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Preparation of porous poly (lactic acid) fibers by medium field electrospinning for tissue engineering applications

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Electrospun fibers find a number of biomedical applications, like scaffolds for tissue engineering, as polymer nanofiber mats can mimic the extracellular matrix (ECM) of the body. In this research, non-woven mats composed of poly(L-lactic acid) (PLLA) fibers were obtained by low voltage (6 kV) electrospinning of PLLA/chloroform (CL)/sodium lauryl ether sulfate (SLES) solution. Tests were performed in two different collector/capillary distances and the produced fibers were evaluated by scanning electron microscopy (SEM), differential scanning calorimetry (DSC) and cell viability. The results show the formation of three dimensional mats composed of porous fibers created by the addition of small amounts of aqueous solution of SLES surfactant; a higher crystallinity degree for longer collector/capillary electrospun distance and positive cell proliferation.

Keywords: *Eletrospinning, Medium field, Tissue engineering, PLLA.*

1. Introduction

Electrospinning is a process by which submicron fibers can be produced[1,2], from polymer solution or melt, by electrostatic forces. These non-woven nanofiber mats have a wide variety of applications, including semi-permeable membranes, and other structures used in tissue engineering[3-5]. These fibers are generally obtained by using a high voltage source (10-20 kV) direct current (DC), with the formation of a polymer jet between a needle and a collector separated by a distance of the order of 10-15cm. This conventional method of electrospinning, producing randomly oriented mats of nanofibers, is also known as Far Field Electrospinning (FFES)[1,6-10].

Greater control over the fibers can be achieved using a modified electrospinning technique, known as Near Field Electrospinning (NFES), which uses an electrode collector distance from 500 μm to 3 mm, in order to take advantage of only the stable jet stage. It is a production method of oriented nanofibers, with standardized deposition[11,12].

One of the most promising application of the nanofibers is in the biomedical field. This membranes can be used as wound dressings and appropriate medium for tissue regeneration like scaffolds for tissue engineering. Due to physical structural similarities to the ECM, with high porosity and large surface area, they provide appropriate drainage and air permeability, comfort in its application and removal, and allow drug-loading and controlled release[13,14].

For this type of application, a mechanically stable three-dimensional matrix, with interconnected pores, is required, so that the cells can grow throughout the structure. As the cells grow and organize, the polymer degrades and is absorbed by the body, leading to a natural tissue replacement[15-17].

This could bring value for people with epithelial injury, preventing bacterial infection even at hospitals with rapid and effective coverage of the wound. Also promoting skin transplants, wich present drawbacks as few donors, the risk of rejection of the implanted material, and the time necessary for complete healing[18-20].

Additionaly, porous features can improve the functionality of the fibers, by increasing surface area, facilitating the cell attachment. Among the mechanisms to explain pore generation in polymer membranes is Phase Separation (PS), where the incorporation of a poor or non-solvent can induce PS and lead to porosity, allowing the formation of circular pores after posterior non-solvent evaporation. This is a simple method, known as non-solvent induced phase separation (NIPS)[4].

Biodegradable polymers have several potential applications in the biomedical field due to its biocompatibility and biodegradability[21]. Among these, the PLLA is a bioresorbable and biocompatible polymer that have been extensively studied for biomedical applications, including scaffolds for epithelial cell regeneration[22-25]. PLLA is a semicrystalline polymer with a glass transition temperature of about 57 ° C, melting point of 174-184 °C; which can be made of 100%

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renewable sources and degrades under natural conditions. It has mechanical properties compatible with the applications in the medical field, with elastic modulus of 3.2 to 3.7 GPa[26-28].

The crystallinty is an important characteristic when considering the material for biomedical applications. The high elongation strains and shear stresses applied on polymer chains during electrospinning are reported to cause alignment of the macromolecular chains along the fibre axis, leading to high degree of molecular orientation. One of the possible influences is related to the effect of crystallinity on the degradation kinetics[29,30].

