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Manufacture of PCL scaffolds through electrospinning technology to accommodate Triple Negative Breast Cancer cells culture

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Abstract

Two-dimensional (2D) cell culture structures are demonstrated to differ from the *in vivo* environment. Therefore, cells adopt a flattened morphology that may lead to a non-physiological behaviour. For instance, 2D culture induces the differentiation of the cancer stem cells (CSCs), a tumorigenic cell niche whose study is crucial in tumors with a high relapse rate such as the triple negative breast cancer (TNBC). As an alternative, electrospun scaffolds which mimic the native extracellular matrix structure have emerged as a three-dimensional culture support. In this work, two different meshes of 7.5 and 15% of poly(ε-caprolactone) (PCL) were fabricated, differentiating in their microstructure. Scaffolds exhibited similar DSC and TGA curves compared with raw PCL, indicating their purity. TNBC MDA-MB-468 cells were seeded on scaffolds and adopted a more elongated morphology when cultured on 15% PCL meshes. Hence, electrospinning PCL scaffolds may become a suitable tool to culture TNBC cells in a more physiological manner.

Keywords: polycaprolactone; scaffolds; electrospinning; three-dimensional cell culture; triple negative breast cancer

1. Introduction

Since some decades ago, disposable polystyrene vessels have been used for cell culture applications due to their practicability [1]. These flat plastic supports enable the foundation of a two-dimensional (2D) cell culture which represents an easy and cheap way to maintain cell division. However, 2D surfaces are clearly in conflict with the physiological surrounding that cells found in the organism. Inside the body, cells are naturally embedded by a three-dimensional (3D) network constituted by fibrous proteins and molecules known as extracellular matrix (ECM) [2]. Actually, the most abundant element is the collagen, a structural protein which is set up in the form of nanofilaments [3]. In this manner, ECM offers a physical support for cells to attach and grow, apart from its role in cell regulation [2], [4]. Cells tend

to adopt an elongated pattern in *in vivo* conditions as they are able to attach different filaments as well as adjacent cells. Nevertheless, in 2D surfaces cells can only proliferate forming a monolayer and they normally adopt a flattened morphology. Cell flatness directly modifies the membrane receptor polarity as well as the cytoskeleton organization, affecting the gene expression and protein synthesis [5].

As an alternative, research has been focused on the development of 3D supports for cell culture purposes, including scaffolds among others. Scaffolds consist on a network of biopolymeric filaments, mimicking the EMC structure [6]. Therefore, they provide a physical structure which enable cells to adopt a more *in vivo* morphology. Lastly, electrospinning process has gained importance in the scaffolds manufacturing field. The main reasons are its potential

regarding the fabrication of small diameters fibers and its customizability [7], [8]. A wide range of polymers can be electrospun to manufacture fibrous meshes, including the poly(ε-caprolactone) (PCL), an FDA approved polyester characterized by its viscoelastic and rheological properties [9].

Cancer research field is taking advantage of the development of 3D structures for cell culture applications. It is demonstrated that cancer cells cultured on 3D structures including scaffolds exhibited a more elongated shape in discordance with monolayer cultured cells [10], [11]. The closeness of 3D-cultured cells morphology to the *in vivo* one makes scaffolds an ideal alternative to study physiological cell behavior. For instance, scaffolds were proved to maintain the differentiation state of cancer cells [10], [12]. Since some decades ago, cancers have been studied as a heterogeneous mixture of different cell types: normal cancer cells and a rare subpopulation of cancer stem cells (CSCs) [13]. CSCs display a key role in cancer development and tumor relapse [14], [15]. Lately, 3D culture supports are used to maintain stemness capacity since monolayer culture induces CSCs differentiation [10], [11], [16], [17].

