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Reactive μCP on ultrathin block copolymer films: Localized chemistry for micro- and nano-scale biomolecular patterning

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Abstract

Three different, complementary soft lithographic approaches for the fabrication of chemical patterns on ultrathin polystyrene-block-poly(*tert*-butyl acrylate) (PS_{690}-b-$PtBA_{1210}$) films are discussed. Central to the methodology is the previously introduced reactive PS_{690}-b-$PtBA_{1210}$ platform that allows one to covalently graft (bio)molecules via robust amide linkages in high densities on flat, as well as on structured, surfaces. As shown in this paper, the combination of the polymer-based platform and reactive microcontact printing (μCP) patterning approaches allows one to obtain patterns of (bio)molecules with (sub)micrometer feature sizes. The μCP approaches comprise: (A) the direct transfer of functional (bio)molecules from an oxidized elastomeric stamp to hydrolyzed and *N*-hydroxysuccinimide (NHS) activated PS_{690}-b-$PtBA_{1210}$; (B) the transfer of a passivating poly(ethylene glycol) layer to hydrolyzed and NHS-activated PS_{690}-b-$PtBA_{1210}$ followed by wet chemical grafting of functional moieties; (C) the local hydrolysis of the PtBA skin layer with trifluoroacetic acid (TFA), followed by NHS activation and wet chemical derivatization. The applicability and the versatility of the combination of the polymer thin film-based platform and soft lithographic methodologies for patterning biologically relevant molecules is demonstrated for polyamidoamine (PAMAM) dendrimers, different proteins, as well as probe DNA. The successful hybridization of target DNA and the immobilization of fibronectin in micropatterns show that ultrahigh density patterns for micro- and nano-arrays, as well as for studies of cell–surface interactions, can be conveniently fabricated based on these approaches and platforms.

Keywords: Block copolymer films; Nanopatterning; Soft lithography; Reactive microcontact printing; Platform fabrication; Biointerfaces

1. Introduction

Biomolecular patterns with feature sizes on the micrometer and nanometer length scales are relevant for the development of advanced bioaffinity screening arrays in genomics [1], proteomics [2], as well as for other related areas [3–5]. Robust patterns can be realized, among others, on self-assembled monolayers (SAMs) [6,7] or thin polymer film-based platforms [8,9] using different patterning approaches. These different patterning methodologies often rely on "top–down" fabrication techniques, such as

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photolithography [1,10] and soft lithography [11–13]. Among these techniques, microcontact printing (μCP) is one of the promising approaches for producing (bio)chemical patterns on various solid substrates [7,14,15].

Pioneering work in this area was extended to the fabrication of tailored patterned and/or structured surfaces for the investigation of cell–surface interactions [16,17]. In particular the work on cell attachment (focal adhesion), cell shape, as well as cell motility and migratory behavior on such surfaces is of considerable interest as fundamental processes of cell biology [16–18] including cell function via protein clustering, may be unraveled or investigated [19].

In general, cells react to topographical and (bio)chemical cues [13,16,17,19,20]; in some cases the modulus of the underlying substrate was found also to be important [17b]. The details of the interaction depend on the type of cell and also on the choice of the proteins or polypeptides exposed on patterned surfaces. Interestingly, pattern sizes and spacings were reported to possess a profound influence on the response of cells [16,19].

Owing to the broad range of length scales involved, ranging from cells on the micrometer length scale to protein clusters or individual proteins and other biomolecules on nanometer length scales, pattering on *multiple length scales* is vitally important in this field. Considering the complexity of cell–surface interactions and the underlying parameters and properties that need to be controlled, it is clear that new approaches are required to further ensure progress in this direction. The definition of patterned surfaces on the molecular scale and in particular the patterning on multiple length scales using multiple types of biomolecules are challenges that cannot yet be addressed satisfactory in massively parallel fabrication on large scales [21].

In the context of patterned platforms, *polymer-based* thin film systems combine a number of distinct advantages that render them complementary to SAM-based approaches. These advantages include, in addition to simple and reproducible fabrication, robustness and stability, the unique possibility to introduce simultaneously topographic and chemical (compositional) patterns, defined and tunable modulus, and nearly unconstrained reactivity of incorporated reactive functional groups in a quasi 3D geometry [22]. Therefore it is possible to selective immobilize (conjugate) biomolecules, such as receptors, antibodies, proteins etc., which show retention of their biological activity. In addition, the non-specific biomolecule (e.g., protein) adsorption can be effectively controlled and eliminated.