No studies specifically reporting the use of SLES surfactant on electrophilic polymer solutions were found in the literature; however, in general, surfactants have been used in a variety of ways due to their ability to lower the surface/interfacial tension of the medium in which they are dissolved[31]. They are also cited as a mean to avoid the formation of grains, as they decrease the surface tension of the solution and help to increase the interaction between the solvent and the polymer chains, avoiding the occurrence of beads on the surface of the fibers[32].

This work aims to obtain PLLA membranes composed of PLLA sub-micro fibers by low voltage electrospinning (6 kV) and validate its use as a scaffold for tissue engineering.

2. Materials and Methods

2.1 Electrospinning apparatus

The apparatus is composed of an infusion pump SDA 1800 (SDAMed), working with a 10 ml syringe and a metal needle with 0.7 mm in diameter by 25 mm length; a high voltage power supply (Leybold), in the 0 to 6 kV range; and a collector, comprising a rotating aluminum cylinder driven by an electric motor, which is powered by a low voltage supply (Minipa PS-500, 0-30V) allowing the collector speed control. The whole apparatus was used in a closed room at ambient temperature.

2.2 Materials

The polymer used in this work was poly (L-lactic acid), PLLA, synthesized at the Faculty of Medical Sciences, Pontifical Catholic University of São Paulo (PUC/Sorocaba), with an weight average molecular mass of 2.78 x 10^5 g.mol^{-1} and polydispersity of 1.58. Chloroform ($CHCl_3$, PA ACS) was purchased from Vetec (Brazil), while aqueous solution of sodium lauryl ether sulfate (SLES) 25 wt%, was supplied by Metaquímica (Brazil).

2.3 Electrospinning of PLLA

8 wt% PLLA solutions were prepared in chloroform at room temperature (25 ± 2 °C). For some experiments, 50 µL of the 25 wt% SLES aqueous solution were added to 33 mL of the PLLA solution. To the complete homogenization, the solutions were stirred at 30 rpm, using a magnetic bar, for at least 1 hour.

Then, the PLLA solution was placed in a 10 mL syringe at the infusion pump. After preliminary tests to set appropriate parameters for the preparation of fiber mats, the electrical voltage applied to the needle was 6 kV, while the flow rate and the collector speed were fixed at 0.5 mL.h^{-1} and 540 rpm, respectively. The two tests were conducted varying the needle to collector distances from 2.0 cm (test 2) to 4.0 cm (test 1). All the experiments were carried out at 25 ± 2 °C.

2.4 Scanning Electron Microscopy

The morphology and diameter of the collected fibers were investigated by scanning electron microscopy (Jeol JSM-6701F), at 10 kV. The fiber mats were coated with gold during 3 mim at 20 mA, using a Denton Vacuum Sputer Coater. The fiber diameters were measured with an image analyzer (ImageJ, v. 1.49p). For each sample, average fiber diameter and standard deviation were determined from 80 measurements of random fibers.

2.5 Differential Scanning Calorimetry-DSC

DSC measurements were performed in a TA Instruments Q20 equipment to the determination of the glass transition (T_g), cold-crystallization (T_{cc}) and melting (T_m) temperatures and the crystallinity degrees of PLLA bulk samples and fiber mats. Samples, with ca. 10 mg, were heated from 30 to 200 °C at a heating rate of 10 °C.min^{-1} under nitrogen atmosphere. The calibration of the DSC was carried out with a standard sample of indium. The glass transition was calculated as the midpoint of the jump in heat capacity (ISO 11357-2). The crystallization (exothermal) and melting (endothermal) were characterized by the onset and peak temperatures, as well as by the area under the corresponding peaks. The crystallinity degree, X (%), was calculated according to the following equation:

$$X\ (\%) = [\Delta H_m - \Delta H_c] / [\Delta H_m^o] \times 100 \quad (1)$$

Where ΔH_m and ΔH_c are the melting and the crystallization enthalpies respectively, and ΔH_m^o is the reference ΔH_m (93.7 J.g^{-1}) of 100% crystalline PLA. The summarized values were calculated by the average of two determinations.

