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Fabrication of Disposable Protein Chip for Simultaneous Sample Detection

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Abstract In this study, we have described a method for the fabrication of a protein chip on silicon substrate using hydrophobic thin film and microfluidic channels, for the simultaneous detection of multiple targets in samples. The use of hydrophobic thin film provides for a physical, chemical, and biological barrier for protein patterning. The microfluidic channels create four protein patterned strips on the silicon surfaces with a high signal-to-noise ratio. The feasibility of the protein chips was determined in order to discriminate between each protein interaction in a mixture sample that included biotin, ovalbumin, hepatitis B antigen, and hepatitis C antigen. In the fabrication of the multiplexed assay system, the utilization of the hydrophobic thin film and the microfluidic networks constitutes a more convenient method for the development of biosensors or biochips. This technique may be applicable to the simultaneous evaluation of multiple protein–protein interactions.

Keywords: protein chip, protein patterning, microchannel, simultaneous detection

INTRODUCTION

Recently, a multiplexed format on a microchip has been applied to broad applications involving the hybridization of nucleic acids or the interactions of proteins with their partners, including small molecules, peptides, nucleic acids, and proteins [1-3]. The diversity of these interactions, as well as variations in the properties of the proteins, have provided new insights into microchip technology [4-6]. The most salient issue with regard to the use of these microchips has been the development of a surface-based assay in which numerous probes are immobilized in a spatially-addressable manner (patterning) [5-7]. Such assay formats have resulted in advances in both miniaturization and multiplexing, which confer advantages in terms of analytical time and the quantity of sample that must be consumed.

The patterning of proteins on solid surfaces, while it prevents the nonspecific binding of unwanted areas and species, constitutes a key technology in the development of biosensors and biochips for the monitoring of specific protein-protein interactions, as well as the immobilization of proteins or cells [5-7]. In the development of diagnostic kits, biohybrid material, biomaterial devices, protein sensors, and protein chips, nonspecific binding frequently represents the bottleneck factor in terms of achieving reliable sensitivity and reproducibility [5-9]. Other desirable methods must fulfill several requirements, such as a minimal number of processing steps, minimal nonspecific binding in the backgrounds, and low production cost for single-use applications.

In order to pattern the proteins onto the chip substrate, several representative methods for protein patterning have been reported, including the photochemical method [10], microcontact printing (μCP) [11,12], and physical spotting [13]. The photochemical method can be repeatedly utilized on the same surface with different proteins, thereby creating multiple protein patterns. However, UV light (265~275 nm) can induce a detrimental effect on protein activity during irradiation, due to the loss of intact conformation.

Microcontact printing (μCP) is a simple and flexible technique, which is generally well adapted for use in research laboratories not equipped with photolithographic tools and clean room facilities. However, the success of transferring biomolecules from stamps to the surfaces of chips is highly dependent upon the processing times required for both the drying of the stamp and the printing of the proteins. The printing process must be optimized in order to obtain reliable data, and the density of printed proteins during batch-to-batch experiments tends to be relatively low.

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Direct spotting is a well-known method for the fabrication of versatile microarrays. Multiple biomolecular arrays could be realized via the use of a robotically-controlled spotter. This is a well-known technique for mass microarray production, but the facilities required for this approach are prohibitively expensive for laboratory research. Therefore, there remains the need for the development of efficient protein immobilization techniques for the orderly patterning of proteins on microfabricated surfaces for protein chips.

In an attempt to ameliorate or circumvent the problems referenced above, we previously developed a novel protein patterning method employing hydrophobic thin film, which reduced nonspecific binding on the background and allowed for the creation of various protein patterns from aqueous solutions at the micron scale [5-7]. Although our proposed approach was well adapted to microfabricated chips, the method was limited in terms of the fabrication of different protein patterns on a single microchip, as the ideas inherent to its initial construction were premised on the use of a single protein solution.

