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Comparative Analysis of Biocompatibility between Poly (L-lactic Acid) (PLLA) and PLDL Purac® Nanofibers for use in Tissue Engineering

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The absorbable polyacid, poly (L-Lactide) (PLLA) is one of the most commonly used and studied materials in tissue engineering, for its biocompatibility and biodegradability. This work will show that the PLLA synthesized at our laboratory has the same biocompatibility as the PLDL Purac®. The PLLA was synthesized through ring-opening polymerization and manufactured into nanofibers through the electrospinning process. The cytotoxicity of the material was evaluated by the calorimetric MTT assay (Sigma), Live/Dead (Molecular Probes), Direct Contact, Elution and Agar Diffusion.The assays were performed using nanofibers membranes made with PLLA and with PLDL Purac®, where the biomaterials were in contact with the fibroblast cell culture, in the sense to compare the results between them. For the MTT, Live/Dead and Elution assays the nanofiber membranes were incubated with the fibroblast cell culture for 24h, 48h and 72h. For the Direct Contact and Agar Diffusion the nanofiber membranes were maintained in contact with the cells for 24h. For the assay controls we used DMEM-LG containing 0.5% phenol as the positive control of toxicity CT(+) and for negative control of toxicity CT(-) DMEM-LG containing 10% FBS. The ANOVA test was used to measure the MTT assay results, and within 24h of exposure, the cells in contact with the PLLA showed a higher proliferation rate than CT(-) and PLDL Purac®, being significantly different ($p < 0,05$). Whatsoever, there were no statistically significant differences between the PLLA, the PLDL Purac®, and the CT(-) after 48h and 72h of culture ($p>0.05$). The morphology of the cells in contact with both biomaterials was also considered normal, showing no signal of cytotoxicity. The high rates of proliferation and viability of the cells in contact with the PLLA, shown by the biocompatibility assays, demonstrate that the PLLA is a biocompatible material. The PLDL Purac® exhibited similar results, as expected. Those findings show that the PLLA synthesized has the same biocompatibility as the PLDL Purac®. Thus, the PLLA synthesized in our laboratory is a high quality biomaterial that can assist in the manufacturing of nanofibers that can be adapted for different biomedical applications.

1. Introduction

Polymers constitute a very wide-ranging class of biomaterials, with diverse uses in the biomedical field. These biomaterials must show suitable mechanical properties as well as biocompatibility characteristics, for use in the human body. Therefore, they should not present any local or systemic adverse biological response. PLA is known for its outstanding biocompatibility and mechanical properties. It also features a diversification of applications, since simple changes in its physical and chemical structure may make it useful in different areas. It can be used as a combination of L-lactic (PLLA) and D, L-lactic (PLDL) acid monomers, being the latter rapidly degraded without formation of crystalline fragments during this process (Fukushima et al. 2008). Therefore, PLA and its copolymers are materials commonly used in tissue engineering. Whereas one of the goals of tissue engineering is to help improve tissue regeneration, the use of nanofiber membranes, combined with their potential for biodegradation, could be of interest for various biomedical applications, since they mimic the natural extracellular matrix (ECM), providing an excellent environment for the cells to attach, grow

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and migrate, thus improving the rate of tissue regeneration (Dong et al. 2009). In the present study, we synthesized PLLA in our laboratory through ring-opening polymerization and manufactured nanofiber membranes with it. To assay the biocompatibility of the material, we performed different biocompatibility experiments, and we compared it with PLDL Purac® nanofibers membranes, a PLA co-polymer commonly used in tissue engineering, with excellent biocompatibility characteristics (Leiggener et al. 2006).

2. Materials and Methods

2.1 Manufacturing of PLLA and PLDL nanofibers

For electrospinning, the biodegradable and biocompatible polymers PLDL Purac® and Poly(L-lactic acid) (PLLA) were used. PLLA was synthesized in our laboratory as described by Lopes (2014). In the nanofibers manufacturing process, the PLLA was dissolved in acetone and chloroform, and PLDL in acetone, both at analytical grade. The solution was then loaded in a 10ml syringe, connected to a polyamide cylinder, attached to a 0.7mm hypodermic needle as a nozzle. The flow rate of the jet (8ml/h) was controlled by using a syringe pump. To charge the solution, a 15kV high voltage power-source was used. The distance between the needle and the collector plate was of 17cm and the time of electrospinning of 2h.