2.6 Cell viability

In the cell viability investigation, the PLLA electrospun mats from tests at 2.0 and 4.0 cm have been previously sterilized by UV germicidal irradiation for 30 min and acclimated in DMEM (Dulbecco's Modified Eagle's Medium) culture for 24 hours. ASC (Human Adipose Stem Cells - Catalog number R7788115, ThermoFisher) were seeded over the electrospun mats at a concentration of 5 x 10^4 cells / mL and maintained in DMEM supplemented with penicillin-streptomycin and 10% fetal bovine serum (FBS) after 1, 5 and 10 days of culture. Cellular constructs were incubated at 37° with 0,5mg/mL MTT (3-(4,5Dimethylthiazol-2-yl)-2,5-Diphenyl tetrazolium Bromide) for 4h. Dimethyl sulfoxide (DMSO) was added to solubilize the MTT-formazan product. Cell viability was quantified by measurement of optical density at 570 nm, using a microplate reader Elx-800-UV (Bio-Tek Instruments, USA. The data were analyzed by non-parametric multiple comparison test Tukey's test with one-way ANOVA.

3. Results and Discussions

3.1 SEM

From the SEM images shown in Figure 1 and 2, it is evidenced the production of uniform, three dimensional mats composed by randomly oriented fibers, so as a homogeneous formation of interstitial spaces between the fibers. Mats produced using a needle-to-collector distance of 2.0 cm (Figure 2) show a more interconnected fiber morphology, than sample produced at 4.0 cm (Figure 1). In both cases, no substantial formation of beads were observed.

Figure 3 emphasizes the porosity found in the electrospun PLLA fibers. It is noted no bead formation, but a great variation of fiber diameters, with the presence of crosslinking fibers (Figure 3b).

The observed fiber porosity is attributed mainly to the addition of a small amount of a surfactant (SLES), in aqueous solution, to the PLLA/CL system. The presence of SLES reduces the surface tension of the solution and helps to increase the interaction between the solvent and the PLLA chains, which prevents the formation of beads on the surface of the fibers, as well as avoid the locking of the syringe.

This alternative was suggested by Pezzin et al.[33], who studied the preparation of polyhydroxybutyrate (PHB) membranes by electrospinning and observed that the addition of sodium dodecyl sulfate (SDS) to a 10% (w/v) PHB solution in chloroform/dimethylformamide (9/1) was important to maintain a constant flow of the solution and increase reproducibility.

Moreover, the little bit of water (ca. 0.1 wt%) added to the PLLA/CL solution together with the surfactant is regarded as the key to the formation of pores during the electrospinning process (NIPS mechanism).

The fiber diameter distribution for a typical mat sample is shown in Figure 4. Most of the fibers (ca. 60%) have diameters between 1.0 and 2.5 μm, while 27.5% are nanofibers with diameters between 90 and 400 nm.

3.2 DSC

The Table 1 summarizes the values for T_g, T_m, ΔH_m, ΔH_{cc} and crystallinity degree obtained by DSC for PLLA bulk samples and fiber mats.

