International Journal of Engineering Research & Technology (IJERT)
ISSN: 2278-0181
Vol. 5 Issue 07, July-2016

Synthesis of PLA Textile Scaffolds by Air Jet Spinning and Their Biocompatibility Characterization

Marco Vladimir Granados-Hernández[1, 2]
[1]Laboratorio de Bioingeniería de Tejidos;
DEPeI, Facultad de Odontología, UNAM. Circuito Exterior s/n. Cd. Universitaria, 04510 Coyoacán, CDMX, México.
[2]Facultad de Ciencias, Posgrado de Ciencias Biológicas, UNAM, Circuito Exterior s/n, CDMX, México.

Juan José Montesinos-Montesinos[4]
[4]Laboratorio de Células Troncales Mesenquimales, Unidad de Investigación de Oncología, Hospital de Oncología, Centro Médico Nacional XXI, IMSS, CDMX, México.

Emmanuel Alejandro Albanes-Ojeda[3]
[3]Instituto de Investigaciones en Materiales, Circuito Exterior s/n. Cd. Universitaria, 04510 Coyoacán, CDMX, México.

Octavio Alvarez-Fregoso[3]
[3]Instituto de Investigaciones en Materiales, Circuito Exterior s/n. Cd. Universitaria, 04510 Coyoacán, CDMX, México

*Marco Antonio Alvarez-Perez[1]
[1]Laboratorio de Bioingeniería de Tejidos;
División de Estudios de Posgrado e Investigación, Facultad de Odontología, UNAM.
Circuito Exterior s/n. Cd. Universitaria, 04510 Coyoacán, CDMX, México

Abstract: **In this article, we have studied the biocompatibility of bone marrow mesenchymal stem cells seeded onto different textile mats of PLA (6, 7, and 10%). PLA nanofibers textile mats were synthesized using the air jet spinning technique. The PLA nanofibers were characterized by X-ray diffraction (XRD) and scanning electron microscopy (SEM). XRD patters showed the semi crystalline fiber structure of PLA and SEM micrographs reveal the morphology and diameter size of prepared PLA fibers membranes scaffold. The biocompatibility were investigated by cell adhesion, cell viability and cell-material interaction assays. Our data indicate that fiber membranes have good biocompatibility and the synthesis method reported has potential for tissue engineering application in manufacturing fibers spun membranes due to ease processing and more economical technology.**

Keywords: Biocompatibility, Air jet spinning, Nanofiber, SEM ananlysis, Biomaterial, Tissue Engineering

I. INTRODUCTION

Tissue engineering (TE) is a multidisciplinary science field where its approach is an inherently sound strategy for regenerating the hierarchical structures of tissues of the human body [1]. Considering the complexity of tissue architecture, TE are focusing on advanced scaffold designs; for study the biological respond and structural function in a spatiotemporal manner for biological replacements or tissue regeneration as potential to improve therapeutic clinical outcomes [2].

Fiber scaffold design is a key in tissue regeneration because the fibers could mimic the native extracellular matrix (ECM) of several tissues. Moreover, fiber scaffolds design could guiding on the acquisition of fundamental knowledge related to the precisely coordinated wound healing response after disease or traumatic injury of damaged tissue by analyzing different functions as structural, chemical and support for proliferation and migration of cells [3-5].

Several techniques used for the manufacturing of fibers scaffolds are reported and one of the most used is electrospinning which is considered as a simple, versatile method for producing fibers from micro- to nanometric scale, offering advantages such as high specific surface area and the ability to incorporate biomolecules components on the fibers [6]. However; electrospinning is limited by its slow rate of production and requires a high voltage supply for the adequate conductivity to form the polymeric jet which will result in the fiber and also the cost and complexity of the process [7-9].

Recently; an alternative technology for manufacturing fiber scaffold is the air jet spinning (AJS). This technique depends that airbrush had to be connected to compressed gas and loaded with polymer solution before use. Thus, the fibers are formed because the instant that shoots the polymer solution from the tip, the solvent begins to evaporate due to the propellant gas competes for oxygen and continues after the fibers have been deposited on the collector. The deposition rate for fibers mats its dependent on the viscosity of the polymer solutions, the concentration of the polymer solutions and the flow rates (gas pressure) [10-12]. Due to the rapid and cheap production rate of fiber scaffolds this technique have tried a variety of polymers. In this regard, poly-(lactic acid) (PLA) is one of the best candidates because it is widely accepted by the medical community for its in vivo and physiological degradation related to PLA ester link hydrolysis,

International Journal of Engineering Research & Technology (IJERT)
ISSN: 2278-0181
Vol. 5 Issue 07, July-2016

thereby creating metabolizable lactic acid by-products. This FDA-approved polymer is economical, can be dissolved in several non-toxic solution and easily processed feature that makes it an excellent candidate for use in the study and application of biomaterials design [13-15].