The main goal of this work is to assess scaffolds potential for three-dimensional breast cancer cell culture applications. For this reason, two different PCL solutions were electrospun since polymer concentration is proved to exert a strong influence on scaffolds structure [8], [18]. Produced meshes were characterized and seeded with a triple negative breast cancer (TNBC) cell line considering that this subtype display the highest proportion of cancer recurrence with a value of 34% [19], an issue directly related to CSCs presence. Cell proliferation and morphology were determined on monolayer culture and 3D scaffolds. Obtained results suggest the suitability of electrospun PCL meshes as a novel tool for 3D breast cancer cell culture. Further studies must be focused on CSC population experimentation with the aim of developing a novel targeted therapy against this malignant niche, useful for TNBC patients.

2. Materials and Methods

2.1. Scaffolds manufacture

Poly(ε-caprolactone) (PCL; 80,000 g/mol; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in acetone (PanReac AppliChem, Gatersleben, Germany). Two different solutions of 7.5 and 15% w/v PCL were produced under 45°C and agitation. Scaffolds were manufactured with an electrospinning machine (Spraybase, Dublin, Ireland) and an 18 G needle emitter (inner diameter of 0.8 mm) located 15 cm above the stationary collector. A voltage of 7 kV was applied and the flow rate was determined at 6 mL/h by the Syringe Pump Pro software (New Era Pump Systems, Farmingdale, NY, USA). Electrospinning process was finished when 10 or 5 mL of PCL-acetone solution were ejected, for 7.5 and 15% PCL concentrations, respectively. Once electrospinning process was finished, resulting scaffolds were cut into squares with a scalpel.

2.2. Scanning Electron Microscopy (SEM) analysis

Microscopic architecture of both scaffolds models was observed through scanning electron microscopy (SEM; Zeiss, Oberkochen, Germany) after carbon coating. Captured images were analyzed with Image J software (National Institutes of Health, Bethesda, MD, USA).

2.3. Differential Scanning Calorimetry (DSC) analysis

Differential scanning calorimetry (DSC; TA Instruments Q2000 V24.4, Newcastle, DE, USA) was carried out on commercial PCL pellets (as received) and on scaffolds from 7.5 and 15% PCL solution. About 5 mg of each sample were placed in aluminum pans. Experiments were conducted from 30°C to 100°C at a rate of 10°C/min. To prevent oxidative degradation, analyses were performed in nitrogen with a flow rate of 50 mL/min.

2.4. Thermal Gravimetric Analysis (TGA)

The thermal degradation of samples was studied through a Thermal Gravimetric Analysis (TGA) using a Mettler Toledo TGA 1 (Columbus, OH, USA). Around 10 mg of non-processed PCL and scaffolds from 7.5 and 15% PCL solution were analyzed. Measurements were performed with a temperature ranging from 30°C to 700°C with a ratio of 10°C/min.

2.5. Cell line

MDA-MB-468 triple negative breast cancer cell line was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). MDA-MB-468 cells were routinely grown 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 37°C and culture medium was changed every 3 days.

2.6. Three-dimensional cell culture

Electrospun meshes were first sterilized with 70% ethanol/water solution overnight, washed with PBS (Gibco) and exposed to UV light for 30 min. Then, scaffolds were placed in a 12-well non-adherent cell culture microplate (Sartstedt, Nümbrecht, Germany). Cell suspension was prepared in a volume of 50 µL and pipetted onto the scaffold center. After an incubation period of 3 hours, 1 mL of medium per well was added. Two-dimensional controls were also performed. For cell proliferation assay, 2 500 cells/well were seeded. In contrast, for cell morphology analysis, the following cell densities were performed (see Table 1). Thus, similar cell confluence was reached among different cell culture supports and culture days.

Table 1. Cell densities performed for cell morphology analysis.

		Cell culture support	
		2D	3D
Cell culture days	3 days	60 000	60 000
	6 days	20 000	30 000
	12 days	4 000	6 000

2.7. Cell proliferation assay

The MTT assay was used to test cell proliferation on the different cell culture supports. After the culture period, medium was removed and scaffolds were put into new wells. They were incubated with 1 mL of medium and 100 µL of MTT (Sigma-Aldrich) for 2 h and 30 min. Resulting formazan crystals were dissolved with 1 mL of dimethyl sulfoxide (DMSO; Sigma-Aldrich). Four aliquots of 100 µL from each sample were transferred into a 96-well plated and placed into a microplate reader (Bio-Rad, Hercules, CA, USA). Monolayer culture samples were equally processed and absorbance was measured at 570 nm.

