 19, 20]. Regulatory T cells (Tregs) play an important role in immune homeostasis and preventing the onset of autoimmune diseases, including HLH[21]. IL-2 plays a crucial role in Treg survival, lineage maintenance, suppressor function, and even cell development while such functions require the binding of IL-2 to its receptors, thereby activating the signal transducer and activator of transcription 5, phosphatidylinositol 3-kinase, and mitogen-activated protein kinase/extracellular signal-regulated kinase pathways[22]. Understanding the role of IL-2 in HLH requires the binding ofIL-2 to its receptor CD25[23]. There are three chains of IL-2 receptors (IL-2R), which incorporate IL-2Rα (CD25), IL-2Rβ (CD122), IL-2Rγ (CD132)[24] and a combination of various forms of IL-2R. In humans, the IL-2R βγ complex binds to IL-2 with a moderate affinity, but the IL-2R αβγ complex constitutes a high-affinity receptor because CD25 adds nearly a 1,000-fold higher ligand affinity to the receptor trimer. In contrast, the heterodimer IL-2Rβγ in murine cells has a low affinity for IL-2. CD25 expression is required to complete the functional receptors of the trimeric structure. The IL-2/CD25 complex has a short half-life in vivo. The binding and dissociation of IL-2/CD25 complex is in equilibrium in vivo[25]. IL-2 can be dissociated from the IL-2/CD25 complex to maintain serum IL-2 levels [26, 27]. An increased CD25level modulates serum IL-2 levels via conjugation with IL-2[28]. Therefore, the IL-2/CD25 interaction is important in complex

formation [29, 30]. Interactions between sCD25 and IL-2 are essential features of signal transduction, and they are important for development and physiological functioning at the cellular level [31, 32].

Taken together, we established a new protein biochip platform for the detection of serological protein-protein interactions between a ligand and its receptor in a forward or reverse manner. The biochip strategy was of an impressive value, simple and timesaving. It could be widely used as a potential substitute for IP and ELISA in protein-specific serological diagnosis and understanding protein interactions in signal transduction pathways. The potential benefits of the biochip format also include its high-throughput assay, reduction of the limitations of IP, and concomitant measurements of multiple protein-protein interactions between ligand-receptor and enzyme-substrate both clinically and pharmacologically.

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Figure legends

Figure 1. Strategies for detecting protein-protein interactions on the biochip. A directon-chip immunoprecipitation workflow detecting matched molecule monomers.

Figure 2. Immunological evaluation for biochip immobilization efficacy on two chemical modification surfaces modified by 16-amino-1-hexadecanethiol hydrochloride(16-AHDT) alone and surface modified by 16-AHDT in combination with sophorolipid (SL)via a direct on-chip immunoassay for serially diluted human IgG protein and Cy3-conjugated goat anti-human IgG antibody. 0.01M PBST-0.1%BSA buffer (pH 7.4) was used as a blank control.

Figure3. Surface chemical characterization of 16-AHDT-SL on gold biochips via attenuated total reflection Fourier-transform infrared spectroscopy (a) and atomic force microscopy: modified surface (b); unmodified surface (c).

Figure 4. Limit of detection of IL-2 and CD25 proteins on the biochip. (a) CD25-based IL-2 protein assay; (b) IL-2-based CD25 protein assay. Serially diluted target proteins were incubated on the receptor- or ligand-based biochips.

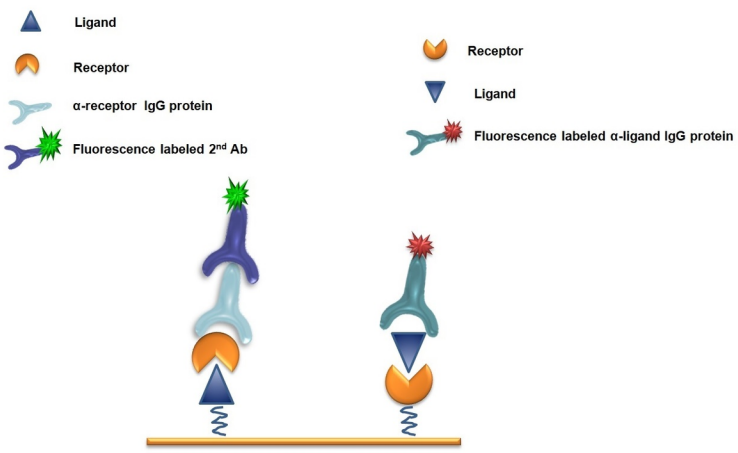
Figure 5. Immunological blocking assays for verifying the detection specificity ofserum ligand IL-2 and its receptor sCD25using the biochip.

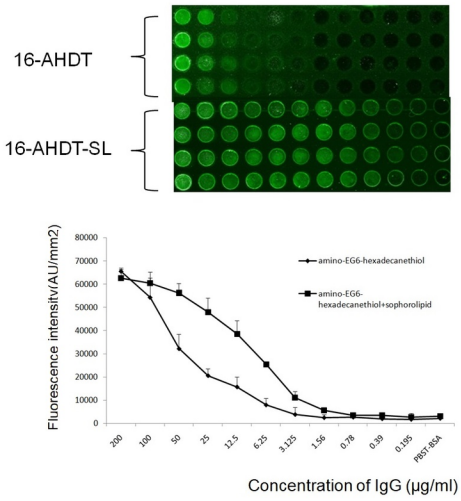
Figure 6. Specific receptor- or ligand-based protein-protein interaction assays in seventy-seven hemophagocytic lymphohistiocytosis patients. (a): CD25-based IL-2 assay; (b): IL-2-based sCD25 assay.

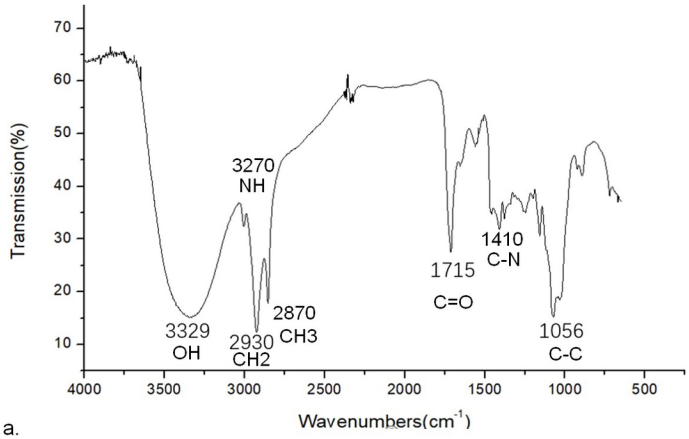
Supplementary Table 1. Values and ratios of IL-2 and sCD25 assays in seventy-seven cases of HLH

Supplementary Table 2. Correlation analysis for serum sCD25 measurement in sixty-five cases of HLH using biochip and CLIA assays (Pearson test).

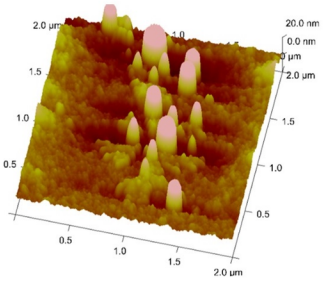
Supplementary Figure for Graphical Abstract.



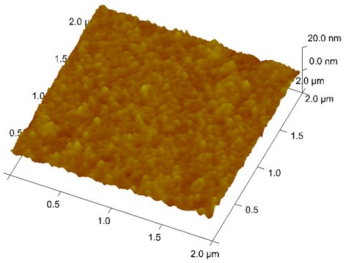




a.



b. modified surface



c unmodified surface