Protein biochips patterned by microcontact printing or by adsorption-soft lithography in two modes

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Patterning of proteins is critical to protein biochips. Printing of layers of proteins is well established, as is adsorption of proteins to surfaces properly modified with surface chemical functionalities. The authors show that simple methods based on soft lithography stamps can be used to prepare functional antibody chips through both these routes. Both methods incorporate transfer of the stamp material poly(dimethylsiloxane) (PDMS) to the biochip, whether intended or not intended. The results indicate that microcontact printing of proteins always includes PDMS transfer, thereby creating a possibility of unspecific adsorption to a hydrophobic domain. © *2008 American Vacuum Society.* [DOI: 10.1116/1.2988771]

I. INTRODUCTION

Protein chips for immunoassays based on ligand-receptor interactions for high throughput screening applications will follow in the footprints of DNA chips.[1] DNA chips have a limited use as a diagnostic tool because many diseases do not express a genetic signature. Compared to DNA, proteins are far more complex and sensitive, denaturation occurs easily, with conformation changes and loss of bioactivity. As a result, protein interactions are usually reduced when immobilized to a solid support.[2] Antibodies are proteins with high ability to bind and discriminate different molecules. Antibodies directed against a large number of molecules are commercially available. Immunoglobulin G (IgG) consists of four polypeptide chains which are cross-linked into a Y-shaped structure where the outer part of the two "arms" (Fab fragments) contains the antigen binding site and the "leg" (FC) is the constant fragment.[3,4] Different approaches for binding the FC part of antibodies giving it a favorable orientation and better accessibility for the antigen to interact specifically have been shown.[5] Nevertheless, adsorption mediated through surface chemistry using thiols,[6] silanes,[7] or poly-L-lysine[8] is the simplest but also the most uncontrolled way to immobilize proteins.[2] However, the orientation and amount of deposited antibody do not necessarily correlate with higher specificity and binding efficiency, since these factors depend on the available binding epitopes.[9] In fact, Vijayendran and Leckband[2] observed a lower density of attached antigens with increasing amount of immobilized antibodies.

One attractive way to pattern proteins to different substrates is microcontact printing (μCP).[10] A relief soft rubber-like stamp is soaked with a protein solution and then transferred from the stamp to a substrate when the stamp is put in conformal contact with a substrate (glass slides, polystyrene dishes, SiO_2, etc.).[6,10–12] The commonly used stamp material, poly(dimethylsiloxane) (PDMS) is an excellent material in many ways; PDMS is transparent, chemically inert, gases can diffuse through it, and its flexibility makes it possible to contact the stamp to a substrate over a large area.[13] A cured PDMS stamp has a hydrophobic surface. By treating it with oxygen plasma, OH groups are introduced to the PDMS rendering a more hydrophilic surface, which is, however, unstable, and the hydrophobic properties are recovered after only a few hours, unless kept submerged in water.[14] However, the ability of PDMS to contaminate the printed pattern by leaving low molecular residuals on the printed pattern could be a big drawback[15,16] or a useful patterning technique. Sharpe *et al.* used the contamination effect as an etch resist printed on gold and found that the contamination was larger when printing with polar ink compared to apolar ink.[17] Graham *et al.* suggested a cleaning procedure approximately one week long,[15] and Thibault *et al.* used a Soxhlet extractor during several hour,[18] and both show results with no or very little transfer of material from the stamp. However, the hydrophobic recovery after oxygen plasma treatment of a "clean" PDMS stamp is only slightly affected if stored in air; hence the stamps probably regain their ability to contaminate and transfer PDMS residuals. Possibly, the contamination could be necessary to achieve a good result using μCP.[18] We have used the PDMS contamination transfer, a technique referred to as PDMS surface energy patterning, to create patterns that differed in wettability.[19] We recently showed that this patterning technique could be used to discriminate between conformational states in biomolecules, and that the enzyme horse radish peroxidase retained its catalytic effect after immobilization to a PDMS surface energy modified chip.[20]

In this article we show that even if PDMS surface energy patterning is a somewhat simple way of creating a hydrophobic pattern on a hydrophilic substrate, it is able to adsorb antibodies while retaining their bioactivity. It is possible to use a single stamp for repeated patterning. When a PDMS stamp is used for μCP of an antibody layer, it will also leave

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some contamination rendering a more hydrophobic protein layer, compared to adsorption onto a PDMS surface energy patterned surface. This contamination could affect the biological activity of the antibody and lead to unspecific binding of antigens.

II. MATERIALS AND METHODS

By placing a relief PDMS stamp in conformal contact with a substrate low molecular weight residuals are transferred to the substrate forming a pattern with alternating surface energy.[19]

A. Materials

All buffers were prepared at the day of use; phosphate buffer (PB) 20 mM, *p*H 7.5 (10 mM Na_2HPO_4, 10 mM NaH_2PO_4) and phosphate-buffered saline (PBS) (20 mM PB, 150 mM NaCl) *p*H 7.4 were prepared in double distilled de-ionized water (18 Ω) (mqH_2O). All proteins used were stored and handled following the supplier instructions. Fluorescein conjugated streptavidin (Streptavidin-FITC) 1 mg ml^{-1} in PBS (absorption of 495 nm, emission of 528 nm) and Texas Red conjugated [rabbit]antistreptavidin IgG (IgG-TxR) 5 mg ml^{-1} in PBS (absorption of 569 nm; emission of 620 nm) (both purchased from Rockland Inc.) delivered as lyophilized powder and restored with de-ionized water to stock solutions, aliquot to avoid cycles of freezing and thawing, stored at −20 °C, and protected from light. [Pig]antirabbit IgG (aIgG-Alexa) (DakoCytomation, Denmark) was conjugated with Molecular Probes Alexa Fluor 350 protein labeling kit (Invitrogen, Sweden, absorption of 346 nm, emission of 442 nm), 1.8 mg ml^{-1}, PBS stock solution stored as aliquots protected from light at 4 °C. The proteins were, if frozen, thawed in 4 °C at the day of use and diluted to incubation concentration in the desired buffer prior to use. Human serum albumin (HSA) 2 mg ml^{-1}, PBS (DakoCytomation, Denmark), was prepared the day of use.