The objective of this study was to produce PLA fiber scaffold by air jet spinning and evaluate the biological respond of the scaffold when combined with human bone marrow mesenchymal stem cells.

II. EXPERIMENTAL METHODS

2.1 Materials

Poly lactic-acid (PLA) pellets ($C_3H_6O_3$; MW 192,000) were purchased from Nature works. Chloroform ($CHCl_3$) and ethyl alcohol absolute anhydrous (CH_3CH_2OH) were supplied by J. T. Baker. All products were used as received without further purifications. Three organic solutions with 6, 7, and 10 %wt of PLA, respectively, were prepared as follow: dissolved in chloroform ($CHCl_3$) and stirring by 20 hours and after that ethanol (CH_3CH_2OH) was added and let the solution stirring for 30 minutes up to obtain an homogeneous solution (polymeric solution). The volume ratio of chloroform/ethanol was 3:1

2.2 Preparation of films and spun membranes

Fibrous spun scaffold were produced via air jet spinning process from PLA polymeric solutions (6, 7, and 10%). In all cases, the polymeric solution was placed in a commercial available airbrush ADIR model 699 with a 0.3 mm nozzle diameter with a gravitational feed of the solution to synthesis the fibers membranes scaffold. The airbrush was connected to a pressurized argon tank (CAS number 7740-37, concentration >99%, PRAXAIR Mexico) and for deposition of the fibers a pressure of 30 psi with 11 cm of distance from the nozzle to the target was held constant. Optimized parameters of 8 mL volume of polymer solution rate with respect to 30 min of time were determined for high quality of fibers deposit.

Films of PLA (6, 7, and 10%) were prepared by deposited the polymeric solution into a specially designed Teflon mould and kept overnight under the fume hood to slowly induce the evaporation of $CHCl_3$/CH3CH_2OH.

2.3 Airbrushed fiber scaffold characterization

The fibers structure were determined by X-ray diffraction (XRD) using a D8 Focus Bruker AXS diffractometer with Cu-kα radiation in the 2Θ range between 10-35º and the morphology and microstructure were observed with a scanning electron microscope (SEM, JEOL JSM-6701F). Measuring of the diameter size was carried out by ImageJ softwareTM.

2.4 Processing Scaffold for cell seeding

Prior to the biological assays, PLA films and PLA spun fiber scaffolds were cut into a round shape (8 mm of diameter), placed in 24 cell culture plates and sterilized by immersion in 70% of ethanol (v/v) with antibiotic solution (streptomycin 100 µg/ml and penicillin 100U/ml) for 30 min. After sterilization, nanofiber scaffolds were rinsed with phosphate-buffered saline (PBS) three times and air dried.

2.5 Culture of human mesenchymal stem cells derived from bone marrow (hBM-MSC).

Biological assays were performed using hBM-MSC cells cultured onto 75 cm2 cell culture flask in Eagle's alpha minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, antibiotic solution (streptomycin 100 µg/ml and penicillin 100U/ml, Sigma Chem. Co) and 2 mM L-glutamine. hBM-MSC were kindly donate by Dr. Juan Jose Montesinos-Montesinos and their isolation and characterization were previously described [16].

2.6 Cell attachment

To establish the effect of PLA scaffolds on cell adhesion hBM-MSC were seeded at $1x10^4$ cell/mL onto films and spun fibers mats of PLA scaffolds at different concentration (6, 7, and 10%), placed in 24 cell culture plates and allowed to adhere in standard cell culture for 2, 4, and 24 h. After the prescribed time period, substrates were rinsed three times using PBS to remove non-adherent cells. Evaluation of cell attachment was performed according to crystal violet assay. Briefly; adherent cells were fixed with 4% paraformaldehyde and incubated with 0.1% crystal violet for 20 min. After that the scaffolds were washed slowly with distilled water for 30 min. The dye was extracted with 0.1% of sodium dodecyl sulfate (SDS) and the optical absorption was quantified at 600 nm with a ChroMate-4300 microplate reader (Awareness Technology). Conventional polystyrene 24-well culture plates were used as a control.

