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Coatings from micropatterned sulfobetaine polymer brushes as substrates for MC3T3-E1 cells

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Abstract In the last decades, polymer brush coatings have proven to be excellent anti-fouling materials by preventing protein adhesion. When using this property to restrict cell growth laterally in cell culture, it is crucial to ensure that other cell functions remain unaffected. The present study therefore examines MC3T3-E1 cell growth and morphology on patterned PSBMA brush substrates and probes their proliferation potential at mRNA level. The osteoblastic cells display a more elongated morphology than cells on the control substrates, but show no sign of elevated levels of the apoptosis marker *p53* or diminished levels of *Ki-67* or *H4*, which serve as indicators of proliferation. Therefore, patterned polymer brushes do not seem to influence cells in their proliferation state and are suitable cell culture substrates. Nevertheless, the use of polymer brush surfaces in long-term cell culture was found to be limited by their instability in cell culture medium.

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1 Introduction

Control over cell adhesion is of interest for a variety of biomedical applications [1]. Whereas biomaterials should provide good conditions for the human cells of the respective tissue and prevent bacterial colonization, surfaces for cell arrays or biosensors should direct cell adhesion without interfering with other cell functions [2–4].

It is well known that protein adsorption is a prerequisite for cell adhesion and it was shown in a number of studies that polymer brushes successfully prevent both protein adsorption and bacterial adhesion [5, 6]. Zwitterionic polymer brushes such as the sulfobetaine poly[*N*-(3-sulfopropyl)-*N*-(methacryloxyethyl)-*N*,*N*-dimethylammonium betaine] (PSBMA) in particular turned out to be effective inhibitors of protein adsorption and biofilm formation [7–9]. Important parameters to control the cells' interaction with surfaces are brush length and density [10–13]. The higher the density and the molecular weight of the polymer brush, the more effective is the prevention of protein adsorption [10–12].

For cell array applications, also the multitude of possibilities to pattern the polymer brush coatings [4, 14–16] is a great advantage, as cells and proteins can thus be conducted accurately to specific areas of a surface. Studies on the colonization of polymer brushes with mammalian cells are rare [1, 17, 18], but Iwata et al. [1] showed that fibroblast adhesion could be restricted by the zwitterionic phosphorylcholine brush poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC).

Yet, although it is known that cells on patterned polymer brush substrates prefer the grooves over the actual brush covered surface, little is known about their proliferation and viability in this situation. Therefore, in the present study the murine osteogenic cell line MC3T3-E1 was

cultured on PSBMA brush-coated substrates with line patterns of 10 and 50 µm line width, respectively. Their morphology was observed and the expression of proliferation and apoptosis markers was analyzed at mRNA level.

2 Materials and methods

2.1 Preparation of polymer brush substrates

Cleansed microscope glass slides (Menzel, Germany) were coated with the initiator 3-(2-bromoisobutyramido)propyl(trimethoxy)silane, following the procedure described in detail by Tugulu et al. [19]. Using a photomask, these initiator-coated substrates were irradiated by deep UV-light to obtain a subsequent structuring. The non-exposed and thus still active areas were polymerized with the zwitterionic monomer *N*-(3-sulfopropyl)-*N*-(methacryloxyethyl)-*N*,*N*-dimethyl-ammonium betaine (SBMA; Merck, Germany) by transferring the irradiated substrate to a vessel containing the polymerization solution. The polymerization conditions were slightly altered from the protocol of the Huck group [20]. Briefly, the monomer SBMA was solved in a water/methanol 1:4 mixture (water obtained from a Millipore Simplicity system; methanol from Merck, Germany) and degassed. A Cu(I)/Cu(II)/bipyridine (CuCl and bipyridine from Aldrich, Germany; $CuCl_2$ from Merck, Germany) catalyst system was added to give a molar ratio of monomer:Cu(I):Cu(II):bipyridine of 100:2:0.2:5. Polymerization was started by adding the initiator coated substrate under nitrogen atmosphere and continued at room temperature for 2 h.

