

Electrospun Poly(L,D-lactide) Scaffolds Support the Growth of Human Embryonic Stem Cell-derived Neuronal Cells

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Abstract: Stem cell-derived cells, especially neural derivatives, are currently attracting considerable attention as a future source of clinical transplantations for the treatment of various neurodegenerative diseases and insults. Human embryonic stem cells (hESC) offer an unlimited source for the production of neural cells. Transplantation of these cells, however, does not necessarily result in optimal cell graft survival, and therefore functional improvement by *in vitro* engineering of the cell grafts is necessary. Here, we evaluated the effects of a clinically relevant substrate, poly(L,D-lactide) (PLDLA), as a growth matrix for hESC-derived neurons on cell viability, growth, and maturation. The results were analyzed using a cell viability test, immunocytochemistry, neuron and neurite outgrowth counts, and scanning electron microscopy. *In vitro* culturing of cells on PLDLA scaffolds induced the differentiation, maturation, and growth of neurites comparable to cells grown on a laminin surface during 7 days follow up. These results suggest that PLDLA is a potential substrate for the preparation of neural tissue-engineered products.

Keywords: Differentiation, Nerve tissue engineering, Neural cell, Neurite outgrowth, Poly(lactide), Scaffold, Stem cell.

INTRODUCTION

Stem cells, especially embryonic stem cells, have an unlimited capacity to proliferate and differentiate into any kind of human body cell type. These unique properties make stem cells a potentially valuable source of living material for tissue engineering, especially for tissues with low self-renewable capacity, like nerve tissue [1]. Regenerative treatments for neurologic deficits are thus currently focused on stem/neural cell transplantations [2]. Human and animal studies indicate that transplantations have some beneficial functional effects, but the survival, migration, maturation, and integration of transplanted cells needs to be enhanced [2, 3]. Thus, more sophisticated approaches toward combining cells and supporting biomaterials, mimicking the extracellular matrix, may lead to tissue engineering applications for both the central and peripheral nervous system [4].

Both natural and synthetic biomaterials have been tested as matrices for neural cells [1, 5-10]. To create functional cell grafts, the supporting matrix should have a structure that enhances cell survival, proliferation, maturation, and targeted growth, which sets up specific requirements for the material. For example, the selected material should enable cell attachment and targeted neurite outgrowth [11] which may be further facilitated by incorporated biomolecules [12].

Further, for clinical relevance, the material(s) should be tested particularly with human-derived neural cells for tissue engineering purposes.

Poly(lactide)-based products are already widely used in clinics. Poly(lactides) can be easily processed into different forms, such as nanofibers *via* phase separation, template synthesis, drawing, or electrospinning [9, 13, 14]. Poly(L-lactide) nano/micro fibrous scaffolds produced by electrospinning have been successfully used for culturing neural stem cells derived from neonatal mouse brain [10]. These studies suggest that the material selected to support the neurite outgrowth should contain fibers that are less than 5 μm in diameter [10, 11]. In particular, the electrospinning technique enables easy and efficient production of nanoscale fibers [3, 13].

Electrospinning is a technique in which an electrostatic field is used to prepare nano-scale fibres from polymer solutions and melts. The principle of the electrospinning method is quite simple - the electrostatic field stretches the polymer solution into jets while the solvent evaporates away. The fibres solidify while collected onto metal collectors of different shapes. By varying the shape of the collector, aligned or randomly oriented fibres, 2D or 3D structures, or certain shaped scaffolds can be processed [9, 15].

Here, we cultured hESC-derived neural cells on electrospun 3D poly(L,D-lactide) (PLDLA) scaffolds for 7 days. Cell viability, attachment, neuronal phenotype, and neurite outgrowth were evaluated. The results indicated that thick 3D PLDLA scaffolds supported neuronal cells as well as the typical culturing matrix laminin.

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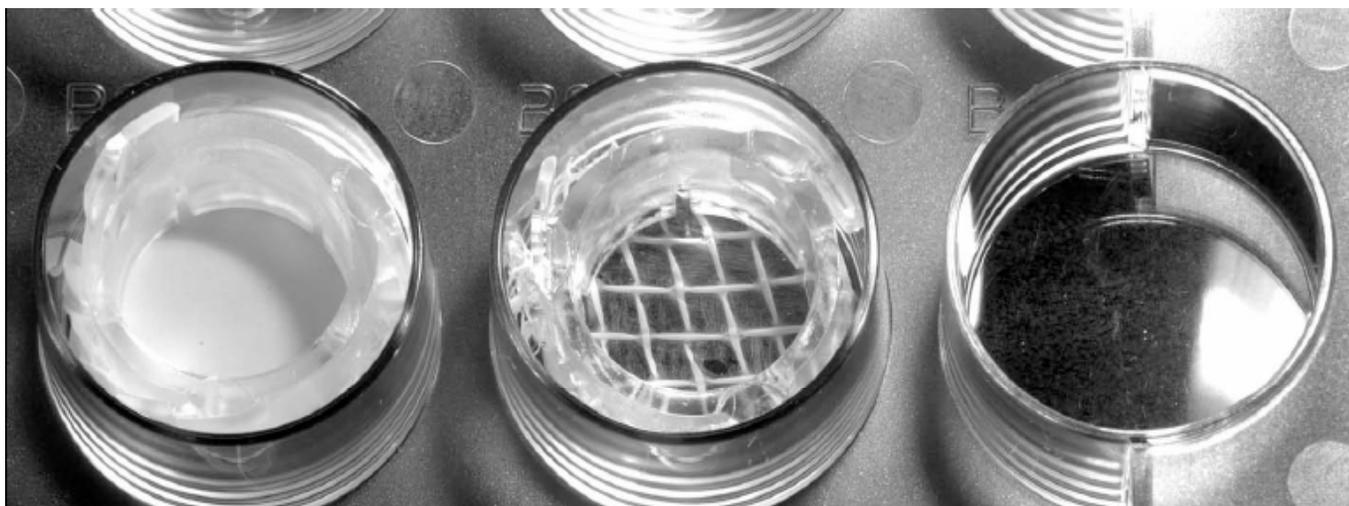


