A MICROFLUIDIC SYSTEM FOR MICROARRAY FABRICATION AND BIOMOLECULE INTERACTION

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Abstract: One kind of microfluidic system was designed for the fabrication of microarray on chemically modified silicon surface, the ligand immobilization in microarray, and the biomolecule interaction of ligand with analyte in solution. By the microfluidic system, many proteins used as ligands could be delivered individually to each area in the microarray and covalently immobilized on surfaces simultaneously. The homogeneous ligand layer in each area could be obtained, which was in favor of getting reliable data for quantitative analysis with the microarray biosensor based on imaging ellipsometry. The mass transport of protein solutions could be effectively minimized with the microfluidic system, and the time for the microarray fabrication and the biomolecule reaction was largely reduced (within 30 min). The areas in the microarray could be connected in series with simple channels junction to react with one analyte solution in turn. An obvious merit of the microfluidic system was repeated use for microarray fabrication and biomolecule reaction, which greatly reduced the cost of protein array, test time and consumption of proteins (15 µl) as well as had a high sensitivity (1 ng/ml for immunoglobulin).

Introduction

The biosensor concept based on imaging ellipsometry for visualization of biomolecular interactions was reported in 1995 [1, 2]. The sensing surface was a kind of biochip with multi-area of bioactivity in microarray, and the interaction result was sampled with the optical imaging technique of imaging ellipsometry. The microchip fabrication was a crucial process for the biosensor. In order to develop it into a practical technique for protein analysis or protein detection, a kind of microfluidic system was developed for the fabrication, ligand immobilization and biomolecule interaction. At the same time, it should be suitable for the quantitative analysis with the biosensor based on the imaging ellipsometry [3].

Materials and Methods

The biosensor principle and the microfluidic technique are presented in this section.

Principle: Imaging ellipsometry is used to visualize the molecule surface concentration distribution of protein layers attached on a patterned surface on a solid substrate. A ligand and its receptor such as an antibody and its corresponding antigen can assemble into complexes due to their affinity. The optical biosensor is based on that each reactant as a ligand is immobilized to a surface to form a monolayer as a bioprobe with its bioactivity. The other reactant as the analyte (or receptor) exists in a solution. The bioprobe is exposed to the solution containing analyte. When the analyte in the solution interacts with its corresponding ligand on the bioprobe and assembles into complex upon their affinity. The molecule surface concentration on the surface where the interaction takes place becomes higher than before exposure to the analyte solution. A significant increase of the layer thickness (mass surface concentration) indicates that the solution contained receptor against the ligand on the surface. With a visualization of imaging ellipsometry, which has a high spatial resolution in the order of 0.1 nano-meter in vertical and micron in lateral, so the increase can be determined, and in this way, the existence of the analyte in the solution can be verified. Many bioprobes arrayed in matrix are used for multi-detection [3-5].

Integrated microfluidic system: In the development of biosensors, the immobilization of biomolecule at interfaces in microarray was important. At the early stages of the biosensor development, a pattern surface was fabricated by photolithography and ligand immobilization was done with robotic spotting or printing technique, and then the whole patterned surface with multi-bioactivity of ligand was inserted into a solution with analyte for biomolecule interaction. The interaction result was sampled with the imaging technique. In this way, it emerged several problems, 1) the surface patterning with photolithography for microarray was expensive since the sensor surface was used only one time; 2) the robotic spotting or printing technique often resulted in poor morphology and unhomogeneity of protein molecule distribution across spot; 3) a large volume of analyte solution was needed for the consumption; and 4) the interaction time (or test time) was quite long, sometimes it's fatal for protein analysis. A kind of method which could solve above problems was urgently needed.

An integrated microfluidic system was developed in our laboratory, which could be used not only to fabricate protein microarray including patterning, ligand immobilization, but also serve as liquid transportation system and packaging system for protein microarray.

