Available online at www.sciencedirect.com
ScienceDirect
Microelectronic Engineering 85 (2008) 1362–1366
MICROELECTRONIC ENGINEERING
www.elsevier.com/locate/mee

Manufacturing substrate nano-grooves for studying cell alignment and adhesion

F.C.M.J.M. van Delft [a,*], F.C. van den Heuvel [a], W.A. Loesberg [b], J. te Riet [b], P. Schön [b], C.G. Figdor [b], S. Speller [b], J.J.W.A. van Loon [c], X.F. Walboomers [b], J.A. Jansen [b]

[a] *Philips Research Europe, MiPlaza, High Tech Campus 4, 5656 AE Eindhoven, The Netherlands*
[b] *Radboud University, Nijmegen, The Netherlands*
[c] *DESC, OCB-ACTA, University of Amsterdam and Vrije Universiteit, Amsterdam, The Netherlands*

Received 21 September 2007; received in revised form 14 December 2007; accepted 10 January 2008
Available online 20 January 2008

Abstract

Nano-scale pattern templates have been manufactured in order to study the differences in cell behaviour between fibroblasts cultured on smooth and on grooved substrata. The pattern templates were made on silicon wafers using electron beam lithography in hydrogen silsesquioxane (HSQ) and subsequent reactive ion etching (RIE). These masters were replicated in polystyrene cell culture material using solvent casting. The replicas were assessed with atomic force microscopy (AFM). After seeding with fibroblasts, morphological characteristics were investigated using scanning electron microscopy (SEM) and light microscopy, in order to obtain qualitative and quantitative information on cell alignment. It appears that both groove depth and width determine the cellular alignment on patterns with a ridge/groove ratio of 1:1. On smooth substrata, cells always spread out in a random fashion. There appears to be a threshold groove barrier size of around 70–80 nm, above which random cell spreading is not possible anymore and contact guidance occurs. It is speculated that this threshold size may be associated with the size of contact molecules at the cell extensions, which grow and find anchoring spots preceding cell spread out and cell alignment.

Keywords: Nanotechnology; Electron beam lithography; HSQ; RIE; Template; Polystyrene; Fibroblast; Cell orientation

1. 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 (altered morphology, orientation, adhesion, or gene regulation). The cellular response to a biomaterial may be enhanced by mimicking the nano-scale surface topography formed by the extra cellular matrix (ECM) components of natural tissue [1]. Previous studies have already addressed micrometer scale topography, and Teixeira et al. have investigated cell behaviour on ridges 70 nm wide, with a pitch of 400 nm and a depth of 600 nm and found cellular alignment along these grooves [2]. Previous in vitro research has shown that nano-columns, produced by colloidal lithography or polymer de-mixing, caused changes in cell morphology, filopodia production, migration, and cytokine release [3]. From the latter studies, it has become clear that topography in the nano-meter scale may be of importance in cell guiding. Despite the amount of control over the dimensions created by colloidal lithography and polymer de-mixing, however, these techniques remain largely random with respect to the placement and orientation of features. Nano-groove patterns with pitches less than 100 nm thus far have not yet been studied and it is unknown to what extent cells will sense and adapt their morphology to an ordered topography if the dimensions become exceedingly small.

* Corresponding author. Tel.: +31 40 2743124; fax: +31 40 2745002.
E-mail address: Falco.van.Delft@philips.com (F.C.M.J.M. van Delft).

0167-9317/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.mee.2008.01.028

In this study, cellular behaviour on nano-groove topography with a 1:1 pitch ratio has been investigated in order not to deviate too far from previously used patterns [4–7]. It was our hypothesis that, if the topography is small enough, a cellular "point break" is reached, where cells no longer display contact guidance along nano-groove patterns. In order to verify this hypothesis, cell responses to such nano-topography fields have been investigated from a morphology point of view, using light microscopy and scanning electron microscopy, and subsequent image analysis.

