



4th International Conference on Tissue Engineering, ICTE2015

Combination of 3D extruded-based poly (ϵ -caprolactone) scaffolds with Mesenchymal Stem/Stromal Cells: strategy optimization

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Abstract

This study uses a combination of 3D extruded-based biodegradable scaffolds and human bone marrow-derived mesenchymal stem/stromal cells to understand how scaffold properties can contribute for efficient cell seeding, proliferation and migration. Highly interconnected porous poly(ϵ -caprolactone) scaffolds were produced by a layer-by-layer extrusion. Two configurations were studied with larger (390 μm) or smaller pore size (190 μm) at the centre. MSC, seeded on the top centre of the scaffold surface, increased 5 folds after 13 days of cultivation for both configurations, allowing us to conclude that in this range of pore sizes pose no limitations for cell migration are observed.

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Peer-review under responsibility of IDMEC-IST

Keywords: Mesenchymal Stem/Stromal Cells; Poly (ϵ -caprolactone); Scaffolds; Tissue Engineering.

1. Introduction

Tissue Engineering (TE) is a multidisciplinary field that uses the combination of materials and cells in order to regenerate repair or restore the functions of tissues. This field was first defined by Langer and Vacanti in 1993 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” [1]. The materials used as support

matrices should fulfil some biological and mechanical requirements, such as biocompatibility, biodegradability, high porosity and interconnectivity, be able to provide biochemical recognition elements, present adequate mechanical properties and superficial finishing and present a good environment for cell adhesion and proliferation [2-4].

In this work we studied poly(ϵ -caprolactone) (PCL) scaffolds produced by an additive manufacturing process called extrusion [5,6]. In this technique, thin thermoplastic filaments are melted by heating and guided by a robotic device controlled by a computer, to form the 3D matrix. 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 structure [7]. These scaffolds were assessed for cell adhesion, proliferation and migration along the whole scaffold using human mesenchymal stem/stromal cells (MSC). MSC are adult tissue-derived multipotent cells with the capacity to differentiate into cells of different lineages, namely osteogenic, chondrogenic, adipogenic, and myogenic [8]. These cells 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) [9]. A major concern on the development of effective strategies that combine the use of stem cells and porous scaffolds is to ensure efficient seeding of the scaffold with cells and subsequently effective cell adhesion and proliferation to cover representative regions of the scaffold. The information available in the literature concerning the adequate pore size to promote effective cell seeding is not consensual [10], therefore the current study is focused on assessing if there is limitations for cell migration and proliferation within the range of pore sizes from 190 to 390 μm .

2. Materials and Methods

2.1. Production of scaffolds

Poly (ϵ -caprolactone) (PCL), Mw 50,000 Da, was used to the production of scaffolds using the Bioextruder (Fig. 1a) [11], based on the extrusion process, an additive biomanufacturing process. In this process, the material is heated until the melting temperature and then is extruded by a nozzle following a layer-by-layer approach.

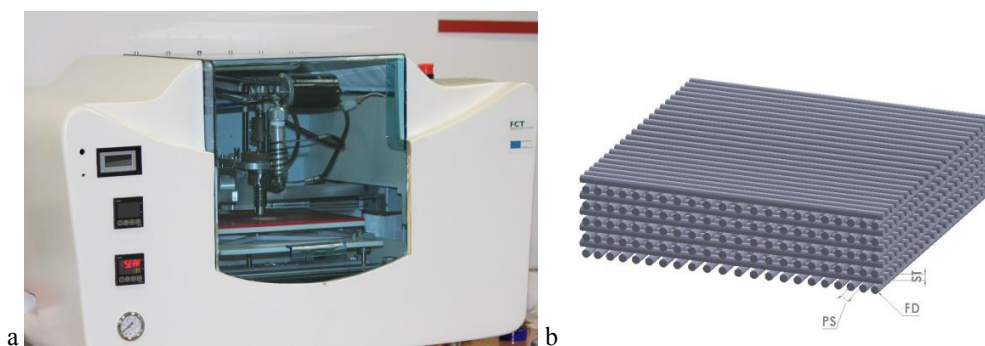


Fig. 1. (a) Bioextruder machine; (b) Scaffold parameters: fibre diameter (FD); slice thickness (ST); pore size (PS).