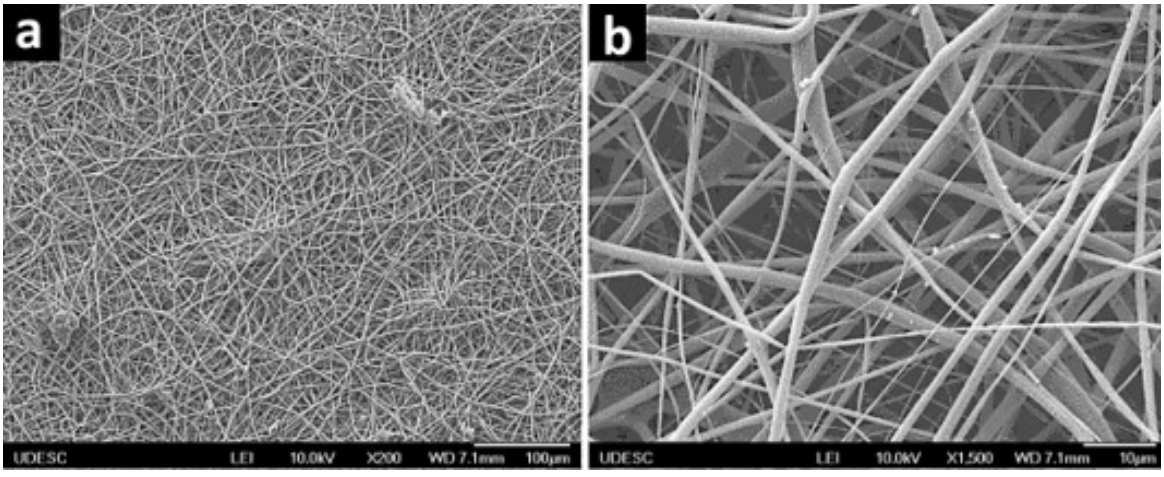

Figure 1. SEM micrographs of test 1: a) fiber mat (200X, b) a closer look (1500X) at the fiber surface. Electrospinning parameters: voltage = 6kV; needle-to-collector distance = 4.0 cm; 8 wt% solution PLLA in chloroform with 0.038 wt% of SLES, T=25± 2 °C.

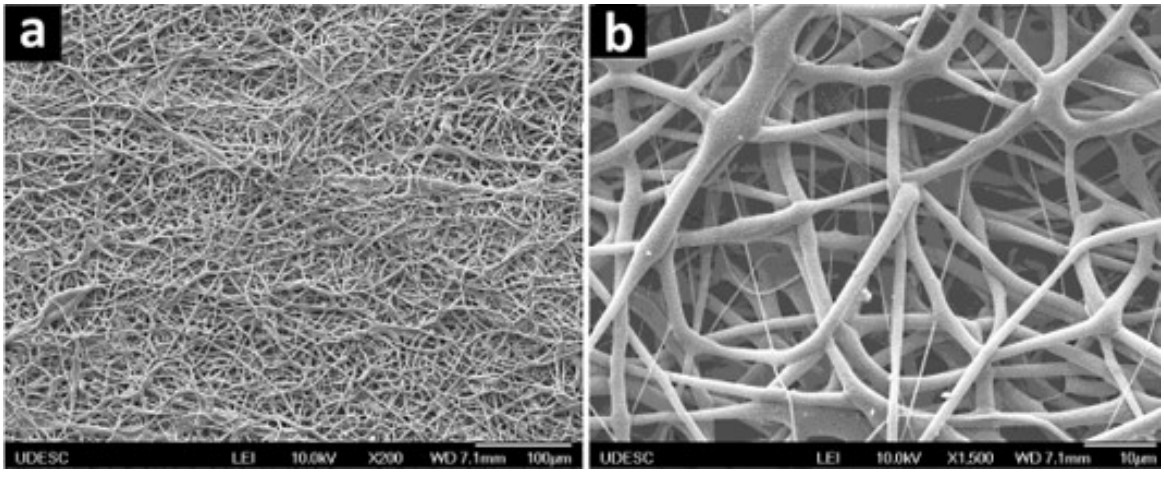

Figure 2. SEM micrographs of test 2: a) fiber mat (200X), b) a closer look (1500X) at the fiber surface. Electrospinning parameters: voltage = 6kV; needle-to-collector distance = 2.0 cm; 8 wt% solution PLLA in chloroform with 0.038 wt% of SLES, T=25± 2 °C.

