Research Article

A label-free protein microfluidic array for parallel immunoassays

A label-free protein microfluidic array for immunoassays based on the combination of imaging ellipsometry and an integrated microfluidic system is presented. Proteins can be patterned homogeneously on substrate in array format by the microfluidic system simultaneously. After preparation, the protein array can be packed in the microfluidic system which is full of buffer so that proteins are not exposed to denaturing conditions. With simple microfluidic channel junction, the protein microfluidic array can be used in serial or parallel format to analyze single or multiple samples simultaneously. Imaging ellipsometry is used for the protein array reading with a label-free format. The biological and medical applications of the label-free protein microfluidic array are demonstrated by screening for antibody–antigen interactions, measuring the concentration of the protein solution and detecting five markers of hepatitis B.

Keywords: Immunoassay / Imaging ellipsometry / Label-free protein microfluidic array

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1 Introduction

Immunoassays are a common and useful means of performing clinical diagnostics, environmental control, and biochemical analysis. RIA, enzyme immunoassay (EIA), fluorescence immunoassay (FIA), and chemiluminescence immunoassay (CLIA) are the most common methods for immunoassays. All these approaches rely on a marker molecule, such as a radioisotope, an enzyme, or a fluorescent probe, or a nanoparticle. Recently, the widespread application of DNA microarray for high-throughput analysis has prompted researchers to develop parallel immunoassays in microarray format [1–3].

Many types of protein microarrays have been designed and most of them were developed by extending technologies used for DNA microarray [4–7], which remain problematic. Microarrays prepared by robotic spotting or printing technique often have spots with poor morphology and inhomogeneity of biomolecular distribution, which is not suitable for quantitative immunoassays [8]. Protein arrays, unlike their DNA counterparts, should not be stored in a dry state which denatures the proteins and alter their conformations considerably [8]. Special packaging systems employing a humid environment have to be developed. In addition, minimized microfluidic device should be developed for microarray reaction to increase speed of analysis and reduce sample consumption [9, 10]. Delamarche and coworkers [11–14] developed microfluidic chips, based on capillary for immunoassays with picomolar sensitivity, using submicroliter volumes of samples. Providing parallel analysis of multiple analytes with economy of samples in short time, the microfluidics system has proved to be a promising technology for miniaturizing biological assays. Nevertheless, fluorescence and other label detection methods used in the above-mentioned protein microarrays are much less convenient due to synthetic challenges, multiple label issues, and the potential for interfering with the binding site [1, 15].

We developed a label-free protein microfluidic array for parallel immunoassays based on the combination of imaging ellipsometry and an integrated microfluidic system. Imaging ellipsometry developed in our laboratory was used for protein array reading. An interesting advantage of ellipsometry is the possibility of the method for determination of the total amount of protein on solid surface in high sensitivity without destruction of the protein and without labeling of the protein [16, 17]. The imaging ellipsometry combines the power of ellipsometry with microscopy and has an x/y-resolution of approximately 1 μm [18, 19]. The field-of-view of the imaging ellipso-
meter is so large (12 × 36 mm²) that all spots at this area on a protein array can be imaged in one step and the amount of protein on all the spots can also be measured simultaneously. Imaging ellipsometry is a similar technique to surface plasmon resonant microscope, but can be used on thin layers in general and is not restricted to films of gold and metals where surface plasmon can be generated [20]. These make the imaging ellipsometer perfectly suited for protein arrays reading.

We designed and developed an integrated microfluidic system that can be used for protein array fabrication, packaging, and reaction. Protein molecules can be patterned homogeneously on substrate to form protein array by the microfluidic system, and the patterning step is local, that is, exposure of protein molecules to the substrate occurs only on targeted areas. Simultaneous immobilizations of proteins on these targeted areas are possible without the introduction of crossinterferences, even where different coupling chemistries are needed. After patterned, the protein array can be packaged in the microfluidic system which is full of buffer so that proteins are not exposed to denaturing conditions. In this design we employed parallel and serial strategies. In parallel format, each spot of protein array can be used individually to detect sample; in serial format, all spots can be connected in series to analyze single sample; in parallel/serial format, spots can be divided into different groups and connected, respectively, in series to react with multiple samples simultaneously. The label-free protein array has been preliminarily used to detect quantitatively CA15-3 which is a breast cancer marker in serum [21]. Sixty sera of patients with different types of breast cancer or breast diseases were detected with the protein array, and the result was in agreement well with that obtained by electrochemiluminescence immunoassay (ECLIA) which is a “golden standard” for clinical test of CA15-3. In addition, the label-free protein array has also been successfully used to detect the C-reactive protein, one of the markers for cardiovascular diseases, and Tie-2, a soluble angiopoietin receptor in patients with acute myocardial infarction [22, 23]. Instead of just giving the preliminary medical application of the protein array, this paper emphasizes the development of the integrated microfluidic system, as well as the biological and medical applications.

