Protein patterning by thermal nanoimprint lithography and NH₃-plasma functionalization of polystyrene

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New approach to create a pattern of specific protein binding sites via nanoimprint lithography is presented. NH₃-plasma functionalized, patterned polystyrene covalently binds a biotin linker to which streptavidin is bound. The streptavidin is used as a versatile anchor for biotinylated functional proteins by means of a sandwich-type quantitative fluorescence immunoassay. Rabbit IgG is the analyte used as a “proof of concept” for the designed system, yielding a detection limit of approximately 50 ng/ml. © 2009 American Vacuum Society. [DOI: 10.1116/1.3123304]

I. INTRODUCTION

The ability to spatially orient and anchor proteins on sub-micron to nanometric sized areas has become a major challenge for the development of bioengineered surfaces. The driving force for the ongoing advances concerning methods for biomolecule array fabrication is their prominent application in a variety of fields, including biomedical diagnostics, DNA analysis, drug discovery, and environmental monitoring. Moreover, many applications of biomolecule patterns can be enhanced by improving the resolution of the protein features. Smaller feature sizes enable, for example, the fabrication of high-density protein arrays for biosensors or proteomic screening, or facilitate studies of cellular interactions with small precisely located clusters of extracellular matrix proteins.

Several strategies have been developed to produce biologically relevant patterns. Among them, a major advantage of nanoimprint lithography (NIL) is that the feature size can be reduced to nanoscale, while still retaining high throughput and reproducibility. Most previous work in protein patterning by NIL^{1–4} has relied on using a sacrificial imprinted polymer and/or a treatment with aminosilanes to create a pattern of specific protein binding sites. The present work demonstrates a new approach: patterned polystyrene (PS) is functionalized with amino groups incorporated via an NH₃/N₂-plasma treatment;^{5,6} a biotin linker covalently bound to the formed amino groups is used to immobilize streptavidin (SAv) onto the PS structures. The SAv is used as a versatile anchor for biotinylated functional proteins, as demonstrated in a sandwich-type immunoassay.

II. EXPERIMENTAL PROCEDURE

The stamps were fabricated by standard UV lithography and reactive ion etching in silicon using a continuous etching process at room temperature in an Oxford Plasmalab ICP-80, with a combination of SF₆ and C₄F₈ gases. The stamp design consisted of five different gratings with periods ranging from 3.3 to 11.3 μ m, 270 nm in depth. All stamps were coated using a fluorosilane-based antiadhesive coating.⁷

The imprinting process was performed by means of the Jenoptik HEX03 press. 4 in. Si wafers having thermally grown SiO₂ on top were used as substrates. A PS 95k (T_g = 94 °C) layer was spin coated on the substrates. That layer was always thick enough to ensure a complete filling of the stamp. The embossing was carried out under vacuum at a temperature of 180 °C. The force (15 000 N) was applied at a speed of 0.2 mm/min and held for 900 s. The demolding temperature was 80 °C.

The residual layer was removed under an O₂ plasma [20 sccm (sccm denotes cubic centimeters per minute at STP), 20 mTorr, and 20 W]. The ammonia plasma treatment was performed in a plasma-enhanced chemical vapor deposition Plasmalab 80 plus for 1 min at 20 W and 200 mTorr in a 4:10 mixture of NH₃ and N₂. X-ray photoelectron spectroscopy (XPS) was carried out in a VG ESCALAB 220iXL spectrometer. The Mg K α x-ray source used was operated at a power of 200 W. For charge correction, the C 1s main peak was set to 284.6 eV.

The chemicals employed were purchased from Abcam and Invitrogen: LC-sulfo-NHS-biotin, 5.6 mg/ml; Alexa-fluor® 488 labeled SAv, 50 μ g/ml; horse-radish peroxidase labeled streptavidin (HRP-SAv), 50 μ g/ml; rabbit immunoglobulin G (IgG) at different concentrations ranging from 50 μ g/ml to 5 ng/ml; biotinylated antirabbit-IgG antibody,

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FIG. 1. XPS C 1s spectra of polystyrene spin coated oxidized silicon wafers: (a) untreated, (b) after NH₃-plasma, (c) after O₂-plasma, and (d) after O₂- and NH₃-plasma treatments.

50 $\mu\text{g}/\text{ml}$; and Alexa-fluor® 488 labeled secondary antibody, 50 $\mu\text{g}/\text{ml}$. The incubations were always performed at room temperature for 1 h.

Fluorescence images were taken with a Zeiss Axiophot II microscope equipped with a Kappa CF 8/1 DXC charge-coupled device camera. The fluorescence intensity was evaluated from microscope images using the scanning probe image processor (SPIP) software.

III. RESULTS AND DISCUSSION

Due to the high reactivity, plasma treatments of polymer materials can lead to the formation of a variety of different species. XPS measurements were done in order to confirm the formation of amino groups on PS surfaces using treatment in NH₃ plasma. We compared samples with and without the additional O₂ plasma step, which is usually performed for window opening of nanoimprinted samples.

