Microconical silicon structures influence NGF-induced PC12 cell morphology†

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Abstract

Micro- and nanofabrication techniques provide the opportunity to develop new types of cell culture platform, where the effect of various topographical cues on cellular functions such as proliferation and differentiation can be studied. In this study, PC12 cells were cultured on patterned silicon (Si) surfaces comprising arrays of microcones (MCs) exhibiting different geometrical characteristics and surface chemistries. It was illustrated that, in the absence of nerve growth factor (NGF), PC12 cells increased proliferation on all types of patterned surface, as compared to flat Si surfaces. However, in the presence of NGF, PC12 cells showed different responses, depending on the plating surface. Unlike low and intermediate rough MC surfaces, highly rough ones exhibiting large distances between MCs did not support PC12 cell differentiation, independently of the MCs’ chemical coatings. These results suggest that the geometrical characteristics of MCs alone can influence specific cellular functions. Tailoring of the physical properties of arrays of Si MCs in order to identify which combinations of MC topologies and spatially defined chemistries are capable of driving specific cellular responses is envisaged. © 2013 The Authors. Journal of Tissue Engineering and Regenerative Medicine Published by John Wiley & Sons, Ltd.

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1. Introduction

Since the beginning of the nineteenth century, when R. G. Harrison established the methodology of tissue culture (Abercrombie, 1961), continuous research has improved not only solutions and environmental conditions for specific cell growth but also solid platforms supporting cell attachment, passing from the two-dimensional (2D) era to three-dimensional (3D) technology. Although the practice of cell culture remains overwhelmingly based on 2D substrates, there is increasing research on the use of 3D scaffolds aimed to provide topographical cues mimicking the natural environment of cells. With the emergence of micro- and nanofabrication techniques, a plethora of approaches to engineering surfaces in a controllable manner are now available (Andersson and Berg, 2004; Park and Shuler, 2003; Voldman et al., 1999). Using techniques such as photolithography, microcontact printing, microfluidic patterning, electrospinning and self-assembly, 3D topographical features of tailored geometry, roughness and orientation, complemented by the desired spatial resolution at micron and submicron scales, can be realized on materials surfaces (Norman and Desai, 2006; Stevens and Georg, 2005). Patterning of culture surfaces has triggered the development of new types of cell culture platform, where the effect of topographical cues on cellular responses can be investigated (Bettiger et al., 2009; Flemming et al., 1999; Martínez et al., 2009).

In addition, precise 3D micro/nanotexturing of biomaterials provides an unprecedented means towards the
manipulation of cell fate. Research over the last decade has shown that biomaterial architecture can drive cellular response via physical and chemical extracellular signals, including topographical and chemical cues at the micro- and/or nanoscales (Lamour et al., 2009; Lee et al., 2011; Psycharakis et al., 2011; Wilkinson et al., 2002), mechanical properties of the substrate (Engler et al., 2006; Mih et al., 2011) and adhesion ligands (Maheshwari et al., 2006; Zhang et al., 1999), essential to defining cell phenotype. Such capability of programming multiple cell instructive cues via 3D structuring is of particular importance in regenerative medicine, where implantable scaffolds actively participate in tissue formation. Besides this, there is great potential to take advantage of biomaterials micro-/nanostructuring, particularly in applications related to biological systems, where cell polarity and organization into a functional network are required.

A prominent example involves the nervous system that is wired up in a unique way via the dynamic formation of elaborate networks in order to ensure intra- and intercommunication signals within the organism. The neuron, which is one of the basic units of the nervous system, elongates and reaches its synaptic target, guided by its growth cone, following multiple guidance signals (Goldberg, 2003; Tessier-Lavigne and Goodman, 1996). In this way, neuronal processes are extended and branched and synapses are generated. For in vitro studies, in the absence of exogenous growth factors and signalling molecules, different topographical features may influence neuronal growth and serve as guidance cues (Franze and Guck, 2010). To this extent, topography could control the directionality of neurite growth and determine the polarity of neuronal junctions. Identification of morphologies that elicit specific neuronal responses will enable the design of intelligent biomedical materials or devices that exploit topographical features to stimulate vital cell functions.

