



Tubular Scaffold Design of Poly(*L*-lactic acid) for Nerve Tissue Engineering: Preparation, Characterization, and In Vitro Assay

Mohammad Taghi Khorasani^{1*}, Hamid Mirzadeh¹, Ali Talebi¹, Shiva Irani¹,
and Morteza Daliri²

(1) Biomaterial Department of Iran Polymer and Petrochemical Institute
P.O. Box: 14965/115, Tehran, Iran

(2) National Research Center for Genetic Engineering and Biotechnology
P.O. Box: 14155-6343, Tehran, Iran

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ABSTRACT

In this study, nerve guidance material was designed and fabricated from crystalline poly(*L*-lactic acid) (PLLA) with microtubules orientation structure by using thermal-induced phase separation (TIPS) technique and dioxane as solvent. Factors such as polymer concentration and temperature-gradient of the system which affect solvent crystallization, microtubules orientation structure, and tubs diameters of the scaffold have been studied. Thus, the tubular morphology, diameter and orientation of the microtubules could be controlled by adjusting the concentration of the polymer solution and temperature-gradient of the system. In vitro P19 mouse embryonic carcinoma cell line was used as a suitable model system to analyze neuronal differentiation. Tubular morphology and differentiation of P19 cells were characterized using scanning electron microscopy (SEM). Results showed that these conditions provide tubular morphology with varying tubs diameters. By evaluating P19 cell differentiation into neuron cells that was confirmed by immunofluorescence staining technique it was shown that most of the cells attached and differentiated on the surface of the scaffold, through which axons penetrated randomly.

INTRODUCTION

Nerve guidance material should provide a physical support for axon extension and be nontoxic and non-irritating. It should also be viable for modification where the growth- and guidance-promoting molecules are loaded [1-2]. In addition, a tubular shape scaffold is preferred because such structure can form a close geometry for the growth of neurons [3]. On the basis of these requirements, various biomaterials, such as, poly(3-hydroxybutyrate) and biodegradable poly (esters) have been reported recently [4-8].

To date, much of the research

effort has been focused on the nerve guidance channels (NGC) to enhance the regeneration across nerve gaps. While they improve regeneration process, when being compared to no intervention, guidance channels rarely approach or match the performance of auto-grafts when the gaps are 10 mm or wider (in rats). This includes numerous studies with varying permeability of the guidance channels [9-11], involving electrically active channels [12-13], as well as degradable guidance channels [14-15]. While a few groups still pursue

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PLLA;
P19 mouse embryonic cell.

(*) To whom correspondence to be addressed.

E-mail: M.Khorasani@ippi.ac.ir

research trying to modify the NGC characteristics, there are emerging consensuses to bridge long peripheral nerve gaps which involve filling NGCs with scaffolds/constructs that promote regeneration.

By now, many techniques have been reported for fabricating the orientation-structured scaffolds [16-18]. One of them is thermal-induced phase separation (TIPS) technique [18]. By using this technique the orientation structure of the scaffold is guided by solvent crystallization under a certain temperature gradient. However, by this technique, the diameter of the formed orientation-structured microtubules is small and less than 120 μm .

For crystallizable polymeric systems (for instance PLLA), three different types of structures can be achieved:

- If phase separation occurs via nucleation of the polymer-rich phase (polymer concentration below the critical point), a globular microporous structure is obtained;

- If during phase separation there is a solvent-rich phase nucleation (polymer concentration above the critical point) a cell-tunnel microporous structure is obtained, where tunnels connecting the previously formed cells are due to the crystallization of the polymer;

- And finally, if polymer concentration is near the critical point, phase separation occurs via spinodal decomposition giving rise to a fibrillar microporous structure [19].

The other technique is electro-spin technique. By this technique, orientated fibrous scaffolds can be made. The orientation of the fibres is good and the fibres are equally distributed in the scaffolds. However, due to the limitation of the electro-spin technique, the diameters of the fibres can only reach several micrometers. Therefore, it is too small for the cells to grow and enter the scaffold [19].

