

Adhesion, proliferation and distribution of human mesenchymal stem/stromal cells (MSCs) in Poly(ϵ -caprolactone) (PCL) scaffolds with different pore sizes

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ABSTRACT: Tissue engineering, combining the use of biomaterials, mesenchymal stem/stromal cells (MSCs) and optimized culture medium formulations, is a very promising research field for tissue regeneration. This work is focused on the application of Poly(ϵ -caprolactone) scaffolds designed to have a spacing gradient between orthogonal fibres with diameters of 0.3 mm with pore sizes ranging from 190 to 390 μm . Human bone marrow (BM)-derived MSCs, were used to evaluate cell adhesion, proliferation and distribution within the scaffolds. Specifically, we hypothesized if seeding MSC in scaffold regions with different pore sizes would influence cell proliferation. Therefore, MSC were seeded at the centre of scaffolds with either larger (390 μm) or smaller pores (190 μm). Results obtained showed similar levels of adhesion and proliferation in both configurations, with fold increase in total cell number of 5 after 13 days of cultivation; which indicates that no limitation for cell proliferation within the range of pore sizes studied is observed.

1 INTRODUCTION

Tissue Engineering (TE) is a multidisciplinary area aiming the development of biological substitutes for regeneration, repair or restore the functions of organs and tissues (Skalak & Fox, 1988); overcoming the limitations of existing clinical treatments for damaged organs or tissues (Chua, *et al.*, 2005). TE was defined in 1993 by Langer and Vacanti as an “interdisciplinary field that applies principles of engineering and life sciences in the development of biological substitutes in order to restore, maintain or improve the function of a tissue” (Langer & Vacanti, 1993).

The more common strategy in TE is the use of support matrices (scaffolds) three-dimensional, biocompatible, biodegradable and highly porous, which constitute the physical substrate required for the process of adhesion, proliferation and cell differentiation (Pereira *et al.*, 2013)

Poly(ϵ -caprolactone), PCL, scaffolds were produced using an additive manufacturing process called extrusion (Bártolo *et al.*, 2012; Melchels *et al.* 2012). The extrusion technique, also referred as Fused Deposition Modelling (FDM) was developed by Crump (1992).

In this technique, thin thermoplastic filaments, in this case PCL, are melted by heating and guided by a robotic device controlled by a computer, to form the 3D object. The material appears in a liquid form and hardens after extrusion in a fibre form. These fibres are extruded layer-by-layer to obtain the desired scaffolds structure (Hao & Harris, 2008).

Mesenchymal stem/stromal cells (MSCs) were discovered by Cohnheim in the 19th century but the term was suggested in 1991 by Caplan (Monteiro *et al.*, 2013). They are adult tissue-derived multipotent cells with the capacity to differentiate into cells of different lineages, namely osteogenic, chondrogenic, adipogenic, and myogenic (Janjanin *et al.*, 2008). They represent only a very small fraction (0.001-0.010%) of the total mononuclear cells in the bone marrow (BM), but they can also be found in other tissues, such as umbilical cord matrix (UCM) and adipose tissue (AT) (Monteiro *et al.*, 2013). A major issue on the development of strategies using stem cells and porous scaffolds is their efficient seeding and cells proliferation to cover representative regions of the scaffold. There is different information on the literature concerning the adequate pore size to promote effective cell seeding (Oh *et al.*, 2007).

The scaffolds tested herein were made of PCL microfibrils orthogonally organised with spacing gradient, which results in different pore sizes within the same scaffold, ranging from 190 μm to 390 μm . Two configurations were tested with either, larger pores at the centre of the scaffold or at their edges. The aim of the work is to assess the adhesion, proliferation and distribution of hMSCs in scaffolds as function of pore size; namely to assess if the latter poses a limitation to cell proliferation. Therefore cells were seeded at the centre of the scaffolds for each configuration, in a region uniform concerning pore size at values of is either 190 μm or 390 μm .

2 MATERIALS AND METHODS

2.1 Production of scaffolds

PCL scaffolds were produced using the Bioextruder (Figure 2), a machine developed in the Centre for Rapid and Sustainable Product Development (CDRsp), based on the extrusion process, an additive biomanufacturing process.



Figure 1 Bioextruder machine (Mota, 2008).