Fig. (1). Experimental design. Two types of PLDLA scaffolds were anchored on the bottom of 12-well plates with CellCrown inserts. Left: thick PLDLA scaffold; Middle: thin PLDLA scaffold; Right: laminin-coated well bottom. Cells were seeded into wells after a 2-day pre-incubation period in cell culture medium.

MATERIALS AND METHODS

PLDLA

Co-polymer PLDLA containing two chiral lactide forms: 4% D-lactide and 96% L-lactide was purchased from Purac Biochem (The Netherlands). The viscosity of the polymer pellets was 2.18 dl/g according the manufacturer's data sheet. The molecular weight of the polymer was 93,732 g/mol, determined by gel permeation chromatography [16].

Fabrication and Preprocessing of Scaffolds

The PLDLA was dissolved in 1,1,1,3,3,3-hexafluoroisopropanol at room temperature to form a 5% polymer solution. The resulting polymer solution was electrospun in a 2-kV/cm electric field and the distance between the spinning needle and collector was 15 cm. The spinning process was performed as described previously [15].

Two types of mesh were processed. The first structure (thin 3D scaffold) was fabricated on a metal grid (wire distance approximately 2.5 mm) so that the electrospun fibres formed a thick mesh on top of the metal wires, whereas thinner mesh were formed between the wires. The structure was highly oriented along the grid wires.

The second structure (thick 3D scaffold) was fabricated on a flat copper dish (smooth collector) resulting in a freely formed randomly-oriented 3D structure. Both types of mesh were processed to samples of 3.5 cm in diameter and sterilized with gamma radiation (26-30 kGy). The formed structures are shown in Fig. (1).

Pre-Incubation of PLDLA Scaffolds

PLDLA scaffolds were placed on the bottom of 12 culture wells (Nunc, Denmark) with CellCrown inserts (Scaffdex, Finland). Scaffolds were pre-incubated with cell culture medium (composition described below) at +37°C for 2 days. The medium pH was measured repeatedly during and after pre-incubation with Duotest pH-paper (Macherey-Nagel, Germany).

Cell Culture and Seeding

The hESCs (HS360 and HS362) were cultured on postnatal human foreskin fibroblasts as previously described [17]. Regea, Institute for Regenerative Medicine, University of Tampere, Finland has the approval of the Ethical Committee of Pirkanmaa Hospital District to culture hESC lines derived at Karolinska Institute (1426/32/300/05). The hESC colonies were cut into small pieces and placed in neural differentiation medium containing Dulbecco's Modified Eagle's Medium/F12 (Gibco, Invitrogen, Finland) and Neurobasal medium (Gibco) 1:1, supplemented with 2 mM GlutaMax (Gibco, USA), 1× B27 (Gibco), 1× N2 (Gibco), 20 ng/ml fibroblast growth factor (R&D Systems, Minneapolis, MN), and penicillin/streptomycin (25 U/ml, Cambrex, Belgium). HESCs formed neurospheres in suspension culture as previously described [18]. The medium was changed 3 times per week and the spheres were mechanically dissected on a weekly basis. Neurospheres were cultured for 6 to 8 weeks prior to seeding onto the scaffolds at the state when they were fully differentiated into neural cells that further differentiate into neurons [19].

Cells were dissociated into a mixed suspension containing both single cells and cell clusters. There after, ~100 000 single cells and 3 to 5 cell clusters were seeded onto PLDLA-scaffolds. In addition, cells were seeded onto laminin (10 µg/ml, Sigma-Aldrich, Finland), which served as a positive growth matrix control. Cells seeded on non-treated polystyrene wells were used as a negative growth matrix control.

PLDLA Scaffolds Functionalized with Laminin

Additional PLDLA scaffolds were treated with 10, 5, 1, and 0.1 µg/ml laminin prior to cell seeding. Briefly, 500 µl of each laminin solution was added to pre-incubated scaffolds and incubated for 2 h at 37°C. For cell experiments, scaffolds were washed 3× with phosphate buffered saline (PBS) prior to medium adding and cell seeding. Cells were allowed to grow onto scaffolds for 7 days prior to analysis. To evaluate the incorporation of laminin by physical

absorption onto PLDLA fibers, FITC labeling kit (Calbiochem, Germany) was used. First, laminin coated PLDLA samples were rinsed carefully with carbonate buffer. Thereafter, FITC solution (1 mg/ml) was added and incubated 5 minutes with samples in room temperature. The unbound FITC was rinsed with PBS and samples were imaged with Olympus IX51 inverted microscope with fluorescence set up combined with Olympus DB71 digital camera (Olympus, Finland) with constant exposure time of 1400 ms.

