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Covalent Microcontact Printing of Proteins for Cell Patterning

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Abstract: We describe a straightforward approach to the covalent immobilization of cytophilic proteins by microcontact printing, which can be used to pattern cells on substrates. Cytophilic proteins are printed in micropatterns on reactive self-assembled monolayers by using imine chemistry. An aldehyde-terminated monolayer on glass or on gold was obtained by the reaction between an amino-terminated monolayer and terephthaldialdehyde. The aldehyde monolayer was employed as a substrate for the direct microcontact printing of bioengineered, collagen-like proteins by using an oxidized poly(dimethylsiloxane) (PDMS) stamp. After immobilization of the proteins into adhesive "islands", the remaining areas were blocked with amino-poly(ethylene glycol), which forms a layer that is resistant to cell adhesion. Human malignant carcinoma (HeLa) cells were seeded and incubated onto the patterned substrate. It was found that these cells adhere to and spread selectively on the protein islands, and avoid the poly(ethylene glycol) (PEG) zones. These findings illustrate the importance of microcontact printing as a method for positioning proteins at surfaces and demonstrate the scope of controlled surface chemistry to direct cell adhesion.

Keywords: cell adhesion · immobilization · microcontact printing · proteins · surface chemistry

Introduction

Controlling cell positioning and adhesion on surfaces is of interest in fundamental cell biology,[1] tissue engineering,[2] cell-based biosensor development,[3] and bioelectronics.[4] Various methods have been used to direct the adhesion of cells to selected areas of a substrate, including micropatterning on polymers,[5] soft lithography,[6] patterning through pores in elastomeric membranes,[7] patterning by using three-dimensional microfluidic systems,[8] laminar-flow patterning,[9] and local oxidation by using microelectrodes.[10] One particularly versatile approach to control cell attachment and patterning is the physical or chemical adsorption of extracellular matrix (ECM) proteins to selected areas of a substrate. ECM proteins are cytophilic in the sense that cells adhere preferentially to any surface coated with these proteins. ECM proteins have been delivered locally to a substrate by, for example, ink-jet printing[11] and electrospray deposition.[12] ECM proteins may also be physisorbed to a substrate patterned with self-assembled monolayers (SAMs) by microcontact printing (μCP) or other forms of lithography.[13] Alternatively, proteins may be microcontact printed on a suitable substrate.[14]

Recently, Huck and co-workers[15] proposed the application of μCP for the in situ synthesis of oligopeptides exclusively in the contact areas between substrate and the poly(dimethylsiloxane) (PDMS) stamp. The advantage of covalent μCP of peptides is that a chemical bond is formed between the protein and the substrate SAM. Hence, there is no diffusion of the printed pattern on the surface. On the other hand, it is unlikely that elaborate protein patterns can be prepared efficiently by multistep peptide synthesis in the confinement between substrate and stamp.

In this work, we use aldehyde-terminated SAMs on gold and silicon oxide substrates as a reactive layer for covalent

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μCP of cytophilic, collagen-like proteins. Specific regions of the SAM were patterned with a cytophilic protein and other areas were made nonadhesive by immobilization of poly(ethylene glycol) (PEG) molecules. Various ECM proteins have been immobilized in patterns on substrates with the aim of directing cell adhesion. Among these proteins are fibronectin, laminin, and collagen.[11–14] Here, we selected collagen-like proteins produced in microbial cells,[16,17] which eliminates the risk of contamination by infectious agents associated with conventional animal products.[18] Moreover, these bioengineered collagens are nonfolding proteins because they lack hydroxyproline. Because these collagens do not fold into helices, they are highly soluble in water, do not form gels, and cannot denature through shear heat. As a result they can be processed easily, also by μCP. Another advantage of the unfolded collagens is that their cell-binding epitopes are very accessible to the medium and are exposed to the cells therein.[18] Furthermore, these molecules are highly polar, which enhances their water solubility and exposure on the surface. A polar surface also reduces unwanted plaque formation, surface fouling, and nonspecific cell attachment.

In particular, we used the gelatin-like, hydrophilic proteins col3a1 and col1a1–1*, which are nonhydroxylated gelatins based on part of the rat type III (col3a1) and mouse type I (col1a1–1*) collagen sequences and produced as heterologous gene products in methylotrophic yeast.[16] The fraction of polar residues in the selected part of col3a1 is some 20% higher than in the selected part of col1a1–1*. In addition to these gelatins, a designer gelatin P4 with a fraction of polar residues 60% higher than that of col1a1–1* was used.[17] The remaining nonpolar residues in this extremely polar construct consisted merely of glycine and proline as the defining residues of collagen-like proteins and gelatins. In contrast to the other two gelatins, the col3a1 construct contains an Arg-Gly-Asp (RGD) cell binding sequence. The RGD sequence is present in many ECM proteins, such as fibronectin, laminin, and collagen. This tripeptide is a minimal sequence required for recognition by cell-surface receptors of the integrin family.[19] Because col3a1 is a random coil protein without any secondary structure,[16] it is likely that the exposure of the RGD sequence on the surface is enhanced and RGD is more readily available for interactions with cell-membrane receptors than in native ECM proteins.

