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The threshold at which substrate nanogroove dimensions may influence fibroblast alignment and adhesion

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INTRODUCTION

Biomaterials for tissue and cell engineering are successfully incorporated into neighbouring tissue when they not only match the tissue's mechanical properties, but also bring forth specific cell responses, thus controlling or guiding the tissue formation in contact with the biomaterial. The cellular response to a biomaterial may be enhanced by mimicking the surface topography formed by the extra cellular matrix (ECM) components of natural tissue [1]. This could be beneficiary in the field of tissue engineering, which aims at the (re)generation of new and functional tissues. These ECM components are of nanometre scale and a first step in this quest is the production of nano(metre scale) topography. Previous studies have already addressed cellular reactions to larger micrometer scale topography. Predominantly groove and ridge patterns were studied, on which cells responded by altering morphology, orientation, adhesion, and gene regulation. Nanogroove patterns of 10-100 nm thus far have not yet been studied, although Teixeira *et al* [2] have studied cell behaviour on ridges 70 nm wide, with a pitch of 400 nm however, and a depth of 600 nm, and found cellular alignment along these grooves. Previous *in vitro* research has investigated nanocolumns produced by colloidal lithography or polymer demixing which caused changes in cell morphology, filopodia production, migration, and cytokine release [3]. From these studies, it has become clear that topography in the nanometre scale may be of importance in cell guiding [1; 3-5]. Despite the amount of control over the dimensions created by these techniques, however, they remain largely random. In addition, it is unknown to what extent cells will sense and adapt their morphology to an ordered topography if the dimensions become exceedingly small.

In this study, a nanogroove topography formed by electron beam lithography has been investigated. In order not to deviate too far from previously used patterns [6-9] ten nanogroove/ridge patterns with a 1:1 pitch ratio have been selected and compared to smooth controls.

We hypothesised that, if the topography is small enough, a cellular "point break" is reached, where cells no longer display contact guidance along nanogroove patterns. In order to verify this hypothesis, cell responses to such nanotopography fields have been investigated from a morphology point of view, using light microscopy and scanning electron microscopy, and subsequent image analysis.

MATERIALS AND METHODS

Substrata

Silicon wafers, 15 cm (6 inch) across, were spin coated with hydrogen silsesquioxane (HSQ) solutions in methyl isobutyl ketone (MIBK) (FOx-12, Dow Corning Corp., Midland, MI, USA) on a Karl Suss spinner at 1000 rpm during 10 s with closed lid, resulting in 100 nm thick HSQ layers. The wafers were exposed in a JEOL Electron Beam Pattern Generator (JBX-9300FS) to a 100 kV beam with a 500 pA beam current (4 nm spot size) using a 4 nm beam step size. The field patterns consisted of squares of 500 × 500 μm^2 containing 1:1 lines and spaces at various pitches. The wafers were developed by manual immersion at 20 °C in a 0.26 M tetra methyl ammonium hydroxide developer (TMA238WA), rinsed in 1:9 v:v TMA238WA:H_2O, rinsed in demineralised water and blown dry with N_2 [10-12]. For obtaining higher master structures, the e-beam patterned HSQ was used as a mask in a Reactive Ion Etching (RIE) process for silicon using a CF_4 /O_2 plasma.

One 250 nm deep groove pattern, with a 2 μm pitch, was made using a photolithographic technique and subsequent etching in a silicon wafer as described by Walboomers *et al.* [6]. Wafers with a smooth surface were used as controls. In all instances, the silicon wafers were used as template for the production of polystyrene (PS) substrata for cell culturing. Polystyrene was solvent cast in a manner described by Chesmel and Black [13]. The dimensions of all substrata are shown in Table 1. Polystyrene replicas were attached to 20 mm diameter cylinders with polystyrene-chloroform adhesive to create a cell culture dish. Shortly before use a radio frequency glow-discharge (RFGD) treatment using Argon was applied for 3 minutes at a pressure of 2.0 x 10^{-2} mbar (Harrick Scientific Corp., Ossining, NY, USA) and a power of 200 W in order to sterilise and promote cell attachment by improving the wettability of the substrata.

Cell culture

Rat dermal fibroblasts (RDF) were obtained from the ventral skin of male Wistar rats as described by Freshney [14]. Cells were cultured in Dulbecco's MEM containing Earle's salts (Gibco, Invitrogen Corp., Paisley, Scotland), L-glutamine, 10% FCS, gentamicin (50 μg/ml), in an incubator set at 37 °C with a humidified atmosphere. Cell passage 4 - 6th was used during the experiments. Onto the various substrata, 3.5×10^4 cells/cm^2 were seeded into the culture dishes.
For the analysis, after 4 or 24 hours, the cell layers were washed three times with phosphate buffered saline (PBS) and prepared for further analysis immediately after retrieval.