Several other techniques have been also developed for the fabrication of porous biodegradable polymers useful for cell transplantation, i.e., solvent casting/salt leaching [18-20], phase separation [21-23], emulsion freeze drying [24], and gas foaming [25-26].

PLLA is the most important synthetic biodegradable polymeric material for fabricating scaffolds in tissue engineering [27-28] on the basis of its good

properties of non-toxicity, absolute biodegradability, adjustable degradation rate, and good biocompatibility [29-33].

In this work, TIPS technique was investigated to fabricate microtubules orientation-structured poly (L-lactide) (PLLA) with adjustable diameter. Novelty of our work is centered on the use of P19 cell as a model for nerve tissue engineering and using high crystalline PLLA through solid-liquid phase inversion method as a tubular scaffold material. The morphology of the scaffolds was identified by scanning electron microscopy (SEM) observations and the effects of the TIPS technique various parameters, such as, concentration of polymer solution and temperature gradient on the morphology of the microtubular orientation-structured scaffolds were studied and discussed.

EXPERIMENTAL

Materials

Poly L-lactic acid (PLLA) or Resomer 207 (L) was purchased from Boehringer Ingelheim (Germany). 1,4-Dioxane (Merck) was used as a solvent for the fabrication of biodegradable polymeric scaffolds. The polymers and solvents were used without further purification. The molecular weight of PLLA ($\bar{M}_w = 281000 \text{ g/mol}$) was determined by Waters gel-permeation chromatography (GPC) equipped with Shodex KF-800 columns at 35°C using THF as a solvent at a flow rate of 1.0 mL/min. The 1,4-dioxane and other reagents were analytical grades and directly used without further treatment.

Methods

DSC Measurements

A Perkin-Elmer differential scanning calorimeter (model DSC-2) was used for studying the thermal behaviour of PLLA. Samples were analyzed in N_2 inert atmosphere with heating rate of 10°C/min between -50°C to 200°C.

Fabrication of Scaffolds

The PLLA was dissolved in 1,4-dioxane to form solutions with predetermined concentration range 1-5 w/v%. The polymer solutions were cast into

Table 1. Scaffolds preparation conditions.

Samples (PDLLA 210)	Solution concentration (w/v%)	Cooling temperature (°C)
a	1.5	5
b	1.5	0
c	1.5	-20
d	1.5	-196
e	2.5	5
f	2.5	0
g	2.5	-20
h	2.5	-196
i	5.0	5
j	5.0	0
k	5.0	-20
l	5.0	-196

polyethylene tubs. The tubs were isolated by polystyrene foam. The phase separation of the polymer solution was performed by cooling the solution from bottom to top of the mould for reaching super-cooling of the polymer solution with carefully controlling of a unidirectional temperature gradient. The details of samples preparation conditions are summarized in Table 1. PLLA solutions were immediately put in refrigerator (5°C), freezer (-20°C) and liquid nitrogen (-196°C) at constant temperature. The phase-separated and solidified polymer/solvent systems were then transferred into a freeze-drying vesse (Crest, GAMMA 2-16 LSC, Germany) under vacuum (less than 45 Pa) and freeze-dried for 72 h. Finally, the produced microtubules orientation-structured scaffolds were further dried under vacuum at room temperature for 48 h to remove the residual solvent and finally preserved in a vacuum dessicator in 4°C refrigerator for further use.

The dry scaffolds were cut into cylindrical shape with a surgical blade (20 mm diameter, thickness 2 mm). Prior to cell seeding, 3D scaffolds were pre-wetted with 70% ethanol for 3 h to be sterilized and enhancing their water uptake. The ethanol was removed by soaking for 1 h, along with agitation and changing the phosphate-buffered saline (PBS) six times. Finally the scaffolds were left in the media overnight.

Characterization of Scaffolds

Scanning electron microscopy was performed on gold coated samples using a Polaron sputter coater. A scanning electron microscopy (VEGA-TE Scan SEM) operating typically at 10 kV was employed for morphology study. Samples and Fracture-frozen cross-sections of the scaffold were mounted onto the sample holder; sputter coated with gold and studied with SEM. Internal diameter of the porous of the scaffold was calculated according to the observed SEM micrographs.

