"Impact of electrospun Tecophilic/gelatin scaffold biofunctionalization on proliferation of vascular smooth muscle cells"

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Abstract

Nanofibrous composite scaffolds based on Tecophilic (TP) and gelatin (gel) were electrospun and further modification of their surfaces was performed by adsorption of gelatin or fibronectin biomolecules. The ability of coated scaffolds to alter the proliferation rate of smooth muscle cells (SMCs) was investigated via various assays and compared to cell proliferation on non-coated scaffolds. The results confirmed the potential of both coated and non-coated composite scaffolds to support SMC growth. Although, the presence of fibronectin increased the proliferation, adsorbed gelatin could reduce the proliferation of SMCs. The success of a tissue engineered vascular graft depends on the ability of the scaffold to control the proliferation rate of SMCs and thus our study provides a better insight towards the fabrication of functional constructs for vascular regeneration.

Keywords: Electrospun scaffold, Smooth muscle cells, Proliferation, Gelatin coating, Fibronectin coating

1. Introduction

Extracellular matrix (ECM) is an intricate network of fibrillar and nonfibrillar macromolecules surrounding the cells consisted of collagen and elastin fibers along with proteoglycans and glycoproteins which provides both structural and regulatory functions to the cells. ECM provides mechanical support to the tissue and affects various aspects of cellular behavior including the adhesion, proliferation, and differentiation of cells through mechanical and biochemical signals [1, 2].

Electrospun nanofibrous scaffolds can mimic the physical dimension and architecture of native ECM in terms of geometry, morphology and topography, while the biological functionality of the nanofibers is considered a major challenge during the development of scaffolds for tissue engineering. Bioactive materials offer a convenient way to control the cell-material interactions [3] depending on the type of tissues or cells.

Vascular smooth muscle cells (SMCs) regulate their phenotype in response to environmental chemical, physical and mechanical signals. During our previous studies we showed that a composite scaffold electrospun using TP and gelatin at a weight ratio of 70:30 could not only support the contractility of SMCs, but also provide appropriate mechanical properties comparable with those of native blood vessels [4, 5]. TP is a family of hydrophilic polyether-based thermoplastic aliphatic polyurethane which is thromboresistance and possesses inherent elasticity [6] suggesting its application as a vascular tissue engineered graft. It was found that addition of gelatin containing many integrin binding sites to TP can improve the potential of the composite scaffold to modulate the behavior of SMCs [4, 5].

The proliferation of SMCs rarely happens under normal physiological conditions but these cells can grow very fast under pathological conditions of some cardiovascular diseases like atherosclerosis [7]. It is essential that a vascular tissue engineered construct possess contractile SMCs such that the proliferation of SMCs be controlled to prevent the recurrence of the disease [8]. Previously we found that the TP(70)/gel(30) scaffold could direct SMCs

towards a contractile phenotype due to its softness and presence of cell adhesion ligands within the fibers [4]. Here, we have studied the proliferation of SMCs in response to biomolecules physically adsorbed on the surface of TP(70)/gel(30) scaffolds. The differences in the interaction of SMCs cultured on TP(70)/gel(30) scaffolds coated with either gelatin or fibronectin in comparison with non-coated TP(70)/gel(30) scaffolds are explored.

2. Materials and methods

2. 1. Materials

TP was a kind gift from Lubrizol. Gelatin type A (300 Bloom) from porcine skin, gelatin solution from bovine skin, 1,1,1,3,3,3-hexafluoro-2-propanol (HFP), and phosphate buffered saline (PBS) were all purchased from Sigma- Aldrich. Fibronectin solution was obtained from Invitrogen. Human aortic SMCs and smooth muscle cell medium (SMCM) were obtained from ScienCell Research Laboratories.

2. 2. Fabrication of nanofibrous scaffolds

The method of electrospinning has been described in our previous studies [4, 5]. Briefly, respective polymer solutions prepared in HFP, either TP alone (8% w/v) or TP/gel solution (8% w/v, with the blend ratio of 70:30 w/w, TP(70)/gel(30)) were individually loaded into a 3 mL plastic syringe fitted with a 27 G blunted stainless steel needle. A high voltage of 10 kV was applied to the polymer solution fed at a constant flow rate of 1 mL/h using a syringe pump. The distance between the syringe needle tip and a grounded flat collector wrapped in aluminum foil was kept at 12 cm, and nanofibers were collected on the 15 mm cover slips placed on the collector. Electrospun scaffolds were transferred to a vacuum desiccator at ambient temperature for at least 48 h to eliminate residual solvents.