Cell Viability and Neurite Outgrowth Studies

During the 7 days of culturing on PLDLA-scaffolds, cell attachment and growth were evaluated with contrast-phase microscopy (Nikon, Finland).

Live/Dead Assay

After 7 days of culturing on the scaffolds or control wells, cells were stained using the Live/Dead Viability/Cytotoxicity Kit for mammalian cells (Molecular Probes/Invitrogen, Finland). Briefly, the kit stains live cells with the green fluorescence dye calcein-AM (emission at 488 nm) and dead cells with the red fluorescence dye ethidium homodimer-1 (emission at 568 nm). Cells were incubated with calcein-AM (0.1 μ M) and ethidium homodimer-1 (0.5 μ M) for 30 minutes and immediately imaged with fluorescence microscope system described above (Olympus). Three representative images (\times 10 objective) were obtained and \sim 100 cells/group were counted.

Immunocytochemical Characterization

Cells were fixed with 4% paraformaldehyde (Fluka, Italy) and stained with neuronal markers. Briefly, cells were blocked with 10% normal donkey serum (NDS, Sigma), 0.1% Triton-X 100 (Sigma), and 1% bovine serum albumin (BSA, Sigma) for 45 min at room temperature. The cells were then washed once with 1% NDS, 0.1% Triton-X, and 1% BSA. Primary antibodies; rabbit anti-microtubule associated protein (MAP-2, 1:800, Chemicon International, Millipore, Massachusetts, MA) or mouse anti-neurofilament 200 kD (NF-200, 1:600, Sigma), in 1% NDS, 0.1% Triton-X, and 1% BSA were incubated at +4°C overnight. Cells were washed three times with 1% BSA and secondary antibodies, Alexa fluor 488 anti-rabbit (1:400, Molecular Probes) and Alexa fluor 488 anti-mouse IgG (1:400, Molecular Probes) in 1% BSA were added for 1 h at room temperature. Finally, cells were washed three times with PBS and twice with phosphate buffer and mounted with Vectashield containing DAPI (4',6-diamidino-2-phenylindole, Vector Laboratories, England), then imaged with the Olympus IX51 system.

Scanning Electron Microscopy

For scanning electron microscopy (SEM), scaffolds samples were fixed with 5% glutaraldehyde overnight at room temperature. Thereafter, samples were washed with PBS and post-fixed with 1% osmium tetroxide. Samples were then dried with a critical point dryer to preserve the cellular structures. Finally, samples were sputtered with gold

and observed with a JEOL Scanning Electron Microscope JSM-5500 with PGT Prism 2000-Si(Li) EDS detector.

Cell and Neurite Length Counting

Neuronal cells and neurites were counted from immunofluorescence images. At least four 10 \times objective views were randomly captured from each sample (1 to 2 samples/group). A total of 417 to 986 cells/group were observed with nuclear staining (DAPI) and the portion of neuronal cells, that is, MAP-2 positive cells, was calculated. The number and length of neurites per neuron were then calculated from at least 70 neurons/group with Cell*-software (Olympus).

Statistics

Statistical analyses were performed using SPSS v.11 software. Differences in neuronal growth and neurite lengths between different sample groups were analyzed using a non-parametric Kruskal Wallis test ($>$ 2 groups) followed by Mann Whitney *post hoc* analysis. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Biocompatibility of PLDLA-Electrospun Scaffold

The fabrication process was optimized so that the mesh was strong enough not to break, but the fiber diameter was as small as possible. Two types of PLDLA-scaffolds forming either a thin 3D structure (2 - 3 fiber thickness) or a thick 3D structure ($>$ 10 fiber thickness) were processed (Fig. 1) which structure thicknesses were estimated from microscopy images. The fiber diameters were analyzed at the end of the study from SEM images. The fiber diameter varied from 800 nm to 5 μ m.

The PLDLA-scaffolds were pre-incubated for 2 days prior to cell seeding. Because PLDLA is biodegradable material, medium pH was monitored prior and during cell experiments. The pH of the media within the PLDLA-scaffolds remained relatively constant, ranging from 7.8 to 8.2, which did not differ from control culture (medium + cells). Moreover, any discernible degradation of the fibers was not detected during the 7 days follow up.

PLDLA-scaffolds were inserted into well plates with CellCrown inserts, which maintained the stability of the scaffolds (Fig. 1). After the cells were cultured onto scaffolds, the following analyses were performed without breaking the scaffold/cell structure.

During culturing, cells were observed with a light microscope. Cells attached to the thick 3D scaffold structures as well as to the laminin-coated wells (Fig. 2D, F). In non-coated wells, attachment was poor (Fig. 2C). Attached cells started to migrate and mature within 3 days in culture.

