

Multiprotein Immunoassay Arrays Fabricated by Microcontact Printing

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Microarrays containing up to three different proteins were fabricated by microcontact printing (μ CP) techniques and tested as a detection system for specific antibodies. After fabrication, immunoassays were successfully performed using the patterned protein microarrays. The developed immunoassays were characterized by fluorescence microscopy and scanning probe microscopy. The characterization revealed the quality of the protein deposition and indicated a high degree of selectivity for the targeted antigen–antibody interactions. The results of this study suggest that μ CP is an inexpensive and effective way to fabricate biologically active substrates that can be of use for multiple reagentless immunosensor applications.

1. Introduction

Over the past decade, microarray technology has evolved from genomics to proteomics. Protein microarrays have the potential to play a fundamental role in the miniaturization of biosensors, clinical immunological assays, and a series of basic research applications ranging from mapping the proteome to examining protein–protein interactions. Unlike DNA microarrays, the technology for generating protein microarrays is still developing.

It is known that proteins can be transferred to a substrate without the loss of biological activity using microcontact printing (μ CP) techniques.¹ The low cost of fabrication, as well as the simplicity of transferring proteins to substrates, when compared to other techniques such as lithography,² pin arrayer printing robots,³ or ink-jet printers,⁴ makes μ CP-fabricated arrays very attractive. The micropatterning of proteins by μ CP on solid substrates is of current interest for this reason.¹

Over the past several years, the μ CP technique, originally developed by Whitesides et al. to transfer alkanethiols onto Au substrates,⁵ has been successfully applied to the patterning of proteins on solid surfaces.^{1,6–9} In the μ CP method, a poly(dimethylsiloxane) (PDMS) stamp coated with a protein solution is dried and brought in contact with a functionalized solid substrate such as glass, silicon, or gold. Proteins are transferred at the places

of direct contact between the stamp surface and the substrate. These prior studies demonstrated that proteins could be transferred in a controlled way onto a variety of different substrates while maintaining their biological activity.⁷ Similar techniques described by Porter et al. rely on optical lithography to create the protein patterns.²

The previous work demonstrated how the patterning of a single protein could be used for a single immunoassay. However, a multiprotein pattern deposited on a substrate is required for microsensor purposes. In addition to the μ CP method, only a microfluidic network (microchannels fabricated in PDMS) has the potential to pattern multiple proteins.^{1,10,11} At the present, μ CP seems to be an easier and more convenient method than microfluidics.

In the work that follows, we expand upon previous efforts by showing that microcontact printing can be used to fabricate platforms capable of sensing multiple antibody–antigen interactions. The immunoassays were detected and characterized by fluorescence microscopy (FM) and scanning probe microscopy (SPM) techniques. This work demonstrates how multiple immunoassay elements can be fabricated by a simple and inexpensive method.

2. Experimental Section

2.1. Stamp Fabrications. PDMS stamps are fabricated by casting and curing Sylgard 184 (Dow Corning, Midland, MI), an elastomeric polymer, against photoresist micropatterned silicone masters (see Figure 1). Masters, which contained a negative relief of the stamp mold, were manufactured at the Stanford University Nanofabrication Facility. These masters were made by first spin coating of a positive photoresist, SPR 220-7 from Shipley (Marlborough, MA), on a silicon wafer. Irradiation with UV light through a chromium mask was used to render the exposed photoresist soluble, which was then removed by washing with the developer LDD26W from Shipley. Two different stamp configurations were used for these experiments. One consisted of 5 mm long \times 2 μ m wide strips separated by 2 μ m. The second consisted of a 10 μ m \times 10 μ m square pattern with a separation of one square from the other by 5 μ m. The PDMS stamps were cured for 2 days at room temperature or 12 h at 60 $^{\circ}$ C and sonicated in an ethanol–water solution before oxidation.