In this study, we suggest another approach for the fabrication of a multiplexed protein microchip using a hydrophobic film pattern as a physical or chemical barrier, coupled with microfluidic channels for the introduction of each protein onto separated strips. In order to determine the feasibility of the multiplexing assay, a mixture of four proteins – ovalbumin, streptavidin, hepatitis B, and hepatitis C antibody – was used as a model system on the protein-patterned strips.

MATERIALS AND METHODS

Materials

All chemicals employed in this study were of analytical or research grade. Silicon nitride (Si_3N_4) substrate was diced into 1.5 × 2 cm pieces. The silicon nitride layer was grown on the silicon substrate via plasma-enhanced chemical vapor deposition (PECVD). Fluorocarbon (FC) polymer, FC 722 and FC 40 (3M's Fluorad™), were obtained from the 3M corporation (Seoul, Korea).

Sulfo-NHS-LC-LC-Biotin was provided by Pierce (Pierce, IL, USA). Biotinylation was conducted in accordance with the protocols recommended by Pierce. FITC (fluorescein isothiocyanate)-labeled BSA (Bovine Serum Albumin), Streptavidin, γ-APS (3-amiopropyl triethoxysilane), Tween 20, glutaraldehyde 25% solution (Grade II), Chicken egg albumin (ovalbumin, Grade V), mouse monoclonal anti-chicken egg albumin antibody, and FITC-conjugated goat anti-mouse IgG were acquired from Sigma (St. Louis, MO, USA). Anti-hepatitis B surface antigen monoclonal antibody, hepatitis B surface antigen, anti-hepatitis C antigen monoclonal antibody, hepatitis C antigen, FITC-anti-hepatitis C antigen polyclonal antibody, and FITC-anti-hepatitis B surface antigen polyclonal antibody were obtained from Biodesign International (Kennebunk, Maine, USA).

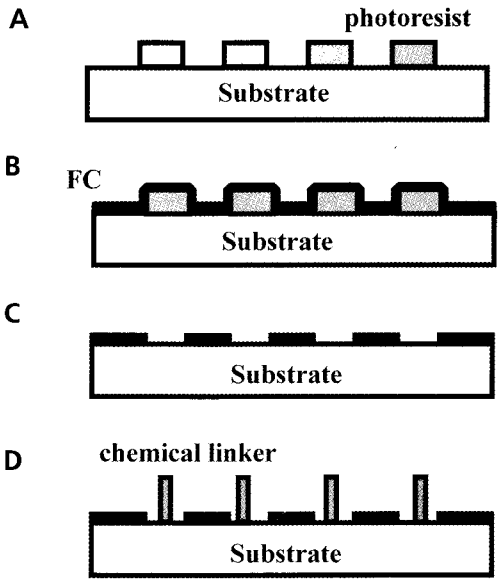

Fig. 1. Schematic diagram of the microfabrication process of FC film patterning on substrates. (A) Thick PR spin-coating and patterning, (B) FC film spin-coating, (C) Hard baking in a convectional oven, (D) PR stripping by an acetone solution in an ultrasonic bath.

Microfabrication of the Hydrophobic Patterned Chip via Lift-off Process

All microfabrication steps were conducted in a clean room (The Inter-university Semiconductor Research Center, Seoul National University). The silicon nitride substrates were precleaned in a mixture of H_2SO_4 and H_2O_2 (4:1) and finally rinsed in deionized water. The FC films were deposited via spin-coating. A mixture of FC 722 and FC 40 (3M's Fluorad™) was used for spin coating.