Porosity Analysis

The density of PDLLA films was determined according to ASTM D1817-90 using pycnometer and then the porosity percentage of PDLLA films was calculated using eqn (1).

$$Porosity (\%) = \frac{\frac{1}{D_s} - \frac{1}{D_o}}{\frac{1}{D_s}} \times 100 \quad (1)$$

D_o = density of the pure PDLLA

D_s = density of the PDLLA scaffold

Cell Culture

The P19 cell line was obtained from the Pasteur Institute of Iran (C422). The cells were cultured and maintained as described by Rudnicki and McBurney [34]. Briefly, P19 cell culturing was started from a frozen stock by thawing cells rapidly at 37°C and then P19 cells were cultured in standard medium, consisting of α -MEM (GIBCO from Germany) supplemented with 10% calf serum and 100U/mL penicillin-streptomycin. The cells were maintained at sub-confluence by sub-culturing them every 48 h into tissue culture grade dishes.

To induce neural P19 cells differentiation, the cells were removed from the culture dishes mechanically. The cells were plated at a density of 5×10^4 cell/mL in a medium supplemented with 0.3 μ M retinoic acid (Sigma) into 100 mm bacteriological grade Petri dishes (0 day) and grown as aggregates until day 4 th. During this period, the medium was replaced after 2 days. The aggregates were plated onto tissue culture grade dishes containing scaffold samples in medium

without RA and culture continued until 10-11 days. After culturing and differentiation of P19 cells onto PDLLA scaffolds, samples were examined using SEM and optical light microscopy techniques (Nikon).

Immunofluorescence

For immunofluorescence staining, P19 cell aggregates were dispersed mechanically and plated on gelatin-coated coverslips. After attachment of cells (at day 7 th), they were directly fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) for 30 min at room temperature.

Following fixation, the coverslips were washed by three changes of PBS for 5 min/wash. Then the coverslips were incubated overnight at 4°C with primary antibody against microtubule-associated protein 2 (MAP-2, 1:100 Sigma). Then the cells were washed three times by PBS/10 min each.

The binding of these antibodies was visualized with polyclonal rabbit anti mouse IgG biotinylated (Dako) for 45 min and Streptavidin/FITC (Dako) for 45 min at room temperature. The coverslips were again washed with PBS for 5 min/wash. The cells were viewed under a Zeiss Axiophot optical microscope.

RESULTS AND DISCUSSION

DSC Analysis

Figure 1 shows the DSC heating curves of the PLLA.

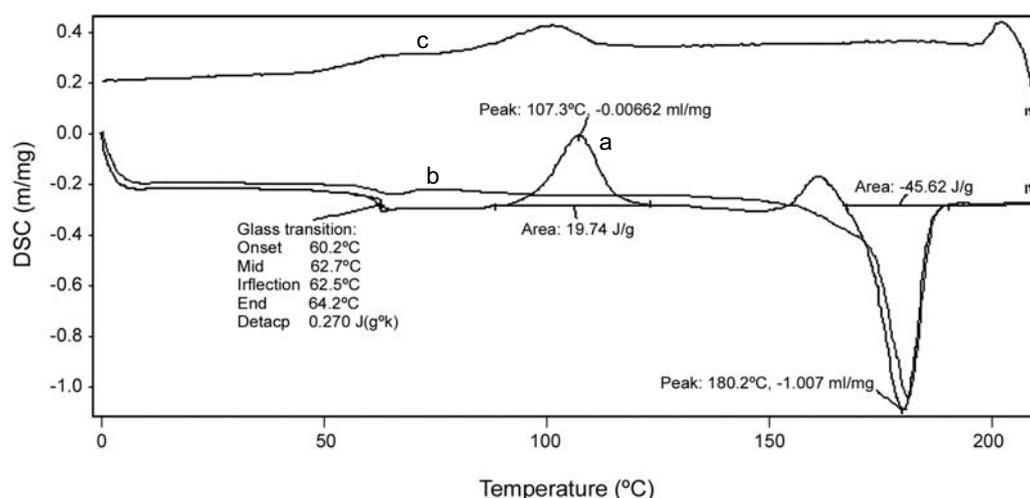
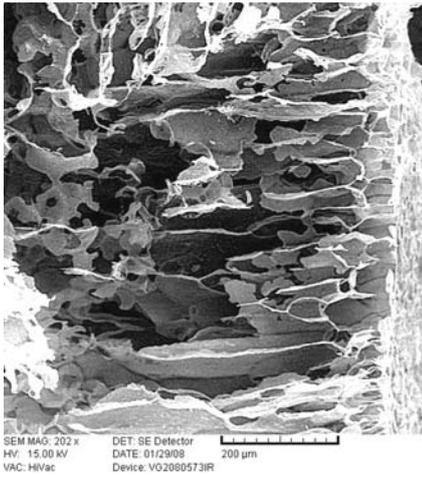