Atomic Force Microscopy

Surface topography was quantitatively evaluated using a Dimension atomic force microscope (AFM; Dimension 3100, Veeco, Santa Barbara, CA). Tapping in ambient air was performed with 118 μm long silicon cantilevers (NW-AR5T-NCHR, NanoWorld AG, Wetzlar, Germany) with average nominal resonant frequencies of 317 kHz and average nominal spring constants of 30 N/m. This type of AFM probe has a high aspect ratio (7:1) portion of the tip with a nominal length of >2 μm and a half-cone angle of <5°. Nominal radius of curvature of the AFM probe tip was less than 10 nm. The probes are especially suited to characterize the manufactured nanogrooves.
Height images of each field/sample were captured in ambient air at 50% humidity at a tapping frequency of 266.4 kHz. The analysed field was scanned at a scan rate of 0.5 Hz and 512 scanning lines. Nanoscope imaging software (version 6.13r1, Veeco) was used to analyze the resulting images. Surface roughness (root mean squared (RMS), nm) and depth (nm) were obtained and averaged for three random fields per substrate.

Scanning electron microscopy (SEM)

SEM graphs of the HSQ master structures were made using a Philips XL40 FEG-SEM. To assess overall morphology of the fibroblasts, SEM was also performed. Cells were rinsed, fixed for 5 minutes in 2% glutaraldehyde, followed by 5 minutes in 0.1 M sodium-cacodylate buffer (pH 7.4), dehydrated in a graded series of ethanol, and dried in tetramethylsilane to air. Specimens were sputter-coated with gold and examined with a Jeol 6310 SEM (Tokyo, Japan). Multiple micrographs were taken of cells on each nanopattern.

Image Analysis

For quantitative image analysis, samples were fixed in paraformaldehyde and stained with Methylene blue followed by examination with a Leica/Leitz DM RBE Microscope (Wetzlar, Germany) with magnification of 20x.
Methylene blue micrographs were analysed with Scion Image software (Beta Version 4.0.2, Scion Corp., Frederick, MY, USA). In several cases, due to low contrast between cell staining and background, the orientation was determined manually with a Falmouth Marine three-limb Course Protractor with a 5-minute accuracy. The orientation of fibroblasts on the different fields and patterns was examined and photographed. The criteria for cell selection were (1) the cell is not in contact with other cells and (2) the cell is not in contact with the image perimeter. The maximum cell diameter was measured as the longest distance between two edges within the cell borders. The

angle between this axis and the grooves (or an arbitrarily selected line for smooth surfaces) was termed the orientation angle. If the average angle was 45 degrees or more, cells were supposed to lie in a random orientation. Cell extensions like filopodia, which could confound the alignment measurement, were not included when assessing the cell orientation. For statistic analysis, cells oriented at <10° from the groove direction were considered to be aligned [15; 16]. The number of cells that were measured per sample group ranged from 24 to 290.

Statistical analysis:
Data acquired from the micrographs of cell alignment were analysed using SPSS for Windows (Release 12.0.1, SPSS Inc., Chicago, USA). The effects of, and the interaction between both time and surface were analysed using analysis of variance (ANOVA), including a modified least significant difference (Bonferroni) multiple range test to detect significant differences between two distinct groups. Probability (p) value ≤ 0.05 was considered to be significant.

Field	Pitch (nm, ratio 1:1)	Depth (nm ± SE)	Roughness (nm ± SE)
A	1000	37.8 ± 0.6	17.3 ± 0.2
B	600	37.3 ± 0.7	16.3 ± 0.1
C	400	34.6 ± 1.4	14.9 ± 0.6
D	300	33.8 ± 1.6	13.5 ± 0.1
E	200	30.6 ± 1.1	11.6 ± 0.1
F	160	30.5 ± 1.1	9.4 ± 0.1
G	100	17.9 ± 1.0	6.7 ± 0.4
H	80	11.0 ± 0.6	4.8 ± 0.1
I	60	7.8 ± 0.6	4.5 ± 0.3
J	40	4.4 ± 0.5	3.6 ± 0.1
K	1000	153.3 ± 2.7	69.1 ± 1.0
L	600	158.0 ± 3.2	64.4 ± 0.7
M	400	149.2 ± 1.2	53.9 ± 1.8
N	300	119.9 ± 2.6	36.7 ± 1.3
O	200	77.4 ± 4.1	22.0 ± 0.5
P	160	51.9 ± 3.4	15.3 ± 0.3
Q	100	17.2 ± 3.5	9.2 ± 0.7
R	80	15.3 ± 1.9	8.7 ± 0.9
S	60	11.6 ± 1.1	8.3 ± 0.1
T	40	10.9 ± 1.1	6.6 ± 0.4
μm-Groove	2000	353.9 ± 8.2	163.3 ± 10.5
Smooth	n/a	n/a	1.24 ± 0.14

Table 1. Feature dimensions of topographically patterned substrata.

