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Assessment of the biocompatibility of the PLLA-PLCL scaffold obtained by electrospinning

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Abstract

Electrospun membranes of poly (L-Lactide) / poly (L-lactide-co-caprolactone) blend were produced and evaluated by physical and mechanical tests to use as a scaffold for cell growth. The membranes were seeded with endothelial cells (HUVEC) and after culturing time it was visualized by confocal laser scanning microscopy and scanning electron microscopy. The results indicate that the process parameters were capable of producing PLLA-PLCL membranes presenting fibers with diameters in the nanometer range. The scaffolds supported cell attachment and growth, indicating the feasibility of producing scaffolds by electrospinning technique, which could be used in tissue engineering applications.

Keywords: polymer; PLLA-PLCL;electrospinning; scaffold; tissue engineering..

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1. Introduction

Tissue engineering (TE) combines concepts of biological sciences and exact sciences whose main objective is the production of biological substitutes that help restore, maintain or even improve the functions of one organ or tissue. TE techniques generally require the use of a porous scaffold, which serves as a three-dimensional (3D) model for initial cell attachment and subsequent tissue formation in vitro and in vivo. Its architecture defines the shape of new cultured tissue. The major challenge in developing the scaffold is to organize the cells or tissue in an appropriate three-dimensional configuration and molecular signals suitable to allow cell growth and tissue formation in a reproducible and economically viable manner [1].

In order to obtain a living tissue replacement, cells can be maintained in culture and expanded in a three-dimensional natural or synthetic matrix, in the presence of growth factors. When provided with proper conditions and chemical signals, cells will secrete various extracellular matrix components to create in fact a living tissue that can be used as replacement tissue to be implanted in the defective site [2-4]. Recent advances in nanotechnology have provided new tools for TE and to the development of 3D cell cultures. According to the requirements of different TE applications, a variety of frameworks with different compositions can be developed. Ideally, a scaffold should be biocompatible, resorbable, porous and presenting a surface that is favorable to adhesion, migration, proliferation and cell differentiation which allow the formation of extracellular matrix and its remodeling [4,5]. Briefly, biocompatibility can be defined as the "ability of a material to perform with an appropriate host response in a specific application, taking into account the interactions between the biomaterial and the host [6].

Various biodegradable polymers have been developed and made available for use as substrates in TE. Both natural polymers such as collagen and chitosan, and synthetic thermoplastic polyester, such as poly (lactic-acid) PLA, poly (glycolic acid) PGA, polylactic-co-glycolic acid copolymers (PLGA) , polycaprolactone (PCL) and polyhydroxy butyrate / valerate have been of great interest due to their biocompatibility, biodegradability and mechanical strength [7-9].

Among several nanotechnology approaches the electrospinning technique stands out as a promising one. It was developed based on the phenomenon of "electrospray", first described in 1882 by Lord Rayleigh, who discovered that a highly charged droplet would break into smaller droplets to pass through a voltage gradient. Instead of producing small droplets as in eletrospraying process, the electrospinning produces long continuous fibers. Although electrospinning is not a conventional technique in cell biology, its set up installation is relatively simple [10]. In the present work the objective was to produce, by electrospinning technique, nanostructured and uniform membranes of blends of poly (L, L-lactide) (PLLA) and poly (L, L-lactide-co-ε-caprolactone) (PLCL) with fibers in the nanometer range, and measure its mechanical and physical features. Also, it was aimed to test this polymer as scaffold to adherent cells and evaluate the events of adhesion and proliferation.

2. Materials and Methods

2.1. Polymers and Electrospinning

The scaffolds were obtained through the electrospinning process. The set up was mounted using a high voltage supplier, a syringe pump (Baxter AS50) with a metal needle and a conductive collector (Figure 1). The system was built in a cabinet for safe solvent evaporation and prevent that the turbulent air interferes in the formation of the polymeric membranes in the collector. The blend was prepared from the copolymer of poly (L, L-lactide-co-ε-caprolactone) (PLCL) with a 60:40 ratio, which was synthesized as described by [11] and poly (L, L-lactide) (PLLA) (PURAC).

