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Optimization of Poli(ε-caprolactone) scaffolds suitable for 3D cancer cell culture

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Abstract

Fused Deposition Model (FDM) as Additive manufacturing (AM) technologies may offer a viable and simpler alternative to manufacture scaffolds for different purposes such as tissue engineering and cells culture. Existing commercial FDM machines are currently being modified to improve their accuracy, capabilities and use. However, for biocompatible and/or bioimplantable materials such as Poli (ε -caprolactone) PCL there is still a lot of work to do to set up process parameters. Cells culture had been carried on 2D without being a proper and real midst. In fact cells do not grow only in two flat directions but in all directions making strong net. Since cells responses to proteins or drugs is important for knowing proliferation or enrichment more real culture in 3D is needed. This work focuses on the study and optimization of open-source 3D printer machine, called RepRap, employed to manufacture biocompatible scaffolds for 3D cells culture of Triple-Negative Breast Cancer (TNBC). It has been shown that scaffolds culture can enhance the Cancer Stem Cell (CSC) population, responsible in part for tumour recurrence after chemotherapy. Mammosphere Forming Index (MFI) was defined in all cell lines to evaluate this population in TNBC cell lines sensible and resistant to chemotherapy. Enriching TNBC cells with CSC after scaffold culture will help to study new therapeutic treatments directed to this population. Several process parameters are tested to manufacture scaffolds and cells culture had been carried out in order to validate the results. Results show that porosity plays an important role in scaffolds manufacture having low cells adhesion and growth. Lower porosity values should be tested to further evaluate MFI index after scaffold culture as cell growth and enrichment indication.

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Keywords: PCL; Scaffold; Additive Manufacturing; Fused deposition model; Cancer Cells; 3D Cell-Culture; Stem Cells

1. Introduction

Cell culture is a technique widely used in cancer research laboratories, for instance to the study of cancer biology and the development of new therapeutic strategies. Nowadays, 2D cell culture is the most frequent technique used. Although interest for 3D cell culture is emerging because cell architecture and interactions simulates better the biology of a tissue compared to the flat-growing 2D systems [1].

The development of accurate constructs made of a matrix or scaffold and living cells to repair and regenerate damaged tissue is a current challenge in the tissue engineering field [2]. Taking advantage of this, scaffold fabrication for 3D cell-culture has emerged using technologies already developed for this purpose. One of these techniques is the additive manufacturing technology, which enables the fabrication of customized scaffolds directly from the patient. For this purpose, existing additive manufacturing machines are currently being modified to improve their accuracy and capabilities. The optimization of process parameters is a major challenge to obtain adequate scaffold morphology and biomechanical behavior, which are to improve cell adhesion and proliferation. Indeed, appropriate porosity, pore size, pore shape, and mechanical strength are required to achieve cell growth and matrix formation [3]. Open source extruders, like the RepRap machine, allow a thorough study of several process parameters involved in the fabrication of scaffolds such as deposition speed, layer thickness, filament distance, deposition pattern, extrusion and bed temperatures and speed movement. Therefore, a precise control over this manufacturing process is required.

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Triple-Negative Breast Cancer (TNBC) is a type of breast cancer which runs an aggressive course and has a poor prognosis. It shows the highest recurrence rate compared to other breast cancer types [4]. TNBC lacks of validated directed therapy, and patients are treated mainly with chemotherapy (anthracyclines and taxans). Even though TNBC shows a good response to these therapies, recurrence at 5 years following diagnosis is about 30% of the cases [5,6].

Recent studies showed that chemoresistance can be achieved by a unique and rare cell niche with stemness features, the so-called Cancer Stem Cells (CSC) [7,8]. These cells, capable of tumor initiation, are not only responsible for tumor recurrence, but also metastasis [9]. Interestingly, scaffolds not only allow cells to interact in a 3D way improving the in-vitro 2D system, but also it has been described that 3D cultures can enhance the CSC population [10,11].

In this study it had been investigated the optimization of the open source and low-cost 3D extruder machine RepRap, employed to fabricate PCL scaffolds suitable for 3D cell culture. Design and manufacturing parameters were determined to ensure the best performance. In addition, this work focuses in 3D scaffolds ability to enrich the CSC population for developing new therapeutic strategies to target this population.

2. Materials and Methods

2.1. Material

A 3mm Poli(ε-caprolactone) (PCL) wire (Perstorp, Malmö, Sweden) was used to manufacture the scaffolds. PCL is biodegradable polyester proven to be biocompatible and free of toxic dye.