The C 1s peaks of all treated samples (Fig. 1) show a tailing at the high binding-energy side, indicating carbon in higher oxidation states due to the formation of carbon-oxygen and carbon-nitrogen bonds. The fact that no distinct peaks were formed is typical for the simultaneous creation of various chemical species.

Quantification of carbon, nitrogen, and oxygen peaks (Table I) indicates an efficient introduction of nitrogen species at the polymer surface in the NH₃-plasma treatment even after pretreatment in O₂ plasma. In fact, the high level of oxygen introduced by the O₂ plasma was drastically re-

TABLE I. Surface concentration of C, N, and O of spin coated polystyrene after treatment in NH₃ and O₂ plasmas determined by XPS.

Sample	C (at. %)	N (at. %)	O (at. %)
Untreated PS	98.1	...	1.9
NH ₃ plasma	88.8	7.6	3.6
O ₂ plasma	79.6	...	20.4
O ₂ plasma, then NH ₃ plasma	83.6	8.5	7.9

FIG. 2. (Color online) Fluorescence micrograph of a sample of spin coated PS after biotin linker and fluorescent-labeled SAV incubation. The sample was half-covered during the NH₃-plasma exposure step.

duced by the subsequent NH₃-plasma treatment. Qualitatively, evidence of the plasma treatments was indicated by the significantly increased wettability of the PS surfaces.

Selective binding of SAV to the activated surfaces is achieved by first binding a biotin linker to an activated carboxylic acid function to the formed amino groups. Binding tests were carried out with fluorescent-labeled SAV. The fluorescence intensity was much higher in the plasma treated PS, while much less protein binding was found when the NH₃-plasma step was omitted, as it can be observed in the fluorescence image in Fig. 2.

For bioanalytical applications, the protein binding selectivity between the functionalized PS and the SiO₂ support is a further key parameter. This was assessed using nonstructured SiO₂ and PS samples (squares of $3.5 \times 3.5 \text{ mm}^2$ in size). The samples were incubated with HRP-SAV and its presence was assessed using enzymatic oxidation of tetramethylbenzidine catalyzed by the HRP. To obtain the intended discrimination between PS and SiO₂ surfaces, it was found that reduction in nonspecific protein adsorption was necessary. Two approaches were tested: (a) treatment with fluorosilanes after the ammonia plasma and (b) addition of bovine serum albumine (BSA) to the HRP-SAV solution. The first approach came to be efficient, but still some adsorption of SAV on the SiO₂ was detected. Addition of a detergent (Tween 20, 0.5%) to all the solutions proved to efficiently suppress the nonspecific binding.

In order to demonstrate the anchoring capabilities of the immobilized SAV, the fluorescence immunoassay depicted in Fig. 3 was followed: first a biotinylated antirabbit-IgG antibody is bound to the immobilized SAV. After incubation of a test sample with the analyte solution, bound rabbit-IgG is detected with a second, fluorescence labeled (Alexa Fluor 488) antibody. The representative fluorescence micrographs in Fig. 4 show the high affinity to the functionalized PS and the low background fluorescence signal. These facts imply a high binding capacity of the immobilized SAV and a low nonspecific protein binding to the fluorosilane treated SiO₂.

FIG. 3. (Color online) Scheme of the developed immunoassay aiming at detecting the presence of rabbit IgG in a solution.

The immunoassay developed was used to quantify the IgG content of several solutions. Figure 5(a) shows the concentration dependence of the recorded fluorescence signal, yielding a detection limit at about 50 ng/ml. In order to re-

FIG. 5. Fluorescence signal with increasing IgG concentration: (a) pure solutions and (b) with 50 $\mu\text{g/ml}$ of BSA.

produce a more realistic system, where the targeted analyte is in solution together with other proteins, the fluorescence intensity for IgG was measured in the presence of BSA. The resulting graph is shown in Fig. 5(b). Comparing the fluorescence intensity for the same IgG concentration, the value is always lower if BSA is present, indicating that BSA blocks surface areas where IgG could adsorb nonspecifically. However, a very similar detection limit for IgG was determined.

IV. CONCLUSIONS

The work presented shows the great potential of NH_3 -plasma functionalized PS for the fabrication of biofunctional structured surfaces, in which amino groups act as attachment points for biomolecules. The use of NIL as the patterning method has the advantage of high throughput and large area patterning. By using shadow masks, the NH_3 -plasma activation can be carried out selectively. This allows defining the specific functional areas on the substrate. Streptavidin is used as a versatile anchor for the selective immobilization of biotinylated functional proteins, as demonstrated for a sandwich-type quantitative fluorescence immunoassay.

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FIG. 4. (Color online) Representative fluorescence micrographs of patterned samples after NH_3 -plasma treatment protein binding according to the scheme in Fig. 3 (IgG 50 $\mu\text{g/ml}$): (a) period of 5.1 μm and (b) period of 3.3 μm .