J Mater Sci: Mater Med (2010) 21:1353Ð1362
DOI 10.1007/s10856-009-3944-5

In vitro astrocyte and cerebral endothelial cell response to electrospun poly(ε-caprolactone) mats of different architecture

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Received: 13 March 2009 / Accepted: 10 November 2009 / Published online: 3 December 2009

Abstract This work focuses on the evaluation of the potential use of electrospun poly(ε-caprolactone) (PCL) micrometric and/or sub-micrometric Þbrous membranes for rat hippocampal astrocyte (HA) and rat cerebro-microvascular endothelial cell (CEC) cultures. Both mats supported cell adhesion, proliferation, cellular phenotype and spreading. MicroÞbrous mats allowed cellular inÞltration, while both HAs and CECs were unable to migrate within the sub-micrometric Þbrous mat, leaving an acellularized inner region. This Þnding was correlated to the presence of larger voids within electrospun PCL microÞbrous mats, suggesting that the morphology should be accurately selected for the realization of a cell environment-mimicking mat. Based on our results, the proper Þber architecture can be regarded as a crucial issue to be considered in order to deal with suitable polymeric mats tailored for speciÞc in vitro application.

1 Introduction

Electrospinning is an efÞcient and cost-effective methodology for the production of polymeric Þbers whose diameters range from microns down to nanometers [1]. The great versatility of electrospinning process allows to fabricate made-on-purpose mats with selected biological and mechanical features. Moreover, electrospinning technique can be regarded as a valuable approach aimed to reproduce a suitable cell environment able to mimic the physical and structural properties of native extracellular matrix (ECM). The Þbrous architecture of ECM acts as cell support and furnishes an instructive background to guide cell behaviour.

The Authors have previously obtained early promising results analyzing the role of topography of different electrospun poly(ε-caprolactone) (PCL) mats as potential platform for in vitro cell culture assay [2]. The inßuence of electrospinning parameters was investigated in order to produce a suitable Þbrous environment for cell adhesion, proliferation and migration in the whole three-dimensional polymeric structure. SpeciÞcally, human umbilical vein endothelial cells (HUVECs) were seeded onto electrospun PCL mats characterized either by micrometric or sub-micrometric Þbers and cell attachment and proliferation were investigated. Our results suggested that polymeric mat properties, such as Þber size and architecture, could inßuence cell response [2].

Recently studies have been focused on the design of in vitro models that are able to reproduce the physiological, anatomical and functional characteristics of the blood–brain barrier (BBB) in order to provide a valid and cost-effective tool for studying new therapeutic approaches [3].

BBB is a complex microanatomical structure that closely regulates the passage of metabolites in the central nervous system (CNS) and it is crucial for normal CNS functions owing to its ability to regulate ion ßux and the supply of nutrients to the brain. Loss of BBB structural integrity and function plays a pivotal role in the pathogenesis of many diseases of the CNS, including brain

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trauma [4], focal brain ischemia [5], meningitis [6], brain tumor [7], stroke [8], inflammation [9], Alzheimer's disease [10], and multiple sclerosis [11].

To date, an in vitro BBB model that allows for permeability variation study and for inexpensive and mass testing of putative CNS therapeutic agents is a relevant issue to be addressed. In fact, several models are based on commercially available polymer membranes that lack certain physical and structural characteristics (such as biodegradability, porosity, pore size, arrangement of pores) resembling natural ECM.

Therefore, bioresorbable fibrous membranes, mimicking the natural 3D environment of the ECM, can be regarded as suitable platform for the culture of specific cell types essential for the establishment of BBB characteristics. BBB characteristics are regulated by complex interactions between capillary endothelial cells, sealed by tight junctions, basement lamina and astrocyte endfeet process, both cell types constituting the BBB itself as well.

On these grounds, the present study focuses on the evaluation of the potential use of electrospun PCL micrometric and/or sub-micrometric fibrous mats as synthetic membranes either for rat hippocampal astrocyte (HA) and rat cerebro-microvascular endothelial cell (CEC) cultures in order to move towards a deeper comprehension of the interaction between cells and electrospun polymeric fibers.

2 Experimental part

2.1 Fabrication of electrospun PCL membranes

Poly(ε-caprolactone) (PCL, $M_n = 80000$) was supplied by Sigma–Aldrich. Tetrahydrofuran (THF), *N*,*N* dimethylformamide (DMF) and chloroform ($CHCl_3$) were supplied by Carlo Erba Reagenti. All reagents were analytical grade and were used as received. PCL granules were solved in (a) THF:DMF (1:1) or (b) $CHCl_3$, the concentration being 14% w/v.

Each polymeric solution was electrospun in air at room temperature through a blunt-tip metal capillary (22G) in the following conditions: applied tension of 12 kV (Spellman, USA), feed rate of 0.4 ml/h (KD Scientific, USA). Polymer mats were collected onto a fixed grounded aluminium target at 15 cm from the tip of the capillary. Matrices were vacuum dried for 48 h and stored in a desiccator. The morphology and the average fiber diameter were determined by scanning electronic microscopy (SEM, Leo Supra 35) before and after the collagen treatment (see par. "Cell cultures on polymeric mats"). Porosity and 2D void size were estimated as previously reported [21]. Briefly, SEM micrographs were processed by means of a custom image analysis software. The size of the 2D voids was evaluated as the diameters of the circles having equivalent area of the bidimensional voids among the fibers.