Our methodology is outlined in Figure 1. In brief, we modified gold and silicon oxide substrates with amino-terminated SAMs and then converted the amino groups into aldehyde groups by reaction with terephthaldialdehyde.[20] Substrates modified in this manner can be patterned directly with cytophilic proteins by means of μCP using an oxidized PDMS stamp. Amino residues, such as lysine, in the protein form imine bonds with the aldehyde SAM. The remaining areas of the aldehyde SAM can be blocked subsequently with PEG to form areas resistant to cell adhesion. Human malignant carcinoma (HeLa) cells were seeded and incubated on the patterned substrates.

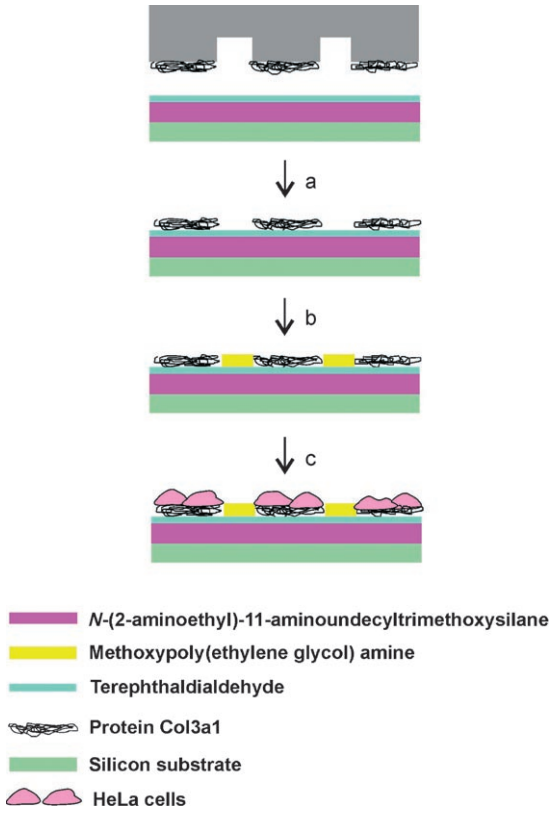

Figure 1. Schematic representation of cell patterning through direct μCP of collagen-like protein col3a1. a) μCP of col3a1 onto aldehyde-terminated SAM. b) Reaction between remaining aldehyde groups and amino-PEG. c) Incubation of HeLa cells.

Results and Discussion

Surface modification for protein and cell attachment: The proteins were immobilized on gold and silicon oxide (glass) surfaces. An outline of the successive steps of the procedure is shown in Figure 2. Firstly, the aldehyde-terminated substrates were prepared according to a procedure published previously.[20] Subsequently, the proteins were attached covalently to aldehyde-terminated substrates **2** and **5** by reaction from a 1 mM solution in phosphate buffered saline (PBS) at room temperature to give protein-covered substrates **3** and **6**. Alternatively, the proteins were immobilized on substrate **5** by direct μCP to give protein-coated substrate **6***. For initial μCP experiments, the ink consisted of a 1 mM solution of col3a1 in PBS buffer and the stamp was a flat, featureless PDMS stamp that was made hydrophilic by treatment with UV/ozone plasma for 30 min. Unmodified PDMS provides a hydrophobic surface that is not suitable for aqueous inks.[14] All monolayers were rinsed extensively with ethanol and/or buffer solution after each reaction step to remove all physisorbed material. The monolayers were characterized by water-contact-angle goniometry, X-ray photoelectron spectroscopy (XPS), Fourier transform infrared reflection-adsorption spectroscopy (FT-IRRAS), and ellipsometry.

