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Electrospinning parameters selection to manufacture polycaprolactone scaffolds for three-dimensional breast cancer cell culture and enrichment

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Abstract

Only a small cell population of a breast tumour presents stem cell characteristics, thus so-called breast cancer stem cells (BCSCs). BCSCs are tumour-initiating cells and chemoresistant and they can grow in non-adherent conditions as mammospheres. Study of BCSCs is a challenge due to their low representation and the inability to propagate them without inducing differentiation. Previous studies have demonstrated that three-dimensional (3D) cell culture models such as scaffolds enhance the BCSCs population.
In this project, scaffolds were fabricated by electrospinning technology. Specific values of voltage and flow rate were fixed to electrospun 7.5 and 15% poly(ε-caprolactone) (PCL) solutions according to process stability. Both scaffolds were seeded with MCF-7 breast cancer cells and 7.5% meshes displayed lower cell proliferation compared with 15% scaffolds. Cells cultured in both scaffolds presented a significant Mammosphere Forming Index (MFI) increase, thus indicating a BCSCs enrichment.
Results show that three-dimensional cell culture with electrospun 15% PCL scaffolds could be useful to expand BCSCs population facilitating the future development of new therapeutic strategies against this tumour subpopulation.

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Keywords: Electrospinning; process parameters; PCL; scaffolds; cell culture

1. Introduction

Breast cancer is the most common tumour diagnosed among women excluding skin cancer. It has been estimated to account for the 28.3% of all new female carcinomas and the 16.8% of female cancer deaths in Europe during 2012 [1]. In addition, tumour relapse occurs in 30% of early-stage breast cancer [2] and it stands for the main cause of deaths related to mammary tumours [3].

Different studies have focused on understanding the heterogeneous nature of tumours, such as breast cancer. It is demonstrated that a small cell population is responsible for tumour initiation and progression. These cells posses several characteristics similar to mammary stem cells, thus termed Breast Cancer Stem Cells (BCSCs) [4,5]. This subpopulation has the ability to undergo self-renewal and differentiate into non-stem breast cancer cells. Furthermore, they are capable of growing in suspension and surviving as non-adherent spheres, termed mamospheres [6,7]. BCSCs can be identified and isolated due to their expression pattern of cells' surface markers such as CD44 and CD24 [5], and their increased activity of the aldehyde dehydrogenase (ALDH) enzyme [8] . Different investigations revealed their radio- [9] and chemoresistance [10] favouring tumour recurrence and metastasis [11]. Thereby, their study is essential to identify new therapeutic targets against cancer stem cell population. However, it has been limited by the inability to propagate them without inducing their differentiation [12] and their low representation within the tumour [5] or cell line [13].

Traditionally, cancer cell culture is performed within two-dimensional (2D) surroundings, providing a quick and cheap way to study their properties *in vitro*. In contrast, 2D systems differ from the *in vivo* environment and cells can only growth in monolayer. Cytoskeleton remodelling caused by cell

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flattening modifies the gene expression and protein [14,15]. Therefore, experiments performed in 2D can not be representative of the physiological cell behaviour leading to, for example, CSCs differentiation [16]. Three-dimensional (3D) cell models simulate the extracellular matrix and maintain the tissue organization. One of the 3D culture systems are the scaffolds, three-dimensional structures mostly made of biopolymeric material such as poly(ε-caprolactone) (PCL). Cells can establish interactions with polymeric filaments and with adjacent cells, maintaining their *in vivo* function and increasing their intracellular signalling. Previous studies demonstrated that 3D culture with scaffolds produced a BCSCs increase [17–19].

Different methodologies are used to produce scaffolds. The Fused Filament Fabrication (FFF) offers a simple and viable way to manufacture these structures. This additive manufacturing technology, widely used by 3D printers [20] and prototypes [21,22], consists on the deposition of the fused material in successive layers. With this procedure, micrometric filaments can be achieved but production of thinner fibres is problematic. Electrospinning is a technology capable of producing nanometric filaments, also used as drug delivery systems [23]. In this technique, polymer is dissolved and placed in a syringe connected to a metallic needle. A high voltage is applied to the biopolymer fluid so the solution droplet becomes charged. When the electric force overcomes the surface tension, a stream of liquid is ejected. This charged structure, called Taylor cone, enables de continuous production of polymer filaments from the needle to the ground collector. When this process is done, the solvent evaporates and nanofibres are randomly formed in the collector.

