Multi-scale engineering for neuronal cell growth and differentiation

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A B S T R A C T

In this paper we investigate the role of micropatterning and molecular coating for cell culture and differentiation of neuronal cells (Neuro2a cell line) on a polydimethylsiloxane substrate. We investigate arrays of micrometric grooves (line and space) capable to guide neurite along their axis. We demonstrate that pattern dimensions play a major role due to the deformation of the cell occasioned by grooves narrower than typical cell dimension. A technological compromise for optimizing cell density, differentiation rate and neurite alignment has been obtained for 20 μm wide grooves which is a dimension comparable with the average cell dimension. This topographical engineered pattern combined with double-wall carbon nanotubes coating enabled us to obtain adherent cell densities in the order of 10^4 cells/cm^2 and a differentiation rate close to 100%.

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Nanotopography
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Carbon nanotubes

1. Introduction

Mechanical cell–substrate interactions can affect many cellular functions such as spreading, migration, differentiation and apoptosis [1–4]. Surface engineering at micro and nanoscale can be employed to fabricate specific surface features for 2D cell culture in the perspective of investigating the inner mechanisms taking place at the cell–substrate interface but can also be envisioned as a process to tune and control cell development. As an example of interest for this work, understanding neurites growth along topographical patterns is important for tissue engineering applications in neurology. Indeed, exploiting neurite guidance by elongated microfabricated ridges can be applied to the fabrication of synthetic surfaces designed to spatially control and direct neurite growth along a pre-defined direction. This achievement can shed some light on neural networks organization but also can contribute to the development of medical devices such as brain engineered implants (tissue engineering scaffolds). Not only physical cues (topographical features) or chemical cues (molecular patterns) are interesting for this kind of application but also their combination seems to be crucial for tuning cell adhesion and differentiation properly. The challenge is to discover the most suited combination of micro/nanostructuration and molecular surface coating for controlling on demand, several extracellular signals acting at different length scales. In this work, topographical micrometric grooves are investigated for promoting alignment of neurites, while surface functionalization with conventional polylysine molecules or Double Wall Carbon Nanotubes (DWCNT) are investigated for promoting cell adherence and differentiation. We want to learn if micro/nanotopographical cues and molecular coating can be associated synergistically for tissue engineering purpose and evaluate the influence of CNTs. In this work, the originality relies on the use of high structural quality CNTs obtained by catalytic Chemical Vapour Deposition (CCVD) exhibiting a very high proportion (80%) of metallic carbon nanotubes which are deposited in a very dense and homogeneous thin layer with a in-plane nanotube axis orientation.

2. Experimental methods

2.1. Engineered substrate fabrication for 2D cell culture

The substrates for the cell culture consisted in a microgrooved polymer, the polydimethylsiloxane (PDMS), which is inert and biocompatible. The PDMS was conventionally microstructured

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using a simple molding process against a silicon master generated by proximity UV lithography and deep Reactive Ion Etching (RIE). An anti-adhesive treatment of the master is carried out using silanisation in liquid phase with octadecyltrichlorosilane (OTS) in order to enable easy demolding of the polymer replica after thermal curing. The PDMS pre-polymer solution containing a mixture of PDMS oligomers and a reticular agent from Sylgard 184 (10:1 mass ratio, Dow Corning) was poured on the silicon master and cured at 80 °C during 3 h. Once demolded, the PDMS substrate is sterilized by UV illumination during 1 h. The patterned polymer sample can be used for 2D cell culture (see Fig. 1) either without any further treatment or after O_2 plasma treatment and coating with polylysine (PLL) by incubation or DWCNT by spray coating. In our experiments we focus on line and space patterns covering mm^2 areas. The line width is varied from 5 to 100 μm and the microgrooves are 25 μm deep (see Fig. 2).

The poly-L-lysine (PLL, Sigma–Aldrich) was dissolved at 1 mg/mL in sterile phosphate-buffered saline solution. To obtain a homogenous polylysine layer on the PDMS surface, we preliminary treat the PDMS in a radio-frequency plasma cleaner (Reactive ion Etchning, Tepla 300 microwave plasma processor, power = 200 W, T = 40 °C, O_2 flow = 1000 mL/min, P = 1.65 mbar) for 30 s and sterilize with UV light before incubating the protein solution for 10 min. The PDMS substrates are finally blow-dried with filtered nitrogen and placed at the bottom of the cell culture wells, as shown in Fig. 1b.