Indeed, there is a growing body of literature demonstrating the effects of artificial micro- and nanotopographical features on nerve cell morphology (Corey et al., 2007; Hoffman-Kim et al., 2010; Klinkhammer et al., 2010; Stenger et al., 1998; Wang et al., 2010), growth and differentiation (Bédouer et al., 2012; Hsu et al., 2009; Jiang et al., 2012; Melissinaki et al., 2011). Silicon-based microfabricated culture substrates with well-defined topographies have been extensively developed in order to create neuronal interfaces for a variety of applications (Pearce and Williams, 2007). Representative examples include the development of grooved surfaces for the study of polarity (Ferrari et al., 2011; Foley et al., 2005), microfluidic culture platforms for the study of biochemical functionality of different nerve cell compartments in vitro (Park et al., 2009; Taylor et al., 2005), and multielectrode devices (neurochips) to monitor the electrical activity of functional neuronal networks (Maher et al., 1999; Merz and Fromherz, 2005).

Concentrating on neuronal cell growth and differentiation, the present study aimed to investigate neuritogenesis of PC12 pheochromocytoma cells on microstructured Si substrates, fabricated by femtosecond laser direct writing. Microstructuring by ultra-short pulsed lasers is an especially attractive approach, because it leads to the formation of arrays of high-aspect ratio microcones (MCs) on solid surfaces. In particular, femtosecond lasers allow patterning through non-linear absorption processes, providing excellent control over the regularity and uniformity of 3D micron and submicron features (Stratakis, 2012). Such MCs present a relatively new type of topographical cue, intended to test the effect of surface architecture on cellular development and morphology, beyond the well-studied horizontal (grooves and ridges) (Cecchini et al., 2007; Mahoney et al., 2005; Taylor et al., 2005) or vertical (pillars, pores) (Bucaro et al., 2012) to the substrate plane features. We have previously shown that MC-patterned surfaces could be used as cell culture platforms for the systematic exploitation of MC morphology on neuron cell adhesion and growth (Papadopoulou et al., 2010). In addition, by controlling micro-/nanomorphology and surface hydrophilicity, MC substrates may constitute a promising culture platform for studying the effect of topography on cellular differentiation and neurite outgrowth of PC12 cells, which upon treatment with nerve growth factor (NGF) differentiate into sympathetic neurons (Greene and Tischler, 1976). The findings presented here show that PC12 cell morphology could be influenced by MC spacing and geometry. The exploitation of the MC arrays for culturing desired cell populations, programming their shape and prescribing their fate, is envisaged.

2. Materials and methods

2.1. Fabrication of microconical structures

Single crystal n-type Si (1 0 0) wafers were subjected to laser irradiation in a vacuum chamber evacuated down to a residual pressure of $10^{-2}$ mbar. A constant SF$_6$ pressure of 500 Torr was maintained during the process through a precision microvalve system. The irradiating laser source was constituted by a regenerative amplified Ti:Sapphire ($\lambda = 800$ nm), delivering 150 fs pulses at a repetition rate of 1 kHz. The sample was mounted on a high-precision X-Y translation stage normal to the incident laser beam. The laser fluence used in these experiments was in the range 0.68–1.50 J/cm$^2$. In order to evaluate cell behaviour under identical cell culture conditions, each MC-structured Si surface ($5 \times 5$ mm) contained three bands, each fabricated at a different laser fluence value.

After laser irradiation, the microstructured surfaces were morphologically characterized by scanning electron microscopy (SEM). An image-processing algorithm (ImageJ, National Institutes of Health, Bethesda, MD, USA) was implemented in order to determine the topological characteristics of the MCs, including height ($h$), base diameter ($d$), aspect ratio ($A$) and roughness ratio ($r$) from top, side-view and cross-sectional SEM images (Table 1). The aspect ratio was calculated by dividing the height by the radius of the cone’s base. The roughness ratio, $r$, was calculated by dividing the actual, unfolded,
surface area of spikes by the total irradiated area. The mean value was calculated from statistics performed at 10 individual surfaces in each case. The data were subjected to one-way ANOVA, followed by Tukey test for multiple comparisons between pairs of means.

Static contact angle measurements were performed using an automated tensiometer, using the sessile drop method. A 2 μl distilled, deionized Millipore water droplet was gently positioned on the surface, using a microsyringe, and images were captured to measure the angle formed at the liquid–solid interface. The mean value was calculated from at least five individual measurements. Successive measurements were reproducible within ± 1°.

In some cases, the surface chemistry of freshly prepared patterned surfaces was altered by a thermally grown, hydrophilic oxide layer cover. Such a layer is known to form high-quality conformal coatings on Si surfaces. For oxidation, the samples were placed in a box furnace and heated at 1000°C for 30 min in air.