2.2. 3D printer machine and software

Printer machine RepRap BCN 3D+ was used to produce three-dimensional scaffolds. It is an open source and modular 3D printer designed by RepRap BCN. This printer uses the Fused Filament Fabrication (FFF) technology so called Fused Depostion Model (FDM). The filament unwound from a coil is supplied to the extruder. Then, at certain temperature and pressure, exerted by a gear, causes the extrusion of the material through the nozzle, which is finally deposited onto a heated computer-controlled Cartesian platform.

The scaffold's design was carry out with the computer-aided design (CAD) software SolidWorks. The designs were saved in STL file formats, which are transferred to a computer-aided manufacturing (CAM) software called Slic3r. This program was used for establishing the printing parameters. It generates G-code files able to command and control the machine in order to obtain the scaffolds printed.

Table 1 shows the process parameters utilized for the experimental set up. Design parameters, such as shape, layer thickness, diameter of filament and distance between filaments, were studied and analysed. Deposition angle between layers was fixed at 0-90º. Shape refers to the basic feature of the scaffold produced. Thickness is the total height of the scaffold and the distance between filaments is the shortest distance between two filaments located within the same layer.

Table 1. Scaffold parameters and levels tested in order to obtain the optimal printing

	Parameters	Levels
DESIGN PARAMETERS	Deposition angle (º)	0-90
	Shape	square, round
	Thickness (mm)	1.8, 3.6
	Diameter of filament (mm)	0.175, 0.30, 0.50
	Distance between filaments (mm)	0.5, 0.7, 1
MANUFACTURING PARAMETERS	Deposition speed (mm/s)	10, 20, 30, 50
	Layer height (mm)	0.15, 0.20, 0.25, 0.28, 0.30, 0.35
	Extrusion temperature (ºC)	65, 75, 80, 85, 90, 95, 105, 110, 115, 120, 130, 150, 155, 160, 180, 200
	Bed temperature (ºC)	25, 30, 33, 35, 37

Other parameters were set, like deposition speed, layer height and temperatures. The deposition speed was defined as the speed for printing movements of the extruder. Layer height is the distance between two connected layers along the Z axis. Finally, temperatures of the extruder and the glass platform were set up as well.

2.3. Cell Culture

MCF-7, MDA-MB-231 and HCC1806 breast carcinoma cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). MDA-MB-231 and HCC1806 are cell lines established from patients with TNBC. MCF-7 are a HER2 positive cell line, (a type of breast cancer that overexpresses the Human Epidermal Growth Factor Receptor 2), used for scaffold validation. Doxorubicin (chemotherapeutic drug) was used to create resistant models from MDA-MB-231 (231**DXR**) and HCC1806 (HCC**DXR**) in our laboratory by treating cells at increasing doses of doxorubicin for 48 hours periods until 6 months.

MCF-7, and MDA-MB-231 and 231**DXR** cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) (Gibco, Walthman, MA, USA) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, 50U/mL penicillin and 50µg/mL streptomycin (HyClone, Logan, UT, USA). HCC1806 and HCC**DXR** cells were cultured in RPMI (Roswell Park Memorial Institute) (Gibco, Walthman, MA, USA) and supplemented as above. All cells were maintained at 37ºC and 5% CO_2 atmosphere.

2.4. Mammosphere-forming assay

In order to evaluate CSC population, the mammosphere-forming technique was performed (Figure 1). Cells from 2D or PCL scaffolds were removed by trypsinitzation. Then cells were counted, and seeded into a 6-well cell culture microplate coated with pHEMA using DMEM/F12 medium supplemented with B27, EGF and FGF (20ng/mL), 1% L-glutamine, 1% sodium pyruvate and 25U/mL penicillin and

25µg/mL streptomycin . Finally, cells were incubated for 5 or 7 days and mammospheres bigger than 50µm were counted using an inverted opical microscope. Mammosphere Forming Index (MFI) was calculated using the formula described below:

$$MFI = \frac{Nº\ mammosphere}{Nº\ cells\ plated} \cdot 100$$

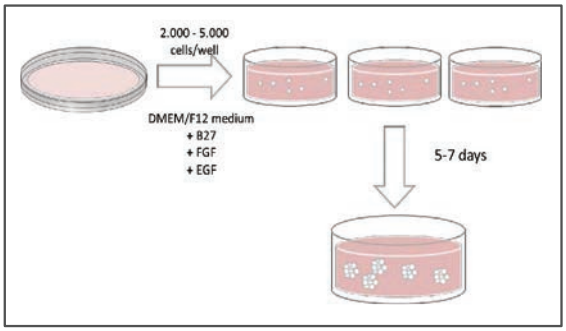


Figure 1. Mammosphere-forming assay protocol

For the mammosphere treatment experiments, doxorubicin was added in the seeding process. Mammosphere Forming Inhibition (MFIn) was calculated, as shown in the formula below:

$$MFIn = 100 - \frac{Nº\ mammospheres_{treatment}}{Nº\ mammospheres_{control}} \cdot 100$$

