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DOI: 10.1002/adma.200601047

Reactive Microcontact Printing on Block Copolymer Films: Exploiting Chemistry in Microcontacts for Sub-micrometer Patterning of Biomolecules**

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Patterned biofunctional interfacial architectures are currently at the focal point of sensor and cell biology research.[1–3] For a wide range of possible applications in the life sciences, in particular, the investigation of controlled cell–surface interactions,[4] (bio)chemical patterning on multiple length scales down to the sub-100 nm level is required. The relevant distances and length scales of ligand clustering in multivalent recognition and those in protein clustering in focal adhesion of certain types of cells on the one hand,[5,6] and the size of biological entities such as bacteria or cells on the other hand, span an enormous range.[7]

For example, the modulation of cell adhesion by altering the spacing of surface-immobilized RGD-sequence-containing (RGD (Arg-Gly-Asp); a tripeptide recognition motif) peptides on Au nanodot patterns prepared by block copolymer self-assembly has been reported.[6] For distances of ≤58 nm and ≥73 nm between the nanodots, the presence and absence, respectively, of focal adhesion was observed, implying a characteristic length scale for integrin clustering in the cell membrane. Similarly, micropatterns of cell-adhesive proteins demonstrated that cell attachment and vitality can be controlled by the size of these patterns.[3,7]

To provide the required patterns, soft lithographic methods[8] as well as embossing and imprinting approaches have been introduced[9] in addition to established optical lithographic techniques.[10] Central requirements for these methods include large-scale parallel fabrication of larger surface areas and control over the (bio)chemical composition of the surface down to the sub-100 nm size regime, together with control over topographical features and substrate modulus.[3,7,11] Traditional microcontact printing (μCP) of low-molecular-mass compounds or direct-transfer μCP of biomolecules may suffer from a number of drawbacks that do not allow one to address the critical length scales mentioned above and to provide the required functionality due to, for example, denaturation of stamped proteins.[8a,12] Thus, new patterning approaches and new platforms are required.

In this Communication we introduce a new reactive microcontact printing strategy to fabricate well-defined functional micro- and nanostructured biointerfaces. Central to our new approach is the exploitation of selective surface chemistry on a reactive block copolymer film by using a volatile and highly diffusive reactant (trifluoroacetic acid: TFA) delivered via an elastomeric stamp (Fig. 1).[13]

The polystyrene-*block*-poly(*tert*-butyl acrylate) (PS_{690}-*b*-P*t*BA_{1210}) block copolymer film platform utilized in this study comprises reactive *tert*-butyl ester moieties at the film surface (skin layer thickness ca. 8 nm) and microphase-separated glassy polystyrene domains in the film interior that provide excellent thermal and processing stability.[14] The microphase separation opens avenues towards structuring on multiple length scales via a synergistic combination of top-down patterning techniques with block copolymer self-assembly.[15]

The volatile and highly diffusive reactant TFA is delivered locally in the (sub-)microcontacts between an elastomeric stamp and a reactive block copolymer film (Fig. 1b). Partitioning of the reactant into the region near the surface (skin layer) of the polymer film results in the highly localized deprotection reaction of *tert*-butyl acrylate (*t*BA) side chains present in the skin layer of the film.[14]

The most notable consequence of our approach is the absence of pattern deterioration by surface diffusion effects on length scales down to (and possibly below) 300 nm. In addition, solventless chemical reactions by direct molecular transfer in the microcontacts between stamps and this platform are characterized by enhanced yields, as compared to solution-phase reactions. Thus, as shown below, sub-micrometer patterns of cell-adhesive fibronectin and nonadhesive PEG_{5000} (PEG: poly(ethylene glycol)) were conveniently fabricated,

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[**] The authors would like to thank Dr. Haoli Zhang (Oxford) for providing us with PDMS stamps with sub-micrometer feature sizes. The support of the EU (NoE Nano2Life) is gratefully acknowledged. This work has been financially supported by the MESA+ Institute for Nanotechnology of the University of Twente, the Council for Chemical Sciences of the Netherlands Organization for Scientific Research (CW-NWO) in the framework of the "vernieuwingsimpuls" program (grant awarded to H.S.) and a BMBF grant (J.S.). Supporting Information is available online from Wiley InterScience or from the author.