B. Master and stamp fabrication

PDMS stamps were molded on a relief master, containing rectangular areas of hollow lines of varying widths from 10 to 400 μm, separated with 100 μm in one area and 50 μm in one area. One area contains circular disks, 50 μm in diameter separated by 200 μm. The depth of the hollow was 20 μm. The master was fabricated with standard photolithography on a silicon wafer with SU-8 (Micro Chem. Corp.) as photoresist. To avoid adhesion between this master and the stamp, silanization is necessary. By submerging the master in a solution of 50 ml xylene and 300 μl dimethyldichlorosilane for 5 min, followed by extensive rinsing with xylene and ultrasonication for 5 min in mqH_2O water, a thin antiadhesion layer is achieved on the master. PDMS is prepared by mixing a two component silicon elastomer Sylgard 184 (Dow Corning Corp.): curing agent and base with mass ratio of 1:10. To avoid trapping of air bubbles in the stamp, the liquid was degassed before casted onto the master and cured in a convection oven for 45 min in 85 °C when used for

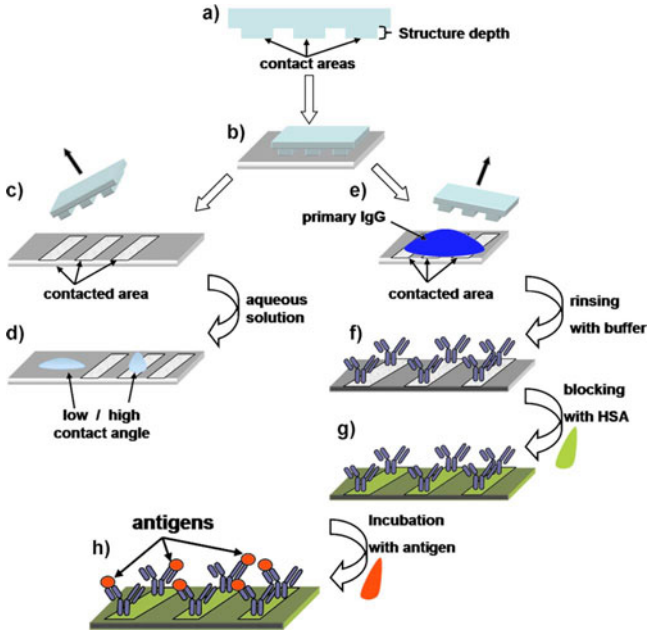

FIG. 1. PDMS surface energy patterning process. A reliefed PDMS stamp (a) was brought in contact with the substrate (b), after removing of the stamp low molecular residuals are transferred to the substrate where contact was achieved (c). The PDMS residuals generate a pattern with alternating surface energy on the substrate (d). Primary antibodies incubated on the PDMS patterned substrate (e) were immobilized on the areas patterned with PDMS residuals (f). After blocking unoccupied space of the substrate with HSA (g), the antigen was incubated on the chip and, after rinsing, complementary antigens was bound to the primary antibodies (h).

PDMS surface energy patterning. After cooling the stamps were gently peeled of from the master and used immediately. Stamps for μCP were cured for 1 h in 120 °C.

C. PDMS surface energy patterning

Standard microscope glass slides or silicon wafers with native oxide layer (~1.5 nm) were used as substrates in fluorescence experiments, respectively, for atomic force microscopy (AFM) and imaging null ellipsometry measurements. All substrates were cleaned by immersion in a 5:1:1 mixture of mqH_2O water, H_2O_2 (30%), and NH_3 (25%) for 10 min at 85 °C (TL-1 wash), followed by extensive rinsing in mqH_2O water and dried in a stream of nitrogen. The substrates were then treated in an oxygen radio frequency (rf) plasma chamber (Pico-RF, Diener Electronic, Germany) for 30 s (175 W, 0.05 Torr). A fresh unused PDMS stamp was put into conformal contact with the substrate for 10 min (if not stated differently) to create the optimal PDMS pattern for protein immobilization. Other contact times up, to 24 h, have been used to evaluate the patterning process [Figs. 1(a)–1(e)].

D. Immobilization of anchor proteins and building of biochips

A droplet (10–40 μl depending on the desirable chip area) of the protein solution to be patterned was placed on the chip. The concentrations used, if not stated in the text,

were for primary antibodies, aIgG-Alexa, or astreptavidin IgG 50 μg ml^{-1} (~333 nM) and streptavidin-FITC 25 μg ml^{-1} (~480 nM), all diluted in PB. The chip was placed in a moist chamber to avoid evaporation. After 60 min incubation the surplus was removed by rinsing with PBS. To avoid unspecific binding to unoccupied spots, the chip surfaces were blocked with HSA (2 mg ml^{-1}, PBS) in 30 min, followed by rinsing with PBS buffer. The complementary or noncomplementary protein (target molecule) was then incubated on the biochip for 60 min. Concentrations of the target molecules were, if not stated in the text, streptavidin-FITC 25 μg ml^{-1} and IgG-TxR 50 μg ml^{-1}, all diluted in PB. The surplus was rinsed away with PBS followed with mqH_2O water and drying in a stream of nitrogen. Rinsing with mqH_2O was always done before the evaluation step even if the sample was removed earlier from the production line (Fig. 1). Texas Red and Alexa 350 show rather good stability against photobleaching. Nevertheless, subdued light was used in the laboratory during preparation of fluorescent solutions and incubation onto the PDMS patterned substrates.

