

IMPACT OF COMPOSITE SUBSTRATES' MODULUS OF ELASTICITY ON CELL BEHAVIOUR

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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 [1,2]. Adhesion, spreading, migration, proliferation and differentiation are the cellular activities that are influenced by material properties.

Titanium, PCL, PCL reinforced with CNTs substrates were manufactured with the scope of creating a tunable modulus of elasticity. The substrates were divided into two groups; the wide range elasticity group (PCL and Titanium) and the low range elasticity group (PCL and PCL reinforced with CNTs). The aforementioned substrates had the same chemical profile as they were coated with the same protein, fibronectin. 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.

Cells' spreading was remarkably increased in the substrates with high modulus of elasticity. The substrates with the wide range elasticity (PCL and Titanium) displayed more significant differences in cell behaviour than the low range group (PCL and PCL reinforced with CNTs).

1. Introduction

Stiffness is a measure of the ability of a material to resist deformation. The influence of the substrate stiffness on human bone cells morphology, motility and differentiation has been known for decades and recently re-emphasized, as an essential in vivo control parameter in cells biochemical signaling [1,2].

In vitro methods employing primary cultures of human cells specific to the implant sites of prostheses are appropriate and suitable tools for evaluating biocompatibility of materials. Furthermore, this kind of approach can provide indications useful in the design of novel materials as well as in improving the characteristics of the already existing ones [3,4].

In the body, tissue stiffness ranges several orders of magnitude, from adipose tissue (Young's Modulus $E \sim$ several kPa) to bone ($E \sim$ GPa). In order to evaluate the importance of substrate elasticity in biomaterial design, it is critical to test a wide variety of substrata that span physiologically relevant ranges of elasticity [5]. However, for valid results, the substrate's chemical profile and topography should be the same.

In this context, the three main steps of this study are: (i) Manufacturing of polymeric substrates with different stiffness, of the order of GPa, and same chemical structure; (ii) Mechanical characterization of the substrates and (iii) Biocompatibility study.

Two groups of substrates were compared; with Low and High range in Elasticity.

2. Materials & Methods

Pellets of Poly-caprolactone (PCL) with average $M_n=80,000$ were purchased from Sigma Aldrich and pristine MWCNTs (purity $\leq 98.5\%$) suitable for bioapplications were purchased from Nanothinx S.A (Greece). PCL was used to manufacture the polymeric substrates. Carbon Nanotubes (CNTs) reinforced the PCL matrix with a concentration of 1% wt. to the weight of the polymer. A 10% PCL solution was prepared in Acetone by gently heating while mixing for about 3h. The polymeric solution was casted in a glass mold. The PCL films were formed in a few hours after the evaporation of the solvent.

The films were tested for the investigation of the modulus of elasticity. Uniaxial tensile mechanical tests up to failure at fracture were performed, using a Minimat (Rheometric Scientific) testing device. According to ASTM D 882-02 Standard, 40mm x 8 mm x 0.8mm specimens were prepared and tested with a span of 30 mm long and a strain rate of 5mm/min. The mean Young's modulus of elasticity was computed from the linear region of the stress-strain curve. Ultimate stress at failure was also determined from the maximum stress of the same curve.

Titanium substrates were also used. Their mechanical properties were obtained from the literature [7], according to the manufacturer information.

To ensure the same chemical profile for the substrates, they were coated with the same protein, fibronectin. Fibronectin contributes to the construction of the Extracellular Matrix (ECM) and, thus influences ECM structure and composition [6].

Human mesenchymal stem cells were obtained from umbilical cord (hMSCs) were cultured in α – MEM (Gibco) with 10 % fetal bovine serum (Biochrome) supplemented with 1% w/v amphotericin B (Biochrome), 0,5% w/v gentamicin (Biochrome), 10^{-7} M Dexamethasone (Sigma), 10 mM β -glycerol phosphate (Sigma), 50 μ g/ml ascorbic acid (Sigma) and incubated at 37°C in humidified atmosphere of 95% air and 5% CO².

The Alkaline Phosphatase (Sigma S0942) reagent was used for detecting the presence of alkaline phosphatase activity. ALP allows for an initial screening of the osteogenic phenotype. The enzyme-linked immunosorbent assay uses antibodies and change of color to recognize a substance. This simple assay to detect alkaline phosphatase activity uses p-nitrophenyl phosphate (pNPP) as the substrate. p-Nitrophenyl phosphate (pNPP) is a soluble substrate for use with alkaline phosphatase conjugates in ELISA procedures. Alkaline phosphatase hydrolyzes pNPP to p-nitro-phenol and inorganic phosphate. During incubation of the alkaline phosphatase sample and substrate at 37 °C, the reaction is followed by monitoring the increase in absorbance at 405 nm. The measurements took place in the F200-TECAN device.