2.2 Cell Incubation

The Vero cells (fibroblast) were seeded (3 x 106 cells/mL) in a culture plate and incubated with 5% CO_2 at 37ºC for 24h. Then, PLLA and PLDL nanofibers were added in wells and cultured for different times. DMEM-LG containing 0.5% phenol and 10% FBS were used as the positive control CT(+) and negative control CT(-) for toxicity, respectively. All experiments were performed at least in triplicate (n=3).

2.3 MTT

The modified Mosmann (1983) method was chosen to perform the MTT assay. For this assay, after periods of incubation, the MTT solution (0.5 mg/mL Sigma) was added, and after 4h of incubation it was withdrawn and 200 µl of dimethyl sulphoxide (DMSO) was added to determine the absorbance values, at an absorption wavelength of λ = 595 nm (FilterMax F5 Multi-Mode Microplate reader, Molecular Probes). The resulting absorbance values were expressed as optical density (OD) (mean value ± standard deviation). The comparison between the values was made through parametric data analysis One-way ANOVA. Analysis with $p < 0.05$ were considered significant. Analyzes were performed by using StatView software (SAS Institute Inc., Cary, NC, USA).

2.4 Elution

The elution assay is a quantitative method designed to show the presence of toxic material eluted from a test sample and the effects on the fibroblast cells cultured in the presence of the extract. For this assay, the materials were incubated in culture medium for 48h. After this period, the extract obtained from incubation of materials was inoculated with the fibroblast cells, prepared as mentioned in item 2.2. After various incubation periods (24, 48 and 72h), the extract was removed and a calorimetric assay was performed, as mentioned in item 2.3.

2.5 Live/Dead®

The qualitative fluorescence assay kit Live/Dead® (Molecular Probes) was used to qualify the fibroblast viability. After the incubation time, the cells were washed with 200 µl of PBS and treated with a solution of Calcein AM and Ethidium homodimer-1 (EthD-1) according to the manufacturer's instructions. The cells were incubated at 37°C for 30 min, then washed with PBS and observed by inverted fluorescence microscopy (Nikon E800) with a specific program (Image Pro-Plus software).

2.6 Direct Contact

For direct contact assay fibroblasts were cultured into a high confluence layer in a 24 wells plate. Then, the culture medium was removed and replaced with fresh one. The nanofiber samples of PLLA and PLDL were placed onto the culture and incubated for 24h, with 5% CO_2 at 37ºC. After this period, the materials and culture medium were removed from the wells. The remaining cells were fixated with alcohol 70% and then stained with Toluidine Blue (TB) and Xylidine Ponceau (XP). The stain only works on the living cells, and thus, the toxicity of the material is indicated by the absence of stained cells around the material.

2.7 Agar Diffusion

The agar diffusion assay is a qualitative test designed to show toxic effects of the material on cell morphology. A near confluent layer of fibroblasts was prepared in a 6-well culture plate. The medium was removed and the cells covered with a solution of 2% agar, which contained vital stain. While the agar solidified, the cells dispersed through it. The PLLA and PLDL nanofibers were then placed onto the surface overlaid of the agar and incubated for 24h at 37 ºC. Latex was chosen as positive control of cytotoxicity CT(+) and Teflon as the negative control of cytotoxicity CT(-). The live cells take up the neutral red vital stain and retain it, while the

dead cells do not. Thus, the toxicity of the material were evaluated by the loss of vital stain under and around the material, allowing differentiating the viable from the lysed cells.

3. Results and Discussion

3.1 Cytotoxicity by MTT assay

Cytotoxicity tests were performed to study the polymer biocompatibility. In this study, we chose the MTT assay where the metabolic activity and the rate of cell growth indicated the degree of cytotoxicity of the PLLA and PLDL in the cell culture. MTT is a yellow salt that is reduced by mitochondrial dehydrogenase activity of the enzyme, resulting in a formazan purple salt. This reduction occurs only in living cells. Thus, cell viability was determined by the intensity of purple color, which is proportional to the amount of formazan crystals formed. After performing the MTT test, the absorbance values obtained generated the curves below (Figure 1). The graph shows the proliferation of fibroblast cells in contact with the PLLA and PLDL after 24, 48 and 72h of exposition.