A Live/Dead assay was used to check the cell viability on thick PLDLA-scaffolds. The cell viability on PLDLA-scaffolds was good, although slightly more dead cells were detected on the PLDLA-scaffold compared to laminin (compare Fig. 2A, B). Most importantly, similar to those

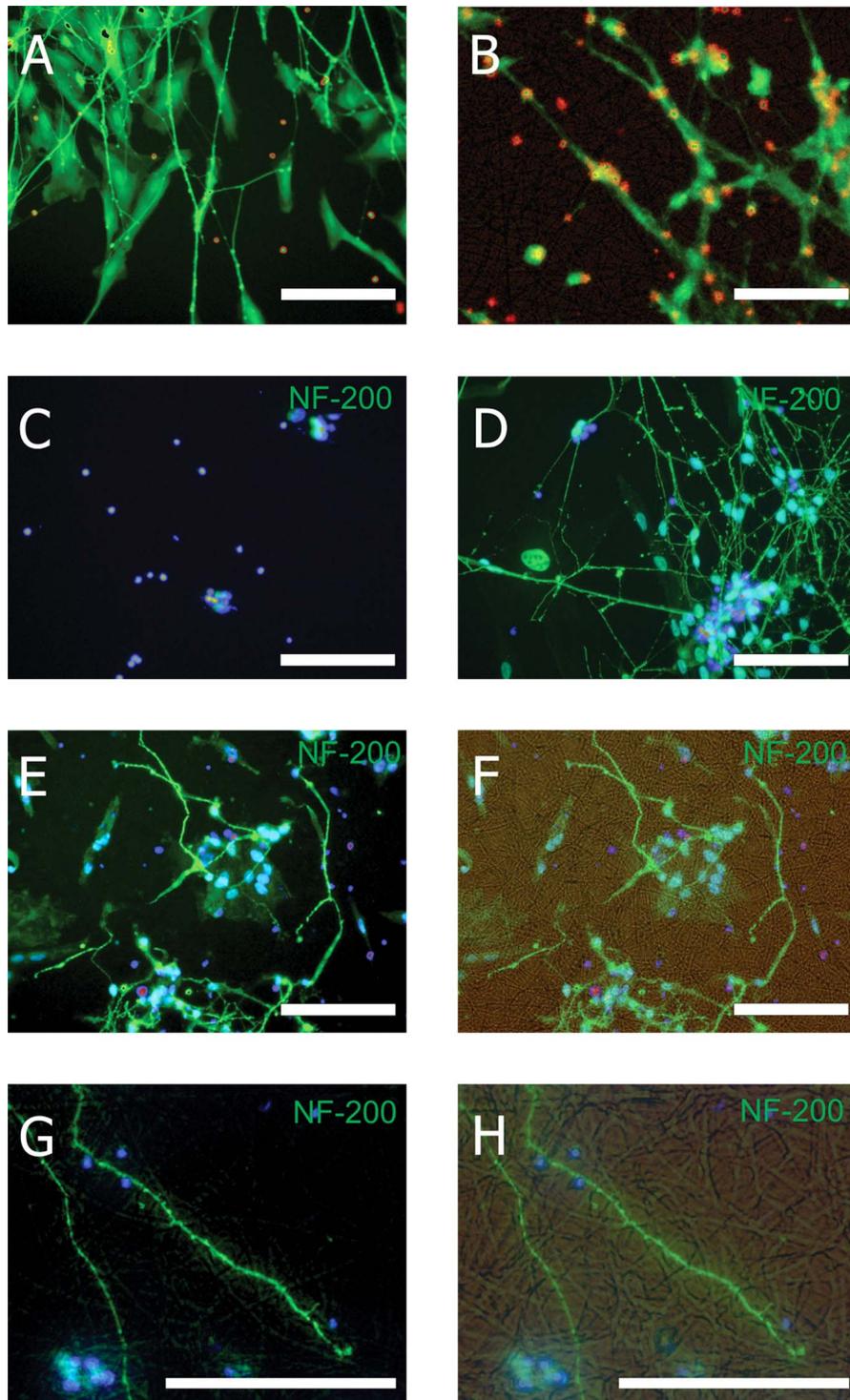


Fig. (2). Neuronal cell growth on thick PLDLA scaffolds. Cells survived well on PLDLA scaffolds (**B**) compared to laminin-grown cells (**A**); green labels viable cells and red dead cells. A neuronal characteristic of cells was confirmed by NF-200 staining (green, **C-H**); Blue: DAPI-positive nuclei. Neurons grew equally well on PLDLA (**E**) and laminin (**D**), whereas growth in nontreated wells was very poor (**C**). Neuronal cells formed neurites that followed PLDLA fibers as shown in the superimposed image (**F**), and in more detail in G-H. Scale bars: 100 μ m.

cells grown on laminin (Fig. 2D), the morphologic characteristics of the cells on PLDLA fibers were typical for neuronal cells (Fig. 2E for NF-200 staining), whereas neuronal growth in non-treated well plates was very poor (Fig. 2C). Moreover, NF-200 positive neurites seemed to

contact the PLA-fibers with tiny filament-like structures (Fig. 2G). Neurites followed the fiber orientation, which was observed as irregular turns in PLDLA fibers (Fig. 2F) compared to the laminin-grown neurites.