### 2.2. Substrate coating

In some cases, substrates were immersed in 0.01% type I collagen (Sigma-Aldrich Chemie GmbH, Munich, Germany) for 2 h at room temperature (RT) and washed in phosphate-buffered saline (PBS) prior to culture initiation. Electron microscopy analysis showed that protein deposition conformed to the underlying topography on the substrates and did not bridge spikes (data not shown). The coated or non-coated substrates were placed directly in 48-well cell culture plates (Sarstedt, Neurberg, Germany).

### 2.3. Cell cultures

The rat pheochromocytoma PC12 cell line (ATCC-LGC, Rockville, MD, USA) was maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Grand Island, NY, USA) supplemented with 10% horse serum (HS; Invitrogen, Carlsbad, CA, USA) and 5% fetal bovine serum (FBS; Biosera, Sussex, UK) (complete medium) at 37°C in a 5% CO2 atmosphere. In order to minimize interassay variability, low passage number (P2–P3) cells were used throughout the study. The collagen-coated MC-structured Si surfaces were placed in 48-well plates and covered with PC12 cells at a density of 0.75 × 10^5 cells/ml. The cells were allowed to adhere in complete culture medium and 24 h later this was either replaced by fresh complete medium or with differentiation medium (DMEM supplemented with 50 ng/ml NGF; 2.5 S, Millipore, Billerica, MA, USA). Complete or differentiation medium was replaced every 2 days. PC12 cells were also grown on collagen-coated flat silicon wafers (negative control condition). Standard polystyrene (PS) tissue culture coverslips (positive control; Sarstedt) were used in order to monitor the progress of the cell culture, since Si substrates are not optically transparent and therefore cannot be viewed via a light microscope. A minimum of three replicates were used for each experiment.

### 2.4. Cell counts

PC12 cells were cultured on control or MC-structured Si surfaces for 4 or 7 days, as described in Section 2.3. Upon culture termination, cell numbers on control or three-band laser-patterned Si substrates were evaluated by counting cell nuclei stained with DAPI reagent. Thus, cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich Chemie GmbH) for 15 min and permeabilized with 0.1% Triton X-100 (Merck KgaA, Darmstadt, Germany) in PBS for 3–5 min. The samples were then washed with PBS and mounted on coverslips with antifade reagent containing DAPI for the staining of nuclei (ProLong® Gold reagent; Molecular Probes, Eugene, OR, USA). Cell imaging was performed using an epifluorescence microscope coupled to a high-resolution Carl Zeiss AxioCam colour camera. Cell number quantification was assessed using an image-processing algorithm (ImageJ).

### 2.5. Immunocytochemical assays

PC12 cells were double-stained for F-actin and β-tubulin. After 4 and 7 days of culture, the cells were fixed with 4% PFA for 15 min and permeabilized with 0.1% Triton X-100 in PBS for 3–5 min. The non-specific binding sites were blocked with 2% BSA in PBS for 30 min. The neuron-specific βIII isoform of tubulin was detected by incubating the cells with the MAB1637 monoclonal antibody (1:900 in PBS–BSA 2%; Millipore, MA, USA) for 1 h.
at RT and subsequent labelling with goat-anti-mouse FITC conjugate secondary antibody (1:200 PBS–BSA 2%; Biotium, USA) for 45 min at RT. Simultaneously, the cells were incubated with tetramethyl rhodamine isothiocyanate-conjugated phalloidin (1:400 in PBS–BSA 2%; Biotium) towards the F-actin staining. The samples were then washed with PBS and mounted on coverslips with antifade reagent containing DAPI for nuclei staining.

Cell imaging was performed using an epifluorescence microscope coupled to a high-resolution Carl Zeiss AxioCam colour camera, while confocal microscopy analysis was performed using a Zeiss AxiosKop 2 plus laser scanning confocal microscope; ×10, ×20 and ×40 objectives were used.

## 2.6. Scanning electron microscopy

The morphology of PC12 cells growing on the patterned surfaces was analysed by SEM. After culture termination, the cells were washed with 0.1 M sodium cacodylate buffer (SCB) and then incubated in the same solution for 15 min. After repeating this step twice, the cells were fixed using 2% glutaraldehyde, 2% formaldehyde in 1% SCB fixative buffer for 1 h at 4°C. All surfaces were then washed twice (for 15 min each time) with 1% SCB at 4°C, dehydrated by immersion in serially graded ethanol solutions (50–100%) and incubated for 15 min in dry 100% ethanol. Prior to electron microscopy examination, the samples were sputter-coated with a 10 nm gold layer. SEM analysis used a JEOL 7000 field emission scanning electron microscope at an acceleration voltage of 15 kV.