RESULTS

Atomic force microscopy

In **Table 1**, the dimension, depth, and roughness as measured by AFM of the patterns in all polystyrene duplicates are given. The nano fields are designated A till T, while the microgrooved and smooth surfaces are designated by their own names. **Figure 1** shows a number of representative images. What can be seen in these images is, that although groove/ridge widths were fairly preserved at the top, at deeper levels the grooves became concave or even V-shaped. The smooth sample has no distinguishable pattern other than a RMS roughness of 1.2 ± 0.1 nm, occasionally a spike is present on the surface, probably the result of blemishes on the original wafer.

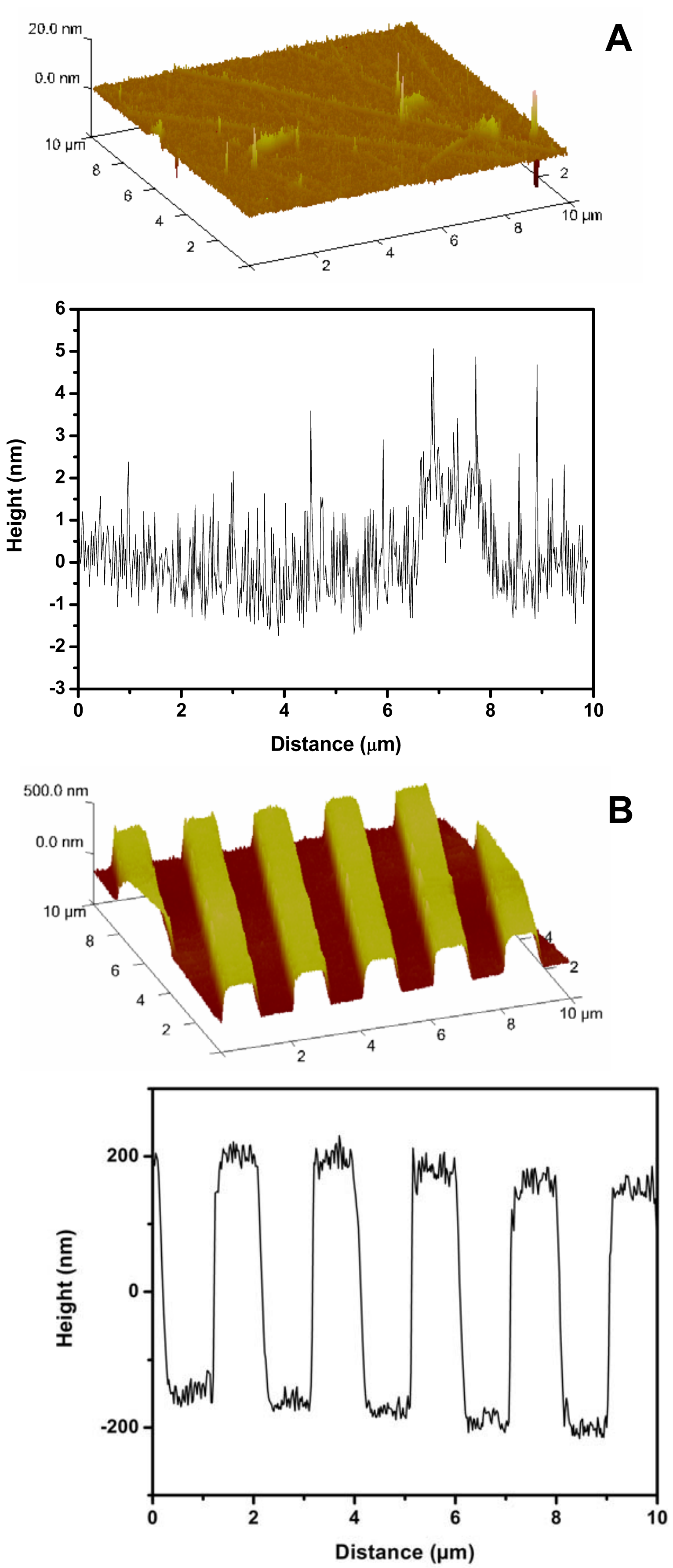
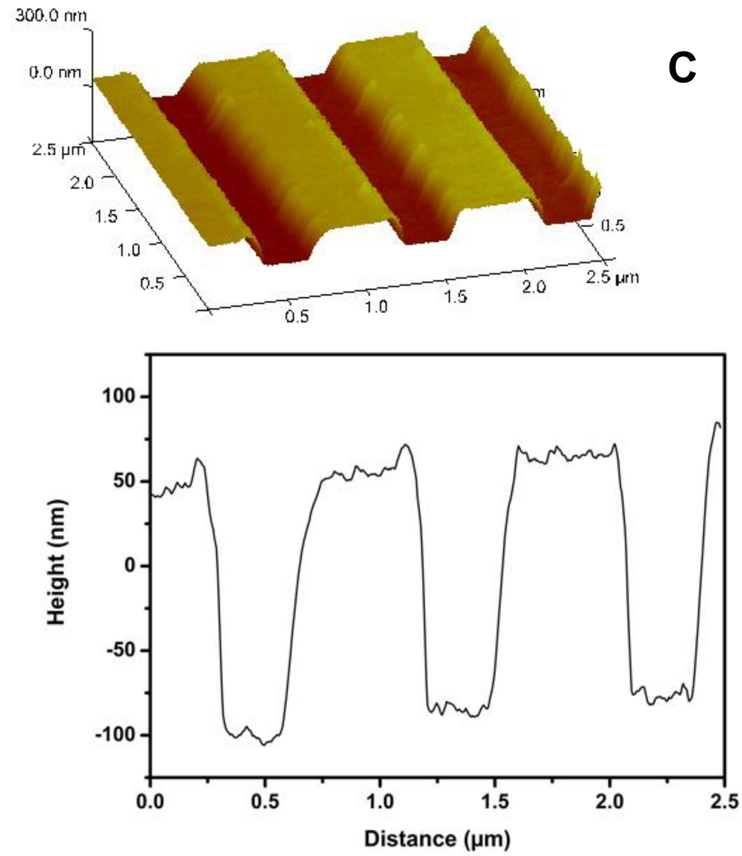
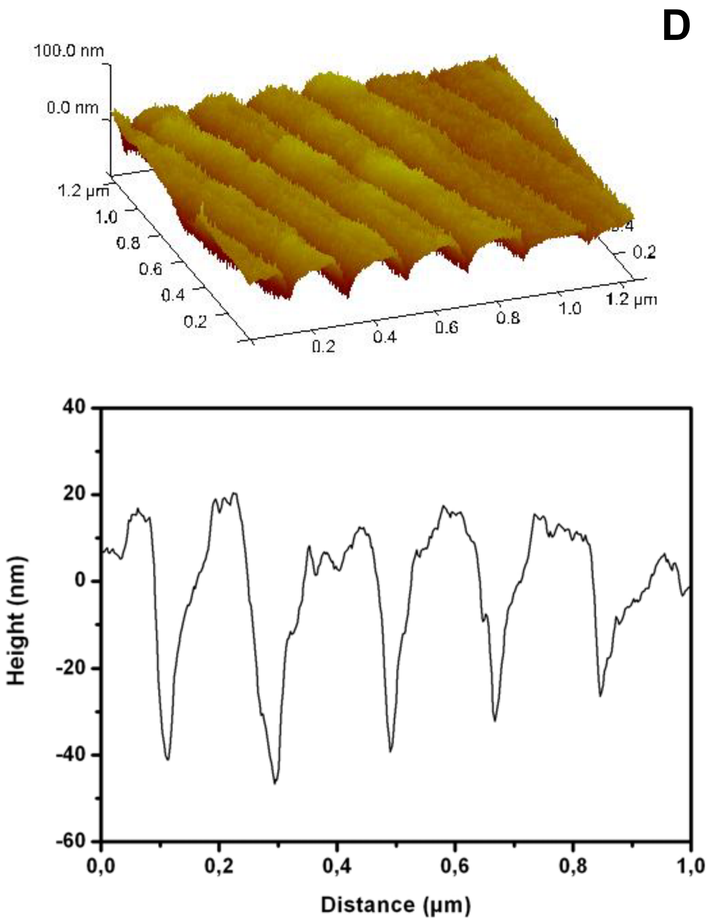


