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Nanofabrication of protein-patterned substrates for future cell adhesion experiments

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Abstract

A method for fabricating sub-micrometer size adhesion sites for future experiments in cell biology is presented. Glass substrates were coated with a thin layer of InSnO and SiO_2. The SiO_2 was structured by means of electron beam lithography and reactive ion etching, exposing sub-micrometer patches of the underlying InSnO. Dodecylphosphate, to which proteins can bind, was selectively adsorbed on these InSnO structures, whereas poly-L-lysine-*g*-poly(ethylene glycol) was used to passivate the surrounding SiO_2 against protein adsorption. The effectiveness of the process was investigated by fluorescent microscopy and scanning near-field optical microscopy on substrates which have been exposed to fluorescently labeled streptavidin.

Keywords: Electron beam lithography; Nanofabrication; Protein patterning; Self-assembling; Protein adsorption; Indium-tin-oxide

1. Introduction

The ability to adhere to other cells and to extracellular matrix is one of the most critical functions of cells in development and physiology. Abnormal adhesion is the cause of innumerable diseases, including many birth defects, mental disorders, immune defects, and cancer. In biology-inspired circuits, like for example in neuron electronics [1], the adhesion site is also a natural electronic interface between cells and silicon devices. Controlling the adhesion of cells and attempting to influence their spreading is, hence, also of interest to novel concepts in device technology.

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Cell adhesion is mediated by several classes of trans-membrane proteins, two of the most important being the integrin and cadherin families. These receptors cluster in specialized organelles, called adhesion sites. The size of an adhesion site varies roughly between 200 and 10 μm side length. The receptors interact with specific ligands, which can be adsorbed at specific areas on cell substrates by applying surface patterning techniques. Stamping these ligands, e.g., by PDMS stamps, is a technique, applied in the micrometer domain [2]. Stamping at the nanometer scale is very challenging and selective molecular assembly patterning (SMAP) was brought into play as another, very flexible approach, particularly in terms of the pattern of geometries obtainable [3]. It is based on the creation of an oxide pattern by lithographic means. The chemical contrast between TiO_2 and SiO_2 areas can subsequently be transferred into an adhesion protein contrast by selective self-assembly of alkane-phosphate onto TiO_2 and a non-interactive polyelectrolyte onto SiO_2. Such substrates will allow the study of cell adhesion and in particular the effect of growth constraints on adhesion sites.

The goal of the present study was to extend this previous work into the nanometer domain. A first step in this direction is the fabrication of metal-oxide patches in an SiO_2 matrix and to observe protein adsorption on such substrates.

2. Sample preparation

We found that the same selective adsorption, which leads to the self-assembly of alkane mono-layers on TiO_2, takes place also on a conductive InSnO (ITO) surface. This opens the additional possibility to electronically address single adhesion sites. Because ITO is transparent, the behavior of the cells can still be studied by means of the well-established optical methods like fluorescent microscopy. In order to further explore nanometer scale range, we decided to use e-beam lithography (EBL) for structuring the samples. Here, it comes at hand that ITO is electronically conductive and that it acts as a discharge layer during EBL.

Commercially available ITO-coated glass slides [4] were covered with a 15 nm thick SiO_2 film by atmospheric-pressure chemical vapor deposition (APCVD). Using a 200 nm thick film of PMMA (950k) as resist spin-coated onto the substrate and pre-baked at 170 °C for 30 min, the structures were delineated by EBL in a Raith 200 e-beam writer. Typical exposure parameters were a dose of 90–100 $\mu C/cm^2$ at 10 keV beam energy. A standard developing in MIBK:IPA 1:2 for 30 s followed by rinsing in IPA was used. The pattern was transferred into the SiO_2 film by means of reactive ion etching (RIE) in SF_6, using an Alcatel GIR 263. The selectivity of PMMA to SiO_2 was found to be about 1:1. Hence, the resolution of the patterns could be further increased by using thinner resist layers. The depth of the etched structure was measured by AFM to be 11 nm. The necessary etching time was only 20 s and therefore controlling the etch depth to better than 1 nm is difficult.