To produce the matrices (scaffolds) the following properties of the Bioextruder were adjusted to obtain a good extrusion of the PCL: temperature at 80°C; deposition velocity of 8 mm/s; rotation velocity 22.5 rpm, slice thickness (ST) of 0.28 mm and the diameter of the nozzle was 0.3mm, which corresponds to the diameter of the fibre (RW) obtained (Figure 3).

Two different samples were produced. They present different pore sizes in two different configurations ranging from 190-390 μm . The decrease/increase of pore size is of 40 μm between two consecutive pore sizes, as showed in Figure 4.

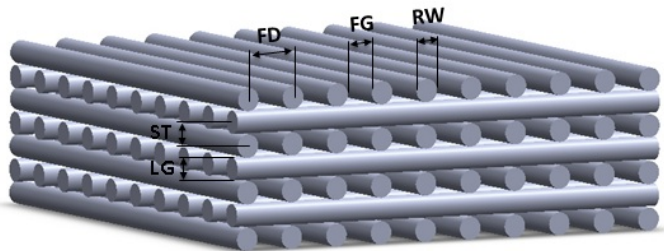


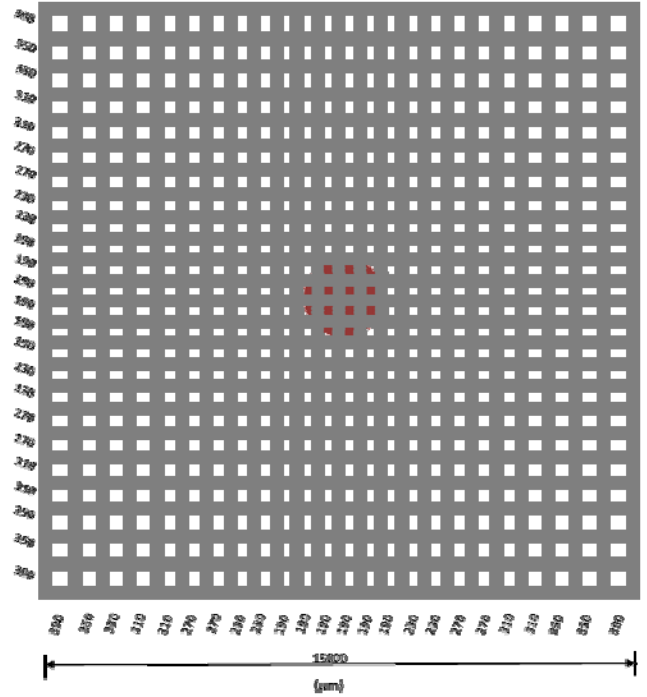
Figure 2 Scaffold parameters: fibre diameter (RW); slice thickness (ST); pore size (FG) (Viana et al, 2013).

2.2 Cell seeding on scaffolds

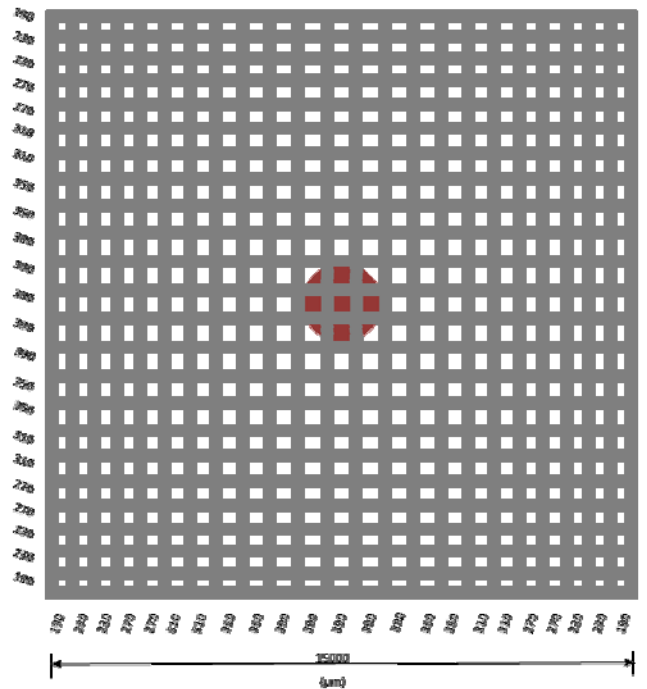
Human MSC obtained under donor consent from the bone marrow were cultured in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM, Gibco®), 10% fetal bovine serum (FBS, Hyclone®), and 1% penicillin/streptomycin (PS, Gibco®). Culture medium was replaced every 3 days.