2 Materials and methods

2.1 Materials

BSA, HSA, goat polyclonal anti-HSA antibody (anti-HSA), human IgG (hIgG), goat polyclonal anti-human IgG antibody (anti-IgG), human fibrinogen (Fib), and goat polyclonal anti-human fibrinogen antibody (anti-Fib) were obtained from Sigma. Hepatitis B surface antigen (HBsAg), mouse monoclonal anti-hepatitis B surface antigen antibody (HBsAb), hepatitis B core antigen (HBcAg), hepatitis B e antigen (HBeAg), and mouse monoclonal anti-hepatitis B e antigen antibody (HBeAb) were purchased from Biodesign (USA). Fetal bovine serum was purchased from Hyclone (USA). Mouse monoclonal anti-CD146 antibody (AA98) was a generous gift from the laboratory of Professor Xiyun Yan, Institute of Biophysics, Chinese Academy of Sciences. All patients sera used in this report were obtained from Peking Union Medical College Hospital, which were detected with ELISA. 3-Aminopropyltriethoxysilane and glutaraldehyde were purchased from Acros. Other chemicals used for the preparation of buffer and substrate were all of analytical grade or better. Silicon wafers were purchased from General Research Institute for Nonferrous Metals (China).

2.2 Protein solution preparation

All protein solutions used for protein array fabrication were prepared at a concentration of 0.1 mg/mL in PBS buffer (PBS: 10 mM phosphate, 0.1 M NaCl, 0.02% sodium azide, pH 7.2). The mixed sera were prepared by diluting anti-hIgG, anti-Fib, anti-HSA, and HBsAg in PBS; each protein concentration was 0.1 mg/mL. Fetal bovine serum was used without dilution.

2.3 Cleaning of silicon wafers

The silicon slides were cut into 10 × 20 mm² pieces and cleaned with a mixture of 30% hydrogen peroxide (H₂O₂) and concentrated sulfuric acid (H₂SO₄) (1:3 v/v) for 30 min. After thoroughly rinsing with water and pure ethanol, the slides were stored in pure ethanol for modification.

2.4 Silanization of cleaned silicon wafers

The cleaned wafers were treated with a fresh ethanol solution of APTES (5% APTES and 95% pure ethanol) for 2 h at room temperature, followed by rinsing with pure ethanol three times and deionized water three times. Before immobilization of proteins, the silicon wafers silanized with 3-aminopropyltriethoxysilane (APTES) were reacted with a 2.5% solution of glutaraldehyde in PBS for 1.5 h, followed by rinsing with PBS buffer. This procedure introduced aldehyde groups on the silicon surface that can react with amino groups of the proteins to immobilize protein molecules covalently on surface.
2.5 Imaging ellipsometry

Imaging ellipsometry was an enhancement of standard single-beam ellipsometry, which combined the power of ellipsometry with microscopy and worked in the off-null mode [18]. The imaging ellipsometry used in this study was an automated one and ellipsometric conditions could be controlled by autoadjusting the polarizer and the analyzer; the angle of incidence was variable from 45 to 90° with a resolution of 0.05°; autofocusing was realized with the standard of the Laplacian algorithm. All these adjustments were carried out automatically with microstepping motors controlled by a computer with homemade software. With the same software, the digital images in grayscale format (8 bits, 0–255 grayscale) could be also automatically captured and processed. The light source was a Xenon lamp, and a specific collimating system was used to provide an expanded parallel probe beam. The beam passed through a polarizer and a compensator (a quarter wave plate) and finally onto the sample at an incident angle of 75°. An optical filter at 632.8 nm wavelength was placed in the incident optical passage to select wavelength in order to increase the ellipsometric contrast of image. The reflection beam passed through an analyzer and an imaging lens with a spatial filter located at its focus plane, and then the ellipsometric image was focused onto the sensing area of the CCD camera with a 768 × 494 pixel array. For a sample with lateral distribution of layer thickness (or surface concentration), null ellipsometry could not be carried out over the entire surface simultaneously due to the fact that different areas would yield different polarization changes. In order to overcome the problem, the optical components were adjusted to fulfill the null conditions on a silicon wafer without adsorbed layers and the off-null ellipsometric principle was used to measure the adsorption layer thickness (or surface concentration). Under this condition [18], the detected intensity “I” was related to the thickness (d) of the layer according to \( I = kd^2 \). As for the same protein and the same ellipsometric conditions, k is a constant and can be determined by the protein layer with known intensity in grayscale and its absolute thickness. In this paper, the absolute thickness of the protein layer was calibrated by conventional ellipsometer. The relationship between surface concentration and protein layer thickness was: surface concentration \( (\mu g/cm^2) = K \times d \) (nm), where \( K \approx 0.12 \) [24].

