PAPER

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Micro patterning of active proteins with perforated PDMS sheets (PDMS sieve)

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Received 16th January 2004, Accepted 16th March 2004
First published as an Advance Article on the web 6th April 2004

We propose a novel technique for patterning active proteins on a glass substrate using a perforated polydimethylsiloxane (PDMS) sheet–sieve. The sieve, which has tapering holes, is fabricated by spin-coating PDMS on a pyramidal-shaped mold. By means of this sieve, FITC (fluorescent isothiocyanate, bovine)–albumin was successfully spotted in a 5×5 μm^2 area in an array. The patterned spots were perfectly isolated, which eliminates the problem of non-specific binding of proteins to undesired areas. To show that proteins maintained their activity after the patterning, we used F_1-ATPase biomolecular motors; their activity can easily be verified by observing their rotary motion after patterning. Selective patterning with three kinds of fluorescent micro beads indicated the possibility of patterning of different proteins on the same substrate by using the sieve.

Introduction

After the completion of human genome sequencing, it has become popular to develop analytical devices for disease diagnosis or drug discovery. For this kind of analysis, it is important to detect protein expression and functions relevant to the diseases and the drugs. Protein array chips are expected to be high-throughput tools for analyzing protein functions and interactions. To improve the effectiveness, we need high-resolution protein patterning which has recently become possible with the aid of micro fabrication techniques.[1–7]

Several MEMS techniques have been proposed for patterning proteins and cells in a specific predefined area of a substrate. The most widely accepted way to pattern proteins is to perform chemical modification at specific locations on the substrate, *e.g.* photochemical techniques[7–10] After such surface modification, the proteins attach to the desired area as a result of chemical interaction. However, proteins are often fused all over the substrate in a non-specific manner. This problem is referred as "non-specific binding" which makes it difficult to fasten the molecules only to specific locations on the substrate.[8,11] Physical methods such as micro-contact printing[12–14] or spraying deposition methods[15] ensure that the molecules do not spread to other locations. However, these methods cause the proteins to dry out or degenerate. Proteins that can keep the activity in a dry state such as antibodies can be used with this method.[14] But it is not suitable for the patterning of enzymes which show activity only in an aqueous environment. Reliable protein chips should have an array of active proteins with a high S/N ratio.[11,16]

In this paper we present a sieve for high-resolution pattering of active proteins. The sieve is a thin polydimethylsiloxane (PDMS) sheet with an array of reverse pyramid-shaped holes. Fig. 1 illustrates the concept of protein patterning with this sieve. The sample solution is poured on to the sieve attached to a glass substrate (Fig. 1a, b). The proteins in the solution bond to the glass substrate through the micro holes and excessive proteins are washed out by a buffer solution (Fig. 1c). The immobilized proteins maintain their position after removal of the sieve (Fig. 1d). In this way, the proteins can be patterned without non-specific binding (Fig. 1e). The drying out of samples, which is a significant problem in micro scale protein patterning, can be avoided by removing the sieve in an aqueous environment.

Experimental

Protein preparation

F_1-ATPase was used as one of the test proteins in this work. This protein was fused with His-tags, which have an affinity to Ni-NTA for the protein purification and fixation to the substrate.[17] *E. coli*, which expresses the F_1-ATPase protein, was cultured in Terrific broth and collected by using a high speed refrigerated centrifuge (CR-21G; HITACHI). The protein was extracted by sonicating the *E. coli* in buffer solution. Then the solution was separated from the precipitate and the supernatant using a micro ultracentrifuge (CR-120GX; HITACHI), the supernatant was purified with a Ni-NTA

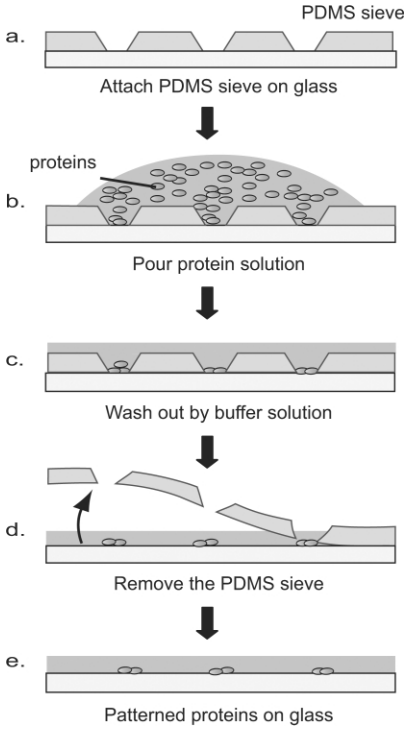

Fig. 1 Sequence of protein patterning using a PDMS perforated structure (PDMS sieve). Protein solution is poured on to the PDMS sieve attached to the glass. The sieve is simply peeled off after washing out excess proteins with a buffer solution.