Figure 1. DSC Thermograph of PLLA 207.

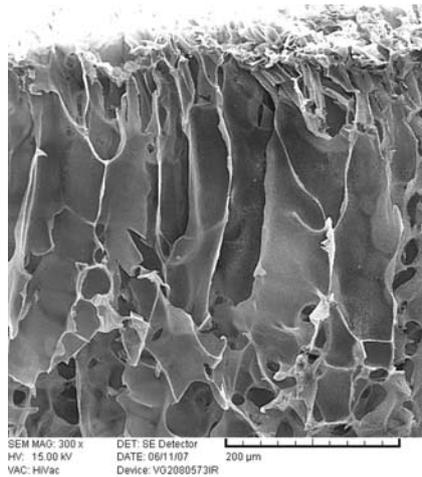
At first, the sample was heated to 200°C then maintained at this temperature for 2 min to remove the thermal history of the polymer (Figure 1 curve a), secondly, the sample was cooled to 0°C (Figure 1 curve b) to detect the crystallization of PLLA. In final cycle the sample was heated again to 200°C (Figure 1 curve c). As it is observed in Figure 1 curve c, a step due to glass transition appears at 62.7°C in PLLA curve. Exothermic peak of crystallization of PLLA (amorphous region) appears at 100°C (above T_g) in the DSC heating curve. In addition to the glass transition peak, the melting peak of PLLA at around 180°C is observed. This melting peak is may be attributed to that of the crystallites formed during DSC heating. The physical and mechanical properties of PLLA 207 crystallizable polymers such as isotactic PLLA are largely dependent on their solid state morphology and level of crystallinity.

Microtubular Architecture

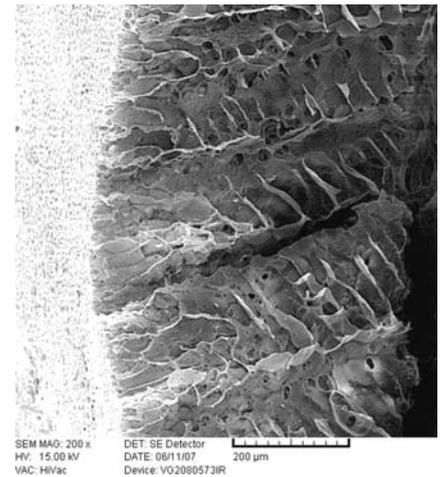
In this work, we have successfully created biodegradable polymer scaffolds with the architecture of a parallel array of open microtubules, and have demonstrated how to control the architectural parameters such as morphology and diameter of the tubules, and the formation of partitions in the tubular architecture with the processing parameters such as polymer concentration and temperature. The data of micro-tubular scaffold preparation conditions are listed in Table 1. When a temperature gradient was maintained uniaxially during the thermally induced phase-



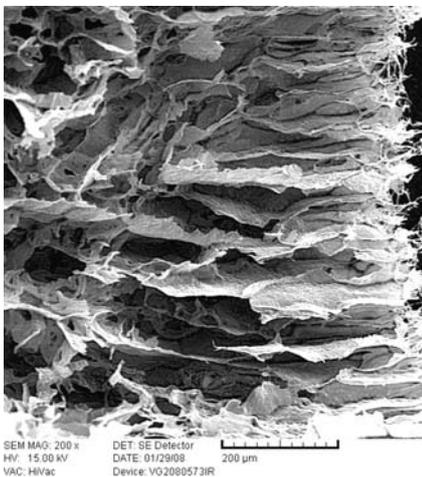
(a)