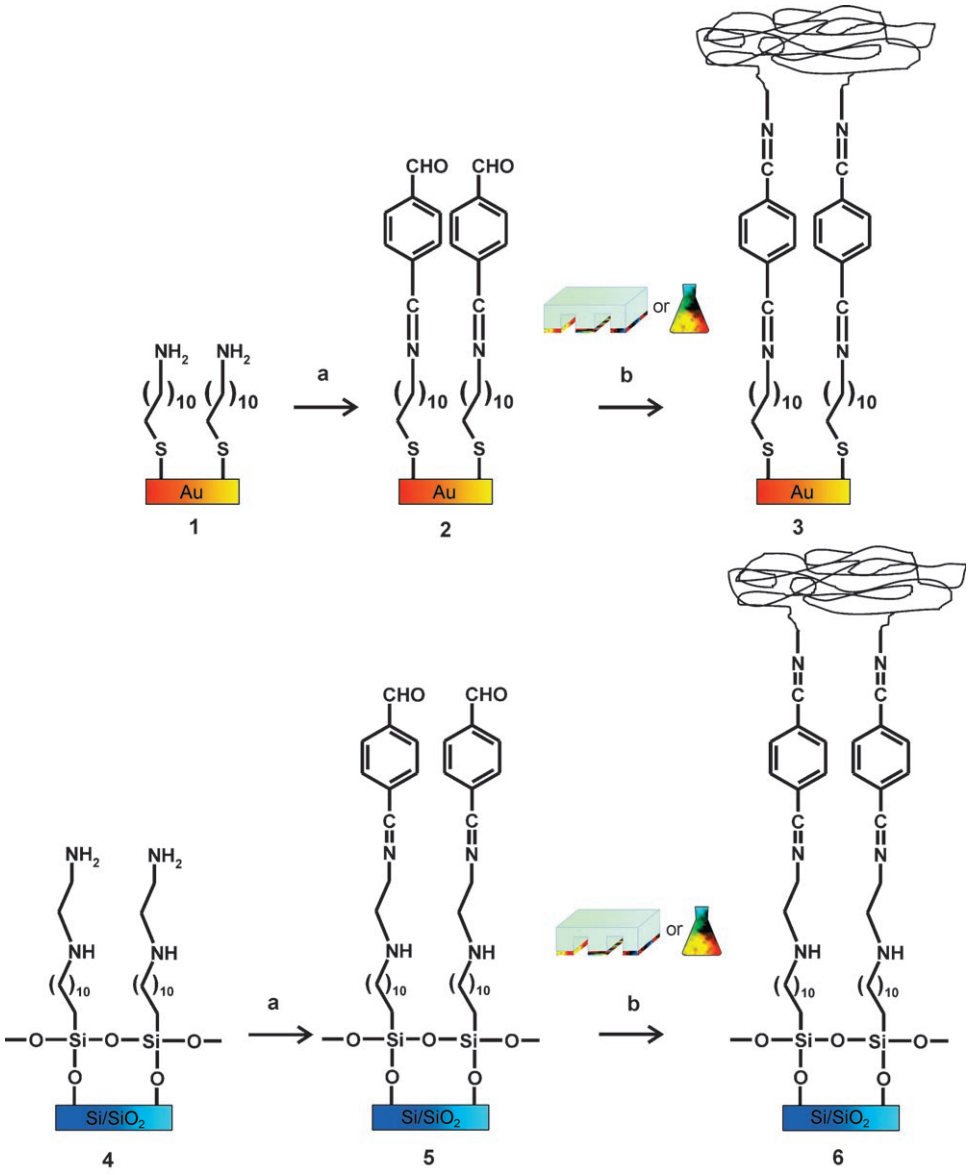

Figure 2. Immobilization of protein col3a1 on gold (**1**–**3**) and on silicon oxide (**4**–**6**) substrates. **1**, **4**: amino-terminated SAMs; **2**, **5**: aldehyde-terminated imine SAMs; **3**, **6**: substrates with immobilized col3a1 protein; a) terephthaldialdehyde; b) col3a1.

Values for the water-contact angle of monolayers **1**–**6** are shown in Table 1. After attachment of the hydrophilic protein col3a1, the water-contact angle decreases dramatically (SAMs **3**, **6**, **6***) and ranges beyond the limit of accurate measurement ($<10°$). The thickness of the monolayers on the silicon substrates **4**–**6*** was investigated by ellipsometry. (Table 1) The thickness of the monolayers increases after each immobilization step and is consistent with the anticipated values. Furthermore, the C:N ratios from XPS measurements for amine and aldehyde SAMs **1**, **2**, **4**, and **5** are in agreement with the molecular composition. The C:N ratio of the protein monolayers **3**, **6**, and **6*** is in accordance with the molecular composition of col3a1.[16] The C:N ratios reported in Table 1 were not corrected for attenuation, which may explain why the observed ratio is usually significantly lower that the calculated ratio. The nitrogen-rich protein is exposed on the surface and shields the underlying carbon-rich monolayer.

After each immobilization step, the surface was rinsed thoroughly with the appropriate solvent (see Experimental Section) and dried under a nitrogen stream before FT-IRRAS spectra were collected (Figure 3). After exposure of the amino-terminated SAM **1** (with its characteristic bands for amino and CH_2 vibrations) to terephthaldialdehyde to give SAM **2**, several changes were observed in the spectra. The broad peak of the amino group disappeared, indicating complete reaction on the surface. Furthermore, several new bands appeared: an imine C=N stretching vibration at 1645 cm^{-1} and a band at 1705 cm^{-1}assigned to the C=O stretching vibration of the second aromatic aldehyde group. The presence of these characteristic imine and aldehyde bands and the absence of the bands assigned to the amine group provide strong evidence that terephthaldialdehyde was attached covalently through an imine bond, leaving reactive aldehyde groups on the surface, consistent with our earlier reports.[20]

After protein immobilization to monolayer **2** several new peaks appeared in the spectra.

Table 1. Advancing and receding water-contact angle, C:N atomic ratio, and thickness of monolayers **1**–**6***.

SAM	θ_{adv} [°]	θ_{rec} [°]	C:N (XPS)	C:N (calcd)	Thickness (ellipsometry) [nm]
1	62±2	50±2	10.6±0.7	11	n.a.[a]
2	77±2	62±2	13.9±0.4	14	n.a.[a]
3	<10	<10	9±1.8	10	n.a.[a]
4	57±2	45±2	8±0.6	6.5	2.4±0.2
5	65±2	54±2	12±0.4	10.5	3.14±0.2
6	<10	<10	7.2±1.5	10	4.62±0.3
6*[b]	<10	<10	8.9±1.2	10	5.3±0.3

[a] Data not available. [b] **6*** proteins immobilized on substrate **6** by μCP with a flat PDMS stamp.

