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Protein Array Fabricated by Microcontact Printing for Miniaturized Immunoassay

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Abstract A protein array was fabricated for a miniaturized immunoassay using microcontact printing (μCP). A polydimethylsiloxane (PDMS) stamp with a 5 μm×5 μm dimension was molded from a silicon master developed by photolithography. Under optimal fabrication conditions, including the baking, incubation, and exposure time, a silicon master was successfully fabricated with a definite aspect ratio. An antibody fragment was utilized as the ink for the μCP, and transferred to an Au substrate because of the Au-thiol (-SH) interaction. The immobilization and antibody-antigen interaction were investigated with fluorescence microscopy. When human serum albumin (HSA) was applied to the protein array fabricated with an antibody against HSA, the detection limit was 100 pg/ml of HSA when using a secondary antibody labeled with a fluorescence tag. The fabricated protein array maintained its activity for 14 days.

Key words: Microcontact printing, poly(dimethylsiloxane), protein array, fluorescence microscopy, sandwich-type immunoassay

Over the past decade, microarray technology has evolved from genomics to proteomics, which offers several advantages. In small volumes, biochemical reactions are often not diffusion-limited, making them more efficient by reducing the amounts of reagent and sample solution used and lowering the costs per test [1, 16]. Miniaturized assays can also be performed quickly and simultaneously in large numbers, plus a small scale can facilitate certain types of assay, *e.g.*, where a laminar flow in capillaries is required.

Although there are already several techniques for creating micron-level two-dimensional arrays of biomolecules on surfaces, such as the conventional pin microarrayer, piezoelectric dispenser, and self-assembled monolayer [4, 10, 13, 14, 17], a major focus of current microarray fabrication research is microcontact printing (μCP) for transferring alkanethiols onto an Au substrate.

In μCP methods, a poly(dimethylsiloxane) (PDMS) stamp is coated with a solution containing the target material for patterning, and then brought into contact with a functionalized solid substrate, such as glass, silicon, or gold. Owing to the low fabrication costs and simplicity of transferring the target material to the substrate, the application of μCP has been expanded to DNA/protein patterning, nanoparticle pattern formation, and patterned cell adhesion [3, 9, 12]. In addition, with the proposal of simultaneous patterning of different types and concentrations of biomolecules [2], μCP has become the leading technology for the high integration and miniaturization of bioassays.

Currently, the most promising method for preparing a protein pattern is to utilize a pre-patterned self-assembled monolayer that is able to induce chemical coupling [8, 11]. The main advantage of this procedure is its reproducibility due to the exclusion of physical adsorption, which otherwise makes the immobilized amount unpredictable. However, the procedure is so complicated that a loss of activity and features of the fabricated pattern can frequently occur in the process of deposition and washing. Therefore, another technique combined with self-assembly (SA) is needed to fabricate a stable and reproducible protein pattern.

Antibody fragments have already been utilized for the fabrication of a protein array with a molecular orientation, and since the molecular affinity of the prepared antibody fragment is known to be relatively high because of the thiol group (-SH), the features and activity of the fabricated array are reproducible in experiments. Nonetheless, while

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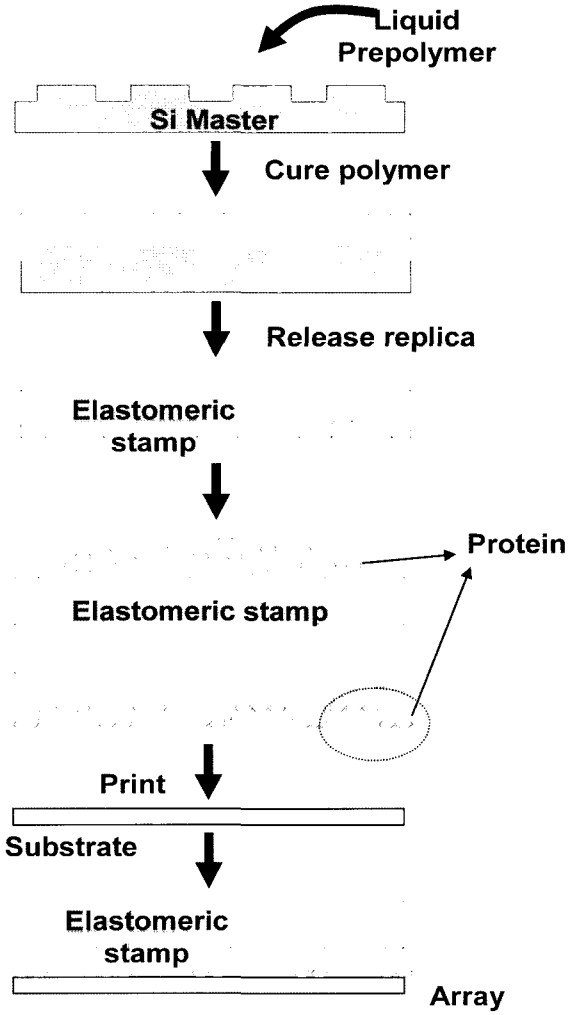