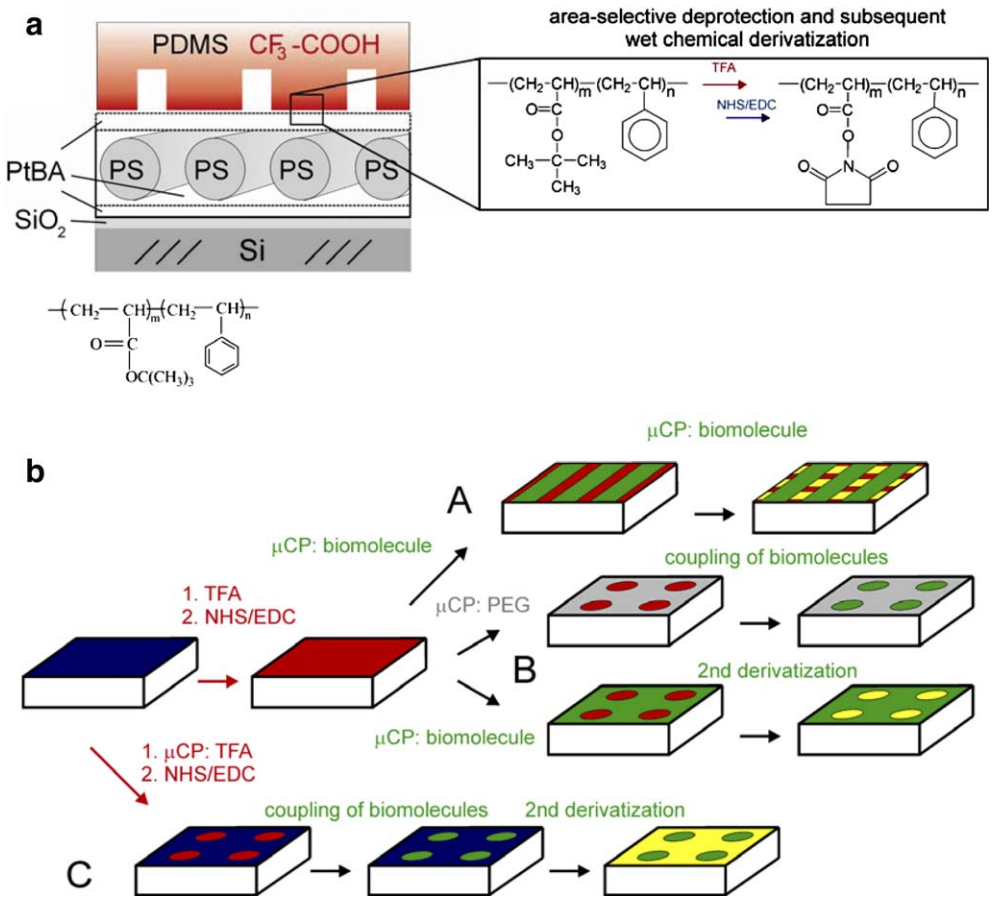
We have recently introduced reactive homopolymer [23,24] and block copolymer [25] thin film platforms that possess these distinct advantages and therefore comprise attractive systems for convenient and reproducible surface modifications that yield robust, functional biointerfaces [26]. These platforms are, as will be discussed in this paper, compatible with existing and new soft lithographic direct print patterning approaches [27]. More importantly, they form the basis for an extension of soft lithographic approaches to functional biochemical patterns that span the mentioned range of length scales from the 10–100 μm down to the sub-100 nm regime. By exploiting the intrinsic self organization of block copolymers [28], it has become possible to match the corresponding length scales with those attainable by new soft lithographic approaches.

In this paper, three patterning approaches are discussed to direct the deposition of biomolecules on PS_{690}-b-$PtBA_{1210}$ diblock copolymer films, as shown in Scheme 1. The patterned polymer films obtained are robust due to the combination of a glassy block (PS) and a protected reactive block (PtBA) in the films that afford film stability and robust bioconjugation via covalent attachment, respectively. Central to the soft lithography approaches is the transfer of molecular species in the conformal microcontact of an elastomeric stamp and the chemically activated polymer thin film (Scheme 1a).

Depending on the particular approach, the molecular species comprise active biomolecules, a passivating polymer or a deprotection agent that induces a local surface chemical reaction. In all cases, the interactions in the microcontact lead to the formation of robust covalent bonds via a reaction of primary amino groups of the transferred biomolecule or passivating polymer with the NHS active ester moieties of the films. Depending on the details of the multistep modification procedure, we can differentiate three complementary approaches.

In approach (A), two types of molecules, a protein (e.g., bovine serum albumin, BSA) and an organic dye (e.g., fluoresceinamine), are directly transferred by stamping onto the polymer films in two sequential reactive μCP steps. Covalent bond formation ensures the fabrication of robust micrometer scale patterns. This approach is conceptually similar to the μCP methods discussed by various authors [29,30].

The shortcomings of this approach, e.g., the exposure of immobilized biomolecules to ambient air and



Scheme 1. (a) Schematic of localized reaction carried out in reactive μCP in the microcontact between a reactant-covered elastomeric poly(dimethyl siloxane) (PDMS) stamp and a substrate-supported reactive polymer film layer (left) and deprotection and activation surface reactions utilized for the PS_{690}-b-$PtBA_{1210}$ diblock copolymer film system discussed in this paper (right); (b) Schematic of the investigated soft lithography patterning approaches of PS_{690}-b-$PtBA_{1210}$ platforms and the subsequent directed covalent coupling of (bio)molecules from buffered aqueous solution. Approach (A): PS_{690}-b-$PtBA_{1210}$ films are homogeneously hydrolyzed with trifluoroacetic acid (TFA) and activated with *N*-hydroxysuccinimide (NHS)/1-ethyl-3-(dimethylamino)-propylcarbodiimide (EDC). Different (bio)molecules are deposited in a sequential manner by direct molecular transfer using μCP. Approach (B) comprises the local passivation of NHS activated PS_{690}-b-$PtBA_{1210}$ films with $PEG_{500}NH_2$ via reactive μCP. This covalently bound PEG layer prevents the non-specific adsorption of (bio) molecules and allows one to directly couple amino-functionalized (bio)molecules to the remaining NHS-activated areas on the polymer films from buffer solution. Approach (C) includes the local hydrolysis of the PtBA skin layer by reactive μCP, followed by activation with NHS/EDC and finally the directed deposition of amino-functionalized (bio)molecules through covalent binding on the patterned surface. Owing to the versatility of the approaches, contact of biomolecules with air can be avoided and positive, as well as negative, images of the stamp can be realized.

the limited choice of stamp geometries [31] is circumvented in the two alternative methods. As shown in (B), a passivation layer of $PEG_{500}NH_2$ is locally transferred onto NHS activated PS_{690}-b-$PtBA_{1210}$ films. The covalent coupling of functional (bio)molecules, confined to the remaining activated NHS functionalized areas, is then carried out from solution.