In order to pattern the FC film onto the surfaces via the lift-off process, 3 μm-thick Photoresist (AZ 4330) was spun onto the substrates and patterned via conventional photolithography (Fig. 1A). Then, the FC thin film was spin-coated onto substrates with Photoresist patterns at a speed of 2,000 rpm (Fig. 1B). After 10 min of hard baking at 110°C, the Photoresist patterns were removed via 2 min of immersion in an acetone solution in an ultrasonic bath. The sonicated specimens were then rinsed with methanol and deionized water in an ultrasonic bath for 2 min each (Fig. 1C). Fig. 1 shows the microfabrication process flow for the patterning of FC films via the lift-off method. Using this lift-off process, we have obtained highly ordered micropatterns on the silicon nitride surfaces.

Fabrication of PDMS Microfluidic Channels

We utilized replica micromolding techniques in order to construct the microchannels. The silicon master was fabricated via silicon deep-reactive ion etching (RIE), using a Photoresist mask. The etching depth was ap-

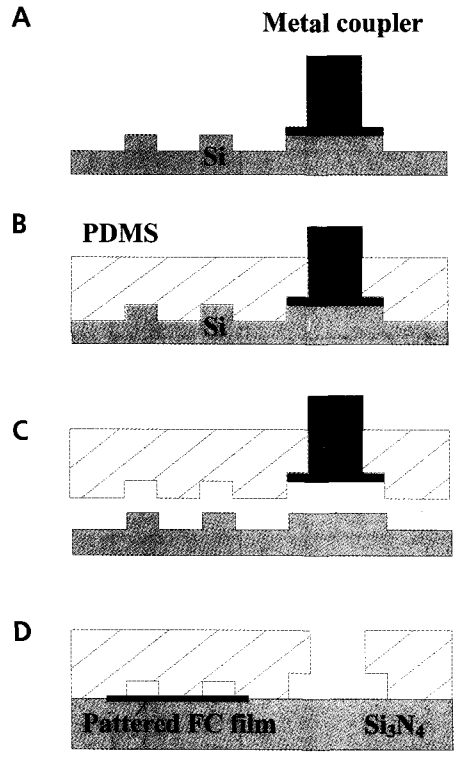

Fig. 2. Fabrication method of PDMS microfluidic channels via replica molding. A silicon master was fabricated via silicon deep RIE and hydrophobic layers were grown via FC thin film deposition. Following the fabrication of the PDMS microfluidic channels, the PDMS microfluidic channels were detached from the silicon master. Finally, the PDMS microfluidic channels were carefully assembled with the patterned hydrophobic chips. (A) A silicon master fabrication by silicon deep RIE and FC thin film deposition, (B) PDMS mixture pouring and curing, (C) Release of PDMS microfluidic channels and surface oxidation, (D) Aligning and bonding of PDMS microfluidic channels on the FC patterned chip.

proximately 100 μm (Fig. 2A). In order to facilitate the release of the cured PDMS, fluorocarbon thin film was deposited on the silicon master surface in C_4F_8 gas plasma using an ICP reactor (Plasma-Therm SLR). A mixture of PDMS prepolymer and curing agent (10:1, Sylgard 184, Dow Corning Co.) was thoroughly stirred, then degassed in a vacuum oven. The degassed PDMS mixture was then poured onto the silicon master and cured at 65°C (Fig. 2B). After curing, the PDMS replica was peeled away from the master (Fig. 2C) [15]. The PDMS microchannels were aligned and bonded to the substrates with the FC patterned surfaces (Fig. 2D). A fluid line coupler was pasted onto the region for the connection of the Teflon tube. Fig. 2 shows the fabrication of microfluidic channels via PDMS replica molding.

Protein Patterning

The substrate was subsequently rinsed in acetone for 10 min, in ethanol for 15 min, and in deionized water for 15 min, in order to remove any residual Photoresist chemicals. The substrate was then dried carefully under blowing nitrogen stream, until the next step.