## 2.7. Image processing

Quantitative information (regarding cell number, neurite length, etc.) was assessed using an image-processing algorithm (ImageJ). Cell numbers and neurite length were measured manually. The number of differentiated cells was determined by visual examination of the field. A differentiated cell was considered to display at least one neurite with a length equal to the cell body diameter. The differentiation ratio was defined as the percentage of differentiated cells after treatment in the presence of NGF over the total number of cells in the field. Neurite growth was determined by manually tracing the contour length of the longest neurite per cell for all cells in a field that had an identifiable neurite. Neurite length was the distance from the edge of the cell soma to the neurite tip. The mean length of the longest neurite per cell in each band of the microstructured area (low, medium or high roughness) was calculated.

## 2.8. Statistical analysis

For statistical analysis, the data were subjected to one way ANOVA followed by Tukey tests for multiple comparisons between pairs of means, using commercially available software (SPSS 21, IBM).

## 3. Results

### 3.1. Fabrication of microstructured Si surfaces

Nerve cells, more than any other type of cells, expand in complicated spatial environments that cannot be facilitated by planar culture substrates. In order to study the effect of platform architecture on nerve cell growth and differentiation, ultra-fast pulsed laser structuring was applied on crystalline Si wafers. This technique offers the advantage of patterning planar surfaces with periodic arrays of topographical features of microscale size, while offering high accuracy and reproducibility (Stratakis, 2012).

In tandem with morphology, surface chemistry can be additionally controlled through proper variation of the laser energy per unit area (fluence), leading to substrates exhibiting different roughness ratios and wettabilities. At low laser fluences, the irradiated surfaces comprised submicron-sized ripples, while at increased laser energy, quasi-periodical arrays of conical MCs (i.e. spikes) were formed (Figure 1B). The enhancement in MC roughness was accompanied by a decrease in surface hydrophilicity (Figure 1C).

The above methodology, compared to traditional planar surfaces, provides reproducibly patterned substrates exhibiting 3D surface characteristics, thus offering an additional parameter to control cell growth and network formation. In this study, three types of patterned surfaces exhibiting different MC morphologies were fabricated using three different laser fluence values. As shown in Figure 1, each culture substrate consisted of these three microstructured areas, irradiated using 0.68 J/cm² (low roughness), 0.95 J/cm² (medium roughness) and 1.50 J/cm² (high roughness), interspaced by an unstructured area (Figure 1A). Thus, each scaffold contained all three types of patterned surfaces, allowing direct comparison of nerve cell growth preferences. As calculated from SEM images, MCs height varied from 1.26 ± 0.28 μm in the low-roughness structures to 8.63 ± 1.17 μm in the high-roughness structures (Table 1). While spike density was lower in the high-roughness structures, the MCs height increased. It is also interesting to note that, although medium to higher roughness MCs maintained the same aspect ratio, their interspike distance increased in a geometrically justifiable manner (Figure 1D, Table 1). Indeed, comparing two series of conical spikes with the same aspect ratio but different heights, it is apparent that when MCs height increases by a factor of two, the interspike distance doubles as well.

In this study, three types of patterned substrate were tested: type A (A1–A3) corresponds to the structured Si substrates described above; type O (O1–O3) corresponds to substrates coated with an inorganic thermal oxide layer; while type AC (AC1–AC3) corresponds to type A substrates immersed in collagen I solution, which is known to facilitate nerve cell growth. As confirmed by SEM analysis (data not shown), both oxide and collagen coatings were conformal and did not influence or disrupt
the microconical-shaped morphology of the initial Si substrates. As evaluated by static contact angle measurements, both coatings rendered the initially hydrophobic MC surfaces superhydrophilic (Table 2).

### 3.2. Effect of substrate roughness on PC12 cell growth

As an initial attempt, PC12 cells were used to assess whether the laser-structured rough Si substrates could support cell growth in the absence of growth factor after 4 and 7 days of culture. Cell growth was assessed by qualitative (via immunocytochemistry, SEM) and quantitative (computer-based image analysis of DAPI nuclear staining) morphological methods. It was shown that the oxidized structures, type O, did not support PC12 cell growth, since minimal adhesion was observed (data not shown). Therefore, further analysis concentrated on the non-oxidized type A and AC structures. Morphological analysis with SEM showed that PC12 cells were grown on all three roughness types, while sharing the same morphological characteristics, including the relatively small and rounded shape cluster formation (Figure 2A).