2.7 MTT assay

Cell viability of hBM-MSC plated at concentration of $1x10^4$ cell/mL in triplicate onto films and spun fibers mats of PLA scaffolds at different concentration (6, 7, and 10%), were checked by the MTT assay for 3, 5, 7, and 14 days of culture. This assay is based on the ability of mitochondrial dehydrogenases of living cells to oxidize a tetrazolium salt (3-[4, 5-dimethylthiazolyl-2-y]-2, 5-diphenyltetrazolium bromide), to an insoluble blue formazan product. The concentration of the blue formazan product is directly proportional to the number of metabolically active cells. The hBM-MSC seeded onto films and spun fibers mats of PLA scaffolds at prescribed time were washed with PBS and incubated with fresh cultured medium containing 0.5 mg/mL of MTT for 4 h at 37°C. Then, the supernatant was removed and 200 µL of dimethyl sulfoxide (DMSO) was added to each well. After 60 minutes of slow shaking the absorbance was quantified by spectrophotometry at 570 nm with a plate reader. During the experiment, the culture medium was changed every two days with fresh media, and all viability experiments were conducted in triplicate.

2.8 Cell morphology

The spreading pattern interaction of hBM-MSC onto PLA 6% fibers scaffolds was evaluated by laser confocal microscopy (CLSM, Leica Mod. DMI4000B; Germany) after staining of hBM-MSC cells. Briefly, hBM-MSC cultured in 75 cm2 cell culture flask were washed with PBS and incubated with CellTraker green CMFDA (Invitrogen Carlsbad, CA) in serum free medium for 30 min. After incubation, the dye staining medium was replaced with fresh completed medium and incubated 2 h at 37°C. Then, hBM-

International Journal of Engineering Research & Technology (IJERT)
ISSN: 2278-0181
Vol. 5 Issue 07, July-2016

MSC cells were washed with PBS, trypsinized and the cell pellet was collected and diluted with cultured medium to get the required cell concentration. hBM-MSC at $1x10^4$ cell/mL were seeded onto PLA 6% fiber scaffolds surfaces that was the better scaffold on biocompatibility and incubated for 24 h. After this time, cells were washed three times with PBS and fixed with 4% formaldehyde in PBS for 15 min at room temperature. hBM-MSC interaction was viewed under confocal microscopy with excitation and emission wavelengths of 492 and 529 nm.

2.9 Statistical Analysis

All numerical data were presented as mean ± standard deviation. All results were subjected to statistical evaluation using ANOVA to determine significant differences between groups. The significance level was set at $p < 0.05$.

III. RESULTS AND DISCUSSION

The structural properties of PLA fibers were evaluated by X-ray diffraction. The results show the presence of amorphous phase where there are some maxims that suggest a poor crystalline phase whit peaks at 16.6, 21.5, and 23° 2Θ distance as result from the stretching of the AJS process (Figure 1). This is important because is reported that amorphous PLA scaffolds are preferred for their properties as faster degradation rates and are good for developing drug delivery vehicles and low-strength scaffolding materials for tissue regeneration [17-19].

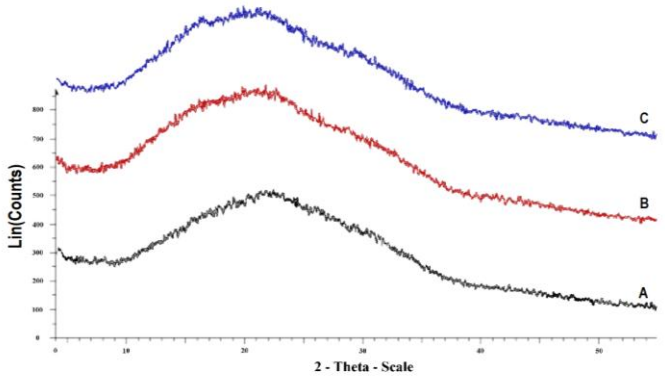

Fig. 1. XRD diffraction pattern of PLA fiber scaffold fabricated via air jet spinning (A) 6%, (B) 7% and (C) 10% of polymer concentration.

