Influence of substrate topography on osteoblasts' behavior: Comparing Solvent Cast and Electrospun Composite Scaffolds

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Abstract

The interaction between cell and material is of crucial importance in the field of biomaterials and tissue engineering. The cell response to surface topography, chemistry and mechanics of the substrate has been extensively investigated [1]. Adhesion, spreading, migration, proliferation and differentiation are the cellular activities that are influenced by material properties.
The present study examines the influence of focuses only on topography. Thus, polymeric composites were manufactured using solvent casting and electrospinning methods. MWCNTs, suitable for bio-applications, were used as a reinforcement. The following substrates, with similar chemistry and different topography, were compared: PCL solvent cast, PCL reinforced with CNTs solvent cast, PCL electrospun and PCL reinforced with CNTs electrospun films. Wharton's Jelly Umbilical Cord Mesenchymal Stem Cells were cultured on the substrates. Proliferation and differentiation of the cells were investigated by determining Alkaline Phosphatase activity and total protein levels. Spreading of MSCs on the substrates was visualized by nuclei and cytoskeleton actin staining.
Randomly distributed fibers of the electrospun scaffolds of 2 μm diameter were clearly shown at SEM micrographs. The CNTs had no remarkable effect on the mechanical properties of the composite materials. Better spreading and proliferation were observed because of the topography of the electrospun fibers and the presence of CNTs.

1. Introduction

The topography of a scaffold appears to influence significantly ~~on~~ cell organization, proliferation, migration and differentiation processes [1]. Electrospinng technique is widely used fabrication method for scaffolds with different topographic textures. Electrospun scaffolds can mimic the hierarchical organized fibrous structure found in the Extracellular Matrix and the fibrous porosity of the scaffolds facilitates nutrient and waste exchange [2].
Poly-caprolactone (PCL), as a biocompatible and non-toxic synthetic aliphatic polyester, can enhance osteoblasts' growth, differentiation and adherence with small manufacturing cost and easy manipulation [3]. In addition, carbon nanostructures (CNTs) can be used for the improvement of the mechanical properties and the cells functions [3].
In this study, four different composite materials were manufactured, PCL film, PCL film reinforced with CNTs, PCL Electrospun and PCL Electrospun reinforced with CNTs, in order to investigate the impact of surface topography on cell behavior.

2. Materials & Methods

2.1 Materials Used

Polycaprolactone (PCL) pellets of Mn = 80,000 g/mol and glacial acetic acid were purchased from Sigma. Pristine MWCNTs suitable for bioapplications were purchased from Nanothinx S.A (Greece). Total protein detection Kits were purchased from Cayman Chemicals (USA) and Alkaline Phosphatase from Sigma. All other chemicals were of reagent grade.

2.2 Fabrication of Scaffolds

Scaffolds were prepared by solvent casting and electrospinning. For PCL scaffolds, a 20% solution was prepared by dissolving Polycaprolactone pellets in glacial acetic acid with gentle heating overnight. For PCL-CNTs nanocomposite scaffolds, MWCNTs in powder form were added in the PCL solution with a ratio of 0.5% wt. CNTs to the polymer weight. PCL-CNTs solutions were left in an ultrasound bath for 4h to help the dispersion of CNTs. Due to hydrolysis of the polymer by acetic acid, all solutions were used within 24 hours.

The electrospinning setup consisted of a syringe pump, a collector and a high voltage supply. For electrospinning PCL solutions, a blunt tip needle with an inner diameter of 0.5mm was used. Due to agglomeration of CNTs in acetic acid and clogging of the needle's tip, a needle with a bigger inner diameter of 0.7mm was used for electrospinning PCL-CNTs solutions. The parameters for both solutions were the same and can be seen in Table 1.

To fabricate solvent cast scaffolds, a glass mold was used. The mold was left on a vibration free, flat surface under a laminar hood for 48h for the solvent to evaporate.

Table 1- Electrospinning Parameters

Parameters	Value
Voltage	20kV
Needle to Collector Distance	20cm
Flow Rate	1ml/h
Temperature	20-25 C
Humidity	40-50%

For further testing, scaffolds were washed in PBS for 24h and sterilized with 70% ethanol solution for 2h and further 30min of UV exposure.