Fig. 1 shows an SEM-image of a cross-structure after RIE. Also, rectangles and dots as small as 75 nm in diameter could be fabricated so far. The SiO_2 is sufficiently thin that it is transparent for the secondary-electrons and, therefore, the roughness of the underlying ITO film can still be observed. This transparency reduces the contrast and makes observing of the small structures difficult. From these pictures, it cannot be concluded whether the ITO-layer was reached.

Time-of-flight secondary-ion mass-spectroscopy (TOF-SIMS) was used to check for the material contrast on simultaneously fabricated 60 μm × 60 μm squares, which are large enough to be resolved in TOF-SIMS imaging (Fig. 2). Based on these images, we conclude that the interface was uncovered.

It is known that dodecylphosphate (DDP) forms self-assembled mono-layers from aqueous solutions on various metal oxide surfaces. On the other hand, DDP does hardly adsorb on SiO_2 [5]. The fact that aqueous solutions can be used is important in view of the future application in biology, where other solvents may not be compatible. The hydrophobic DDP layer on ITO blocks the adsorption of poly-L-lysine-*g*-poly(ethylene glycol) (PLL-*g*-PEG) to which the substrates were subsequently exposed. PLL-*g*-PEG, however,

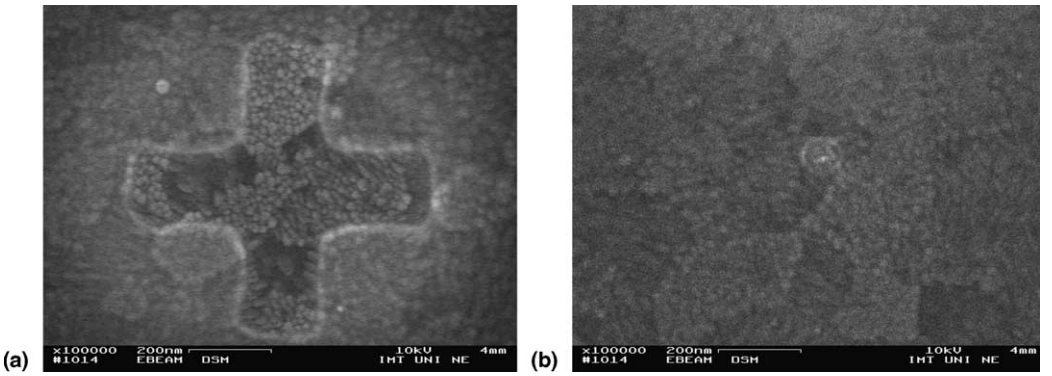

Fig. 1. (a) SEM image of a cross, etched into SiO_2. The size of the scale bar is 200 nm. The morphology of the underlying ITO film can be observed through the 15 nm thick SiO_2 film. This makes observing of the small circular structure in (b) difficult. It has a diameter of 85 nm.