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Protein molecule as ligand could be patterned homogeneously on each area in microarray. The patterning step was at first to localize the exposure of protein molecules as ligand to targeted areas on substrate. Simultaneous immobilization of proteins on these targeted areas was possible without the introduction of cross-interferences, even where different coupling chemistries are needed. And then, the protein array could be stored in a packaging system which was full of buffer so that proteins were not exposed to denaturing condition. The spots in protein microarray could be divided into different groups and connected to each other, respectively in series by a liquid handling system to react with different samples simultaneously under dynamic condition, instead of reacting with single sample under static condition just like conventional protein array. In this way, it's possible to detect several analyte in one sample, or one kind of analyte for several samples simultaneously.

The microfluidic system was fabricated by multilayer soft lithography [6], which consisted three parts. The first part was an 8×6 elliptic microcells array (EMCA) (Figure 1A) fabricated by two-level soft lithography with poly (dimethylsiloxane) (PDMS) (Sylgard 184, Dow Corning). The volume of each cell was 18 nl, depth 50 µm, and cells gap 700 µm. Each cell has two access holes (diameter 300 µm) for solution input and output. The second part of the microfluidic system was an 8×12 Teflon tube array (TTA) (Figure 1B), the tubes were fixed into a polymethyl methacrylate slab punched with a drillpress, and the distribution and the size of the TTA were same as that of the access holes array in EMCA. Four permanent magnetic discs (Ni-plated-NdFeB, 3mm diameter) were embedded into the polymethyl methacrylate slab. The third part of the microfluidic system was a connecting array (CA) (Figure 1C) fabricated by two-level soft lithography with PDMS, which could serve as a connecter to connect the cells in EMCA in series. There were different CAs, different CA could divide the cells into different groups and connect the cells of each group in series, respectively.

In general, a performance with four steps was required to fabricate protein microarray with the microfluidic system (Figure 1B): (i) the EMCA was placed in contact with the silicon wafer surface, for example, which was previously activated by formation of aldehyde groups to achieve chemical coupling with pendant amino groups common to proteins; (ii) the TTA was aligned on the EMCA to make the tubes connect with the access holes and a polymethyl methacrylate slab with four permanent magnetic discs was put under the substrate, the TTA, the EMCA and the substrate were tightly fixed by magnetic force; (iii) proteins solutions were sucked into different cells simultaneously by a multi-channel peristaltic pump (ISMATEC Corp., Swiss); and (iv) after allowing the proteins to bind on the substrate surface for a set period of time, the channels were flushed thoroughly with phosphate buffer, so that homogeneous ligand layers were prepared on each areas in the microarray.

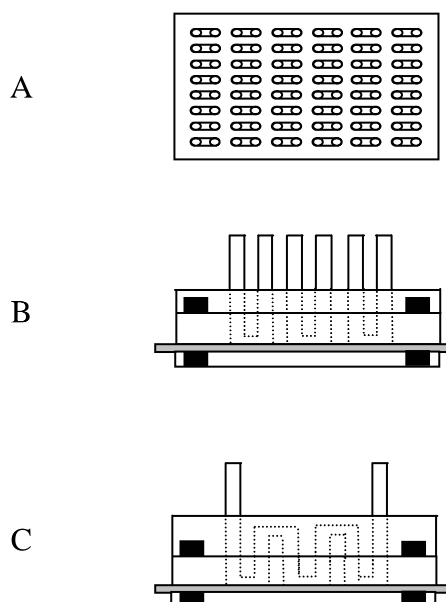

Figure 1: A microfluidic system used for protein microarray fabrication and biomolecule reaction, (A) schematic of an 8×6 elliptic microcells array (EMCA), cell volume 18 nl, depth 50 µm, cells gap 700 µm. Each cell has two access holes (diameter 300 µm) for liquid input and output. (B) schematic of microfluidic setup for protein array fabrication. EMCA is placed in contact with a substrate surface chemically modified, and an 8×12 Teflon tube array (TTA) was aligned on the EMCA to make the tubes (fixed in a polymethyl methacrylate slab) connect with the access holes in EMCA. TTA, EMCA and substrate are fixed tightly by exterior magnetic force from permanent magnetic discs embedded in TTA and a polymethyl methacrylate slab under the substrate. Solutions of proteins are sucked into cells through channels in the array. (C) schematic of microfluidic setup for protein microarray reaction. TTA is replaced by connecting array (CA) which can divide the cells in EMCA into several groups and connect the cells of each group in series, and multiple analyte in solution flow through cells in different groups to react with proteins in microarray, respectively.