In approach (C), reactive μCP is used to locally hydrolyze PS_{690}-b-$PtBA_{1210}$ films followed by activation and bioconjugation from buffer [25]. As discussed in this paper, these three approaches comprise a versatile toolbox to produce (bio)reactive patterns based on polymeric film platforms down to sub-micrometer length scales, and thus lay the foundations to fabricate ultrahigh density biomolecular arrays.

2. Experimental

2.1. Materials

PS_{690}-b-$PtBA_{1210}$ diblock copolymers were purchased from Polymer Source Company (Dorval,

Canada) and used as received. The number average molar mass and polydispersity were 202.4 kg/mol and 1.03, respectively. Amino end-labeled PEG (denoted as $PEG_{500}NH_2$), purchased from Nektar UK Company ($M_n = 500$ g/mol, PDI = 1.1), fluoresceinamine (Molecular Probes, Inc. The Netherlands) and the DNA samples (probe DNA: P: 25 mer 5′-GGA ATG TGC CAT ACC GAA TCC GTG T-3′; Cy5-labeled target DNA: 5′-CAC GGA TTC GGC ATG-3′, Cy5-labeled mismatch DNA: 5′-TGT GCC TAA GCC ATA-3′, MWG BIOTEC AG, Ebersberg, Germany) were used as received. DNA samples were stored at −4 °C until use. Fifth-generation (G5) amino-terminated PAMAM dendrimers were obtained from Aldrich as a 5 wt% methanolic solution [32]. Bovine serum albumin (BSA) with Alexa Fluor®594 conjugate was bought from Molecular Probes Inc. and was used as received. Fibronectin, purchased from Roche Diagnostics GmbH (Penzberg, Germany), was stored at 4 °C until use. Lissamine rhodamine B sulfonyl chloride was bought from Molecular Probes Inc.

2.2. Preparation of thin films

Silicon (111) wafers (Okmetric N/P (100) wafers, thickness 381 ± 15 μm) and glass cover slides (Menzel–Glaser, Braunschweig, Germany) were used as substrates. These substrates were cleaned prior to use by an oxygen plasma treatment (pressure of O_2: 0.5 bar; current: 30 mA) using an Elektrotech twin system PF 340 apparatus or a SPI Plasma Prep™ Plasma Cleaner (Structure Probe Inc, West Chester, USA). Alternatively, the substrates were cleaned in piranha solution (solution of 1:3 (v/v) 30% H_2O_2 and concentrated H_2SO_4) for 15 min and then rinsed with copious amounts of high-purity water (Millipore Milli-Q water). *Caution*: Piranha solution should be handled with extreme caution; it has been reported to detonate unexpectedly.

Thin films were prepared by spin-coating polymer solutions in toluene (conc. 10 mg/mL) onto oxygen plasma cleaned silicon wafers or glass substrates. The samples were spun at 3000 rpm for 30 s using a P6700 spin coater (Specialty Coating Systems Inc). All spin-coated samples were annealed at 135 °C for 24 h in vacuum before analysis. A film thickness of 90 ± 5 nm was determined using a custom-built spectroscopic ellipsometer using a He–Ne ($\lambda = 632.8$ nm) laser. The refractive index of the block copolymer film was approximated as 1.50. The refractive index of the PEG films was approximated as 1.4638 [33].

2.3. Approach A

2.3.1. Homogeneous Hydrolysis

The polymer films were hydrolyzed in neat trifluoroacetic acid for 20 min, rinsed three times using Milli-Q water, and activated by immersion into an aqueous solution of 1-ethyl-3-(dimethylamino)-propylcarbodiimide (EDC, 1 M) and *N*-hydroxysuccinimide (NHS, 0.2 M) for 30 min. The samples were then rinsed with Milli-Q water and dried in a stream of nitrogen.

2.3.2. Microcontact printing

The PDMS stamps (prepared according to the literature [14]) were mildly oxidized in an UV ozone plasma reactor for 50 min [34]. Firstly, oxidized PDMS stamps were inked by soaking them in a 100 μM solution of fluoresceinamine in PB (pH = 7.4) for 60 min. Before reactive microcontact printing, the stamps were blown dry in a stream of nitrogen. Then the stamps were applied for 30 min on the films. After careful removal of the stamp, the film surface was rinsed with Milli-Q water. Secondly, oxidized PDMS stamps were inked by soaking them in a 200 μM solution of BSA in PB solution (pH = 7.4) for 60 min. After drying in a stream of nitrogen, the stamps were applied for 30 min on the films in perpendicular direction. After removal of the stamp, the film surface was rinsed with Milli-Q water again.

2.4. Approach B

Oxidized PDMS stamps (see above) were inked by soaking them in a 200 μM solution of $PEG_{500}NH_2$ in PB (pH = 7.4) for 60 min. Before reactive microcontact printing, the stamps were blown dry in a stream of nitrogen. Then the PEG–NH_2 loaded stamps were applied for 180 min on the films (the stamp, surface area 1 cm^2, was loaded with a mass of 30 g). After careful removal of the stamp, the film surface was rinsed with Milli-Q water. The PEG patterned films were then immersed in fluoresceinamine solution (100 μM, PB: pH = 7.4), BSA solution (200 μM, PB: pH = 7.4), fibronectin solution (100 μM in PB, pH = 7.4), or G5 PAMAM solution (5.0×10^{-4} M solution in methanol), respectively. After the specified reaction time (30 min, 30 min, 180 min, and 180 min, for

fluoresceinamine, BSA, fibronectin, and G5 PAMAM, respectively) the samples were taken out, rinsed with PB buffer and Milli-Q water, and dried in a stream of nitrogen. Finally, the samples were dried inside a vacuum oven for one day prior to the fluorescence microscopy experiments. DNA immobilization (100 nM) in phosphate buffer on $PEG_{500}NH_2$ patterned film followed the same procedure as coupling fluoresceinamine or BSA. Afterwards, the hybridization of target DNA with dye (100 nM) was carried out in phosphate buffer. Then the films were rinsed with PB buffer (pH = 7.4) and Milli-Q water, and dried in a stream of nitrogen. All experiments were carried out at $T = 25 \pm 2°$.