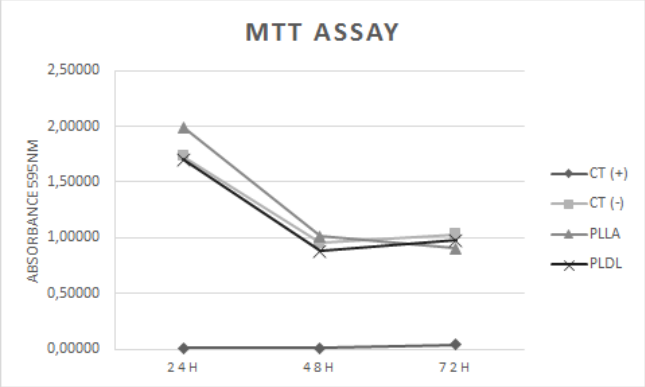

Figure 1. MTT assay for fibroblast cells cultured with PLLA and PLDL, negative control for toxicity CT(-) and positive control for toxicity CT(+) for 24 h, 48 h and 72 h.

According to the ANOVA test, after 24h of exposure, there were statistical differences ($p < 0,05$) between CT(-), PLLA and PLDL, with PLLA showing a higher rate of cell proliferation than PLDL and CT(-). Whatsoever, there were no statistically significant differences between the PLLA, PLDL and the (CT-) after 48h and 72h of culture ($p > 0.05$). Those results appoint that at the initial exposure time, where the medium is rich in nutrients and space for the cells to proliferate, there is a high rate of cellular growth. The presence of the nanofibers stimulate the cell development, which explains the higher amount of cells when cultured with the PLLA and PLDL nanofibers, than on the CT(-). Those findings show that the synthesized PLLA and the PLDL do not present cytotoxic behavior with respect to the fibroblast cells in the evaluated periods, in accordance with the MTT studies of Sarasua et al. (2011) and Wu et al. (2014).

3.2 Elution

The elution assay is a quantitative technique designed to show the incidence of toxic material eluted from a test sample into the culture medium. Figure 2 shows the kinetics of the cells exposed to the PLLA and PLDL nanofibers. As observed, the positive control of cytotoxicity CT(+) shows a very little presence of live cells. As for the negative control CT(-), cells with PLLA nanofibers and cells with PLDL nanofibers, both showed a high rate of proliferation at 24h of incubation, with no statistical difference between them, where at 24h of incubation there was a high amount of space and nutrients for the cells to multiply. At 48h there was a decrease in cell proliferation for CT(-), PLLA and PLDL, probably due to the increasing amount of metabolites and the decreased quantity of space and nutrients for the cells. The same happened at 72 h of incubation. These findings show that the synthesized PLLA and the PLDL does not negatively affect the fibroblast cell viability in the evaluated time periods.

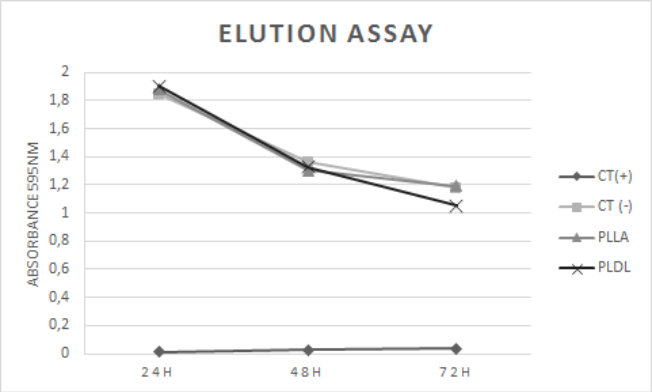

Figure 2. Elution assay for fibroblast cells cultured with PLLA and PLDL, negative control for toxicity CT(-) and positive control for toxicity CT(+) for 24 h, 48 h and 72 h.

3.3 Viability by LIVE/DEAD®

The Live/Dead® assay provides a qualitative evaluation of the polymer biocompatibility when in contact with the cells. The fibroblast were cultured with the biomaterials at three different times: 24, 48 and 72h, along with it controls of toxicity. The calcein present in live cells produces an intense green fluorescence, which is determined by the enzymatic conversion of the non-fluorescent cell-permeant agent, calcein AM. EthD-1 enters cells with damaged membranes undergoing a strong fluorescence enhancement upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells. EthD-1 is excluded by the intact plasma membrane of live cells.