2.8. Cell morphology analysis

MDA-MB-468 cells were seeded on adherent coverslips (Sarstedt) and 7.5% and 15% PCL scaffolds. After incubation, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) for 20 min. Then, samples were washed and incubated with 0.2% Triton X-100 (Sigma-Aldrich) for 10 min to permeabilize the cells. Blocking buffer (PBS with 3% bovine serum albumin [BSA; Sigma-Aldrich]) was added for 20 min. Later, cells were incubated for 20 min with rhodamine-phalloidin (Cytoskeleton Inc., Denver, CO, USA) (1:250) to stain actin cytoskeleton and then with 4,6-diamidino-2-phenylindole (DAPI; BD Pharmingen, Franklin Lakes, NJ, USA) (1:1000) for 10 min to stain nuclei. All incubations were performed at room temperature. Fluorescent samples were imaged with a confocal microscope (Nikon A1R, Nikon, Tokyo, Japan). Camera settings (illumination intensity, quality, resolution and colour) were standardized for all photographs. Rhodamine-phalloidin and DAPI, for red and blue fluorescence respectively, were captured and merged with Image J software (National Institutes of Health). This software was also used to determine nuclear and cytoplasmic elongation factors. Briefly, five cells of ten different pictures were randomly selected and their length and width of the nucleus and cytoplasm were measured. Finally, the following formula was used:

$$Nuclear/Cytoplasmic\ Elongation\ Factor = \frac{length\ nucleus/cytoplasma}{width\ nucleus/cytoplasma}$$

2.9. Statistics analysis

All data are expressed as mean ± standard error (SE). Data were analyzed using IBM SPSS (Version 21.0; SPSS Inc., Chicago, IL, USA) through ANOVA and post hoc tests. Statistical significant levels were $p < 0.05$ (denoted as *), $p <$ 0.01 (**) and $p < 0.001$ (***). All observations were confirmed by at least three independent experiments.

3. Results and discussion

3.1. Electrospun PCL scaffolds characterization

Once scaffolds were produced, they were characterized through several approaches. Meshes from 7.5 and 15% PCL-acetone solution were observed under a scanning electron microscopy (Fig. 1).
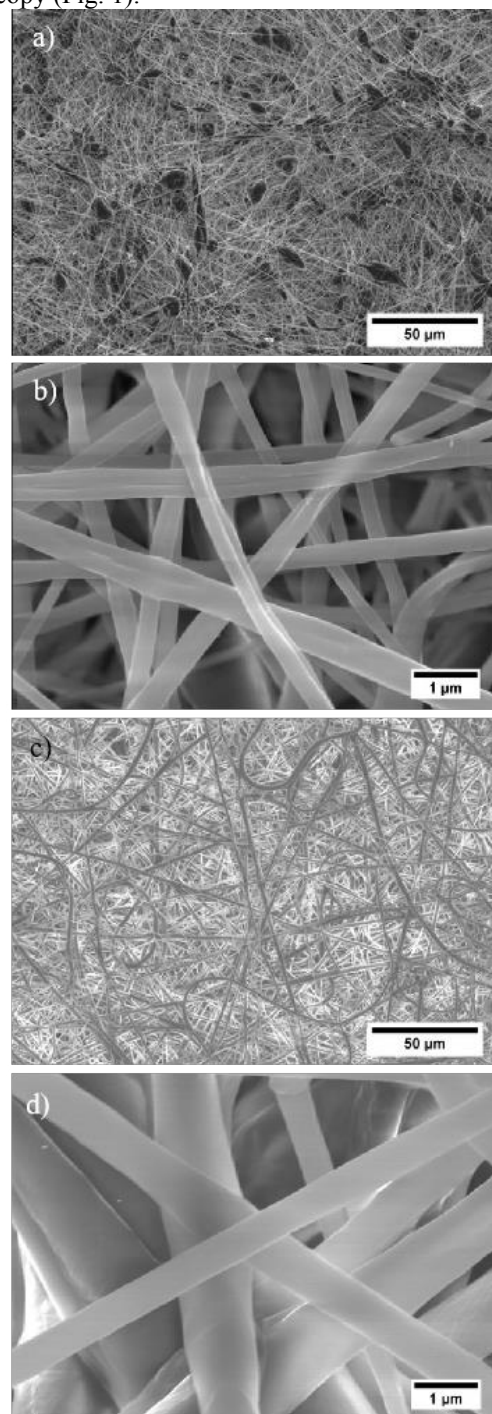