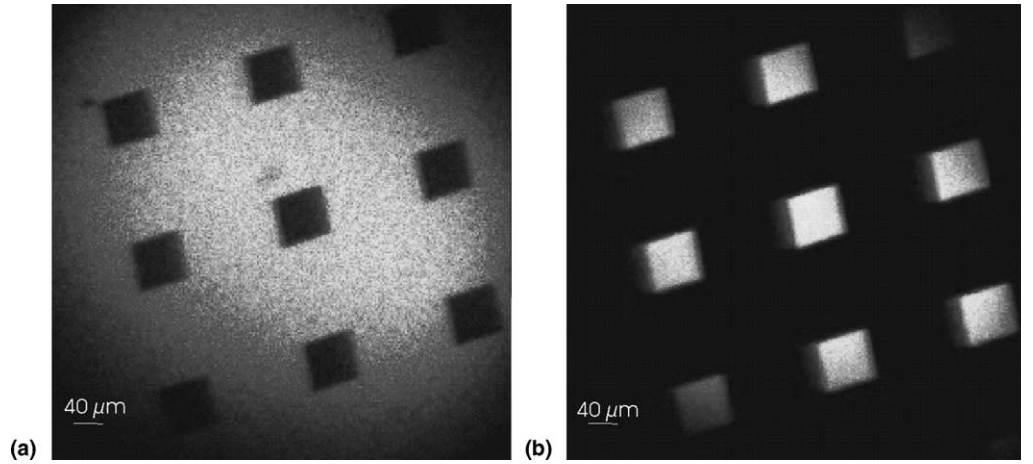

Fig. 2. TOF-SIMS image of an array of 60 μm × 60 μm large squares, etched into SiO_2 on an ITO film. The left image shows Si^+ (m/z = 28) contrast, whereas the right one represents the In^+ (m/z = 115) contrast. The scale bar is 40 μm.

adsorbs on SiO_2, where it forms an effective barrier against protein adsorption. The fluorescently labeled proteins (Streptavidin-AF488) could therefore be selectively adsorbed on the DDP or the ITO areas correspondingly.

3. Evaluation

A first indication of the selectivity of DDP and PLL-*g*-PEG adsorption, respectively, could be gained by TOF-SIMS imaging again, when looking at the characteristic m/z-lines for $SiOH^+$ and PO^{2-}, respectively. Fluorescent microscopy images on the large patterns also show a high contrast. Fig. 3 shows fluorescence microscope images of an array of crosses, rectangles and dots, respectively, with a good contrast and a low background. The dimensions are at the resolution limit of our microscope, but still the shapes can be discerned. Using scanning near-field optical microscopy (SNOM), a clear protein contrast at the sub-micron level was observed. Fig. 4 shows an SNOM image and a simultaneously recorded AFM image of a sample with crosses. The size of the SNOM probe apex was about 300 nm. Therefore, the

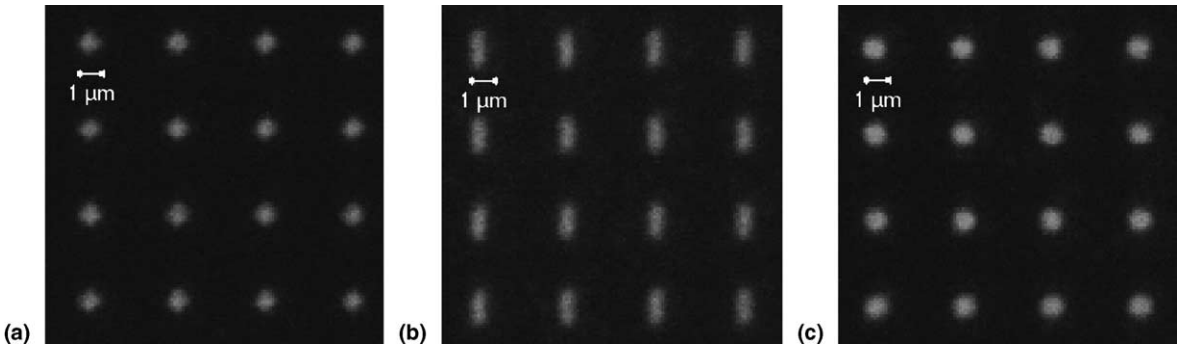

Fig. 3. Fluorescence microscope images of selectively adsorbed streptavidin labeled with AF488. The crosses in the left picture (a) were designed to have a width of 200 nm and a length of 1 µm, the rectangles in the center (b) measured 0.4 µm × 1.6 µm, and the dots in (c) have a diameter of 670 nm.