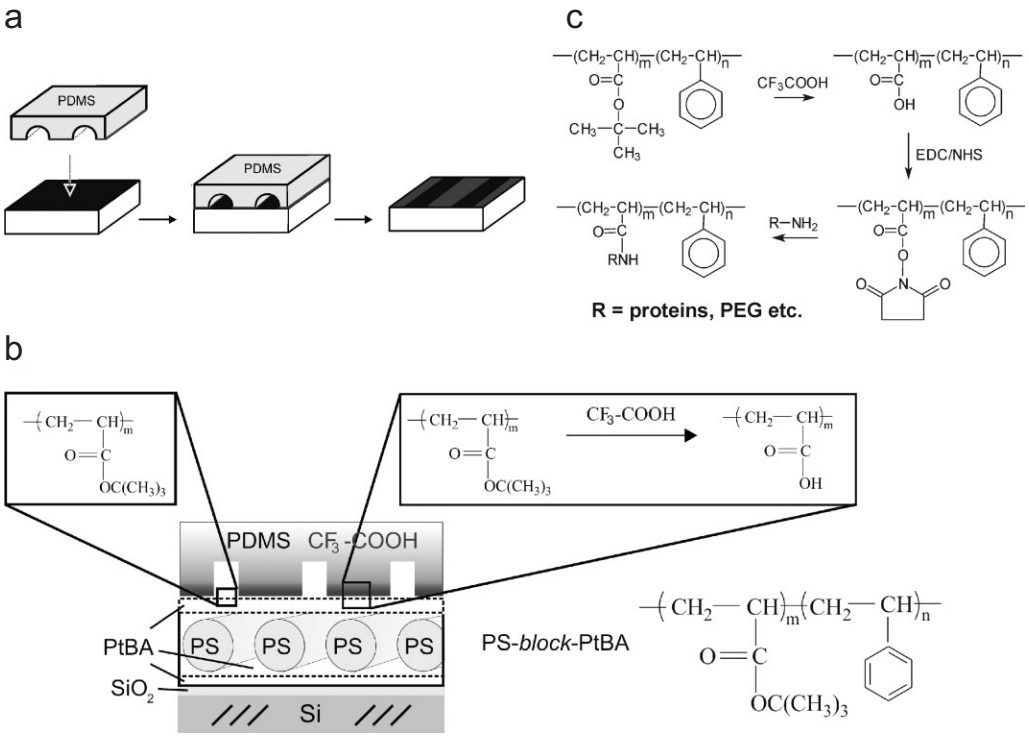

Figure 1. Schematic of our new reactive microcontact printing strategy on block copolymer films. a) Localized reaction carried out by reactive μCP in the conformal microcontact between a reactant-covered elastomeric poly(dimethyl siloxane) (PDMS) stamp and a substrate-supported reactive polymer film. b) Schematic of a localized deprotection reaction carried out in the contact areas of the stamp and the P*t*BA skin layer. c) Sequence of surface reactions utilized for the PS_n-*b*-P*t*BA_m diblock copolymer film system (here: $m = 1210$; $n = 690$) discussed in this Communication. Alternatively to the localized deprotection chemistry shown, reactants, such as proteins or amino end-functionalized poly(ethylene glycol) (PEG-NH_2), can be transferred in the contact areas of the stamp and an *N*-hydroxysuccinimide-activated (NHS) film, resulting in covalent attachment of the reactants in high coverages.

and their interaction with highly metastatic pancreatic cancer cells was unveiled. Ultimately, this methodology can be extended to micro- and nanoscale patterning on the surface of microphase separated block copolymer films that expose both constituent blocks. Patterning on multiple length scales thus becomes possible, as the length scales of the top-down (reactive μCP) and bottom-up (80–100 nm) approaches can indeed overlap.[16]