Cayman detection kits were used to determine Total Protein levels, as a measure of cell proliferation.

ALP was divided per Total Protein for normalization of differentiation according to cell number.

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 detect cell spreading.

3. Results & Discussion

The substrates were divided into two groups according to the magnitude of elasticity. The first group refers to high range of elasticity and consists of PCL and Titanium substrates. PCL and PCL reinforced with CNTs are included in the second group with the low range of elasticity. The chemical profile of the substrates was kept constant by coating their surface with fibronectin.

3.1 Mechanical properties

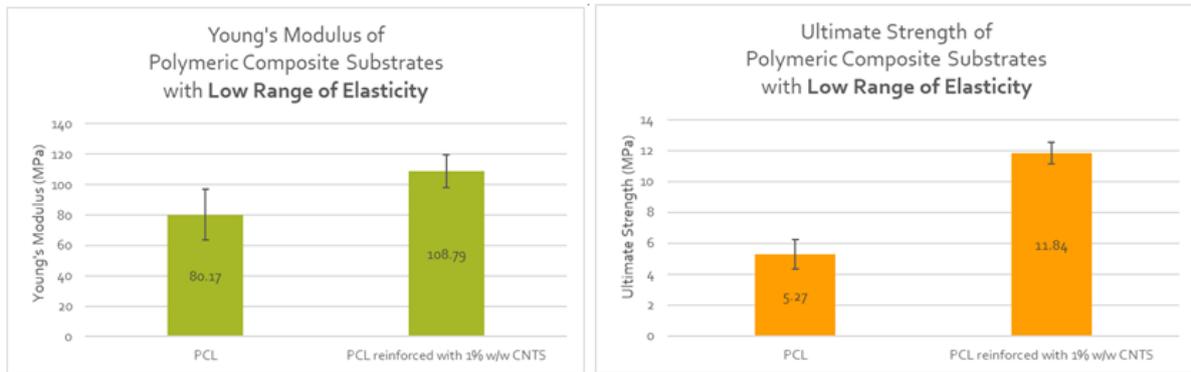


Figure 1- Young's Modulus of the substrate groups: Wide and Low Range of Elasticity



Figure 2- Ultimate Strength of the substrate groups: Wide and Low Range of Elasticity

The CNTs reinforcement had a significant impact on the mechanical properties of the PCL. Specifically, a 36% increase in the modulus of elasticity was observed due to the presence of 1% wt. of CNTs in the PCL matrix (figure 1). The ultimate strength of the PCL matrix was increased up to two times, because of the CNTs reinforcement (figure 2).

As for the group with the wide range of elasticity, the Titanium elasticity was 3 orders of magnitude greater than the PCL substrate (figure 1). The same trend was observed for the ultimate strength of the substrates with the low range group (figure 2).