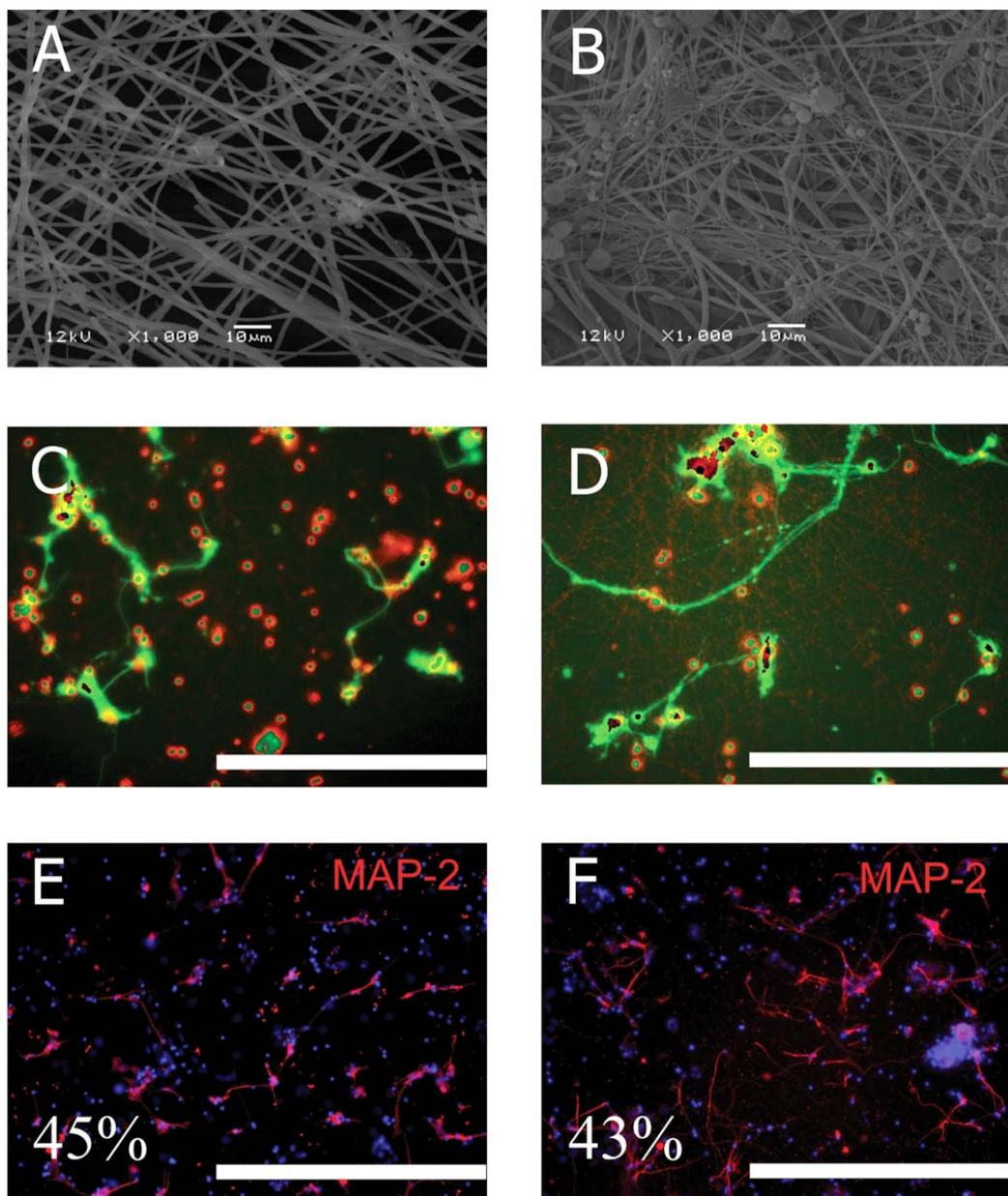


Fig. (3). Neuronal cell growth on thin and thick 3D PLDLA scaffolds. Neuronal cells grew better on thick 3D PLDLA scaffolds than on thin 3D PLDLA scaffolds, as shown in the SEM images (**B** vs. **A**), live death analysis (**D** vs. **C**), and in MAP-2 staining (**F** vs. **E**). Scale bars: (**A**-**B**) 10 µm, (**C**-**F**) 200 µm.

Comparison of Thin and Thick 3D Scaffold Structures

The thin 3D structure comprised an approximately 3-fiber thick structure (Fig. 3A) whereas the thick 3D structure comprised an approximately 10-fiber thick structure (Fig. 3B). The amount of MAP-2 positive neuronal cells was similar among the laminin-coated controls, thin 3D structure, and thick 3D structure (36% vs. 45% vs. 43%, respectively; compare Fig. 5A to Fig. 3E, F). The total neurite length/cell (median: 116.0 µm vs. 111.5 µm vs. 126.9 µm) was also similar ($p > 0.05$, Kruskal-Wallis) among neurons grown on the laminin, thin 3D structure, or the thick 3D structure, respectively. Average neurite lengths were also similar (median: 58 µm vs. 55.7 µm vs. 62.8 µm, $p > 0.05$, Kruskal-

Wallis) between groups. Attachment of the cells, however, was better on the thick 3D structure than on the thin 3D structure (Fig. 3A vs. B) and therefore we focus on the thick 3D structure on the following analysis.