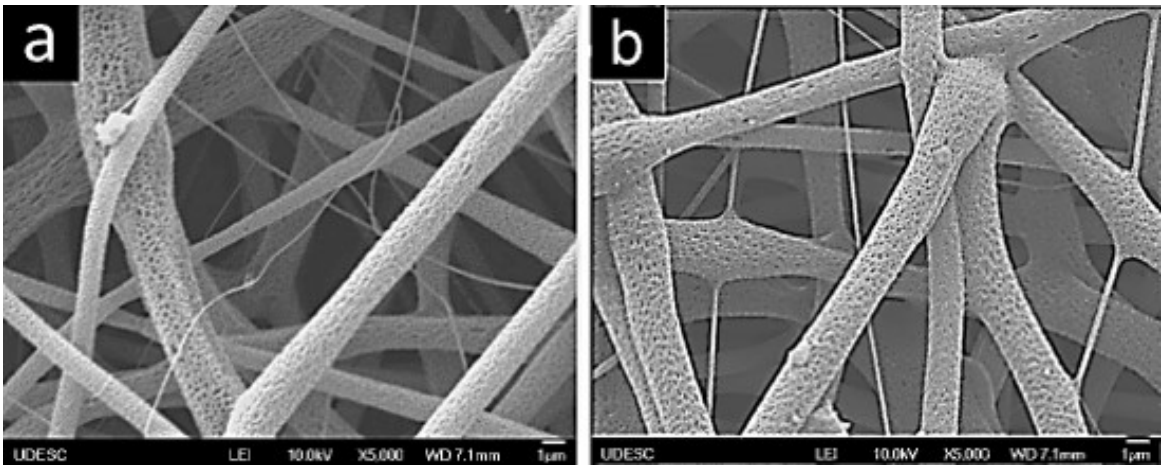

Figure 3. SEM micrographs emphasizing fiber surface porosity. a) test1(needle-to-collector distance = 4.0 cm) and b) test 2 (needle-to-collector distance 2.0 cm).

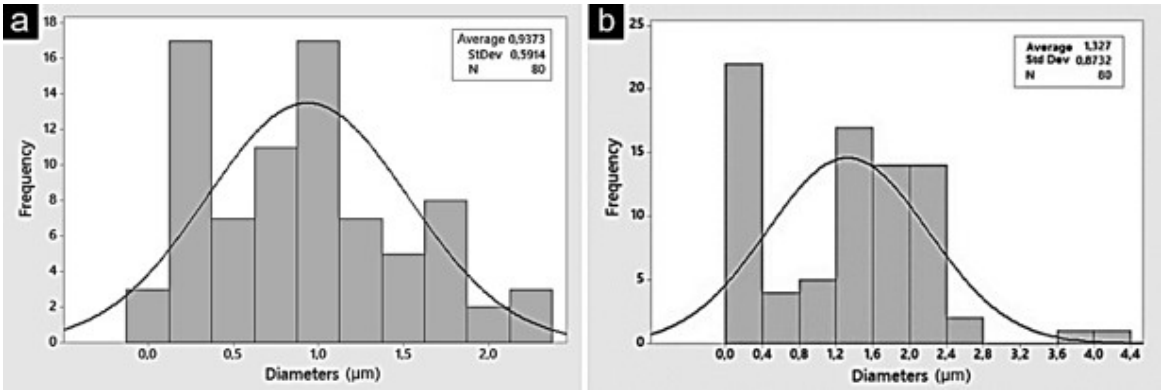

Figure 4. Fiber diameter distribution of as-spun PLLA fibers, a) test1 (needle-to-collector distance = 4.0 cm) and b) test 2 (needle-to-collector distance 2.0 cm).