These polymers were dissolved in chloroform P.A. (MERCK). The solution concentration, the ratio of the polymers and the process parameters, such as applied voltage and flow rate were varied during the procedure in order to obtain a membrane presenting uniform fibers. The electrospun membranes were then placed in a fan oven (50 °C, 24 h).
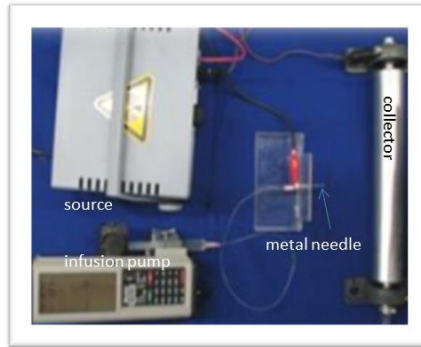
Figure 1: Electrospinning set up used to produce PLLA-PLCL membranes

2.2. Differential Scanning Calorimetry (DSC)

Measurement of heat flow variations in the polymeric sample was compared to a reference by DSC analyses making it possible to observe thermal transitions: melting temperature (Tm), glass transition (T_g) and crystallization transition (T_c). The samples were heated in an inert nitrogen atmosphere (50 ml / min flow rate) from - 80 °C to 210 °C at a rate of 20 °C min^{-1}. Plots of heat flow versus temperature were obtained and the percent crystallization of the material was calculated according to Equation 1, where ΔHm = melting enthalpy (J/g); 93 and 142 are the melting enthalpy (J/g) for PLLA and PCL 100% crystalline respectively and c = concentration (%) of the PLLA or PLCL in the copolymer.

$$\text{crystallization (\%)} = \frac{100.\Delta H_m}{93.c_{PLLA} + 142.c_{PLCL}} \tag{1}$$

2.3. Tensile Testing

The mechanical properties were evaluated by tensile testing using a tensile test machine (INSTRON 5565). In the tensile test the sample is subjected to an effort that tends to lengthen it to rupture. Eight test samples were cut according to the standard ASTM F2150 (Standard Guide for Characterization and Testing of Biomaterial Scaffolds Used in Tissue-Engineered Medical Products). The samples were cut in a way to prevent flaws and tears formation which could cause premature damage to the sample. The 10 kg load cell was used and the cross-head speed was set to 1mm/min.

The results as force (F), length variation (ΔL) and stress curve (σ) versus deformation (ε) were calculated using Equation (2) and (3), where A = area, T = thickness and L_0 = initial sample length.

$$\sigma = \frac{F}{A} \text{ and } A = w.t \tag{2}$$

$$\varepsilon = \frac{\Delta L}{L_0} \tag{3}$$

2.4. Surface Characterization of PLLA-PLCL Membranes

Scanning electron microscopy (SEM) (Hitachi TM 3000, Japan) was carried out to examine the morphology, microstructure, fiber diameter and fiber integrity of the PLLA-PLCL blend obtained by electrospinning, at an accelerating voltage of 5 kV. A total of five samples with 5.5 mm diameter were visualized and imaged. The mean value (N = 5; n = 100) of fibers diameter were determined with the ZEN 2012 software (Carl Zeiss, Germany) and is expressed as mean ± standard deviation (SD).

2.5. Culture Conditions and Cell Growth on PLLA-PLCL Scaffolds

In order to check the feasibility of using PLLA-PLCL as scaffolds human umbilical vein endothelial cells (HUVEC) (Heart Institute, Brazil) were used. The cells were cultured with RPMI medium 1640 (Gibco, Life Technologies, USA) supplemented with 10% (v/v) bovine foetal serum and 1% (v/v) penicillin/streptomycin in polystyrene T-25 cm^2 culture flasks. The T-25 flasks were maintained at 37 °C under static condition in a moisturized atmosphere with 5% CO_2 over ten subculture cycles.