Thick 3D PLDLA Structure

Representative neuronal growth on the thick 3D PLDLA scaffolds is shown in Fig. (4). An SEM image of the typical morphology of bipolar neurons grown on the thick 3D PLDLA scaffold is shown in Fig. (4E). Moreover, cells grew on fibers and they matured as neurites outgrew along the fibers, and the neuronal cells formed networks (Fig. 4). The

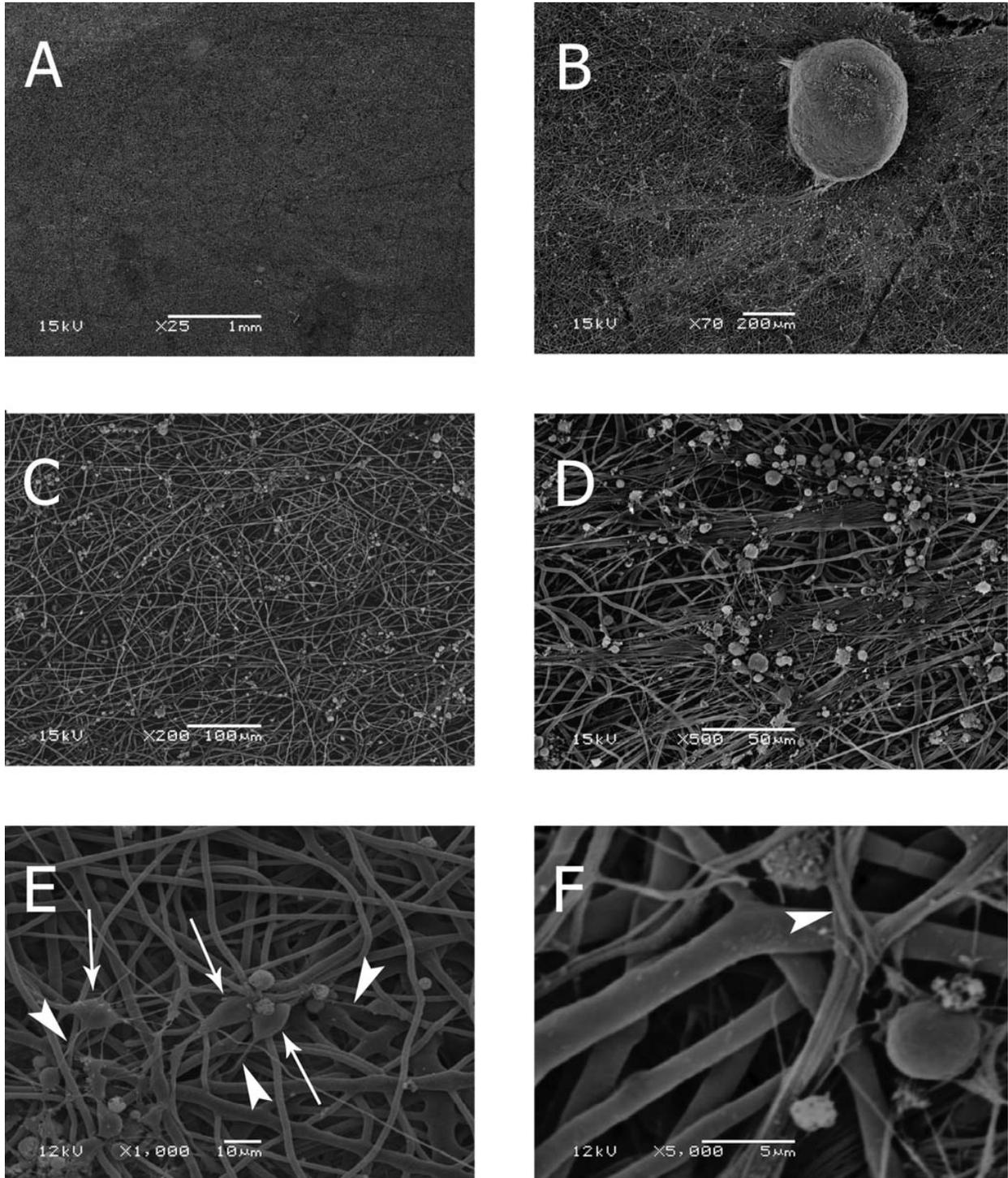


Fig. (4). Representative SEM images of neurons grown on thick 3D PLDLA scaffolds. Neurons grew well on PLDLA scaffolds (A-C), formed networks (D) and neurite outgrowth at least partially followed the fiber alignment (E-F). Scale bars: (A) 1 mm, (B) 200 μm , (C) 100 μm , (D) 50 μm , (E) 10 μm , (F) 5 μm .

neuronal cells migrated from the attached neural cell clusters as shown in Fig. (4B).