Microfabrication and protein patterning were conducted in accordance with the previously reported procedures (Fig. 1D) [5-7]. First, the oxidation process was conducted using modified piranha solution to preserve the hydrophobic thin film. After the substrates were soaked for 3 h in the modified piranha solution, they were carefully dried in a convection oven for 3 h at 110°C. Secondly, the introduction of amine functional group onto the surface was effected using a 10% solution of 3-aminopropyl triethoxysilane in absolute ethanol at room temperature for 12 h. Thirdly, the amino-silanized substrate was allowed to react with 10% glutaraldehyde in 1 mM PBS buffer (pH 7.5) at 30°C for 1 h. After several rinses in deionized water, the fabricated microchannel was assembled with the aldehyde-modified silicon chip. Each protein solution was then continuously introduced to aldehyde-modified strips at room temperature for 1 h. Schiff bases were formed between the amine groups in the proteins and the aldehyde groups on the surface. Feeding was maintained at a constant rate of 1 mL/h with a microsyringe pump (PHD2000, Harvard Apparatus, MA, USA). Finally, 1% BSA solution was applied in order to inactivate the residual aldehyde groups on the patterns.

Protein Interaction Assay

In the streptavidin-biotin system, FITC-BSA-biotin solution was allowed to react with streptavidin. For quantitative analysis, the amount of immobilized proteins on the desired patterns (100 × 200 μm) was measured indirectly with FITC-BSA-biotin, and variances of the fluorescence intensity were calculated from both the intra- and inter-patterns (batch to batch trials).

For the immunoassay, after ovalbumin (1 μg/mL) was initially patterned on the surface in accordance with the above procedure, mouse anti ovalbumin monoclonal antibody (1 μg/mL) was allowed to interact with the patterned ovalbumin for 15 min at room temperature. The final binding event was induced via the addition of FITC-labeled anti-mouse IgG secondary antibody (1 μg/mL) for 15 min at room temperature. In the hepatitis immunoassay, hepatitis B surface antigen antibody (1 μg/mL) and hepatitis C antigen antibody (1 μg/mL) were initially immobilized. Then, hepatitis B surface antigen (1 μg/mL) and hepatitis C antigen (1 μg/mL) were introduced for 15 min at room temperature. Finally, the FITC-labeled anti-hepatitis B surface antigen secondary antibody (1.25 μg/mL) and FITC-labeled anti-hepatitis C antigen secondary antibody (1.25 μg/mL) were added for 15 min at room temperature.

Fluorescence Analysis

Fluorescence images were acquired with laser-scanning confocal fluorescence microscopy (BioRad MRC 1024, Bio-Rad Laboratories Inc., CA, USA). FITC was excited

at 488 nm with a krypton-argon laser. The micropatterned substrates were viewed with the 10 × objective. As the fluorescence evidences an emission peak in a frequency corresponding to the color green, only the green component of the image was analyzed.

Contact Angle

Prior to the measurement of contact angle analysis, the substrates were rinsed in deionized water and dried under a stream of nitrogen. The contact angles of the substrates were determined after each reaction step within 24 h. Krüss G10 contact angle analyzer (Krüss GmbH, Hamburg, Germany) was employed in order to determine the wettability and surface tension of the hydrophobic thin film. All measurements were conducted at room temperature and ambient humidity. Each reported value was the average of the contact angles measured five times.

RESULT AND DISCUSSION

Chip Design

Many microfluidic chips are composed of PDMS and glass [14,15]. Although it is easy to create permanent bonds between PDMS microchannels and glass substrates via oxygen plasma bonding [16], cartridge-type biochips present difficulties with regard to this process, owing to their usage of different materials, including silicon or polymers. As the silicon chips used in this study were coated with hydrophobic thin film originating from an anti-stiction agent, it is difficult to create a bond between the PDMS microchannel and the silicon chip via the conventional oxygen plasma method.