E. μCP of proteins

Glass substrates were cleaned by submerging in NH_3-acid (65%) for 24 h, rinsed in flowing mqH_2O for 20 min, dried with N_2, and finally air plasma treated (30 s, 175 W). PDMS stamps were ultrasonically cleaned in 50% EtOH for 10 min, rinsed in multiple baths of mqH_2O and dried in a stream of nitrogen before 50 μl IgG-Alexa (50 μg ml^{-1}, PB) was incubated on the stamp in 60 min. The stamp used consisted of parallel lines separated with 100 μm. The antibody inked stamps were then rinsed in three sequential PBS baths and dried with flowing N_2 gas. Stamps were put in contact with the glass slides for 60 min. Substrates were incubated with secondary protein solution, IgG-TxR as complementary, or streptavidin-FITC as noncomplementary antigen, for 60 min. All substrates were then washed with PBS to remove weakly bound biomolecules. A final wash with de-ionized water was done to remove salt residues prior to drying with N_2 flow and microscopy (Fig. 2).

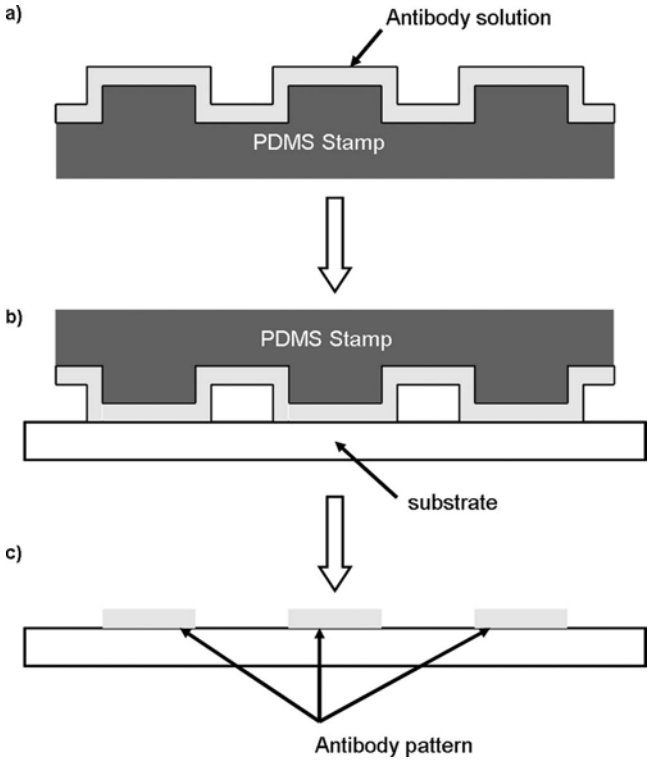

FIG. 2. Clean reliefed PDMS stamp is "inked" with an antibody layer (a). The stamp is brought into conformal contact with the substrate (b). After removing the stamp the antibodies are transferred from the stamp to the substrate where contact has been achieved.

F. Characterization with imaging ellipsometry

Imaging null ellipsometer EP^3 (Nanofilm surface analyze, Germany) was used to analyze protein adsorption and the PDMS surface energy patterning. The EP^3 is an ordinary null ellipsometer fitted with an X, Y, Z sample holder and charge coupled device (CCD) camera as detector. This makes it possible to calculate the nulling conditions in every pixel individually with the software (EP^3 VIEW V2.05) and display a map of the ellipsometer parameter, Δ and Ψ angles, in each pixel. By building a model system of the sample and addressing known refractive indexes [n=1.5 protein standard value, n =1.44 PDMS (Ref. 21)] and thicknesses for the different layers in the sample, it is also possible to calculate and display a map of the layer thickness. The objective of the CCD camera determines the lateral resolution of the system. In optimal conditions with 10× objective, this is 1 μm, and the thickness resolution is 0.1 nm. The imaging null ellipsometer measures the film thickness as an average over a defined region of interest (ROI). All thickness measurements of antibody layers adsorbed to a PDMS surface energy pattern have been calculated as an average of at least five ROI (approximately 100×100 μm) measured on five PDMS patterned chips giving a spatially averaged thickness. The samples were characterized directly after patterning. A nonpatterned clean area of the substrate was used to determine the SiO_2 thickness of each substrate and used in the model. In the same manner was the PDMS residual film thickness measured for different contact times and used in the ellispometric model when measuring the adsorbed antibody thickness. The average thickness and standard deviation of SiO_2 and PDMS residual were calculated from at least ten measurements. All experiments were performed in a clean room environment with controlled temperature and humidity.

G. Characterization with AFM

Atomic force microscope, Dimension 3000 (Veeco, Digital Instruments), was used to analyze the μCP protein pattern. NSG 10 cantilevers (NT-MDT, Netherlands) were used in tapping mode and the measurements were evaluated with the software V5.30 (Digital Instruments).