The protein microarray fabricated with the microfluidic system was used for protein analytes with the following three steps (Figure 1C): (i) TTA was removed and the given CA was aligned on the EMCA to divide the cells into several groups according to requirement and connect the cells of each group in series, respectively; (ii) tested solution with analyte were sucked into different groups, respectively and flowed through each cell of each group in turn to react with immobilized ligand in the microarray for a set period of time to make the analyte-ligand reaction to a

complete level; and (iii) the phosphate buffer was used thoroughly to wash the channels to remove all unbinding proteins.

Results

Four proteins, human immunoglobulin G (hIgG), human fibrinogen (Fib), human serum albumin (HSA), and monoclonal antibody of hepatitis B surface antigen (HBsAb) were selected for the fabrication of a protein array as a demonstration with the fluidic system. The four protein solutions were sucked into the microfluidic system, each 0.2 µl, and allowed to bind in separated areas with aldehyde groups modified surface for 10 min, respectively in an array.

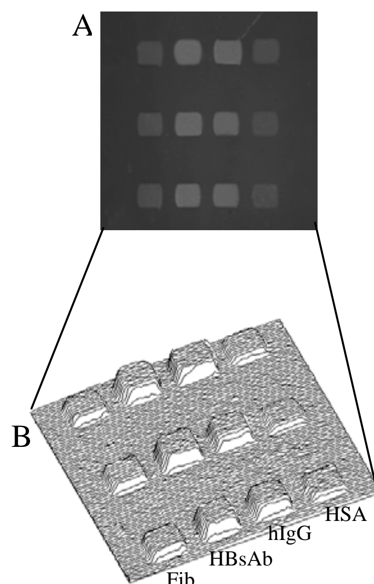

Figure 2: protein microarray with 12 spots fabricated by the microfluidic system shown with an image in grayscale (A) and the image in three-dimension (B), Fib, HBsAb, hIgG and HSA were arrayed on the substrate in triplicate, respectively.

After washed with phosphate buffer, the slide with the four proteins array was taken from the microfluidic system and detected. The digital image of protein array captured by the imaging ellipsometer was shown in Figure 2A. The image of protein array in grayscale format (Figure 2A) was converted into three-dimension image (Figure 2B) according to the relationship between the intensity (I) and the thickness (d) ($I = kd^2$, k was a constant [2]). The protein array consisted of 12 spots and each protein was arrayed in triplicate. The thicknesses of layers on three spots of each protein were measured with ellipsometer, and the thickness deviation was within 0.1 nm. Five areas (each 0.1 mm^2) on each spot were selected randomly and measured for the layer thicknesses, the deviation was also within 0.1 nm. The protein array with homogeneous thickness distribution spots was in favor of obtaining reliable quantitative result.

For the microfluidic system as a micro-reactor of protein array for protein detection, at first the protein array as the method described above was fabricated, and then a CA was used to divide the spots of the protein array into two groups and connect the spots of each group in series. 10 µl of serum containing anti-hIgG, anti-Fib, anti-HSA and HBsAg was sucked into one of groups to react with proteins on spots at a flow rate of 1µl/min, and another group was left as reference. After washed with phosphate buffer, the substrate was taken from the microfluidic system and detected with imaging ellipsometer. The result was shown in Figure 3. The thickness of protein layers on the eight spots with the four ligands in duplicate reacted with the serum increased obviously, and the thickness of protein layers on the spots with Fib layers increased from 2.58 nm to 8.28 nm, HBsAb 4.17 nm to 7.37 nm, hIgG 4.05 nm to 6.44 nm, and HSA 2.48 nm to 5.06 nm, respectively.