PCL scaffolds were sterilized with alcohol and UV light overnight and placed in a low attachment 24-well plate (VWR). Cells (80000 cells/scaffold)

were placed on the top centre of the scaffold for both configurations in a region of no more than 2 mm diameter of a total of 15 mm scaffold square side, ensuring that the cells were seeded on a region with the same pore size (Figure 4). Cells were left to incubate for 1 hour in order to allow cell adhesion before the scaffold be submerge with media. Experiments were performed in static conditions in an incubator (37°C and 5% CO₂).



A



B

Figure 3 Schematic image of the pore size gradient variation in which A. represents the scaffolds with a smaller centre (190 μm) and B. scaffolds with a higher centre (390 μm). Red dots in both images represent the seeding place of the cells.

2.3 Adhesion and proliferation assay

Cell adhesion and proliferation was estimated indirectly using a commercially available kit Alamar-Blue™ (AB) (Alfagene), where resazurin is reduced to bright red–fluorescent resorufin by metabolic active cells. The original resazurin was diluted in Dulbecco’s modified Eagle’s medium (DMEM, Gibco®) with 10% fetal bovine serum (FBS, Hyclone®) solution (1:10) and cells were incubated for 2 hours with such solution. Florescence of the samples was measured using a multiplate fluorometer with excitation and emission wavelengths set-up to 560 nm and 590 nm, respectively. All AB assays were the result of three readings for each sample taken. Equivalent cells were estimated through a calibration curve prepared using different cell densities seeding in regular culture wells and correlating cell counts after 3 days against AB estimations. Three scaffolds of each configuration were seeded in parallel assays and indirect cell numbers estimated, every 3 days for a total 13 days.

3 RESULTS AND DISCUSSION

3.1 Production of scaffolds

Two different scaffold samples were produced (Figure 5), both with dimensions of 15 x 15 x 3 mm. Scaffolds are made of orthogonal PCL fibres of 0,300 mm diameter, therefore the pores can be assumed to be cuboids with square bases and constant high (in z axis) defined by the fibre diameter of 300 μm, which is similar throughout all the scaffold.

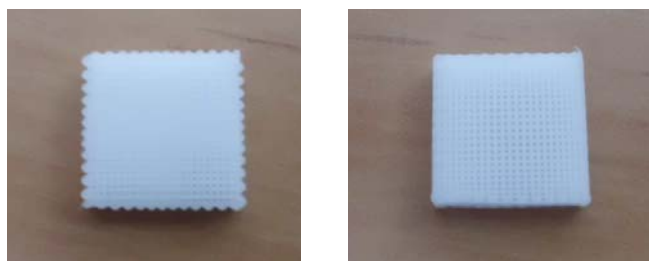


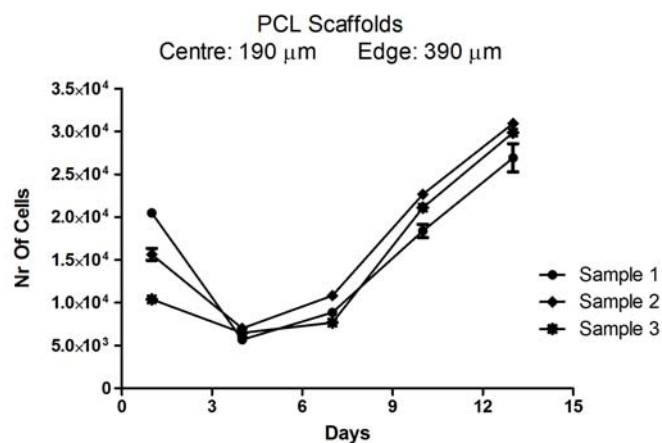
Figure 4 PCL scaffolds with 190-390 μm of pore size: A. 190 μm in the centre and 390 μm in the border; B. 390 μm in the centre and 190 μm in the border.