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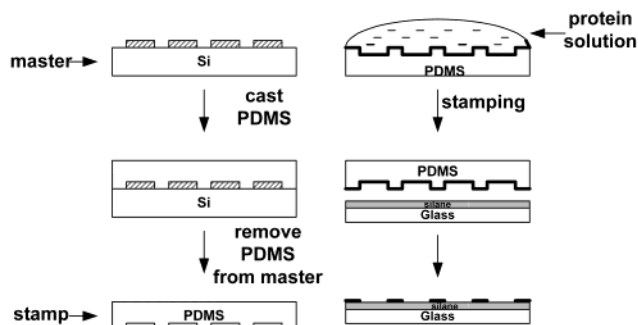


Figure 1. The PDMS stamp is fabricated by molding it from a silicone master and used for microprinting.

2.2. Substrate Functionalization. For this study, proteins were physically immobilized on thick glass slides. The glass slide offered a transparent substrate on which to deposit proteins that were later studied by fluorescence microscopy.

Glass substrates (Gold Seal, Hungary) were degreased by a brief wash in acetone and methanol. They were then cleaned in hot (120 °C) sulfochromic solution for 30 min, washed with water, and dried in an oven at 120 °C. The substrates were immediately silanized after drying by immersing the clean glass slides in a solution of chlorodimethyloctadecylsilane (0.02 M) in an anhydrous toluene solution overnight. Preparation of the silane solution and silanization were carried out in a drybox under nitrogen.¹² After silanization, the glass slides were washed with toluene and later with methanol to wash out any nonbonded chlorodimethyloctadecylsilane and then dried in an oven. The root-mean-square (rms) surface roughness of the glass substrates used in this study was found to be 1.28 nm using SPM. The roughness of the substrate plays a critical role in the determination of morphological uncertainties when probing the substrates with SPM.

2.3. Chemicals and Reagents. Immunoassay reagents included rabbit IgG, mouse IgG, human IgG, donkey anti-rabbit IgG fluorescein labeled, goat anti-mouse IgG fluorescein labeled, and goat anti-human IgG fluorescein labeled, all of which were obtained from Pierce, Rockford, IL.

Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). Chlorodimethyloctadecylsilane and anhydrous toluene were received from Aldrich.

2.4. Microprinting: Stamp and Sample Preparations. The PDMS elastomer commonly used for μ CP, after appropriate surface treatment, appears to be an acceptable material for the directed transfer of proteins from solution to a micropattern on a solid substrate. The process of loading a protein onto a PDMS stamp will be referred to as "inking" and the protein loaded onto the stamp or substrate as the "inking protein". The mechanism of transfer is not known at this time, but the most probable explanation is that binding between the hydrophobic surface of silanized glass and proteins is stronger than the adhesion force between the proteins and the PDMS surface.

Under proper conditions, a monolayer of protein can be easily patterned onto a glass substrate.⁷ There are two critical conditions required for high-quality transfers: (1) oxidation of the PDMS stamp (making the surface hydrophilic to ensure uniform wetting by the aqueous protein solution) and (2) the drying time of the protein solution on the stamp surface. Attention to both of these conditions was crucial for the success of the stamping process.

The surface of the PDMS stamp was rendered hydrophilic by treating the surface of the stamp in a low-temperature plasma cleaner (Harrick Scientific) prior to use. The surface of the PDMS stamp was exposed to solutions of the inking proteins (100–200 μ g/mL, 6.6×10^{-7} to 1.3×10^{-6} M) for various lengths of time. Excess solution was removed, and the stamp was dried under a stream of nitrogen gas. After inking, the stamp was brought into contact with the glass substrate and a very small amount of force was applied to make a better contact between both surfaces (Figure 1). The stamp was removed after 1–2 min, and the glass

slide was washed with a phosphate-buffered saline solution (PBS) and deionized water.

Fabrication of an array with two proteins involved taking a patterned substrate already prepared in the way described above and exposing it to a solution of a second protein of about the same concentration as the first protein (200 μ g/mL). During immersion in the solution, the second inking protein becomes immobilized in the nonpatterned region of the functionalized substrate. Substrates prepared in this manner allowed for the detection of two proteins that have a specific binding affinity with the proteins immobilized on the substrate.

Arrays capable of detecting three proteins were fabricated by another approach. A PDMS stamp with a pattern consisting of long thin strips, each having a width of 2 μ m and separated by 2 μ m from another stripe, was used twice to pattern proteins in orthogonal directions. To produce the substrate, the first protein was deposited in a single direction with the PDMS stamp. Next, a second protein was deposited with the same PDMS pattern, but now the pattern was stamped in a perpendicular direction with respect to the first protein. The nonpatterned regions were then exposed to a solution of a third protein, producing a patterned substrate containing three separate proteins.