For the stable construction of microfluidic chip, the protein chip assembly used a plastic housing in order to prevent any leakage of the protein solution while protein patterning was conducted with the PDMS microchannels. The assembled system consisted of an acrylate housing, a silicon chip, a PDMS microchannel, and PEEK tubing (Fig. 3A). As is shown in Fig. 3B, the tight seal between the cover and silicon chip was reinforced using an acrylic housing with screw bolting. The fabricated silicon chip has 20 rectangular patterns per strip, and contains four strips. In order to discriminate each strip, the dot patterns were inscribed on each line (Fig. 3C). They are sandwiched between a top and a bottom microfluidic network fabricated via soft-lithography (Fig. 3). As is shown in Fig. 3A, the four strips were designed for multiple assays, respectively. The expansion of the strip numbers may potentially provide perfectly identical illumination conditions for the assays. Also, the additional increase of multiple strips will allow for multiplexing capabilities.

In order to seal the microfluidic chip and maintain the relatively efficient flow rates required for protein patterning on each strip, the pressure effect must be considered. As the protein solution flows through the microfluidic channel for selective immobilization onto the patterned

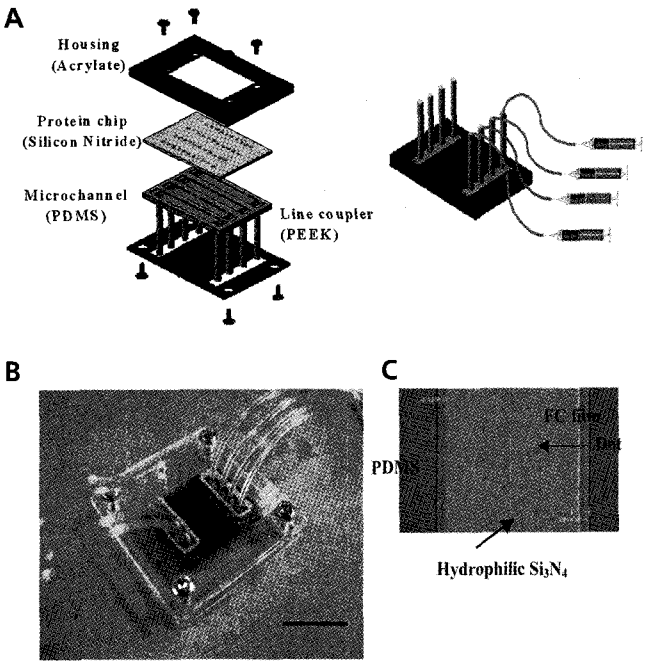

Fig. 3. The images of chip construction. (A) The schematic diagram of the microfluidic chip. The microfluidic chip consists of an inner layer composed of a silicon protein chip, sandwiched between a top and a bottom microfluidic network fabricated via soft-lithography, which is a cartridge type of protein chip. The final assembled microfluidic chip was connected with a syringe pump to introduce proteins into each lane. For the protein interaction assay, the microfluidic chip can be readily disassembled. (B) The snapshot of fully assembled chip. Scale bar indicates 1 cm. (C) The enlarged image of the patterned regions in each lane. The patterning of the dots can be used for the differentiation of the lanes, respectively.

silicon chip, pressure accumulates within the microchannel. The internal pressure, coupled with the external holding pressure, can effect the deformation of the microfluidic channel, which then leads to leakage of the protein solution. However, the overload of external pressure results in the collapse of the microfluidic channel, thereby leading to a blockage of the protein solution flow. The dimensional design of the microfluidic chip was determined using ANSYS fine element analysis software, considering major factors including microfluidic flow pressure, holding pressure, and channel deformation. Young's modulus and the Poisson ratio of the PDMS were approximately 3 MPa and 0.49 [6], respectively. As the maximum microfluidic pressure was determined to be less than 125 kPa in the previous study [6,14], the simulation was conducted at 125, 62.5, and 31.25 kPa. According to the results of finite elemental analysis, increases in the external pressure result in the collapse of the microchannel, and leakage occurs at high flow rates (>0.2 mL/min), and the optimum channel's height is 100 μm below 125 kPa (data not shown). Thus, the dimensions of the PDMS microchannels were selected as 0.8 × 0.1 × 14 mm (width, height, length).