DWCNT used in this work are prepared in-house by CCVD, by decomposition of CH_4 at 1000 °C (H_2:CH_4 atm.) [5]. After catalyst removal (HCl), the sample contains approximately 80% of DWCNT, the rest being mainly SWCNT (∼15%) and triple-walled nanotubes. The large proportion of metallic CNT [6] may present some advantages for neuronal tissue engineering allowing future electrical stimulations of the cells. Purified DWCNTs and carboxymethylcellulose were mixed with ultra-pure water with a mass ratio of 1:10 in mass (DWCNTs: 0.1%, CMC: 1%). The mixture was sonicated for 30 min (Sonics Vibra Cell) at a power of 150 W, under cooling in an ice bath. The mixture then appears as a stable black suspension which is re-suspended in ultra-pure water (centrifugation 16,000 rpm during 30 min).

The DWCNT suspension was then sprayed on the PDMS surface, which has been exposed to O_2 plasma. The hydrophilicity of the treated PDMS surface allows a good spreading of the ejected droplets resulting in a homogeneous dense layer of DWCNTs on the polymer surface as shown in Fig. 2. It is worth noticing that a control experiment (not shown here) consisting in culturing the cells on a PDMS substrate sprayed with a pure CMC solution without any DWCNTs, showed no noticeable effects on the cell adherence and morphology.

2.2. Cell culture

Neuroblastoma N2a mouse cells were grown in DMEM medium supplemented with 10% fetal bovine serum (PAA Laboratories) and 1% penicillin–streptomycin (GIBCO) in petri dishes (FALCON). Cells were subcultured twice a week, and maintained at 37 °C and 5% CO_2. All reported experiments were performed using cells with less than 20 passages. Neuro2a cells were seeded on PDMS substrates at a density of 1.2×10^4 cells/cm^2. Cells were incubated 24 h then differentiated by switching the medium DMEM to a prewarmed DMEM containing 0.1% BSA (bovine serum albumine, *Euromedex*). Cells were then maintained at 37 °C during 48 h before being fixed with 3.5% paraformaldehyde (Sigma) for characterization.

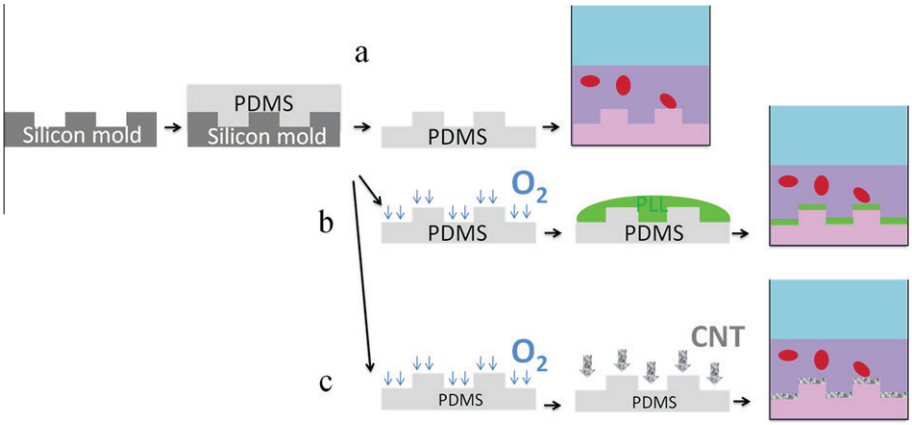

Fig. 1. Schematic representation of PDMS substrate fabrication for 2D cell culture. (a) The PDMS substrate is directly used for cell culture. (b) The PDMS substrate receives a poly-L-lysine coating. (c) The PDMS substrate receives a DWCNT coating before being used for cell culture.

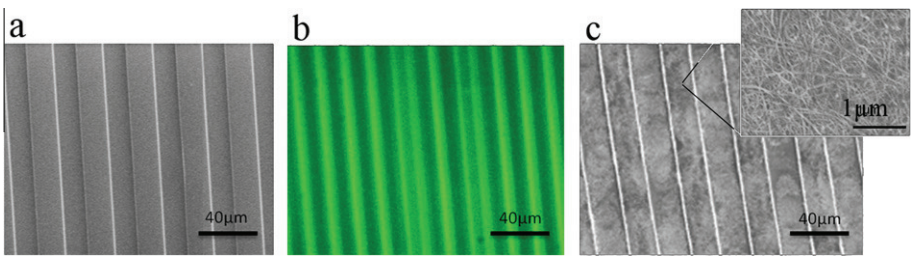

Fig. 2. Typical PDMS surfaces prepared for 2D cell culture. (a) SEM image of a microgrooved surface of PDMS, (b) fluorescence image of a microgrooved PDMS surface coated with a homogeneous layer of fluorescent polylysine. We have checked that both top and bottom of the PDMS surface are coated with polylysine (c). SEM image of a microgrooved PDMS surface covered by a layer of DWCNTs. The 50–150 nm thick DWNT-layer is dense.