3.2 Cell behaviour

3.2.1. Cell number/proliferation

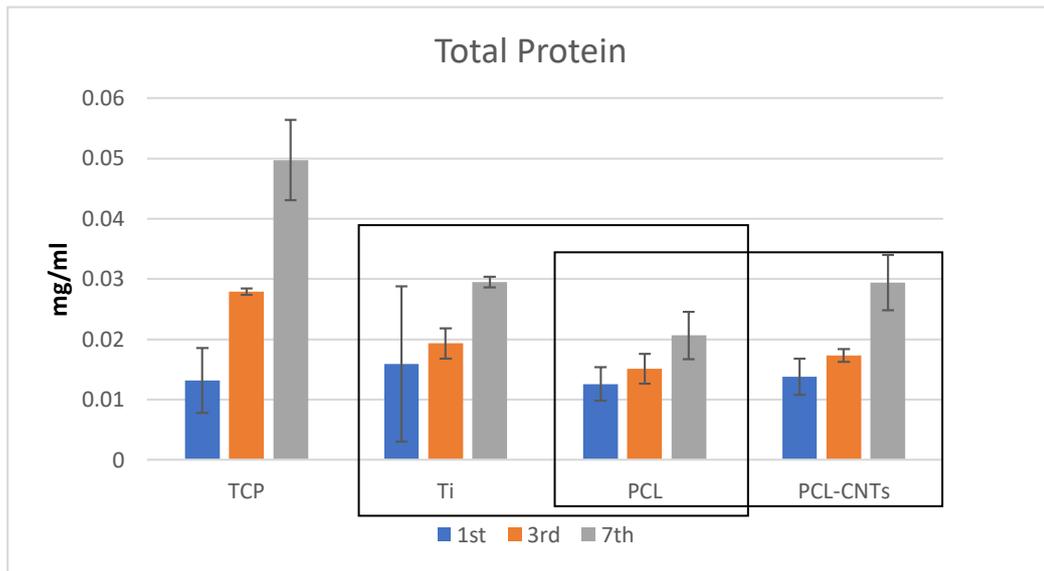


Figure 3- Cell proliferation: Measurement of Total Protein Levels in 1, 3 and 7 days of cell culture on substrates of wide (first frame) and low (second frame) elasticity.

Total Protein levels were measured as an indicator of cell proliferation for the first, third and seventh day of cell culture. Tissue culture plastic (TCP) was used as a control substrate. The first frame contains Titanium and PCL substrates, which have a wide range of elasticity. On the contrary, the second frame with the PCL and PCL reinforced with CNTs substrates represents the group of lower range of elasticity.

The cell proliferation for all the substrates increased for the duration of the experiment, which is a good indicator of cell metabolism. In the first frame, greater proliferation was observed on the substrate with the greater elasticity. Similar trend was also observed in the group with the low range of elasticity.

3.3.2. Cell differentiation

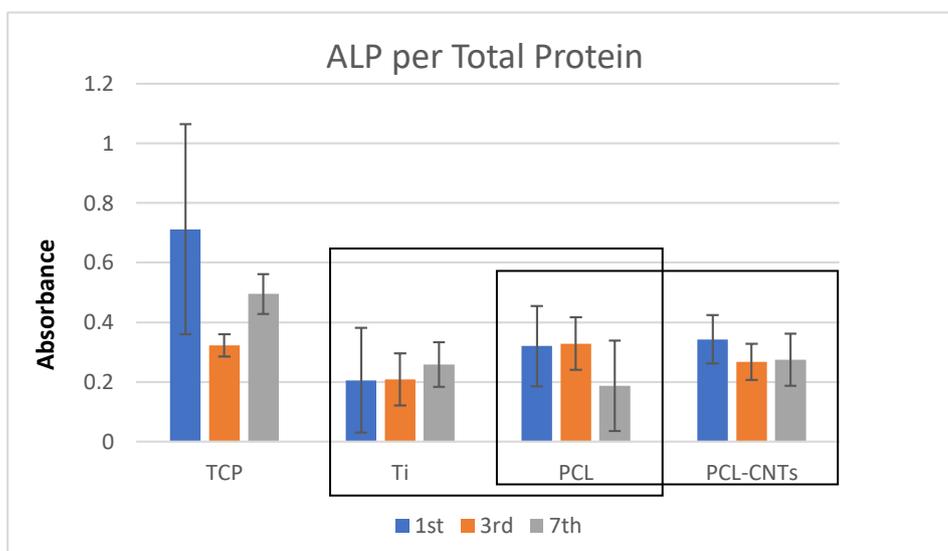


Figure 4- Cell differentiation: Measurement of ALP per Total Protein Levels in 1, 3 and 7 days of cell culture on substrates of wide (first frame) and low (second frame) elasticity

ALP per Total Protein levels were measured as an indicator of cell differentiation for the first, third and seventh day of cell culture. Tissue culture plastic (TCP) was used as a control substrate. The first frame contains Titanium and PCL substrates, which have a wide range of elasticity. On the contrary, the second frame with the PCL and PCL reinforced with CNTs substrates represents the group of lower range of elasticity.

In the group of the first frame, proliferation was prioritized over differentiation on the substrate with the greater elasticity (titanium). As for the second group with the low range of elasticity, slight differences were observed between the less (PCL) and the more (PCL-CNTS) stiff substrates.

3.2.3. Cell Spreading - Staining

MSCs spreading was investigated by staining the nucleus and the cytoskeleton for 3 and 7 days of culture. In figures 5 to 8, the third day of culture is illustrated on TCP (Control), PCL, PCL reinforced with CNTs and Titanium respectively. As for the seventh day of culture, the results are depicted on figures 9 to 12. It should be mentioned that PCL substrate is simultaneously part of the group of low and of the wide range of elasticity.