DOI: 10.1039/b400774c

super flow affinity column (QIAGEN) and gel filtration HPLC (Superdex 200 column (Amersham Pharmacia Biotech)). The protein was modified by biotin-PEAC5-maleimide (Dojindo) to bind with 0.49 μm streptavidin beads (PreActive Streptavidin Coated Microspheres; Bangs Laboratories, Inc.).

Fabrication

In order to observe the patterned samples using an bright field inverted microscope (IX-70, Olympus), transparent materials such as glass must be used for substrates. PDMS is suitable for patterning proteins on glass because it shows good adhesion to glass, but it can be smoothly peeled off from the surface. The fabrication process of the PDMS sieve starts with forming pyramidal silicon structures (Fig. 2a). A thermally-oxidized silicon wafer (500 μm in thickness, with a 0.5 μm thick oxide layer, Ferrotec Silicon Inc.) was used as substrate. After etching the oxide layer with BHF, the silicon layer was anisotropically etched with 22% TMAH at 75 °C. To lower the adhesion of PDMS, the silicon was first treated with CHF_3 plasma, then PDMS (SYLGARD 184, DOW CORNING) was poured over the pits on the etched silicon substrate. This way we obtained a PDMS mold with pyramidal structures (Fig. 3a). We found that Parylene (Specialty Coating Systems) works well as separation layer between the PDMS pyramidal mold and the sieve. A 0.5 μm thick layer of Parylene was evaporated on the mold using a Parylene coater (PDS2010; Specialty Coating Systems). After a 10 : 2 PDMS–curing agent mixture was spun (MIKASA SPINNER IH-D2) and cured on the pyramids, a sheet with a thickness less than 100 μm was obtained.[18] Since liquid PDMS is a viscous solution, the small apertures at the top of the pyramids were not smooth after peeling off. However, the edges were thoroughly smoothed out by SF_6 plasma etching (10 ml min^{-1}, 50 W, 5–10 min) (Fig. 2b). This way, tapered holes were made in the PDMS sheet as shown in Fig. 3c and d. Fig. 3b, c, and d show a peeled PDMS sieve.

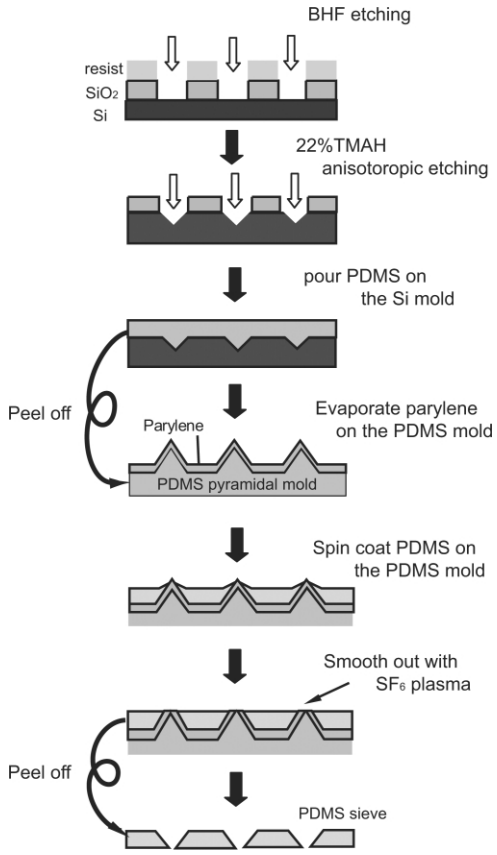

Fig. 2 Fabrication process of the PDMS sieve. The sieve is formed by spin-coating PDMS on a pyramidal mold.

The aperture size can be controlled by changing the spinning speed. Fig. 4 shows the correlation between the aperture size and spinning speed. When the speed is less than 500 rpm, the pyramid mold is completely covered by the PDMS sheet, and no aperture is observed. Using spin speeds between 600 and 1100 rpm, an aperture size of less than 10 μm can be obtained. The minimum feasible size of the aperture is about 3×3 μm^2. At speeds over 1500 rpm, the aperture size increases to over 20×20 μm^2, but the thickness of the sheet becomes less than 50 μm, which is too thin to be used. In this experiment, the speed was set to 1000 rpm; as a result, we obtained a sheet less than 100 μm thick.