PLA fiber scaffold morphology and diameter size were evaluated by scanning electron microscopy (Figure 2). The micrographs show that the spinning process of different concentration of polymeric solution of PLA (6, 7, and 10%) have the same fiber morphology in the scaffolds with random directions and with a noticeable change in uniformity when the concentration of PLA is increased. Moreover, the differences were found in the diameter sizes distribution of fibers in the scaffolds. Fiber scaffold with 6%wt of PLA present a mean diameter of 800 nm (Figure 2a), fiber scaffold with 7%wt of PLA show a mean diameter of 600 nm (Figure 2b) and finally, the fibers with high concentration of PLA, 10%wt, present a mean diameter of 700 nm (Figure 2c). It means that the concentration of PLA have an effect on diameter size of the nanofibers and also stretches them to a regular diameter with a quality surface morphology deposited by air jet spinning. Recent studies have validated the manufacturing of nanofibers and also suggested that cells can be attached and organized better in nanostructured materials [17, 21-23].

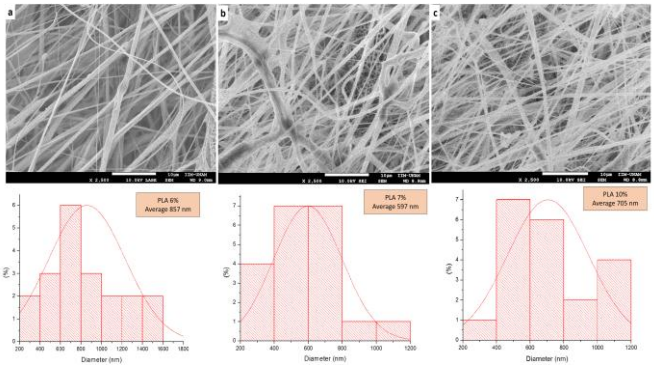

Fig. 2. SEM images and diameter size of PLA spun fiber scaffolds. (a) 6%, (b) 7% and (c) 10% of polymer concentration spinning by AJS technique.

Cell interactions with their surrounding extracellular matrix play an important role in regulating cellular functions as basic as attachment, spreading and proliferation [24]. In our study we evaluated the effect of fiber topology in terms of biocompatibility response. As shown in Fig. 3, the adhesion of hBM-MSC cells with different concentration (6, 7, and 10%) of PLA films and PLA nanofiber surface scaffolds was measured at 2, 4, and 24 h after seeding. The number of hBM-MSC cells attaching onto 6% of PLA nanofibers surface scaffold showed an increase from 83 to 130%, for 7% of PLA nanofibers from 44 to 75% and for 10% of PLA nanofibers from 85 to 91% when compared to PLA films scaffolds that maintain a constant adhesion value approximately of 50% in the culture time. However, this increased in adhesion of the hBM-MSC cells could be favored for fibrillary morphology of the PLA scaffolds. The nanoscale fiber as showed by the images of the topography by SEM has a significant contribution on the cell functionality and biocompatibility. Moreover, nanofiber morphology could influenced the cell respond and hBM-MSC attach more readily and efficiently to the 6% PLA fiber scaffold when compared with the PLA 7 and 10%. Similar results have been reported that cellular adhesion of mesenchymal stem cells is regulated by the surface topography and the nanoscale of the materials [25-27].

International Journal of Engineering Research & Technology (IJERT)
ISSN: 2278-0181
Vol. 5 Issue 07, July-2016

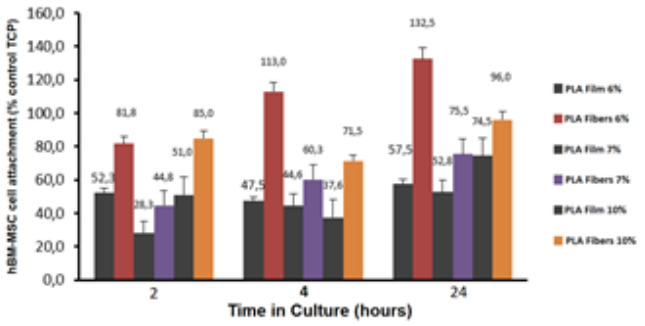

Fig. 3. Adhesion of hBM-MSC cells cultured onto PLA films and PLA nanofiber scaffolds after an incubation period of 2, 4, and 24 hours expressed as the percentage of attached cells in comparison with control culture tissue plate.