2.2 Cell and culture conditions

The experimental protocol was approved by the Ethics Committee of the University of Padua for Animal Testing (CEASA).

In this study HAs and CECs derived from 1 to 3-day-old Sprague–Dawley rat pups (Charles-River, Como, Italy) were used. Primary cultures of HAs were prepared according to McCarthy [12]. Pups were killed by cervical dislocation, hippocampal was removed, stripped of meninges and cut into small pieces. Cells were enzymatically treated with trypsin solution (0.8 mg/ml) and after subsequent treatment with trypsin inhibitor solution (0.5 mg/ml), plated on poly-lysine-coated Petri dishes at a density of 0.8–1.0 $\times 10^5$ cells/cm^2. The culture medium consisted of Eagle's basal medium (BME) supplemented with 2 mM glutamine, 20 mM $NaHCO_3$, 25 mM KCl, 10% fetal calf serum (FCS) and streptomycin (0.1 mg/ml). After the cultures reached confluence, an enriched population of type-1 astrocytes was prepared by means of a 20 h continuous shaking. CECs were isolated and cultured as previously described [13]. Briefly, the grey matter of rat brains was dissected, chopped and centrifuged to separate microvessel fragments. In order to separate microvessels from other components, tissue was digested by means of 0.1% collagenase/dispase solution for 1 h at 37°C, cells were resuspended in 25% BSA and the mixture was centrifuged. The cells were resuspended in cell basal medium MV2 (PromoCell, Heidelberg, Germany) and seeded on Petri dishes coated with fibronectin (1 μg/cm^2). Confluence cultures were exposed to immunoseparation by means of Dynabeads M-450 Tosylactivated coated with CD31 in order to obtain pure CEC cultures.

2.3 Characterization of cultured HAs and CECs

Purity of HA cultures was estimated by immuno-characterization of glial fibrillary acidic protein (GFAP), marker of glial filaments in astrocyte cytoplasm. Cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 1% Triton in PBS for 5 min at room temperature. Cells were incubated, at room temperature, with 0.5% BSA in PBS for 1 h to block non-specific binding sites, with the primary antibody mouse anti-GFAP monoclonal antibody (1:800, Chemicon International, Temecula, CA) for 1 h, and then with the secondary texas red anti mouse IgG (1:200, Vector Laboratories, Burlingame, CA) for 30 min. Each incubation was followed by 3 washes with PBS. The samples were mounted with VECTASHIELD Mounting Medium

for ßuorescence with DAPI (Vector Laboratories, Burlingame, CA) and observed by means of a ßuorescence microscope (Olympus BH Series), excitation wavelength 350 nm. Negative controls were carried out omitting the primary antibody.

CEC cultures have been characterized for morphology, phenotype (expression of von Willerbrand factor), and formation of capillary-like structures, as previously reported [13].

2.4 Cell cultures on polymeric mats

PCL disks (12 mm diameter) were previously sterilized by immersion in 70% v/v ethanol solution overnight and dried at room temperature in a sterile hood. Thereafter, in order to facilitate cell attachment on Þbers, the mats were treated with rat tail collagen solution (1 $\mu g/cm^2$) (SigmaÐAldrich, St Louis, MO) dissolved in 0.05% acetic acid overnight and washed twice with PBS prior to cell seeding. HAs and CECs were seeded at a density of 10^5 and 5×10^4 cells/well, respectively, on polymeric disks in a 24-well microtiter plate, and cultured under standard conditions with the medium replaced every 2Ð3 days.

2.5 Viability study

Cell viability was monitored after 1, 7 and 14 days by the colorimetric MTS assay 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (CellTiter 96 Aqueous Assay, Promega). Metabolically active cells react with a tetrazolium salt in the MTS reagent to produce a soluble formazan dye that can be observed at wavelength 490 nm. The cellular constructs were rinsed with PBS (phosphate buffer solution) in order to wash out unattached cells, transferred into a new well in order to take in account only cells attached to the mat, and Þnally incubated with 20% MTS reagent in culture medium for 90 min. Thereafter, aliquots were pipetted into 96 well plates and the sample were read at 490 nm in a microplate autoreader EL13 (Bio-tek Instruments).

2.6 Morphology study

In order to investigate the cellular morphology on polymeric mats, after 14 days incubation period, cellular constructs were washed once with PBS and Þxed with 4% formaldehyde. Successively the samples were incubated with 4Õ-6-diamidino-2-phenylindole (DAPI), as a ßuorescence nucleic acid stain (VECTASHIELD Mounting Medium with DAPI, Vector Laboratories, CA, USA) (excitation wavelength 350 nm, emission wavelength 460 nm) for 30 min at room temperature in darkness. The nuclei of the cells were observed by means of a confocal laser scanning microscopy (Leica SP5, HCX PL Apo 63x/0.50 oil objective) and imagines were captured using LAS AF software.

To observe cells interaction with the PCL substrates, live cells staining procedure involving PKH26 Red ßuorescent cell linker kit (Sigma, St. Louis, MO) was used. The kit uses membrane labelling technology to stably incorporate a ßuorescent dye (PKH26) (excitation wavelength 551 nm, emission wavelength 567 nm) into lipid regions of the cell membrane. Labelling conditions employed were essentially as described by the manufacturer. Brießy, cells were resuspended in Diluent C (Sigma) at 10^7 cells/ml and diluted in the same volume of 2 μM PKH26 dye stock (Sigma). The cell suspension was incubated for 5 min at room temperature, then added to an equal volume of heat-inactivated fetal calf serum (FCS) and incubated at room temperature for 1 min to stop the labelling. Cells were then washed three times in cell basal medium, resuspended and seeded on polymeric disks. After 14 days incubation period, cellular constructs were washed once with PBS and Þxed with 4% formaldehyde. Then, the specimens were observed under a confocal laser scanning microscopy (Leica SP5, HCX PL Apo 63x/0.50 oil objective) and imagines were captured using LAS AF software.