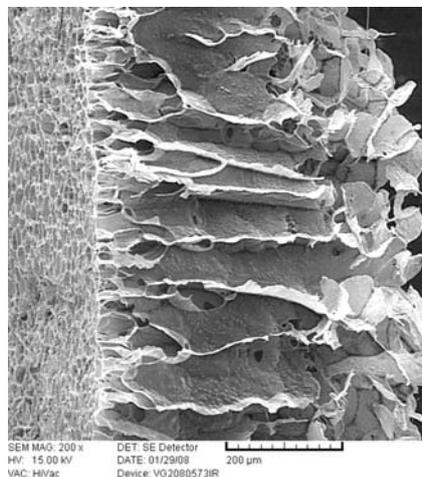
(b)



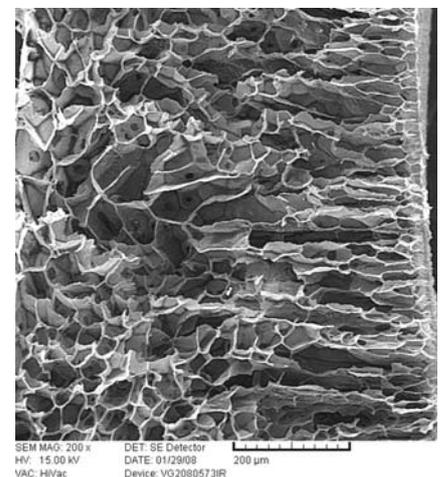
(c)



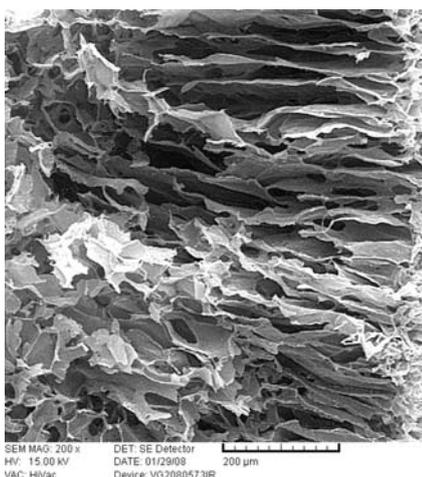
(d)



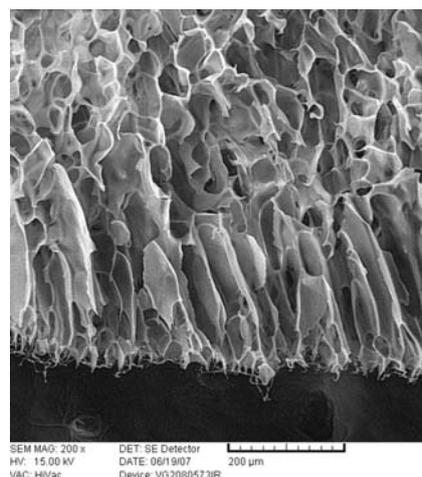
(e)



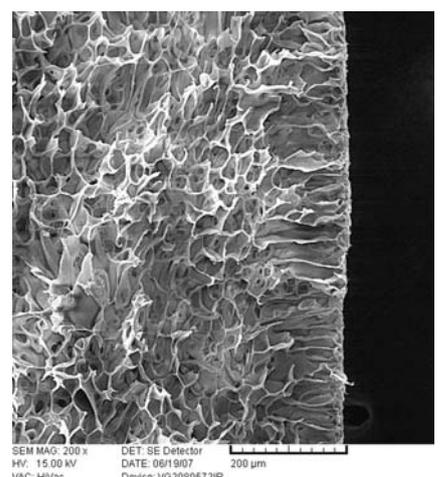
(f)



(g)



(h)



(i)

