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Production of Polycaprolactone Microcarriers using Electrospray for Fibroblast Cultures

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In order to culture anchorage-dependent cells in bioreactors, is required to produce microcarriers on which cells can adhere and proliferate. Current tissue engineering studies have introduced electrospray as a technique to manufacture nano and micro size structures. Since the material employed to build these structures has to display biocompatibility, poly-ε-caprolactone was utilized as it is a biopolymer that shows a good response by the host cells. This research focused on developing poly-ε-caprolactone microcarriers by using electrospray technique evaluating the diameter, porosity, and cytotoxicity thereof. Microcarriers were yielded at two chloroform concentrations, flows, and distances respectively from a syringe to a collector. Every test performed was carried out at 8 kV. The best findings were at both a concentration of 8 % w/v, flow of 2.0 mL/h, and diameter range from 79.39 µm to 143.54 µm (mean ~~at~~ of 112.39 µm). The scanning electronic microscopy runs depict low porosity, which is an essential characteristic in order to form an appropriated surface for the cellular adhesion and proliferation in spinner bioreactors. The cytotoxicity assays did not exhibit adverse effects on the cells when commercial fibroblast murine was employed. These findings allow carrying on the edge-cutting development of human fibroblasts cultured in spinner reactors.

1. Introduction

Currently, impairment and subsequent loss of organs and tissues is a worldwide public health issue. The development of tissue engineering and biomaterials field has allowed obtaining different materials for the scaffolds fabrication (Hutmacher, 2001; Williams, 2008). Scaffolds are essential because they adhere and proliferate onto anchorage-dependent cells. Traditionally, scaffolds have been fabricated as capsules, and cells proliferate under their surface until covering the totality of available superficial area (Hutmacher, 2001). Nevertheless, this alternative does not guarantee the oxygen and nutrients transportation towards the cells inside of the scaffold. One solution to this problem is the development of microcarriers and employment of bioreactors (Leong and Wang, 2015).

Microcarriers are employed in tissues engineering (Leong and Wang, 2015; Malagón-Romero et al., 2015) as containers to culture monoclonal antibodies or vaccines (Kiremit et al., 1989). They are microspheres made of different materials on which cells adhere and proliferate covering the entire surface (Malagón-Romero et al., 2015). Several materials are employed for their fabrication such as polymers (Shivakumar et al., 1989) (dextran, polystyrene, polyacrylamide, polyacroleine, polymetacrylate), glass (alone or covered with plastics o polystyrene), gelatin (Drury and Mooney, 2003; Rungsiyanont et al., 2012) (alone or cover with dextran) or collagen (Freshney, 2011; Karimian et al., 2016). The diameter range of microcarriers is 100-400 µm (Karimian et al., 2016), offering a specific surface area from 380 cm^2/g to 440 cm^2/g with a density of 1.02-1.10 g/mL (Malda and Frondoza, 2006). Thus, depending on the size and density of the microspheres by using microcarriers, it is possible to increase its surface-to-volume ratio to 90,000 cm^2/L, (Freshney, 2011).

In this manuscript, poly-ε-caprolactone (PCL) was selected to engineer microcarriers. This polymer, which has been increasingly employed in the last years (Dash and Konkimalla, 2012; Woodruff and Hutmacher, 2010), is an aliphatic and semi-crystalline polyester with a boiling point of 59-64 ºC and with a glass transition temperature near to –60 ºC (Abedalwafa et al., 2013). Further, PCL is used for fabrication of biodegradable

wound dressing because it has biocompatibility and biodegradability characteristics (Chen et al., 2000; Labet and Thielemans, 2009).
Different methods have been reported for fabrication of microcarriers, such as the reported by Wang et al. (2008) and Gorodetsky et al. (1999) who employed water in oil emulsions with gellan gum and fibrinogen respectively. Kwon and Peng (2002) synthesized microcarriers by dripping propylene glycol alginate solution onto calcium chloride. Malagón et al. (2015) fabricated microcarriers from a mixture of calcium alginate and fibrinogen present in human blood plasma. Among the researchers who work with PCL, Zhou et al. (2016), Enayati et al. (2010) and Bock et al. (2011) reported the fabrication of microcarriers synthesis by electrospray.
The aim of this research regards the experimental conditions to obtain microcarriers by employing PCL through electrospray technique which will be employed in spinner reactors for culturing human cells.