Fig. 1. Schematic description of microcontact printing for protein array fabrication.

the binding event of antigens on the fabricated pattern has been investigated by the current authors [5], a miniaturized sandwich-type immunoassay based on an antibody fragment and secondary antibody has not yet been reported.

Accordingly, this study fabricated a protein array on the basis of an antibody fragment that enabled the formation of a stable and rigid thin film. Figure 1 shows a schematic description of the protein patterning based on μCP. On the patterned surface, the proposed assay system can be visualized using a fluorescence-labeled protein, such as a secondary antibody tagged with fluorescein isothiocyanate (FITC) or Cy3. The optimal fabrication conditions for the silicon master were also investigated to produce a PDMS stamp with clear features. The performance of the fabricated protein array was examined using fluorescence microscopy. Finally, the variation in activity of the immobilized antibody fragment was considered relative to the preservation time.

MATERIALS AND METHODS

Materials

All the solutions were prepared with Millipore (Milli-Q) water. The human serum albumin (HSA) and fluorescence isothiocyanate (FITC)-labeled HSA were purchased from Sigma (St. Louis, MO, U.S.A.) and AbCam (U.K.), respectively. The monoclonal and polyclonal antibodies against HSA were also purchased from Sigma. A Cy3-labeled antibody against rabbit IgG was utilized as the secondary antibody and purchased from Amersham Biochemistry (U.K.). The antibody was immobilized on a gold (Au) surface that had been pre-cleaned with a piranha solution. The antibody fragmentation was carried out on the basis of cited references [6, 7]. The other chemicals used in this study were obtained as reagent grade.

Preparation of Master Mold

The chromium mask was purchased and prepared by Micro Image Co., Ltd. (Korea). Before the mask was fabricated, an array of 5 μm×5 μm squares was organized by computer aided design (CAD). The masters, containing a positive relief of the stamp mold, were manufactured by the Nanofabrication Center at the Korea Institute of Science and Technology (KIST), which involved spin-coating a positive photoresist, AZ7220 from Shipley (Marlborough, MA, U.S.A.), onto a silicon wafer and irradiation with UV light through a chromium mask to render the exposed photoresist soluble, allowing it to be removed by washing with a developer, LDD26 from Shipley.

Stamp Fabrication

The PDMS stamps were fabricated by casting and curing Sylgard 184 (Dow Corning, Midland, MI, U.S.A.), an elastomeric polymer, against the silicon master with 3-μm thick features made by photolithography. The prepolymer and curing reagent were mixed using a volume ratio of 10:1, and the mixture poured onto the silicon master. The viscous polymer solution was then degassed in a vacuum station (Bernant, IL, U.S.A.) for 2 h, and after curing at 60°C overnight, the elastomeric stamp bearing the complementary pattern of the master was peeled off, sonicated with 70% ethanol for washing, and dried under nitrogen blowing.

Microcontact Printing: Sample Preparation

As the surface property of the prepared PDMS elastomer is hydrophobic, this allowed the proteins fabricated by μCP to be easily adsorbed on the PDMS surface. The process of loading proteins onto the stamp surface is referred to as "inking." Thus, after inking the PDMS surface, the stamp was incubated for approximately 1 min and dried using a slide centrifuge (Fisher, U.K.). The Au surface was then

Table 1. Summarized procedure for fabrication of a silicon-based master using AZ7220 photoresist.

Photoresist (P/R) name	Process name	Thickness (μm s)	Spin speed (rpm)	Viscosity (cSt)
AZ 7220	1. P/R coating	1.5	4,000	
		2	3,500	45
		3	3,000	
	2. Soft baking	Thickness (μm s)	Pre-bake (at 65°C, min)	Soft bake (at 100°C, min)
		1.5	1	1
		2	1	2
		5	1	3
	3. Exposure		Time	
			4 sec	
	4. Development		Time	
			1 min	
	5. Rinse, dry, & hard bake	Thickness (μm s)		Hard bake (at 110°C, min)
		1.5		2
		2		3
		5		5

placed in contact with the dried PDMS stamp. The mechanism of transfer at this point has not yet been determined, although the most probable explanation is that the binding affinity between the thiolate molecules and the gold surface is stronger than the adhesion force between the PDMS surface and the thiolated molecules [15, 18]. After 30 sec, the solid substrate fabricated with the PDMS stamp was released and subjected to further processing.