Continued

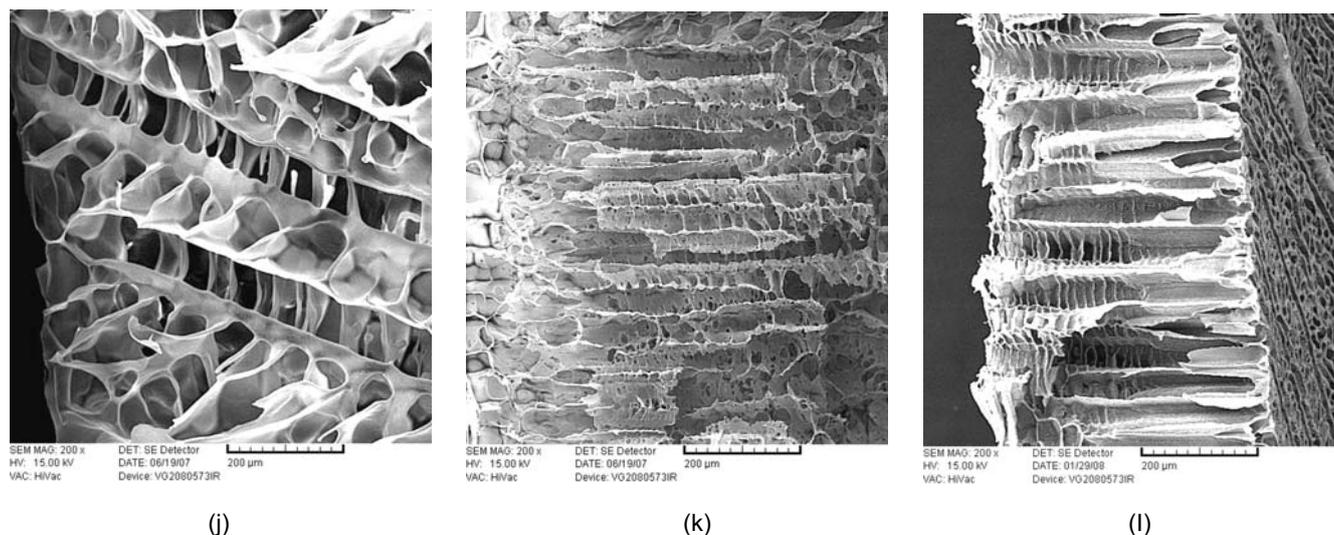


Figure 2. SEM photomicrographs (a-l) of cross sections of scaffolds that conditions of preparations are listed in Table 1 (scale bar is 200 μm).

separation process, the characteristic architecture of an array of parallel microtubules was achieved as shown in SEM micrographs in Figure 2 showing the cross-section of the same samples, prepared at different temperatures (5, 0, -20, and -196°C) and different polymer solution concentrations (1.5, 2.5, and 5 w/v%). Characteristics of scaffolds are summarized in Table 2. As shown in this table, the internal diameters of the pores are between 50 to 200 μm . SEM micrographs observation and results in Table 2 demonstrate that decrease in freezing temperature, leads to decreasing pores diameters and increasing porosity wall thickness.

Figures 2a-2l show the SEM micrographs of the cross-section of the samples. As it is evident in these micrographs, the morphology of tubular scaffolds is variform. Figure 2j shows that increasing solution concentration has resulted in decreasing pores diameters. In some cases, the increasing polymer concentration and phase-separation temperature have favoured ladder-like tubule formation over the open tubular architecture. These figures show that by decreasing freezing temperature from 5°C to -196°C, the diameter of the pores decreases. As it is noticeable in these micrographs, in each freezing temperature, 2.5% solution concentration gives better tubular morphology, but by using higher concentration (w/v%) an unfavourable morphology is produced. Therefore, 2.5% solution concentration is the optimum concen-

tration for tubular morphology structure for in vitro study in our study.

Different morphologies with different tubular diameters were obtained. We believe that crystallization of 1,4-dioxan at oriented temperature gradient depends on solution concentration and temperature [35]. By using a lower temperature-cooling agent, a higher temperature gradient would produce which leads to formation of more crystal nuclei through a higher crystallization speed as the results are shown in Figures 2g-2l. In contrast, by using a higher temperature-cooling agent, a lower temperature gradient

Table 2. Characteristics of scaffolds.