Figure 1. AFM graphs of topographies and height profiles of smooth (A), microgrooved (B), and nanogrooved substrates Field K and P (C and D, respectively).


C
300.0 nm
0.0 nm
2.5 µm
2.0
1.5
1.0
0.5
0.5
1.0
1.5
2.0
2.5 µm
0.5
Height (nm)
100
50
0
-50
-100
0.0
0.5
1.0
1.5
2.0
2.5
Distance (µm)


D
100.0 nm
0.0 nm
1.2 µm
1.0
0.8
0.6
0.4
0.2
0.2
0.4
0.6
0.8
1.0
1.2 µm
0.2
Height (nm)
40
20
0
-20
-40
-60
0,0
0,2
0,4
0,6
0,8
1,0
Distance (µm)

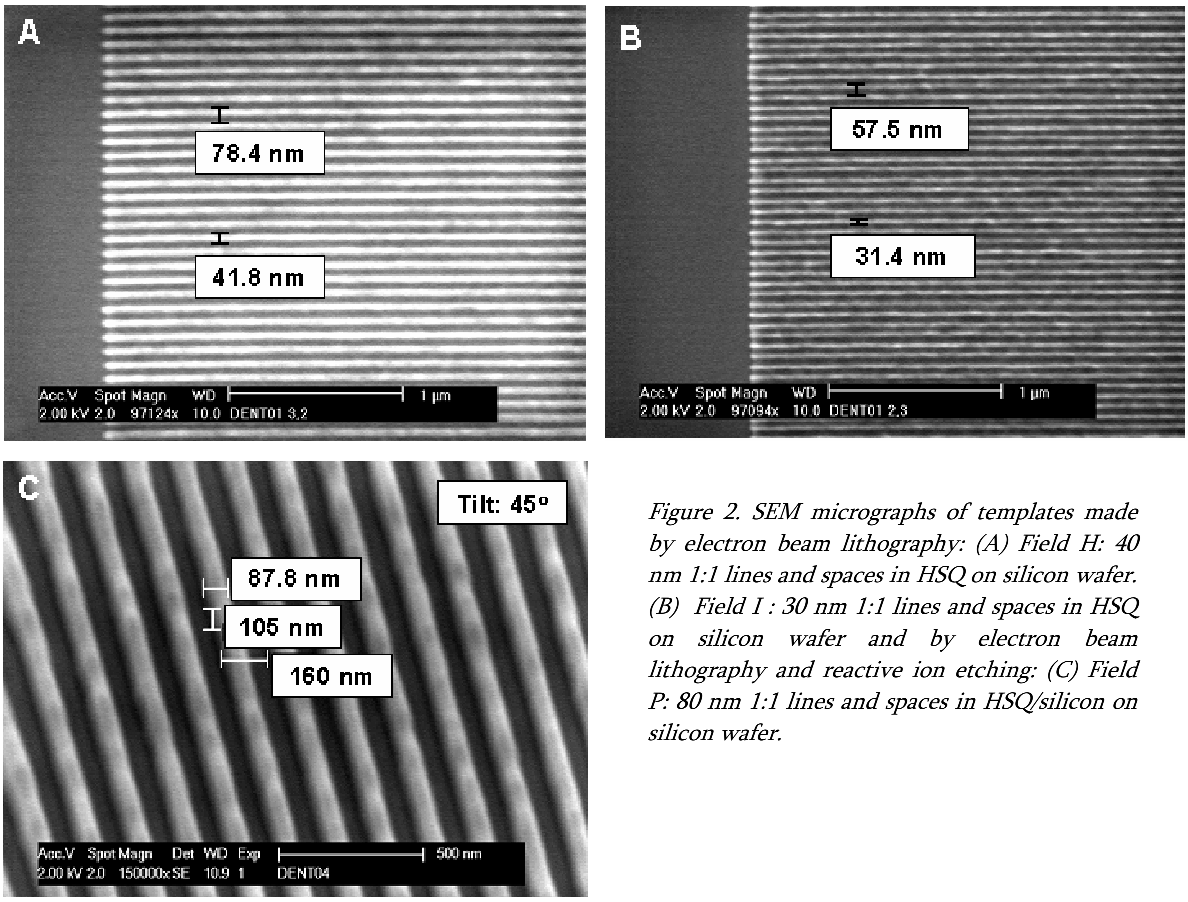


Figure 2. SEM micrographs of templates made by electron beam lithography: (A) Field H: 40 nm 1:1 lines and spaces in HSQ on silicon wafer. (B) Field I : 30 nm 1:1 lines and spaces in HSQ on silicon wafer and by electron beam lithography and reactive ion etching: (C) Field P: 80 nm 1:1 lines and spaces in HSQ/silicon on silicon wafer.

Scanning electron microscopy

Figure 2 shows a number of representative SEM graphs of the HSQ on silicon template structures as made by means of electron beam lithography (and Reactive Ion Ethching).

The various nano topographies were accurately replicated in the polystyrene substrata. When observing cell morphology, fibroblasts cultured on smooth substrata displayed a cell spreading which is considered random (**Figure 3A**), while an orientation along the groove direction was observed when cells are cultured on micro-groove substrata (**Figure 3B**). With decreasing pitch visual inspection made it clear that alignment was reduced. From field K to N, alignment of cells was still observed (**Figure 3C**), on fields O till Q however cellular orientation was observed only rarely. Fields with the smallest pitch values (R, S, and T) resulted in cell morphology undistinguishable from the cells growing on the planar surface surrounding the textured fields, or the smooth control samples (**Figure 3D**). Fibroblast alignment on fields A to J was virtually non-existent as opposed to, fields with the deeper groove depth (K, etcetera) that did elicit alignment. Interestingly, time had a beneficiary effect on cellular alignment; in all cases increased culture times (24 hrs vs. 4 hrs) lead to an increase in orientation and/or more fields triggering alignment on the cell population (Field A in **Figure 3E & F**).

Image analysis

Image analysis and subsequent statistical analysis performed confirmed that topography and culturing time were both significant factors in eliciting cellular orientation. **Figure 4** lists the various patterns and the mean angle of orientation they enticed to the fibroblasts. To designate the turning point after which alignment does not occur anymore, polynomic trend lines were drawn in the figures and the steepest parts were calculated.