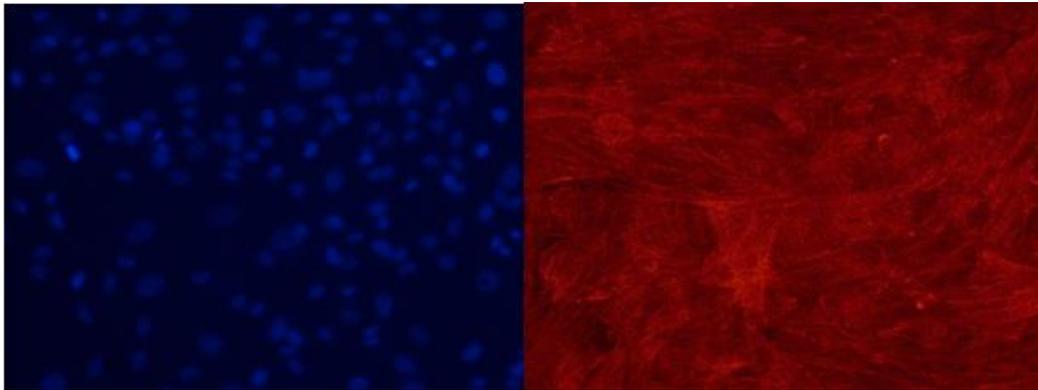


Figure 5- 3rd Day of culture- Cells' nucleus (stained with DAPI- Blue) & Cytoskeleton Actin (stained with Phalloidin- Red) on Tissue Culture Plastic - Control substrate (Magnification 10x)

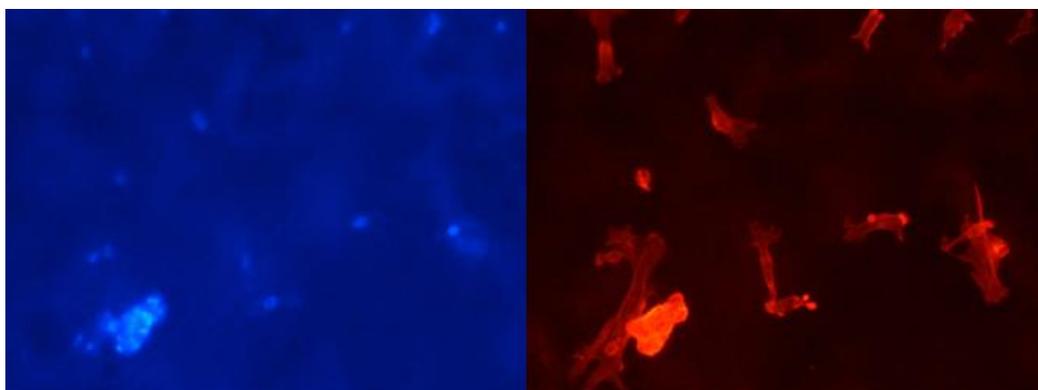


Figure 6- 3rd Day of culture- Cells' nucleus (stained with DAPI- Blue) & Cytoskeleton Actin (stained with Phalloidin- Red) on PCL substrate (Magnification 10x)

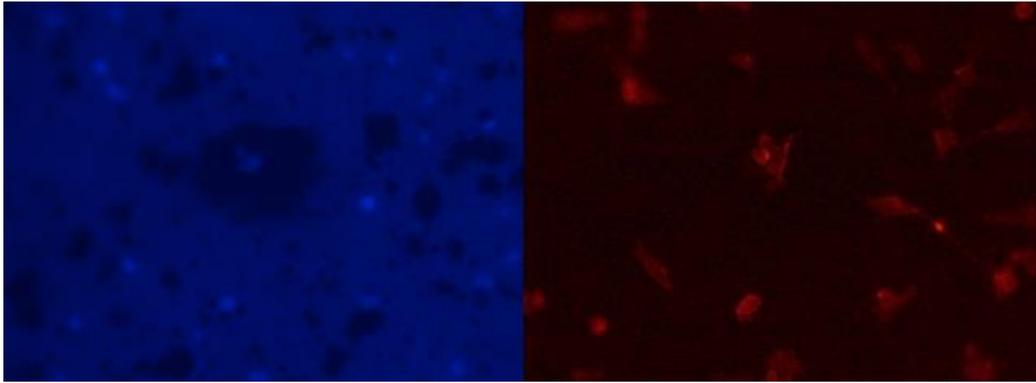


Figure 7- 3rd Day of culture- Cells' nucleus (stained with DAPI- Blue) & Cytoskeleton Actin (stained with Phalloidin- Red) on PCL reinforced with CNTs substrate (Magnification 10x)