The experiments were carried out in 96 well polystyrene plates incubated under the same culturing conditions. Sterile PLLA-PLCL samples with 0.35 cm^2 and 5.5 mm diameter were placed inside experimental microplate wells and allowed to equilibrate with culture medium for two hours prior to the beginning of the experiments. To perform the assay, the cells were enzimatically dissociated from T-25 culture flasks with 0.5% (w/v) tripsin (Sigma, USA) and 0.2% (w/v) EDTA (Sigma, USA) in phosphate buffer saline pH 7.4 (PBS), centrifuged (200 x *g*, 5 min.) and resuspended with RPMI. Cell concentration (cells/mL) was determined by cell counting with Neubauer hemocytometer and proper dilution was prepared in order to seed 5000 cells per sample (quadruplicates) at the start of the experiment. The experimental groups were as follows: 1) polystyrene microplate wells (positive control) and 2) PLLA-PLCL scaffold. This experiment was also used to prepare samples to microscopy studies described in the following sections

2.6. Proliferation Assay

To determine the number of viable cells onto the PLLA-PLCL scaffolds, according to standard ASTM F2739-8 (Standard Guide for Quantitating Cell Viability within Biomaterial Scaffolds) it was used the cell proliferation assay Promega kit (USA). This reagent kit contains an electron coupling reagent and a tetrazolium compound (MTS) which can be bioreduced by mitochondrial enzymes of viable cells into a colored formazan product that is soluble in culture medium. Following three, 24, 48 and 72 hours from the initial seeding, cells adhered to scaffolds were incubated with 317 µg/mL MTS for three hours. At the end of incubation time the quantity of formazan product formed was measured by absorbance readings at 490 nm using a FluoStar Omega Plate Reader (BMG LABTECH, Germany) and it is proportional to the number of living cells. The background absorbance resulted from the blank well (RPMI and MTS, without cells) was subtracted from the samples data. A standard curve was determined at the start of the experiment, under the same culture conditions. This calibration curve was performed by seeding different numbers of cells, as previously determined by hemocytometer quantification, in quadruplicate, in the culture plate wells. By plotting the recorded absorbances against the known number of cells seeded a calibration curve was obtained and the number of cells adhered to the scaffolds was estimated.

2.7. Scanning Electron Microscopy

SEM study was carried out on cultured HUVEC adhered to PLLA-PLCL scaffold at three, 24, 48 and 72 hours after the initial seeding. The samples were rinsed twice with PBS, and fixed with 100 µL of 4% paraformaldehyde (PFA) in PBS for 30 minutes. The samples were rinsed with deionized water and allowed to dry at room temperature. A total of three samples from each culture time were examined and imaged with a Hitachi TM 3000 (Japan) at an accelerate voltage of 5 kV.

2.8. Immunofluorescence Staining

In order to verify cell distribution onto the scaffold, three hours after the start of the experiment samples were fixed with 4% PFA for 30 minutes, washed with PBS, permeabilized with 0.1% Igepal (Sigma, USA) at 37°C for 30 minutes and blocked with 2% bovine serum albumin at 37°C for another 30 minutes. Fluorescence staining was done with DAPI 25 µg (Sigma; nuclei staining) and Alexa Fluor 488 phalloidin 1: 300 (Life Thechnologies; F-actin staining, USA), incubated overnight at 4°C for 18 hours. After that samples were washed with PBS and placed between two glass coverslips with glycerol / PBS (1:1) and observed under the confocal laser scanning microscope (CLSM) (Zeiss LSM510-Meta, Germany).

Results were tested for normality using a Kolmogorov test. A statistical analysis was performed with one-way analysis of variances (ANOVA) (Sigma Stat 3.1, USA) followed by a post Hoc Tukey test for comparison of the PLLA-PLCL scaffold group versus control group. Results were considered significantly different at $P < 0.05$. Data are expressed as means ± standard deviations.