Analysis

One of the most common techniques used to detect a binding event in an immunological array is to utilize a secondary reagent, such a second antibody or antigen that is fluorescent-labeled. Thus, the patterns of a fluorescent-labeled specific secondary antibody were used in the present study and detected using fluorescence microscopy (DML/HCS, Leica Microsystems Wetzar GmbH, Germany), including a charge-coupled device (CCD). A mercury lamp with an optical filter at 488 nm and 530 nm was used to induce the fluorescence of the fluoresceine isothiocyanate (FITC) and Cy3 functional groups conjugated to anti-IgG molecules.

Results and Discussion

Optimal Fabrication of Master for Stamp Preparation

Before preparing the PDMS stamp, the silicon master was fabricated using a photolithographic technique. The AZ7220 photoresist was selected to obtain a high aspect ratio with a micrometer film thickness. The photoresist was coated on a *p*-type silicon wafer and baked softly at 65°C and then at 100°C. Next, the prepared substrate was irradiated for 4 sec below the photomask, and the subsequent development was carried out for 1 min for selective removal of the coated photoresist. When the process time was more than the determined value, this resulted in undercut phenomena across the substrate (data not shown). The prepared Si master was then utilized as a mold to generate a patterned stamp. Figure 2 shows micrographs of the photomask, master mold, and fabricated stamp. The shape and configuration of the processed master mold (Fig. 2B) was exactly transferred, *i.e.*, equal to that of the prepared photomask (Fig. 2A). As such, the PDMS stamp generated from the mold had exactly the same pattern as the shape and configuration of the fabricated master.

The performance of the fabricated PDMS stamp was evaluated by fluorescence microscopy. Anti-monkey IgG labeled with FITC was utilized as the ink for the stamp, and then transferred to the Au surface. Figure 3A shows the fabricated protein pattern. The ink was successfully transferred to the solid surface, and the size of the transferred features maintained throughout. Figure 3B shows a microscopic view of the fluorescence generated by the HSA-FITC bound to the fabricated protein pattern with Mab against HSA. As the concentration of the antigen decreased, the fluorescence intensity decreased accordingly, and when the HSA concentration applied to the fabricated pattern was below 1 μg/ml, no HSA was observed by fluorescence microscopy.

Miniaturized Sandwich Immunoassay of μCPed Pattern

Using the patterned antibody surface, the antibody-antigen interaction was investigated using fluorescence microscopy. An antibody fragment was utilized for the oriented immobilization [18], and Fig. 4A shows a microscopic