2.7 Cryosectioning

In order to investigate cellular inÞltration within PCL mats, after 14 days incubation period, cellular constructs were Þxed in 4% PBS-buffered paraformaldehyde for 15 min, washed three times with PBS and inÞltrated with 5% agarose. Specimens were cryosectioned at 8 μm thickness using the Leica CM 1850 UV Cryostat. Samples were then incubated with 4Õ-6-diamidino-2-phenylindole (DAPI), as a ßuorescence nucleic acid stain (VECTASHIELD Mounting Medium with DAPI, Vector Laboratories, CA, USA) for 30 min at room temperature in darkness. The nuclei of the cells were observed by means of a ßuorescence microscope (Olympus BH Series), excitation wavelength 350 nm.

2.8 Scanning electronic microscopy (SEM)

In order to investigate cellular interaction with micrometric polymeric Þbers, after 14 days incubation period, cellular constructs were Þxed for 24 h with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Following dehydration in 70, 80, 95% and absolute ethanol and gold sputtering, samples were examined by a scanning electron microscope (Stereoscan-205 S, Cambridge, UK).

2.9 Immunocytochemistry staining

The expression of the HA and CEC markers (GFAP and vWF, respectively) was assessed by immunocytochemistry staining. Sections were treated with 1% Triton in PBS for 5 min at room temperature. Cells were incubated, at room temperature, with 0.5% BSA in PBS for 1 h to block non-specific binding sites, with the primary antibodies: mouse anti-GFAP monoclonal antibody (1:800, Chemicon International, Temecula, CA) and rabbit anti-vWF polyclonal antibody (1:800, GeneTex Inc., San Antonio, TX) for 1 h. Following PBS washing, a second incubation was performed for 30 min at room temperature with the secondary antibodies: texas red anti mouse IgG (1:200, Vector Laboratories, Burlingame, CA) for GFAP and fluorescein anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA) for vWF. The samples were mounted with VECTASHIELD Mounting Medium for fluorescence with DAPI (Vector Laboratories, Burlingame, CA) and observed by means of a fluorescence microscope (Olympus BH Series). Negative controls were carried out omitting the primary antibody.

2.10 Statistical analysis

Data are expressed as mean ± standard deviation. Statistical analysis was performed by means of non parametric tests (SPSS 12.0, SPSS Inc, USA). Assessment of differences between groups was performed by means of the Mann–Whitney U test. Significant level was set at $P < 0.05$.

3 Results

3.1 Morphology of electrospun PCL mats

Electrospun mats were composed of uniform and homogeneous randomly arranged PCL fibers (Fig. 1). Average fiber diameter was 3.6 ± 0.6 and 0.8 ± 0.2 μm ($P < 0.05$) for membranes derived from $CHCl_3$ and THF:DMF, respectively. Estimated porosity was for all samples around 90%. The average size of the 2D voids was 9 ± 4 μm and 4 ± 1 μm ($P < 0.05$) for micrometric and sub-micrometric mats, respectively. Microstructural and mechanical properties of electrospun mats are reported in details elsewhere [21]. As a final consideration, it has to be pointed out that SEM investigations of collagen-soaked electrospun membranes revealed the same overall morphology observed for the respective as-spun samples, either in terms of average fiber diameter (i.e. 3.3 ± 0.9 μm and 0.7 ± 0.2 μm, for micrometric and sub-micrometric mats, respectively) and in terms of the average 2D void size (i.e. 13 ± 4 μm, 3 ± 1 μm for micrometric and sub-micrometric mats, respectively).

3.2 Characterization of HA and CEC cultures

Rat HAs proliferated at the first passage and displayed characteristic flattened morphology, as assessed when observed using phase-contrast microscopy (Fig. 2a). Moreover, after shaking, cultures of astrocytes expressed GFAP and were routinely >95% pure (Fig. 2b). Astrocytes at first passage were used for our experiments.