2.5. Growth inhibition assay

MDA-MB-231, HCC1806, 231**DXR** and HCC**DXR** were plated out at a density of $5x10^3$ cells/2mL/well in 6-well plates. Posterior overnight cell adherence, fresh medium along with the corresponding doxorubicin concentration was added to the cultures. Following treatment, media was replaced by drug-free medium (1mL/well) containing MTT (3,4,5-dimetylthiazol-2-yl-2,5-diphenyltetraolim bromide, Sigma) solution, and incubation was prolonged for 2 hours at 37ºC. Formazan crystals formed by metabolically viable cells were dissolved in DMSO (300µL/well) and absorbance was determined at 570nm in a multi-well plate reader (Model Anthos Labtec 2010 1.7). Using control OD values (*C*) and test OD values (*T*), % of Cell Proliferation Inhibition (CPI) was calculated from the equation below:

$$CPI = 100 - \frac{T}{C} \cdot 100$$

Data presented are from two separate wells per assay and the assay was performed at least three times.

2.6. Scaffold sterilization

Scaffolds were sterilized with 70% ethanol/water solution overnight, washed with PBS (Gibco, Walthman, MA, USA) and finally exposed to UV light for 90 minutes.

2.7. Cell culture in scaffolds

Scaffolds were placed into a 12-well cell culture microplate. First, 250µL of cell suspension (10.000-100.000 cells) were placed onto the centre of its surface to allow cells attach on the scaffold. After 1 hour incubation period, 1.5mL of fresh medium was added to cover the scaffold. Cells were incubated for 72 hours and then counted. (Figure 2).

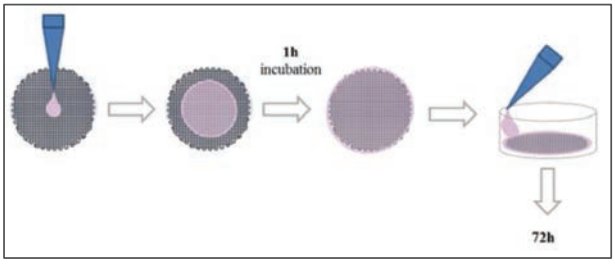


Figure 2. Cell seeding protocol on PCL scaffolds.

To quantify the cells attached, the scaffold was placed in a new well, washed with PBS and 1mL of trypsin was added. After incubation, 2.5mL of fresh medium were added and the cell suspension was collected and centrifuged at 1500rpm for 5 min. Finally, the supernatant was discarded and cells were counted using a Neubauer Chamber and an inverted optical microscope. The same procedure was done to obtain the cells attached at the well where the scaffold was placed.

2.8. Statistical analysis

All data are expressed as mean ± standard error (SE). Data were analyzed by Student *t* test. Statistical significant levels were $p < 0.05$ (denoted as *), $p < 0.01$ (denoted as **) and $p < 0.001$ (denoted as ***). *p-value* is shown in results when significance is reached ($p < 0.05$).

3. Results

3.1. Scaffolds design and manufacturing

Scaffolds were designed with a round shape, with the size of 19mm diameter to allow their use in regular cell culture plate-dishes of 12 wells. The final designs had 1.8mm of thickness, composed of 6 different layers of polymeric material, being 0.3mm of thickness each layer. The distance between filaments was 0.7mm and the deposition angle was established at 0-90º (Table 2).

Scaffolds manufacturing parameters were optimized by screening experiments to print the scaffolds efficiently and properly for cells culture (Table 3).

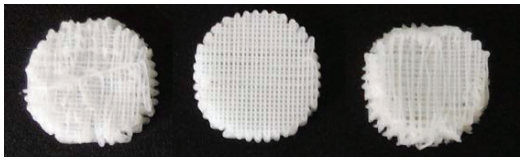
The deposition speed took a small value to optimize the material's deposition. The nozzle tip size was fixed at 0.35mm. The printed filaments had a diameter of 0.30mm. With visual screening, it was proved that the optimal extrusion temperature was 85ºC (Figure 3). Finally, scaffolds were fabricated with a bed temperature of 35ºC, to guarantee their adhesion to the printing platform.