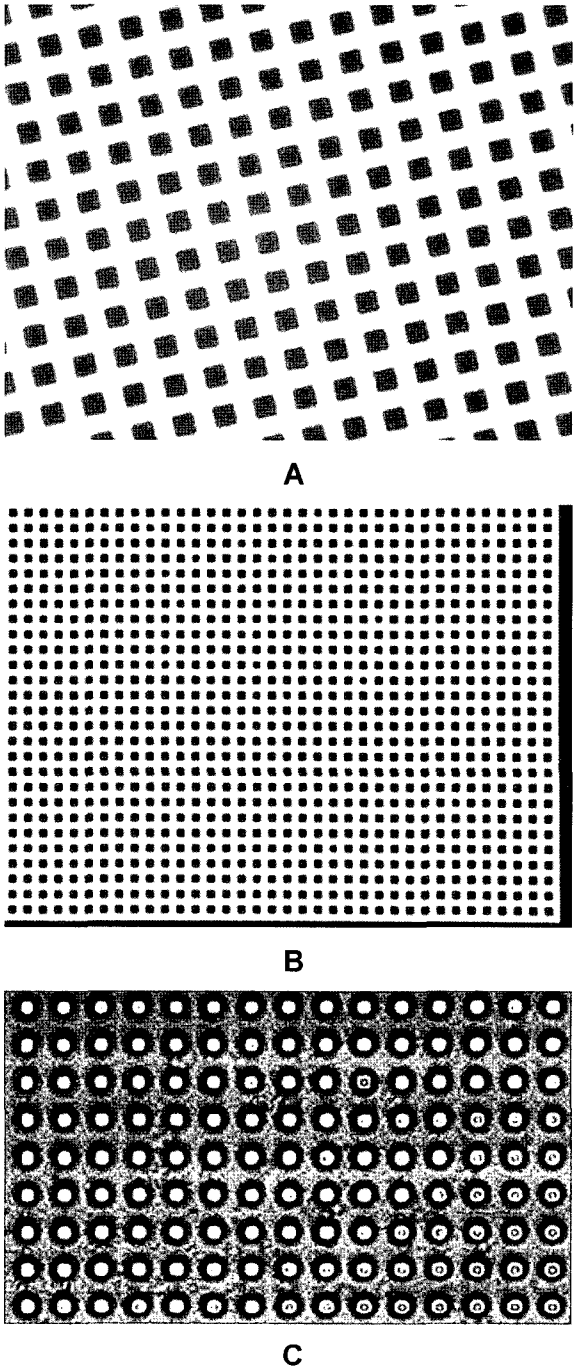

Fig. 2. Optical micrographs of (**A**) patterned chromium photomask, (**B**) fabricated Si master, and (**C**) fabricated PDMS stamp. The square dimensions were 5 μm×5 μm.

view of the fluorescence generated by the labeled secondary antibody when a sandwich-type immunoassay was carried out in a miniaturized form, as shown in Fig. 4B. To eliminate any nonspecific binding between the bare Au surface and the protein, casein was applied to the surface patterned with the antibody fragment. As too much

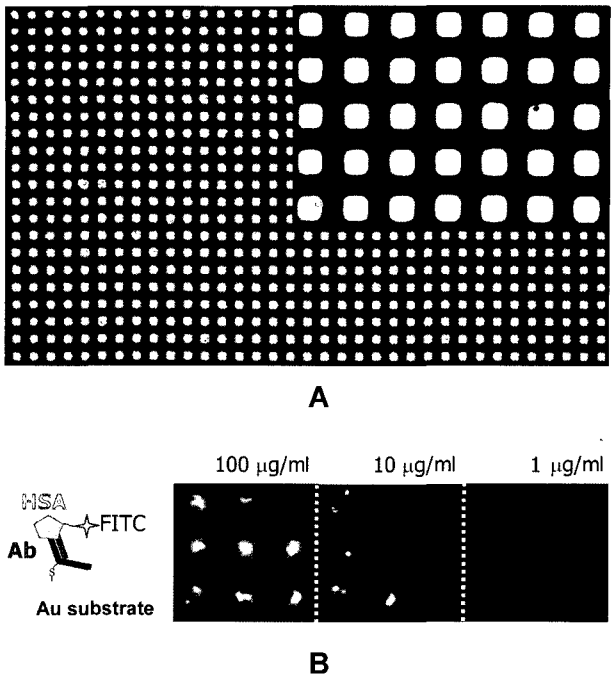

Fig. 3. Fluorescence micrographs of the (**A**) pattern of FITC-labeled antibody fabricated by μCP and (**B**) FITC-labeled HSA bound to the fabricated antibody pattern.

incubation time in the blocking solution can disrupt the patterned protein surface, controlling the immersion was important to maintain the array features and size. Therefore, the prepared antibody pattern was immersed for 10 sec in the solution with 1% (w/v) of casein, and then immediately washed with phosphate-buffered saline (PBS).

The variation of the fluorescence emission in the proposed immunoassay was investigated relative to the concentration of the antigen. When comparing the fluorescence measured in the vicinity of a high concentration region, such as 100 μg/ml, or 10 μg/ml of HSA, the intensity of the emitted fluorescence decreased with a reduction of the antigen concentration. When an antigen concentration of less than 100 pg/ml of HSA was applied to the surface, it could not be visualized by fluorescence microscopy. Therefore, the detection limit in this experiment was determined as 100 pg/ml of HSA. In addition, the miniaturized system with a sandwich-type configuration induced a 10^4-fold improvement in the detection limit for the proposed immunoassay (on the basis of HSA).

To investigate the lifetime of the prepared protein array, the fabricated antibody fragment was preserved in a temperature-controlled chamber that was maintained at 4°C. Figure 5 shows the variation in the fluorescence emitted by the proposed sandwich-type immunoassay relative to the preservation time. The assay procedure was the same as the schematic description shown in Fig. 4B. As previously, any nonspecific binding was excluded by a