2.3 Physicochemical Tests

SEM analysis was employed for the investigation of the substrate topography (JEOL-JSM 6300). Fiber diameter for the electrospun substrates was calculated using ImageJ software (National Institutes of Health, USA).

Tensile tests were performed up to failure using a Minimat (Rheometric Scientific) testing device. The mean Young's modulus of elasticity and the ultimate stress at failure of the substrates were determined according to ASTM D 882-02 Standard with some modifications. Strips with dimensions 7 mm x 35mm were cut and mounted on the device. The free length was 12mm and the strain rate was 5mm/min.

2.4 Biological Tests

Wharton's Jelly Umbilical Cord Mesenchymal Stem Cells (hMSCs) were donated from Biomedical Research foundation (Academy of Athens). Cells were cultured in appropriate culture medium to

promote their osteogenic differentiation. The culture medium consisted of a-MEM supplemented with 10% FBS, 2mM l-glutamine, 50 μg/ml l-ascorbic acid, 1% v/v amphotericin B, 0.5% v/v gentamicin, 10mM β-glycerophosphate and 10^{-7} M dexamethasone. A 14 mm circle was punched from each film for cell culture. Specimens were placed in 24-well plates and cells were seeded at a density of 50,000 cells/cm^2. Tissue Culture Plastic (TCP) was used as control in all experiments.

Alkaline Phosphatase (ALP) activity was measured using a colorimetric assay after 3 and 7 days of cell culture. The assay is based on the conversion of p-nitrophenyl phosphate (pNPP) into p-nitrophenol, a reaction catalyzed by ALP, which results in the development of a yellow colour in alkaline conditions. A reaction solution was prepared by dissolving a 5mg tablet of pNPP in 5 ml of assay buffer. Samples were removed from culture medium, rinsed twice with PBS and lysed in 1 ml of 0.1% Triton-X. Then, 100μl of lysate was transferred into a 96-well plate and 100μl of reaction solution were added in each well. The plate was incubated in 37 ℃ for 1h and the absorbance was measured in 405nm using a microplate reader (TECAN F200). Total protein was measured using a Total Protein Detection Kit (Cayman,). ALP was divided per Total Protein for normalization of differentiation according to cell number.

Immunocytochemistry was used to visualize the spreading of cells on the substrates at the 7th day of culture. The specimens were removed from culture medium, rinsed twice with PBS, fixed with 4% paraformaldehyde, permeabilized with Triton-X and blocked with bovine albumin. The Actin Cytoskeleton and Focal Adhesion Staining Kit (FAK100; Millipore) which contains a fluorescent-labeled phalloidin (TRITC-conjugated phalloidin) and 4′,6-diamidino-2-phenylindole (DAPI) was used to visualize the cells' actin filaments and nuclei respectively.

3. Results & Discussion

3.1 SEM Analysis

Images taken with a Scanning Electron Microscope showed uniform fiber formation for both PCL and PCL-CNTs scaffolds and can be seen in Figure 1. Fiber diameter and surface pore size are listed in Table 2. Electrospun PCL-CNTs scaffolds had larger fiber diameter in comparison to plain PCL due to the fact that a needle with a larger diameter had to be used in order to achieve clogging free electrospinning of the solution.