Table 2. Scaffold desig parameters.

Parameters	
Diameter	19mm
Shape	Round
Thickness	1.8mm
Number of layers	6
Distance between filaments	0.7mm
Deposition angle	0-90º
Diameter of filament	0.30mm

Table 3. Scaffold manufacturing parameters

Parameters	
Deposition velocity	10mm/s
Layer height	0.30mm
Nozzle tip size	0.35mm
Extrusion temperature	85ºC
Bed temperature	35ºC



(a) (b) (c)

Figure 3. Scaffold manufacturing at different extrusion temperature (a) 80ºC, (b) 85ºC, (c) 90ºC.

3.2. Cancer Stem Cells population characterization

Cancer Stem Cells are a very rare population within the tumor. These cells have been demonstrated to have the ability to survive and propagate in a non-adherent way, forming spheres called mammospheres [7,12,13].

To determine CSC population, the mammosphere-forming assay was performed in cell lines MCF-7, MDA-MB-231, HCC1806, and resistant models 231DXR and HCCDXR (Figure 4). MFI was then calculated (Table 4).

CSC represents a small population in all cell lines, with values ranging from 0.67% to 2.47% of MFI. When comparing the ability to form mammosphere between parental and resistant cells to doxorubucin, no increase of MFI was observed in the two models.

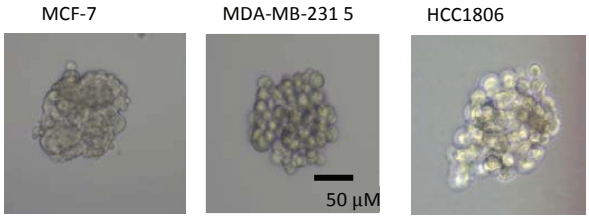


Figure 4. Mammospheres under optic microscope for MCF-7, MDA-MB-231 and HCC1806.

Table 4. Mammosphere Forming Index (MFI).

Cell Line	**Cells seeded (cells/well)**	**Days**	**MFI (%)**
MCF-7	2.000	7	2.47±0.26
MDA-MB-231	5.000	5	1.78±0.24
HCC1806	5.000	5	0.92±0.17
231**DXR**	5.000	5	1.75±0.27
HCC**DXR**	5.000	5	0.67±0.04

Data are shown as mean ±SEM.

As cell plasticity plays an important role in tumor biology and drug resistance, it was checked the ability to form mammospheres under doxorubicin pressure [14-16]. To do so, the experiments were repeated in presence of doxorubicin in both sensible and resistant cell lines and MFIn was calculated for each model. The same experiment was performed in adherent conditions using the MTT assay as described in Material and Methods section. Intrinsic resistance of CSC to doxorubicin was observed in all models (Figure 4). In adherent conditions, cell lines proliferation inhibition (% CPI) ranged from 84.2% to 89.5%. Otherwise, MFIn values showed significant lower inhibition in all cell lines, ranging from 20.6% to 60.7% (*p-values* < 0.001).

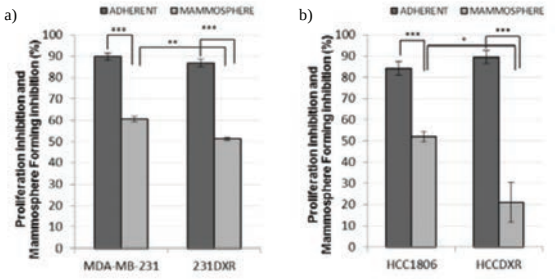


Figure 5. Proliferation inhibition and Mammposphere forming inhibition (MFIn) under doxorubicin treatment for 5 days. (a) MDA-MB-231 and 231**DXR** with doxorubicin (70nM). (b) HCC1806 and HCC**DXR** with doxorubicin (140nM). Experiments were performed at least three times in duplicate. *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$) indicate levels of statistically significance.

The cytotoxic effect of doxorubicin was then evaluated in CSC population comparing parental and resistant models (Figure 4). MDA-MB-231 and 231**DXR** showed an MFIn of 60.66% and 51.29% (*p value*: 0.005) respectively. HCC1806 MFIn was 51.97% and 20.61% for HCC**DXR** (*p value*: 0.036). CSC population from resistant models HCC**DXR** and 231**DXR** showed significance resistant to doxorubicin, maybe due to an enrichment of this population in this models [8].