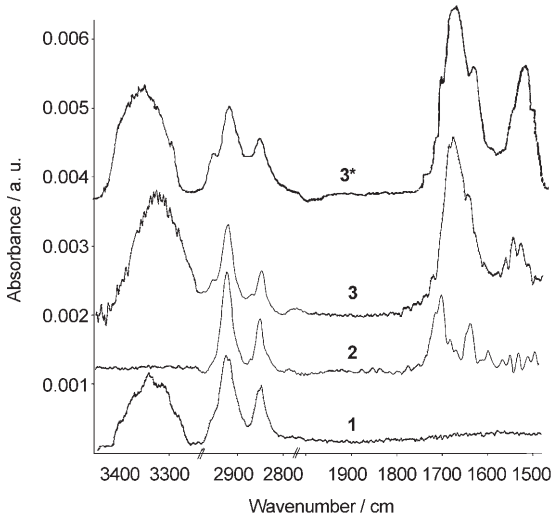

Figure 3. FT-IRRAS spectra of functionalized monolayers obtained by sequential exposure of Au surface to 11-aminoundecylthiol (**1**), terephthaldialdehyde (**2**), and protein col3a1 immobilized from solution (**3**) and by μCP with a featureless stamp (**3***).

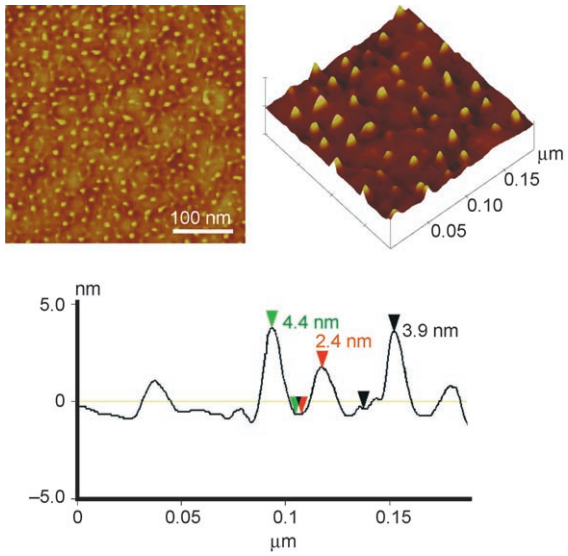

Figure 4. Tapping-mode AFM height images of immobilized protein col3a1 on aldehyde-terminated gold substrate (SAM **3**).

The broad band at approximately 3350 cm^{-1} can be assigned to the N–H stretching vibration of the amino groups and amides in the protein (Figure 3, spectrum **3**). Bands at 2926 and 2854 cm^{-1} indicate CH_2 asymmetrical and symmetrical stretching vibrations, respectively, of the substrate SAM. Furthermore, a shoulder at 1645 cm^{-1} overlapping with the 1678 cm^{-1} amide I band can be assigned to the C=N stretching vibration of the imine group, resulting from the reaction between the protein amine groups and the aldehyde groups from the SAM. Finally, the band at 1546 cm^{-1} was assigned to the amide II stretching vibration. Spectra of microcontact-printed protein monolayers were also obtained (Figure 3, spectrum **3***). Col3a1 was printed onto aldehyde-terminated SAM **2** with a flat, oxidized PDMS stamp. The FT-IRRAS spectra of SAM **3** (obtained by 1 h of chemisorption of col3a1 from solution) and SAM **3*** (obtained by 15 min of μCP with a flat, featureless stamp) are virtually identical, once more confirming the remarkable efficiency of the immobilization reaction in the confinement between stamp and substrate.[15,20] However, the amine band is shifted to higher wavenumbers and also the ratios of amide I to amide II are not equal for SAM **3** and SAM **3***, which could indicate differences in hydration and orientation of the protein layers.

To investigate the structure and distribution of the protein onto the aldehyde-terminated SAM **2**, tapping-mode AFM images were taken directly after immobilization of protein col3a1 (Figure 4). The molecules that were not attached covalently to the surface were removed by sonication and thorough rinsing of the surface. Col3a1 forms a homogeneous, but relatively low-coverage layer on top of the aldehyde-terminated SAM **2**. The average height of the col3a1 molecules ranges from 1 to 4 nm, consistent with the data from ellipsometry (Table 1). Each molecule of protein is attached to the surface and avoids the neighboring protein molecules as a consequence of electrostatic and steric repulsion.