2. 3. Analysis of nanofibers

The morphology of the electrospun scaffolds was observed under scanning electron microscope (SEM; FEI-QUANTA 200FQ12, Netherlands) at an accelerating voltage of 10

kV. Before observation, each sample was coated with gold by a sputter coater (JEOL JFC-1600 Auto fine coater, Japan). Based on the SEM images, fiber diameter was determined using image analysis program (Image J, National Institutes of Health, USA).

The surface roughness of the substrates was quantitatively evaluated using atomic force microscopy (AFM, Dimension 3100, Digital Instruments, USA) in a dynamic force mode. Silicon nanoprobe tips (Nanosensors, Switzerland) with a resonant frequency near 330 kHz were used. The root mean square (RMS) roughness as one of the roughness parameters in three random areas of each surface with dimensions of 50 μm × 50 μm (x, y directions) analyzed at scan rate of 0.5 Hz and 256 scanning lines was calculated and reported by Nanoscope imaging software (version 5.30, Digital Instrument, Inc.).

The chemical composition of the nanofibrous surface was characterized using ATR-FTIR spectroscopy (Thermo Nicolet, Waltham, MA). The transmittance of samples was recorded between 400 cm^{-1} and 4000 cm^{-1}, with a resolution of 4 cm^{-1}.

2. 4. Scaffold functionalization and cell seeding

Human aortic SMCs were normally cultured in a 150 cm2 cell-culture flask using smooth muscle cell medium (SMCM) containing basal medium complemented with 2% fetal bovine serum (FBS), 1% smooth muscle cell growth supplement (SMCGS), and 1% penicillin/streptomycin solution (P/S). The SMCs were incubated at 37º C in a humidified atmosphere containing 5% CO2 for 7 days and the culture medium was changed once in every 3 days.

The electrospun nanofibrous scaffolds collected on glass cover slips of 15-mm diameter were placed in 24-well plates and pressed with a stainless steel ring to prevent swelling and shrinkage. The fibers were sterilized under UV light for 2 h and washed thrice with PBS for 5 min each in order to remove any residual solvent. The composite samples (TP(70)/gel(30)) were divided into three groups. Subsequently, electrospun TP and one group of

TP(70)/gel(30) scaffolds along with tissue culture polystyrene (TCP) were immersed in 200 µL of SMCM overnight before cell seeding. Furthermore, two other groups of composite scaffolds were submerged in 200 µL of either gelatin solution or fibronectin solution (diluted in SMCM to obtain 10 µg/well) overnight before cell seeding. The cultured SMCs were trypsinized by trypsin-EDTA, counted with trypan blue using hemocytometer and 200 µL of cell suspension containing 1 × 104 cells were pipetted onto the upper surface of each well and allowed to attach for 24 h before adding extra SMCM to reach 1 mL medium/well.

2. 5. Cell viability

After 7 days of culturing cells on the scaffolds or control wells, cells were stained using the Live/Dead Viability/Cytotoxicity Kit for mammalian cells (Molecular Probes, Invitrogen) which contains calcein AM and ethidium homodimer-1 (EthD-1) to stain live and dead cells with green and red fluorescent dyes, respectively. The staining solution was prepared by adding 20 µL of 2 mM EthD-1 solution and 5 µL of 4 mM calcein to 10 mL of PBS. Cell-scaffold constructs cultured for 7 days were washed with PBS and incubated with 200 µL of staining solution for 30 min at 37° C. Then, samples were again washed with PBS and imaged with a Leica DM IRB fluorescent microscope.

2. 6. Cell proliferation

The adhesion and proliferation of cultured SMCs on coated and non-coated scaffolds as well as TCP were evaluated by the colorimetric MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay using CellTiter 96 AQueous One solution (Promega, Madison, WI). After culturing the cells for a period of 1, 4, 7, and 10 days, cell-seeded constructs were rinsed with PBS to remove unattached cells and incubated with 1 mL of serum free medium containing 20 % of MTS reagent at 37° C for 3 h. Absorbance of the obtained dye was measured using a spectrophotometric plate reader (FLUOstar Optima, BMG Lab Technologies, Offenburg, Germany) at 490 nm.