The local deprotection of the *t*BA groups in the P*t*BA skin layer by TFA proceeded rapidly, as was shown in independent studies.[17] In our current work, it was found that TFA can be administered by a TFA-loaded oxidized PDMS stamp. Optimized TFA loading of the stamp ensured that no liquid acid was present and that the reaction occurred in the stamp/film contact area. The deprotection and subsequent activation and derivatization reactions were studied on films hydrolyzed using featureless stamps by contact angle, ellipsometric, atomic force microscopy (AFM), Fourier transform infrared (FTIR), and X-ray photoelectron spectroscopy (XPS) methods and showed reproducible functionalization and high grafting densities.[17] The transfer of low-molecular-mass silicones was ruled out on the basis of XPS data acquired on the polymer films after stamping (see Supporting Information). Micropatterns fabricated according to the reactive μCP procedure shown in Figure 1 were investigated by fluorescence microscopy (Fig. 2a). In addition, the thickness of passivating $PEG_{5000}NH_2$ layers that were grafted via direct-transfer μCP was assessed by ellipsometry (Fig. 2b).

The fluorescence microscopy image shows a homogeneous coverage of the covalently coupled dye and a very sharply defined pattern (see also fluorescence emission intensity cross-sectional plot). The coverage of fluoresceinamine in the nonhydrolyzed (and activated) areas is virtually zero, and the near-constant fluorescence emission intensity observed in the fluorescent stripes implies that the hydrolysis, activation, and subsequent derivatization by grafting proceeded homogeneously. Pronounced material transport, i.e., polymer film deformation by capillary forces or spreading of TFA (on the optically accessible length scale), were absent.[18]

Interestingly, the thickness of PEG_{5000} grafted by direct-transfer μCP is 20–25 % larger than that of $PEG_{5000}NH_2$ coupled from solution (Fig. 2b). This result indicates that the high local concentration (and pressure) under the solventless conditions of μCP improve the coupling efficiency.[19] The higher grafting densities thus obtained are linked to improved suppression of nonspecific protein adsorption.[20]

To investigate the limitations of the approach, the localized hydrolysis of PS_{690}-*b*-P*t*BA_{1210} films using reactive μCP of

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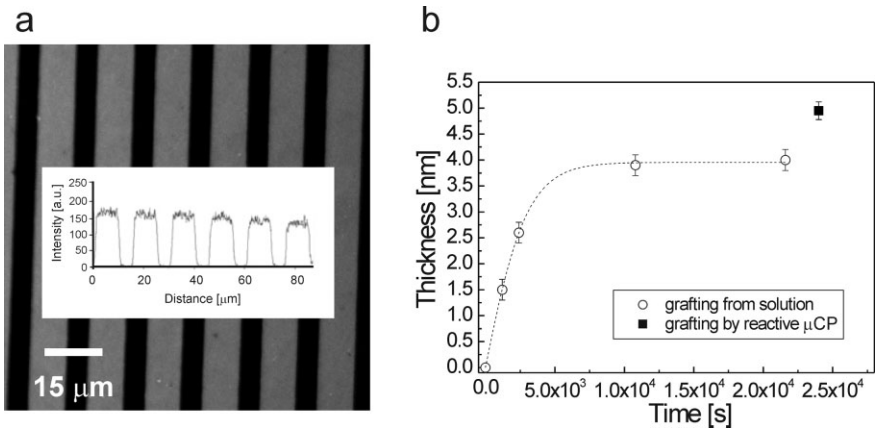

Figure 2. a) Fluorescence microscopy image and corresponding cross-sectional intensity plot (inset) for PS_{690}-*b*-P*t*BA_{1210} films patterned with a stamp that possessed line features (10 μm × 5 μm). Details for (a): The block copolymer film was hydrolyzed by placing a TFA-covered oxidized PDMS stamp in conformal contact for 20 min, followed by activation of the carboxylic acid groups formed in the contact areas with NHS/EDC (EDC: 1-ethyl-3-(dimethylamino)-propylcarbodiimide) and grafting of fluoresceinamine from phosphate buffer (PB). b) Ellipsometric thickness of grafted PEG_{5000} layers obtained on prehydrolyzed and NHS-activated PS_{690}-*b*-P*t*BA_{1210} films versus reaction time (○), and maximum grafted thickness for a PEG_{5000} layer grafted by reactive μCP transfer (■) using an excess of $PEG_{5000}NH_2$ on the stamp.