All protein solutions were made in PBS at pH = 7.4. The substrates with patterned antigens were incubated with an analyte solution containing complementary antibodies (anti-IgG) for 40–60 min and washed with PBS buffer and water before fluorescence and atomic force microscopy (AFM) studies. Concentrations of all antibodies were in the range of 100–200 μ g/mL (10^{-7} – 10^{-6} M). The concentration of BSA used as a blocking agent was 5–10 mg/mL (10^{-5} – 10^{-4} M).

2.5. Detection. 2.5.1. Fluorescence Microscopy. One of the most common techniques used for sensing binding on an immunological array is to use a secondary reagent such as a second antibody or antigen that is fluorescently labeled. Patterns of fluorescently labeled specific secondary antibody were used in this work and were detected by fluorescence microscopy using a confocal microscope (Nikon Inverted Eclipse TE-300 optical microscope from Fryer Co. (Huntley, IL)) equipped with a CCD camera. A mercury lamp with a principal line at 488 nm was used to induce fluorescence of fluorescein isothiocyanate (FITC) functional groups conjugated to anti-IgG molecules.

2.5.2. Scanning Probe Techniques. Precise verification of the quality of printing and the efficiency of the complementary antibody binding were obtained using SPM. A simple measurement of morphological changes is often sufficient for the detection of antibody–antigen reactions, since a binding event results in an increase in height.

High-resolution SPM images of the patterned proteins were obtained using two commercially available atomic force microscopes: a PicoSPM manufactured by Molecular Imaging¹³ and an SPM system produced by Nanotec.¹⁴ The design of the PicoSPM allows for the imaging of a substrate under an aqueous solution, making it possible to monitor the absorption of proteins while simultaneously imaging the substrate. The substrates were imaged in both contact and noncontact modes. For noncontact images, cantilevers with a nominal spring constant of 2 N/m were used.¹⁵ In the contact mode, the spring constant of the cantilevers had a nominal value of 0.06 N/m.¹⁶

3. Results and Discussion

The binding of various proteins was determined by morphological and optical examination of targeted regions on the substrate after both printing and immunological complex formation.

To optimize conditions for protein transfer, we characterized the μ CP method by stamping only BSA with a fluorescence tag onto a silanized glass substrate. Fluorescence spectra and a noncontact SPM image¹⁷ of the

(13) PicoSPM manufactured by Molecular Imaging, Phoenix, AZ 85044, www.molec.com.

(14) The Nanotec scanning force system from Nanotec, Universidad Autonoma de Madrid, E-28049 Madrid (Spain), www.nanotec.es.

(15) ThermoMicroscopes Non-Contact Ultralevers (ULNC-AUHW).

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(17) Topographic images obtained using the Nanotec SPM system.

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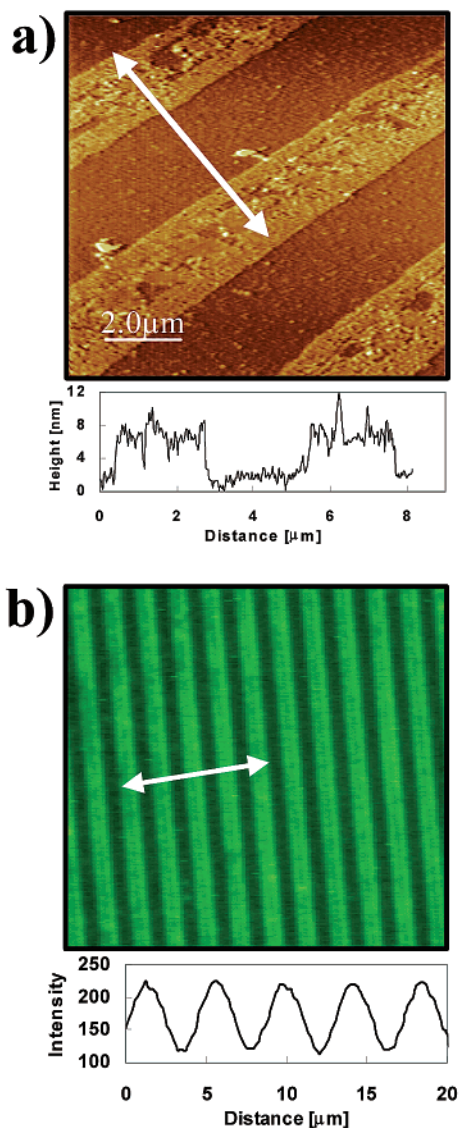