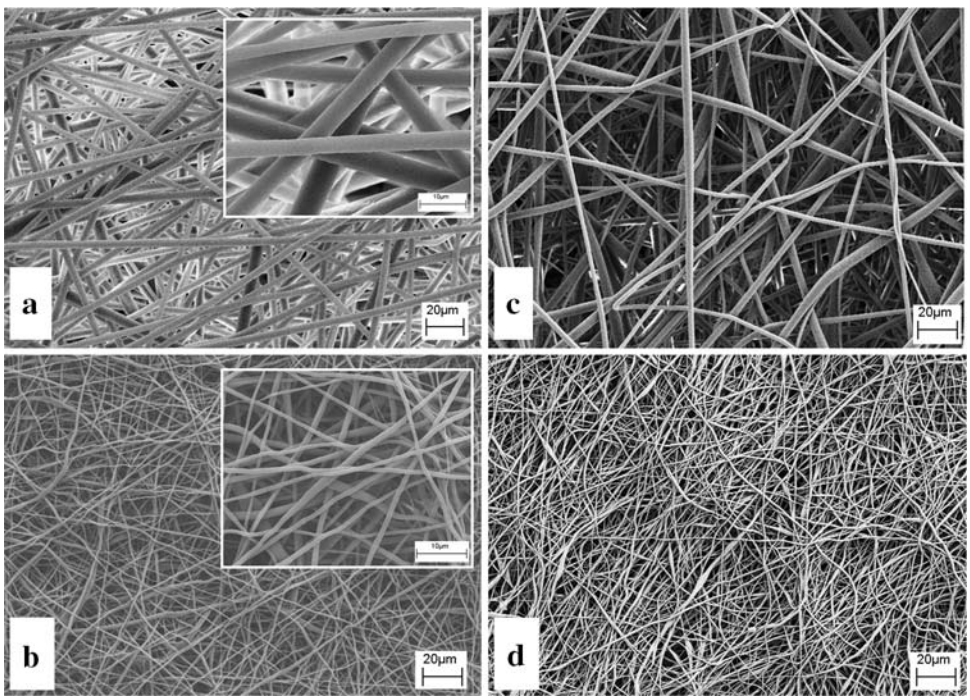

Fig. 1 SEM micrographs of PCL fibers: as-spun micrometric (a), as-spun submicrometric (b), collagen-soaked micrometric (c) and collagen-soaked submicrometric (d) mats (magnification × 1000; insert magnification × 5000)

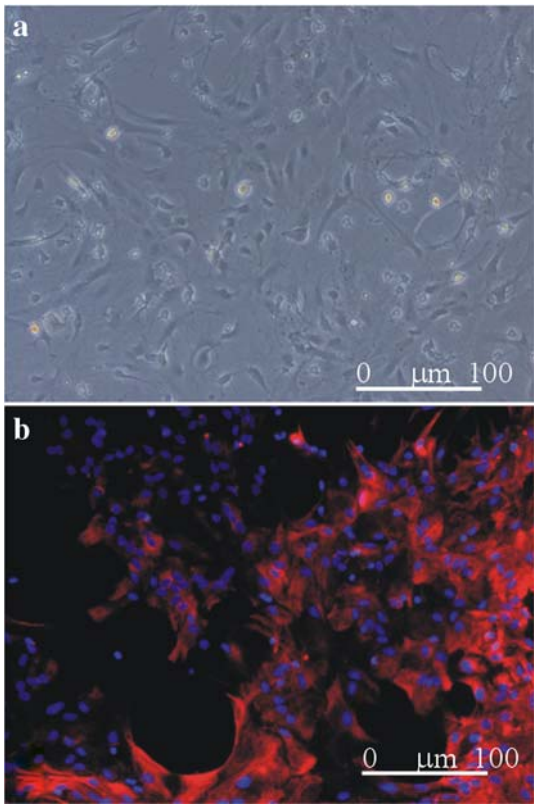

Fig. 2 Rat HAs 5 days after isolation at passage 1 before puriÞcation (a) and their immunoßuorescence characterization for GFAP after puriÞcation (b) (original magniÞcation×100)

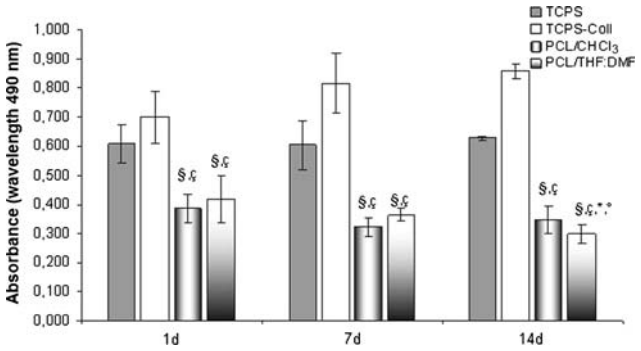

Fig. 3 HA viability evaluated with MTS assay for tissue culture plate scaffold (TCPS), collagen-coated TCPS, microÞbrous (PCL/$CHCl_3$) and submicroÞbrous matrices (PCL/THF:DMF) ($P < 0.05$ with respect to TCPS; § $P < 0.05$ with respect to collagen-coated TCPS; * $P < 0.05$ with respect to 1 day; ° $P < 0.05$ with respect to 7 days)

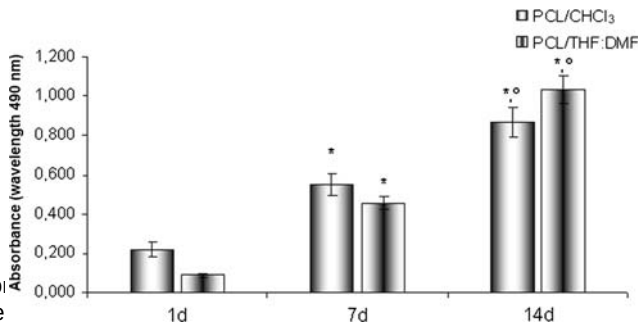

Fig. 4 CEC viability evaluated with MTS assay for microÞbrous (PCL/$CHCl_3$) and submicroÞbrous matrices (PCL/THF:DMF) (* $P < 0.05$ with respect to 1 day; ° $P < 0.05$ with respect to 7 days)

Rat CECs were isolated, cultured and characterized as previously described in Conconi et al. [13]: cultured CECs displayed characteristic oval nuclei, were spindle shaped when conßuent and maintained their phenotype and in vitro pro-angiogenic capability until the Þfth passage (data not shown). Hence, cultures from the second to the fourth passage were chosen for the experiments with the polymeric electrospun mats.