The basis (in x and y axis) of the pore ranges from squares with sides of 190 μm to 390 μm, with increments of about 40 μm in square sides size (Figure 4). Two configuration types of scaffolds were prepared with either pore bases increasing from the centre to the edge or from the edge to the centre. Figure 5A present a smaller pore size in the middle of the scaffolds (190 μm) increasing this value to the edges which present the highest pore size of 390 μm. On

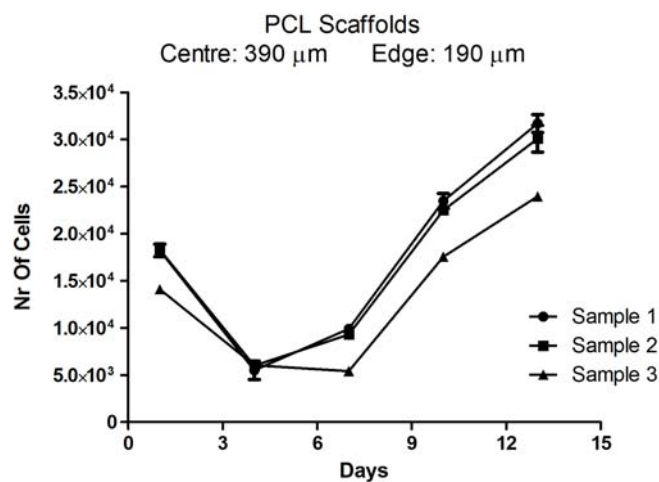
the other hand, Figure 5B present the opposite configuration, higher pore size in the middle (390 μm) which decreases to the edges until 190 μm.

3.2 Cell adhesion and proliferation assay

The results obtained are presented in Figure 6, where cell numbers are estimated indirectly as described before. Three samples of the two configurations were used.



A



B

Figure 5 Adhesion and proliferation assay of scaffolds with A. a smaller pore size at the centre; B. higher pore size at the centre.

Figure 6A shows the results of the scaffolds with a smaller pore size at the centre. It is possible to observe that the number of cells which adhering to scaffold are different (varying in about 30%). Despite this difference, in the three samples, until day 4, there is a decrease in cell number, and in fact this number at that time is similar for the three samples. Then, cell number started to increase at a similar rate in the three samples.

Figure 6B shows the results of the scaffolds with a higher pore size at the centre. Cell proliferation presented the same behaviour in both configurations.

The number of cells which adhered to the scaffolds was similar for the first two samples and slightly lower for the third one (less than 10%). As previously observed, at day 4 the cell number is similar to the three scaffolds samples and after this, that day cell numbers increase with similar proliferation rate, but with a delay for sample 3.

Comparing scaffolds with the two different configurations, we observed that they present the same pattern for both configuration concerning cell adhesion as well as its proliferation rate (Figure 7).

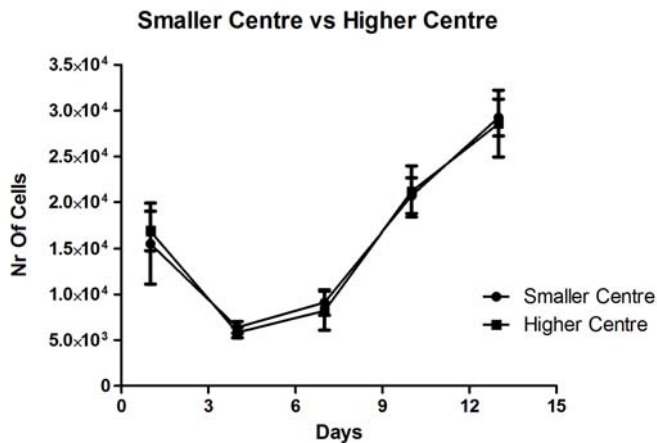


Figure 6 Comparison between cells adhesion and proliferation in scaffolds with two different configurations. Results are averages of the three assays presented in Figure 6.

At day 1 the number of cells was estimated to be about 15000 cells/scaffold, which means only 20% of adhesion. Using this number as indication of the cells that after seeding were able to successfully adhere to the scaffold, it is possible to observe a decrease to approximately 40% in cell number until day 4, most probably due to cells lost during the migration through the scaffolds. After this time point, and until the end of the experiment (day 13) we observed an increase in cell number. Taking into account the proliferation period of 9 days the cell population increased by 5 fold (Figure 8).