2. Experimental

Silicon wafers (6 in.) 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 $500 \times 500\ \mu m^2$ containing a.o. 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) during 2 min, rinsed in 1:9 v:v TMA238WA:H_2O (for 5 s), rinsed in demineralised water (5 s) and blown dry with N_2 [8,9]. For obtaining higher master structures, the e-beam patterned HSQ was used as a mask in a standard reactive ion etching (RIE) process for silicon in a Surface Technology Systems (STS) multiplex RIE cluster tool. SEM graphs of the HSQ master structures were made using a Philips XL40 FEG-SEM. 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 [10]. Surface topography was quantitatively evaluated using a Dimension atomic force microscope (AFM; Dimension 3100, Veeco, Santa Barbara, CA) [11]. 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°. The nominal radius of curvature of the tip was less than 10 nm. 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 using 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 the depth (nm) were obtained and averaged of three random fields per substrate.

The 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 min at a pressure of 2.0×10^{-2} mbar (Harrick Scientific Corp., Ossining, NY, USA) in order to promote cell attachment by improving the wettability of the substrata.

Rat dermal fibroblasts (RDF) were obtained from the ventral skin of male Wistar rats as described by Freshney [12]. Cells were cultured on the replicas in an incubator set at 37 °C with a humidified atmosphere, as described in detail by Loesberg et al. [11]. To asses overall morphology of the fibroblasts, also SEM was performed in a JEOL 6310 (Tokyo, Japan) [11].

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) at a magnification of 20×. 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°, 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.

Detailed information on the statistical analysis of the cell alignment can be found in Ref. [11,13,14].

3. Results and discussion

Fig. 1a shows a representative SEM graph of the HSQ on silicon template structures as made by means of electron beam lithography. Fig. 1b shows a template obtained after subsequent RIE of silicon. Fig. 1c and d show an example of an AFM measurement of a replicated polystyrene substrate, featuring non-rectangular profiles. As is generally known, diffusion limitation in the smallest grooves during RIE can result in shallower depths or grooves becoming more concave. The characteristics of the actual wafers have not been investigated thoroughly, as our main interest is in the substrata the cells are cultured on. 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 [15,16]. Another possible explanation for the concave appearance of the grooves 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