3. Results and Discussion

3.1. *Electrospun PLLA-PLCL Blend – Morphology and Mechanical Properties*

According to [12] the process consists of applying a high voltage at the tip of a syringe needle, where a polymer solution droplet by the surface tension is maintained. The increase of the electric field causes charge to be induced within the polymer solution resulting in charge repulsion and the formation of electrostatic forces. These forces are opposed to the surface tension and further increasing the change in the droplet shape - the hemispherical droplet becomes conical, which is referred to as a Taylor Cone. When a critical stress is reached, the charge repulsion overcomes the surface tension, causing initiation of the jet. The polymer jet travels in random motion to the metal collector (counter electrode), the solvent evaporated, and the result is a substrate of nonwoven fibers [12-14]. With the purpose of obtaining fibers with controlled diameters and reproducible physical and chemical properties, it is necessary to standardize technical parameters [12, 15].

The high quality membrane PLLA-PLCL blend at 5% in chloroform solution was obtained under the following conditions: distance between poles of 7 cm, applied voltage of 17 kV and flow rate of 1.0 ml/h. The parameters were chosen based on the uniformity and minimum defects of the membranes visualized.

From SEM image for PLLA-PLCL it can be observed fibres with 0.55 µm mean diameter (± 0.22), obtained by averaging a total of 100 fibres from five samples, and randomly chosen. As it is seen in Figure 2, the fibers formed 3D structure.
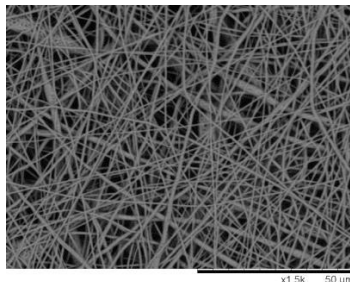

Figure 2. Scaffold obtained from electrospinning of PLLA: PLCL (50:50); parameters used: 17 kV, 7 cm and 1 mL/h, needle diameter of 1,8mm. Scale bar = 50 µm; magnification x1500.

The DSC curves from the first heating for electrospun scaffolds with different proportions of PLLA/PLCL (Figure 3) were similar, since they were produced from the same material. The observed shift to higher temperature of the PLLA melting temperature (T_{m2}) when mixtures containing lower content of PLLA 10:90 and 30:70 were compared with 50:50 blend, while the cold crystallization temperature showed the opposite behavior. This may be explained by separation and crystallization induced by higher concentrations of PLLA, leading to more stable crystals. The inhibition of crystallization in polymers is restricted due to the limitation of segmental motion and phase separation of the different segments, lactide and caprolactone [16, 17]. To 50:50 PLLA-PLCL 5% presented main transitions at T_{m1}=54.5 °C (PLCL), Tc = 69.5°C (PLLA) and T_{m2}=174.3 °C (PLLA).

Table 1 shows the results of DSC analysis. T_{m1} and T_{m2} refer to the melting temperatures of poly (ε caprolactone) crystallites in PLCL and PLLA, respectively.

Table 1 – Results of DSC analyses for scaffolds samples.

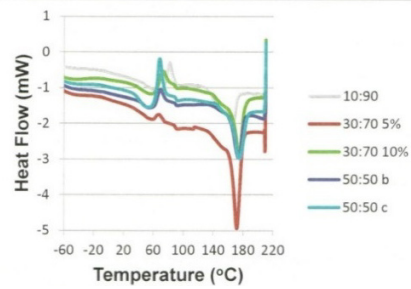|	Tm$_1$ (ºC)	Tc (ºC)	Tm$_2$ (ºC)	ΔHm (J/g)	Crystallinity (%)
10:90	64,8	80,5	166,4	28,9	30,43
30:70	56,6	68	172,5	28,81	29,14
50:50 b	54,5	69,5	174,3	24,72	24,05
50:50 c	52,5	68,1	174,9	27,36	26,61
PLCL		33,6	114,5	11,2	9,95

Figure 3 – DSC analyses for different scaffolds produced by electrospinning in different proportions of PLLA/PLCL.