2.2 Analysis of polymer brush substrates

The obtained patterned polymer brushes were analyzed with optical microscopy employing differential interference contrast (DIC; Olympus IX70). Thus, the homogeneity of the polymerization and the sharpness of the pattern could be clearly observed. The thickness of the polymer brushes in air and water could be determined by atomic force measurements (AFM; Agilent 5500). In air, the brushes could simply be scanned in the intermittent contact mode (silicon cantilever 42 N/m), whereas the swollen brushes in water were very deformable. Thus, very soft cantilevers (SiNi 0.32 N/m) and tips with a large diameter (SiO_2 spheres with $d = 1$ µm) were necessary to determine the brush heights in solution. To compare the height profiles in air with the ones in water, the brushes were scanned in contact mode with a force of 8 nN. The samples were incubated in water for 20 min prior to measurement. The water contact angle of the coating was measured employing the contact angle measuring system G10 (Krüss, Germany). To see possible surface modifications in cell culture medium caused by the multitude of ingredients therein, PSBMA brush samples were measured again after a two weeks immersion time in cell culture medium.

2.3 Cell culture

Cell culture experiments were performed with the murine osteogenic cell line MC3T3-E1 (German Collection of Microorganisms and Cell Cultures, Germany). The cells were cultured in expansion medium consisting of α-minimal essential medium (α-MEM, Biochrom, Germany) supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Germany), 4 mm L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin (all from Biochrom, Germany) at 37 °C, 5% CO_2 and saturated humidity. The culture medium was changed twice a week. At subconfluence, cells were sub-cultured by 0.05% trypsin/0.02% EDTA (Biochrom, Germany) treatment. Cells of passage 8 were used for the experiment. MC3T3-E1 cells were seeded at 10^4 cm^{-2} onto cell culture plastic, the glass control or the PSBMA brush substrates (line patterns with 10 or 50 µm width) in 12-well cell culture plates and were cultured in expansion medium for 21 days. Medium was changed twice a week.

2.4 Cell morphology

Live cells were examined light-microscopically (DMI6000 B, Leica, Germany). Micrographs were taken with a DFC420C camera (Leica, Germany) at days 1 and 21. To analyze cell morphology more closely, scanning electron microscopy (SEM) was performed at day 21. For this purpose, cells were fixed with 2.5% glutaraldehyde in 1 mm PBS (pH 7.3) containing 1% saccharose, washed with PBS and then fixed with 2% aqueous osmium tetroxide. Subsequently, the samples were dehydrated in a graded series of 1-propanol. Then, the cells were critical point dried using carbon dioxide as translation medium, mounted on a specimen holder and coated with platinum (3–4 nm) by electron beam evaporation. Cells were imaged with a scanning electron microscope (S-5200, Hitachi Europe GmbH, Germany) at an accelerating voltage of 10 kV.

2.5 Real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR)

Quantitative effects on gene expression were examined by real-time RT-PCR. Briefly, RNA was isolated from the cells after 21 days of cultivation using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer instructions. 1 µg RNA was transcribed into cDNA using

Table 1 Primer sequences used for real-time RT-PCR

mRNA	Primer sequence	PCR product size (bp)
GAPDH forward	5′-ACCCAGAAGACTGTGGATGG-3′	81
GAPDH reverse	5′-GGATGCAGGGATGATGTTCT-3′	
H4 forward	5′-ATGTCAGGACGAGGAAAAGG-3′	96
H4 reverse	5′-CTTGGTAATGCCCTGGATGT-3′	
Ki-67 forward	5′-GACAGCTTCCAAAGCTCACC-3′	214
Ki-67 reverse	5′-GTGTCCTTAGCTGCCTCCTG-3′	
p53 forward	5′-GGAAATTTGTATCCCGAGTATCTG-3′	61
p53 reverse	5′-GTCTTCCAGTGTGATGATGGTAA-3′	

the Omniscript RT Kit (Qiagen, Germany). Specific primer pairs (Table 1, Thermo Electron GmbH, Germany) were designed using published gene sequences (PubMed, NCBI Entrez Nucleotide Database) and used for real-time RT-PCR (StepOnePlusTM Real-Time PCR System, Applied Biosystems, Germany).

