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Influence of micro-patterned PLLA membranes on outgrowth and orientation of hippocampal neurites

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ABSTRACT

In neuronal tissue engineering many efforts are focused on creating biomaterials with physical and chemical pathways for controlling cellular proliferation and orientation. Neurons have the ability to respond to topographical features in their microenvironment causing among others, axons to proliferate along surface features such as substrate grooves in micro-and nanoscales. As a consequence these neuronal elements are able to correctly adhere, migrate and orient within their new environment during growth. Here we explored the polarization and orientation of hippocampal neuronal cells on non-patterned and micro-patterned biodegradable poly(L-lactic acid) (PLLA) membranes with highly selective permeable properties. Dense and porous nonpatterned and micro-patterned membranes were prepared from PLLA by Phase Separation Micromolding. The micro-patterned membranes have a three-dimensional structure consisting of channels and ridges and of bricks of different widths. Nonpatterned and patterned membranes were used for hippocampal neuronal cultures isolated from postnatal days 1–3 hamsters and the neurite length, orientation and specific functions of cells were investigated up to 12 days of culture. Neurite outgrowth, length plus orientation tightly overlapped the pattern of the membrane surface. Cell distribution occurred only in correspondence to membrane grooves characterized by continuous channels whereas on membranes with interconnected channels, cells not only adhered to and elongated their cellular processes in the grooves but also in the breaking points. High orientation degrees of cells were determined particularly on the patterned porous membranes with channel width of 20 μm and ridges of 17 μm whereas on dense nonpatterned membranes as well as on polystyrene culture dish (PSCD) controls, a larger number of primary developed neurites were distributed. Based on these results, PLLA patterned membranes may directly improve the guidance of neurite extension and thereby enhancing their orientation with a consequently highly ordered neuronal cell matrix, which may have strong bearings on the elucidation of regeneration mechanisms.

1. Introduction

In neuronal network formation, neurite outgrowth and the orientation of cellular growth are two important processes that can be facilitated by designing a well-defined cellular pattern. The control of a neuronal network architecture can be utilized not only to reproduce structural features and signaling of the brain but also to study alterations of circuitry patterns. Neuronal microscale patterns have been shown to be useful in applications ranging from drug screening or tissue engineering, biosensors to neuronal culture platform serving basic research interests.

Neurons have the capacity of responding to topographical features in their microenvironment and they have been shown to adhere, migrate, and orient their axons in the same direction of surface features. Artificial substrates with microstructures (e.g., microchannels, ridges and grooves) have been designed in an attempt to mimic the natural physical cues of CNS elements represented by fibrous proteins, cell bodies and cell processes that direct the growth of developing neurites. To this purpose, recently several methods have been introduced with the intent of generating structural surfaces such as microfabrication technology,

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photolithography and microcontact printing [1–3]. These approaches have successfully guaranteed neuronal growth on laminin, lysine, growth factor and other adhesive molecules patterned surfaces [4–7].

It is worthy to note that a wide range of patterns have been explored in the culture of neuronal cells. Mahoney studying the effects of polyimide microchannels of 20 and 60 μm in width and 11 μm in depth on PC-12 cells displayed that neurites were directed along the axis of the grooves [8]. In addition, micro-patterned polystyrene modified with grooves width of 16 μm and grooves spacing of 13 μm directed growth and differentiation of neural progenitor cells [9]. Similarly, polylactic acid in configuration of thin films guided PC-12 and chick sympathetic neurites by unidirectional grooves in 1 and 2 μm in width and 100 nm in height [10] and as nanofiber scaffold supported the growth and the alignment of dorsal root ganglion neurons (DRG) primary sensory neurons and E 15 primary motor neurons [11]. Another matrix and precisely protein micro-patterns of various widths have been applied to affect the outgrowth rates of DRG [12]. Moreover, combinations of topographical (e.g., microchannels) and molecular cues (e.g., NGF, laminin) proved to synergically effect neuronal polarization and neurite growth [9,13,14].

Aside the usual physical and biochemical cues, some specific physical parameters of the biomaterial such as permeability must be taken into consideration when designing a substrate to be used in tissue engineering. Among the recommended biomaterials that are used for neuronal regeneration, membranes with selective permeable properties have shown to be particularly advantageous because of the diffusion of nutrients and molecules, which are important for cell growth besides offering a large surface area for cell attachment. Previously we showed that synthetic polymeric membranes were able to support the axonal outgrowth and differentiation of neuronal cells [15,16]. Here we explored the polarization and orientation of hippocampal neuronal cells on nonpatterned and micro-patterned biodegradable poly(L-lactic acid) (PLLA) membranes, which are developed by Phase Separation Micromolding [17]. The micro-patterned membranes have a three-dimensional structure consisting of channels plus ridges and bricks whereas the nonpatterned membranes have a flat surface. This polymer offers distinct advantages in that its bio-compatibility as well as its sterilization procedures have been widely documented [18–20]. Furthermore, its degradation rate can be designed so that it matches that of the new neuronal tissue formation. In order to study these micro-patterned membranes, hippocampal neuronal cells were selected as our experimental model because they are the most common and best characterized model for investigating polarization events that usually occurs spontaneously during the first 48–72 h of culture [21,22]. Neurite length, orientation and specific functions of hippocampal cells were investigated on micro-patterned membranes with ridges and channels of different width and with bricks up to 12 days of culture. Changes in morphological and functional behaviours of cells were compared on the one hand with those of cells cultured on nonpatterned membranes characterized by porous and dense surfaces and on the other hand with polystyrene culture dish (PSCD) as a reference substrate.