In order to produce the matrices (scaffolds), the following properties of the machine were adjusted to obtain an effective extrusion of the PCL with the following parameters: temperature 80°C; deposition velocity 8 mm/s; rotation velocity 22.5 rpm, slice thickness (ST) 280 μm ; and nozzle diameter 300 μm (corresponding to the fibre diameter (FD) obtained (Fig. 1b)).

Different samples were produced in order to evaluate the cell behaviour in terms of cell adhesion, proliferation and migration. Samples produced for this assessment presented pore size variations, between 190-390 μm , following two different configurations: (a) pore size increases from the centre to the edge or (b) pore size decreases from the centre to the edge. This increase/decrease between fibres is of 40 μm and it takes place every two pores in each side of the scaffold. The exception is the centre of the scaffold, which comprises a region of 2 mm with fibres equally spaced.

2.2. Cell seeding and culture in scaffolds

Human BM-derived MSC were recovered from cryopreservation [12] and cultured in culture medium consisting of low-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco®), 10% fetal bovine serum (FBS, Hyclone®), and 1% penicillin/streptomycin and fungizone (PS, Gibco®). Culture medium was replaced every 3 days.

PCL scaffolds were sterilized with 70% ethanol (v/v) (Merck) and UV light overnight and placed on an ultra-low attachment 24-well plate (VWR).

For assessment of the pore size influence in cell behaviour in terms of migration, cells (8.0×10^4 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 2). Cells were left to incubate for 1 hour in order to allow cell adhesion; then culture medium was added to immerse the scaffold. Experiments were performed under static conditions in an incubator under controlled atmospheric conditions (37°C, 5% CO₂ and 20% O₂).

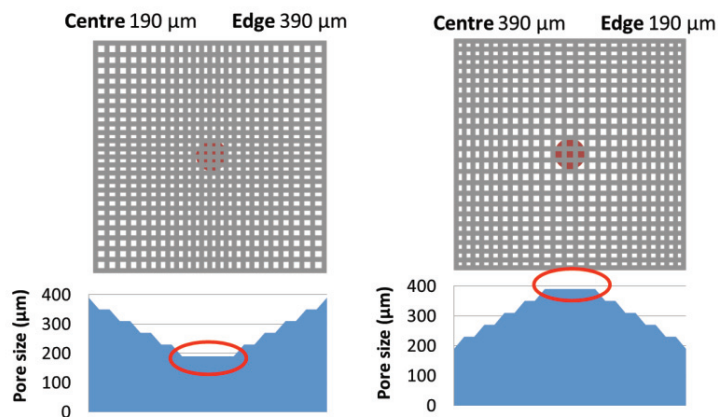


Fig. 2. Schematic image of the pore size gradient variation. Red dots represent the seeding place of the cells.

Cell adhesion and proliferation was estimated using an indirect method, Alamar-Blue™ (AB) (Invitrogen), where Resazurin is reduced to the bright red–fluorescent Resorufin form by metabolic active cells. AB solution was diluted in DMEM+10%FBS solution (1:10) and cells were incubated in this solution for 2 hours. Fluorescence of the samples was measured using a multiplate fluorometer (Infinite 200 PRO, TECAN) with excitation and emission wavelengths set-up to 560 nm and 590 nm, respectively. Three readings for each sample were taken. Equivalent cells number are estimated through a calibration curve that correlates cell counts after 3 days against AB estimations. Three scaffolds of each configuration were seeded in parallel assays and cell numbers estimated every 3 days during 13 days.

2.3. Cell Migration Assessment

4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich®) and Rhodamine Phalloidin (Sigma-Aldrich®) staining was performed to assess cell migration along the scaffold. DAPI labels the nuclear DNA and Rhodamine Phalloidin binds to cell actin filaments.