3.3 Characterization of HA and CEC cultures on PCL mats: viability assay

HA viability on days 1, 7 and 14 after seeding was revealed on tissue culture plate support (TCPS), collagen-coated TCPS, and on both investigated electrospun PCL matrices. As can be observed in Fig. 3, astrocytes were vital on both PCL mats, even if their viability was signiÞcantly ($P < 0.05$) lower in comparison to TCPS and collagen-coated TCPS. Moreover their viability on PCL mats did not vary during the observation period, the same behaviour has been observed for HAs seeded on either TCPS or collagen-coated TCPS.

CEC viability on days 1, 7 and 14 after seeding was observed only in the case of the two investigated electrospun matrices (Fig. 4). In fact, using the same seeding density, CECs cultured on TCPS became conßuent after 5 days. The results showed that CECs were vital and proliferate on both PCL mats, and a signiÞcant ($P < 0.05$) increasing trend was observed during the observation period. On the other side, cells seeded on the submicroÞbrous net after 14 days showed higher viability, even if not signiÞcant, with respect to those seeded on microÞbrous one.

3.4 Characterization of HA and CEC cultures on PCL mats: morphology study

As can be observed in Fig. 5, the nuclei of HAs displayed a characteristic oval morphology and were well distributed on the surface of both mats (Fig. 5a, d). These observations were also conÞrmed by membrane labelling (PKH26) presented in Fig. 5b, e: both PCL mats supported cell growth. Mats cryosectioning allowed us to investigate cellular inÞltration within PCL mats, as DAPI staining demonstrated HAs grown on microÞbrous mats were present onto and inside the polymeric surface, suggesting a cellular inÞltration and a three-dimensional cell growth (Fig. 5c). On the other side, HAs grown on

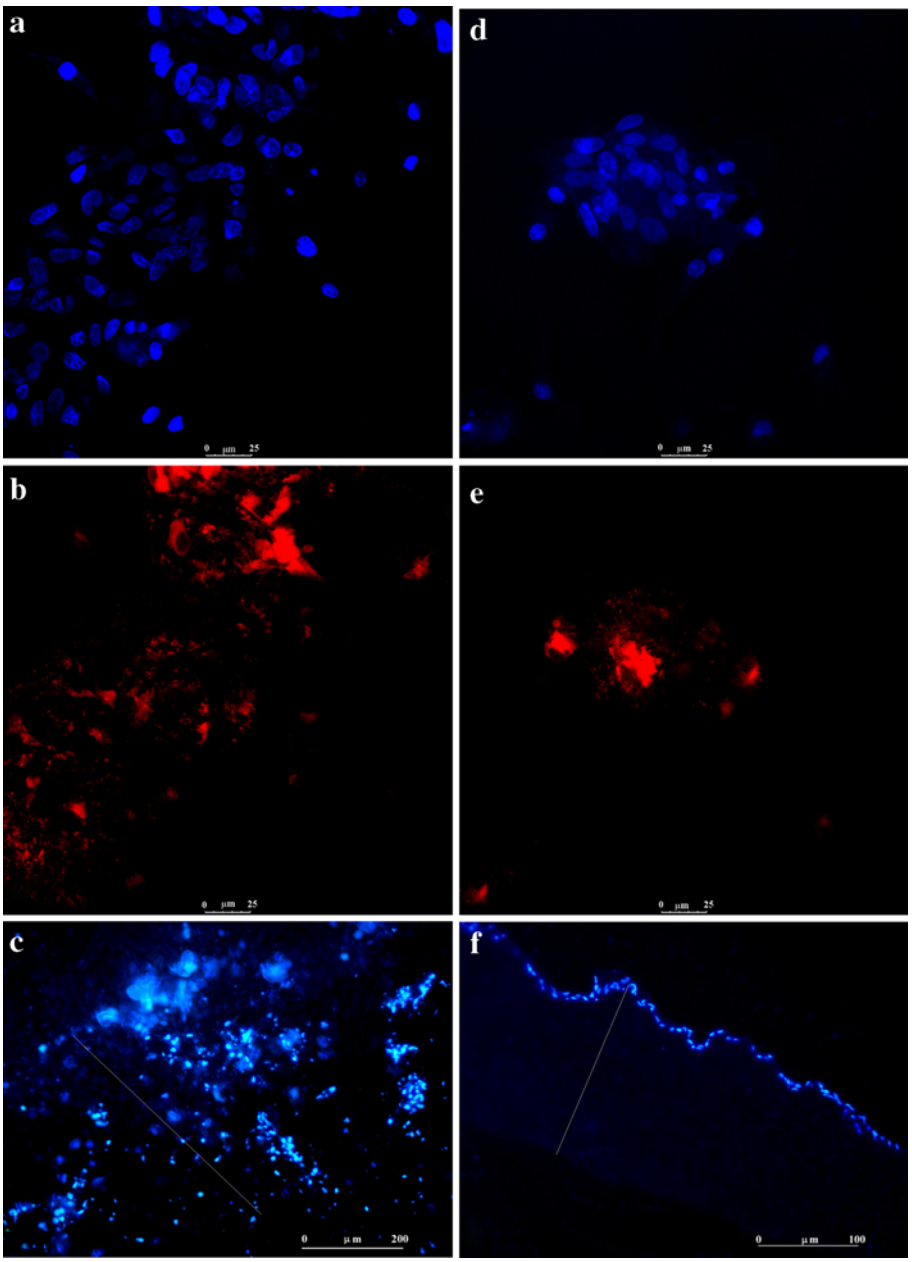

Fig. 5 HA cultures 14 days after seeding on a–c PCL:$CHCl_3$ mat and d–f on PCL:THF:DMF mat, stained with a, d DAPI (×630), b, e PKH26 (×630), c, f cryosections stained with DAPI (broken lines identify cross-section of the mat) (a–e ×50, f ×100)

submicroþbrous mats were unable to migrate, the inner part of the constructs remaining acellularized (Fig. 5f).