2. Experimental procedures

2.1 Preparation of solutions.

Chloroform has been reported as an excellent solvent for PCL, taking 2 h for dissolution of a PCL solution concentration of 10 % (Georgiadou et al., 2015). Therefore, PCL (Aldrich with a molecular weight Mn=80,000) was dissolved in chloroform (Merck-CAS 67-66- 3) at two concentrations. For increasing the solubilization of the polymer into a solvent, solutions were subjected to ultrasound in a sonicator (ATU- ATM40-2LCD) with a frequency of 50 Hz for 10 minutes at room temperature (17 °C). Then, solutions were stored at 5 °C until the development of the tests during one week, according to experimental design (Table 1). The viscosity of each solution was measured in a rotational viscometer (Cannon LV Model 2020) employing a spindle at room temperature (17°C).

2.2 Production of microcarriers.

The microcarriers were obtained in an electrospray equipment that works with a 9 W power source and has a voltage capacity of 30 kV (CZE1000R, Spellman). Every test was developed at 8 kV. Additionally, the equipment is composed of a syringe pump (KDS100), a high voltage probe (HVP-40DM, Pintek), a nozzle, and a collector. The distance between nozzle and collector was modified according to the respective test (Table 1). In the collector, ethanol (Sharlau, 99 %, CAS N° 64-17- 5) was employed to gather the microcarriers. Microspheres obtained in the electrospray equipment were washed with Hank´s isotonic sterile solution and resuspended in it.
The microcarrier sterilization was carried out by applying ultraviolet light for 1 h in a laminar flow cabinet. Then, microspheres were washed twice with a sterile phosphate-buffered saline solution to eliminate residual ethanol. Finally, microcarriers were suspended in a Dulbecco's modified Eagles's medium (DMEM) and maintained at 37 °C for 24 h.

Table 1: Experimental Design used for microcarriers engineering

Test	Flow (mL/h)	Distance from nozzle to collector (cm)	Concentration (% w/v)
A	2.5	30	7
B	2.5	25	7
C	2.5	30	9
D	2.5	25	9
E	2.0	30	7
F	2.0	25	7
G	2.0	30	9
H	2.0	25	9

2.3 Analysis of microcarriers.

A sample of the microspheres was fixed onto a microscope slide and later analyzed by an inverted microscope equipped with a camera. 5 photos with 10 X magnification were taken for each test. By using the free software ImageJ (https://imagej.nih.gov/ij/; Schindelin et al., 2012; Schindelin et al., 2015; Schneider et al., 2012), the microcarrier diameter distribution was obtained for calculating the mean and the standard deviation. Another sample of microcarriers was dehydrated in a graded series of ethanol (50 %, 70 %, 90 % and 100 %). Once the microcarriers were suspended in absolute ethanol, were drying in a desiccator for 24 h.

Samples were sputter coated with gold (108 Auto/SE 7008-220 Sputter Coater) for 40 s and examined in Scanning Electronic Microscopy (SEM) (Phenom ProX, Switzerland).

2.3 Determination of cytotoxicity

Cytotoxicity evaluation employed commercial murine fibroblasts (CRL 476). Fibroblasts were cultured in 96-well microculture plates. The microcarrier concentration was determined by counting the number of microspheres present in the taken photos. Concentration was adjusted to 6 cm^2/mL in the DMEM, according to ISO 10993-12. Solutions were kept in an incubator at 37 °C for 24 h to guarantee extraction of components from the material. Each well was loaded with different extract volumes (10 μL, 20 μL, and 30 μL) with a micropippete. Control assays were cells without extract. Viability was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 24, 48 and 72 h. 10 μl of MTT was added to each well and was incubated for 4 h at 37 °C. The supernatant was removed and formazan crystals were extracted with 200 μl of dimethyl sulfoxide (DMSO). Absorbance was determined for each well at 570 nm in an ELISA equipment. Then, cellular viability was calculated for each well vs negative control (cells without extract).

3. Results and Analysis

Viscosity obtained was 93 cP and 490 cP for 7 % w/v and 9 % w/v solution concentrations respectively. This result expresses an increment of the viscosity as solution concentration increases. Viscosity is an important parameter for classification of the solutions, so rheological characteristics of the solution (dilute, non-entangled semi-dilute, and entangled semi-dilute) determine the production of spheres or fibers (Zhou et al., 2016); these regimens are the result of the combination of both molecular weight and concentration. For obtaining spheres with the electrospray technique, is necessary to guarantee a semi-dilute entangled (Bock et al., 2011). This regime is a key characteristic of the electrospinning and electrospray techniques difference. In the electrospray technique, PCL has to be in solution and then it is discharged through the nozzle of the electrospray. The combination of both solution concentration and electric field allows the particle diameter (Maeng et al., 2009; Xie et al., 2015).