2.5. Approach C

2.5.1. Local hydrolysis and derivatization

A drop (50 μL) of trifluoroacetic acid was applied to the stamp surface (1×1 cm^2) and the acid was left to dry in air for 4 min. Then the stamp was brought to contact with the surface of the polymer films and was kept in contact for 10 min. After peeling off the stamp, the films were rinsed three times using Milli-Q water, subsequently activated with NHS/EDC and reacted with fluoresceinamine (details see above).

2.6. Labeling of adsorbates

Labeling of surface-immobilized fibronectin and PAMAM dendrimers was carried out by reaction with lissamine rhodamine B sulfonyl chloride (100 μM in PB, pH = 7.4) for 30 min. Afterwards the samples were rinsed thoroughly with PB, followed by Milli-Q water and finally dried in a stream of nitrogen.

2.7. 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® $63\times/1.4$ NA oil-immersion objective. Fluoresceinamine was excited with the 488 nm line of an Ar^+ laser, and Cy5-labeled DNA and BSA conjugated with Alexa Fluor® 594 with a 633 nm HeNe laser. The fluorescence emission of these dyes was recorded with photomultiplier tubes (Hamamatsu R6357) after spectral filtering with a 500–550 nm bandpass filter and a 650 nm longpass filter for fluoresceinamine and Cy5-labeled DNA and BSA-Alexa Fluor® 594, respectively. Images were acquired with maximum pinhole diameters. Fluorescence micrographs were also taken using a Olympus IX 70 fluorescence microscope equipped with a U-MWG-2 fluorescent filter and a BA590 filterblock.

2.8. Atomic force microscopy (AFM)

The contact mode AFM measurements were carried out with a NanoScope O multimode AFM (Digital Instruments/Veeco, Santa Barbara, CA) using a 100 μm scanner and microfabricated silicon nitride tips/cantilevers (Nanoprobes, Veeco) in ambient atmosphere (ca. 30% relative humidity, 24 °C temperature).

3. Results and discussion

The direct transfer of biomolecules using μCP is an established micropatterning strategy [29,35] and has been recently expanded to include reactive *polymer* thin film-based platforms [36,37]. We demonstrate here that the recently introduced PS-b-PtBA block copolymer thin film system is fully compatible with this approach, as well as with the two new approaches described here, and yields robust assemblies owing to the covalent attachment of the immobilized species.

3.1. Fabrication of multimolecular arrays by direct molecular transfer (Method A)

To directly fabricate multimolecular arrays by μCP, two different kinds of molecules were sequentially printed and coupled to a chemically activated PS_{690}-b-$PtBA_{1210}$ film. The PtBA skin layer in the films was initially homogeneously hydrolyzed with TFA and activated with NHS/EDC. Subsequently, a fluoresceinamine-covered oxidized PDMS stamp (lines: 10×5 μm^2) was brought to contact with the film. After a contact time of 30 min, the stamp was peeled off and dye-labeled BSA was deposited by a second printing step on the pre-patterned film (the stamping direction was perpendicular to the original fluoresceinamine pattern). Fig. 1a shows the resulting fluorescence microscopic image of the fluoresceinamine emission, while Fig. 1b displays the corresponding image for the emission of the dye-label on the transferred BSA.

The patterns were very homogeneous, thus indicating good conformal stamp–polymer film contact

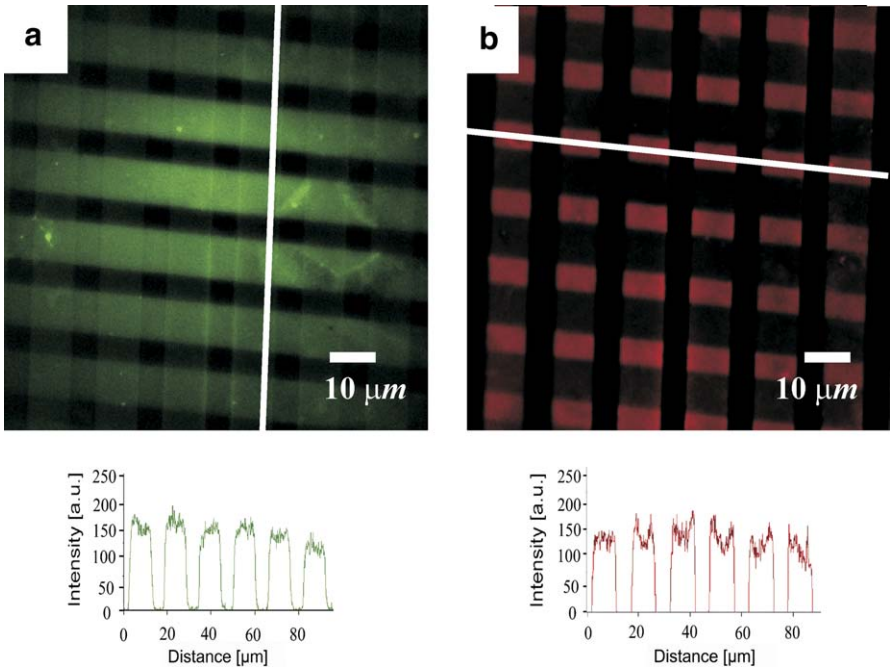


Fig. 1. Fluorescence microscopic images (top) and cross-sectional intensity profiles (bottom) of a hydrolyzed and NHS-activated PS_{690}-b-$PtBA_{1210}$ film on glass that was area-selectively functionalized in two sequential reactive μCP steps: (a) Fluorescence emission of fluoresceinamine; (b) fluorescence emission of Alexa Fluor®594 label on BSA.