After 13 days of culture, culture medium was discharged and cells were rinsed with phosphate buffered saline (PBS) (Gibco). Afterwards those cells were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich®) during 20 min

at room temperature (RT). Then, a solution of Triton X and PBS (1 µg/mL) was used for 10min, followed by 45 min in dark of Rhodamine Phalloidin with PBS (1 µg/mL). Samples were then washed with Triton X and PBS again and 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich®) (1,5 µg/mL) was added for 5 min. After discarding the DAPI solution, the samples were submerged in PBS and photographs were taken using an inverted fluorescence microscope (LEICA® DMI 3000B, Germany).

2.4. Statistical analysis

Statistical analysis was performed using the statistical analysis features of Microsoft Excel and GraphPad Prism.

3. Results and Discussion

Different samples of scaffolds were produced (Fig. 3). Samples present dimensions of 15 x 15 x 3 mm with different pore sizes: (a) increase from the centre to the edge and (b) decrease from the centre to the edge. All scaffolds are made of PCL fibres with 300 µm diameter. 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 every two pores in each side of the scaffold. This increment scheme is valid for all scaffold regions except for its centre, which comprises a region of 2 mm with fibres equally spaced. These samples were produced to assess pore size limitations to cell migration along the matrices.

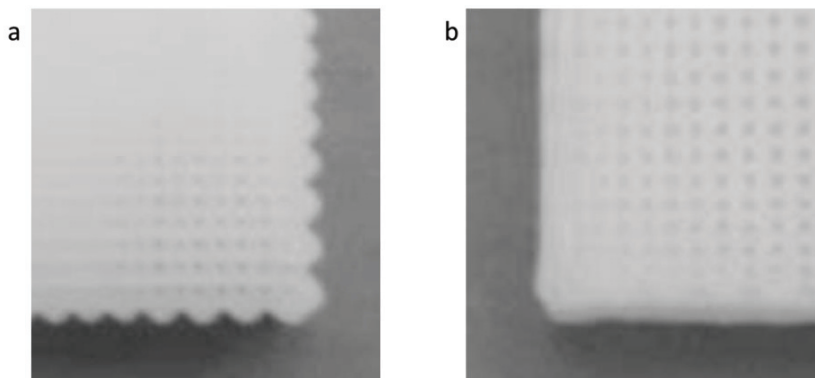


Fig. 3 PCL scaffolds produced by extrusion. Different pore sizes: (a) 190 µm in the centre and 390 µm in the edge; (b) 390 µm in the centre and 190 µm in the edge.

Results for cell adhesion and proliferation are presented in Fig. 4, where cell numbers are estimated indirectly as described in Materials and Methods in biological triplicates (from the same donor). Each experiment is represented as an independent bar.

Fig. 4a shows the results obtained with scaffolds with a smaller pore size at the centre ($P < 0.0001$). According to the results presented, there is a difference between biological triplicates of about 30% in cell adhesion. However, at day 4 of culture, all the triplicated samples tend to decrease in cell numbers converging to the same cell number. After this point and until the end of the experiment there is an increase in cell number in a similar profile for all samples. This period corresponds to a proliferation phase.

Fig. 4b shows the results obtained for the other configurations of scaffolds studied, with a higher pore size at the centre ($P < 0.0001$). Here, cell adhesion and proliferation presented the same behaviour as in the previous

configuration and between triplicates despite a slightly variation in the third sample which presents a small delay in the start of the proliferation phase.

Cell Adhesion and Proliferation

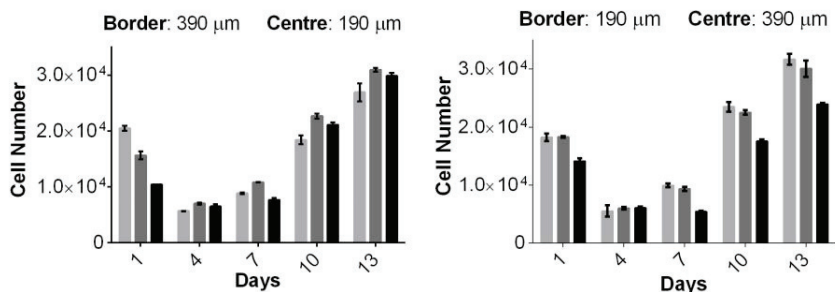


Fig. 4. Adhesion and proliferation assay of scaffolds with (a) smaller pore size at the centre; (b) higher pore size at the centre. Each color bar represents a sample of biological triplicates (from the same donor).