2.6 Ellipsometer

The thickness of biomolecular layers was calibrated with an ellipsometer of rotating analyzer type (SE 400, SENS-TECH, Germany) equipped with a He–Ne laser (\( \lambda = 632.8 \) nm). The measurements were carried out at an angle of incidence of 70°.

3 Results and discussion

3.1 Microfluidic system fabrication and operation

The microfluidic system consists of three main parts: an 8 × 6 elliptic microcells array (EMCA) (Fig. 1A); an 8 × 12 stainless-steel tube array (TA) (Fig. 1B) and a connecting array (CA) (Fig. 1D).

Figure 1. Schematic of the microfluidic system used for protein array fabrication, packaging, and reaction. (A) EMCA; (B) microfluidic setup for protein array fabrication. A TA, EMCA, and substrate are fixed tight by magnetic force from permanent magnetic disks embedded in TA and a polymethyl methacrylate (PMS) slab under the substrate; (C) microfluidic setup for protein array packaging; (D) microfluidic setup for protein array reaction, the cells in EMCA are connected by the CA.
The volume of each elliptic cell of EMCA is 18 nL, with a depth of 50 μm, and cells gap of 700 μm. Each cell has two access holes (diameter 300 μm, depth 250 μm), and solution can pass in and out of the cell through them. The EMCA was fabricated with PDMS (Sylgard 184, Dow Corning) as described previously [25, 26]. Briefly, the EMCA was created by replication from a 3-D silicon wafer master that was created photolithographically from 2-D chrome masks pattern. The chrome masks were created using e-beam photolithography by the group of Professor Liangqiang Peng of the Institute of Microelectronics, Chinese Academy of Sciences. The 3-D silicon wafer master with two levels of features was made with a negative photoresist (SU-8 50) using two-level photolithography by the group of Professor Futing Yi of the Institute of High Energy Physics, Chinese Academy of Sciences. To fabricate the EMCA, we sandwiched a drop of PDMS prepolymer between the master and a Teflon sheet, and allowed it to cure under pressure at 70°C. Pressures in the range of 10–50 kPa were required to ensure that the access holes were not blocked by a thin underlayer of PDMS that seeped between the Teflon and the features during curing. Once the EMCA had cured, the Teflon sheet was peeled away and the EMCA was removed from the master.

The TA was fabricated in polymethyl methacrylate (PMS) by microfabrication technique. The thru hole array was drilled with a digitally programed machine tool. The distribution of the thru holes in PMS was the same as that of the access holes in EMCA. Stainless-steel tubes were fixed into the thru holes to form TA. Four permanent magnetic disks (Ni-plated-NdFeB, 3 mm diameter) were embedded into the PMS (Fig. 1B).

The third part of the microfluidic system is a CA (Fig. 1D) that was created in PDMS by same fabrication technique as that of EMCA. There are different CAs which can connect cells in EMCA in different formats, such as connecting all cells in series or dividing cells into different groups and connect the cells of each group in series, respectively.

In general, four steps were required to fabricate a protein array with the microfluidic system (Fig. 1B): (i) the EMCA was placed in contact with the silicon wafer surface previously activated by formation of aldehyde groups to achieve chemical coupling with pendant amino groups common to proteins; (ii) the TA was aligned on the EMCA to make the tubes connect with the access holes and a PMS slab with four permanent magnetic disks was put under the substrate, the TA, the EMCA and the substrate were fixed tight by the magnetic force; (iii) proteins solutions were sucked into different cells simultaneously by a multichannel peristaltic pump (ISMATEC, Swiss); and (iv) after allowing the proteins to adsorb on the substrate surface for a set period of time, the channels were flushed thoroughly with phosphate buffer.