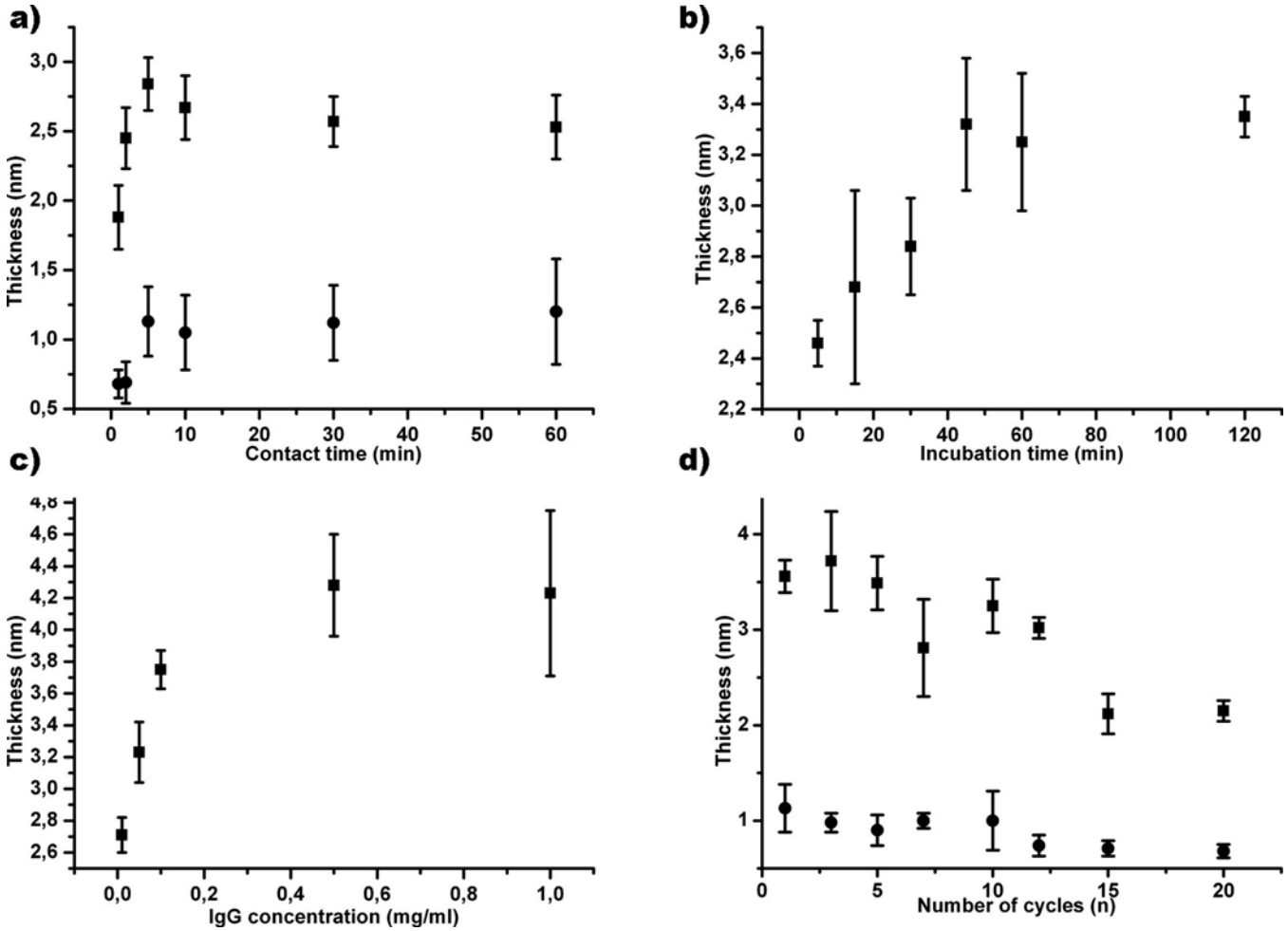

FIG. 3. Thickness measurement determined with imaging ellipsometry of the antibody film adsorbed to a PDMS pattern depending on (a) contact time (IgG concentrationof 50 μg ml^{-1}, and 30 min incubation time), (b) incubation time (contact time of 5 min and IgG concentration of 50 μg ml^{-1}), (c) incubation concentration (contact time of 5 min and 45 min incubation time), and (d) subsequent printing with a single stamp (contact time of 10 min, IgG concentration of 50 μg ml^{-1}, and 30 min incubation time). Film thicknesses of (●) only the PDMS residual pattern and (□) only the adsorbed antibody layer.

H. Characterization with contact angle goniometry

Contact angle goniometry with a CAM 200 goniometry (KSV Instruments, Helsinki, Finland) was used to analyze the surface wettability of the protein chips, using fresh mqH_2O in room temperature and humidity. The protein chips was patterned, both by PDMS surface energy modification and μCP as described above, both with patterned and unpatterned flat stamps. Water droplets of similar size were placed on the protein chip, and the static water contact angle with the surfaces was measured. The average contact angle was measured and averaged on at least ten samples.

I. Fluorescence microscopic evaluation

Fluorescence evaluation of the chips was done with a Zeiss Axiovert 200M inverted light microscope, a mercury lamp (HBO 100) as light source, and equipped with an AxioCam HRc CCD camera. All samples were immediately analyzed after preparation and illuminated as little as possible in the fluorescence microscope to minimize photobleaching. The microscope was focused on the sample at the edge of the pattern and moved to an unexposed area before capturing an image. Microscopic photoluminescence pictures were recorded in reflection mode using either 365/12, 470/40, or 546/12 nm band pass excitation and corresponding long pass emission filters at 397, 515, or 590 nm. The exposure time was set by the software to give the best result from each experiment. Hence low fluorescence intensity results in longer exposure times, making the exposure time a rough indication of the intensity. The images were analyzed using Zeiss AXIOVISION v3.1 software.

III. RESULTS AND DISCUSSION

A. Optimization of protein adsorption to a surface modified by PDMS stamp

The thickness of the adsorbed antibody layer is depending on a number of parameters, the time of stamp contact being one of them. After only 5 min contact time was a maximum antibody film thickness of 2.8 ± 0.2 nm, on top of the underlying PDMS residual thickness achieved [Fig. 3(a) and Fig. 4(a)]. In this experiment, a fresh PDMS stamp was brought

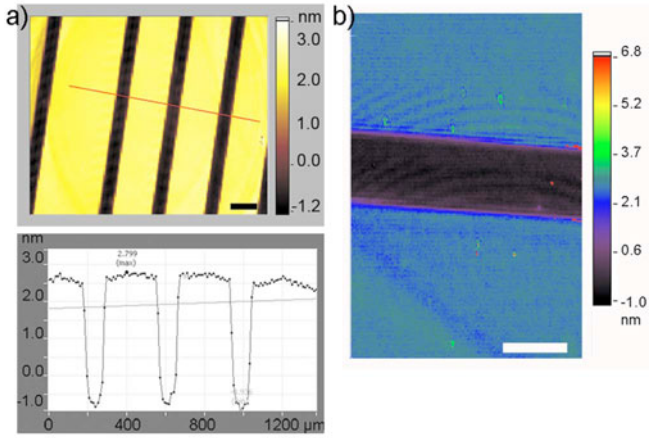

FIG. 4. Imaging ellipsometry micrograph showing the calculated thickness of adsorbed antibody film onto the PDMS surface energy pattern (scale bar: 100 μm) with a profile along the red line (a) and a zoom of the valley between two PDMS surface energy patterned areas (b) (scale bar: 100 μm). The underlying PDMS residual film ($\sim 0.7 \pm 0.1$ nm thick) was used as baseline in the model and corresponds to 0.0 nm thickness.