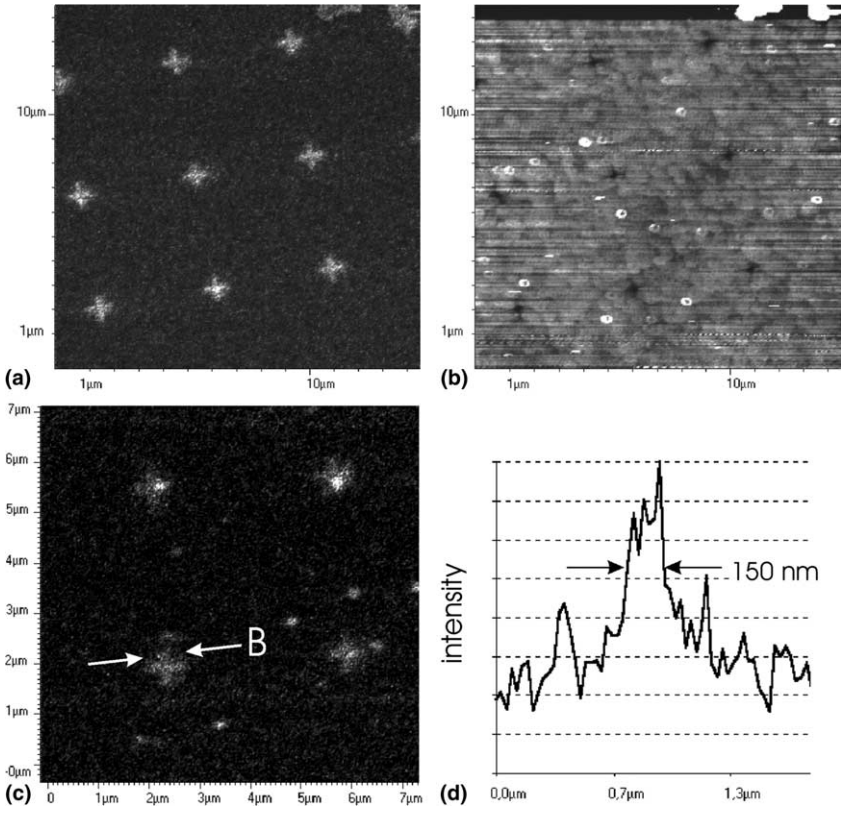

Fig. 4. (a) SNOM and simultaneously recorded AFM images (b) of an array of the same crosses, shown in Fig. 3(a). The picture size is 15.5 µm × 15.5 µm, the bar width of the crosses was designed to be 0.2 µm and the length 1 µm. The crosses in the SNOM image on (c) have a bar width of 140 nm and a length of 660 nm, the image represents an area of 7.3µm × 7.3µm. In (d), a cross-section of the optical intensity between the two arrows indicated in (c) is shown.

smaller structures are no longer observable in the topography image but still show up in the optical contrast. The designed values were 140 nm bare width and 660 nm length. The cross-section through the optical signal in Fig. 4(c) and (d) shows a width of 120 nm and a length of 480 nm, respectively. It is possible that the cross was not completely coated with Streptavidin, which would explain the relatively large difference in length.

4. Summary and conclusion

We have successfully structured 15 nm thick SiO_2 films on a InSnO–glass substrate. The resulting material contrast in the 100 nm scale range could be converted into a corresponding protein contrast by using self-assembling of a dodecylphosphate monolayer on the InSnO patches and passivating of the surrounding SiO_2-matrix by means of poly-L-lysine-*g*-poly(ethylene glycol). It is known that such thin SiO_2 films do have pinholes and it was not clear whether these pinholes could lead to unwanted protein adsorption. At the present level of detection sensitivity, we can conclude that protein rejection by the PLL-*g*-PEG was strong enough to block also eventual pin-holes. The effectiveness was controlled by fluorescence microscopy and SNOM observing the distribution of fluorescently labeled streptavidin.

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