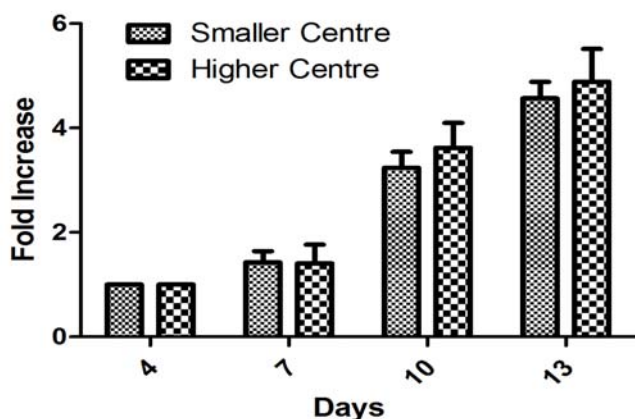


Figure 7 Proliferation rate of hMSCs in PCL Scaffolds with different pore sizes.

4 CONCLUSIONS

In this work PCL scaffolds with microfibrils orthogonally organised with spacing gradient were produced by an additive biomanufacturing process, the extrusion, and tested for adhesion, proliferation and distribution of BM hMSCs. Comparative results obtained show similar levels of adhesion and proliferation for both configurations, with fold increases of 5 after 13 days of cultivation. We concluded that adhesion of hMSC to PCL scaffolds is possible as well as its proliferation. The results in this study suggest that pore size do not pose a limitation to cell proliferation within the range studied (190-300 μm), since the final cell output is similar for both cases.

This work is expected to contribute to the understanding of biomaterial and cell interactions for tissue regeneration.

5 ACKNOWLEDGMENTS

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6 REFERENCES

- Bártolo, P.J., Kruth, J., Silva, J., Levy, G., Malshe, A., Rajurkar, K., Mitsuishi, M., Ciurana, J., & Leu, M. 2012. Bio-medical production of implants by additive electro-chemical and physical processes, *CIRP Annals - Manufacturing Technology* 61, 635–655.
- Chua, C.K., Leong, K.F. & Yeong, W.Y. 2005. Rapid Prototyping in Tissue Engineering: A State of the Art Report in *Virtual Modeling and Rapid Manufacturing*, Edited by P.J. Bártolo et al, Taylor&Francis.
- Crump, S. The extrusion process of fused deposition modeling. In: *Dayton (OH): Proceedings of the third international conference on rapid prototyping*. 1992. p. 91-102.
- Hao, L. & Harris, R. 2008, Customised Implants for Bone Replacement and Growth, in *Bio-Materials and Prototyping Applications in Medicine*, Edited by P.J. Bártolo & Bidanda, B, Springer.
- Janjanin, S., Li, W.-J., Morgan, M. T., Shanti, R. M. & Tuan, R. S. 2008. Mold-Shaped, Nanofiber Scaffold-Based Cartilage Engineering Using Human Mesenchymal Stem Cells and Bioreactor. *Journal of Surgical Research*, 149, 47-56.
- Langer, R. & Vacanti, J.P. 1993. Tissue engineering. *Science*, 260, 920-926.
- Melchels, F.P.W., Domingos, M.A.N., Klein, T.J., Malda, J., Bártolo, P.J. & Huttmacher, D.W., 2012. Additive manufacturing of tissues and organs. *Progress in Polymer Science* 37, 1079– 1104.

Monteiro, B. J.C., Guerreiro, J. D. T., dos Santos, F., Cabral, J.S.M., da Silva, C. L. & Ferreira, F. C., 2013, Study of the effects of electrospun poly(ϵ -caprolactone)/gelatin matrices on human mesenchymal stem cell culture, *IEEE 3rd Portuguese Meeting in Bioengineering* 1-6, ISBN: 978-1-4673-4859-1.

Mota, C. 2008. Bioextrusão para Engenharia de Tecidos. Master degree, Polytechnic Institute of Leiria.

Oh, S.H., Park, K., Kim, J.M. & Lee, J.H. 2007, In vitro and in vivo characteristics of PCL scaffolds with pore size gradient fabricated by a centrifugation method, *Biomaterials* 28, 1664–1671.

Pereira, R.F., Barrias, C.C., Granja, P.L. & Bártolo, P.J. 2013, Advanced biofabrication strategies for skin regeneration and repair, *Nanomedicine* 8(4), 603–621.

Skalak, R. & Fox, C.F. 1988. Tissue engineering, Alan R. Liss, New York.

Viana, T., Biscaia, S., Almeida, H. A. & Bártolo, P. J. 2013. Permeability Evaluation of Lay-down Patterns and Pore Size of PCL Scaffolds. *Procedia Engineering* 59: 255 – 262.