HeLa cell adhesion to protein substrates: We investigated the adhesion of human malignant carcinoma (HeLa) cells onto substrates coated with different proteins. Cells were incubated over protein-modified substrates for 24 h at 37 °C at an initial cell density of 3×10^4 cells cm^{-2}. We compared the attachment of HeLa cells onto glass slides that had been modified from solution with various types of gelatins, differing in polarity, in folding and in the presence of the cell-binding sequence RGD (Figure 5). These gelatins were the gel-forming animal gelatin type B, and three non-gel-forming gelatins produced in yeast cells, namely, col3a1,[16] col1a1–1*,[16] and the hydrophilic designer gelatin P4.[17] The highest number of attached cells was found on the col3a1-modified substrate (980 cells cm^{-2}). Conventional B-type animal gelatin gives the lowest adhesion of HeLa cells of all substrates studied (435 cells cm^{-2}). Both col3a1 and B-type animal gelatin contain RGD, however, col3a1 contains more RGD per gram of protein and, moreover, col3a1 is completely unfolded. A significant number of cells was also found on the P4-modified substrate (790 cells cm^{-2}) and on the col1a1–1*-modified substrate (500 cells cm^{-2}), which is surprising as neither of these proteins contains the RGD sequence. It is unlikely that P4 induces more unspecific adhesion than col1a1–1*, as P4 is considerably more polar than col1a1–1*.[16,17] However, it is possible that P4 (and maybe also col1a1–1*) contains unidentified cell-binding sequences other than RGD.[19]

Protein and cell patterning: Col3a1 was microcontact printed in 3-μm lines at 5 μm intervals by using a hydrophilic, oxidized PDMS stamp (spin coated on a silicon master, cured, treated with UV/ozone plasma for 30 min and stored

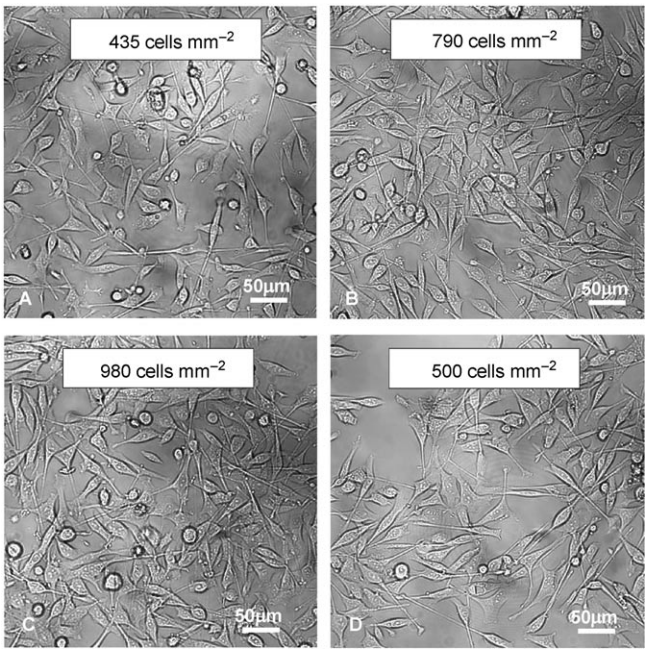

Figure 5. HeLa cells on glass slides modified with A) B-type animal gelatin, B) P4 polar gelatin, C) col3a1, D) col1a1–1*.

in water) onto aldehyde-terminated SAM **2**. The ink (i.e., col3a1) concentration was 1 mM in PBS and the contact time was 15 min. After μCP, the substrate was sonicated in PBS for 5 min and rinsed thoroughly with PBS and water to remove all physisorbed material, and finally dried with nitrogen. The surface was imaged with tapping-mode AFM (Figure 6A). The contrast in the height images confirms the formation of a protein pattern on the surface. The average height of the printed protein layer is 1.3 nm, which is consistent with ellipsometry (Table 1) and also with the observed height of chemisorbed col3a1 (see above). Although the proteins could not be removed at neutral pH by either rinsing or sonication, the proteins could be removed efficiently through acid-catalyzed hydrolysis.[20] Indeed, after hydrolysis no pattern was observed on the SAM (Figure 6B). This observation confirms that, most probably, immobilization of proteins occurs through covalent imine bonds (and not physisorption).

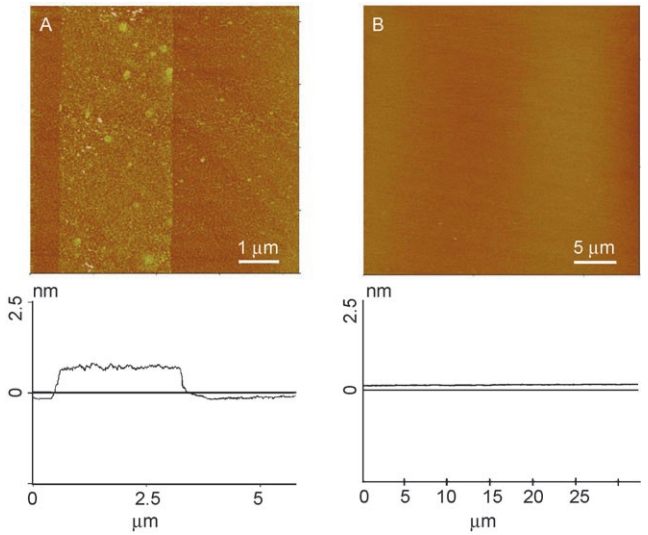

Figure 6. Tapping-mode AFM images of microcontact-printed col3a1 onto aldehyde-terminated substrate **2**: A) by using hydrophilic, oxidized PDMS with 3-μm lines separated by 5 μm, B) after acid-catalyzed hydrolytic release of microcontact-printed proteins.