3.3. Scaffolds and cell culture

The scaffold with deposition angles of 0-90º (Figure 2a) was printed and tested for cell culture (Figure 5).

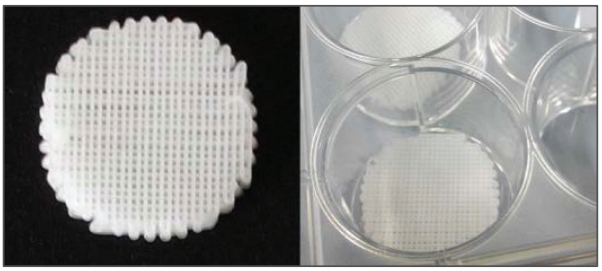


Figure 5. Scaffold fabricated with RepRap Machine a) 19mm diameter, 1.8mm thick, and deposition angles of 0-90º b) the scaffold plated in a 12 well-plate.

Before its use, scaffolds were sterilized as mentioned in Material and Methods section. Then MCF-7 cells were seeded at different densities (10.000, 50.000 and 100.000 cells/well) during 72 hours. Attached and non-attached cells were counted.

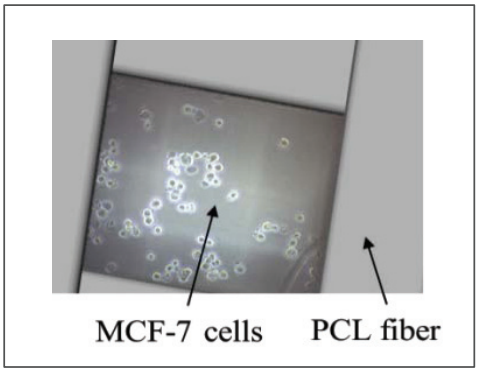


Figure 6. Optical microscope images of MCF-7 cells attached at the bottom of the well. Scaffold fiber has been drawn on the top.

In this first attempt, no cells were attached to the scaffold. As it was described and then observed under the microscope, this scaffold has large pores. The fibers of the different layers have the same disposition angle and, for that reason, cells can easily fall to the bottom of the well before they can get attached to PCL fibers (Figure 6).

As PCL fibers have already been tested in cell culture [10], new goal is set up focusing on designing and testing new scaffolds with different deposition angles between layers to achieve different and smaller pore sizes.

Two more designs have been performed, where the deposition angles were variable, taking the values of 0-60-120º and 0-45-90-135º (Figure 7). The variation of the angle deposition between layers results in a different pore size and shapes between the scaffolds (Table 5).

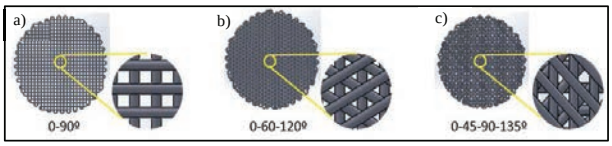


Figure 7. Scaffolds designs with different deposition angles: (a) 0-90º, (b) 0-60-120º, (c) 0-45-90-135º.

Table 5. Pore characteristics depending on the deposition angle

Deposition angles	Pores shape	Area
0-90º	Square	0.15mm^2
0-60-120º	Equilateral triangle	0.1256mm^2
0-45-90-135º	6 variable forms (triangles and irregular polygons)	1.98x10^{-4} to 0.13mm^2

4. Conclusions

In this study, the parameters of the open source RepRap 3D printer have been optimized to fabricate scaffolds of PCL suitable for cell culture.

Parameters, like deposition speed, diameter of the filament and extrusion and bed temperatures have been determined to obtain an optimal manufacture.

Design parameters, such are round shape, diameter and thickness were thought to allow its use in regular 12 well-cell culture plates. Three different designs have been performed, with different angles between layers, obtaining different pores sizes in all designs.

MCF-7 and TNBC cell lines had the ability to form mammospheres with low values of MFI, showing that CSC represents a very small population in these cell lines. CSC enriched population showed also intrinsic resistance to chemotherapy when compared to adherent culture. On the other hand, CSC population in resistant cell models developed in our lab (HCC**DXR** and 231**DXR**) showed increased ability to form mammospheres compared to the parental models under doxorubicin treatment.

MCF-7 cell line was used to test the scaffolds printed with deposition angles of 0-90º. Because of the same distribution of the fibers between layers and the large pores size of this design, cells drifted at the bottom of the well without attaching the scaffold. The other two designs, with smallest pore size will be tested in further studies.

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