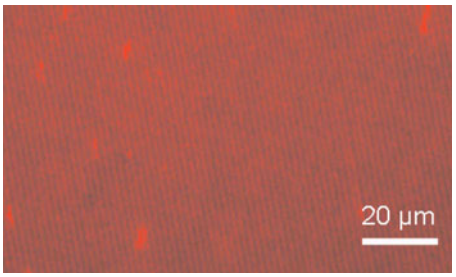

FIG. 5. Fluorescence microscopy image of IgG-TxR adsorbed on a PDMS surface energy pattern glass slide. The stamp used was molded on a optical grating: the period are 1,67 μm (excitation of 546 nm, exposure time of 2.7 s, scale bar of 20 μm).

into conformal contact with a clean substrate. The contact time between the stamp and the substrate was varied from 1 min up to 60 min. An antibody solution (IgG-TxR, 50 μg ml^{-1}, PB) was incubated as a drop on to the PDMS surface energy pattern in 30 min. The thickness of the antibody layer, adsorbed to the PDMS, was determined using imaging ellipsometry after the sample was rinsed and dried. Longer contact time instead resulted in a slightly thinner layer of adsorbed antibodies, despite the fact that the thickness of the underlying transferred PDMS residual layer was increased from 0.7 ± 0.1 nm, with 1 min contact time, to 1.3 ± 0.3 nm after 60 min contact time. Fluorescence microscopy pictures show that long contact time (>60 min) results in blurred patterns with increasing fluorescence from the areas where the stamp has not been in contact with the substrate (see supplementary Fig. S1).[22] The width of the valleys in the transferred pattern was measured to 100 ± 5 μm [Fig. 4(b)]. This corresponds to the width of the space separating the lines on the stamp and could therefore be determined as the parts where the stamp has not been in contact with the substrate. Thin traces of what could be unspecific adsorbed antibodies just as well as PDMS residuals were detected in this valleys with imaging ellipsometry [Fig. 4(b)]. But as only week fluorescence is visible when examined with fluorescence microscopy was this traces determined to contain adsorbed antibodies. However, is contrast, both in antibody thickness and fluorescence, between the hydrophobic PDMS surface energy patterned areas and the bare hydrophilic substrate evident.

Using the same procedure described, but varying the incubation time of the antibody solution (5–120 min) and keeping the contact time and incubation concentration constant (5 min contact time, 50 μg ml^{-1}), a maximum antibody film thickness of 3.3 ± 0.3 nm was obtained after 45 min incubation time. Longer incubation time did not increase the adsorbed antibody film thickness [Fig. 3(b)]. In the same way was the optimal concentration of antibodies in the incubation solution determined to 500 μg ml^{-1}, resulting in a 4.3 ± 0.3 nm thick antibody film (contact time of 5 min; incubation time of 45 min) [Fig. 3(c)].

The IgG antibody used in this study has a Y-shaped structure, ~ 10 nm long, and 3–5 nm wide, determined with x-ray crystallography.[3] Therefore we assume that the antibodies are evenly distributed and partly laying flat on the surface, also considering that the ellipsometer is measuring the average thickness in each pixel, with a size of ~ 2 μm^2.

The patterns of antibodies adsorbed onto PDMS patterns were all generated with the first stamping with a fresh stamp. However, repeated patterning using the same stamp is possible. One single stamp was used in a sequential stamping process. The stamp was brought in conformal contact with the substrate and moved to the next substrate after 10 min contact time. Antibody solution (IgG-TxR, 50 μg ml^{-1}) was incubated on the pattern for 30 min, and this cycle was repeated up to 20 times. The thickness of the transferred PDMS layer decreased from 1.1 ± 0.2 to 0.7 ± 0.1 nm after 20 stamping cycles. The film thickness of adsorbed antibodies decreased even more, 3.6 ± 0.2 nm after the first cycle to 2.2 ± 0.1 nm after 20 cycles [Fig. 3(d)]. (See supplementary Fig. S2 for fluorescence microscope pictures.) The adsorbed antibody layer is relatively strongly bonded to the PDMS pattern after the first rinsing step of the surplus. Further rinsing shows just a small weakening in fluorescence intensity, detected by increased exposure time. This weakening could, however, also be derived from photobleaching.

B. The resolution limits of PDMS surface energy patterning for protein adsorption

By using commercial optical grating as templates for stamp fabrication could PDMS was PDMS stamps with lines at periods of 1.67 μm prepared and used for PDMS surface energy patterning. An adsorbed IgG-TxR pattern was easily resolved with fluorescence microscopy (Fig. 5). It is probably possible to decrease the pattern size further, but we have recently shown that transferred PDMS residuals could behave as a fluid on the patterned substrate; thin submicrometer lines could be unstable and breaks up into short parts further contracting into drops and sets the PDMS surface energy patterning limits.[23] This could be compared to work done by

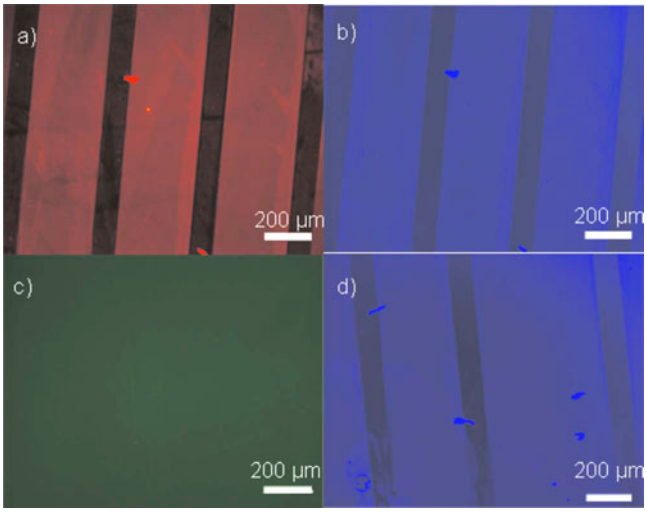

FIG. 6. Fluorescence microscopy image of two PDMS patterned glass slide with immobilized aIgG-Alexa, subsequently blocked against unspecific binding with HSA. Incubated with (a) IgG-TxR (excitation of 546 nm and exposure time of 4 s) and (b) streptavidin-FITC (excitation of 470 nm and exposure time of 10 s). [(b) and (d)] Excitation at 365 nm of the chip surface in (a) and (b), respectively; exposure time of 1.6 s in both. The stamps used were molded on the SU-8 master with 100 μm separation of the lines.