After fabrication, the TA was removed and a PMS slab with four permanent magnetic disks was put on the EMCA to cover the nozzles of channels which are full of buffer (Fig. 1C). The microfluidic system served as a packaging system for protein array to avoid exposing dry condition.

If the protein array was used to analyze samples in parallel format, TA was aligned on the EMCA and 48 different samples can be detected simultaneously. If it was used to analyze samples in serial or parallel/serial format, CA was aligned on the EMCA to connect all cells in series or divide the cells into several groups and connect the cells of each group in series to detect single sample or multiple samples (Fig. 1D).

3.2 Protein array fabrication and reaction

To determine whether the microfluidic system could be used for protein array fabrication and reaction, we selected four proteins, hIgG, Fib, HSA, and HBsAb. The four protein solutions were sucked into the microfluidic system, each 1 μL, and allowed to adsorb on the substrate surface with aldehyde groups for 10 min, then all the spots were blocked with BSA (20 mg/mL) for 30 min. After washing with phosphate buffer, the substrate with the four proteins array was taken from the microfluidic system and detected with imaging ellipsometer. An image in grayscale of the protein array is shown in Fig. 2A, the intensity of each spot is listed in Table 1, and the thickness distribution (Fig. 2B) of the protein array was deduced from the image in grayscale according to the relationship between the intensity and thickness [18]. The protein array consisted of 12 spots and each protein was arrayed in triplicate. Ten regions (each 0.02 mm²) were selected randomly on each spot, and the average thickness of each such region was determined. Both relative deviations of layer thickness within each spot and over three spots of each protein were less than 2%.

In order to demonstrate the microfluidic system as a microreactor for protein array, we first fabricated the same protein array by the method described above, and then a CA was used to divide the spots on the protein array into three groups and connect the spots of each group in series. After all the spots were blocked with BSA (20 mg/mL) for 30 min, 10 μL of mixed sera containing anti-hIgG, anti-Fib, anti-HSA, and HBsAg was sucked into group 1 to react with proteins on spots at a flow rate of 1 μL/min, the same volume of fetal bovine serum was...
Figure 2. Protein array with 12 spots fabricated by the microfluidic system shown in grayscale image (A), and the corresponding thickness distribution (B). Fib, HBsAb, hIgG, and HSA were arrayed on the substrate surface in triplicate, respectively.

Table 1. Spot intensity in grayscale of the protein array in Fig. 2

<table>
<thead>
<tr>
<th></th>
<th>Fib</th>
<th>HBsAb</th>
<th>hIgG</th>
<th>HSA</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>51.8</td>
<td>74.6</td>
<td>71.5</td>
<td>52.1</td>
</tr>
<tr>
<td>II</td>
<td>52.6</td>
<td>74.5</td>
<td>71.7</td>
<td>52.4</td>
</tr>
<tr>
<td>III</td>
<td>52.7</td>
<td>74.1</td>
<td>71.7</td>
<td>52.6</td>
</tr>
</tbody>
</table>

sucked to react with spots in group II in the same time and group III was left for reference. After washing with phosphate buffer, the substrate was taken from the microfluidic system and detected with imaging ellipsometer. The protein array after reaction is shown in Fig. 3 and the intensity in grayscale of each spot is listed in Table 2. The intensity of the spots reacted with the mixed sera increased obviously due to the forming of antibody–antigen complexes.

Table 2. Spot intensity in grayscale of the protein array in Fig. 3

<table>
<thead>
<tr>
<th></th>
<th>Fib</th>
<th>HBsAb</th>
<th>hIgG</th>
<th>HSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>165.8</td>
<td>136.4</td>
<td>110.8</td>
<td>79.8</td>
</tr>
<tr>
<td>II</td>
<td>52.2</td>
<td>74.8</td>
<td>71.9</td>
<td>51.8</td>
</tr>
<tr>
<td>III</td>
<td>51.7</td>
<td>74.3</td>
<td>72.3</td>
<td>51.4</td>
</tr>
</tbody>
</table>

Figure 3. Detection of anti-Fib, HBsAg, anti-hIgG, and anti-HSA with a label-free protein array. Fib, HBsAb, hIgG, and HSA were arrayed on the substrate surface in triplicate, respectively. Twelve spots on the protein array were divided into three groups, I, II, and III. Group I reacted with mixed sera containing anti-hIgG, anti-Fib, anti-HAS, and HBsAg, group II reacted with fetal bovine serum and group III left for reference.