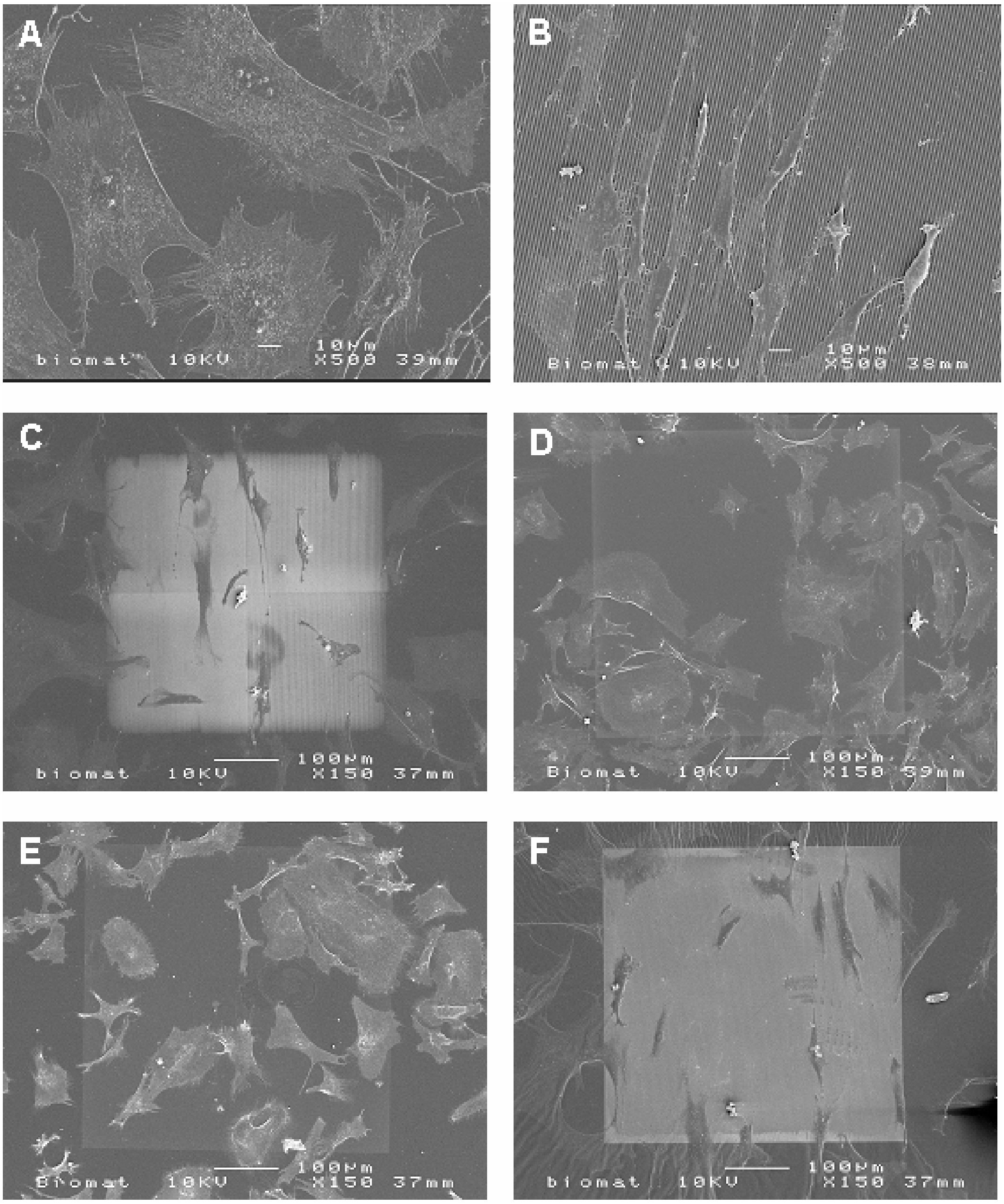


Figure 3. SEM micrographs of RDFs cultured under various conditions. (A) smooth substratum with cells in a random fashion, (B) 1 µm wide (350 nm deep) grooved substratum displaying aligned cells. (C) Field N (width 150 nm, depth 120 nm) still elicits cellular alignment to a majority of the cells compared to (D) Field R (width 40 nm, depth 15 nm) on which cells lay randomly spread. (E) Field A (width 500 nm, depth 35 nm) after 4 hours of culturing and Field A after 24 hours of culturing (F). All micrographs were taken from 24 hrs samples, unless otherwise stated.