2. Materials and methods

2.1. Preparation of patterned and nonpatterned membranes

The membranes were cast from poly(L-lactic acid) (PLLA) of $M_w = 1.6 \times 10^5$ g/mol. Both porous and dense membranes were manufactured. The porous PLLA films were cast from a 5 wt% PLLA in 1,4-dioxane solution using ethanol as nonsolvent at 3–4 °C. A 10 wt% PLLA in chloroform solution was used to prepare dense PLLA membranes. In fact, there the chloroform was allowed to evaporate for 30–60 s and then the membranes were immersed in ethanol bath.

For patterned membranes the initial casting thicknesses was 225 μm, whereas for nonpatterned ones it was 150 μm. The membranes were kept in a nonsolvent bath for 1 day and subsequently washed for 5–8 h in Milli-Q water, which was replaced 2–3 times. Finally, they were dried in a controlled atmosphere ($T = 18–21$ °C). All solvents and nonsolvent were purchased from Merck (Germany).

Samples were cut from the sheets to fit the specific well-plates. Sterilization was accomplished by samples being immersed in a 70% ethanol solution for 20–30 min, then the ethanol was allowed to evaporate and the samples were placed in well-plates. Isopropanol (70%) was sprayed on the samples within a laminar flow cabinet and left to evaporate. To ensure complete wetting of the sheets, the samples were kept in PBS for at least 4–8 h in an incubator (37 °C, humid atmosphere with 5% CO_2).

2.2. Membrane characterization

For the patterned membranes, 2 molds were used with distinct patterns. One pattern consisted of 30 × 20 × 20 μm channels (channel width × ridge width × channel dept). The second pattern consisted of interconnected channels with dimensions of 20 × 10 × 15 μm; every 100 μm the ridges are interrupted for 10 μm to create the interconnected pattern. Scanning electron microscopy (SEM, JEOL 5600LV) was used to visualize the sheet's pattern, dimensions and porosity. Prior to imaging, the samples were sputter-coated with a nm-thin gold layer. It is important to point out that during casting and phase separation the membranes shrunk and so the dimensions of these patterns resulted to be slightly smaller that the dimensions of the mold. Nonetheless, shrinkage is reproducible and uniform and is therefore taken into account with mold design.

The investigated membranes were identified on the basis of their characteristics: dense nonpatterned (d-np) and porous nonpatterned (p-np). The patterned membranes were distinguished on the basis of the measured channel width and ridge width (Table 1) in: d-p 20/25, p-p 20/17 and ic-p 17/7. FITC labelled Poly-L-lysine (PLL) (Sigma) was used for the visualization and quantification of the membrane coating. Imaging of the FITC labelled coated membranes were obtained by using an Olympus Fluoview FV300 Laser Confocal Scanning Microscope (LCSM) (Olympus Italia). Quantitative analysis was performed on the different areas of 3 samples of investigated coated membranes using Fluoview 5.0 software (Olympus Corporation) by measuring the fluorescence average intensity. A calibration curve of FITC labelled PLL was obtained by casting known quantities of the fluorescent protein on defined areas of polystyrene dishes calculating the surface concentrations and capturing confocal images of the dry samples. The thickness of the PLL coating resulted to be 1.2 ± 0.09 μm, as measured with FITC labelled PLL by *Z*-direction scanning at the LCSM.

2.3. Cell isolation and culture

The hippocampus of both hemispheres was dissected from the brain of postnatal days 1–3 (PND1-3) hamsters (*Mesocricetus auratus*), removed and collected in falcon tubes in Neurobasal medium A (Invitrogen Corporation, Milan, Italy) containing 0.02% BSA (Sigma, Milan, Italy). The tissue was digested in a Neurobasal medium A containing 0.1% papain (Sigma) and 0.02% BSA (Sigma) for 20 min at 37 °C [23]. Ten minutes after digestion, the tube containing the tissue was mixed and at the end of digestion, the supernatant containing papain was removed and Neurobasal medium A supplemented with B27 (2% v/v; Invitrogen Corporation, Milan, Italy) penicillin–streptomycin (100 U/mL), glutamine 0.5 mM (Biochrom AG), 5 ng/mL basic fibroblast growth factor (b-FGF; Sigma) was added to the remaining pellet. Samples were gently triturated mechanically by using a sterile Pasteur pipette with a wide opening to dissociate larger aggregates. After sedimentation of the aggregates the supernatant was removed and transferred into tubes containing 1% papain inhibitor in Neurobasal medium A and 1% BSA, as described elsewhere [15,16]. The samples were centrifuged at 1300 rpm for 10 min at room temperature and cell pellets were gently re-suspended in Neurobasal medium A containing B27 supplement, penicillin–streptomycin, 0.5 M glutamine, 5 ng/mL b-FGF. Serum-free B27

Table 1
Specificities of the micro-patterned polymer sheet replicas.

	Channel width (μm)	Ridge width (μm)	Channel height (μm)	Remarks
d-np	–	–	–	Dense nonpatterned
p-np	–	–	–	Porous nonpatterned
d-p 20/25	20	25	20	Dense continuous channels
p-p 20/17	20	17	17	Porous continuous channels
ic-p 17/7	17	7	7	Interconnected channels