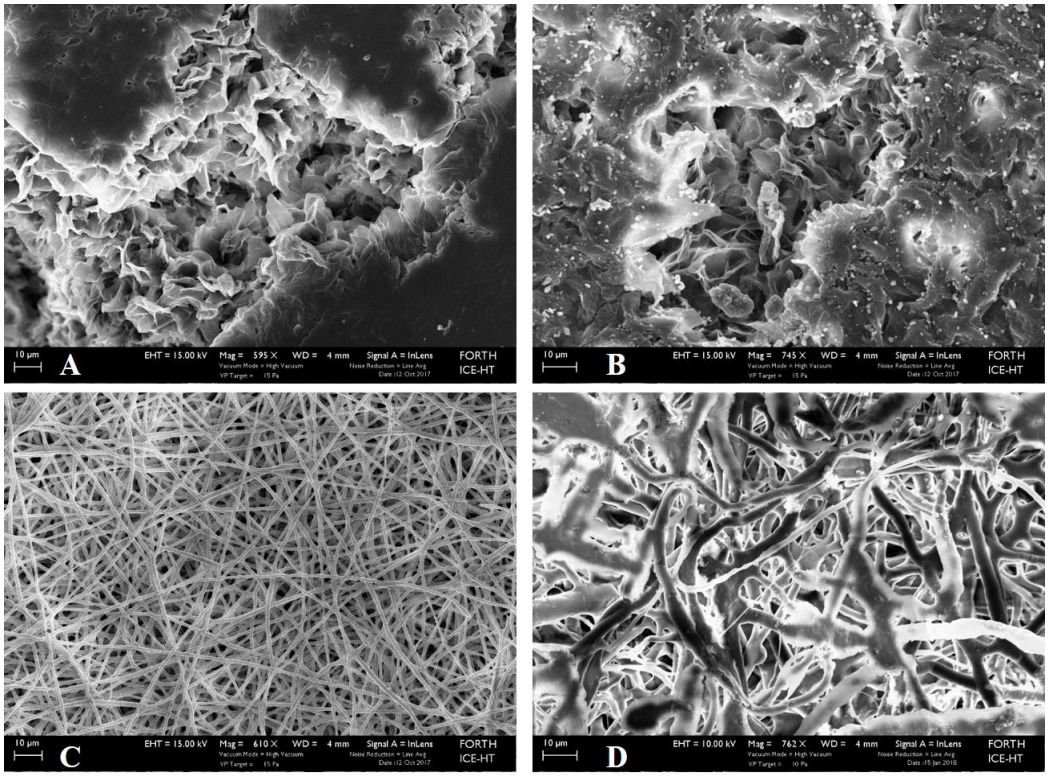

Figure 1- SEM images of scaffolds: a) PCL solvent cast film (PCL SC), b) PCL-CNTs solvent cast film (PCL-CNTs SC), c) PCL electrospun film (PCL ES) and d) PCL-CNTs electrospun film (PCL-CNTs ES).

Table 2- Fiber Diameter and Surface Pore Size for electrospun films

	PCL ES	PCL-CNTS ES
Fiber Diameter (μm)	1.33 ± 0.45	3.13 ± 1.49
Surface Pore (μm)	13.93 ± 3.16	29.47 ± 7.55

3.2 Mechanical Properties

The addition of CNTs did not enhance the mechanical properties of the scaffolds (Table 3), since, in acetic acid, CNTs have very poor dispersion. On the contrary, solvent cast PCL and PCL-CNTs scaffolds displayed much higher mechanical properties in comparison to the corresponding electrospun films.

Table 3- Mechanical Properties of Scaffolds

	PCL SC	PCL-CNTs SC	PCL ES	PCL-CNTs ES
Young's Modulus (MPa)	141.07 ± 26.3	128.59 ± 8.43	8.58 ± 2.87	7.6 ± 2.87
Ultimate Strength (MPa)	11.78 ± 2.11	10.55 ± 3.02	1.51 ± 0.9	2.17 ± 0.91