The amount of each respective amplification product was determined via the $\Delta\Delta C_T$ method [21]. The average C_T value of the respective gene of interest of each sample measured (in duplicate, resulting in $n = 2 - 4$) was normalized to the average C_T value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the respective sample:

$$\Delta C_T = C_T(\text{gene of interest}) - C_T(\text{GAPDH}) \quad (1)$$

$\Delta\Delta C_T$ values were calculated by referring ΔC_T values to the cell culture plastic control:

$$\Delta\Delta C_T = \Delta C_T(\text{glass or PSBMA brush substrate}) - \Delta C_T(\text{cell culture plastic}) \quad (2)$$

The relative mRNA expression was then calculated by the term

$$2^{-\Delta\Delta C_T}. \quad (3)$$

The experiment was performed once in duplicate (except for the line pattern with 50 µm width, where only one mRNA sample could be obtained). mRNA expression data are expressed as mean of measurements. Statistical analysis was omitted due to the small number of measurements.

3 Results

3.1 Polymer brush substrates

The PSBMA brush line patterns were surveyed after polymerization, employing the DIC mode of a light microscope. A clearly visible structure with sharp edges (Fig. 1a, b) was taken as an indicator for a successful patterning and polymerization.

AFM measurements showed an equal PSBMA brush height of around 50 µm for line patterns with 5 µm line width or more (Fig. 1c). To cover different pattern sizes, but nevertheless have comparable heights of the PSBMA brush pattern, substrates with a line width of 10 and 50 µm, respectively, were chosen for cell culture experiments. To provide a better picture of the polymer brushes in the aqueous environment, AFM scans in deionized water were conducted (Fig. 2). The PSBMA brush line patterns swelled to about twice their dry height and were softer than in their dry state.

Contact angle measurements indicated that the PSBMA brushes with a static contact angle of 15° (2° standard deviation) offer a hydrophilic environment to the cells. After two weeks immersion in cell culture medium, the substrates were even more hydrophilic. These surfaces were not measurable in five out of nine cases, as no stable drop was formed. The samples that were measured showed a higher variation of data than before immersion, indicating a much less defined surface than before exposure to cell culture medium.

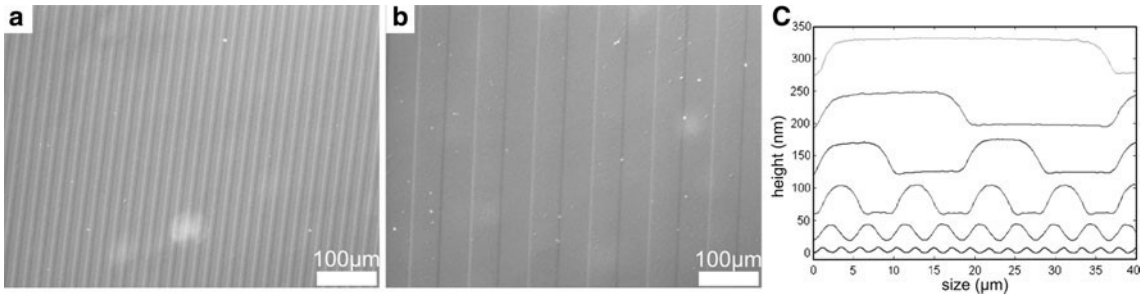

Fig. 1 DIC micrograph of patterned PSBMA brush coatings with 10 µm (**a**) and 50 µm line width (**b**) and AFM line diagram of the different pattern widths' profiles (**c**)