Figure 6 shows CEC attachment and growth on PCL electrospun mats on days 14 after seeding. It can be seen that cells adhered and spread on the surface of micrometric and submicrometric þbrous mats, demonstrating that both mats allowed adhesion and net interaction with endothelial cells. At day 14, DAPI staining allowed to observe that CECs on both mats had intact, rounded shape nuclei (Fig. 6a, d). Membrane labelling (Fig. 6b, e) demonstrates that CECs grew on microþbrous mat in a scattered way adopting an elongated shape, spread on along the þbers (Fig. 6b). Otherwise, CECs cultured on submicroþbrous network formed clusters and spread on the mat surface side by side (Fig. 6e). DAPI staining of mat cryosections conþrmed the results highlighted for HAs: microþbrous mats allowed CEC inþltration and three-dimensional cell growth (Fig. 6c). On the other side, the small size of the voids delimited by the submicrometric þbers unabled cells grown and migration and, as a consequence, the inner part of the constructs remained acellularized (Fig. 6f).

On the basis of these overall results, only micrometric PCL mats were further investigated.

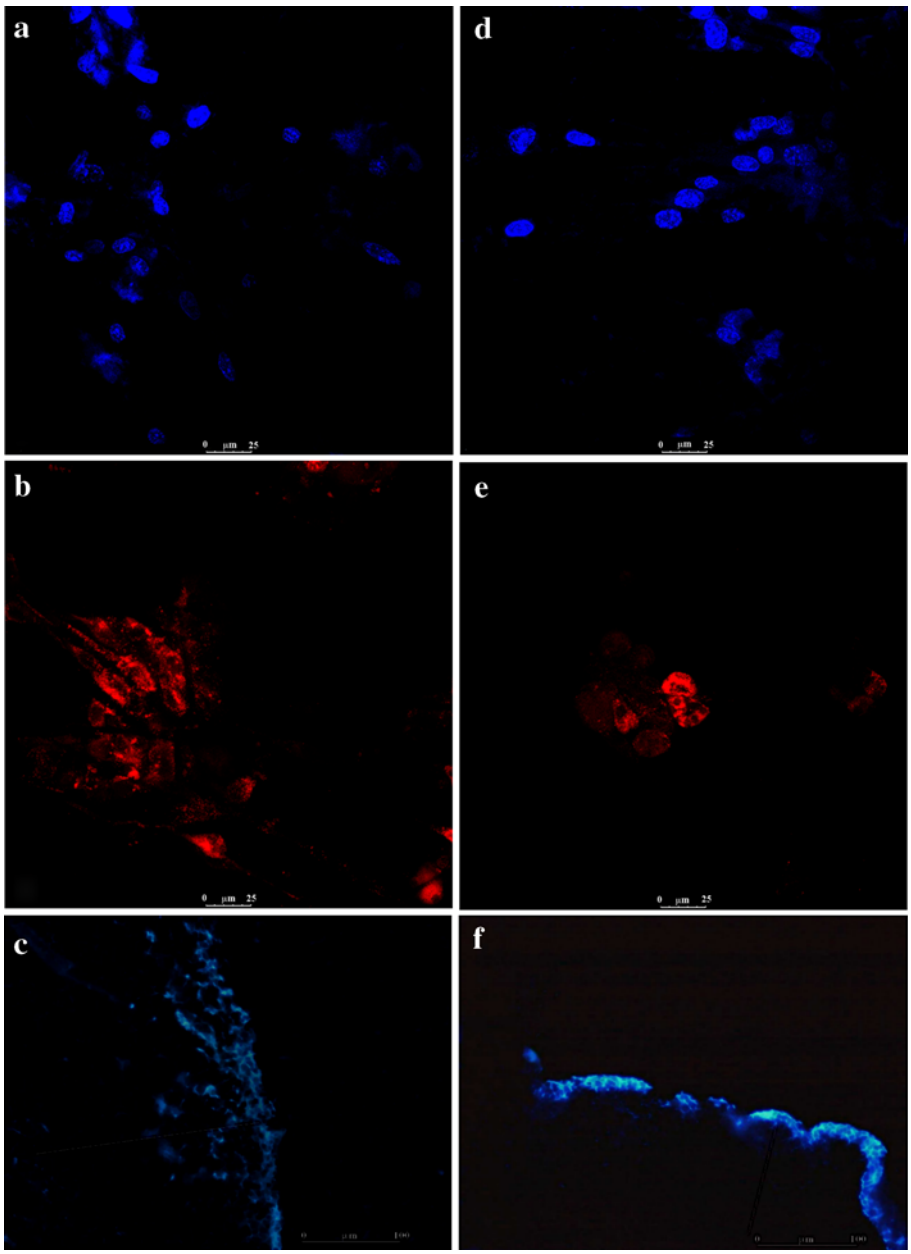

Fig. 6 CEC cultures 14 days after seeding on a–c PCL:$CHCl_3$ mat and d–f on PCL:THF:DMF mat, stained with a, d DAPI (×630), b, e PKH26 (×630), and c, f cryosections stained with DAPI (broken lines identify cross-section of the mat) (original magnification ×100))