TFA with PDMS stamps exposing submicrometer-sized line features was studied using friction-mode AFM (Fig. 3a). In addition, the interactions of adherent PaTu8988T pancreas adenocarcinoma cells with nanopatterned fibronectin–PEG functionalized PS_{690}-*b*-P*t*BA_{1210} films were investigated by wide-field optical microscopy (Fig. 3b).[21,22] The key observations are twofold: The patterns of the stamps were faithfully transferred in the reactive μCP step, also on length scales of 500 nm × 300 nm, and binary cell-adhesive/cell-repulsive nanopatterns were found to affect the cancer cell morphology.[22]

The sharp line-edge definition observed in the friction-mode AFM images as well as the pronounced friction-force contrast between the hydrophilic and hydrophobic areas suggest the occurrence of a laterally homogeneous

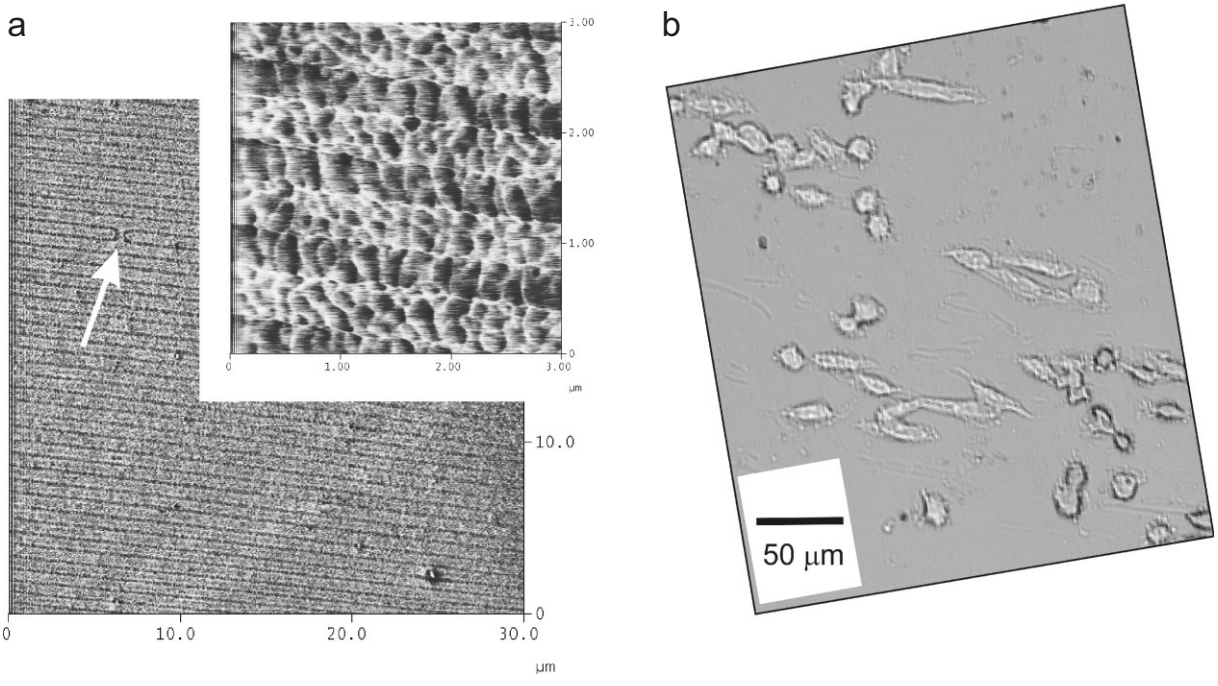

Figure 3. a) AFM friction image acquired on a PS_{690}-*b*-P*t*BA_{1210} film after local hydrolysis by reactive μCP (inset: high resolution AFM friction image). The stripes with high friction-force contrast (width ca. 500 nm) are ascribed to the hydrophilic part (poly (acrylic acid): PAA), the low friction-force stripes (width ca. 300 nm) are ascribed to the hydrophobic part (P*t*BA). Note in (a) how a defect in the stamp has been faithfully reproduced (the defect is highlighted by the arrow). For a friction-mode AFM image of a block copolymer film after transfer of PEG, see the Supporting Information. b) Optical microscopy image of PaTu8988T pancreatic cancer cells on a pattern of fibronectin (300 nm line width) and $PEG_{5000}NH_2$ (500 nm line width) on a block copolymer film prepared by direct reactive μCP of $PEG_{5000}NH_2$ on a TFA-prehydrolyzed and an NHS/EDC-activated PS_{690}-b-P*t*BA_{1210} film, followed by functionalization of the unreacted areas with fibronectin (the microscopy image has been rotated to ensure the same orientation of the features in (a) and (b)). Cells were cultured for 24 h on the nanopattern. Blank experiments on unpatterned fibronectin-functionalized films showed much fewer elongated cells, which also exhibited a smaller aspect ratio (see Supporting Information).

and well-defined deprotection reaction. The chemical contrast is in full agreement with previously reported data for a related polymer platform and data obtained on functionalized PS_{690}-*b*-P*t*BA_{1210} films in micrometer-scale patterns.[17,23] No evidence for pronounced TFA diffusion was observed at these length scales, indicating that the minimum feature size is limited by the feature dimensions on the elastomeric stamp at this point.