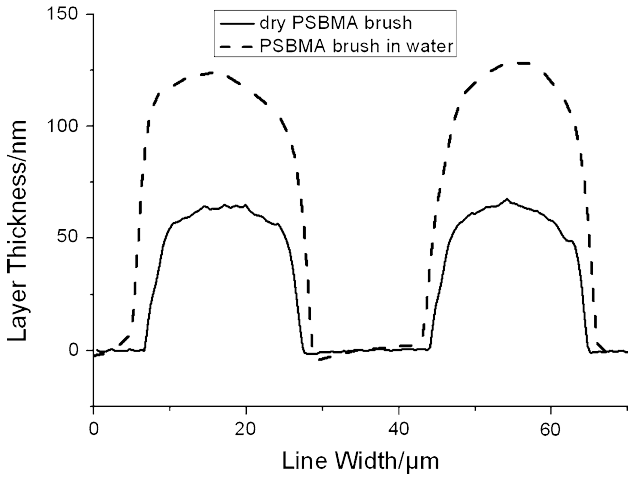

Fig. 2 AFM line diagram of patterned PSBMA brushes in dry state (*continuous line*) and swollen in water (*dashed line*)

3.2 Cell morphology

MC3T3-E1 cell growth and morphology were observed light-microscopically throughout the experiment. At day 1, the cells showed undirected growth and a widespread, osteoblast-like morphology on the cell culture plastic control (Fig. 3a) and on the glass control (Fig. 3b) featuring a higher cell density on plastic. In contrast, cells on the PSBMA brush line patterns with 10 µm width (Fig. 3c) and 50 µm width (Fig. 3d) grew highly aligned with a very elongated cell morphology. Based on earlier experiments [17], cells were known to grow between the PSBMA brush lines on the deactivated initiator surface layer. At the end of the experiment at day 21, the cell culture plastic control (Fig. 3e) and the glass control (Fig. 3f) were completely overgrown with cells in an undirected manner. However, on the PSBMA brush line patterns with 10 µm (Fig. 3g) and 50 µm width (Fig. 3h) cells were still well aligned. Whereas cells on the line pattern with 50 µm width (Fig. 3h) only grew between the PSBMA brush lines, the cells had overgrown some of the line pattern with 10 µm width (Fig. 3g, right part).

MC3T3-E1 cell morphology was analyzed more closely by SEM at day 21 (Fig. 4) confirming the more outspread morphology of the cells on the cell culture plastic control (Fig. 4a) and on the glass control (Fig. 4b) than of the elongated, aligned cells on the PSBMA brush line patterns with 10 µm width (Fig. 4c) and 50 µm width (Fig. 4d). However, all the cells seemed to have been viable and had produced considerable amounts of extracellular matrix (Fig. 4, triangles).

3.3 Cellular proliferation potential

To investigate the possibility of an influence of the patterned PSBMA brush substrates on the proliferation potential of the cells, the mRNA expression of the apoptosis marker *p53* [22] as well as of *Ki-67*, a nuclear protein present in all phases of the cell cycle in proliferating cells [23] and of the cell cycle-associated gene *H4* encoding a histone [24] was analyzed at day 21 (Table 2). Relative to the cell culture plastic control, *p53* mRNA expression was not elevated in cells cultured on the PSBMA brush substrates. Moreover, based on the present initial analysis, the cell proliferation did not seem to be constrained by the PSBMA brush substrates as reflected by the mRNA expression of the proliferation markers *Ki-67* and *H4* relative to the cell culture plastic control. Cells on the glass control yielded similar results. On the PSBMA brush line pattern with 50 µm width, proliferation even seemed to be slightly induced.

4 Discussion

The present study evaluates patterned polymer brush coatings and their influence on fundamental cell functions. Osteoblastic MC3T3-E1 cell culture on zwitterionic PSBMA brush substrates with a linear stripe pattern demonstrates that cell viability appears not to be influenced by the polymer brushes, although the cells differ in morphology from those on control substrates (Fig. 3).