Samples (PLLA 207)	Porosity presents ($\pm 5\%$)	Average internal pore diameter (μm)
a	97.8	212 \pm 10
b	97.5	114 \pm 7
c	98.1	100 \pm 7
d	96.8	63 \pm 5
e	94.7	202 \pm 8
f	95.5	85 \pm 6
g	94.2	73 \pm 6
h	93.5	31 \pm 4
i	92.3	83 \pm 6
j	91.8	66 \pm 6
k	90.8	54 \pm 5
l	90.2	25 \pm 4

would produced which only leads to formation of fewer crystal nuclei by slower crystallization rate. As a result, fewer and larger crystals have been produced (Figures 2a and 2b). For the present system, the microtubules direction-structured scaffold with internal diameter in a range of 25-200 μm could be fabricated by means of adjusting cooling agent or temperature-gradient of the TIPS technique.

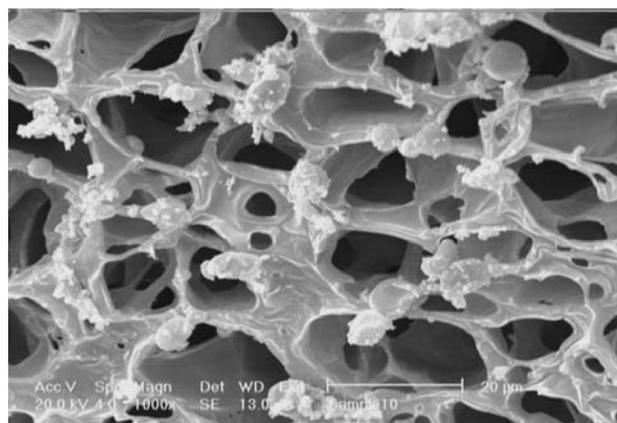
By means of choosing a suitable temperature gradient, super-cooling temperature by its careful control and solution concentration, the average diameter of the microtubules-like pores would be much higher than 200 μm . This orientation-structured scaffold with such diameter of the microtubules is suitable for nerve tissue engineering.

In Vitro Study

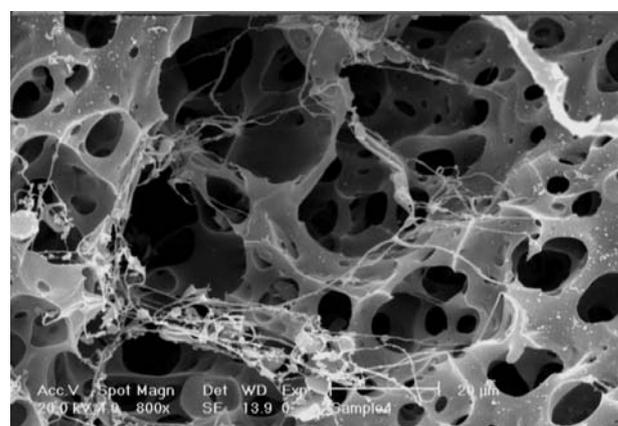
In this study, we found that one of the biodegradable polymers, PLLA, could be used as a scaffold for tissue engineering, in particular, to support neuron differentiation. Nerve stem cells play a unique and obligatory role in nerve repair and pertaining to enable the CNS to be regenerated in the body.

Figures 3a and 3b show the SEM micrographs of undifferentiated and differentiated cells to neuron cells on the PLLA scaffold (Table 1, sample a) As can be seen in Figure 3a, after 4 days there are some cells attached on the scaffold and begun to differentiate. While, Figure 3b shows the differentiated cells to neurons which have appeared grown onto the scaffold after 8 days. We have also observed the presence of neuron-like cells based on morphological criteria as early as 4 day post-RA treatment. Therefore, SEM examination demonstrated the attachment of the cells on the porous scaffold and differentiated to neuron cells with random axon and obviously the penetration of axons into the pores.