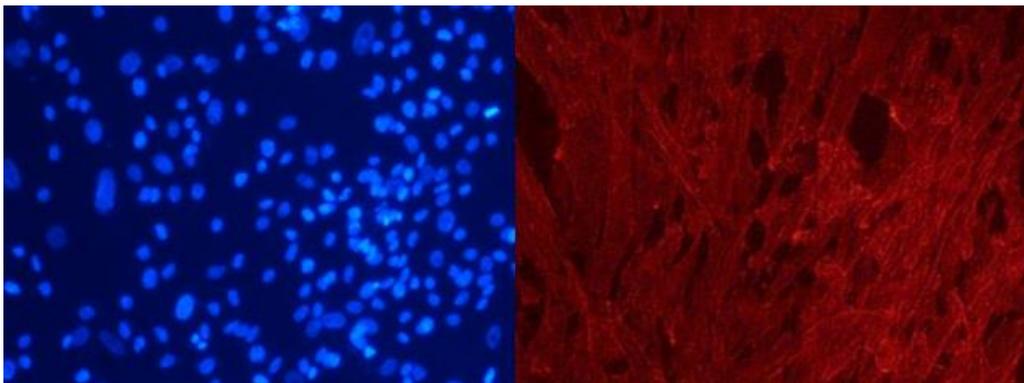


Figure 8- 3rd Day of culture- Cells' nucleus (stained with DAPI- Blue) & Cytoskeleton Actin (stained with Phalloidin- Red) on Titanium substrate (Magnification 10x)

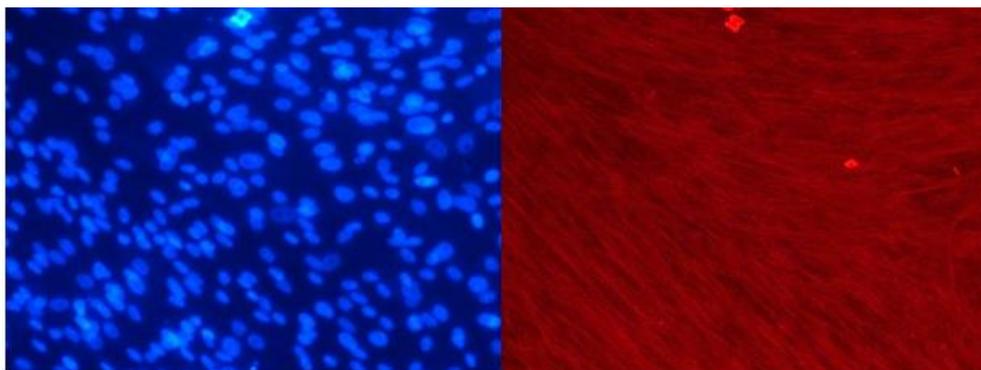


Figure 9- 7th Day of culture- Cells' nucleus (stained with DAPI- Blue) & Cytoskeleton Actin (stained with Phalloidin- Red) on Tissue Culture Plastic- Control substrate (Magnification 10x)

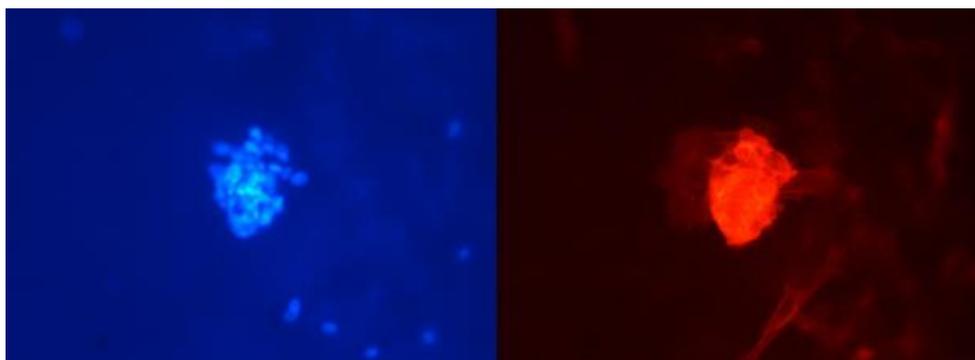


Figure 10- 7th Day of culture- Cells' nucleus (stained with DAPI- Blue) & Cytoskeleton Actin (stained with Phalloidin- Red) on PCL substrate (Magnification 10x)