The D1708 (Test Method for tensile properties of plastics by use of microtensile specimens) was used as reference because the thickness and dimensions of the substrates are very thin and fragile. The thickness of the substrates varied between 0.065 and 0.08 mm. Figure 4 shows individual results. Pores appeared to have a non-uniform distribution in the substrate. The mechanical strength and porosity of scaffold depend on fiber composition, fiber diameter and density [18]. The average stress and elongation were 181.09 kN.mm^{-2} and 9.04% respectively.

Table 2 – Results of maximum strain (ε) and stress (σ) before failure and average for 9 specimens tested.

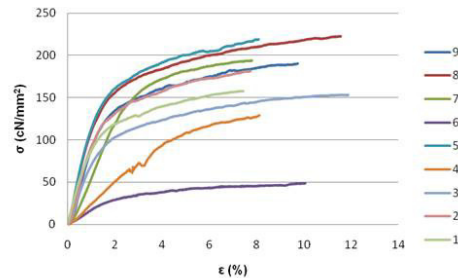| Specimens	ε(%)	σ(cN/mm^2)
1	7,42	157,62
2	7,76	181,74
3	11,89	153,54
4	8,12	129,45
5	8,08	219,13
6	10,05	48,49
7	7,8	194,25
8	11,55	222,74
9	9,74	190,29
Average ± SD	9,04 ± 1,79	181,09 ± 32,55

Figure 4 – Stress (cN/mm^2) X Strain (%) curve for 9 membranes samples with 5% concentration of PLLA: PLCL 50:50 presenting different fibers diameter and porosity produced by electrospinning..

3.2. Cell Growth on PLLA-PLCL scaffolds – SEM and CLSM

When producing scaffolds for tissue engineering it is crucial to be suitable in supporting cell attachment and growth [19-20]. From images obtained by SEM (Figure 5, A-D) it is possible to observe cell adhesion and growth after three, 24, 48 and 72 hours from the initial seeding. The scaffold fibers obtained with diameters in the nanometer range allowed cellular attachment as wells as proliferation [10]. From higher magnifications images (Figure 5, E-H) it also can be seen penetration of cells within the 3D structure of the scaffold which is confirmed by CLSM images (Figure 6 B).