In order to quantitatively evaluate cell outgrowth on the different substrates, upon culture termination, scaffolds were stained with DAPI and the number of nuclei/mm² surface area was evaluated using ImageJ (Figure 2B). The mean cell number in each band of the microstructured area (flat, low, medium and high roughness) was calculated and expressed as a percentage over

### Table 2. General properties of the different samples used for this study

| Sample | Flat | Flat, oxidized | A1 | AC1 | O1 | A2 | AC2 | O2 | A3 | AC3 | O3 |
|--------|------|----------------|----|-----|----|----|-----|----|----|-----|----|
| Laser fluence (J/cm²) | 74 | 25 | 110 | 0 | 21 | 124 | 0 | 21 | 152 | 0 | 0 |
| Wetting angle (°) | 74 | 25 | 110 | 0 | 21 | 124 | 0 | 21 | 152 | 0 | 0 |

Series A, as-prepared patterned Si surfaces; series O, patterned surfaces after thermal oxidation; series AC, non-oxidized patterned Si surfaces with collagen coating. The substrates were fabricated using different laser fluences and exhibited different wetting properties, as measured directly by static contact angle values. The mean value was calculated from at least five individual measurements. Successive measurements were reproducible within ± 1°.
the total number of cells counted onto the whole microstructured area. Thus, after 4 days of culture, type A substrates supported growth of 47.5% of the cells in the low-roughness band, 30.7% in the medium-roughness band and 18.9% in the high-roughness band, while only 5% in the flat band. At day 7 of culture, the percentage of cells on the low-roughness band increased to 51.8%, while on the other bands cell growth did not show any statistically significant change (Figure 2Ba). A detailed report of the absolute values of the cell density range for the different rough surfaces at the different culture times follows. For non-coated substrates (A substrates), the cell number density among experiments varied: 620–3301 N cells/mm² for low-, 525–1522 N cells/mm² for medium- and 295–782 N cells/mm² for high-roughness substrates (4DIV); 1988 N cells/mm² for low-, 145–1490 N cells/mm² for medium- and 70–1219 N cells/mm² for high-roughness substrates (7DIV). For collagen-coated substrates (AC substrates), the cell number density among experiments varied: 131–1660 N cells/mm² for low-, 408–1575 N cells/mm² for medium- and 75–1345 N cells/mm² for high-roughness substrates (4DIV); 601–1988 N cells/mm² for low-, 1037–2052 N cells/mm² for medium- and 141–2566 N cells/mm² for high-roughness substrates (7DIV). Thus, in the absence of NGF, although PC12 cells did not differentiate to develop neurites, they seemed to prefer the low-roughness structures for growth. Using the AC type substrates, cells were shown to grow better on the medium-roughness bands (47.3% of total cells) at day 4 of culture, while they did not seem to have any preference on band roughness at day 7 of culture (Figure 2Bb). On the contrary, despite the presence of NGF stimulus, cells did not appear to differentiate on the MC surfaces showing that at low (Figure 3Ca, Cd) and intermediate (Figure 3Bc) roughness parts of the substrates, the cells could not differentiate to develop neurites, they seemed to prefer the low-roughness structures for growth. Using the AC type substrates, cells were shown to grow better on the medium-roughness bands (47.3% of total cells) at day 4 of culture, while they did not seem to have any preference on band roughness at day 7 of culture (Figure 2Bb). It is interesting to note that at day 4 of culture MC surfaces were largely preferred to flat ones (two- to eight-fold higher proliferation; Figure 2B), while flat surfaces could not support cell growth after day 7 of culture.

3.3. Effect of substrate roughness on PC12 cell growth in the presence of NGF

PC12 cells were placed onto the Si structures and induced to differentiate towards the neuronal lineage by stimulation with NGF, which is considered to be the classical inducer of PC12 cell differentiation into a sympathetic neuron phenotype (Greene and Tischler, 1976). NGF-induced (50 ng/ml) differentiation of PC12 cells was assessed by qualitative (via immunocytochemistry, SEM) and quantitative (computer-based image analysis) morphological methods.