Figure 2. (a) AFM image of BSA patterned by μ CP on a glass substrate accompanied by a profile trace showing the height of the BSA to be approximately 6 nm. (b) Fluorescence image of the same sample (BSA patterned by μ CP) accompanied by an optical profile.

protein transfer are shown in Figure 2. The noncontact SPM measurements were made in an ambient environment and showed that the average height of the BSA monolayer was 6 ± 2 nm, in agreement with the structural data reported in the literature.¹⁸ The AFM images and profiles provided information on the quality of the transfer process. Protein molecules appear to be densely packed, and the edges of the pattern are well-defined. The transitions from protein-coated regions to the glass substrate were found to have an average lateral dimension of 120 nm. The width of the strip fluctuated by 150 nm along its length. This information indicates that the resolution of the transfer was on the submicron length scale. This is also evident from the FM image, albeit at lower resolution, which shows that the BSA protein is confined only within the pattern over a large area of the substrate.

To characterize the efficiency of protein adsorption from solution onto a silanized glass substrate, an atomic force microscope operating in contact mode¹⁹ in an aqueous

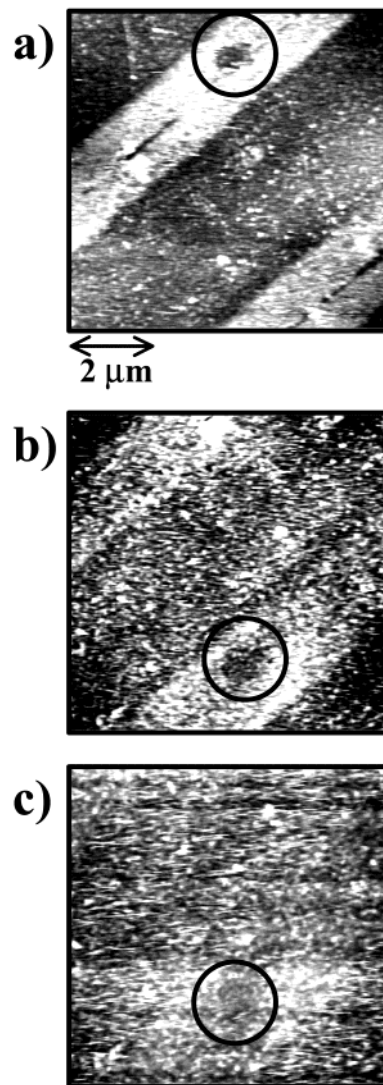


Figure 3. AFM topography image of a μ CP-printed mouse IgG array in (a) PBS buffer solution, (b) PBS buffer with bovine serum albumin (5 mg/mL) 15 min after BSA injection, and (c) PBS buffer with bovine serum albumin (5 mg/mL) 20 min after BSA was added.

environment was used to monitor the in situ adsorption of BSA as a function of time. The results are shown in Figure 3.

Figure 3a shows an image of mouse IgG under a buffer solution. The strips of protein are easily distinguished and are used as a reference to gauge the amount of BSA that has been immobilized in the open regions between the mouse IgG strips. A short time after acquiring the initial image under the buffer solution, a 5 mg/mL concentration of BSA was injected into the buffer solution. Images b and c of Figure 3 show the substrate approximately 15 and 20 min after the BSA injection. These images clearly show that the BSA begins to adsorb onto the exposed regions of the substrate, resulting in a reduction in the contrast of the mouse IgG strip as a function of time. The defect area on the mouse IgG strip (see the circle in Figure 3) confirmed that the atomic force microscope was imaging in approximately the same region during the measurements. This information is useful because BSA is commonly used as a blocking agent to prevent nonspecific adsorption of protein to substrates.

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(19) Topographic images obtained using the Molecular Imaging SPM system.