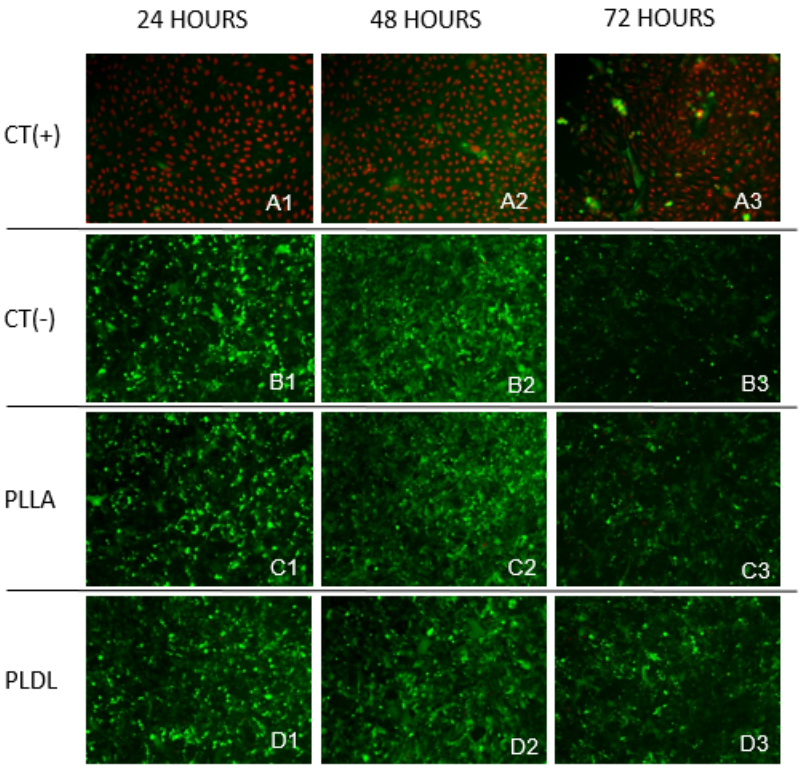

Figure 3. Live/Dead assay of fibroblast in contact with PLLA and PLDL at different times. A – Positive control CT(+). B – Negative control CT(-). C – PLLA nanofibers cultured with fibroblasts. D – PLDL nanofibers cultured with fibroblasts.

Figure 3 presents the results of the Live/Dead assay. Images A show the positive control of toxicity CT(+) where the cells were red stained, showing cell death at all times. Images B shown the negative control of toxicity CT(-), where, at different times, cells were stained in fluorescent green indicating that the cell membrane was intact. Images C show the cells in contact with PLLA for 24h, 48h and 72 h, respectively. They all showed normal morphology, as the ones at CT(-); however, at 72h we can see some cells red stained, showing that there are some dead cells. Whatsoever, at 72h of incubation, there are less nutrient and space for the cells to keep proliferating, with a high amount of metabolites in the culture medium. This can be the reason for the appearance of a higher number of dead cells in the culture. The same results were obtained for the cells in contact with PLDL, as shown in Images D. Those results show that the presence of PLLA and PLDL nanofibers did not affect negatively cell proliferation and morphology, instead improved cell proliferation, in line with the results previously shown by Bernstein et al. (2012), who tested PLLA with different formats, such as screws and pins through LIVE/DEAD® assay. Hence the qualitative assay of Live/Dead corroborate the quantitatively cytotoxicity assay MTT.

3.4 Direct Contact

The cells were directly exposed to the PLLA and PLDL nanofibers to assay if their presence would cause toxic effects on cell morphology. Since only live cells are stained, we can measure the toxicity of the material through the lack of stain around the area exposed to the material. Figure 4 presents the results of the assay; Images A report the control, where no material was in contact with the fibroblast, showing normal cell morphology (A1 and A2). Images B show the cells in contact with PLLA nanofibers and stained with Toluidine Blue (B1) and Xylidine Ponceau (B2), both images show a normal cell morphology, indicating that the PLLA nanofibers present no toxicity for the fibroblasts. Images C show the cells in contact with PLDL nanofibers and stained with Toluidine Blue (C1) and Xylidine Ponceau (C2) indicating that the PLDL nanofibers also present no toxicity for the fibroblasts.