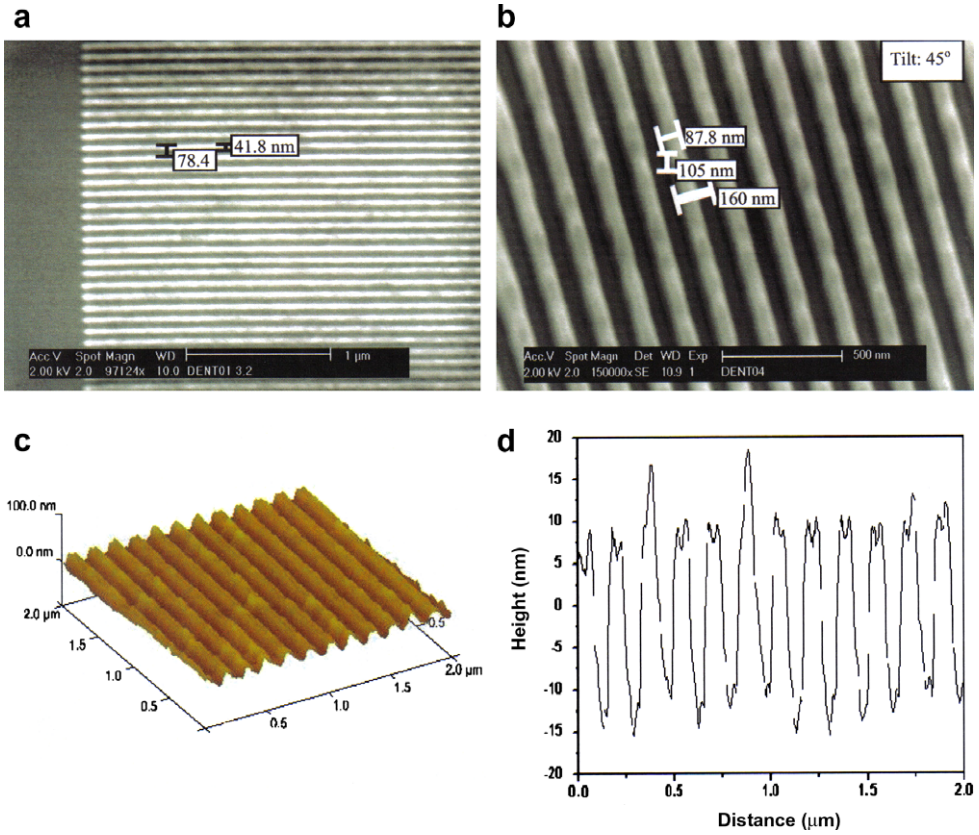

Fig. 1. (a) SEM graph of 40 nm 1:1 lines and spaces in HSQ on Si, (b) SEM graph of 80 nm 1:1 lines and spaces after RIE in Si, (c) AFM graph of 80 nm 1:1 lines and spaces in polystyrene replica, and (d) its matching height measurement cross section.

during our AFM measurements, however, AFM cantilevers with the smallest possible scanning tip radius in combination with a high aspect ratio have been employed. Grooves with aspect ratios of up to 3 should be measurable for >50 nm width, and up to 1.5 for widths >30 nm. Over the whole range measured here, the aspect ratio never surpassed 1, suggesting that the AFM measurements were not significantly influenced by convolution phenomena. In our AFM measurements the smooth reference substrata showed no distinguishable features other than 1 nm roughness amplitudes.

After seeding the replicas with fibroblasts, morphological characteristics were investigated using scanning electron microscopy (SEM) and light microscopy, in order to obtain qualitative and quantitative information on cell alignment. Microscopy and image analysis showed that fibroblasts after 4 h had adjusted their shape according to nano-topographical features down to cut-off values of 100 nm width and 70 nm depth.

In Fig. 2, SEM graphs are shown of fibroblasts after 24 h culturing time; in this case, fibroblasts would even align themselves on grooves of 35 nm depth and 200 nm width. It appears that both depth (d) and width (w) determine the cellular alignment on groove patterns with a ridge/groove ratio of 1:1, as shown in Fig. 3a. On the smooth substrata, cells always spread out in a random fashion, resulting in a mean orientation angle $\theta = 45°$. Note that full alignment alongside the grooves would result in $\theta = 0°$, and an orientation perpendicular to the grooves would result in $\theta = 90°$. In Fig. 3b, the orientation parameter $\cos(2\theta)$ (giving 0 for random orientation, 1 for full alignment, and -1 for perpendicular alignment) is shown as a function of the groove barrier size $(d \cdot w)^{0.5}$; the groove barrier size can be used as a sort of molecular yard-stick. There appears to be a threshold groove barrier size of around 70–80 nm, above which random cell spreading is not possible anymore and contact guidance occurs. It is speculated (in Fig. 3c) that this threshold size may be associated with the size of contact molecules (like vitronectin and fibronectin) at the cell extensions, which grow and find anchoring spots preceding cell spread out and cell alignment. In case the grooves are too deep and too wide, it is energetically unfavourable for the contact molecules to descend to the bottom of the groove or to cross the groove; as a consequence, the cell extensions will predominantly grow in the ridge direction and, hence, eventually the cell is also aligned in the ridge direction.

Current e-beam lithography permits the reproducible fabrication of 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 [17,18]. This study shows that fibroblast cells display meagre alignment on fields with ridge/groove widths much less than 100 nm.