On the nanopatterned substrates exposing 300 nm wide lines of fibronectin and 500 nm wide lines of PEG_{5000}, the cells were observed to spread in highly unusual very elongated shapes. While only (4 ± 2) % of the cells showed an elongated morphology (mean aspect ratio: (2.2 ± 0.5)) on unpatterned fibronectin, (31 ± 9) % of the cells were found to be elongated on the nanopatterns (mean aspect ratio (2.9 ± 0.8), see Supporting Information for distributions). More strikingly, the order parameter *S* of 0.97 indicated near-perfect alignment for the elongated cells on the nanopatterns compared with near-isotropic orientation, i.e., no preferred orientation, on the unpatterned films.[24] These observations indicate that the cells are indeed forced to stretch along the sub-micrometer-sized pattern of cell adhesive (fibronectin) and nonadhesive (PEG_{5000}) stripes and can differentiate the in-plane directionality of the anisotropic surface chemistry. As a consequence, the cells spread out in the direction of the highest fibronectin coverage (lowest PEG coverage).

Extrapolating from the results shown above, it appears that the fabrication of smaller feature sizes is feasible by using appropriate stamps with smaller features and optimized mechanical properties. By joining this top-down approach with the bottom-up approach of block copolymer self-organization, multiple functions with defined nanoscale spacings can be introduced that, in conjunction with systematic studies of the effect of feature type and spacing, exposed proteins, and different cell types, open the way to study and understand the cell biology related to cancer-cell–surface interactions and cell migratory behavior.

In summary, we have shown that highly localized, selective deprotection chemistry and efficient grafting reactions can be carried out in the microcontact region between an elastomeric stamp and a reactive PS_{690}-*b*-P*t*BA_{1210} diblock copolymer film. Partitioning of the volatile deprotection reagent TFA into the polymer film reduced the lateral diffusion of the reactant and thereby allowed the fabrication of sub-micrometer scale features. Subsequent wet-chemical functionalization, which avoided the exposure of sensitive material to ambient conditions, afforded very robust and functional nanopatterned biointerfaces. Pancreas cancer cells were observed to spread in highly spatially directed, pronounced elongated shapes on nanopatterned platforms exposing 300 nm wide lines of fibronectin and 500 nm wide lines of PEG_{5000}, thus indicating that tailored surfaces that allow one to acquire important insight into cell behavior at surfaces are now readily accessible.