The covalent μCP of proteins was investigated further by fluorescence microscopy. Firstly, col3a1 was printed in 100-μm dots onto an aldehyde-terminated glass slide. The substrate was sonicated briefly in PBS and rinsed thoroughly with PBS and water (Milli-Q). Next, the remaining aldehyde gaps were reacted with methoxypoly(ethylene glycol) amine. Immobilized protein col3a1 was then labeled with a fluorescent dye, Lissamine rhodamine B, from ethanolic solution for 1 h at room temperature. It is evident from the fluorescence image (Figure 7) that Lissamine-labeled col3a1 is present exclusively in the contact regions (100-μm dots) and not in the poly(ethylene glycol)-coated areas in between.

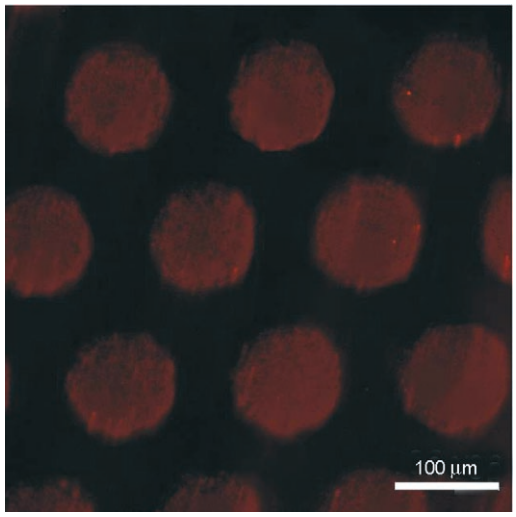

Figure 7. Confocal microscopy image of printed col3a1 patterns (100-μm dots separated by 100 μm) labeled with the fluorescent dye Lissamine rhodamine B.

To pattern HeLa cells we used substrates that were patterned with col3a1 proteins obtained by the direct μCP of protein onto aldehyde-terminated SAM and subsequent blocking of the remaining aldehyde groups with amino-PEG (see Figure 1). It is important to select the correct dimension of the pattern on the surface. Cells are constrained and inhibited if they are confined within areas similar to or smaller than their natural dimensions. If the separation between the patterned cells is insufficient, cells readily occupy the space between the patterns. Here, we chose 100-μm dots with 100-μm spacing between dots. HeLa cells were seeded onto the substrate patterned with adhesive "islands" and incubated

for 24 h at 37 °C at an initial concentration of 3×10^4 cells cm^{-2}. The HeLa cells attach to each island, spread to the limits of the printed pattern, and stay on the pattern even after vigorous rinsing of the substrate with PBS (Figure 8). On average, more than nine out of ten cells

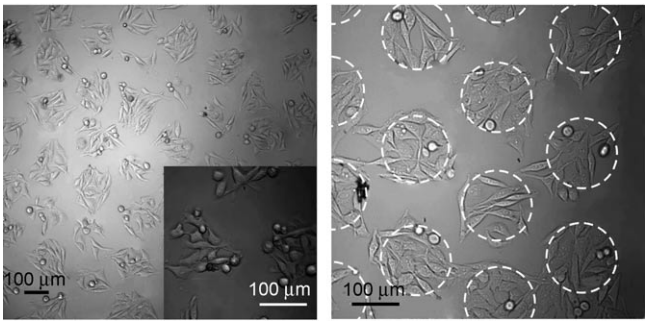

Figure 8. Patterns of HeLa cells obtained by µCP of protein col3a1 (100-µm dots separated by 100 µm).

adhere to the islands, whereas less than one out of ten adhere outside the islands. As a control experiment we used a glass substrate that was covered homogenously with col3a1 proteins. Cells were seeded at a concentration of 3×10^4 cells cm^{-2} and, after incubation (24 h at 37 °C), cells attached to the substrate without forming any pattern (Figure 5).

We also investigated the stability of the cell pattern after 24 h, 48 h, and 72 h (Figure 9). After 24 h of incubation the pattern was apparent and cells adhered preferentially (>90 %) to the part of the substrate at which col3a1 was immobilized. After 48 h of incubation cells penetrated the regions between the protein islands. After about 72 h the cells approached confluency and spread onto the entire nonadhesive area, so that the original pattern was no longer visible. After 48 h of incubation, elongated (>50 µm in length) membrane protrusions and actin tubules were observed. HeLa cells migrated onto cell-resistant areas (Figure 10). This might indicate cell-to-cell communication between cells on the pattern, as well as the possible secretion of ECM proteins by the HeLa cells that, similarly to col3a1, enhance adhesion of the cells to the substrate. Alternatively, the HeLa

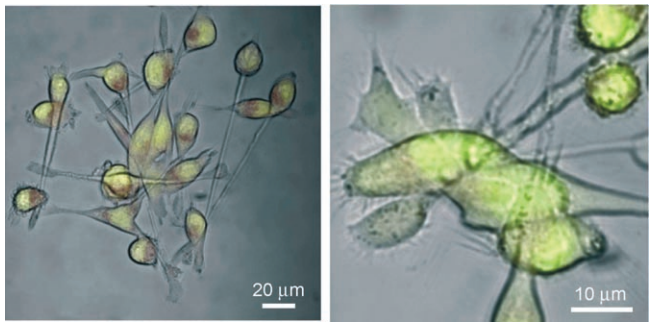

Figure 10. Patterned substrates with HeLa cells after 48 h incubation. Cells show long (>50 µm in length) actin tubules and migrate towards cell-resistant areas.