2.7. Cell morphology

After 7 days of cell culture, morphological characteristics of SMCs seeded on electrospun scaffolds and TCP were studied by SEM. Cell-seeded constructs were washed with PBS and fixed with 3 % glutaraldehyde (Sigma-Aldrich) for 3 h. Fixed samples were further rinsed with deionized water and exposed to a gradient of ethanol (50%, 70%, 90%, and 100%) followed by treating with hexamethyldisilazane. Finally, specimens were air dried in a fume hood and examined using SEM.

2.8. Statistical analysis

All data presented are expressed as mean ± standard deviation (SD) of the mean. One-way ANOVA combined with Tukey post hoc tests was carried out for multiple-comparison of different samples. A value of $p<0.05$ was considered statistically significant.

3. Results and discussion

3.1. Characterization of nanofibrous scaffolds

Using electrospinning technique, nanoscale and beadless TP fibers and TP/gel blended fibers were fabricated. Figure 1 provided SEM micrographs of porous and uniform interconnected structures of electrospun scaffolds along with fiber diameter and size distribution.

SEM images clearly demonstrated that the fiber diameter significantly decreased from 881±228 nm to 409±150 nm and the diameter distribution broadened after adding gelatin to composition which might be due to the role of amino acids provided by gelatin to improve the stretching force and self-repulsion and decreasing the viscosity of the composite solution[9].

The arterial wall tissue possesses a high degree of nanometer surface roughness [10] and electrospun fibers can provide surface roughness on a nanometer scale [11]. The roughness of a biomaterial based graft is an important factor due to its effect on various cellular behaviors such as protein adsorption, cell attachment and cell proliferation [12, 13]. Similar to results

reported by Milleret et al. [14], surface roughness increased with increasing constituent fiber diameter (Figure 2). The averaged RMS roughness of the electrospun TP and TP(70)/gel(30) surfaces was 861±90 nm and 514±37 nm, respectively which corroborated significant smoother surface of the composite scaffold compared to pure one.

The FTIR spectra of the electrospun scaffolds are shown in Figure 3. For pure TP, the bands at about 2935 cm-1 and 2856 cm-1 attributed to asymmetric and symmetric stretching vibration of – CH2 –, respectively. Urethane C = O stretching was visible at 1703 cm-1 and the bands at 1530 cm-1 and 1310 cm-1 were both responsible for urethane N – H bending and C – N stretching. In addition, the band at 1450 cm-1 was assigned to – CH2 – bending vibration. The strong characteristic bands appeared at about 1110 cm-1 and 1080 cm-1 arose from stretching of C – O – C bond, respectively in aliphatic ether of soft and hard segments of TP. The composite scaffold exhibited similar IR peaks with those of the pure TP matrix [15-17]. In addition, two typical peaks were observed at 1650 cm−1 and 1540 cm−1 of the composite scaffold spectrum corresponding to amide I band and amide II band of the gelatin, respectively [9, 18].

3.2. Cell growth and morphology

Using biomolecules mimicking the natural ECM is a promising approach for tissue engineering. The behavior of cells including cell adhesion, proliferation and differentiation in contact with bioactive motifs of ECM or other biomolecules attached to the surface of the scaffold can partially influence the profile of tissue reconstruction. Vasoactivity of contractile SMCs through vascular regeneration may enable the production of functional tissue engineered blood vessels. We explained the potential of the scaffolds to control the phenotypic modulation of SMCs in our previous studies and indicated preservation of SMCs contractility on electrospun TP(70)/gel(30) scaffolds [4, 5]. While, proliferation of SMCs seeded into scaffolds is generally essential to regenerate a vascular tissue, uncontrollable

proliferation in the implanted graft can cause vessel wall thickening and narrowing of the vessel lumen [7]. In this study, we focused on the surface modification of electrospun TP(70)/gel(30) scaffolds with either gelatin or fibronectin physically adsorbed on the surface to investigate their effects towards SMCs proliferation.