Fig. 1. Microscopic characterization of 7.5 (a and b) and 15% PCL (c and d) electrospun scaffolds. Specimens were imaged at 500 X (a and c) and 15000 X magnifications (b and d).

As previously described, both models exhibited similar surface porosity whereas 15% PCL specimens showed larger pores compared with 7.5% PCL scaffolds [11]. A previous study determined the fiber diameter of the different specimens, with an average value of 295.12 ± 148.45 nm and 701.13 ± 401.89 nm for 7.5 and 15% PCL scaffolds, respectively [11]. Interestingly, meshes from 7.5% PCL solution presented spherical structures made by non-filamentous polymer, apart from the nanofibers (Fig. 1A). These formations, called beads, are related with low polymer concentrations [20].

A Differential Scanning Calorimetry (DSC) analysis was later performed with the electrospun scaffolds in order to study their thermal behavior (see Fig. 2.).
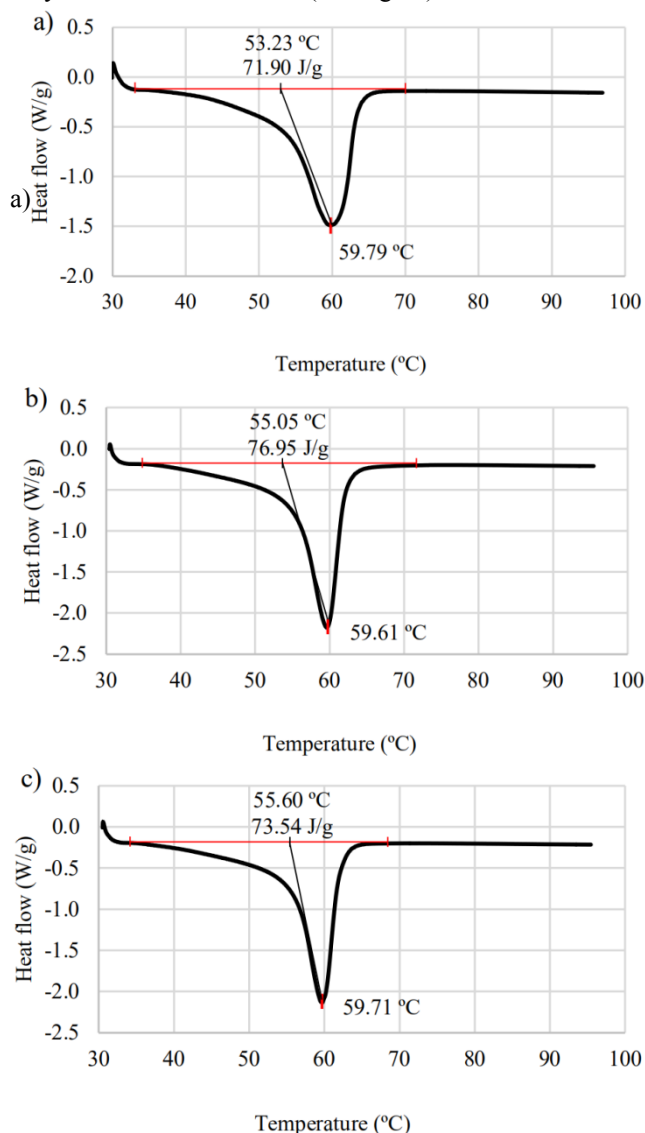

Fig. 2. DSC curves of PCL pellets (a) and 3D scaffolds from 7.5% (b) and 15% PCL solution (c).