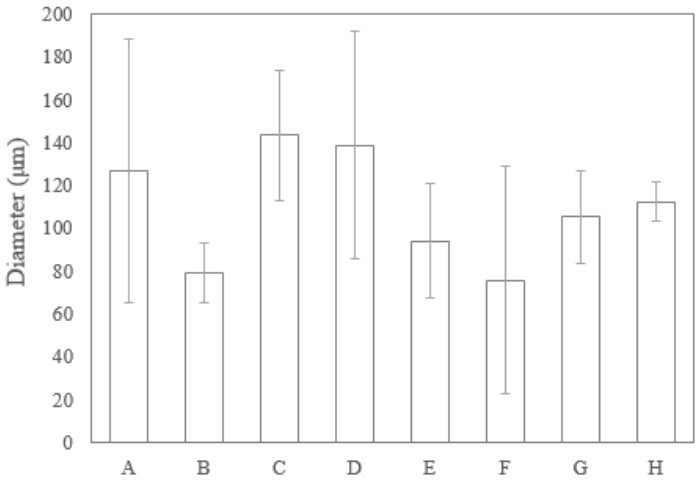

Figure 1: Diameter of the obtained PCL microcarriers for each test.

Diameter mean for each test is presented in Figure 1. The flow rate increase does not affect the PCL microcarrier diameter considerably. This result is different the reported one by Meng et al. (2009), the authors of the current manuscript state it is due to a small change in the flow rate (2.0 mL/h and 2.5 mL/h). On the other hand, PCL solution concentration affects the diameter, so higher concentrations mean larger diameters. Bock et al. (2011) published that particles have a spherical morphology when solutions with high concentrations (about 9-10 % w/v) are employed. The results obtained in this manuscript agree with that study. Finally, the distance from nozzle to collector do not evidence effect on the diameter of the microspheres. Literature reports that is possible to obtain PCL microspheres by working in a nozzle-collector distance in the range of 20-25 cm for a flow rate range 0.2 mL/h - 0.5 mL/h (Zhou et al., 2016), so in this study was confirmed this experimental information because we employed distances in the range 25-30 cm.

In the microcarriers yield is relevant avoid the generation of microfibers. Zhou et al. (2016) reported that for avoiding the formation of fibers, a high concentration of polymer solution must be guaranteed to lead to a reduction of the molecular weight (*sine qua non* conditions for a regime semi-dilute entangled). Results obtained in SEM are presented in Figure 2, and they allow to conclude that both performed solution concentrations with a molecular weight of Mn=80,000 guarantee an entangled semi-dilute regime, because every test presented the predominant spherical over fiber shape.