Cell cultures carried out in the two different configurations present the same cell adhesion pattern, as well as proliferation rate ($P < 0.0001$). At day 1, the number of cells was estimated to be 1.5×10^4 cells/scaffold, approximately, which represents $\sim 20\%$ of adhesion. This low value of cell adhesion follows values previously reported in the literature at the range of 15%. After seeding, a decrease of approximately 40% in cell number until day 4 is observed; after this time point, and until the end of the experiment (day 13), we observed an increase in cell number, which corresponds to an increase in cell number of 5 folds for the 9 days of proliferation phase.

Images obtained from DAPI and Rhodamine Phalloidin staining (Fig. 5) corroborates the previous results showing that cells are able to migrate along the whole scaffold, and that the range of pore size used do not pose a limitation for cell migration. Moreover, these results show that cells adhesion is effective with cells stretching themselves along the PCL fibres.

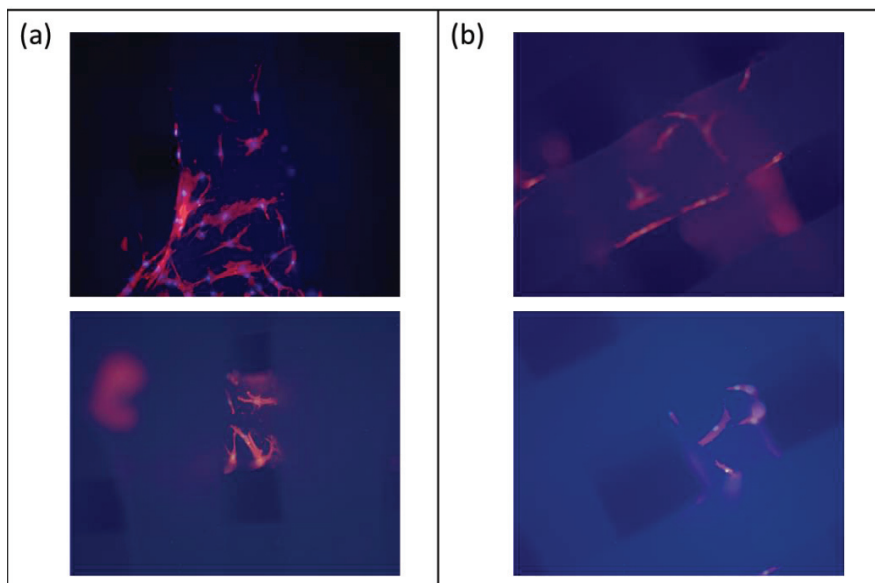


Fig. 5 Scaffolds with DAPI and Rhodamine Phalloidin staining, (a) 190 μm of pore size; (b) 390 μm of pore size.

4. Conclusions

In this work PCL scaffolds with a spacing gradient were produced by a layer-by-layer technique, FDM or extrusion, and tested for adhesion, proliferation and distribution of human BM MSC. This work shows successful adhesion and proliferation of MSC to/in the developed scaffolds. Comparative results obtained show similar levels of adhesion and proliferation for both configurations, with fold increase values of 5 in terms of total cell number after 13 days of cultivation. Since similar cell numbers were obtained in both scaffold configurations assessed, this study suggests that this range of pore size (190-300 μm) does not pose a limitation to cell proliferation and migration for these cells. Further studies are needed targeting the study of altering scaffolds configurations and properties to obtain a better understanding of cell behaviour when in contact with different environments *ex-vivo*.

Acknowledgements

This research is sponsored by the Portuguese Foundation for Science and Technology through SFRH/BD/73970/2010, IF/00442/2012 and MIT Portugal Program, Bioengineering Systems Focus Area.

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