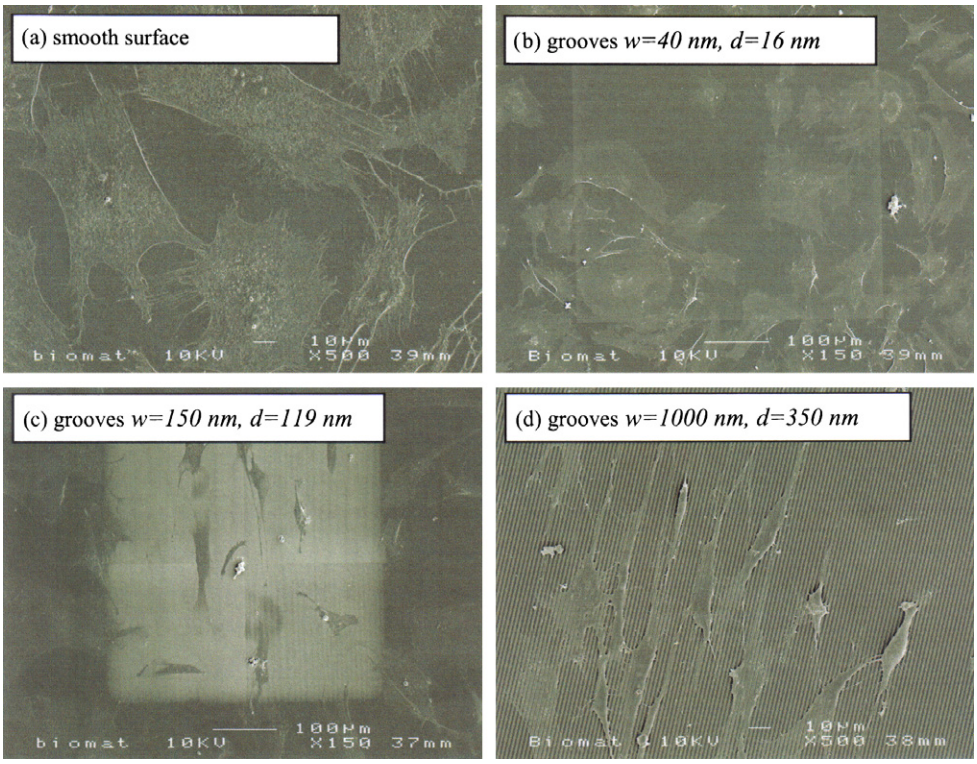

Fig. 2. SEM graphs of Fibroblast cell orientation after 24 h: (a) smooth surface, (b) grooves $w = 40$ nm, $d = 16$ nm, (c) grooves $w = 150$ nm, $d = 119$ nm, and (d) grooves $w = 1000$ nm, $d = 350$ nm (made by photolithography and etching in a silicon wafer [4]).

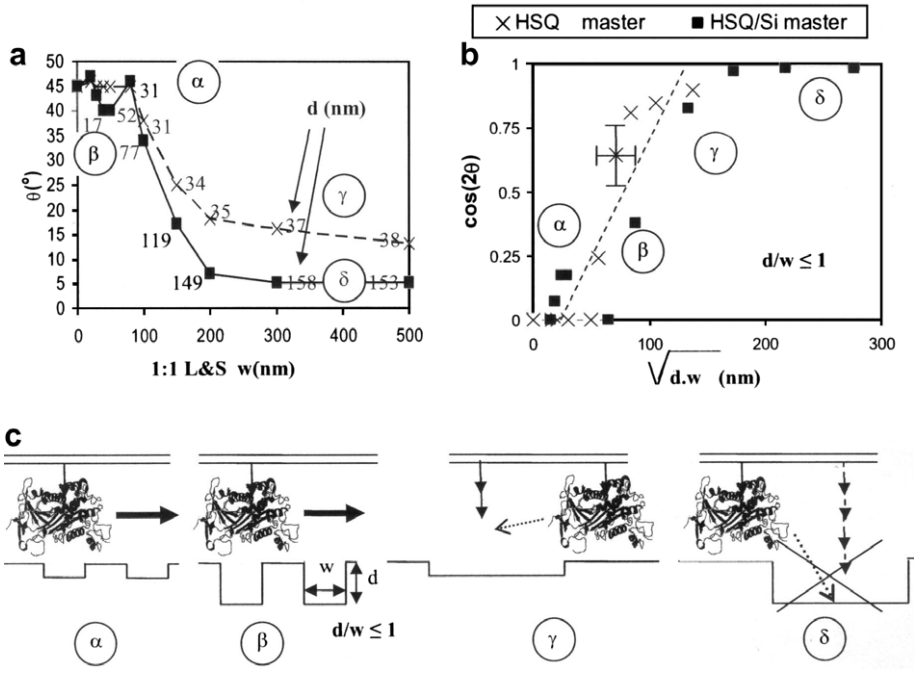

Fig. 3. (a) Fibroblast 24 h mean orientation angle θ as a function of groove width (w) and depth (d); (b) Orientation ($\cos 2\theta$) as a function of groove barrier size $(d \cdot w)^{0.5}$ for $d/w \leqslant 1$; estimated error bars for the orientation- and groove barrier size-parameters are indicated; (c) Cross section of grooves and contact molecules; for narrow grooves: no barrier $\Rightarrow$ no contact guidance (α, β), for wider/deeper grooves: barrier sensed $\Rightarrow$ contact guidance (γ, δ).