3.3. Proliferation Assay

The number of viable cells onto the PLLA-PLCL scaffolds during culture time was determined after three, 24, 48 and 72 hours. Figure 7 shows an increase of the number of cells indicating proliferation. As it can be observed, growth rate was slower until 48 hours of culturing. After 72 hours the number of viable cells was five times greater than at the beginning of the experiment demonstrating the feasibility of using this blend as substrate to cultivate adherent cells.
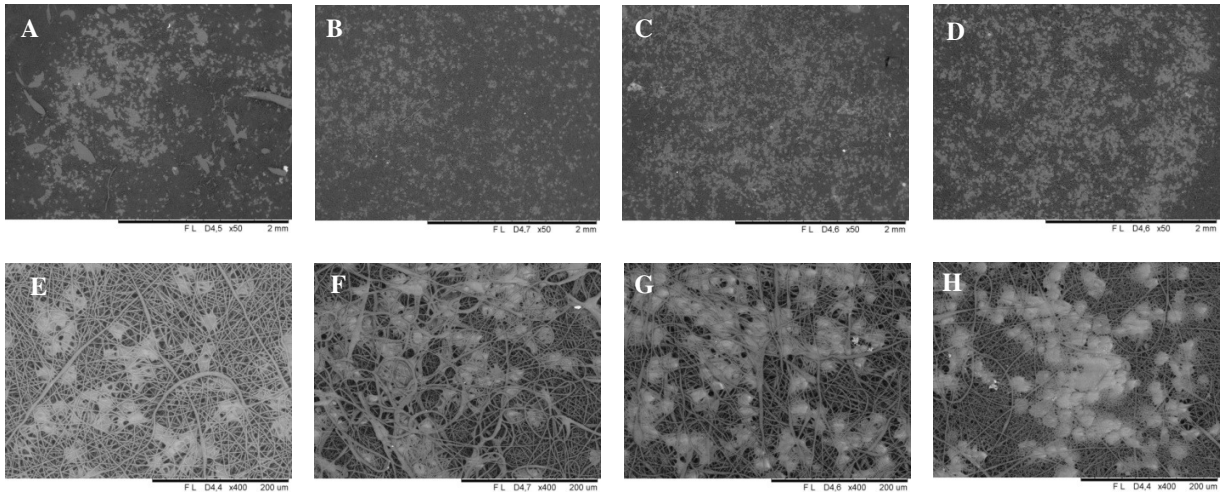
Figura 5: SEM images of PLLA-PLCL scaffolds showing cultured HUVEC after three (A, E); 24 hs (B, F); 48 hs (C, G) and 72 hs (D, H) from the initial seeding. A, B, C, D: Scale bar = 2 mm; magnification x50. E, F, G, H: Scale bar = 200 µm; magnification x400.
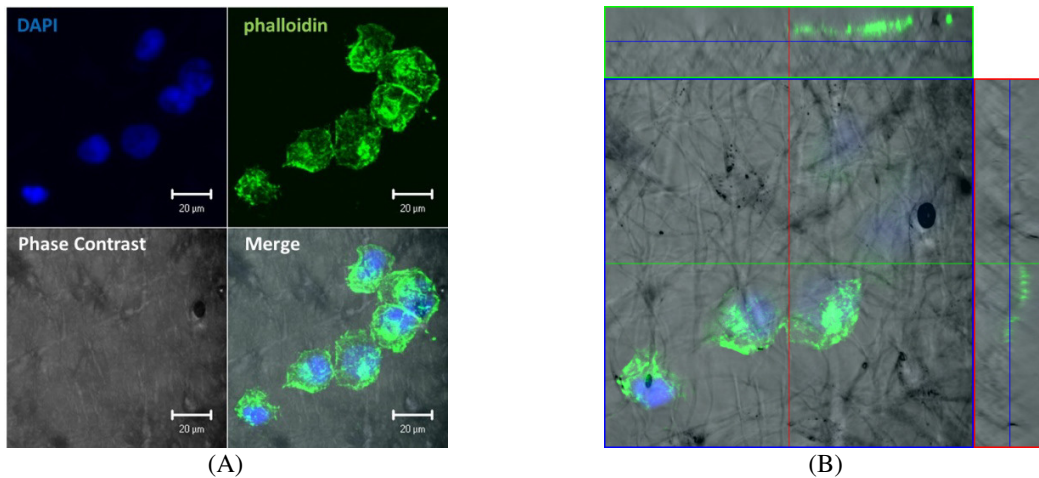

(A)   (B)

Figure 6: Confocal analysis of HUVEC adhesion on PLLA-PLCL scaffold. Immunofluorescence staining of F-actin is shown in *green*, nuclei staining in *blue* and phase contrast image in grey scale. A) Image represents the sum of all slices of gallery from the three different channels isolated and also the overlay (Merge); B) Representation of gallery images in an orthogonal sections (triplet of mutually perpendicular sectional images). Objective: x40; Zoom: x2. Scale bars = 20 µm.
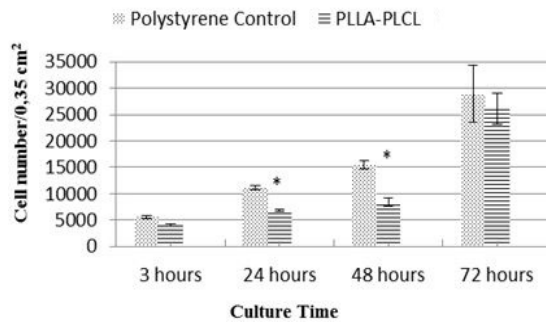

Figure 7. MTS proliferation assay. HUVEC cultured onto PLLA-PLCL scaffold. (*) denotes statistical significance ($P < 0.001$) versus control.

4. Conclusion

The parameters defined in the electrospinning process were satisfactory in producing PLLA-PLCL membranes with fibers in nanometer scale and forming a 3D structure.

The results show that PLLA-PLCL scaffold has biocompatible properties which allowed cellular attachment, adhesion and proliferation over the time tested.

Acknowledgements

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