Qualitative analysis included immunocytochemical staining for specific protein expression and morphology assessment using SEM imaging. Immunocytochemical studies indicated that cells growing on the low- and medium-roughness MCs could differentiate towards the neuronal cell lineage, showing increased, flattened cellular bodies, sprouting neuritic processes. These processes expressed neuron-associated βIII-tubulin (green staining in Figure 3A, 3Ba, 3Bb), with the distal end regions showing thin filaments sprouting outwards the expressed actin (red filaments in Figure 3A, 3Ba, 3Bb). Such actin-based extensions that facilitate axon development and guidance could represent the terminal filopodia of growth cones. On the contrary, despite the presence of NGF stimulus, cells did not appear to differentiate on the MC surfaces of the highest roughness. In this case, the cells displayed round morphology, were covered with microvilli, exhibited short or no processes and grew to form cell clusters (Figure 3Bc), resembling untreated PC12 cells. This differential cell response in the presence of NGF on the different substrates was also confirmed by SEM imaging, showing that at low (Figure 3Ca, Cd) and intermediate (Figure 3Cb, Ce) roughness parts of the substrates, the cells...
mostly exhibited a bipolar shape, forming an elegant neurite network, while at the highest roughness (Figure 3Cc, Cf) the cells mostly maintained their round shape.

In order to quantitatively evaluate PC12 cell differentiation, the percentage of differentiated cells and neurite lengths in the presence of NGF were estimated. As already noted in the Methods section, the percentage of differentiated cells was calculated from the ratio of cells displaying neuron-like processes over the total number of cells. The results showed that, for the AC series, cell differentiation was promoted only by patterned surfaces of low and medium roughness (26.4% and 18.3% at day 4 of culture and 24.9% and 12.66% at day 7 of culture, respectively), whereas it was strongly inhibited on surfaces of high roughness (only 4.14% and 0.96% at days 4 and 7 of culture, respectively; Figure 4). Similar results were obtained with the A series (data not shown).

Furthermore, it is important to note that the observed differentiation ratio was independent of the substrate’s hydrophilicity, since type A (hydrophobic and superhydrophobic) and AC (superhydrophilic) series gave similar results.

Figure 3. (A) Effect of NGF on PC12 cell morphology: morphological analysis of PC12 cells with fluorescence microscopy before (a) and after (b) treatment with NGF. Double labelling with β-tubulin (green) and actin (red) of PC12 cells; blue, DAPI nuclear staining (4 DOC). (B, C) Effect of surface roughness on PC12 cell differentiation: confocal microscopy (B) and SEM images (C) of NGF-treated PC12 cells on laser-patterned Si substrates of different roughness after 4 DOC: low-roughness substrates (AC1) (a, d); medium-roughness substrates (AC2) (b, e); high-roughness substrates (AC3) (c, f)

Figure 4. Effect of NGF treatment on differentiation: NGF-induced differentiation and growth of PC12 cells on laser-patterned Si substrates coated with collagen (AC series). PC12 cells were treated with 50 ng/ml NGF for 4 and 7 DOC. Cells with at least one neurite with a length equal to the cell body diameter were counted and expressed as a percentage of the total number of cells in a field (± SE). The significance levels (p) were calculated using ANOVA followed by Tukey test for multiple comparisons between pairs of means (* p < 0.01, n = 30 fields of view for each roughness type and time of culture). The difference between surfaces of low and medium roughness and the flat surface was highly significant (p < 0.001)
Neurite lengths of differentiated cells grown on the AC substrates at day 4 of culture were shown to be higher at low roughness (66.7 ± 9.6 μm) compared to 41.1 ± 3.7 μm and 28.0 ± 4.2 μm at medium and high roughness, respectively, while at day 7 of culture low and medium roughness were shown to support equally well longer neurite growth (79.0 ± 7.4 μm and 82.7 ± 12.3 μm as compared to 30.1 ± 5.5 μm at high roughness, respectively) (Table 3). It has to be noted that at high roughness MCs, the number of cells analysed from the images was very small (n = 8 and n = 11 for 4 and 7 DIV, respectively), since, as previously mentioned, this surface could not support PC12 cell differentiation. Non-coated, type A surfaces of low and medium roughness could equally well support neurite growth at day 4 of culture, while no neurites grew on high-roughness substrates (data not shown). In this case, at day 7 of culture neurite growth was limited, since differentiation was obtained in only a few cells (data not shown).