A preceding study of our research group showed a BCSCs expansion after 3D culture with FFF scaffolds [24–26] and other groups demonstrated this enrichment using electrospun scaffolds [17–19]. The present study has focused on the characterization of electrospinning process and produced PCL scaffolds. Different scaffolds and culture conditions have been tested to improve three-dimensional breast cancer cell proliferation. The final objective of this work is to evaluate the impact of 3D cell culture on BCSCs population. According to the background, three-dimensional cell culture with electrospun scaffolds can be a useful way to enrich BCSCs for developing new therapeutic strategies to target this malignant population.

2. Materials and Methods

2.1. Scaffolds Manufacture

Poly(ε-caprolactone) (PCL; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in acetone (PanReac AppliChem, Gatersleben, Germany) to obtain 7.5 and 15% w/v PCL concentrations. An electrospinning apparatus (Spraybase, Dublin, Ireland) connected to a 18G needle was used to produce scaffolds. PCL solution was supplied to the emitter with a flow rate controlled by the Syringe Pump Pro software (New Era Pump Systems, Farmingdale, NY, USA) and different voltages were applied. Distance between emitter and collector was fixed at 15 cm. Room relative humidity and temperature were fixed at 55-60% and 20ºC, respectively, to avoid solvent evaporation. Emitter tip images were captured with a Chamaleon camera (Point Grey Research, Richmond, BC, Canada). Process was stopped when 5 mL of solution were ejected. Then scaffolds were cut into squares of 16 mm to allow their use in 12-wells cell culture microplates.

2.2. Scaffolds Characterization

Scaffolds weight and thickness were measured with an analytical balance (Sartorius, Göttingen, Germany) and a digital micrometer (Mahr GmbH, Göttingen, Germany), respectively. Six specimens from three different batches were evaluated to calculate the average value.

2.3. Cell Line

MCF-7 breast cancer cell line was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, 50 U/mL penicillin, and 50 µg/mL streptomycin (HyClone, Logan, UT, USA). Cells were maintained in a 5% CO_2 humidified incubator at 37ºC.

2.4. Three-Dimensional Cell Culture

Scaffolds were sterilized with 70% ethanol/water solution overnight, washed with PBS (Gibco) and exposed to UV light for 30 min. Then scaffolds were placed in 12-well non-adherent cell culture microplates (Sartstedt, Nümbrecht, Germany). Cell densities of 50 000, 100 000 and 300 000 cells/well were prepared in 50 µL volume. Cell suspension was pipetted onto the scaffold centre. After 3 hours' incubation, 1.5 mL of medium was added. Two-dimensional controls with same cell densities were performed.

2.5. Cell Proliferation Assay

The (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (MTT) assay was used to test cell proliferation. MTT is a yellow tetrazolium salt which can be reduced by metabolically active cells resulting in water-insoluble purple crystals of formazan. The formazan crystals can be solubilized by dimethyl sulfoxide (DMSO) into a colored solution. Therefore, the absorbance of formazan solution is directly related to the initial cell amount.

Thus, scaffolds were washed with PBS and put into new wells. They were incubated with 1 mL medium and 100 µL MTT (Sigma-Aldrich) for 2 h 30 min. Formazan crystals were dissolved with 1 mL DMSO. Four 100 µL aliquots from each well were pipetted into a 96-well plate and placed into a microplate reader (Bio-Rad, Hercules, CA, USA). Absorbance was measured at 570 nm.

2.6. Mammosphere-Forming Assay

Cells from 2D culture and scaffolds were trypsinized and suspended with DMEM/F12 medium (HyClone) supplemented with B27 (Gibco), EGF and FGF (20 ng/mL; Miltenyi Biotec, Bergisch Gladbach, Germany), 1% L-glutamine and 1% sodium pyruvate. Suspended cells were seeded into a 6-well non-adherent cell culture microplate (Sarstedt) at a density of 2 000 cells/well. Cells were incubated for 7 days and mammospheres bigger than 50 µm were counted. Mammosphere Forming Index (MFI) was calculated using the formula described below (1).

$$MFI\ (\%) = \frac{nº\ mammospheres}{nº\ seeded\ cells} \text{x}\ 100 \quad (1)$$

2.7. Statistical Analysis

All data are expressed as mean ± standard error (SE). Data were analysed by Student t test. Statistical significant levels were $p<0.05$ (denoted as *), $p<0.01$ (**) and $p<0.001$ (***).