Simultaneous Proteins Assay

The protein chip on silicon substrate was adapted and expanded herein for the development of a protein interaction assay containing protein-small molecule, antigen-antibody, and antibody-antigen. Firstly, the hydrophilic patterns (silicon nitride) with hydrophobic background (FC film) are created via conventional photolithography, after which the aldehyde-modified surfaces on the hydrophilic region are introduced in order to effect the selective immobilization of proteins in accordance with the previously developed methods prior to the assembly of each component [5-7]. The fabricated PDMS microfluidic channels, which included four lanes (width: 0.8 mm, height: 0.1 mm, length: 14 mm) were mounted for the delivery of each protein solution to each strip. As the capillarity of protein solution is dependent on the surface properties of the microfluidic channel, the microfluidic channels were exposed briefly to oxygen plasma in order to introduce the hydrophilic functional groups to the surfaces of PDMS (inherent contact angle 119°), thus allowing for a positive capillary action on liquids, due to changes in the properties of the hydrophilic surfaces (contact angle 15°). For multiple protein patterning, each of the protein solutions (1 μg/mL) was introduced via injection through the separated inlet lines in Fig. 3A. These multiple protein strips function as a miniaturized assay with selectivity determined by the specificity of the capturing proteins. Consequently, the protein strips, in the current format, evidence a potential capacity to capture and detect up to four different protein interactions, using a single drop of sample fluid (~50 μL). Identification and quantification of analytes occur via fluorescent signals acquired using a CCD camera, thereby allowing for the digital analysis of the binding event.

In order to determine whether the protein strips might be utilized for the interaction assays, we selected three representative types of studies; streptavidin-biotin (lane 1) for protein-small molecule interactions (Fig. 4A); ovalbumin–anti-ovalbumin antibody for indirect immunoassay (lane 2) (Fig. 4B); and hepatitis antibody – hepatitis antigen for direct immunoassay (lanes 3 and 4) (Figs. 4C and 4D). After the first layer protein of each pair was arrayed on the aldehyde-modified silicon chip using the microfluidic channels, the microfluidic chip was disassempled, thereby releasing the protein chip. Their partners were then loaded into the protein chip.

The fluorescent image shown in Fig. 4A was probed with FITC-BSA-biotin as a model system for interactions between proteins and small molecules. As had been anticipated, only patterns harboring streptavidins in lane 1 were detected, and the other patterns evidenced no fluorescence signals, due to the lack of an interaction partner. This finding indicates that the immobilized protein was capable of retaining its functional activity on the silicon surface, and non-specific binding to other proteins is negligible.

The immunoassay is known to be one of the most important analytical methods, and is widely employed in clinical diagnoses [17,18]. In this study, we utilize two