Renault *et al.*[11] using high-resolution PDMS stamp structured with grids of 40 nm wide lines separated by 800 or 100 nm wide posts able of printing single or a few antibodies on one spot. Poly(methyl methacrylate) have recently been used as stamp material with good result able to create protein patterns of ~150 nm in size and with aspect ratios up to 300.[24]

C. The ability of adsorbed protein to bind its complementary protein

Two PDMS patterned glass substrates with a layer of adsorbed aIgG-Alexa were prepared as described above (10 min contact time, 50 μg ml^{-1} IgG concentration, and 45 min incubation time). Possible free sites on the samples were blocked with HSA to prevent unspecific protein adsorption, before they were exposed to a third protein solution of the antigen. IgG-TxR from rabbit was used as complementary antigen and streptavidin-FITC as noncomplementary antigen. aIgG-Alexa is not expected to bind streptavidin. The samples were rinsed with PBS and gently blown dry with N_2 before analysis with fluorescence microscopy. A distinct red pattern was visible when the first sample, incubated with IgG-TxR, was illuminated at 546 nm [Fig. 6(a)]. No pattern was visible when the second sample, incubated with streptavidin-FITC, was illuminated at 470 nm, only background fluorescence could be detected (exposure time 10 s) [Fig. 6(c)]. When the two samples instead were illuminated at 365 nm distinct lines of blue fluorescence, deriving from the underlying aIgG-Alexa becomes visible [Fig. 6(b) complementary antigen; Fig. 6(d) noncomplementary antigen]. This shows that the interaction between immobilized antibodies to PDMS patterned chips is specific with its complementary antigen and that the bioactivity is preserved.

TABLE I. The total film thickness of proteins adsorbed to a PDMS patterned substrate determined with imaging ellipsometry.

Sample type	First adsorbed antibody layer	Second adsorbed antigen layer	Thickness of bilayer (nm)
1	IgG	···	3.2 ± 0.2
2	aStreptavidin IgG	Streptavidin	3.9 ± 0.1
3	aIgG	IgG	4.9 ± 0.1
4	IgG	aIgG	8.0 ± 0.4

PDMS surface energy patterning was performed on clean SiO_2 substrates by putting a fresh PDMS stamp, with 100 μm separation between lines, in conformal contact for 5 min. Three samples with different antibodies adsorbed, IgG, aIgG, or aStreptavidin IgG, were prepared on the PDMS patterned SiO_2 substrates as described above. The thickness of adsorbed IgG was determined with imaging ellipsometry to 3.2 ± 0.2 nm (Table I). Complementary antigen, streptavidin, aIgG, or IgG was incubated as drops on the adsorbed primary antibody chips. After rinsing and drying the total protein thickness was determined with imaging ellipsometry from at least ten measurements, summarized in Table I. The protein layer was increased with 4.8 nm when aIgG was bound as a secondary antibody to the adsorbed IgG pattern, compared to only 1.7 nm when IgG was bound to a primary aIgG pattern. If instead streptavidin was bound to a layer of astreptavidin IgG, an increment of the protein film thickness of 0.7 nm was measured. The size of streptavidin has been determined to ~5 nm.[25] This indicates that the primary antibodies are mainly laying flat on the PDMS modified surface with the antigen binding epitopes (Fab) unable to efficiently bind the antigen and consequentially making the binding epitopes for secondary antibodies easily accessible. This results in a higher amount of bound secondary antibodies, hence the average protein thickness.

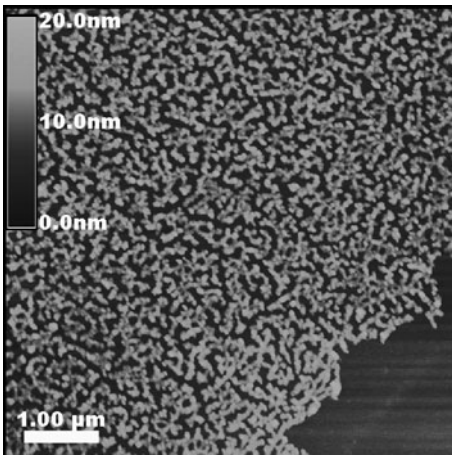

FIG. 7. AFM micrograph of μCP antibody pattern on SiO_2 wafer.

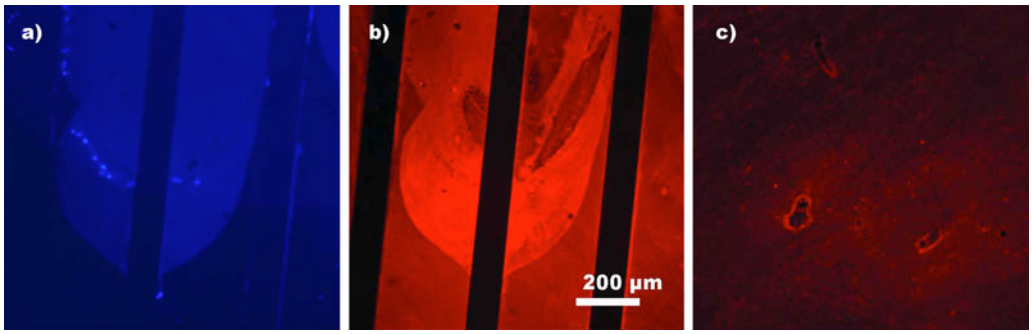

FIG. 8. Fluorescence microscopy picture of aIgG-Alexa μCP onto glass substrates followed by incubation with IgG-TxR illuminated at (a) 365 nm and (b) 546 nm. (c) aIgG-Alexa bath adsorbed onto nonpatterned glass substrates followed with bath adsorption of IgG-TxR illuminated at 546 nm. Exposure time of 1 s and scale bar of 200 μm in all parts.