and good molecular transfer. The cross-sectional plots of the images demonstrate that the corresponding molecular ink has been faithfully transferred in the stamp–film contact region (width 10 μm). As concluded from a detailed cross-sectional analysis (see Supporting Information), cross-contamination is not pronounced.

This and similar patterns with two different types of (bio)molecules could be conveniently fabricated over large areas up to the maximum stamp size utilized in this study (1 cm^2). Using this simple approach, it is hence also possible to prepare multimolecular biomolecular patterns on the PS-b-PtBA block copolymer platforms.

3.2. Local passivation and functionalization of PS_{690}-b-$PtBA_{1210}$ films (Method B)

One possible disadvantage of the direct molecular transfer (strategy A) is that biomolecules are brought into contact with air, which may lead to their denaturation. To overcome this limitation, the area-selective deposition of (bio)molecules *from solution* has been studied, as described in approach (B), via the local passivation of NHS activated PS_{690}-b-$PtBA_{1210}$ films with $PEG_{500}NH_2$ [26]. The surface of PS_{690}-b-$PtBA_{1210}$ films was firstly homogeneously hydrolyzed using TFA, followed by activation with NHS/EDC. By reactive μCP using a $PEG_{500}NH_2$ soaked PDMS stamp (circular depressions with 15 μm diameter and 30 μm spacing in a square geometry), a $PEG_{500}NH_2$ layer was successfully transferred on the activated films. This antifouling PEG layer prevents locally the immobilization of solution-borne amino-functionalized (bio)molecules, such as fluoresceinamine, dye-labeled BSA, and probe DNA, while the coupling proceeds efficiently in the remaining NHS activated areas. The thus obtained patterned films were analyzed by fluorescence microscopy (Fig. 2).

Fig. 2 shows the results of fluorescence microscopy experiments after various (bio)molecules were covalently coupled from PB solution onto $PEG_{500}NH_2$ pre-patterned films. For comparison, the data of the $PEG_{500}NH_2$ pre-patterned film are also shown. For the PEG patterns, negligible background fluorescence emission was observed (Fig. 2a). After coupling fluoresceinamine and G5 PAMAM dendrimers to the patterned films from solution, highly fluorescent features were observed (Fig. 2b and d). The covalent coupling of proteins to the NHS activated areas proceeded also