To assess how the fiber diameter and surface chemistry affect cell viability, MTT assay of hBM-MSC seeded onto PLA films and PLA fibers scaffolds was determined after 3, 5, 7, 14, and 21 days in culture. The results of the MTT assay are presented as the optical absorbance at 570 nm as shown in Figure 4. We found high levels of MTT conversion, comparing it to the control on day 5 and continue until day 21. This increment is directly proportional to the increase of metabolic active cells on the fibrillary surface of PLA scaffolds and inversely proportional to the toxicity effect of the surface topography of the material. Moreover, from the graphic the cellular viability on the fiber surface could be influenced by the surface morphology, i.e., fiber diameters because on cell viability onto PLA films showed slow rate of growth in all time of cell culture [28]. Moreover, statistical significances in MTT activity were found between hBM-MSC culture onto PLA fiber scaffolds and hBM-MSC culture in PLA films at $p < 0.05$.

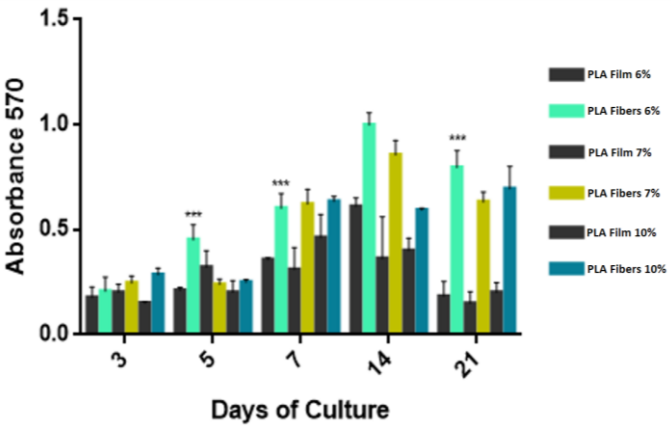

Fig. 4. Cell viability of hBM-MSC (MTT assay) cultured onto PLA films and PLA nanofiber scaffolds after an incubation period of 3, 5, 7, 14, and 21 days expressed as the absorbance at 570 nm. Asterisks indicate statistical significance ($p < 0.05$).

Figure 5 shows the morphology of the cell-material interaction of hBM-MSC cells cultured onto 6% of PLA nanofiber scaffold. The images show a well attached cell to the nanofiber surface covering long extensions of area with very spreading, elongated morphology. Our results showed that the nanotexturing of fibers has a significant influence on cell morphology. These results are supported by previous works where it has been reported that the distance between fibers plays a crucial role in the response of cells and also the contact guidance that refers to the tendency of cells to be guided in the direction of the nanofibrilar shape [25-28]

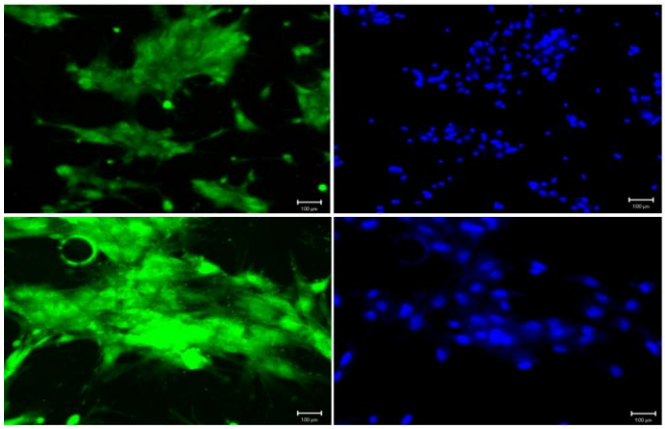

Fig. 5. Confocal images of cell material interaction of hBM-MSC cells cultured onto 6% PLA nanofiber scaffolds after an incubation period of 24h. The images showed a flattened and spreaded cell morphology indicated that cell is oriented along the fibers of the PLA scaffold.