D. Microcontact printing protein layers with soft lithography

To compare the adsorption of antibodies onto a PDMS pattern with standard microcontact printing, two sets of samples were studied. One set was printed with a protein ink from a PDMS stamp. The pattern on the PDMS stamp consists of disks of 50 μm in diameter separated with 200 μm. A drop of antibody solution (IgG-TxR 50 μg ml^{-1}, PBS) was deposited on the EtOH washed PDMS stamp surface. The antibody inked stamp was after rinsing and drying placed in conformal contact with a clean SiO_2 substrate and gently removed after 60 min. The resulting antibody pattern was on average 5.0 ± 0.5 nm thick, as observed with imaging ellipsometry. Evaluation of the same samples with AFM reveals a more detailed nanostructure, where the printed antibodies form a distinct network with high peaks separated with deep valleys reaching all the way down to the SiO_2 surface (Fig. 7). The thickness of the protein network was determined to be 9.8 nm with a rms roughness of 3.5 nm.

Comparative fluorescence studies were performed on aIgG-Alexa μCP on two clean hydrophilic glass slides. The stamp used consisted of parallel lines separated with 100 μm. Two clean glass slides were simultaneously submerged (bath incubated) in a solution of aIgG-Alexa (50 μg ml^{-1}, PB) as control. Complementary antigen, IgG-TxR (50 μg ml^{-1}, PB), was incubated on one of the μCP aIgG-Alexa sample and on one of the glass slides with adsorbed aIgG-Alexa. The two other samples were similarly handled but instead incubated with noncomplementary streptavidin-FITC (25 μg ml^{-1}, PB) as control. The sample was examined with fluorescence microscopy after rinsing and drying. The fluorescence from the primary aIgG-Alexa was rather weak when excited at 365 nm [Figs. 8(a) and 9(a)]. Nevertheless, a distinct bright red pattern was visible from the IgG-TxR bound to the μCP primary antibody, when excited at 546 nm [Fig. 8(b)]. In areas where the stamp, due to pattern design or stamp defects, had not been in contact with the substrate, neither the primary aIgG-Alexa nor the antigen IgG-TxR was detected. Comparing Fig. 8(c) with Fig. 8(b) it is easy to see that the printing process clearly affects the adsorption. More IgG-TxR is adsorbed to the μCP sample [Fig. 8(b)] than to the bath adsorbed layer [Fig. 8(c)], regardless of printed species. This indicates that changes in the substrate surface due to intrinsic properties of the stamp are more important for antigen binding than the layer transferred by μCP of primary antibodies. The samples incubated with streptavidin-FITC [Fig. 9(b)] were clearly fluorescent where the stamp has been in contact during the μCP, indicating that the streptavidin was adsorbed to the μCP PDMS pattern. As there is no biological specificity between streptavidin and aIgG, the driving force for streptavidin adsorption must be something else. This could be compared to the bath adsorbed reference [Fig. 9(c)]. No pattern is visible, and the fluorescence seen in Fig. 9(b) from the non-

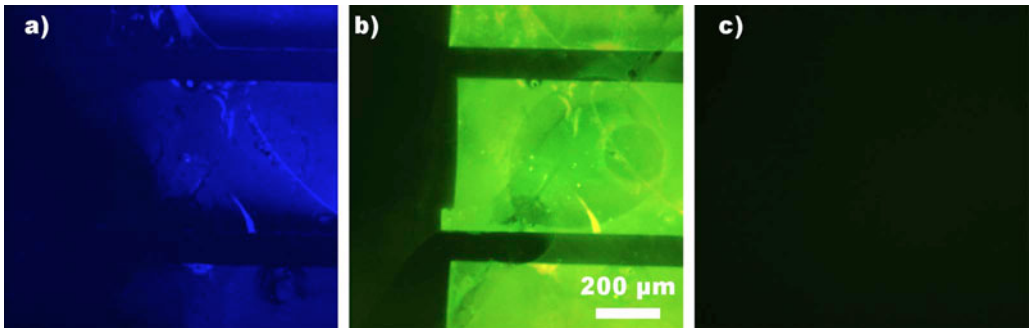

FIG. 9. Fluorescence microscopy picture of aIgG-Alexa μCP onto glass substrates followed by incubation with streptavidin-FITC illuminated at (a) 365 nm and (b) 470 nm. (c) aIgG-Alexa bath adsorbed onto glass substrates followed with bath adsorption of streptavidin-FITC illuminated at 470 nm. Exposure time of 1 s and scale bar 200 μm in all parts.

complementary streptavidin-FITC is nowhere to be seen in Fig. 9(c), indicating that the printing process itself is enough to create a discriminating pattern for adsorption.

The static water contact angle measured on a protein film on chips fabricated with μCP of the primary antibody using a flat stamp to $81° \pm 17°$ compared to only $31° \pm 30°$ when using PDMS surface energy with a flat stamp. An adsorbed layer of proteins could make the surface more hydrophilic,[26] as the case with the antibodies adsorbed to the PDMS surface energy pattern above in contrast to the μCP antibodies.