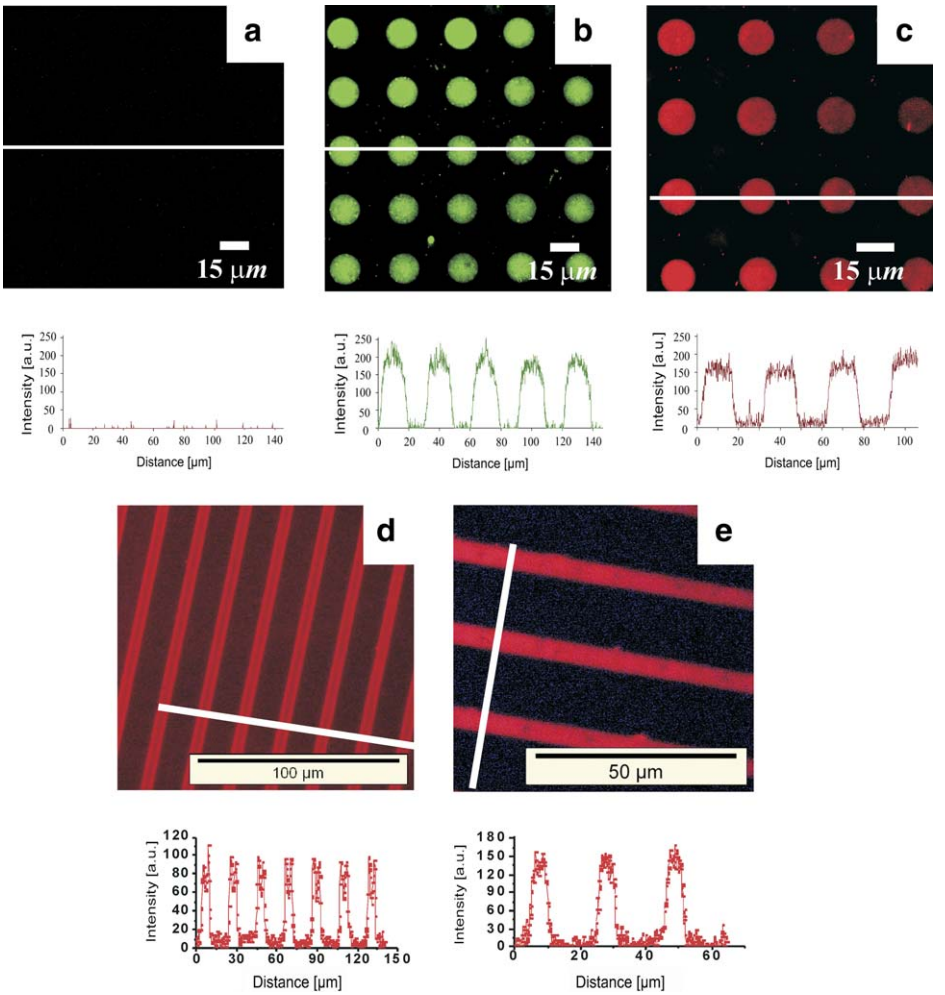


Fig. 2. Fluorescence microscopy images (top) and cross-sectional intensity profiles (bottom) of PS_{690}-b-$PtBA_{1210}$ (a) after grafting of $PEG_{500}NH_2$ (stamp–sample contact time: 180 min); (b) following reaction with fluoresceinamine; (c) following immobilization of BSA; (d) following reaction with G5 PAMAM (labeled afterwards with lissamine rhodamine B); (e) following reaction with fibronectin from solution (labeled afterwards with lissamine rhodamine B).

area-selectively as shown in for BSA and fibronectin (Fig. 2c and e). The profiles show well-defined spots (lines) that do not suffer from pronounced inhomogeneous biomolecule distributions [38] that have been reported for direct spotting techniques, e.g., in commercially available DNA microarrays [39].

These data show that organic dyes, hyperbranched macromolecules (as highly reactive, quasi-3D matrix) [40], as well as relevant proteins, including fibronectin, which is of importance for cell–surface interaction studies [41], can be conveniently coupled to PEG pre-passivated patterns with features on the micrometer scale [42]. The patterned polymer films obtained are robust due to the combination of the glassy PS block and the protected reactive PtBA block in the films that afford film stability and robust bioconjugation via covalent attachment, respectively. In addition, the bioconjugation reactions are carried out in PB solution, thus possible denaturation of proteins is minimized compared to the conventional direct transfer in ambient air.

Subsequently, the hybridization of target DNA on probe DNA pre-patterned PS_{690}-b-$PtBA_{1210}$ films was investigated to demonstrate the functionality of the patterned surfaces fabricated. Amino end-functionalized 25-mer probe DNA was firstly covalently grafted onto $PEG_{500}NH_2$ pre-patterned films from PB solution. The films were then immersed in a solution containing the fully complementary target DNA.

Films functionalized with probe DNA showed negligible fluorescence emission (Fig. 3a). After

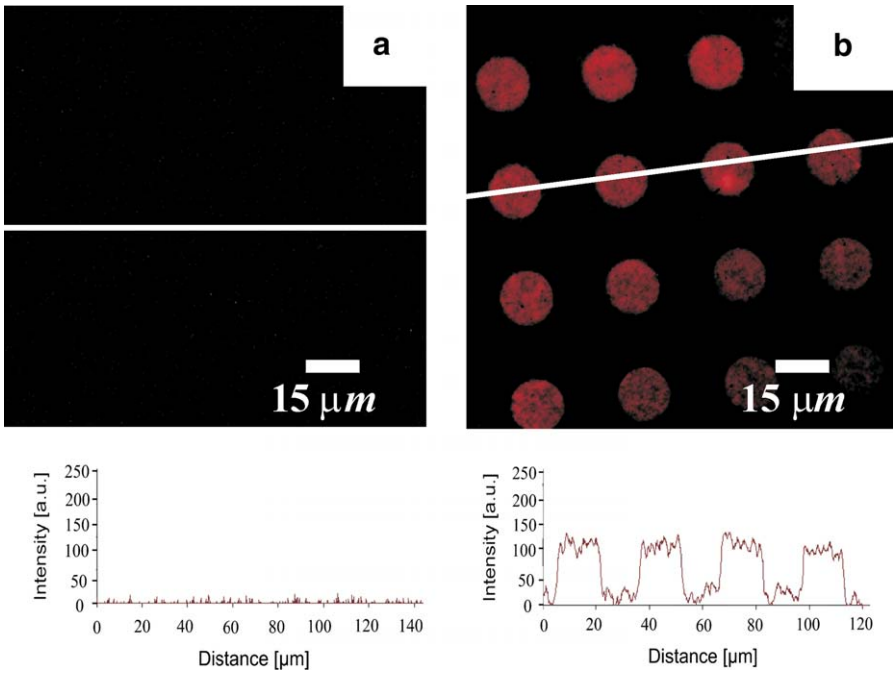


Fig. 3. Fluorescence microscopy images (top) and cross-sectional intensity profiles (bottom) of (a) a hydrolyzed and NHS-activated PS_{690}-b-$PtBA_{1210}$ surface that was patterned with $PEG_{500}NH_2$ and functionalized with probe DNA from solution; (b) a PEG-probe DNA patterned PS_{690}-b-$PtBA_{1210}$ surface following hybridization with complementary target DNA.

hybridization with dye-labeled fully complementary target DNA, a regular fluorescent pattern (diameter of circular dots: 15 μm) was detected (Fig. 3b). By contrast, when the probe DNA pattern was treated with mismatch target DNA using identical conditions, only negligible background fluorescence emission was observed (see Supporting Information).

These experiments show that the directed deposition of probe DNA was achieved similar to the directed deposition of fluoresceinamine, BSA etc., and that such polymer films may function as affinity biosensors to detect, among others, DNA hybridization or pathogenic bacteria [43] also in patterned, array-based formats.

The quality of the patterns and their performance is comparable to the NHS-homopolymer systems reported previously [23] however, owing to the presence of the glassy block *inside* the films, the PS_{690}-b-$PtBA_{1210}$ platform is more robust during, e.g., multistep processing.