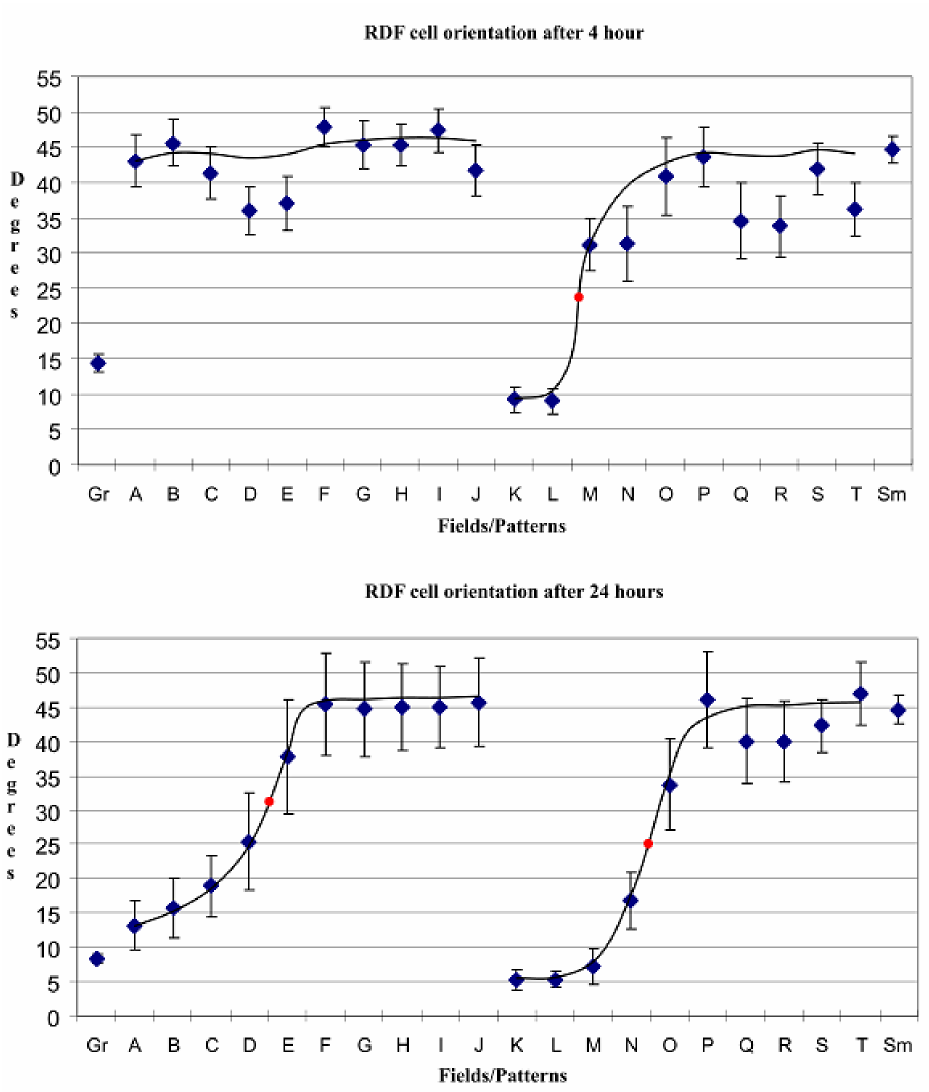


Figure 4. The mean angle and standard error of the mean showing the distribution of cellular orientation divided over time. (A) Cellular orientation after 4 hours culture shows alignment for the groove pattern and fields K and L. All other surfaces, smooth pattern and fields with small groove dimensions do not elicit orientation at this point. (B) After 24 hours cell alignment has appeared or improved on the several patterns and fields. Polynomic trend line in black with the turning point in red. Gr = 1 μm Groove pattern, Sm = Smooth pattern, nanometric Fields A through T (see also: Table 1).

Figure 4 presents these trend lines and illustrates the turning point between 23 and 31 degrees. Regarding topography; RDFs cultured on smooth substrata displayed a random cell spreading (average orientation angle around 45°), while those cultured on the 1 μm wide groove pattern showed a clear alignment along the grooves for 82% of the cells. Nanopatterns with depth 80 – 150 nm also resulted in cellular orientation on fields with larger pitch values (Fields K, L, M, and N). Nanopatterns with depths of 35 nm did not result in orientation of the fibroblasts at 4 hours, however when cultured for a prolonged period of time, the fields A and B did elicit cell alignment. Time played a key part in cell response towards the grooves; 24 hours groups showed improved alignment compared to 4 hours samples. As mentioned earlier, analysis of cells cultured on the deeper grooved nanopattern for 24 hours showed an orientation trend, which gradually decreased with decreasing pitch width. After 24 hours of culturing, the deep nanopattern resulted in enhanced alignment with the cut-off at 100 nm (Field O), after which cells appeared not to be aligned anymore.

Discussion

The aim of this study was to understand the morphological cell response of fibroblasts *in vitro*, particularly their orientation along a nanogroove pattern. Fibroblasts were cultured on polystyrene substrata, both smooth and grooved. In the latter case, both width and depth of the grooves were varied. The patterns were characterized and cell behavior was analyzed using microscopic techniques. From our data it can be concluded that RDFs adjust their shape according to nano topographical features down to a cut-off value of 100 nm width and a depth of 70 nm, which is a novel finding. Given sufficient culturing time, fibroblasts will even align themselves on groove depths as shallow as 35 nm, provided that ample ridge-surface (150 nm wide ridges) is available to the cells. It appears depth is the most essential parameter in cellular alignment on groove patterns with a pitch ratio of 1:1.