Table 1. DSC parameters for PLLA bulk samples and fiber mats produced at different needle-to-collector distances: Tg - glass transition temperature at the transition mid-point; Tm – melting temperature; ΔHm – enthalpy of melting; ΔHc – enthalpy of crystallization

Sample	T_g (°C)	T_m (°C)	ΔH_m (J/g)	ΔH_c (J/g)	Crystallinity Degree (%)
Bulk powder	63	180	32	20	37
Fiber mat – 4.0 cm	61	175	54	16	40
Fiber mat – 2.0 cm	63	174	37	15	23

The T_g and T_m values for PLLA as powder or fiber mats does not differ significantly, and are in agreement with the literature[26–28]. On the other hand, the crystallinity degree is higher for the fiber mats (40%) at the 4.0 cm test, than for the powder form (37%). This is due to the elongation of the solution, during the electric field application, in the form of fine fibers, causing the polymer chains to elongate parallel to the jet direction. The polymer chains extend in the direction of the forming filament, giving rise to the fibers. It is also verified that the needle-to-collector distance considerably affects the crystallinity degree. Fibers obtained at a distance of 2.0 cm are 16% less crystalline than those produced at 4.0 cm.

In fact, the degree of crystallinity of a polymer depends on its cooling rate during the solidification process. During crystallization, the highly randomly oriented and entangled chains in the solution need to assume an orderly configuration. For this to occur, sufficient time must be given for the chains to move and align in relation to each other.

Thus, it is estimated that the distance (collector/capillary) of 2.0 cm featuring a shorter time for fiber formation and solvent evaporation provided a less crystalline fiber than those produced at 4.0 cm, wich has a greater distance, allowing a longer time to the alignment of the polymer chains and, thereby, featuring a higher degree of crystallinity. For a better understanding, the respectives DSC graphs are shown in the Figure 5.

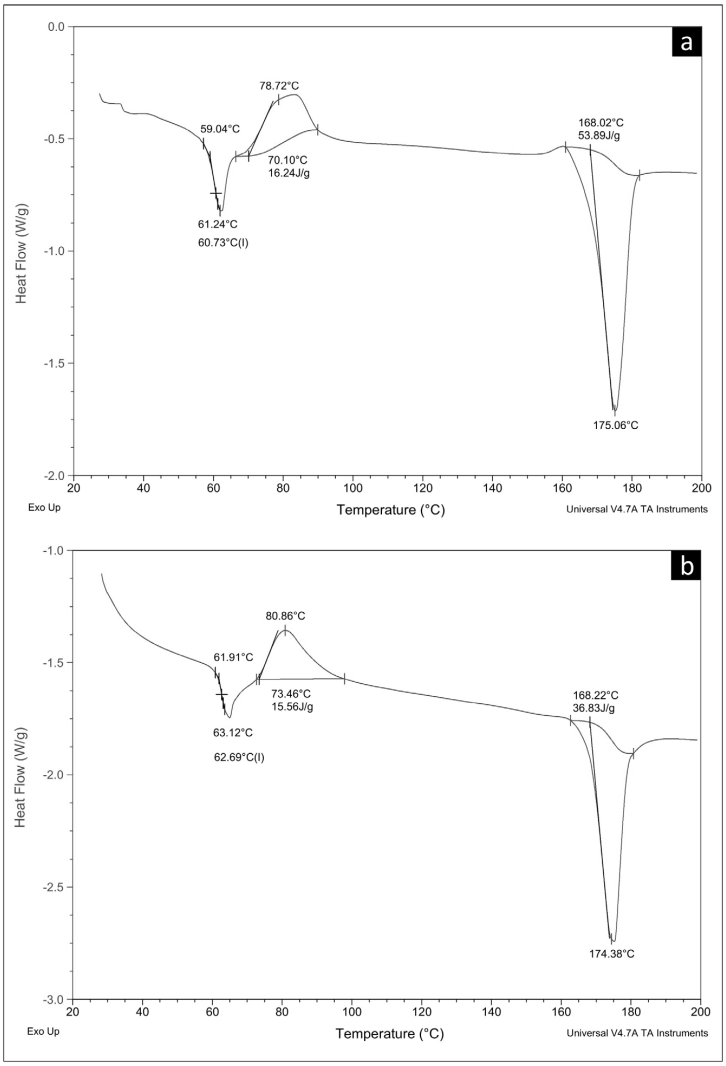

Figure 5. DSC curves for: a) test1 (needle-to-collector distance = 4.0 cm) and b) test 2 (needle-to-collector distance 2.0 cm).