3.3 Cell Proliferation

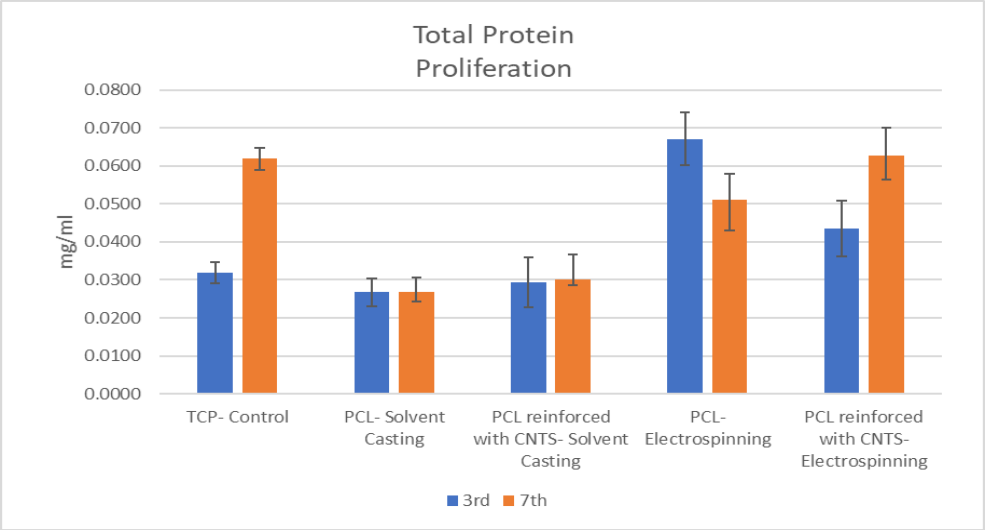

Figure 2- Cell proliferation: Measurement of Total Protein Levels in 3 and 7 days of cell culture on PCL - Solvent Casting, PCL reinforced with CNTs - Solvent Casting, PCL - Electrospinning and PCL reinforced with CNTs – Electrospinning.

Total protein levels were measured as an indicator of cell proliferation for the third and seventh day of cell culture (figure 2). Tissue culture plastic (TCP) was used as a control substrate. The total protein levels on both electrospun substrates, PCL and PCL reinforced with CNTs, were remarkably increased compared to those from the solvent cast scaffolds. (figure 2), cell proliferation was increased up to 7th day.

3.4 Cell Differentiation

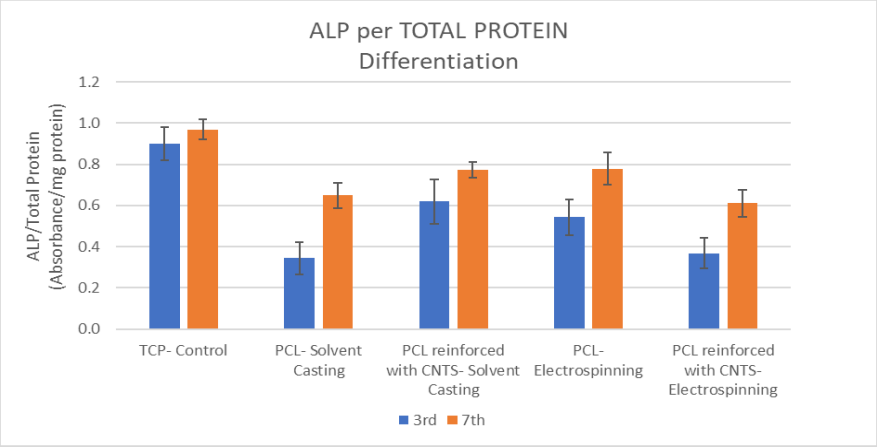

Figure 3- Cell Differentiation: Measurement of Total Protein Levels in 3 and 7 days of cell culture on PCL - Solvent Casting, PCL reinforced with CNTs - Solvent Casting, PCL - Electrospinning and PCL reinforced with CNTs – Electrospinning.

ALP per total protein levels were measured as an indicator of cell differentiation for the 3rd and 7th days of culture (figure 3). TCP was used as a control substrate. Increase in differentiation was

observed on all substrates with time. Slight differences were noticed as for the differentiation of cells because of the method used.

3.5 Cell Spreading

MSCs spreading was investigated by staining the nucleus and the cytoskeleton. In figures 4 to 7, the seventh day of culture is illustrated on PCL solvent cast film, PCL reinforced with CNTs solvent cast film, PCL electrospun film and PCL reinforced with CNTs electrospun film.

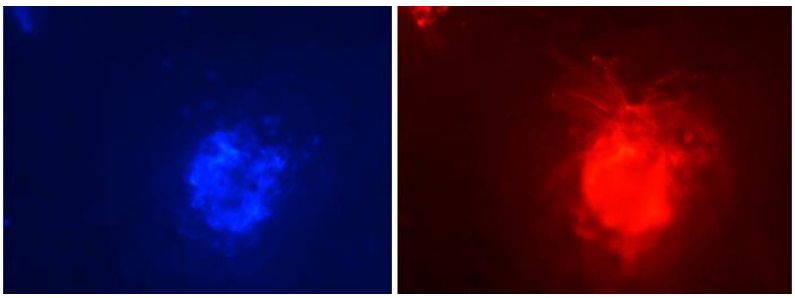

Figure 4- Cells' **nucleus** (stained with DAPI- Blue) **& Cytoskeleton** Actin (stained with Phalloidin- Red) on **PCL** substrate- **Solvent Casting** (Magnification 10x)