IV. CONCLUSION

In this study PLA were spun by AJS from different polymer solutions to form random fiber topographies and mean fiber diameters ranging from 600 to 800 nm. These surfaces were investigated for evaluating the biological response of hBM-MSC under culture conditions and our results did not show any cytotoxicity effects, because hBM-MSC were able to exhibited good cell functionality as cellular adhesion, cellular viability and cell-material interactions. Our results suggested that fiber spun mat manufacturing by air jet spinning have a great potential application on the tissue engineering field.

ACKNOWLEDGMENT

Authors want to thank to Raul Reyes Ortíz and Adriana Cruz Tejeda for their technical assistance. MVGH thanks to CONACYT (No. 299078) and EAAO thanks to CONACYT (No. 277810) for their doctoral scholarship during the course of this study. This research was financially supported by funds from DGAPA-UNAM: PAPIIT IN210815 project.

International Journal of Engineering Research & Technology (IJERT)
ISSN: 2278-0181
Vol. 5 Issue 07, July-2016

REFERENCES

[1] R.E. Horch, A. Boccaccini, A. Arkudas, Regenerative Medicine and Tissue Engineering: How Far Are We?, *J Tissue Sci Eng*, **2013**, 4 e 121.

[2] M.B. Fisher, R.L. Mauck, Tissue engineering and regenerative medicine: recent innovations and the transition to translation, *Tissue Eng Part B Rev*, **2013**, 19, 1-13.

[3] . R. Langer, D.A. Tirrell, Designing materials for biology and medicine, *Nature*, **2004**, 428, 487-492.

[4] B.M. Baker, A.M. Handorf, L.C. Ionescu, W.J. Li, R.L. Mauck, New directions in nanofibrous scaffolds for soft tissue engineering and regeneration, *Expert Rev Med Devices*, **2009**; 6(5), 515-532.

[5] S.K. Vimal, N. Ahamad, D.S. Katti, A simple method for fabrication of electrospun fibers with controlled degree of alignment having potential for nerve regeneration applications, *Materials Science and Engineering C*, **2016**, 63, 616-627.

[6] G.C. Ingavle, J.K. Leach, Advancements in electrospinning of polymeric nanofibrous scaffolds for tissue engineering, *Tissue Eng. B Rev*, **2013**, 20(4), 277-293.

[7] H. Liu, X. Ding, G. Zhou, P. Li, X.Wei, Y. Fan, Electrospinning of nanofibers for tissue engineering applications, *J. Nanomater*, **2013**, 2013, ID 495708, 11.

[8] A. Martins, R. Reis, N. Neves, Electrospinning: processing technique for tissue engineering scaffolding, *Int. Mater. Rev*, **2008**, 53, 257-274.

[9] . Yao, C.W.m. Bastiaansen, T. Peijs, High Strength and High Modulus Electrospun Nanofibers, *Fibers*, **2014**, 2, 158-187.

[10] M.R. Badrossamay, H.A. McIlwee, J.A. Goss, K.K. Parker, Nanofiber assembly by rotary jet-spinning, *Nano Lett*, **2010**, 10(6), 2257-2261

[11] W. Tutak, S. Sarkar, S. Lin-Gibson, T.M. Farooque, G. Jyotsnendu, D. Wang, J. Kohn, D. Bolikal, C.G. Simon Jr., The support of bone marrow stromal cell differentiation by airbrushed nanofiber scaffolds, *Biomaterials*, **2013**, 34, 2389-2398.

[12] A. Abdal-Hay, A.S. Hamdy, K.A. Khalil, J.H. Lim, A novel simple one-step air jet spinning approach for deposition of poly(vinyl acetate)/hydroxyapatite composite nanofibers on Ti implants, *Mater Sci Eng C Mater Biol Appl*, **2015**, 49, 681-690.

[13] H. Tsuji, Poly(lactide) stereocomplexes: formation, structure, properties, degradation, and applications, *Macromolecular Bioscience*, **2005**, 5, 569-597.

[14] G. Sabbatier, D. Le Nouën, P. Chevallier, B. Durand, G. Laroche, F. Dieval, Air spun poly(lactic acid) nanofiber scaffold degradation for vascular tissue engineering: A 1H NMR study, *Polymer Degradation and Stability*, **2012**, 97, 1520-1526.

[15] S. Francois, N. Chakfe, B. Durand, G. Laroche, A poly(L-lactic acid) nanofibre mesh scaffold for endothelial cells on vascular prostheses, *Acta Biomaterialia*, **2009**, 5, 2418-2428.