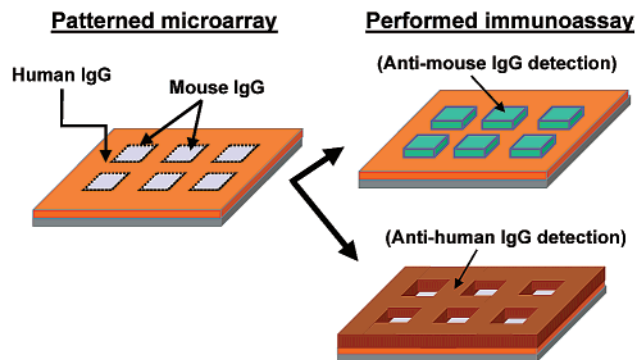


Figure 4. Performing the immunoassay for a two-protein array.

The experiment described above indicated that within 25 min the uncovered area was blocked with BSA.

After completion of these initial studies to characterize the quality of transferred protein regions using the μ CP method, samples designed to detect two antibodies were prepared in the manner described above. Samples configured to detect two specific antigen–antibody interactions were made by first patterning one antigen by μ CP (Ag1, i.e., mouse IgG) and then backfilling the region between the Ag1 pattern with proteins immobilized from a solution of the second antigen (Ag2, i.e., human IgG). The sample with both antigens patterned on the substrate was exposed to a solution containing an antibody specific to Ag1 or Ag2 (respectively, Ab1 or Ab2). A schematic illustrating this approach is shown in Figure 4.

Verification and characterization of the μ CP-deposited proteins were conducted using fluorescence microscopy and SPM. SPM data showed that microprinted monolayers of human IgG and mouse IgG had a similar height of 5 ± 2 nm with respect to the underlying substrate (data not shown). This is consistent with sizes of IgG's reported in the literature.^{7,2} Similar results were observed when IgG was printed and the unprinted area was blocked with BSA; only a small difference in topography (less than 1 nm) was observed. Therefore, a microarray of human IgG and mouse IgG should show no difference in height prior to incubation in anti-mouse IgG or anti-human IgG.

Figure 5a shows a false-colored topographic SPM image (acquired in contact mode) of the substrate after exposure to anti-mouse IgG. The mouse–anti-mouse IgG coated regions (squares) were found to be 4 ± 2 nm higher (brighter in the SPM image) than the surrounding regions containing only human IgG. This verifies that a specific binding occurred only in the targeted regions.

The SPM images show numerous objects located between the mouse–anti-mouse IgG coated regions. These objects have heights that are 3–5 times larger than the square regions containing the mouse–anti-mouse IgG. However, there were also numerous objects with dimensions on the order of the anti-mouse IgG. These could be regions of cross-reaction. Unfortunately, the SPM could not determine the nature of these objects. However, the anti-mouse IgG was labeled with a fluorescence tag. Therefore, if these objects are residues related to the anti-mouse–human IgG cross-reaction, they would be detected by fluorescence microscopy.

Figure 5b is a fluorescence microscopy image of the same sample studied by SPM. The images show fluorescence in the square regions containing mouse–anti-mouse IgG. This, in addition to the SPM data, shows once again that a targeted protein interaction occurred. Careful examination of the fluorescence microscopy images provides evidence that the residues observed in the SPM images