The thick 3D structure was then functionalized with 10, 5, 1, or 0.1 $\mu\text{g/ml}$ laminin and neuronal cell growth was analyzed. FITC-labeling showed that laminin incorporated

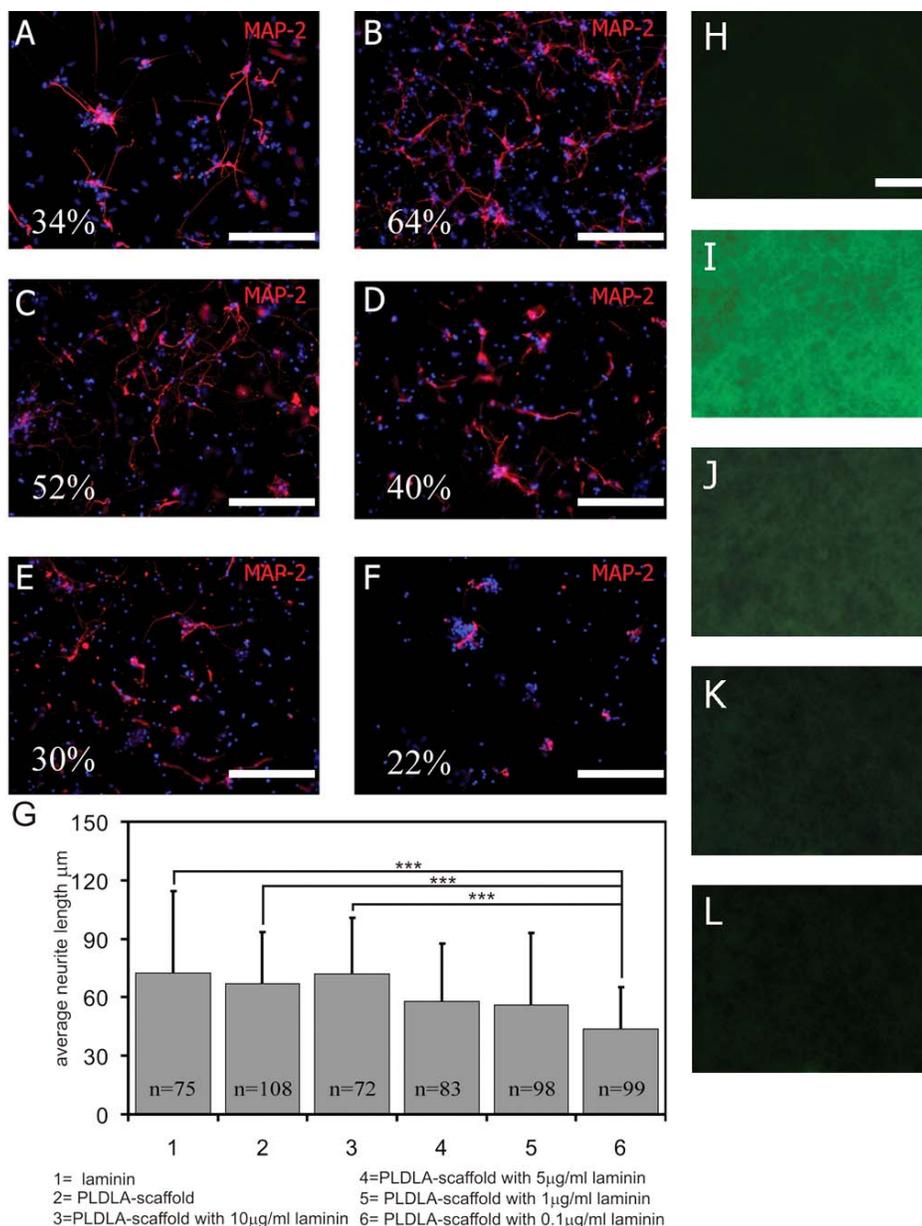


Fig. (5). Functionalization of PLDLA scaffolds. MAP-2 positive neurons (red) grew equally well on laminin (A) and PLDLA scaffolds treated with 10 $\mu\text{g/ml}$ laminin (B). Reduction of laminin to 5 $\mu\text{g/ml}$ (C), 1 $\mu\text{g/ml}$ (D), and 0.1 $\mu\text{g/ml}$ (E) resulted in poorer neuronal cell growth. Neuronal growth in nontreated polystyrene wells was very poor (F). Average neurite length in laminin, PLDLA, or PLDLA + 10 $\mu\text{g/ml}$ laminin was similar, but neurons grown on PLDLA + 0.1 $\mu\text{g/ml}$ formed significantly shorter neurites (G). The attachment of laminin to PLDLA fibers were evaluated with FITC-labeling. Labeled fibers is presented in figures H-L: H= without laminin, I=10 $\mu\text{g/ml}$, J= 5 $\mu\text{g/ml}$, K= 1 $\mu\text{g/ml}$, and L= 0.1 $\mu\text{g/ml}$. Scale bars: from A to F: 100 μm , from H to L: 50 μm . n = amount of MAP-2 positive cells counted, errors bars present standard deviation, *** $p < 0.001$ Mann-Whitney U-test.

onto fibers (Fig. 5H-L) and intensity of the labeling correlated with the laminin concentration used. Cell viability and the amount of MAP-2 positive cells were similar to that of cells on the nonfunctionalized thick 3D structures in scaffolds functionalized with 10 or 5 $\mu\text{g/ml}$ of laminin (see Fig. 5). Of all the functionalized scaffolds, cell viability and neuronal phenotype was lowest in thick 3D structures treated with 0.1 $\mu\text{g/ml}$ laminin (Fig. 5E). The neurite lengths are presented in Fig. (5G). The mean neurite amount/cell was 2. Neurite outgrowth on the laminin-treated thick 3D structures

differed significantly from that on nonfunctionalized scaffolds ($p < 0.000$, Kruskal-Wallis). The thick 3D scaffolds not treated with laminin and those treated with 10 $\mu\text{g/ml}$ laminin supported neurite outgrowth well and both the total neurite length and average neurite length were similar among cells grown on these matrices ($p > 0.05$). Both the total neurite length and mean length gradually decreased when the concentration of the laminin coating was decreased (see Fig. 5G for details).