3.3. Local deprotection and functionalization of PS_{690}-b-$PtBA_{1210}$ films (Method C)

The patterning of reactive surfaces by means of molecular transfer of either active species (method A) or passivating species followed by wet chemical coupling (method B) has been very recently complemented by a new reactive microcontact printing approach that exploits the deprotection of the *tert*-butyl ester groups in PtBA by TFA in micrometer and sub-micrometer stamp–film contacts [25b]. As shown in a mechanistic study [25c], faithful pattern transfer can be realized using this approach, if the presence of liquid acid on the elastomeric stamp is avoided. The partitioning of TFA into the skin layer of the polymer film enables one to achieve a level of minimum feature size and resolution unattainable with molecular inks of similar molar mass and volatility [29,30].

As shown below, it is possible to replicate the patterns present on the stamps faithfully by reactive μCP on the micrometer scale in the deprotection step (*tert*-butyl ester hydrolysis), when the loading of TFA on the oxidized PDMS stamps is well controlled. To validate the analysis for sub-wavelength scale patterns using an alternative viable method, micrometer scale patterned films were first investigated using contact mode AFM. Fig. 4 shows height and friction images obtained simultaneously on such patterned films. No pronounced morphology is observed here. However, a clear friction force

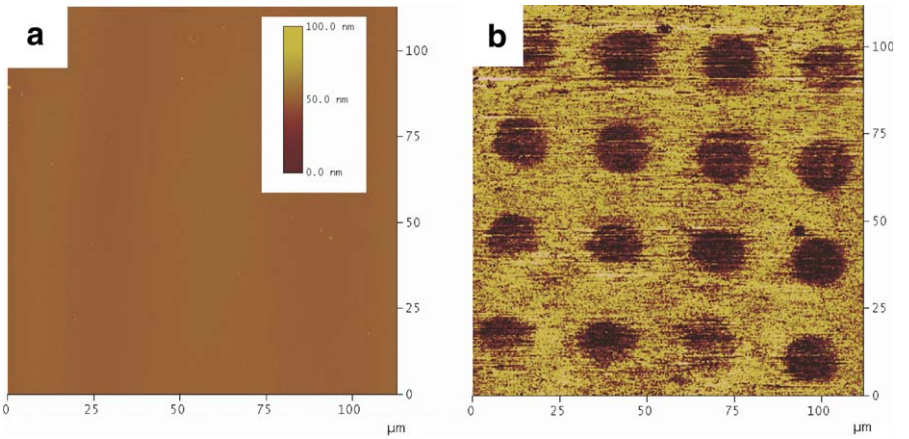


Fig. 4. AFM height (a) and friction (b) images acquired after local hydrolysis by reactive μCP. The areas with high friction force contrast (matrix) is ascribed to the hydrophilic part (PAA); the area (circles) with low friction force contrast is ascribed to the hydrophobic part (PtBA).

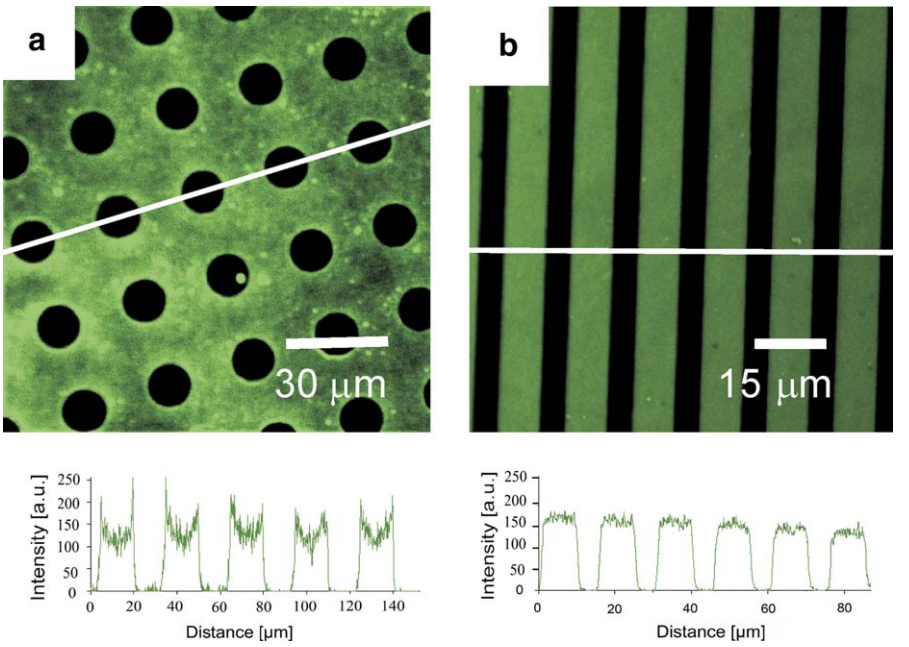


Fig. 5. Fluorescence microscopy images (top) and corresponding cross-sectional intensity plots (bottom) for PS-b-PtBA films patterned with stamps that possessed circular (15 μm) and line (10×5 μm^2) features.

contrast is observed in Fig. 4b, i.e., low friction force circles in a high friction force matrix. Here, the low friction force areas are ascribed to the more hydrophobic surface functionality (PtBA) and the high friction force areas to the more hydrophilic component (PAA) [44]. The data suggest that the reaction occurred selectively in the area where the acid-soaked stamp contacted the polymer surface.

Films similar to the one shown in Fig. 4 were subsequently activated with NHS/EDC, followed by reaction with fluoresceinamine in PB. Optimized regular patterns with homogeneous fluorescence emission were observed with feature sizes that correspond to the corresponding sizes on the PDMS stamps, as shown in Fig. 5a and b, respectively.