The MC3T3-E1 cells on the patterned PSBMA substrates produced extracellular matrix (Fig. 4) and did not seem to be impaired in growth by the polymer brush coating, although their morphology was less widespread and more elongated than the one of the cells on the control substrates (Fig. 4). An elongated morphology of MC3T3-E1 cells on polymer brush substrates was already observed by Tomlinson et al. [18] and was associated with an reduced amount of adsorbed fibronectin. Nevertheless, based on the number of experiments already performed, at the mRNA level (Table 2), there were no tendencies of diminished proliferation—as represented by *Ki-67* and *H4*—or increased apoptosis, for which *p53* served as a marker [22–24]. Taking into account the mRNA values concerning viability and the morphological observations, PSBMA brushes appear to be a promising tool to restrict cell growth to certain areas.

The distribution of the MC3T3-E1 cell adhesion on the patterned substrates (Fig. 3c, d, g, h) confirmed earlier studies [1, 10, 17], which showed that cells where unable to colonize polymer brush surfaces of high grafting density and over 5 nm layer thickness. Results are consistent in this respect, although these studies differ in terms of cell type and polymer chemistry. The high chain mobility and excluded volume of the polymer brushes are thought to be the reasons for their ability to diminish protein and cell adhesion [10]. Theoretical models show in accordance with