cells may secrete enzymes that can affect the patterned substrate. It was reported previously that cells can also indirectly degrade nonadhesive surfaces by exerting local physical stresses, such as mechanical strains or pH changes.[21]

Conclusion

Covalent µCP of collagen-type protein col3a1 onto aldehyde-terminated substrates is useful to obtain protein patterns that can be applied to direct cell adhesion. Col3a1 shows the highest adhesion upon comparison with other, similar proteins, such as col1a1–1*, P4, or bovine gelatin. Direct covalent immobilization of proteins by µCP delivers protein in well-defined, spatially and geometrically controlled areas to the substrate. By blocking nonprinted areas on the substrate, cells can be positioned and separated in confined domains. Cells adhere preferentially on the col3a1 patterns. After long incubation times (72 h) the HeLa cells proliferate and migrate on the pattern and fill in the cell-resistant areas. There are several advantages of covalent µCP of proteins to direct cell adhesion: it is easy, inexpensive, fast, and straightforward. The pattern can be tailored according to the desired application by designing the geometry of the master for PDMS-stamp fabrication. This method can be useful in generating large areas of addressable arrays of cells in a variety of shapes dependent on the stamp. In addition, this methodology to pattern cells can be useful in un-

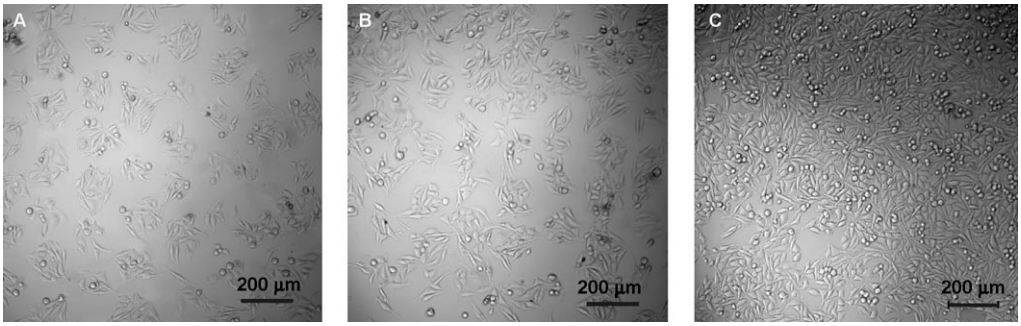

Figure 9. HeLa cells on the substrate with col3a1 printed in 100-µm dots A) after 24 h incubation, B) after 48 h incubation, C) after 72 h incubation.

derstanding how spatial or geometric modification of active surfaces influences cellular behavior, such as cell–cell interaction, signaling between cells, and cell motility.

Experimental Section

Materials: The following materials and chemicals were used as received: 11-aminoundecanethiol (Dojindo Laboratories), terephthaldialdehyde 99% (Aldrich), poly(dimethylsiloxane) (PDMS) (Dow Corning), *N*-(2-aminoethyl)-11-aminoundecyltrimethoxysilane (AEAUTMS) (Gelest), Lissamine rhodamine B (Molecular Probes), methoxypoly(ethylene glycol) amine (Fluka). Proteins col3a1, col1a1–1*, and P4 were biosynthesized at Wageningen UR.[16,17] Gelatin type B from bovine skin was purchased form Sigma–Aldrich. Serum and materials for cell culture were purchased from Gibco or Invitrogen. All solvents were of HPLC grade, and all other reagents were of analytical grade. Other solvents or reagents were purchased from either Aldrich or Sigma.

Monolayer formation: Monolayers on gold and silicon substrates were prepared according to procedures published previously.[20] The procedure for immobilization of proteins is identical for gold and silicon surfaces. The substrates were immersed for 1 h in protein solution (1 mM in PBS). Subsequently, the substrates were rinsed copiously with Milli-Q water and dried under a stream of nitrogen. In the direct μCP method (substrates with full coverage of proteins), a flat, featureless PDMS stamp (oxidized for 30 min with a UV/ozone plasma and stored under Milli-Q water) was inked with a 1 mM solution of col3a1 proteins in buffer (PBS), dried under a stream of nitrogen, and placed on aldehyde-terminated substrate for 15 min at 35 °C. After reaction time the stamp was lifted off and the substrate was sonicated in PBS for 5 min, rinsed copiously with Milli-Q water (to remove all physisorbed material), and dried under the stream of nitrogen.