Survival and retention of SMCs on the coated and non-coated composite scaffolds were determined by cell viability assay after 7 days of cell culture. In this assay live cells are distinguished by the presence of ubiquitous intracellular esterase activity determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within the live cells, producing an intense uniform green fluorescence in live cells. EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence thereby producing a bright red fluorescence in dead cells upon binding to nucleic acids. EthD-1 is excluded by the intact plasma membrane of live cells. As evident in Figure 4, cells survived well and a small number of dead cells was observed on all scaffolds confirming their biocompatibility without producing toxic effects, however the lack of sufficient cell recognition ligands on the pure TP scaffold resulted in the poor attachment and proliferation of cells on this construct. SMCs demonstrated aligned orientation and parallel alignment on composite scaffolds rather than on pure TP and TCP confirming the maintenance of contractile phenotype of SMCs on coated and non-coated electrospun TP(70)/gel(30) scaffolds. The growth and mitochondrial metabolic activity of SMCs on the scaffolds assessed by MTS assay after 1, 4, 7, and 10 days of cell culture were shown in Figure 5. It was observed that cells on all scaffolds adhered to some extent, began to proliferate after culturing for 1 day and continued to increase in number over time. The results illustrated that non-coated, gelatin coated, and fibronectin coated composite scaffolds were able to support SMCs proliferation in a greater level than on pure TP scaffolds. The proliferation of SMCs on

TP(70)/gel(30)-gelatin coated and TP(70)/gel(30)-fibronectin coated scaffolds displayed significantly lower and higher level, respectively, compared to on TP(70)/gel(30)-non coated on days 4 and 7. After culturing for 10 days, significant differences in cell number on non-coated and gelatin coated scaffolds were also observed. Although there was an increase in cell proliferation on fibronectin coated scaffolds compared to non-coated ones, this increase was not statistically meaningful at day 10. Generally, among all the composite scaffolds, the highest cell proliferation rate was obtained on fibronectin coated constructs while it was significantly lower than proliferation on TCP at days 4, 7, and 10. This findings suggested that TP(70)/gel(30) scaffolds can provide a suitable substrate for SMCs, and physically adsorption of various biomolecules on their surface makes researchers able to obtain desirable proliferation rate. This finding in accordance with finding of other researchers [19-21] confirmed the behavioral regulation of the cells cultured on scaffolds via the involvement of specific integrin/ECM interaction. Additionally, as suggested by Kim et al., it was observed that initial interaction of SMCs with the scaffold can influence the long-term behavior of the cells which is due to setting off a chain of signaling events through the initial cell contact with the scaffold led to long-term changes in gene expression and tissue composition [21].

The morphology of SMCs on electrospun scaffolds was further investigated via SEM. Results presented in Figure 6 revealed SMCs attachment on composite scaffolds after 7 days of cell culture which is consistent with results of viability and MTS assays. Although the SMCs spread on coated and non-coated constructs showed similar morphologies with aligned organization compared to the cell morphology appeared on TP and TCP, cell proliferation rate on these composite constructs (covered area by cells) was the main difference of them.

The proliferation of CMCs on composite scaffolds increased in the following order; TP(70)/gel(30)-fibronectin coated> TP(70)/gel(30)-non coated> TP(70)/gel(30)-gelatin

coated suggesting the ability of biomolecules to control proliferation of SMCs seeded on the same scaffolds with only different biomolecules modifying their surfaces. This simple method might have a valuable outcome for appropriate regeneration of vascular tissues engineered grafts.

4. Conclusion

This study has demonstrated important role of the specific cell adhesion biomolecules including gelatin and fibronectin coupled with the surface of electrospun TP(70)/gel(30) scaffolds in the control of SMC proliferation to engineer new functional vascular tissues. Results showed that fouling of surface by adsorption of fibronectin biomolecules may promote the surface for enhanced cell growth while presence of gelatin biomolecules on the surface of the scaffold leads to a decreasing propensity in proliferation of SMCs. Therefore, TP(70)/gel(30)-gelatin coated scaffolds can be a more attractive candidate for development of a functional vascular graft with low proliferated SMCs in the wall.