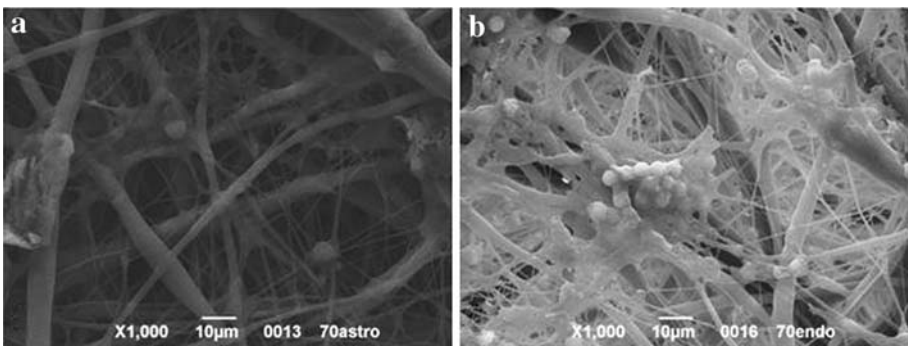

Fig. 7 SEM micrograph of HAs (a) and CECs (b) after 14 days seeding on PCL:CH_3Cl mat

SEM analysis allowed us to better observe cell-fiber interactions. Both HAs (Fig. 7a) and CECs (Fig. 7b) cultured on micrometric PCL mat, appeared flattened forming fibers bridges to join the fibers. These results suggested that both cell types well interacted and integrated with surrounding fibers.

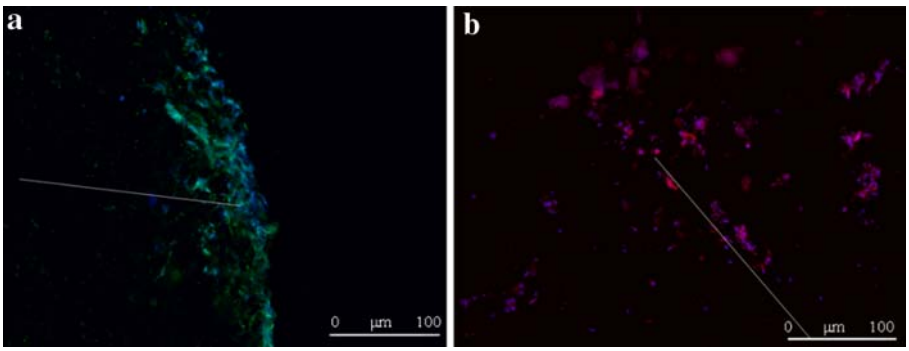

Fig. 8 HA (a) and CEC (b) culture cryosections, after 14 days seeding, on PCL:CH_2Cl_2 mat stained with anti-GFAP and DAPI (a) and with anti-von Willebrand factor and DAPI (b). (broken lines identify cross-section of the mat) (original magnification ×100)

Moreover, fluorescence immunocytochemistry staining (Fig. 8) showed that both HAs and CECs displayed typical markers expression, GFAP (Fig. 8a) and vWF (Fig. 8b) for astrocytes and endothelial cells, respectively.

4 Discussion

Aim of the present study was to assess whether micrometric and submicrometric PCL electrospun mats could promote the survival, proliferation and culture of HAs and CECs.

Electrospinning is a versatile technique of producing micro- and nano-fibrous mats [14]. Electrospun fibrous meshes show an organized architecture mimicking the ECM and, holding the promise to provide the topographic cues to the seeded cells, might enhance tissue regeneration. In a previous study, cytotoxicity evaluation showed that electrospun PCL mats were free of toxic amounts of residual solvent/s and were biocompatible for HUVEC cultures [2].

In this study, the rationale was to evaluate the cytocompatibility of electrospun PCL micro and sub-micro fibers for rat HAs and CECs, both cell types playing an active role in the formation and maintenance of the BBB.

In our study, cell response demonstrated that both micrometric and sub-micrometric mats supported adhesion, proliferation, cellular phenotype and spreading of rat HAs and CECs. After 14 culture days, HA viability did not significantly increased, following a trend comparable to that observed for cultures on TCPS. We hypothesize that astrocytes were in a normally quiescent condition considering that for culturing we used serum-addicted medium without added growth factors that could enhanced their viability [15]. At our knowledge, a very few number of literature data concerning astrocyte cultures on PCL mats were carried out and the most part of these studies were realized in in vivo experiments. Recently Gerardo-Navarro et al. [16] demonstrated that human neural progenitor-derived astrocytes increased the adhesion and migration rate in the blended nanofibers mats (25% collagen Type I, 75% PCL) in comparison to PCL nanofibers, while proliferation was not affected. Moreover Brynda et al. [17] demonstrated the importance of the presence of collagen layers on hydrogel surfaces in association with laminin or fibronectin, enhancing the adhesion and growth of astrocytes on the hydrogel in comparison to cell-non-adhesive hydrogel surface. These results confirmed previously reported data for which conditioning with cell adhesion proteins, such as collagen or fibronectin, prior to the cell seeding is a recognized optimal in vitro culture condition aimed to facilitate cell adhesion. In our experiments PCL mats were soaked in collagen solution, the presence of collagen onto and/or within electrospun fibers only assisted cell attachment. In fact, the viability of HAs on both the collagen-treated tissue culture plate support (TCPS) and on the untreated one, was comparable, the difference being not statistically significant.