The plots presented the typical DSC curves of a partially crystalline polymer with no impurities. The endothermic peak indicates the melting temperature, which displayed no differences among samples ranging from 59.61 to 59.79 ºC. According to the obtained results, the melting temperature of pure PCL was reported to be around 60ºC [21], [22]. These facts indicated the absence of impurities and, therefore, that manufacturing process did not affect the integrity of the

biopolymer. The onset melting temperature of raw PCL pellets was 53.23ºC whereas both scaffold models displayed slightly higher values between 55.05 and 55.60 ºC. Non-processed PCL exhibited a melting enthalpy of 71.90 J/g. Manufactured PCL scaffolds displayed moderately greater values of 76.95 and 73.54 J/g for 7.5 and 15% PCL meshes respectively, in agreement with previous analyses [23].

Then, thermal degradation of samples was studied through a Thermal Gravimetric Analysis (TGA). TGA curves of non-processed PCL pellet and electrospun 7.5 and 15% PCL scaffolds are presented in Fig. 3. All specimens exhibited a single step curve, indicating a stable degradation in a simple process. No differences were observed among pristine PCL and manufactured PCL scaffolds since their TGA curves appeared overlapped (Fig. 3). Moreover, degradation process of raw PCL and PCL meshes started around 350ºC, as reported by the literature [24], [25]. These data support the hypothesis that electrospinning manufacturing process did not affect the integrity of PCL on resulting scaffolds and no impurities were detected.
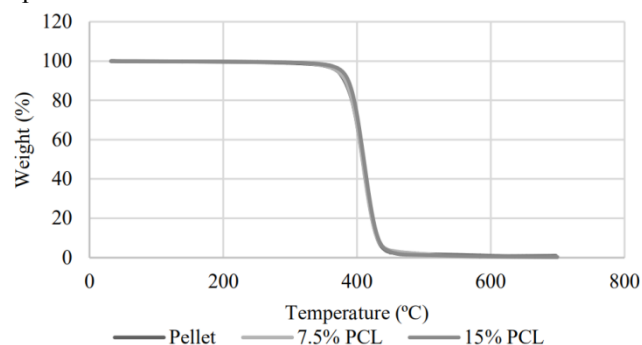

Fig. 3. TGA thermographs of PCL pellets and 3D scaffolds from 7.5% and 15% PCL solution.
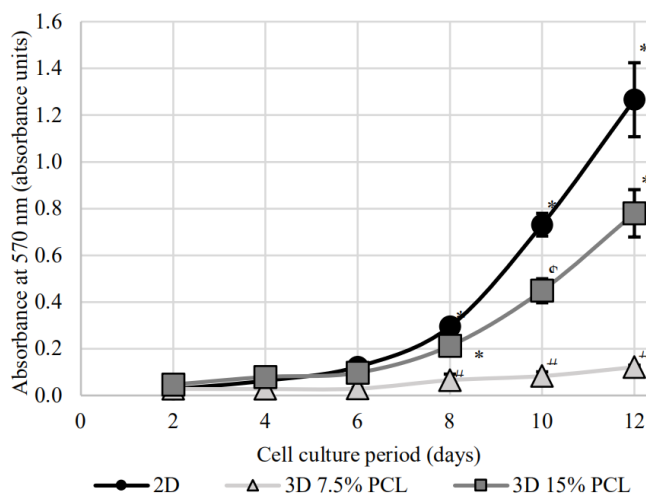

Fig. 4. Cell proliferation analysis for MDA-MB-468 triple negative breast cancer cells cultured on two-dimensional surfaces (2D) and on 7.5% and 15% PCL scaffolds (3D). (*), (#), and ($) symbols represent significant ($p < 0.05$) differences among groups.