3.3 Biomedical applications

In biological research, clinical diagnosis or bioindustries, especially fields related with proteins, the concentration often needs to be detected. Many methods are used currently, such as Biuret, ELISA, RIA, LC, and electrophoresis. The label-free protein array can serve as an alternative method to conveniently detect protein concentration. Samples can be detected directly without any labeling, which avoids disturbances from conjugated markers or handling with radioactive materials. The detection time can be largely reduced without the time consumed for protein labeling. The typical assay procedure with the label-free protein array is similar to any other
assay: optimize assay conditions for the desired range of analyte concentrations, establish a calibration curve using known concentrations of analyte to cover the required range, and measure the unknown concentration of analyte. We used mAb AA98 [27] to measure the concentration of human CD146 in serum. AA98 was first immobilized covalently on protein array, about 1.2 ng/spot. A calibration curve for human CD146 bulk solution concentration detection was obtained using a chip fabricated with the microfluidic device described in this paper (Fig. 4). Each data was obtained by averaging three replicate experiments. 0.1 ng/mL is the current LOD of the protein array. The result was shown here only for a demonstration purpose. In a real application, the relevant protein should be selected, the proper concentration range should be chosen and detection conditions should be optimized.

Hepatitis B is a serious disease caused by a virus that attacks the liver. There are over 100 million hepatitis B virus carriers in China, and over 30 million are already chronically infected with hepatitis B. Over 100 million blood samples need detection for the diagnosis of Hepatitis B per year in China. HBsAg, HBsAb, hepatitis B core antibody (HbcAb), HBeAg, and HBeAb are five markers for Hepatitis B diagnosis, and they are detected one by one with ELISA in Chinese hospitals.

The feasibility of using the label-free protein array for detecting five markers of hepatitis B was investigated. HBsAg, HBeAg, HbcAg, HbsAb, and HBeAb were arrayed in triplicate on one substrate with the microfluidic system, and the spots on the protein array were divided into three groups, I, II, and III. After blocking with BSA, 30 μL of a patient’s serum was sucked into the micro-fluidic system to react with spots in group II at a flow rate of 1 μL/min, the same volume of negative serum was sucked to react with spots in group I in the same time and group III was left for reference. A detection result with intensity value relative to the control spot larger than 2 grayscale is considered being positive, otherwise being negative. The quantitative result (Fig. 5 and Table 3) indicated that HBsAg, HbeAg, and HbcAb were positive in this patient’s serum. Thirty-five sera of patients have been detected with the protein array and the results of 33 sera were in agree with that obtained by ELISA in positive or negative aspect (Fig. 6). Each result from protein array showed in Fig. 6 was obtained based on at least three replicate experiments.

Table 3. Spot intensity in grayscale of the protein array in Fig. 5

<table>
<thead>
<tr>
<th></th>
<th>HBsAb</th>
<th>HBeAb</th>
<th>HbcAg</th>
<th>HBsAg</th>
<th>HBeAg</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>73.8</td>
<td>73.3</td>
<td>63.4</td>
<td>83.1</td>
<td>56.1</td>
</tr>
<tr>
<td>II</td>
<td>126.7</td>
<td>111.1</td>
<td>124.6</td>
<td>82.4</td>
<td>55.7</td>
</tr>
<tr>
<td>III</td>
<td>74.2</td>
<td>73.7</td>
<td>63.2</td>
<td>81.4</td>
<td>55.3</td>
</tr>
</tbody>
</table>

4 Concluding remarks

A label-free protein microfluidic array based on the combination of imaging ellipsometry and an integrated microfluidic system was developed for immunoassays. Fabrication, packaging, and reaction of protein array can be carried out within an integrated microfluidic system.
Figure 6. Detection results of 35 patient sera obtained with the protein array (---) and with ELISA (…Δ…) for HBsAg (A), HBsAb (B), HBeAg (C), HBeAb (D), and HBcAb (E), respectively. The different detection results obtained by these two detection methods were labeled with * (B and E).

Imaging ellipsometry was used for protein array reading in a label-free format. The applications of the label-free protein microfluidic array for screening of antibody-antigen interactions, measuring concentration of protein solution and detecting five markers of hepatitis B demonstrated in this paper indicated that it is a promising technology for parallel immunoassays.

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5 References