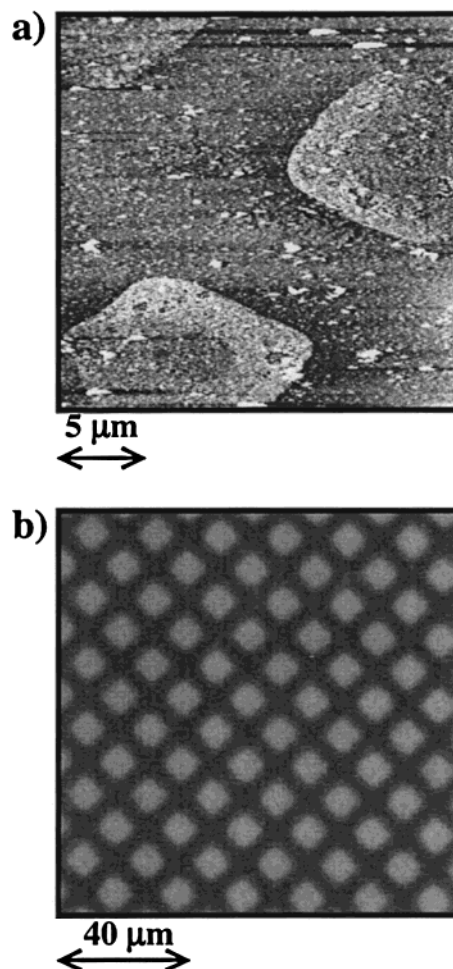


Figure 5. (a) AFM image of printed mouse IgG (square regions) and human IgG (regions between squares) after incubation in a solution of FTIC-labeled anti-mouse IgG. The image shows an increase in the height of only the printed areas (bright square areas of printed mouse IgG), which confirms specific binding of the anti-mouse IgG antibody to the mouse IgG antigen. (b) Fluorescence image of the same sample confirming bonding of FTIC-labeled anti-mouse IgG only in areas printed with mouse IgG (bright color).

are related to cross-reaction between anti-mouse and human IgG. From these images, we estimate that 10–15% of the surface is affected by unwanted cross-reaction products.

Figure 6a,b shows the detection of anti-human IgG (bright surrounding regions). Again, our SPM studies indicate conclusively that for anti-human IgG detection, the region surrounding the mouse IgG squares is 3 ± 2 nm higher (see Figure 6a). The AFM images also show that regions of antigen bonding are not uniform. Once again, minimal cross-reaction between anti-mouse and human IgG patterns can be seen from the fluorescence microscopy data.

Advancing this methodology further, we prepared substrates designed to detect three specific antibodies (Figure 7a). To fabricate this substrate, we patterned strips of two different antigens perpendicular to each other and then filled the third antigen in the regions between the strips by exposing the substrate to a solution of a third protein. In this approach, the first antigen stamped was mouse IgG and the next antigen (human IgG) was printed perpendicular to the mouse IgG. The area not covered by mouse and human IgG was blocked with a third protein,

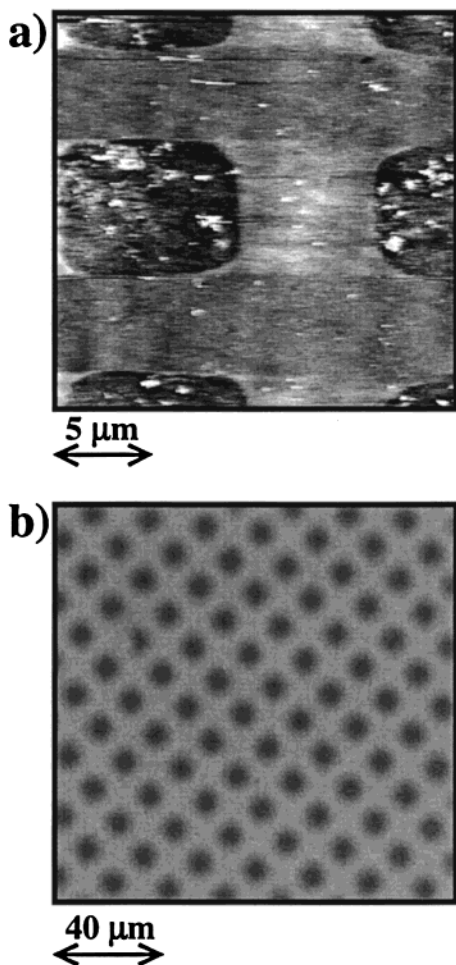


Figure 6. (a) AFM image of a printed mouse IgG and human IgG substrate after incubation in a solution of FTIC-labeled anti-human IgG. The image shows an increase in the height only the unprinted areas (bright regions surrounding the square areas), which confirms specific binding of the anti-human IgG antibody to the human IgG antigen. (b) Fluorescence image of the same sample confirming bonding of FTIC-labeled anti-human IgG only in the unprinted areas covered with human IgG (bright color).

BSA. After the preparation, the substrate was exposed to a solution containing a specific antibody.