There exist a number of similar μCP protocols for patterning proteins,[10,12,27] where fluorescence labeled secondary aIgG is interacting with μCP IgG. In this article we show that protein without biospecificity could under some conditions adsorb to a printed IgG pattern. The differences in the resulting contact angle between the two patterning methods used, together with the distinct difference in fluorescence intensity in Fig. 9(b) from the areas where the stamp has been in contact with the printed antibodies compared to the bath incubated, show that change in surface characteristics induced by residues from the PDMS stamp is affecting the μCP protein pattern, possibly due to simultaneous transfer of PDMS and protein in the microcontact printing process turning the areas with patterned antibodies more hydrophobic. Since proteins are well known to more easily adsorb to a hydrophobic surface compared to a hydrophilic surface,[9,28] will the streptavidin adsorb faster and in a more dense layer to the hydrophobic PDMS contaminated μCP aIgG-Alexa pattern. This results in a more intense fluorescence picture compared to the hydrophilic parts where the stamp was not in contact with the substrate or the hydrophilic bath incubated glass substrates. On this areas the proteins also adheres but in a lower amount resulting in weaker intensity. We suggest that the mechanism responsible for the larger unspecific interaction of streptavidin with the aIgG pattern could be the PDMS surface energy modification.

IV. CONCLUSIONS

We have in this work shown that antibodies can be adsorbed in patterns due to the PDMS surface energy modification of a hydrophilic substrate. The antibodies are physisorbed and retain the ability to specifically bind its complementary antigen despite the random orientation. It is possible to use the same stamp for up to 20 steps at least. We also show that μCP antibodies using PDMS as stamp material, PDMS residuals, could leave contamination from the stamp onto the printed antibody pattern, rendering a more hydrophobic protein layer compared to when PDMS surface energy patterning is used. This PDMS contamination could be a reason to unspecific adsorption of proteins to the μCP antibody pattern.

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[1] A. Lueking, D. J. Cahill, and S. Mullner, Drug Discovery Today **10**, 789 (2005); W. Kusnezow and J. D. Hoheisel, J. Mol. Recognit. **16**, 165 (2003).
[2] R. A. Vijayendran and D. E. Leckband, Anal. Chem. **73**, 471 (2001).
[3] E. W. Silverton, M. A. Navia, and D. R. Davies, Proc. Natl. Acad. Sci. U.S.A. **74**, 5140 (1977).
[4] A. K. Abbas and A. H. Lichtman, in *Cellular and Molecular Immunology*, 5th ed., edited by W. R. Schmitt and H. Krehling (Saunders, Philadelphia, 2005), p. 43.
[5] A. A. Karyakin, G. V. Presnova, M. Y. Rubtsova, and A. M. Egorov, Anal. Chem. **72**, 3805 (2000); W. Zhang and M. J. Czupryn, Biotechnol. Prog. **18**, 509 (2002); D. Gao, N. McBean, J. S. Schultz, Y. S. Yan, A. Mulchandani, and W. F. Chen, J. Am. Chem. Soc. **128**, 676 (2006).
[6] J. L. Tan, J. Tien, and C. S. Chen, Langmuir **18**, 519 (2002).
[7] S. W. Howell, H. D. Inerowicz, F. E. Regnier, and R. Reifenberger, Langmuir **19**, 436 (2003).
[8] B. Haab, M. Dunham, and P. Brown, Genome Biol. **2**, research0004.1 (2001).
[9] H. Elwing, Biomaterials **19**, 397 (1998).
[10] A. Bernard, E. Delamarche, H. Schmid, B. Michel, H. R. Bosshard, and H. Biebuyck, Langmuir **14**, 2225 (1998).
[11] J. P. Renault, A. Bernard, A. Bietsch, B. Michel, H. R. Bosshard, E. Delamarche, M. Kreiter, B. Hecht, and U. P. Wild, J. Phys. Chem. B **107**, 703 (2003).
[12] J. R. LaGraff and Q. Chu-LaGraff, Langmuir **22**, 4685 (2006).
[13] E. Delamarche, H. Schmid, B. Michel, and H. Biebuyck, Adv. Mater. (Weinheim, Ger.) **9**, 741 (1997).
[14] M. J. Owen and P. J. Smith, J. Adhes. Sci. Technol. **8**, 1063 (1994); T. Murakami, S.-i. Kuroda, and Z. Osawa, J. Colloid Interface Sci. **202**, 37 (1998).
[15] D. J. Graham, D. D. Price, and B. D. Ratner, Langmuir **18**, 1518 (2002).
[16] K. Glasmastar, J. Gold, A. S. Andersson, D. S. Sutherland, and B. Kasemo, Langmuir **19**, 5475 (2003).
[17] R. B. A. Sharpe, D. Burdinski, C. van der Marel, J. A. J. Jansen, J. Huskens, H. J. W. Zandvliet, D. N. Reinhoudt, and B. Poelsema, Langmuir **22**, 5945 (2006).
[18] C. Thibault, C. Severac, A. F. Mingotaud, C. Vieu, and M. Mauzac, Langmuir **23**, 10706 (2007).
[19] X. J. Wang, M. Ostblom, T. Johansson, and O. Inganas, Thin Solid Films **449**, 125 (2004).
[20] P. Asberg, K. P. R. Nilsson, and O. Inganas, Langmuir **22**, 2205 (2006).
[21] N. K. Persson and O. Inganas, Sol. Energy Mater. Sol. Cells **90**, 3491 (2006).
[22] See EPAPS Document No. E-BJIOBN-3-009803 for facts and figures describing the impact of contact time on the adsorbed protein pattern and sequential PDMS surface energy pattering with a single stamp. For more information on EPAPS, see http://www.aip.org/pubservs/epaps.html.
[23] J. A. Wigenius, M. Hamedi, and O. Inganäs, Adv. Funct. Mater. **18**, 2563 (2008).
[24] M. Pla-Roca, J. G. Fernandez, C. A. Mills, E. Martinez, and J. Samitier, Langmuir **23**, 8614 (2007).
[25] S. A. Darst, M. Ahlers, P. H. Meller, E. W. Kubalek, R. Blankenburg, H. O. Ribi, H. Ringsdorf, and R. D. Kornberg, Biophys. J. **59**, 387 (1991).
[26] L. Vroman, Nature (London) **196**, 476 (1962).
[27] D. J. Graber, T. J. Zieziulewicz, D. A. Lawrence, W. Shain, and J. N. Turner, Langmuir **19**, 5431 (2003).
[28] G. B. Sigal, M. Mrksich, and G. M. Whitesides, J. Am. Chem. Soc. **120**, 3464 (1998).