In order to obtain nanogroove patterns, electron beam lithography and reactive ion etching have been employed. In the latter technique, the phenomenon of diffusion limitation plays a role, especially in smaller grooves; the chemical reactants will insufficiently reach the bottom of the proposed design of the grooves, resulting in shallower depths or grooves becoming more concave. Simply increasing the etching time in RIE may result in the desired depth locally. However, this would lead to the top edges of the ridges enduring more etching, resulting in a reduced sharpness and somewhat convex and concave profiles of, respectively, the wafer ridges and the ensuing polystyrene duplicates. The characteristics of the actual wafers were not the focus of our study, as our main interest is in the substrata onto which the cells are cultured. In addition, polystyrene casting could be influenced by capillary forces elicited by the nano-grooves which may affect the reproduction accuracy, although literature data concerning imprint lithography techniques suggest that 20 nm details can easily be accomplished when pressing a mould into polymers [17; 18].

Another possible explanation for the concave appearance of the grooves, from fields E and O onwards, is the intrinsic limitation of AFM measurements related to tip convolution. Also, this phenomenon can have an effect on the reliability of the depth measurement. In order to minimize these effects during our AFM measurement, a high aspect ratio tip especially designed for scanning grooves was used.

Scanning electron microscopy, cell staining, and subsequent image analysis all confirmed that fibroblasts were oriented on nanogrooved surfaces. Because the pitch dimensions used on both nanopattern wafers were equal, the difference in rate of cellular orientation was predominantly determined by the groove depth. These results are in accordance with work of Clark *et al.* on micrometric textures [15; 16]. They concluded that groove depth is much more important in alignment of cells than the spacing of the grooves. Even when nano-scale patterns were used, an increase in groove depth led to better orientation [19]. Teixeira *et al.* [2], who cultured epithelial cells on patterned substrata showed an approximate 35% of cells aligned; this value could be the result of shallow ridge width (70 nm, pitch 400 nm) with appropriate depth (600 nm). However, it could also be that cell orientation is the result of both (or more) parameters. Current e-beam lithography permits the fabrication of large areas of features comparable in size to those found in fibrillar ECM. Individual collagen fibrils have diameters that are commonly in the range 20 – 100 nm although they often form larger aggregates [19; 20]. This study shows that fibroblast cells display meagre alignment on fields with ridge/groove widths of 100 nm or less. So, it is possibly a combination of factors that lead to cell guidance by the environment (be it substrata or tissue) rather than simply one parameter.

Similar to Teixeira's results and our own work [9], in this study it was observed that cells extend themselves while probing the substrata, since the grooves patterns are too narrow for the cells to get into contact with the bottom. The cellular extensions, probing the substrate surface, only found the ridges, resulting in extension of the cellular body along these ridges. Early responses of fibroblasts growing on textured substrata have been described before [21]; and cell orientation starts with the cell exploring the surroundings with the appearance of membrane extensions. Connective tissue cells need ECM in order to survive; since none is present, the aforementioned extensions are produced in all directions. Within hours, fitting anchor points are found and focal adhesion contacts are established with deposited ECM material, and the cell flattens and spreads, followed by the formation of filaments in the longitudinal direction [22; 23]. This study showed it was clear that cell alignment occurred immediately on Fields K, L, and 1 μm grooves during cell spreading and in accordance with the grooves. An alternative view concerning cell guidance on such small features is that not the increasing amount of ECM proteins with time, but the lag time between guidance on a small scale (filopodia, lamellapodia) and the influence on the entire cell might perhaps explain the increase in orientation [24; 25].

As explained earlier, having the ability to control cell behaviour is of great advantage in tissue engineering. And topography is just one method of gaining control. It is now clear that cells as a whole respond strongly to structured nano topography to a cut-off value of 35 nm. However, as Dalby *et al.* [25] pointed out for filopodia and to some extent lamellapodia, this value may be as low as 10 nm when random distributed “nano islands” are applied to induce interaction.

By using a pattern design which surface is large enough to extract quantitative data, future research will, besides visualisation of cell cytoskeleton components and quantification of the cell’s responses on a molecular level, look at the interaction of these groove dimensions and a dynamic force, like mechanical strain or fluid flow on cell conduct.

Conclusion

Since e-beam lithography is able to generate diverse patterns, a new approach was made possible to conduct research on cell behaviour on a wide variety of fields. This in turn allowed us to ascertain the cellular divide as presented in this study. The gradual decrease in dimension values allowed us to investigate in more detail the fibroblasts response towards topography. It is proposed that criteria concerning cellular orientation go beyond an arbitrary value, since those values are derived from measurements on specified topographies. By approaching natural dimensions these theoretical standards are no longer valid. Cellular alignment is triggered by a combination of ridge width and groove depth and most likely by other external (dynamic) factors as well, such as mechanical stress or compression. In this static study, design groove depths below 35 nm or ridge widths smaller than 100 nm do not result in fibroblast alignment.

It is concluded that fibroblast cells, cultured upon increasingly smaller nano-scale topography, experience, in accordance with our hypothesis, a decisive point where they no longer demonstrate contact guidance. This threshold seems to be at a 35 nm for whole cell alignment.

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