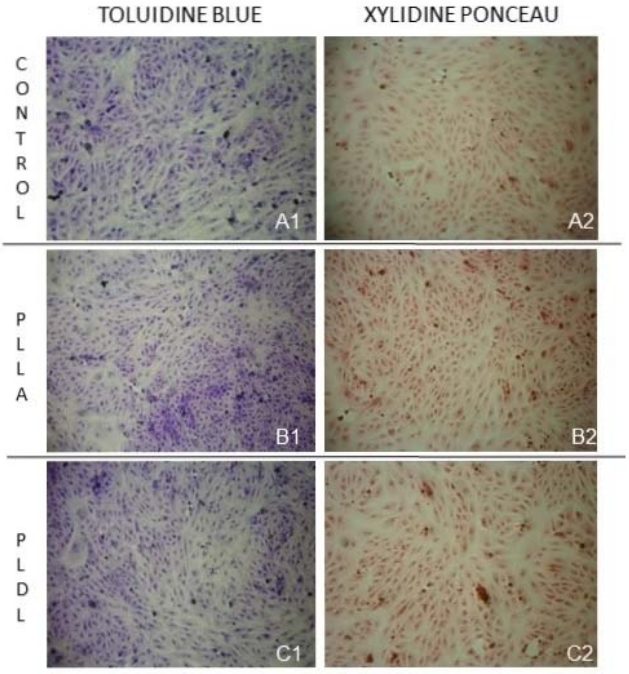

Figure 4. Direct Contact assay. Cells in contact with PLLA and PLDL stained with Toluidine Blue and Xylidine Ponceau.

3.5 Agar Diffusion

The agar diffusion assay is a qualitative test, where one can assay the toxicity of a material from the lack of dye around it, since only live cells can be stained. Figure 5 show the fibroblast cells in contact with PLLA, PLDL and their respective controls. On image A we can see the lack of dye of the cells in contact with the Latex CT(+), showing cell death. On image B, the cells were all stained with vital stain neutral red when in contact with the Teflon CT(-), showing that the cells were alive and with normal morphology. Image C show the cells in contact with PLLA nanofiber and image D with PLDL nanofibers. On both images, the cells were stained, both presenting normal morphology. This demonstrated that both materials do not present any toxic effect on cell culture.

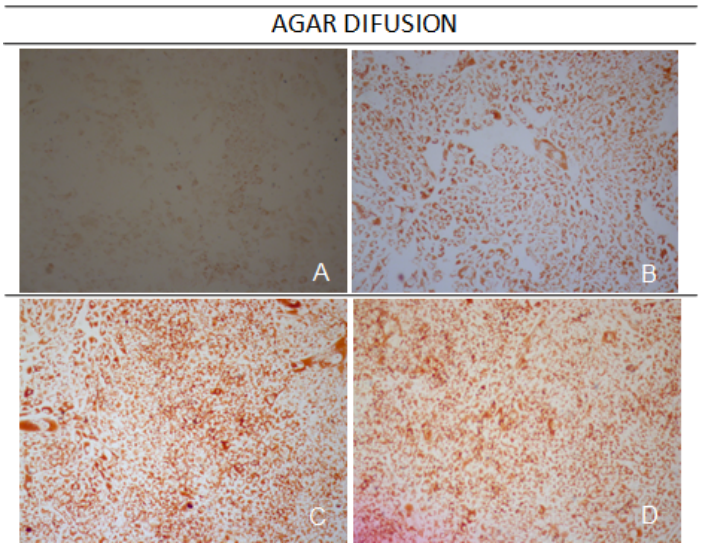

Figure 5. A – Positive Control of Toxicity (Latex). B – Negative control of toxicity (Teflon). C- Cells exposed to PLLA nanofibers. D- Cells exposed with PLDL nanofibers.

4. Conclusion

The results of the MTT assay, Live/Dead, Direct Contact, Elution and Agar Diffusion bring to the conclusion, that both PLLA nanofibers and PLDL® nanofibers do not present toxic effect on fibroblast cell cultures. Moreover, there were no statistical differences between the quantitatively results of PLLA and PLDL® on the MTT and Elution assays. This show that the PLLA synthesized in our laboratory has the same biocompatibility characteristics than the PLDL®, a material available on the market.

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