4. Conclusions

It is concluded that fibroblast cells, cultured upon increasingly smaller nanoscale topography, experience, in accordance with our hypothesis, a decisive point where they no longer demonstrate contact guidance. This point seems to be around a 70–80 nm threshold.

Acknowledgements

SEM on fibroblast morphology was performed at the Microscopic Imaging Centre (MIC) of the Nijmegen Centre for Molecular Life Sciences (NCMLS), The Netherlands. S.S. acknowledges Financial support by NanoNed, the Dutch nanotechnology programme of the Ministry of Economic Affairs.

References

[1] D.C. Miller, A. Thapa, K.M. Haberstroh, T.J. Webster, Biomaterials 25 (2004) 53–61.
[2] A.I. Teixeira, G.A. Abrams, P.J. Bertics, C.J. Murphy, P.F. Nealey, J. Cell. Sci. 116 (2003) 1881–1892.
[3] M.J. Dalby, M.O. Riehle, D.S. Sutherland, H. Agheli, A.S. Curtis, Eur. Cell. Mater. 9 (2005) 1–8 (discussion 8).
[4] X.F. Walboomers, H.J. Croes, L.A. Ginsel, J.A. Jansen, Biomaterials 19 (1998) 1861–1868.
[5] E.T. den Braber, J.E. de Ruijter, H.T. Smits, L.A. Ginsel, A.F. von Recum, J.A. Jansen, Biomaterials 17 (1996) 1093–1099.
[6] D.M. Brunette, B. Chehroudi, J. Biomech. Eng. 121 (1999) 49–57.
[7] W.A. Loesberg, X.F. Walboomers, J.J. van Loon, J.A. Jansen, J. Biomed. Mater. Res. A (2005) 723–732.
[8] F.C.M.J.M. van Delft, J.P. Weterings, A.K. van Langen-Suurling, J. Romijn, J. Vac. Sci. Technol. B 18 (6) (2000) 3419–3423.
[9] F.C.M.J.M. van Delft, J Vac Sci Technol B 20 (6) (2002) 2932–2936.
[10] K.D. Chesmel, J. Black, J. Biomed. Mater. Res. 29 (1995) 1089–1099.
[11] W.A. Loesberg, J. te Riet, F.C.M.J.M. van Delft, P. Schön, C.G. Figdor, S. Speller, J.J.W.A. van Loon, X.F. Walboomers, J.A. Jansen, Biomaterials 28 (2007) 3944–3951.
[12] R.I. Freshney, Culture of Animal Cells: A Multimedia Guide, John Wiley & Sons Ltd., Chichester, 1999.
[13] P. Clark, P. Connolly, A.S. Curtis, J.A. Dow, C.D. Wilkinson, Development 99 (1987) 439–448.
[14] P. Clark, P. Connolly, A.S. Curtis, J.A. Dow, C.D. Wilkinson, Development 108 (1990) 635–644.
[15] J. Haisma, M. Verheijen, C.A. van den Heuvel, J. van den Berg, J. Vac. Sci. Technol. B 14 (6) (1996) 4124–4128.
[16] S.Y. Chou, P.R. Krauss, P.J. Renstrom, J. Vac. Sci. Technol. B 14 (6) (1996) 4129–4133.
[17] P. Clark, P. Connolly, A.S. Curtis, J.A. Dow, C.D. Wilkinson, J. Cell. Sci. 99 (Pt 1) (1991) 73–77.
[18] G.A. Dunn, J.P. Heath, Exp. Cell. Res. 101 (1976) 1–14.