| Roughness type | 4 days of culture, L<sub>neurite</sub> ± SE (μm) | 7 days of culture, L<sub>neurite</sub> ± SE (μm) |
|----------------|---------------------------------------------|---------------------------------------------|
| Low            | 66.7 ± 9.6                                  | 79.0 ± 7.4                                  |
| Medium         | 41.1 ± 3.7                                  | 82.7 ± 12.3                                 |
| High           | 28.0 ± 4.2                                  | 30.1 ± 5.5                                  |

*NGF-induced differentiation and growth of PC12 cells on laser-patterned Si substrates coated with collagen (AC series). PC12 cells were treated with 50 ng/ml NGF for 4 and 7 days of culture. The length of the longest neurite was determined for cells with at least one identified neurite. The results are expressed as mean length ± SE. Experiments were repeated at least five times using cultures prepared on separate days (n = 30 cells for each roughness type and time of culture).

4. Discussion

The control of neuronal cell outgrowth is of critical importance in a wide spectrum of neuroscience applications, including tissue-engineering scaffolds and neuron electrophysiology studies. Although information regarding neuron cell outgrowth on more complex topographies remains limited, evidence of phenotype alteration in stem cells (McNamara et al., 2010; Ortinau et al., 2010) or neuronal cells (Christopherson et al., 2009; Schindler et al., 2006) upon culture on 3D scaffolds, as compared to traditional flat substrates lacking structural cues, emphasizes the necessity to shift from 2D to 3D or multi-scale culture models. The aim of the present study was to investigate the cellular growth and differentiation pattern (neuritogenesis) of the PC12 cell line on laser-textured Si surfaces comprising arrays of MCs with different geometrical characteristics. The construction of a single scaffold containing all three different roughness ratios allowed direct comparison of the effect of MCs topology on cell growth preference.

Using the same culture conditions, the cells were shown to largely prefer MCs surfaces for growth, as compared to flat substrates. In the absence of NGF stimulation, PC12 cells cultured on MCs maintained their round shape and small nucleus size, while clustering in a 3D manner, characteristics similar to those well documented by Greene and Tischler (1976). In this case, it was shown that MC Si substrates could support PC12 cell adherence without any coating, while cell behaviour could change upon altering the MCs surface energy using a chemical coating. Although cell growth was inhibited on oxide-coated MCs, it became slightly improved on collagen-coated structures. The above results demonstrated the useful utilization of Si MCs in supporting nerve cell adherence and neurite growth.

Upon treatment with NGF, the cells flattened and extended processes with tubulin-rich terminals, surrounded by actin-expressing lamellipodia and filopodia resembling the already described growth cones of primary neurons (Lin and Forscher, 1993; Mingorance-Le Meur et al., 2009). Although differentiated cells grew on top of the surfaces, it seemed that they sensed the scaffold features via the tips of their neurite extensions (Figure 5). As previously proposed (Yamada et al., 1970), the actin-based motile part at the growth cone of these filopodia could be responsible for probing. Although PC12 cells responded to NGF treatment on the patterned Si surfaces, they displayed a differential behaviour, depending on the MCs topology. Since all three different rough surfaces were structured within a single substrate and placed in a single well, ensuring that all different rough surfaces were exposed to exactly the same culture conditions, it can be concluded that, upon NGF treatment, cell differentiation was promoted on low- and intermediate-roughness textures, whereas it was strongly inhibited on the high-rough ones. Since this differential response was observed for different MCs’ surface chemistries, the effect should be associated with MCs topology. This observation implies that the topographical cue imposed by specific MCs topology restricted neuronal precursors to exit the cell cycle and differentiate.