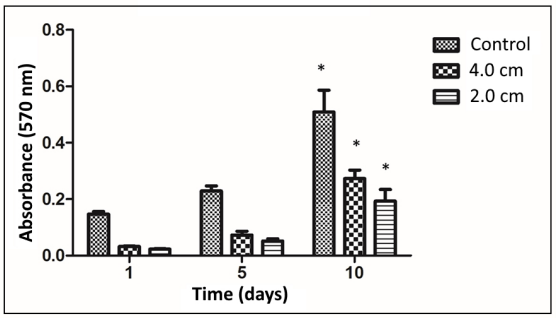

Figure 6. Cell viability graph.

3.3 Cell viability

In Figure 6 it is shown the graph characterizing the cell viability. In this assay, cell growth was noted after 10 days of cultivation. The polystyrene plate was used as control. Values are expressed as mean ± SD, n = 5. * P <0.01.

Both mats allowed the proliferation of ASC's, with a higher viability value for the 4 cm test. There was significant cell growth ($p < 0.01$) in both control group as in the mats, when compared the 10 days assay with the others. Thus, there was obtained a positive result considering the main purpose of application of this technique, i.e. to allow cell proliferation in the polymer framework.

One reason for the effective growth of cells produced in the membrane, may be the porosity on the surface of the fibers. In fact, porous fibers have been studied in various fields due to a large surface area and high porosity[16,22]. Furthermore, the type of roughness of these fibers determine the number and types of anchorage of the cells with its substrate. It is observed that on rough surfaces, the cells form layers covering the valleys between the peaks of these pores[34].

According to literature[14] nonwoven polymer nanofiber are among the most promising biomaterials analogs as extracellular matrix, for applications such as scaffold for cell growth. Theoretically, these mats are ideal, because the cells are sensitive to the topography of the support surface,

although the exact reasons for this are yet not clear. Thus the search for surfaces with beneficial influences on cell growth has been the subject of recent research[35].

4. Conclusions

A three-dimensional fiber network with highly porous surface, was obtained by MFES. Interconnected pores and thinner diameters were obtained using a greater collector/capillary distance (4.0 cm).

The PLLA, proved to be viable to the scaffold application, due to its biocompatibility with the type of implanted cell. It was also verified that the presence of a small amount of water, acting as a nonsolvent which evaporates during the electrospinning process, favors the formation of fibers with high surface porosity, adequate for cell attachment, proliferation, and diferentation which is ideal for cell growth.

The theory of the cristalinity increase through the electrospinning process was confirmed, with fiber mats presenting a higher cristalinity degree than the bulk powder test as well as a higher degree when using a greater collector/capillary distance.

Another important observation is about the electrospinning process, which can be assumed as a novel medium field electrospinning technique, (MFES), once it is not in the range of the FFES (10-15cm and 10-20kV) nor in the range of NFES (500µm-3mm), by using intermediate needle-to-collector distances (2-4 cm) and potential (6 kV) to the production o PLLA fiber mats.

The use of this relatively low voltage, medium electrical field, and rotating collector was not able to align the fibers; thus, it can be conclude that even with the reduced voltage and collector/capillary distance, the fibers were collected in its unstable fase, resulting in a aleatory deposition.

In order to provide a scaffold with the most suitable proprerties to the tissue engineering application, the material should mimic the ECM as much as possible, thus the obtained fiber mats are potentially useful for tissue regeneration applications.

5. Acknowledgements

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