Experimental

Materials: PS_{690}-*b*-P*t*BA_{1210} diblock copolymers (number-average molecular weight (M_n) 202.4 kg mol^{-1}; polydispersity index (PDI) 1.03) were purchased from Polymer Source Company (Dorval, Canada) and used as received. Amino end-labeled PEG ($PEG_{5000}NH_2$) was purchased from Nektar UK Company (M_n = 5000 g mol^{-1}, PDI = 1.1), fluoresceinamine was purchased from Molecular Probes, Inc. (The Netherlands), and fibronectin was purchased from Roche Diagnostics GmbH (Penzberg, Germany).

Preparation of Thin Films: Thin films were prepared as reported previously by spin-coating filtered polymer solutions in toluene (10 mg mL^{-1}) onto oxygen plasma-cleaned silicon (111) wafers and glass cover slides, followed by annealing at 135 °C for 24 h in vacuum [14]. A film thickness of (90 ± 5) nm was determined using a custom-built spectroscopic ellipsometer using a He–Ne laser (λ = 632.8 nm) [14].

Reactive Microcontact Printing: 50 μL of TFA was applied to the surface of a PDMS stamp (1 cm × 1 cm), which was preoxidized for 60 min in an oxygen plasma according to a procedure published earlier [25], followed by drying in air for 4 min. The stamp was then brought into contact with the surface of the polymer films, and the contact was kept for 20 min. After peeling off the stamp, the films were rinsed three times using Milli-Q water, activated with NHS/EDC (i.e, treated in an aqueous solution of EDC (1 M) and NHS (0.2 M) for 30 min, and rinsed with Milli-Q water), and reacted with fluoresceinamine, $PEG_{5000}NH_2$, or fibronectin (100 μM in PB, pH 7.4). Before the direct transfer of $PEG_{5000}NH_2$ (carried out according to a published procedure [23]), the polymer films were hydrolyzed in neat TFA for 20 min, rinsed three times using Milli-Q water, and activated with NHS/EDC. After soaking in the $PEG_{5000}NH_2$ solution for 60 min, the stamp was loaded with an excess of PEG by solution-casting a $PEG_{5000}NH_2$ solution onto the top of the stamp.

Fluorescence Microscopy: Fluorescence microscopy images of dry samples on glass cover slips were recorded at room temperature on a Zeiss LSM 510 confocal laser scanning microscope using a Plan–Apochromat 63×/1.4 NA (numerical aperture) oil-immersion objective, the 488 nm line of an Ar^+ laser as excitation wavelength, a 500–550 nm bandpass filter, and a Hamamatsu R6357 photomultiplier tube.

Optical Microscopy: Optical microscopy was performed using an Olympus BX 60 (standard setup).

Atomic Force Microscopy: The contact-mode AFM measurements were carried out with a NanoScope III multimode AFM (Digital Instruments/Veeco, Santa Barbara, CA) using silicon nitride tips/cantilevers in ambient atmosphere, as described earlier [26].

Cell Culture: The investigated human pancreatic ductal adenocarcinoma cell line PaTu 8988T was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Pancreas tumor cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 5 % fetal calf serum (FCS), 5 % horse serum and 2 mM L-glutamine at 5 % CO_2. For analysis of adherence, cells were trypsinized, seeded sub-confluent on manufactured substrates, cultured for 24 h, and fixated with 1 % glutaraldehyde in phosphate-buffered saline (PBS). After PBS washing, cell adherence was studied using optical microscopy.

Received: May 12, 2006
Revised: August 16, 2006

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[24] Ten areas of 250 μm^2 were analyzed and the arithmetic mean was calculated ± standard deviation. The order parameter S was calculated as $S = 1/2\,(3 \langle \cos^2 \theta \rangle - 1)$, where θ denotes the angle between the orientation direction of the long axis of the elongated cells with respect to the director (line direction of the pattern). For the unpatterned samples no preferential orientation was observed (see Supporting Information); the very limited number of data points renders the estimate of the order parameter in this case (the average value of the order parameter was (0.26 ± 0.16) less appropriate).

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