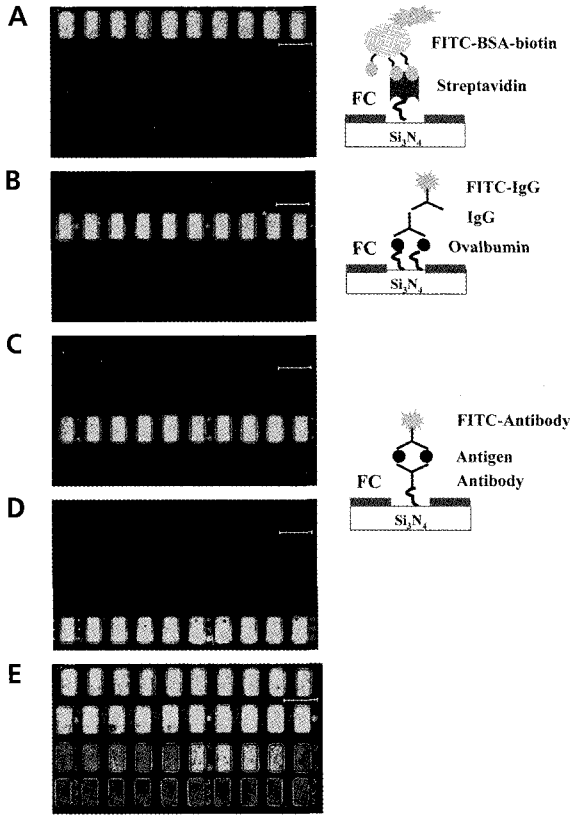

Fig. 4. The confocal fluorescence image of the protein interaction assay. The images clearly show lane 1: streptavidin – FITC-BSA-biotin, lane 2: ovalbumin – mouse anti ovalbumin monoclonal antibody – FITC-labeled anti-mouse IgG secondary antibody, lane 3: hepatitis B surface antigen antibody – hepatitis B surface antigen – FITC-labeled anti hepatitis B surface antigen secondary antibody, and lane 4: hepatitis C antigen antibody – hepatitis C antigen – FITC-labeled anti-hepatitis C antigen secondary antibody. The results of a single interaction assay were respectively shown from Fig. 4A to 4B. Fig. 4E indicates the simultaneous detection of biotin, ovalbumin, hepatitis B, and hepatitis C. Scale bar indicates 200 μm.

different immunoassay methods as a model system for protein-protein interactions. First, for the indirect immunoassay, after lane 2 was treated with ovalbumin, the protein chip was preferentially probed with anti-ovalbumin monoclonal antibody, after which FITC-anti IgG secondary antibody was added for 30 min at room temperature, which generated a fluorescent signal originating from binding with ovalbumin antibody (Fig. 4B). Similarly, only patterns harboring ovalbumin were visible after the binding of the captured ovalbumin antibody and fluorescent secondary antibody, in accordance with the format of the indirect immunoassay (Fig. 4B). Finally, in the direct immunoassay of hepatitis B and C (lanes 3 and 4), the capturing antibody interacted with each antigen and binding was confirmed using fluorescent secondary hepatitis antibody (Figs. 4C and 4D). As infections with the hepatitis B virus (HBV) and hepatitis C virus (HCV) are the primary causes of chronic liver inflammation, result-

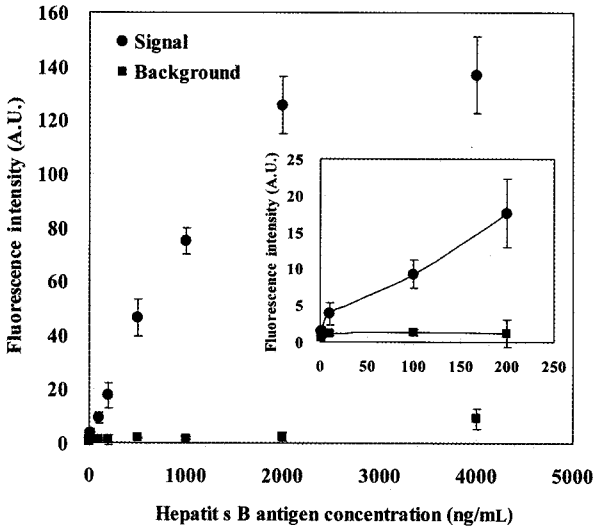

Fig. 5. Quantitative analysis of hepatitis B detection; signal (●) and background (■). In the inset graph, the enlarged data was shown in a range from 1 to 200 ng/mL of the hepatitis B antigen.

ing in cirrhosis and hepatocellular carcinoma, the analysis of hepatitis might prove extremely valuable. This approach might also prove useful in the selection of appropriate treatment and the early-stage diagnosis and monitoring of these diseases [19-22].