3.2. Breast cancer cell proliferation

Electrospun PCL scaffolds could become a suitable physical support for 3D cell culture. Indeed, scaffold

microarchitecture exert a high influence on cell adhesion and growth [11], [26], [27]. For this reason, adherent 2D surfaces and 7.5 and 15% PCL meshes were seeded with MDA-MB-468 breast cancer cells in order to evaluate cell efficiency (Fig. 4). Differences among cell culture models were found from 8 days of culture, when cell growth rate on 2D control and 15% PCL scaffolds adopted higher values compared to 7.5% PCL samples. Meshes from 7.5% PCL displayed the significantly lowest cell proliferation rate with almost a linear trend. In contrast, 15% PCL model served as the optimal support, with high absorbance values close to the monolayer ones.

3.3. Breast cancer cell morphology

Three-dimensional scaffolds provide a physical support which mimics the extracellular matrix structure. Therefore, cells can establish interactions with adjacent cells and with polymeric filaments, adopting a more *in vivo* morphology. For this reason, MDA-MB-468 cells morphology among different culture supports was studied through fluorescence microscopy during 3, 6 and 12 culture days. As can be seen in Fig. 5, MDA-MB-468 cells seeded on 2D surfaces exhibited a rounded shape since their cytoplasm remained flattened. Cells on 7.5% PCL scaffolds displayed a morphology similar to the aforementioned, with a globular appearance. In contrast, a fraction of cells cultured on 15% PCL meshes showed a higher number of cytoplasmic prolongations, adopting as a result a more elongated shape compared with the other culture supports. Regarding nuclear appearance, no qualitative differences were observed among conditions.

In order to analyze possible quantitative differences concerning morphology, nuclear and cytoplasmic elongation factor were calculated (Fig. 6). As previously seen, no differences were observed on nuclear elongation. However, statistically significant differences were detected on cytoplasmic elongation analysis in agreement with previous observations. MDA-MB-468 cells when cultured on 15% PCL scaffolds adopted a more elongated cytoplasm along time in dealing with the other conditions (Fig. 6B).
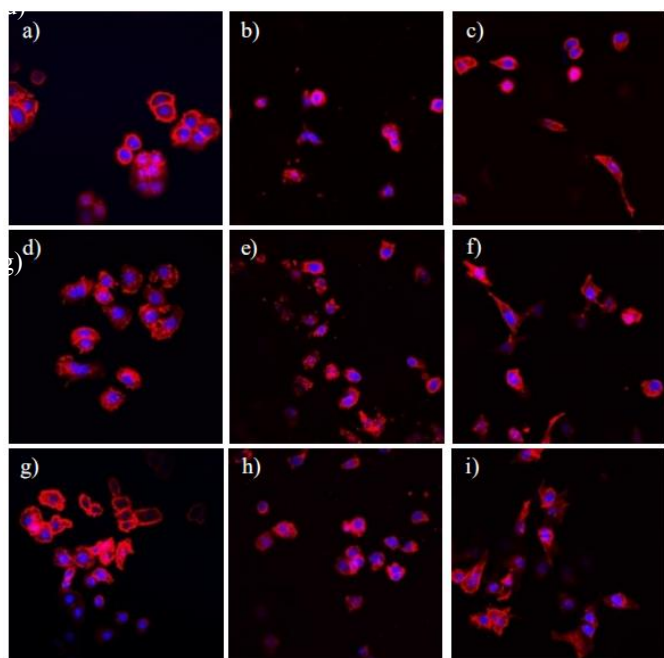