3. Results

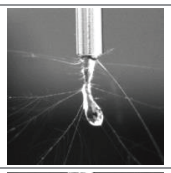
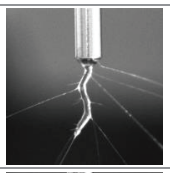
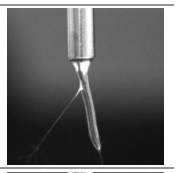
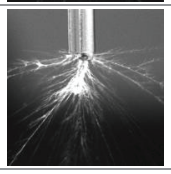
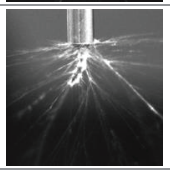
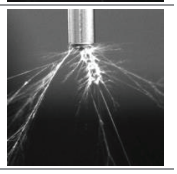
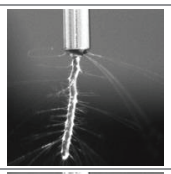
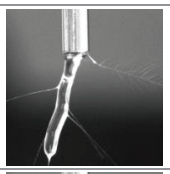
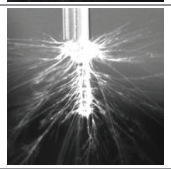
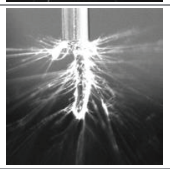
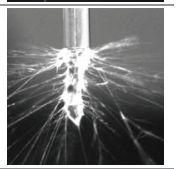
Electrospinning is a technology highly influenced by fabrication parameters such as polymer concentration, applied voltage and polymer solution flow rate. Literature shows a wide range of electrospun specifications [17–19,27–29]. Therefore, different parameters values were tested to reach a stable Taylor cone and a steady stream ~~(Figure 1)~~.

Poly(ε-caprolactone) (PCL) and acetone were chosen as biopolymer and non-toxic solvent, respectively. Two different concentrations of 7.5 and 15% PCL were produced and voltages of 7 and 15 kV were applied to analyse Taylor cone formation. Polymer fluid was supplied with flow rates of 1, 3 and 6 mL/h. Images from needle tip were taken 90 seconds after process beginning, shown in Table 1.

In all conditions, the polymeric solution droplet solidified once exposed to air, producing a stalactite-like form. These structures were also produced with different room humidity (50-60%) and temperature values (19-25ºC) (data not shown). Only the stream located at the end of this structure was functional. The other ones, produced by the voltage, remained as solidified PCL filaments. Voltage of 15 kV produced unstable Taylor cones and a higher number of non-functional streams in all tested parameters instead of 7 kV. Flow rate was also related to droplet morphology. Higher speeds exhibited thicker structures with few non-functional streams, remaining the Taylor cone stable. Both polymer concentrations presented a similar trend during screening. However, 15% PCL solution showed thicker stalactite-like structures in response to the high amount of polymer.

As the main aim is to obtain a stable Taylor cone and produce continuous filaments, voltage of 7 kV and flow rate of 6 mL/h were selected to produce scaffolds suitable for 3D cell culture. Both PCL concentrations (7.5 and 15%) were chosen to test different scaffolds. Considering electrospinning background, polymer concentration is directly related to fibre morphology [30]. Hence, two different meshes can be evaluated through 3D cell culture and BCSCs enrichment.

Table 1. Taylor cone formation of PCL-acetone solutions under different electrospinning parameters (PCL concentration, flow rate and applied voltage). Emitter tip images were taken 90 seconds after electrospinning start.

[PCL]	7.5% w/v		
	1 mL/h	3 mL/h	6 mL/h
7 kV			
15 kV			
[PCL]	15% w/v		
	1 mL/h	3 mL/h	6 mL/h
7 kV			
15 kV			

A 5 mL PCL solution volume was processed with electrospinning thus a membrane made of filaments was obtained. The PCL film was cut with squares shapes of 16 mm to obtain final scaffolds (Figure 1). Specimens weight and thickness were measured to perform a macroscopic characterization. Weight and thickness values from different electrospinning processes did not statistically differ, with *p*-values ranging from 0.16 to 0.98 (data not shown). Grouped data is presented in Table 2. Scaffolds of 7.5% PCL solution were less heavy ($p= 8.86\text{x}10^{-28}$) and thinner ($p= 3.94\text{x}10^{-7}$) than 15% ones, in agreement with the PCL amount in each solution.

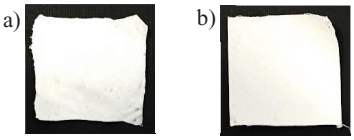


Figure 1. Electrospun scaffolds cut into squares of 16 mm side from 7.5% (a) and 15% w/v PCL solution (b).