2.3. Fluorescence microscopy and image analysis

To observe the cells and quantify their behavior, we stained the actin cytoskeleton and the cells nucleus. Cells were fixed with 3.5% paraformaldehyde in PBS and sucrose (1:1) for 30 min at room temperature, rinsed three times with PBS and permeabilized (2 min in a 50 mM solution of NH_4Cl in PBS and 10 min in a PBS–Triton solution (0.3% in volume). Actin cytoskeleton was stained with tetramethylrhodamine(TRITC)-conjugated phalloidin (molecular probes) at a dilution of 1:200. Cell nucleus are stained with the 4′-6-diamidine-2-phenyl indole (DAPI) used at 1:100, targeting DNA. Fluorescence images were taken with a fluorescent microscope Leica with a 40× objective. Images of at least 300 cells on five randomly chosen observation fields were captured and analyzed for each experimental condition. Each experiment was repeated three times. Cytoplasmic extensions longer than or equal to 10 μm were counted as neurite. A cell was considered as differentiated when equipped with at least one neurite.

3. Results and discussion

3.1. Cell localization

One spectacular effect of topographical patterns is the cell localization inside the polymer grooves. This effect was quantitatively analyzed and a summary of the results is presented in Fig. 3a as a function of groove width and surface coating (no coating, PLL coating, DWCNT coating). The graph plots the proportion of cells growing in the grooves. It is clear that by adapting the groove width around 10–20 μm, it is possible to obtain near hundred percent of the cells located in the grooves. However, we observed that the surface treatment can influence this score. Indeed, by providing favorable conditions of adherence inside the grooves and on the terraces, surface coating attenuates the preference of cells for the cavities. This attenuation is highest for DWCNT coating which seems to indicate that this material enhance cell adherence whatever the surface topography, as previously reported [7]. This graph thus reveals a clear competition between topographical induced effects (tending to localize the cells in the grooves) and coating effects (tending to a delocalization). Cell localization preferentially occurs when the width of the grooves are comparable with the average dimension of the cell body. This result is in good agreement with previous reports showing that for dimension frustrating cell plasticity the number of adherent cells dramatically decreases (see also Fig. 3b). These observations suggest that when the cells are cultured on an adequate microgroove having a width comparable with cell size, the cells reach the best growing conditions in maximizing their contact surface with the PDMS (bottom groove, and lateral walls) and also grow in the grooves. However, when the grooves are smaller than the cell size, the cells need to deform their cytoskeleton and their nucleus. These conditions appear to be unfavorable for cell adherence and differentiation (see also Fig. 3b). Finally, for the widest microgrooves (50 and 100 μm), the cells do not develop preferentially in the grooves because they experiment a topographical landscape looking like a flat surface.

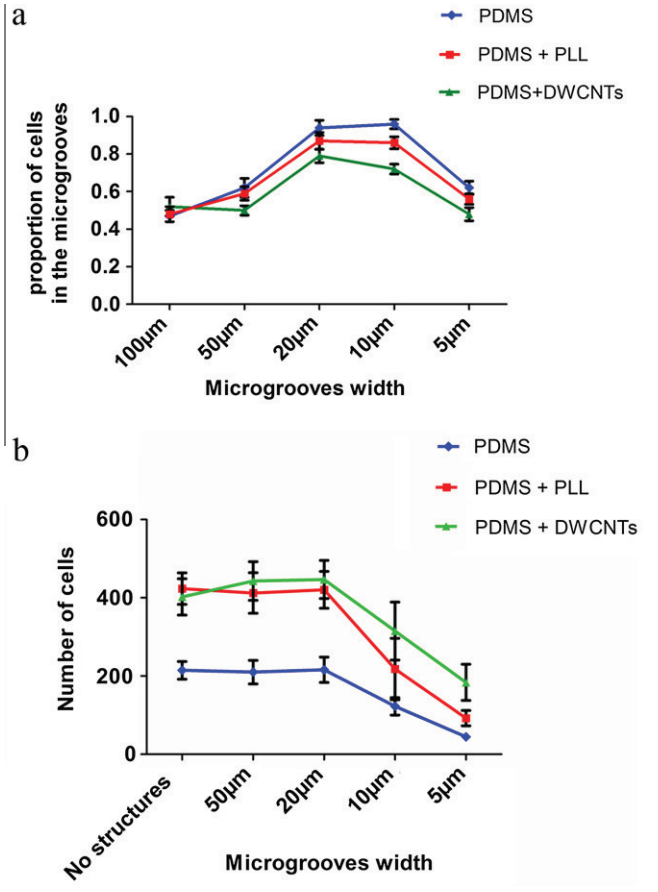

Fig. 3. (a) Statistical quantitative analysis of the proportion of Neuro2a cells growing in the microgrooves as a function of groove width and surface coating. (b) Number of Neuro2a cells adhering onto different PDMS surfaces after 48 h of culture as a function of groove width and surface coating.