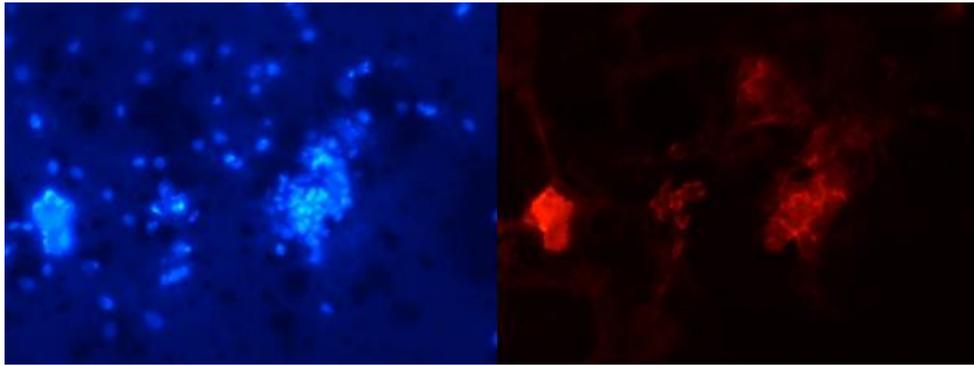


Figure 11- 7th Day of culture- Cells' **nucleus** (stained with DAPI- Blue) & **Cytoskeleton Actin** (stained with Phalloidin- Red) on **PCL reinforced with CNTs** substrate (Magnification 10x)

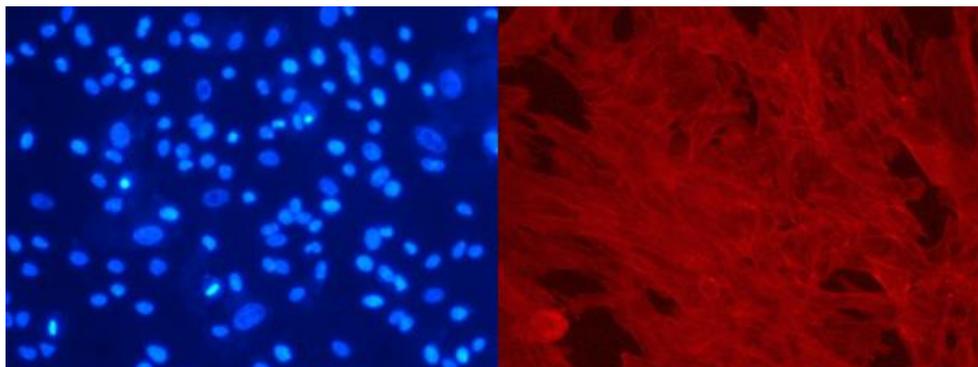


Figure 12- 7th Day of culture- Cells' **nucleus** (stained with DAPI- Blue) & **Cytoskeleton Actin** on **Titanium** substrate (stained with Phalloidin- Red) (Magnification 10x)

MSCs cultured on PCL and PCL reinforced with CNTs substrates tend to agglomerate (figures 6,7, 9,10). Comparing the 3rd and the 7th day of culture of each substrate, cell proliferation and spreading was multiplied the 7th day, which indicates the viability of MSCs.

Between PCL and PCL reinforced with CNTs, cell spreading was slightly enhanced on the stiffer substrate. Titanium substrate increased remarkably the cell spreading comparing with the PCL substrate, at both 3rd and 7th day of culture.

4. Conclusions

The CNTs are a remarkably good reinforcement for the PCL matrix, as an increase in the Modulus of Elasticity and in the Ultimate Strength was detected.

MSCs cultured on the substrates appear to have increased Total Protein levels in the course of time, which is a good indicator of cell metabolism. Greater proliferation was observed on the substrate with the greater elasticity. As for the differentiation, slight differences were observed among the substrates.

In addition, different cell spreading areas were noticed among the substrates with various Modulus of Elasticity. Cell spreading was enhanced in the substrate with the highest Stiffness.

Finally, the substrates with the wide range elasticity (PCL and Titanium) have shown more significant differences in cell behaviour than the low range group (PCL and PCL reinforced with CNTs), whereas the substrates of the low range group has the same matrix.

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