Table 2. Macroscopic characterization of 7.5 and 15% electrospun PCL scaffolds (7 kV, 6 mL/h) after cutting them into 16 mm squares. Values are expressed as mean ± standard error.

	7.5% PCL	15% PCL
Weight (mg)	6.71 ± 0.17	14.11 ± 0.30
Thickness (µm)	147.22 ± 5.00	196 ± 4.65

Scaffolds were tested with 3D culture of MCF-7 breast cancer cells. Sterilized scaffolds were placed in non-adherent 12-well microplates. Meshes were seeded with three different cell densities (50 000, 100 000 and 300 000 cells) and incubated during 3 days. After incubation, MTT cell proliferation assay was carried out (Figure 2). In all culture conditions, 15% scaffolds showed a significant increase in cell proliferation compared with 7.5% specimens. Scaffolds exhibited major cell efficiency when seeded with low cell density (50 000 cells) compared with 2D control. Both scaffolds seeded with 100 000 cells showed significant reductions in normalized cell proliferation (p=5.48x10^{-3} for 7.5% specimens and p=5.90x10^{-3} for 15%) compared with 50 000 cells. Scaffolds from 15% PCL solution incubated with 300 000 cells also exhibited significant decreased efficiency (p=5.11x10^{-3}) compared with low cell density.

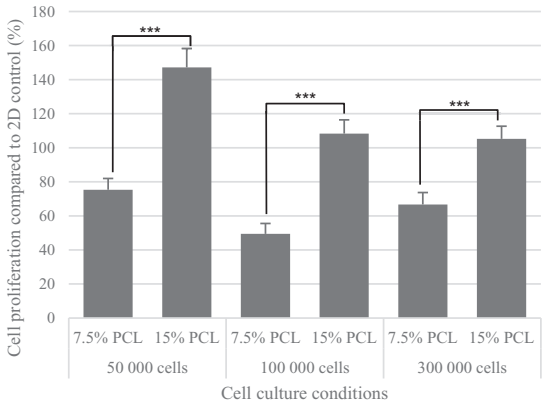


Figure 2. Cell proliferation analysis after 3 culture days of 7.5 and 15% PCL scaffolds seeded with three different cell densities. Adherent 2D control was also performed with same culture conditions. Statistical significant level was p<0.001 (***).

Mammosphere forming assay was performed to study the effect of 3D cell culture on BCSCs population (Figure 3). BCSCs can survive and proliferate in non-adherent surfaces, forming spherical colonies called mammospheres. Therefore, mammospheres number is a correlate of BCSCs abundance in the sample [6,7]. Cells previously cultured in 2D and 3D models were seeded in non-adherent microplates. After 7 days' incubation, mammospheres were visualized and counted. Mammospheres from cells cultured in 2D and 3D did not display different morphology (Figure 3a-c). Regarding mammospheres number, 3D cultured cells showed a significant higher Mammosphere Forming Index (MFI) compared with 2D cultured ones (Figure 3d), indicating a BCSCs enrichment. Both scaffolds exhibited similar MFI values, with a 3-fold increase compared with the control.

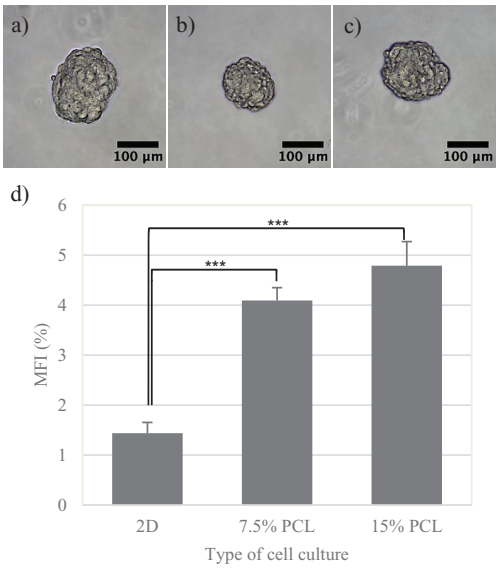


Figure 3. Mammosphere forming assay. (a-c) Mammosphere images from 2D cultured cells (a), 3D cultured with 7.5% PCL scaffolds (b) and 3D cultured with 15% PCL scaffolds (c). (d) Mammosphere Forming Index (MFI) of MCF-7 cells after 2D and 3D culture. Statistical significant level was p<0.001 (***).