Results and discussion

Patterning test with albumin

FITC (fluorescent isothiocyanate, bovine)–albumin (5 mg ml^{-1}, Sigma) was used to test protein patterning by this method. Since it is difficult to introduce protein solution into micro holes from which the air cannot be purged by water, the solution was initially degassed in a vacuum chamber. As a result of this treatment, the air remaining in the micro holes was absorbed by the degassed solution, and the holes were immediately filled with the solution. After patterning the proteins, an array of fluorescent spots, less than 5×5 μm^2, could clearly be observed (Fig. 5a); the spot size appeared to be larger due to scattering. The brightness distribution of these spots (Fig. 5b) confirmed that there were no leaks or diffusion of proteins. This indicates that non-specific binding is avoided by this method due to the excellent adhesion properties of the PDMS sieve. For detection of an antigen–antibody reaction, the non-specific binding problem can be also avoided by removing the sieve after the addition of the antibody to the antigen. The sieve can be reused for patterning by washing it with detergent using a sonicator.

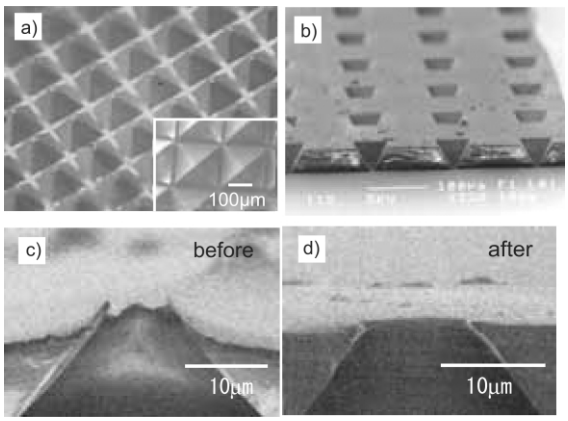

Fig. 3 a) Array of PDMS pyramidal mold. b) SEM photograph of the PDMS sieve with its cross-section. c) Close-up view of the smaller part of the aperture before SF_6 plasma treatment. d) Close-up view of the smaller part of the aperture after SF_6 plasma treatment.

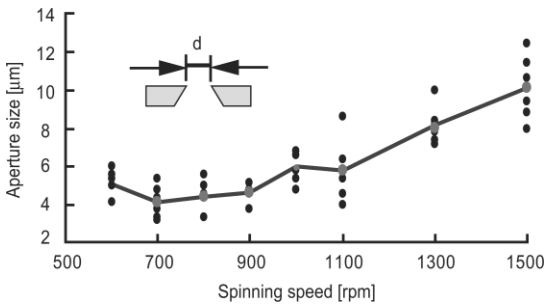

Fig. 4 Dependency of the aperture size on the spin-coating speed.

Patterning of active enzymes

The activity of patterned proteins is investigated. A biomolecular rotary motor (F_1-ATPase) was used for this experiment. The activity of this protein can easily be verified by observing its rotary motion after the patterning. F_1-ATPase was immobilized on a glass surface through chemical interaction between His-tags and Ni-NTA. Streptavidin-coated micro beads were connected with biotin at the rotation axis of F_1-ATPase (Fig. 6a). The activity of the protein was assessed by observing the rotation of the micro beads attached to the protein.[17] When ATP (adenosine triphosphate) solution containing 2 mM $MgCl_2$ was introduced into the holes, rotation of the beads inside a $10 \times 10\ \mu m^2$ hole was observed (Fig. 6b). The rotation was maintained even when the sieve was peeled off. This indicates that our patterning method can be used for patterning biologically active proteins.

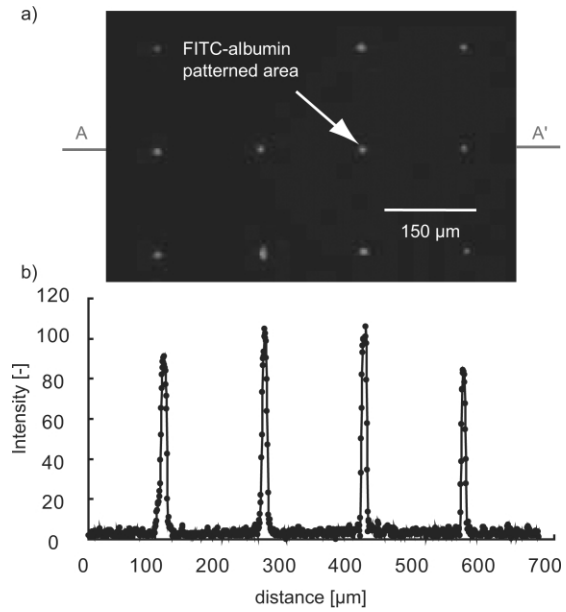

Fig. 5 Protein patterning test. a) Fluorescent image of FITC–albumin patterned on a glass substrate using the PDMS sieve. b) The intensity distribution along line AA′ shows no non-specific binding after peeling off the sieve.