Figure 5. ‘Sensing’ substrate roughness: SEM image of differentiated PC12 cells after NGF treatment on laser-patterned Si substrates. Inset is a higher magnification image focusing on sprouting filopodia (pseudocoloured)
Further insight into the topography-mediated inhibition of cell differentiation could be provided by focusing on the geometrical features of the MCs, presented in Table 1, in comparison with the cell size and shape observed by SEM imaging (Figure 2A). In particular, in the case of high-roughness surfaces, the distance among MCs, c, is higher (4.48 ± 0.96 µm) compared to the low and medium roughness ones (1.73 ± 0.28 and 2.06 ± 0.53 µm, respectively). Accordingly, for low- and medium-roughness surfaces, undifferentiated cell bodies remained on top of the microstructures (Figure 2Aa, b), while on the high-roughness ones cells could be ’trapped’ among MCs (Figure 2Ac), because the size of undifferentiated cells is comparable to the interspikes’ space. It thus appeared that among the different morphological parameters of MCs, the interspike distance could be considered to be a critical characteristic affecting cell differentiation. On the contrary, cell differentiation appeared to be insensitive to the aspect ratio, A, since although both medium- and high-roughness surfaces exhibited similar values for A, they showed very different behaviour with regard to cell differentiation. Based on these results, a geometrical model could be proposed, suggesting that there is a critical interspike distance over which cells are not allowed to elongate, develop extensions and finally differentiate. This distance should depend on the cell size. Indeed, in the case of low- and medium-roughness surfaces, PC12 cell bodies would lie on top of many MCs, while on the highest roughness surfaces, exhibiting lower MC density and larger interspike space, the cells would merely stay upon a few, or even trapped within, spikes (Figure 2A). In addition, the characteristics of cell differentiation on periodical arrays of Si MCs may exemplify an ergonomic way of neuritic sprouting, where neuritic extension would be dictated by the shortest interspike distance. Accordingly, for small interspike distances (spacing below ~2 µm), the cells would spread readily, since they could reach MCs to establish the next focal adhesion contacts in all directions and, consequently, the cells would acquire a flattened phenotype. Upon increasing MCs spacing, the probability of reaching the next spike would decrease.

Another important observation that complies with previous reports (Ranella et al., 2010) is that in the low- and medium-roughness surfaces, many filopodia sprouted from cellular bodies, whereas this was not the case for cells growing on the highest roughness surfaces. It can be suggested that the absence of such filopodia sproutings from the cells could inhibit the differentiation process. According to Luckenbill-Edds et al. (1979), both control and NGF-treated PC12 cells shared the same sprouts, named extensions, and the same machinery of organelles. However, after NGF treatment, these extensions stabilized into processes, filled with microtubules and elongated, resembling the morphology of growth cones in primary neurons. Therefore, it could be argued that for cells cultured on high-roughness surfaces, the absence of the very first extensions disabled NGF from exerting its effect towards cell differentiation.

A number of studies envisage correlating specific geometrical characteristics of the topography with the induced cell morphological changes, as well as the effect of surface topography and neurotropic signals, such as NGF, on PC12 cell differentiation. Most of the proposed models imply parallel aligned topographical cues, in the form of alternating grooves andridges (Cecchini et al., 2007; Foley et al., 2005; Mahoney et al., 2005), or electrospun fibres (Lee et al., 2009; Liu et al., 2010). According to these studies, the morphology of the differentiated cells depend upon the dimensions of the features (width, height and interf Feature distance). As an example, it can be stated that morphology could switch from bipolar to multipolar upon shifting from submicron- to micron-width ridges (Ferrari et al., 2011; Yao et al., 2009). An increasing number of studies highlight the sensitivity of cell morphology to vertically orientated structures at microscale (Bucaro et al., 2012; Su et al., 2013) and, more recently, to submicron and nanoscale (Haq et al., 2007). In these cases, a differential response could be obtained when shifting from micro- to submicron-scale features. For example, arrays of pillars in the microscale range patterned on Si wafers could support PC12 cell differentiation towards a highly branched neurite phenotype in the presence (Su et al., 2013) or absence of neurotrophic factors (Bucaro et al., 2012). On the contrary, gold nanopillars limited neurite outgrowth of PC12 cells compared to smooth substrates (Haq et al., 2007). To the best of our knowledge, the effect of MCs topography on the differentiation fate of PC12 cells into a neuronal phenotype has not been exploited. The results presented here suggest that a specific type of vertically aligned topographical cue (microconical structures) could influence the differentiation fate of PC12 cells in the presence of optimal neurotropic signals.

Conclusions

In this study, laser-fabricated arrays of Si MCs exhibiting different geometrical characteristics were used as PC12 cell culture substrates. It was shown that MCs could influence the differentiation of PC12 cells in the presence of neurotrophic signals. Notably, among the different topographical characteristics of MCs, the interspike space effectively influenced the PC12 cells’ differentiation fate. Although PC12 cells were treated with the neurotrophic factor NGF, cell differentiation was inhibited on the high-roughness MC surfaces. The results presented here define a useful experimental approach to influencing cell differentiation by proper selection of surface microtexture. The patterned substrates presented here could potentially be used as model scaffolds for the systematic exploration of the role of 3D microtopography on cell differentiation and neural network outgrowth.

Conflict of interest

The authors have declared that there is no conflict of interest.
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