Preparation of protein-modified substrates for cell attachment: Aldehyde-terminated glass slides were prepared as described.[20] The substrates were immersed for 1 h in protein solution (1 mM col3a1, col1a1–1*, or P4 in PBS solution, 0.1 mM aqueous gelatin solution). Subsequently, the substrates were rinsed copiously with Milli-Q water and dried under a stream of nitrogen. In a control experiment, the imine monolayer that was formed by the reaction between amino-terminated SAM and terephthaldialdehyde and col3a1 (SAM **3**) was hydrolyzed by immersing the substrate in aqueous acetic acid solution (pH 3) for 1 h at RT.[20]

Microcontact printing of proteins on aldehyde-terminated substrates: Aldehyde-terminated glass slides (or silicon oxide on silicon wafers) and gold substrates were prepared according to procedures mentioned above. PDMS stamps (obtained by spin casting on silicon masters and curing) were oxidized in a commercial UV/ozone plasma reactor (Ultra-Violet Products, model PR-100) for 30 min at a distance of about 2 cm from the plasma source. This reactor contains a low-pressure mercury UV light operating with UV emissions at 185 nm (1.5 mW cm^{-2}) and 254 nm (15 mW cm^{-2}) to generate molecular oxygen. Oxidized stamps were placed in Milli-Q water to maintain their hydrophilicity. Subsequently, the stamp was inked with a 1 mM solution of protein in PBS, dried with N_2, and brought into conformal contact with the substrate for 15 min at 35 °C. After this time the stamp was removed and the substrate was sonicated in buffer for 5 min and rinsed thoroughly with PBS and Milli-Q water. The remaining gaps on the substrate (containing aldehyde groups) were reacted with 1 mM methoxy-PEG amine aqueous solution with triethylamine for 30 min at RT. After reaction time the substrate was rinsed with water and dried with nitrogen. The reaction of col3a1 with Lissamine rhodamine B was performed by soaking the substrate with immobilized protein into a 1 mM ethanolic solution of the dye for 1 h. Subsequently, the surface was rinsed thoroughly with ethanol and water and dried under nitrogen.

Cell culture and seeding onto the substrates: HeLa cells (human cervix epithelial cell line) were cultured with Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (Gibco 16000), 2 mM L-glutamine, and 1% antibiotics/antimycotic solution. The cells were trypsinized in a 0.05% trypsin–EDTA solution and seeded onto the substrate slides at a concentration of 3×10^4 cells cm^{-2} in Iscove's modified Dulbecco's medium. Substrates with cells were incubated for 24 h at 37 °C with 5% CO_2. Following the incubation substrates were rinsed with PBS to wash away unattached cells. The same incubation protocol was used for both homogeneous and patterned protein substrates.

Instrumentation

Contact-angle measurements: Contact angles were measured by using a Krüss G10 goniometer, equipped with a CCD camera. Advancing and receding contact angles (θ_{adv} and θ_{rec}) were determined automatically during growth and shrinkage of the droplet by a drop-shape analysis. Milli-Q water (18.4 MΩ cm^{-1}) was used as a probe liquid. Both angles (advancing and receding) were measured for at least three different locations on each sample.

Ellipsometry: Ellipsometric-layer thickness was measured by using a Plasmos Ellipsometer ($\lambda = 632.8$ nm) assuming a refractive index of 1.5 for the monolayers and 1.465 for the underlying native oxide. The thickness of the SiO_2 layer was measured separately on an unmodified part of the same wafer and subtracted from the total layer thickness determined for the monolayer-covered silicon substrate.

FT-IRRAS spectroscopy: FT-IRRAS spectra in the mid-IR region of 1024 scans at 4 cm^{-1} resolution, 20 kHz speed, were recorded by using a BIO-RAD FTS-60A spectrometer with a liquid-nitrogen-cooled cryogenic, external mercury–cadmium–telluride (MCT) detector, with its sample area modified to accommodate an external reflection-sampling geometry. The sample area was purged by dry nitrogen. Background spectra consisting of 1024 averaged scans were taken before collecting each sample spectra.

AFM: AFM measurements were carried out by using a digital multimode Nanoscope III (Digital Instruments, Santa Barbara, CA, USA) scanning force microscope in tapping mode, with 512×512 data acquisitions, using n(+)-silicon AFM pointprobes tips, type NCH-W with nominal spring constant 37–56 N m^{-1} (Nanoprobes, Digital Instruments) and E-scanner. Typical scan rates of 0.8–1 Hz were used to acquire the data. All imaging was conducted at RT in air.

Laser scanning confocal microscopy/optical microscopy: Microcontact-printed substrates with and without cells were imaged by using a Carl Zeiss LSM 510 scanning confocal microscope with an excitation HeNe laser beam of wavelength 543 nm and a 10, 40, and 60× objective was used. The emitted fluorescence was collected by using a R6357 spectrophotometer. All confocal microscopy images were acquired in liquid.

XPS analysis: XPS spectra were obtained by using a Physical Electronics Quantera Scanning X-ray Multiprobe instrument, equipped with a monochromatic $Al_{K\alpha}$ X-ray source operated at 1486.7 eV and 25 W. Spectra were referenced to the main C 1s peak set at 284.0 eV. XPS data were collected from a surface area of 700 μm×300 μm with a pass energy of 224 eV and a step energy of 0.8 eV for survey scans, and 0.4 eV for high-resolution scans at a 45° takeoff angle, whereas the angle between sample surface and the X-ray beam was 90°. For quantitative analysis, the sensitivity factors used to correct the number of counts under each peak were as follows: C 1s, 1.00; N 1s, 1.59. The calculated C:N ratios (Table 1) are not corrected for attenuation. Charge neutralization was achieved by low-energy electrons and low-energy argon ions.

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