4. Discussion

Voltage, flow rate and PCL concentration values were tested to achieve a stable Taylor cone (Table 1). In all screening tests, PCL solution partially solidified producing a stalactite-like form. Apparently, acetone was evaporated due to its high volatility producing this lengthened formation at the needle tip. Some authors electrospun PCL-acetone solution, but solvent evaporation in the emitter tip was not mentioned as Taylor cone structure was not studied [27–29, 31]. Low voltage of 7 kV and high flow rate of 6 mL/h produced a more stable Taylor cone. Relation between applied electric field and droplet morphology was superficially studied by Zong et al. using polylactic acid (PLA) dissolved in dimethyl formamide (DMF). They assumed that high voltage removed quickly the solution droplet from the tip. Thus, the droplet became smaller and the Taylor cone oscillated due to the high voltage. The same authors indicated the existence of a minimum solution volume available in the needle tip to reach a steady Taylor cone [32]. However, fewer differences were observed in droplet morphology varying flow rate parameter compared with voltage.

Scaffolds weight and thickness were presented in Table 2. Values of scaffolds from different electrospinning processes did not present significant differences demonstrating reproducibility between batches in regard to physical characterization. Furthermore, macroscopic characterization results were in agreement with PCL amount in each solution, being the 7.5% scaffold weight the half than 15% specimen.

Scaffolds from 15% PCL solution displayed a significant cell proliferation increase in all cell densities compared with

7.5% meshes (Figure 2). In summary, cells showed higher growth kinetics in scaffolds from higher PCL concentration. Opposed to these results, a human cancer cell study asserted that higher cell proliferations can be obtained with small fibres diameters from low biopolymer concentration scaffolds [33]. Cell proliferation differences can be attributed to filament framework characteristics. It was demonstrated that low PCL concentration resulted in meshes with spherical structures made by non-filamented polymer, called beads. The presence of beads led to fibroblast proliferation decrease [28, 31]. Moreover, other variables such as porosity and morphology could directly affect cell adhesion and proliferation. Regarding cell density, both scaffolds presented higher normalized cell efficiency at 50 000 seeded cells (Figure 2). Cell viability in scaffolds was proven to be influenced by initial cell amount [34]. Proliferating cells narrow the pores space, decreasing oxygen and nutrient diffusivity [35]. Thus, cell densities of 100 000 and 300 000 cells appeared slightly larger to allow a perfect mass exchange.

Cells cultured in 7.5 and 15% scaffolds exhibited a 3-fold increased MFI compared with cells cultured in monolayer (Figure 3). Both meshes showed similar MFI values while 15% PCL scaffolds presented a significant increased cell proliferation. As mentioned above, differences on their structure can explain the contrast of cell proliferation. Although these characteristics, both scaffolds provide a three-dimensional environment. This fact allows the cell to establish interactions with filaments in different plans of the space, enabling the cytoskeleton reorganization and gene expression regulation.

Regarding CSCs expansion, Feng et al. published a 2-fold MFI increase with MCF-7 cells cultured in electrospun PCL scaffolds [17]. These authors also noticed that 3D culture enhanced EMT markers expression and cell invasion. Relation between scaffolds culture and EMT was previously reported by Saha et al. H605 mouse mammary tumour cells showed higher expression of EMT markers when cultured in electrospun PCL fibers. Authors established that H605 cells cultured in scaffolds underwent EMT-like transitions [18]. In addition, Sims-Mourtada et al. described BCSCs enrichment since MCF-7 cells cultured in scaffolds showed a double MFI than 2D cultured cells. The authors suggested that enrichment was on account of an inhibition of BCSCs differentiation [19]. The same work described that 3D cultured cells exhibited increased chemoresistance. Consequently, three-dimensional cell culture could be an alternative to perform cytotoxicity experiments to obtain more reliable and physiological results.

Taking into account all data, this work proved that electrospinning is a potent technology useful in diverse fields. Controlling process parameters allowed the production of different meshes, demonstrating its potential for nanotechnology research. Produced scaffolds have been proven to allow cell adhesion and proliferation. Scaffolds mimic the extracellular matrix, allowing the cells to adopt a more *in vivo* morphology and behaviour. Finally, cell culture with electrospun PCL scaffolds has proven to expand BCSCs population. Therefore, 3D cell culture could facilitate the development of new therapeutic strategies against this malignant subpopulation.

Acknowledgements

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