Unfortunately we did not detect the cells and axons in cross-section of the scaffolds by SEM technique. However, only a few P19 cells were able to differentiate into neurons. Figure 4 shows the cell differentiation into the scaffold by immunochemical staining for neuronal-specific marker protein MAP-2 (microtubule-associated protein 2) antibody. Cells were fixed at various developmental stages and stained with anti MAP-2. Figure 4 shows the morphology of the cells stained with this antibody and



(a)

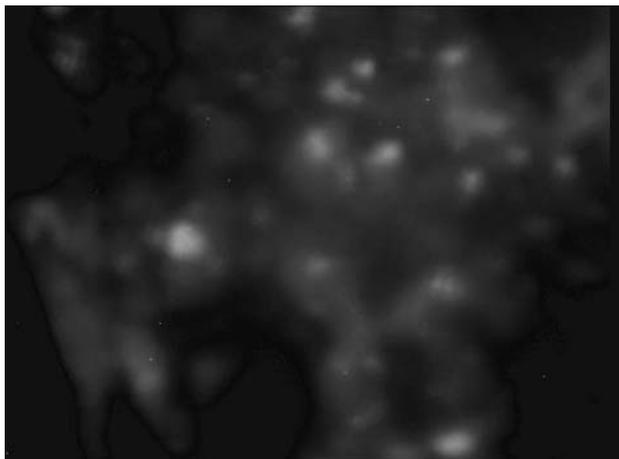


(b)

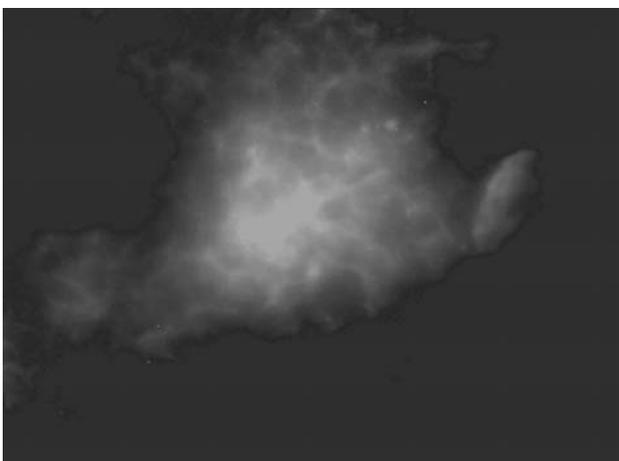
Figure 3. SEM photomicrographs of differentiated P19 cells to neuron: (a) attachment of cells on the scaffold and (b) differentiated cells (neuron) growing onto the scaffold (scale bar is 20 μm).

confirm the differentiation of P19 cells into neuron cells. Staining of MAP-2 was detectable on the scaffold surface after the 8th day. Cells on the scaffolds were strongly immunopositive for MAP-2. In our investigation, we have demonstrated that PLLA scaffold is suitable for P19 cell adhesion and differentiation to nerve cells.

Research in this area has led to deeper understanding of cell-matrix interactions. However, the cells are surrounded by other cells in the body, and the extracellular matrix in a three-dimensional environment. There is little understanding of cell-cell interactions and cell-matrix interactions in these three-dimensional systems. These works have demonstrated that crystalline PLLA tubular scaffold and differentiation



(a)



(b)

Figure 4. Fluorescent image of differentiated P19 cells into neuron on the PLLA 207 scaffold: (a) 8 days and (b) 12 days culturing.

of P19 cells on it is a favorable model for studying nerve tissue engineering which needs to make way for oriented penetration of axons in tubular scaffolds.

CONCLUSION

The crystalline PLLA polymer which is biodegradable and biocompatible has been converted to tubular scaffold by gradient temperature technique using solid-liquid phase separation method. The morphology and diameter of tubes can be controlled. Therefore, we can design tubular scaffolds with the capability of controlling a variety of factors such as freezing temperature, concentration of polymer solutions, and

temperature gradient. The scaffolds can be used as a model for the differentiation of P19 cells into neuron cells. Therefore, the scaffolds can be tailored into synthetic conduits for nerve repair. Our results conclude that samples with internal pore diameters between 100 to 220 μm are suitable for nerve tissue engineering.

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