The ability to measure more samples under the same conditions is promising because it might reduce both analytical time and analytical cost, and may also effect improvements in the statistical properties of the data. For the blind test, a sample mixture of FITC-BSA-biotin, ovalbumin antibody, hepatitis B antigen, and hepatitis C antigen was allowed to interact with the patterned silicon chip. Then, a mixture of FITC-antibody was probed in order to measure the fluorescence signals. This result is shown in Fig. 4E. As had been expected, the multiple protein strips were capable of accurately identifying the blind samples in the simultaneous assay. As the four distinctive markers from 1 to 4 lane alleviate the positions of the patterned proteins, we were able to detect these interactions simultaneously (Fig. 4E). The measured signal intensity values are similar in lanes 1 and 2. However, a reduction in the signal intensity was observed in the cases of lanes 3 and 4, which may be attributable to from a slight lower avidity or specificity of hepatitis antibody, or to a cross-reaction occurring with other proteins in solution when the mixture was added to the protein chip.

Quantitative Analysis

Another prerequisite for the protein chip is a capability of quantitative analysis below a certain level [23]. In order to assess the performance of our quantitative analyses, 1 μg/mL of unlabeled hepatitis B antibody was utilized for protein patterning, and then different concentrations of hepatitis B surface antigen were incubated with the patterned antibody surface. Finally, FITC-hepatitis B antibody was utilized in order to quantify the binding of hepatitis B surface antigen concentrations. As the antibody and antigen evidence a high binding constant, this assay is, in general, the most powerful method in molecular biology, immunoassays, diagnostics, and biosensors. The feasibility of the antibody and antigen reaction system is a fundamental criterion in studies of biosensors, protein chips, and other miniaturized analytical devices. By varying the concentration of hepatitis B antigen (the proteins being captured), the fluorescence intensity of the patterns began to become saturated at above 2 μg/mL. Fig. 5 indicates the magnitude of the fluorescence intensity obtained via the analysis of 20 patterned rectangles per lane from three separated batch experiments. The fluorescence signal intensity increases in a linear fashion with increases in the concentration of the antigen, up to 2 μg/mL (Fig. 5). This result indicates that the protein chip had a quantitative capability from 10 ng/mL (~500 pM) to 2 μg/mL, and that concentrations in this range were accessible not only with purified proteins, but also with cell lysates or blood samples. The detection limit was 10 ng/mL (inset graph of Fig. 5), comparable to that of our recently reported protein assay system with regard to the signal-to-noise ratio (*i.e.* S/N 3.31 ± 0.26). The level of the signal-to-noise ratio (>2.0) indicates a reliable range of detection in the chip-based assay [5,21]. Therefore, if the specific interaction is defined, the protein chip may potentially be exploited for the quantification and detection of proteins in actual samples.

In addition, four of the fabricated protein strips evidenced low background signals, at below 2 μg/mL, thereby indicating the prevention of nonspecific binding from the capability of the hydrophobic thin film. Low background signals indicate that the hydrophobic thin film functions as a physical, chemical, and biological constraint, such that a highly ordered protein pattern at micron level can be obtained, coupled with the blockage of nonspecific binding. This result is strongly correlated with the findings of our previous studies [5-7].

CONCLUSION

In this study, we have demonstrated the patterning of multiple proteins with the prevention of nonspecific binding using a hydrophobic thin film (Fluorocarbon) coated onto silicon surfaces and microfluidic channels.

One of the principal advantages of the protein chip is its disposability. Via micromolding techniques, PDMS microchannels can be easily and massively fabricated. The primary cost of the chip is due to the fabrication of the protein chip on a silicon substrate, which can also be made of plastic. However, the protein chip on silicon substrate can be produced on the large scale in a common silicon facility at reasonable cost, and can be easily separated as diposable biosensor.

We expect the convenience, rapid preparation, and robustness of protein chip to prove valuable in practical usage, as sample mixtures can be readily detected by our proposed method. Our approach represents a step to-

wards mass production while retaining exceptional assay characteristics.

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