3.2. Cell adhesion and differentiation

In order to determine what are the most suitable conditions to obtain a large number of adherent and differentiated cells, we compared the number of adherent cells on microgrooved PDMS surfaces as a function of groove width and surface coating (no coating, PLL coating, DWCNT coating). We observed that Neuro2a cells do not adhere well on virgin PDMS while DWCNT or PLL coatings double cell density in our experimental conditions. In parallel, the microstructures dimension plays an important role, showing once again that groove widths smaller than cell dimension reduce drastically the probability of adhesion on the surface. The number of adherent cells, the proportion of cells developing neurites and the ratio between the nucleus area and the cellular nucleus perimeter were analyzed. The measured values normalized with the control surface without structures are presented in Table 1.

We clearly evidence the change in the geometry of cell nucleus in small grooves. This effect occurs for dimensions below 20 μm. This observation is correlated with a very small adhesion rate and also with a very poor differentiation rate (correlation coefficient of 0.98). In summary, for 20 μm wide grooves on a PDMS substrate coated with DWCNTs we obtain a cell density as large as 10^4/cm^2 and nearly 100% of differentiation.

Table 1
Observation of the number of cells, the proportion of cells having neurites and the ratio between the cells nucleus area and perimeter expressed in μm. The PDMS surface received no coating in this case but the same trends have been observed with PLL coating and DWCNT coating.

	No structures	50 μm	20 μm	10 μm	5 μm
Normalized number of cells	1	0.98	1	0.57	0.21
Proportion of differentiated cells	1	0.99	1	0.75	0.04
Ratio area/perimeter of the cell's nucleus (μm)	1	0.91	0.93	0.76	0.5

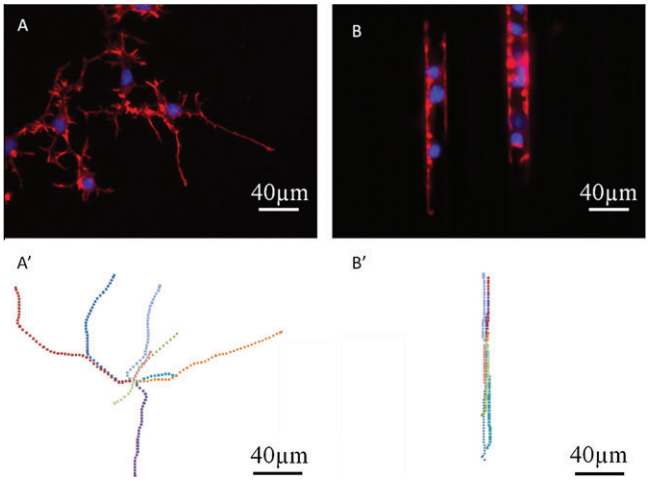

Fig. 4. (A) and (A′) Neuro2a cells cultured on a flat PDMS surface. (A) Optical fluorescence image, in red: immunostaining of actin with tetramethylrhodamine(TRITC)-conjugated phalloidin and in blue: immunostaining of cell nucleus with 4′-6-diamidine-2-phenyl indole (DAPI). (A′) Polar visualization of the neurite orientation of 7 characteristic cells. (B) and (B′) Neuro2a cells cultured on a microgrooved PDMS surface (20 μm wide), (B) optical fluorescence image immunostaining of actin (red) and cell nucleus (blue), (B′) polar visualization of the neurite orientation of 7 characteristic cells, showing a perfect alignment along the grooves axis.

3.3. Neurite guidance

Fig. 4 shows the effects of the microstructures on neurite guidance. As can be seen from fluorescence analysis, we obtain a clear guidance effect of the neurites along the grooves axis. On a flat PDMS, the direction of neurites is random while guidance was systematically observed as soon as grooves are patterned at the PDMS surface. For wide microstructures, the filaments are not guided from their origin because the cell dimensions are much smaller than the microgrooves' width. When the cells dimension and the microgrooves' width are comparable, the alignment of the filaments can start from their origin and this alignment is kept over distances as long as 100 μm. Finally, the best alignment was obtained with microgrooves measuring 20 μm wide, allowing to align near 100% of the filaments. The same observation on the alignment was made whatever the surface coating.

4. Conclusion

In this work, we have combined micro/nanostructuration and molecular surface coating for controlling neuronal cell (cell line Neuro2a) adherence, differentiation and neurite alignment. PDMS topographical micrometric grooves turned out to promote strongly the alignment of neurites along the groove avis, while surface functionalization with conventional polylysine molecules or Double Wall Carbon Nanotubes (DWCNT) increases significantly cell adhesion and differentiation. DWCNTs turned out to be a good alternative to the usual polylysine coating for promoting cell attachment and could be later used for electrical stimulation due to their metallic behaviour. Pattern dimensions were found also to play a major role. A technological compromise for optimizing cell density, differentiation rate and neurite alignment has been obtained for 20 μm wide grooves which is a dimension comparable with the average cell dimension.

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