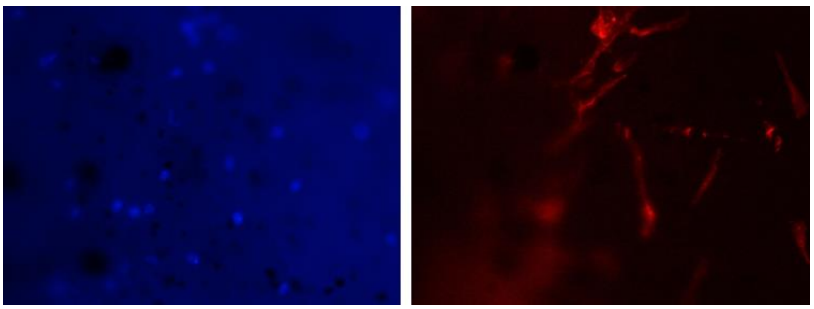

Figure 5- Cells' **nucleus** (stained with DAPI- Blue) **& Cytoskeleton** Actin (stained with Phalloidin- Red) on **PCL reinforced with CNTs** substrate- **Solvent Casting** (Magnification 10x)

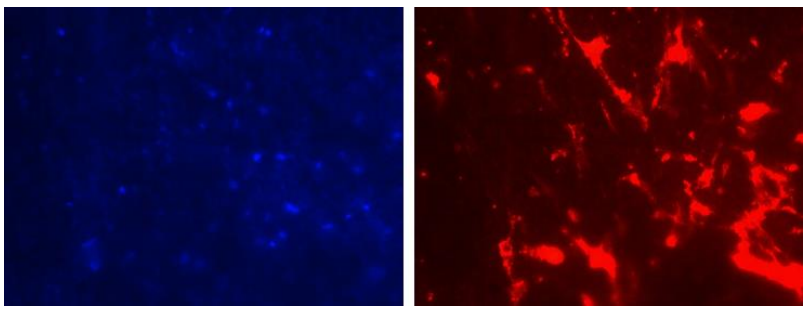

Figure 6- Cells' **nucleus** (stained with DAPI- Blue) **& Cytoskeleton** Actin (stained with Phalloidin- Red) on **PCL** substrate- **Electrospinning** (Magnification 10x)

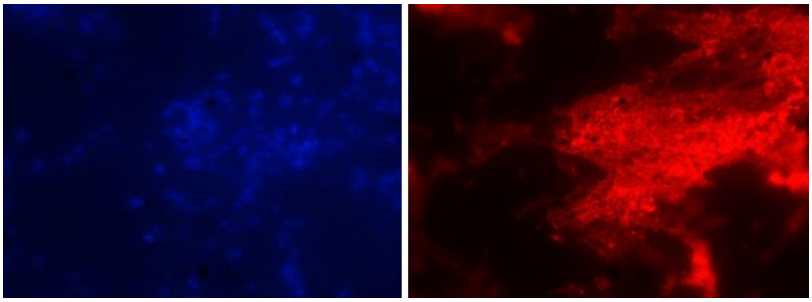

Figure 7- Cells' **nucleus** (stained with DAPI- Blue) **& Cytoskeleton** Actin (stained with Phalloidin- Red) on **PCL reinforced with CNTs** substrate- **Electrospinning** (Magnification 10x)

In the case of PCL solvent cast film, cell spheroids were formed (figure 4). The addition of CNTs in PCL matrix (solvent cast) resulted in better spreading, according to figure 5. It should be mentioned that, topography has a great impact on cell spreading, as depicted in figure 6 and 7.

3. Conclusions

Film formation from a PCL in Acetic Acid solution has proven to be a rather complex procedure, mainly due to the fact that PCL should not stay in solution for too long due to acid hydrolysis.
Randomly distributed fibers of the electrospun scaffolds were clearly shown at SEM micrographs (figure 1). In addition, the CNTs did not have a remarkable effect on the mechanical properties of the composite materials, due to poor dispersion.
Concerning cell behavior, both cell proliferation and differentiation increased on the electrospun fibers and in the presence of CNTs. Spreading was favoured by nanotopography, whereas plain PCL led to spherical shape and agglomeration of the cells.

Acknowledgments

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