In the case of CEC viability, a significant increasing trend was found. In particular, after 14 days, higher cell viability was observed for sub-microscale mat with respect to the micro-fibrous one. This behaviour can be related to the surface topography. Xu et al. [18] proved that the proliferation of vascular endothelial cells (ECs) was enhanced on smooth poly(-lactic acid) surface rather than on a rough one. They suggested that, depending on mat roughness, cell interaction with substrate surface could activate different focal contact structure connected with the control of cytoskeletal arrangements and of cell behaviour (such as cell proliferation). Furthermore, a different cell response to the surface roughness has been demonstrated by a number of researchers [19, 20]. The two investigated electrospun mats were comprised of fibers of different size and topography, the microfibers showed a nanoporous surface while submicrofibers presented a rather smooth surface (Fig. 4). This effect can be attributed to a thermodynamic instability during the electrospinning process in which phase separation takes place. The phase separation yields both solvent-rich and polymer-rich regions, being the nanopores a consequence of the solvent-rich regions within the jet [21]. Jarusuwannapoom et al. [22] investigated the effect of the

solvents on the electrospun polystyrene mats, observing that the rough surface of the collected Þbers may be a result of the very low boiling point of the chloroform (61.2°C), while smooth Þbers were obtained using DMF as solvent and for any spinning condition. However, the exact mechanism behind cell response to different surface topography needs to be further investigated.

The presented results demonstrated that the different cell-mat/cellÐcell interactions depended on mat structural architecture. By comparing cell morphology and cryosection staining of the two constructs, it can be observed that both HAs and CECs elongate on the microÞbrous mat following Þber direction instead of adopting a random orientation and spread-out morphology (similar to that observed on two-dimensional smooth substrates) when cultured on submicroÞbrous net. Moreover, SEM analysis demonstrated the interaction and integration of both cell types with PCL microÞbers. Additionally, microÞbrous environment favoured cellular inÞltration with respect to submicroÞber one, where HAs and CECs were unable to migrate within the mat, leaving an acellularized internal region.

The Þber dimension and the pore size resulted hence to be critical mat features, affecting cell attachment, proliferation, migration and ingrowth. Kwon et al. [23] found that HUVECs proliferated better on scaffold with Þber diameter of 1.16 ± 0.17 μm with respect to scaffold with the same porosity and Þber dimension of 7.02 ± 1.03 μm, and suggested that highly packed fabrics or high-surface-density Þbers provide an extremely high surface-to-volume ratio, which favours cell attachment and proliferation [24]. The decrease in the Þber size of fabric resulted in an increased Þber density and mechanical strength that seem to favour cells adhesion and proliferation. However, our results suggest that a decreased Þber size resulted in a 3D architecture characterized by lower average 2D void size that limit cell inÞltration and 3D colonization. Carampin et al. [25] described that electrospun polyphosphazene nanoÞber (Þber diameter 0.8 ± 0.1 μm) scaffolds, seeded with CECs, showed a cell monolayer having 100% confluence. However, even if nanoÞbers supported cell adhesion and proliferation, ECs were unable to migrate through the wall thickness and the inner part of the scaffold. In a previous study it has been shown that HUVEC behaviour was remarkably affected by morphological features of PCL mats, demonstrating that microÞbrous network was a more suitable environment for cell colonization with respect to the submicroÞbrous one [2]. Here presented results confirmed that submicroÞbrous polymeric mat could favour cell attachment, proliferation and spreading but their small average 2D void size, usually smaller than normal cell size, might limit 3D cell colonisation and inhibit cell inÞltration and migration [26]. On the other side, microÞbrous mat, even if not the same size scale as ECM, could be advantageous since it comprised larger voids that allow and facilitate cellular inÞltration and/or diffusion of nutrients enhancing their potential 3D application [27].

5 Conclusions

In this work the response of the blood brain barrier (BBB) cell types to the three-dimensional architecture of electrospun PCL mats was investigated. It was observed that hippocampal astrocytes (HAs) and cerebromicrovascular endothelial cells (CECs) adhered, were viable and grew on micro- and submicro-electrospun PCL Þbrous mats. Moreover, cells maintained their speciÞc phenotype on both mats, indicating a biological function of the cells. However, cell inÞltration and 3D growth were inßuenced by Þber dimension and 2D void size: microÞbrous network appeared, indeed, to be a more suitable environment for cell colonization. Based on our results, the proper Þber architecture can be regarded as a crucial issue to be considered in order to deal with suitable polymeric mats tailored for speciÞc in vitro application.

Acknowledgements This research has been supported by PRIN 2006 fundings ÔÔProgettazione e realizzazione di scaffolds nanostrutturati organici, inorganici ed ibridi da utilizzare in medicina rigenerativa come substrati per il differenziamento di cellule staminaliÕÕ. The authors wish to thank Dr. Francesca Nanni and Prof. Giampiero Montesperelli (Department of Chemical Science and Technology, University of Rome ÔÔTor VergataÕÕ) for SEM analysis.

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