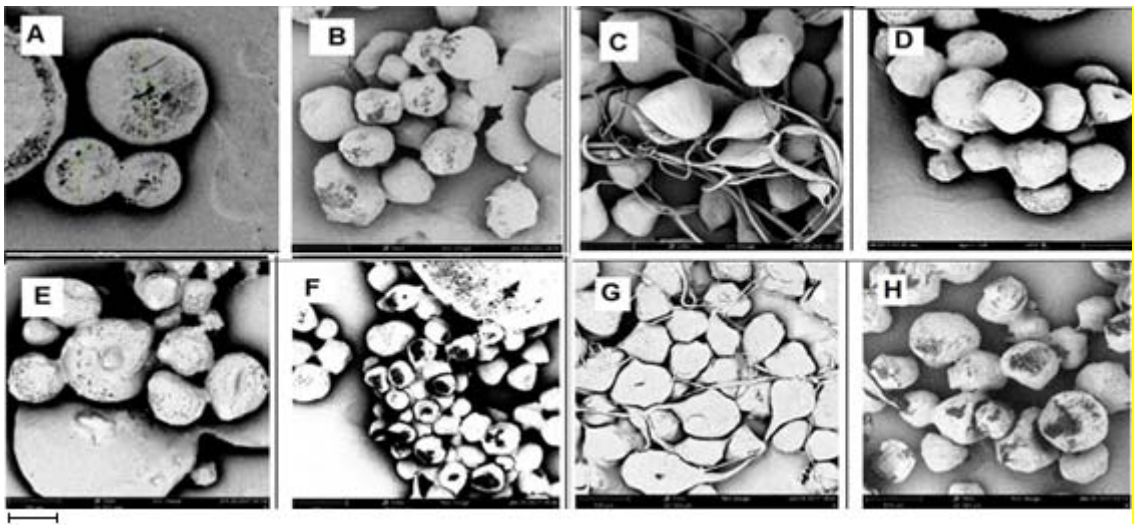

Figure 2: Diameter of the obtained PCL microcarriers for each test. (the scale sing down the figure is for 100 µm)

Additionally, evaporation of chloroform produced a rough surface in the microcarriers, allowing a better cell adhesion. Zhou et al. (2016) reported that concentration of solution has a greater effect on the formation and morphology of the particles. C and G tests presented the formation of the microcarriers and the generation of some fibers. These tests correspond to a nozzle-collector distance of 30 cm and a concentration of 9 % w/v with a flow of 2.5 mL/h (C) and 2.0 mL/h (D). From these tests is possible to conclude as formation of fibers increases, PCL solution concentration increases. Similarly, an increment in the nozzle-collector distance generates fibers formation. This result is similar to the reported by Zhou et al. (2016).
For the E and F tests, particles have finished joining together, forming a bunch. In the other tests (A, B, D, y H, see Figure 1), microparticles are in the expected range for the diameter which is from 90 to 300 µm (Freshney, 2011). From the obtained results, H test was chosen as the best (9 % w/v, 2 mL/h, 25 cm of distance and 8 kV) because the diameter was 112.39 µm and it presented the lowest standard deviation (9.17 µm).
Recuperation of the microcarriers was developed in absolute ethanol for avoiding bunch formation. Zhou et al. (2016) have reported some strategies employed while recollection of the microparticles. It is common to employee water, air or metallic springs. Wu and Clark (2007) reported the employment of water for recovering porous particles when a PCL was dissolved in a mixture of chloroform and acetone. In this manuscript, ethanol was employed due to low density compared with polycaprolactone. Thus, once the particle was in contact with the solvent, precipitated into the solvent due to the high solubility of chloroform in ethanol. Another solvent evaluated was water, but microparticles were less dense than that of water, so the particles would float on the water surface and form bunches. This alternative was appropriated for collecting microparticles. However, some particles have finished in a bunch. In future works, other alternatives for collecting will be evaluated, similar to reported by Zhou et al. (2016).

4. Cytotoxicity assessment

Results for the cell viability are presented in Table 2 for Microcarriers obtained in the H-test conditions. According to table data, the microcarriers are not toxic to cells. Additionally, the assay was developed during 72 h and microcarriers were stables and did not present changes in their morphology. This is important for the culture in bioreactors where the stability for a long time is necessary (longer than one week). This behavior is similar to reported by Bock et al. (2011) who met no toxic residues while culturing NIH3T3 fibroblast in contact with microspheres of PCL.

Table 2: Cell viability for murine fibroblasts cultured on PCL microcarriers in DMEM.

Time (h)	Volume of eluded 10 µL	Volume of eluded 20 µL	Volume of eluded 30 µL
24	89.32 ± 11.98	118.0 ± 14.82	117.1 ± 8.410
48	100.4 ± 1.230	84.00 ± 0.3200	92.10 ± 0.3700
72	92.29 ± 30.03	167.1 ± 75.97	103.6 ± 3.330

5. Conclusions

A good design and building of scaffold is a key issue for cellular culture. The technique employed to engineer the scaffolds must take into consideration the material employed because it needs to have biocompatibility and biodegradability characteristics. This research studied some favorable conditions in order to obtain microcarriers of poly-ε-caprolactone by using the electrospray technique. Results evidence the relationship between diameter as a function of flow rate, concentration, and collection distance.

According to the results, the collection distance did not prove to be a determining factor for the production of microspheres of a certain size, as demonstrated by the concentration and the flow rate of PCL solutions, which have a direct relationship. In other research were find out the relationship between the voltage applied to the particles and the shape, and also the size and porosity of the particles respectively.

As engineered microcarriers did not present toxicity and were stable into the culture medium, this work evidenced the possibility to obtain microparticles that could be employed for culturing anchorage-dependent cells in spinner bioreactors.

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