DISCUSSION

The results of the present study demonstrated that an unorganized 3D nanofibrous PLDLA scaffold is a promising growth matrix for hESC-derived neurons. The cells were able to attach, grow, differentiate, and migrate on PLDLA scaffolds as well as on their normal culture matrix, that is, laminin-coated polystyrene well plates.

Our results indicated that the electrospun 3D PLDLA scaffolds are suitable for culturing hESC-derived neuronal cells. These observations are consistent with earlier studies in which the biocompatibility of polylactide-scaffolds and neuronal cells was examined [10, 20]. The normal extracellular cell matrix is a complex 3D biopolymer structure that surrounds cells in tissue. By mimicking that structure, it is possible to create a structure that enhances cell survival, proliferation, and maturation [21]. The process of electrospinning produces a highly porous structure containing very thin, even less than 70 nm diameter, fibers [15]. This type of structure mimics the extracellular matrix and is therefore suitable material for scaffolds, as demonstrated here. The pores initiate the cell growth while the fibres provide the structure for the system. A wide range of different synthetic polymers can be processed *via* electrospinning [9]. PLDLA fibers with a diameter ranging from 800 nm to 5 μ m support neuronal cell growth, although the thinner fibers are better for orienting neurite growth, as reported previously [14].

Unlike most previous studies, we used a co-polymer of L-lactide and D-lactide that is slowly biodegradable [22] [23]. The mix of 4% D-lactide and 96% L-lactide co-polymer has better degradation properties in the body than PLLA, which does not necessarily fully degrade or at least degrades very slowly in a biologic environment [22]. Yang and colleagues (2005) followed the growth of neonatal mouse neurons on PLLA scaffolds for 2 days [10]. Here, we extended the follow-up period to 1 week and the results showed that neuronal cells grew well on PLDLA scaffolds after a longer culturing period. At the end of this follow-up time, biodegradation of the material was not observed which is in line with previous study reporting only minor degradation of PLDLA fibers during first 5 weeks *in vitro* conditions [23]. In addition, the pH of the culturing media was monitored during the culturing and remained constant during the follow-up. Based on these findings, PLDLA is a suitable scaffold material for neuronal cells. Whether neuronal cells grown on PLDLA scaffold form functional networks as shown for laminin grown neurons [24] will be addressed in the future studies.

The cells grew well on the randomly-oriented PLDLA scaffolds. Both aligned and randomly-oriented fibers have been examined in previous studies. The results of these studies suggest that, compared to randomly-oriented fibers, aligned fibers form a better scaffold for neuronal cells due to better neurite outgrowth [14, 25]. In the present study, however, the neurites grew equally well on randomly-oriented fibers and in laminin-coated control wells, the surface of which are known to support neurite outgrowth [18]. Randomly orientated fibers seemed to guide neurite growth (neurites grew along the fibers) to some extent. Processed fibers can be modified by functionalizing them with different substrates to increase cell attachment and

maturation [12, 26-28]. Previously, Chiu and colleagues (2007) evaluated the functionalization of nanofibrous poly-L-lactide with collagen for supporting osteoblast growth whereas Koh and colleagues (2008) reported nanofibrous poly-L-lactide with laminin for supporting PC12 neurite growth. Here, we functionalized PLDLA fibers by laminin treatment which was verified by FITC-labeling. Unexpectedly, coating the PLDLA fibers with laminin did not increase neurite outgrowth, which further suggests that the randomly-orientated PLDLA scaffolds supports neurite outgrowth as well as laminin alone. In previous study, functionalization of PLLA with laminin using various techniques showed that electrospun blended laminin-polymer nanofibers supported PC12 cells growth better than PLLA by itself but still the poly-L-lysine treated cell culture wells were superior [29]. The functionalization of PLDLA with laminin seemed to support the maintenance of MAP-2 positive neurons in higher concentrations (10 or 5 μ g/ml) whereas the lower concentrations (1 to 0.1 μ g/ml) resulted in poorer maintenance. This suggests that laminin used for functionalization works at similar concentrations as used for normal cell culture well coatings and thus no clear positive effect (better growth of the cells or reduced use of laminin) is gained by the functionalization. As the FITC labeling showed that the 10 μ g/ml of laminin incorporates very smoothly on PLDLA scaffolds and supports the neuronal cell growth, it may suggest that incorporation of less amount of laminin disrupts the nano-topography of PLDLA and therefore results in poorer neuronal cell attachment and neurite outgrowth. Thus, as PLDLA as such seems to work well without any further modifications, there is no need to add expensive human laminin which makes processing of the scaffold also less complicated.

The results of the present study indicate that hESC-derived neurons grow and mature well on 3D PLDLA scaffolds, and the functionalization fibers with laminin do not bring any extra value. These findings suggest that a more controlled and transplantable scaffolds can be processed for human-derived neurons which opens new avenues for *in vitro* studies of neuronal network functionality and the development of tissue-engineered products for clinical purposes.

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