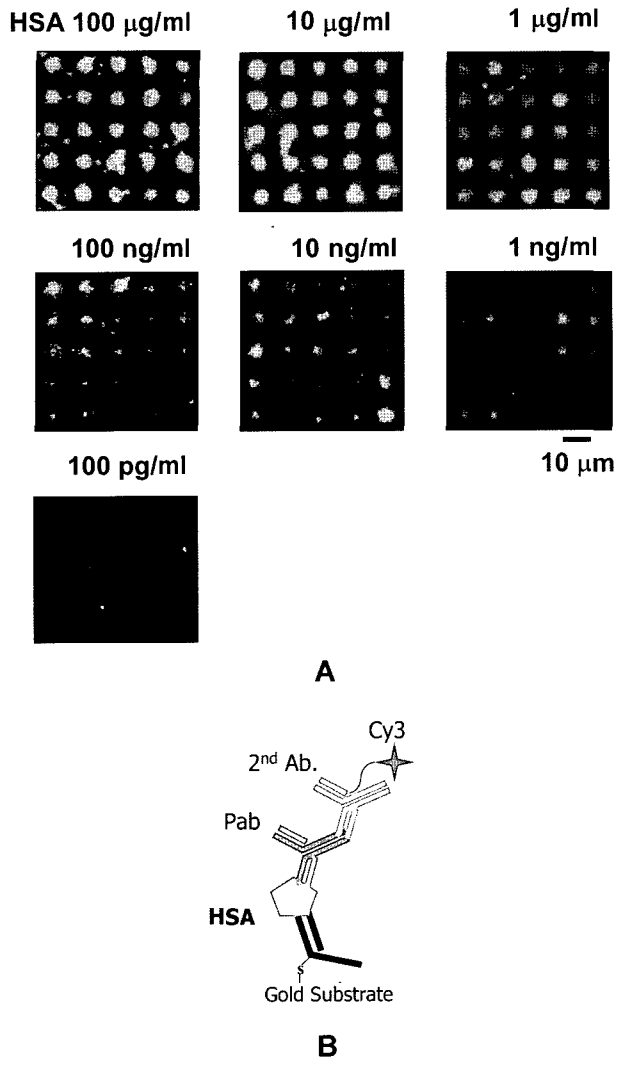

Fig. 4. Fluorescence micrographs of sandwich-type immunoassay using Cy3-labeled secondary antibody relative to HSA concentration.

blocking material, and the shape and configuration of the fabricated array were conserved, although not perfectly matched with the original protein pattern, as shown in Fig. 3A.

When 1 ng/ml of HSA was applied to the fabricated surface, the fluorescence emission from the miniaturized spot remained for over 2 weeks. When the prepared pattern composed of the antibody fragment was preserved over 20 days, no meaningful fluorescence micrograph was acquired when applying antigen of 1 ng/ml. Therefore, when applying a sandwich-type immunoassay to the fabricated protein array, the miniaturized detection of the target antigen must be performed within approximately 2 weeks. Consequently, this study successfully performed a miniaturized immunoassay with a sandwich-type configuration, where the detection limit was 100 pg/ml of HSA.

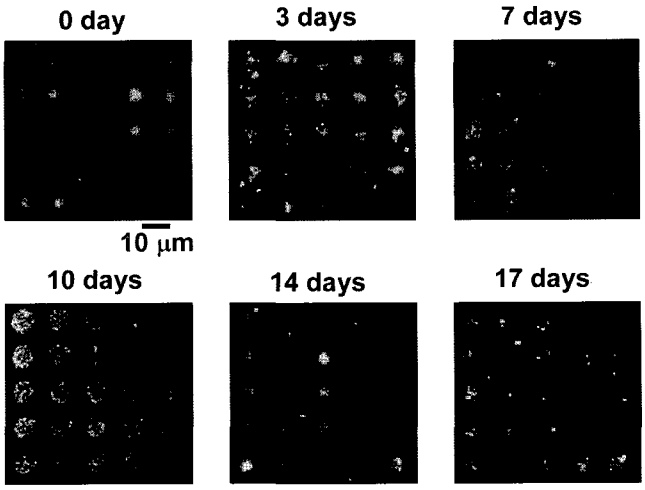

Fig. 5. Variation in biological affinity of fabricated protein array relative to preservation time. The immunoassay was carried out using 1 ng/ml of HSA.

Microcontact printing (μCP) was applied to the formation of a protein array and miniaturized immunoassay. A 5 μm ×5 μm PDMS stamp bearing a complementary pattern to the silicon master was successfully fabricated. The elastomer was coated with a solution containing an antibody fragment for patterning, and brought into contact with an Au substrate. The use of the fragmented antibody meant the antibody was immobilized on the Au surface in an oriented way. The pattern formation was visually investigated using fluorescence microscopy. When decreasing the concentration of the target protein, the fluorescence intensity also decreased, and the detection limit was 100 pg/ml of HSA. The biological activity of the patterned antibody fragment was maintained over 2 weeks (with respect to 1 ng/ml of HSA). Accordingly, the present experimental results suggest that a protein array fabricated using the proposed miniaturized format can be successfully applied to the measurement of various proteins.

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