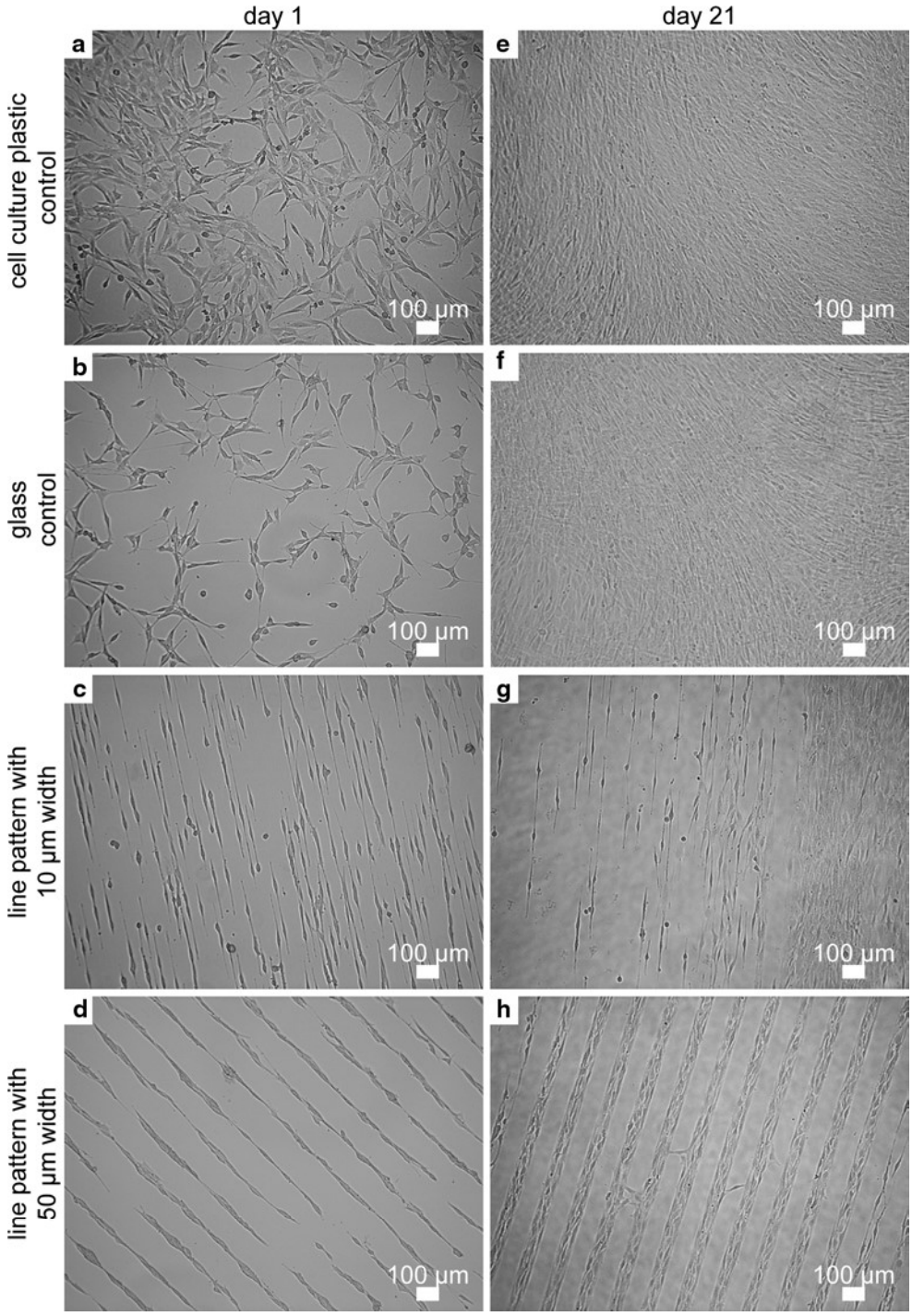

Fig. 3 Cell morphology of live MC3T3-E1 cells on the cell culture plastic control (**a**, **e**), the glass control (**b**, **f**) and the line patterns with 10 µm width (**c**, **g**) and 50 µm width (**d**, **h**) after 1 day (*left panel*, **a-d**) and after 21 days (*right panel*, **e-h**) of culture in expansion medium via light microscopy

experiments that surface tethered polymers also compete against proteins for the interaction with the surface, when blocking protein adsorption [13]. The ability to swell and the consequently highly hydrated state of the polymer brushes is also accounted for the resistance to cell attachment [17, 25, 26]. Mendelsohn et al. [25] described the large effect that swelling behavior of surface coatings could have on cell adhesion. With the same chemical composition the coatings with higher swelling ability exhibited cytophobic properties. Considering the capability