[16] J.J. Montesinos, E. Flores-Figueroa, S. Castillo-Medina, P. Flores-Guzmán, E. Hernández-Estévez E, G. Fajardo-Orduña, S. Orozco, H. Mayani, Human mesenchymal stromal cells from adult and neonatal sources: comparative analysis of their morphology, immunophenotype, differentiation patterns and neural protein expression, *Cytotherapy*, **2009**,11,163-176.

[17] E.Y. Gómez-Pachón, R. Vera-Graziano, R. Montiel-Campos, Structure of poly(lactic-acid) PLA nanofibers scaffolds prepared by electrospinning, *IOP Conf. Series: Materials Science and Engineering*, **2014**, 59, 012003.

[18] T.T Lin, X. Liu, C.B. He, Ab Initio Elasticity of Poly(lactic acid) Crystals, *J. Phys. Chem. B*, **2010**, 114(9), 3133-3139.

[19] M.S. Davachi, B. Kaffashi, Polylactic Acid in Medicine, *Polymer-Plastics Technology and Engineering*, **2015**, 54(9), 944-967.

[20] R. Mehta, V. Kumar, V. Bhunia, S.N. Upadhyay, Synthesis of Poly(Lactic Acid): A Review, *Jl of Macromolecular Science, Part C: Polymer Reviews*, **2005**, 45(4), 325-349.

[21] S. Francois, CH. Sarra-Bournet, A. Jaffre, N. Chakfe, B. Durand, G. Laroche, Characterization of an air-spun poly(L-lactic acid) nanofiber mesh, *J Biomed Mater Res Part B: Appl Biomater*, **2010**, 93B, 531-543.

[22] V. Guarino, V. Cirillo, P. TaddeI, M.A. Alvarez-Perez, L. Ambrosio, Tuning size scale and crystallinity of PCL electrospun fibres via solvent permittivity to address hMSC response, *Macromol Biosci*, **2011**, 11(12),1694-705.

[23] E. Tayton, M. Purcell, A. Aarvold, J.O. Smith, A. Briscoe, J.M. Kanczler, K.M. Shakesheff, S.M. Howdle, D.G. Dunlop, R.O.C. Oreffo, A comparison of polymer and polymer-hydroxyapatite composite tissue engineered scaffolds for use in bone regeneration. An in vitro and in vivo study, *J Biomed Mater Res Part A*, **2014**, 102(8), 2613-2624.

[24] B. Geiger, A. Bershadsky, R. Pankov, K.M. Yamada, Transmembrane crosstalk between the extracellular matrixecytoskeleton crosstalk, *Nat Rev Mol Cell Biol* **2001,** 2, 793-805.

[25] P. Viswanathan, M.G. Ondeck, S. Chirasatitsin, K. Ngamkham, G.C. Reilly, A.J. Engler, G. Battaglia, 3D surface topology guides stem cell adhesion and differentiation, *Biomaterials*, **2015**, 52, 140-147.

[26] R.J. McMurray, N. Gadegaard, P.M. Tsimbouri, K.V. Burgess, L.E. McNamara, R. Tare, K. Murawski, E. Kingham, R.O. Oreffo, M.J. Dalby, Nanoscale surfaces for the long-term maintenance of mesenchymal stem cell phenotype and multipotency, *Nat Mater*, **2011**, 10(8), 637-644.

[27] M.J. Curran, R. Chen, R. Stokes, E. Irvine, D. Graham, E. Gubbins, D. Delaney, N. Amro, R. Sanedrin, H. Jamil, J.A. Hunt, Nanoscale definition of substrate materials to direct human adult stem cells towards tissue specific populations, *J Mater Sci Mater Med*, **2010**, 21(3), 1021-1029.

[28] K. Fujikura, A. Obata, T. Kasuga, Cellular Migration to Electrospun Poly(Lactic Acid) Fibermats, *Journal of Biomaterials Science*, **2012**, 23, 1939-1950.

Corresponding Author:

*Marco Antonio Alvarez-Perez
Laboratorio de Bioingeniería de Tejidos; División de Estudios de Posgrado e Investigación, Facultad de Odontología, UNAM. Circuito Exterior s/n. Cd. Universitaria, 04510 Coyoacán, CDMX, México