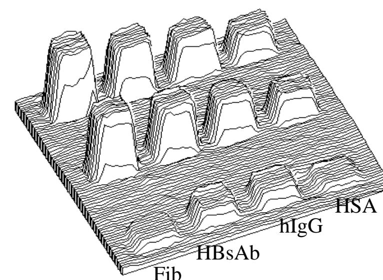

Figure 3: Detection of anti-Fib, HBsAg, anti-hIgG and anti-HSA with a protein array of Fib, HBsAb, hIgG and HSA arrayed in triplicate, respectively. 12 spots on the protein array were divided into two groups with the microfluidic system, and one group with eight spots in a series was connected to react with a serum containing these four proteins (each protein concentration 25 µg/ml) and another group with four spots was left as a reference. The thickness of protein layers on the eight spots reacted with serum increased obviously due to the formation of antibody-antigen complex.

For some potential clinic applications, a microarray with five probes for Hepatitis B detection was demonstrated here. Hepatitis B was a serious disease caused by a virus that attacked the liver. The virus, which was called hepatitis B virus (HBV), could cause lifelong infection, the cirrhosis (scarring) of liver, the liver cancer, the liver failure, and even death. The World Health Organization estimated that 400 million people worldwide were already chronically infected with hepatitis B, about Seventy five percent lived in Asia and the Western Pacific, with China having the highest incidence of the disease in the world. The diagnosis of HBV infection was generally made on the basis of serology. In china, hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), hepatitis B core antibody (HBcAb), hepatitis B e

antigen (HBeAg), and hepatitis B e antibody (HBeAb) were five markers for Hepatitis B diagnosis, and they were detected one by one with Enzyme-Linked Immunosorbent Assay (ELISA).

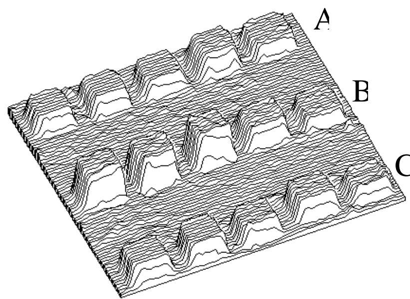

Figure 4: Detection of five markers for the diagnosis of hepatitis B with a single label-free protein array. HBsAb, HBeAb, HBcAg, HBsAg and HBeAg were first arrayed in triplicate on a substrate surface and blocked with BSA (20 mg/ml). 15 spots on the protein array were divided into three groups, A, B and C, and connected in series respectively with the microfluidic system. A group reacted with negative serum, B group reacted with patient's serum and C group served as reference.

A protein array with HBsAg, HBeAg, HBcAg, HBsAb and HBeAb ligand as probes were arrayed in triplicate, respectively in the protein array, and the spots on the protein array were divided into three groups, A, B and C. The spots in three groups were first blocked with bovine serum albumin (BSA) (20 mg/ml) for 30 min, and then incubated in a patient's serum 30 µl with the microfluidic system to react with probes in B group, the same volume of negative serum was sucked to react with probes in A group at the same time, and C group was left for reference. The detection result was shown in Figure 4. In comparison with the spots in A and C groups, the thicknesses of protein layers on the spots with HBsAb, HBeAb and HBcAg in B group significantly increased, but almost no change occurred on the spots with HBsAg and HBeAg in B group. The result indicated that HBsAg, HBeAg and HBcAb were positive in this patient's serum, which was in agreement with the result obtained by ELISA. In terms of sensitivity, ellipsometry had a thickness resolution of 0.01 nm or better and therefore sensitive enough for detection of molecule layers on solid surfaces. Standard sample of HBsAg (1 ng/ml) detection was achieved by the protein array in our laboratory (data not shown), which reached to the requirement of sensitivity for hepatitis B diagnosis in China. Over 200-million blood samples needed detecting for the diagnosis of Hepatitis B every year in China, therefore the development of new detection method with low cost and short time was quite valuable.

Conclusions

With the combination of the imaging ellipsometer and the microfluidics, the integrated microfluidic system may realize the protein array fabrication with homogenous ligand spots conveniently, and a micro-reactor for protein detection, as well as multiple samples analysis simultaneously. Compared with present clinic techniques, the detection time and the consumption of samples may be largely reduced with the microfluidic system. The demonstration results show a simple label-free protein array with high sensitivity as a promising technique for medical diagnosis.

Acknowledgements

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