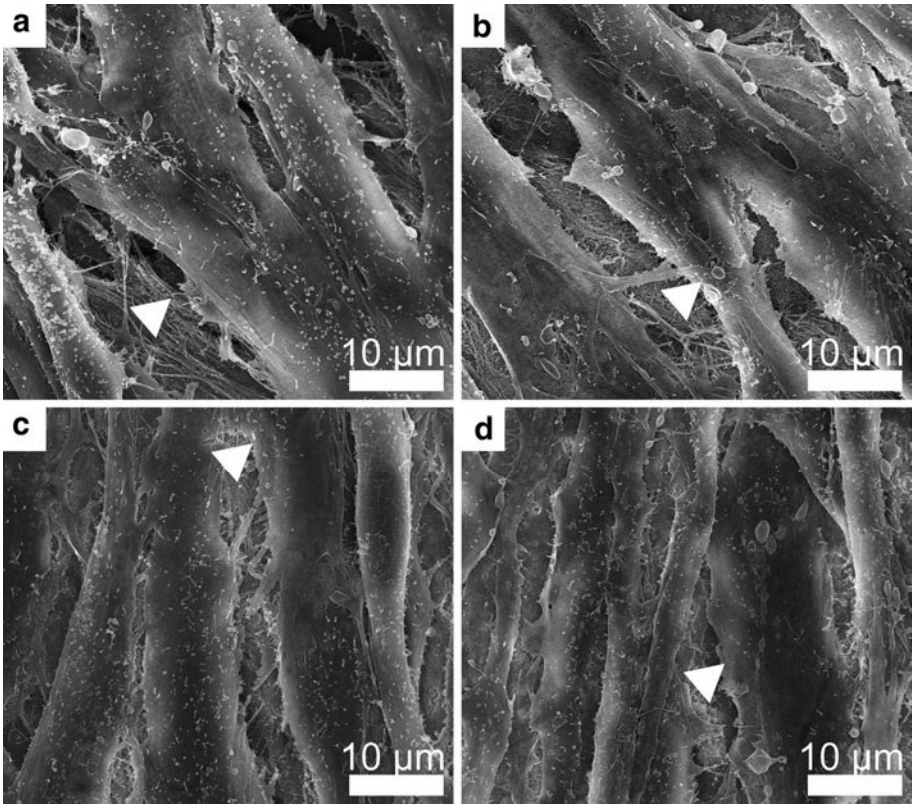

Fig. 4 Cell morphology of MC3T3-E1 cells on the cell culture plastic control (**a**), the glass control (**b**) and the line patterns with 10 µm width (**c**) and 50 µm width (**d**) after 21 days of culture in expansion medium via SEM. Triangles indicate extracellular matrix

Table 2 Normalised mRNA expression of the apoptosis marker *p53* and of the proliferation markers *Ki-67* and *H4* relative to the cell culture plastic control in MC3T3-E1 cells cultured on the glass control and on the line patterns with 10 µm width and 50 µm width at day 21 of the experiment. mRNA expression on the cell culture plastic control was set at 1. Statistical analysis was omitted due to the small number of measurements ($n = 2 - 4$)

Substrates	Gene of interest		
	p53	*Ki-67*	*H4*
glass control	0.44 ± 0.05	0.51 ± 0.04	0.37 ± 0.08
line pattern with 10µm width	0.79 ± 0.05	1.22 ± 0.24	0.84 ± 0.42
line pattern with 50µm width	0.94	2.10	4.84

of the PSBMA brushes in water to swell to a height about twice of that in dry state (Fig. 2), this could also be a reason for their anti-adhesive properties.

Despite the polymer brushes' established property to prevent cell adhesion, it should be noted, that in many studies on polymer brushes, cell adhesion on patterned polymer brush substrates was observed for short periods [1, 9, 10, 12, 27]. In the present study, long term cell culture experiments were conducted for 21 days. After this time, cells were partly able to overgrow PSBMA brush regions of the 10 µm line pattern (Fig. 3g, right part of the micrograph). The cells seem to induce favorable surface modifications for themselves, even on polymer brush coatings, by the great amount of extracellular matrix produced during cell culture (Fig. 4c, d). For the deposition of extracellular matrix the detachment of polymer brushes might also be beneficial, which takes place after incubation in cell culture media for polymer brushes of high densities [28]. The contact angle measurements point into a similar direction. In the present study, a 2 weeks' immersion sufficed to make PSBMA covered substrates less defined and so hydrophilic that the water contact angle could not be measured accurately. This detachment results in a lower brush density and thickness, which diminishes the polymer brush's ability to resist protein adsorption [10–13]. Thus, even without cells, proteins and electrolyte included in cell culture medium are able to modify the polymer brush surface over a longer period of time. Both effects, the lacking long term stability of high density polymer brushes and the actively modifying extracellular matrix production of osteoblastic cells, are the most probable reasons for the overgrowth of smaller polymer brush structures.

Seeing the whole picture, polymer brushes still present themselves as feasible means to laterally control cell growth for biomedical applications without hampering essential cell functions. Based on the small number of experiments, further studies will be necessary to confirm that there are no minor effects on cell viability or apoptosis, which might only be unveiled by a greater number of

samples. Improvements of polymer brush stability are required, when proceeding to long term cell culture. Otherwise, control of cell adhesion is not ensured. Future studies will show, if other aspects of cellular function such as osteoblastic differentiation are affected by sulfobetaine polymer brush substrates.

5 Conclusion

In the present study, polymer brushes again present themselves as bioinert material, which can direct cell adhesion to certain areas without hampering the viability of the cell material. This was shown by observing the cell morphology and extracellular matrix production by light and scanning electron microscopy. At the mRNA level, no signs of affected proliferation or increased apoptosis could be observed. The only constraint for this material is the lacking stability in cell culture media, which makes it as yet unsuitable for long-term applications.

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