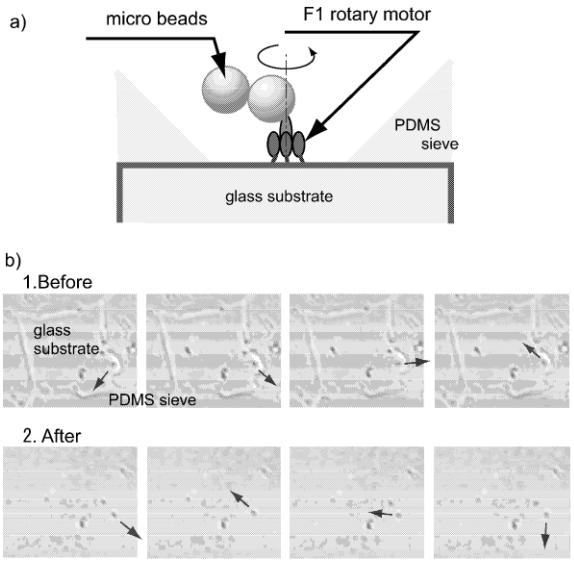

Fig. 6 a) Cross-sectional schematic of the sieve with F_1-ATPase. b) Sequential photographs of the rotational F_1-ATPase immobilized on the glass in a hole. (b-1) Photos of rotation of F_1-ATPase before the sieve removal (every 0.6 s). (b-2) Photos after the peeling (every 0.6 s). Excess proteins and beads are eliminated after the removal. The protein keeps rotating unless drying or ATP depletion of the buffer.

Selective patterning

Since the injection aperture of this sieve is 10 times larger than the spotting area, different solutions can be injected into individual openings by using a conventional glass-pipette dispenser. In this study, three kinds of fluorescent micro beads (FluoSpheres Fluorescent Microspheres; Molecular probes) with green, red, and blue fluorescent dyes were used to demonstrate selective patterning of proteins. The bead diameters are 0.2 μm for the green and red ones, 1 μm for the blue ones. No leaks to nearby holes could be observed when the solution was manually introduced into each hole with a dispenser. Fig. 7 shows an RGB pattern of the beads with different fluorescent dyes attached to $15 \times 15\ \mu m^2$ spots with 270 μm intervals on the same substrate after peeling off the sieve. Different proteins can be arrayed with this method on the same substrate surface.

Conclusion

A PDMS pyramidal mold and a PDMS sieve with tapered micro pores were designed and fabricated. In this process, the Parylene provides a good separation between the pyramids and the sieve. With the sieve, the patterned area was less than $5 \times 5\ \mu m^2$, and non-specific binding on unintended areas could be avoided. The observation of the F_1-ATPase rotary motion showed that the activity of the proteins was maintained during the patterning process. Selective patterning of different fluorescent beads was also realized on a substrate. These results indicate that this method allows the realization of a micro array of different active proteins for protein chips.

Acknowledgement

We thank Dr Hiroaki Suzuki, Dr Kazuhito Tabata and Dr Kuniyuki Kakushima for helpful discussion. Part of this research was supported by the PROgram for the Promotion of Basic Research

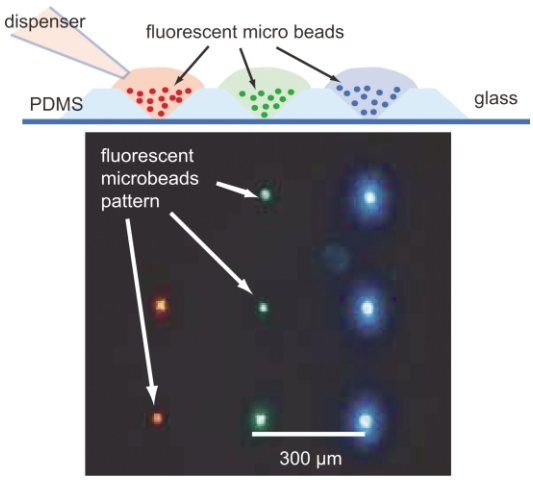

Fig. 7 Selective patterning of micro beads with three different fluorescent dyes (red, green, and blue). The photograph was taken after PDMS sieve removal. The blue beads are bigger than the beads of the other two colors.

Activities for Innovative Biosciences (PROBRAIN) under the supervision of the Ministry of Agriculture and Fisheries in Japan.

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