Fig. 5. Fluorescence images of MDA-MB-468 cells cultured during 3 (a-c), 6 (d-f), and 12 days (g-i). Cells were cultured on two-dimensional (2D) adherent coverslips (a, d, and g), or on three-dimensional scaffolds from 7.5% PCL solution (b, e, and h) and 15% PCL solution (c, f, and i). Actin cytoskeleton was stained with rhodamine-phalloidin (red) and nucleus was stained with 4,6-diamidino-2-phenylindole (DAPI; blue). Pictures were captured at a magnification of 400X.
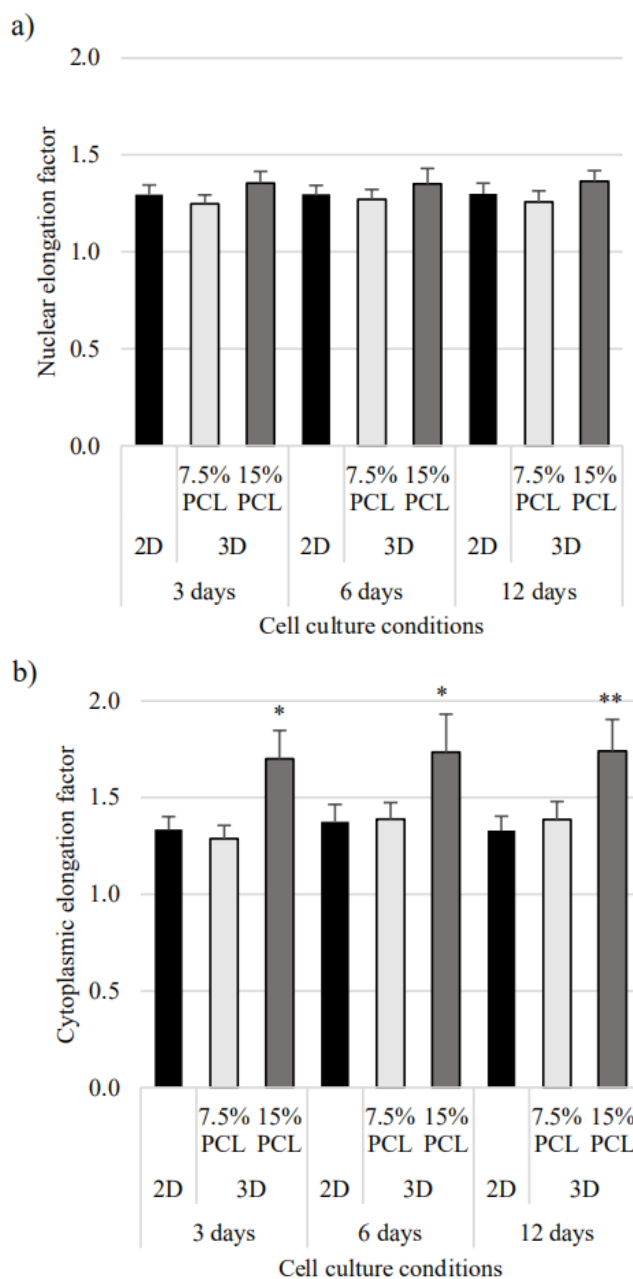
Fig. 6. MDA-MB-468 cell morphology analysis cultured on bidimensional surfaces (2D) and on 7.5% and 15% PCL scaffolds. Nuclear (a) and cytoplasmic elongation (b) were measured. Elongation values from scaffolds were compared with monolayer values and levels of statistically significance were indicated as * ($p < 0.05$) and ** ($p < 0.01$).

4. Conclusion

In this work, electrospun PCL scaffolds are presented as an alternative to monolayer culture regarding CSCs study. Presented data verify the capability of electrospinning as a scaffolds manufacturing process for cell culture applications. DSC and TGA curves of scaffolds almost overlap raw PCL data, indicating the absence of material modification neither contamination. Meshes from 7.5% PCL solution exhibited smaller fiber diameters and the presence of beads. In contrast, 15% PCL scaffolds exhibited greater filament diameters and beads absence. These discrepancies were reflected on cell proliferation analysis. MDA-MB-468 cells presented a higher cell growth rate when cultured on 15% PCL meshes compared with the 7.5% PCL model, as well as a strongest cytoplasmic elongation. Taking all into account, electrospun PCL scaffolds were able to accommodate TNBC cells culture. Therefore, cancer research could take benefit from 3D scaffolds for culture and stemness studies.

Acknowledgements

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