Results shown in Figure 7b represent the fluorescence spectra after the sample was exposed to a solution of anti-mouse IgG. From the data, it is clearly seen that only one strip pattern is fluorescent, therefore indicating the detection of a specific antibody. Next, the same sample was exposed to a solution containing anti-human IgG. Figure 7c shows the fluorescence spectra after this exposure. The data indicate that another patterned strip (perpendicular to the one in Figure 7b) showed optical activity, revealing the detection of a second antibody. To detect a third antibody, one only has to replace the BSA with another biologically active antigen. SPM images of the crossed stamped protein strips showed that the height of the orthogonal strips was 5 ± 2 nm with respect to the BSA region in between. These data correspond to the height of anti-mouse IgG or anti-human IgG bound to the strips of corresponding mouse or human IgG. In the intersections of two strips, the height of the proteins was 9 ± 2 nm with respect to the BSA layer. This suggests that two overlapping layers of printed protein, mouse IgG and human IgG, may be present.

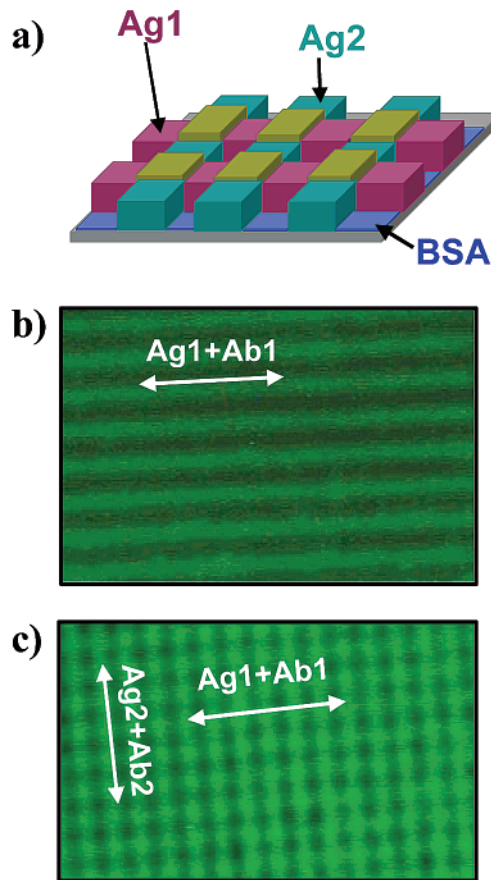


Figure 7. (a) Substrate design to detect three proteins. For this experiment, two printed antigens were used, mouse IgG (Ag1) and human IgG (Ag2). The regions between the IgG-printed areas were blocked with BSA. (b) Fluorescence image of the detection array after incubation in only a solution of FTIC-labeled anti-mouse IgG (Ab1). The image shows optical activity only in the Ag1 + Ab1 direction (bright areas) and no activity in the Ag2 direction (image size, $30 \times 45 \mu\text{m}$). (c) Fluorescence image of the same substrate after incubation in a solution of FTIC-labeled anti-human IgG (Ab2). Now optical activity can be seen in both the Ag1 + Ab1 and Ag2 + Ab2 directions. These data indicate once again that only specific protein binding occurred (image size, $34 \times 56 \mu\text{m}$).

4. Conclusions

We have described a simple protocol for producing micron-size arrays of proteins from the immunoglobulin family using μCP techniques. The long-range integrity of the fabricated arrays was verified using fluorescence microscopy. Ambient SPM was used to inspect the microscopic quality of printed array elements. After fabrication, the arrays were backfilled with a second protein to produce a platform capable of binding two separate proteins via antibody–antigen interactions.

Fabricated antibody arrays were subsequently used to detect the presence of two separate proteins after ~ 1 h exposure to solutions containing the appropriate complementary antigens. The formed immunoassays were studied using fluorescence microscopy and subjected to detailed topographic analysis using a variety of SPM techniques. Cross-reaction and nonspecific binding studies were performed and revealed minor (~ 10 – 15%) coverage. The techniques developed were subsequently extended to fabricate an immunoassay capable of detecting three separate proteins. We conclude that microcontact printing technology is a simple and inexpensive way to make a microarray capable of detecting two or more proteins simultaneously. These results may have important im-

plications for the production of inexpensive and sensitive biosensing immunoassays.

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