The results shown in Fig. 5 demonstrate that micrometer-sized features can be replicated faithfully. In addition, a comparison of the fluorescence microscopy data shown in Figs. 2b and 5a shows that patterns that correspond to the negative and

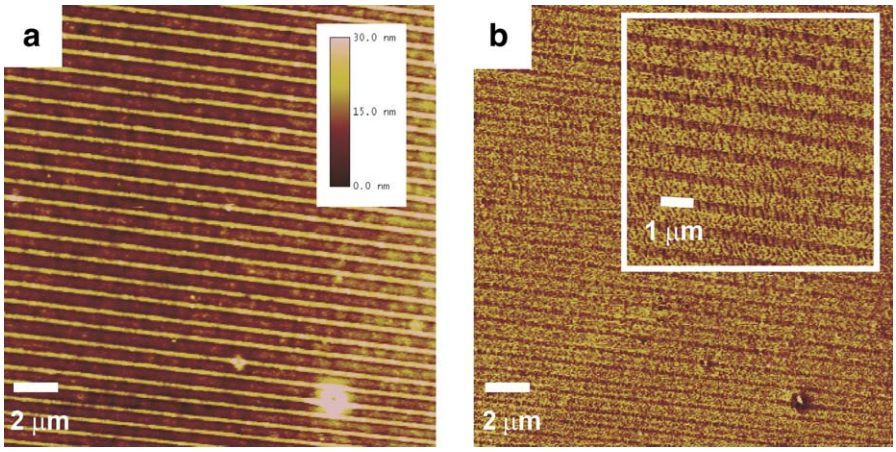


Fig. 6. AFM height (a) and friction (b) images of PS-b-PtBA film after local hydrolysis by reactive μCP using TFA (inset in (a) height scale; inset in (b) high resolution friction image). The stripes with high friction force contrast (width ~500 nm) are ascribed to the hydrophilic part (PAA); the low friction force stripes (width ~300 nm) are ascribed to the hydrophobic part (PtBA).

the positive images of the features on one particular type of stamp, respectively, can be replicated using the procedures described in this paper. Hence, problems of mechanical instabilities of thin posts in PDMS stamps etc. [31] can be circumvented by choosing the complementary stamp geometry and the appropriate printing approach. In particular, using the versatile reactive μCP approach (C) described here one can fabricate patters with small biomolecule features that possess large lateral spacing. These features make the methodology attractive for the fabrication of tailored structured surfaces for investigations of cell–surface interactions and the corresponding cell biology.

For reduced pattern dimensions (sub-micrometer length scales), however, liquid spreading, surface diffusion and diffusion via the gas phase may become a limiting factor. Using appropriate TFA loading on the stamps, these pathways of pattern deterioration can be avoided [25c]. Using AFM, the patterning was tested down to the 300 nm level. Fig. 6 shows AFM images of PS_{690}-b-$PtBA_{1210}$ films that were chemically patterned by local hydrolysis using a stamp with 500 nm × 300 nm line features. The AFM height and friction images were captured simultaneously.

The height difference due to capillary force effects [45] was very small (<7 nm), while the friction image in Fig. 6b shows a regular pattern of alternating lines with high and low friction forces. The width of the single stripe of the bright area is 500 ± 20 nm and the width of the single stripe of the dark area is 300 ± 20 nm. The bright area corresponds to high friction forces (attributed to the hydrophilic reaction product PAA) and the dark area corresponds to low friction forces (attributed to the more hydrophobic PtBA).

Such patterned surfaces were successfully activated with NHS and derivatized by grafting reactions with primary amines, such as *n*-butyl amine (see Supporting Information) and various biomolecules, as judged from various spectroscopic and force microscopic analysis methods (no data shown) [28].

Smaller features are very likely accessible via this reactive μCP route. The smallest features produced to date were limited by the dimensions of the elastomeric stamps available. Due to the unavailability of suitable stamps with smaller feature sizes, we have not been able to verify the limits of this methodology yet. Furthermore, the behavior of different organic acids, with different spreading and diffusion properties, has not been investigated in detail. Thus, it can be expected that the ultimately attainable length scales match the natural length scale of the self organization of the utilized block copolymers (periodicity ~90 nm) [28]. Thereby these combined approaches may open unique possibilities for controlled biomolecular patterning on multiple length scales and with multiple types of proteins at predefined separation distances and symmetries, e.g., for the investigation of cell–surface interactions.

4. Conclusions

Thin films of PS_{690}-b-$PtBA_{1210}$ on solid supports, which are stable in a wide range of different environments and processing conditions, were successfully

deprotected and subsequently derivatizated *locally* in aqueous media *without* dissolution or removal of the film. The PtBA skin layer present can be easily locally hydrolyzed by reactive microcontact printing, followed by activation with NHS ester groups. Using three different complementary approaches, the area-selective covalent coupling of fluoresceinamine, PAMAM dendrimers, BSA, fibronectin, and probe DNA, as well as successful hybridization on the polymer films, showed that PS_{690}-b-$PtBA_{1210}$ films can be used as an alternative substrate for developing robust patterned biochips. In particular, the covalent immobilization of (bio)molecules *via* amide linkage formation in *aqueous media, i.e., without the need to expose biomolecules to air,* provides an efficient and secure attachment of molecules on substrate compared to mere physical interactions [30]. Finally, reactive μCP on PS-b-PtBA film-based platforms comprises a simple way to produce reactive patterns down to sub-micrometer length scales, as shown by using stamps with sub-micrometer features. Thus, based on these approaches and platforms ultrahigh density patterns can be conveniently fabricated.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.eurpolymj.2006.06.025.

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