5. References

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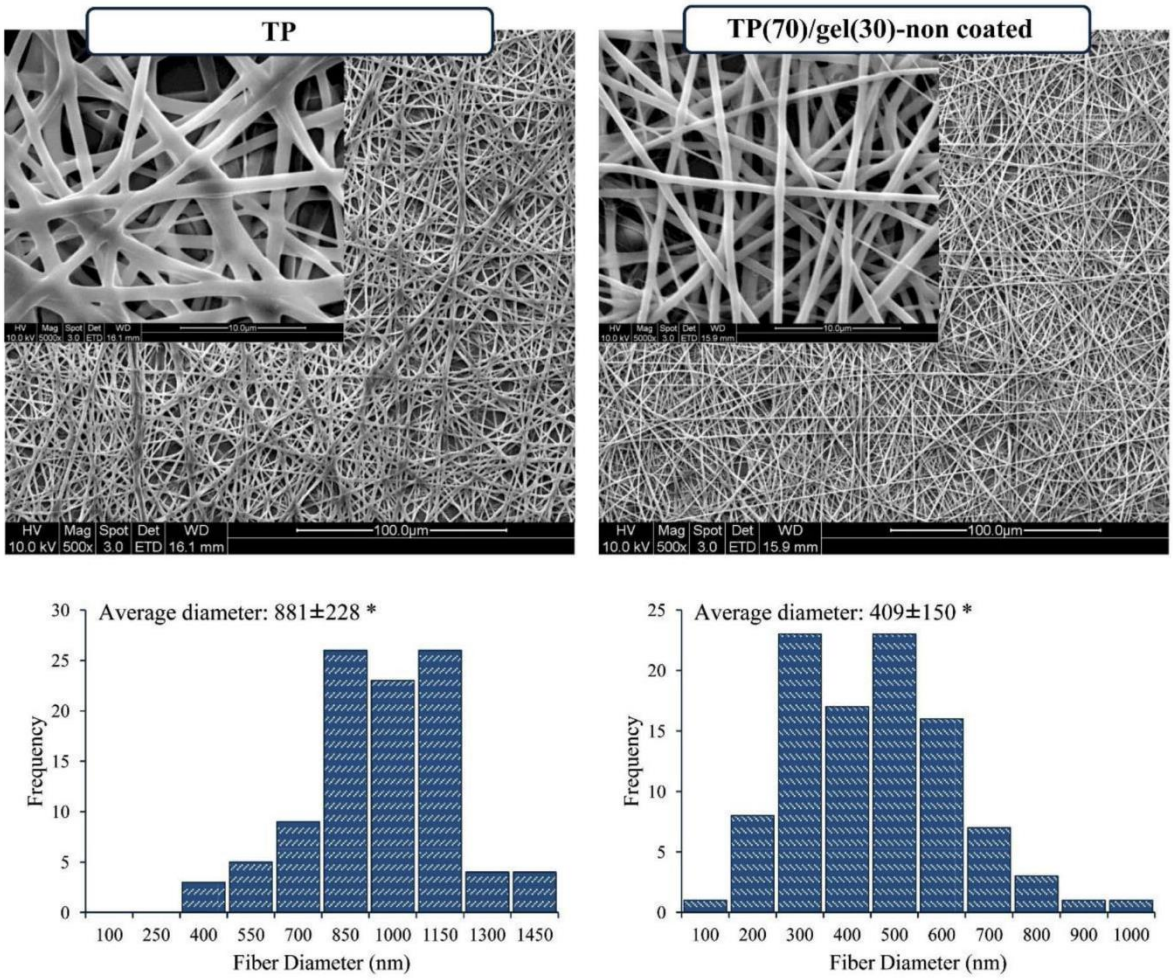

Figure 1. Morphology and fiber diameter distribution histogram of electrospun TP and TP(70)/gel(30) scaffolds. * Significant differences at $p<0.05$

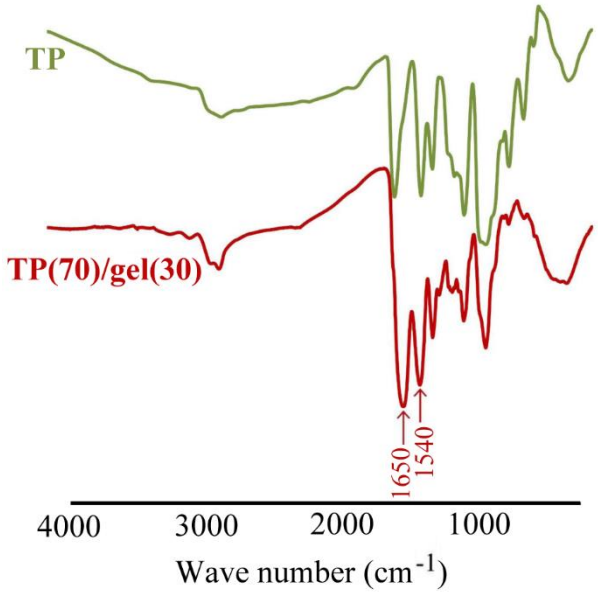

Figure 2. FTIR spectra of electrospun TP and TP(70)/gel(30) nanofibers.

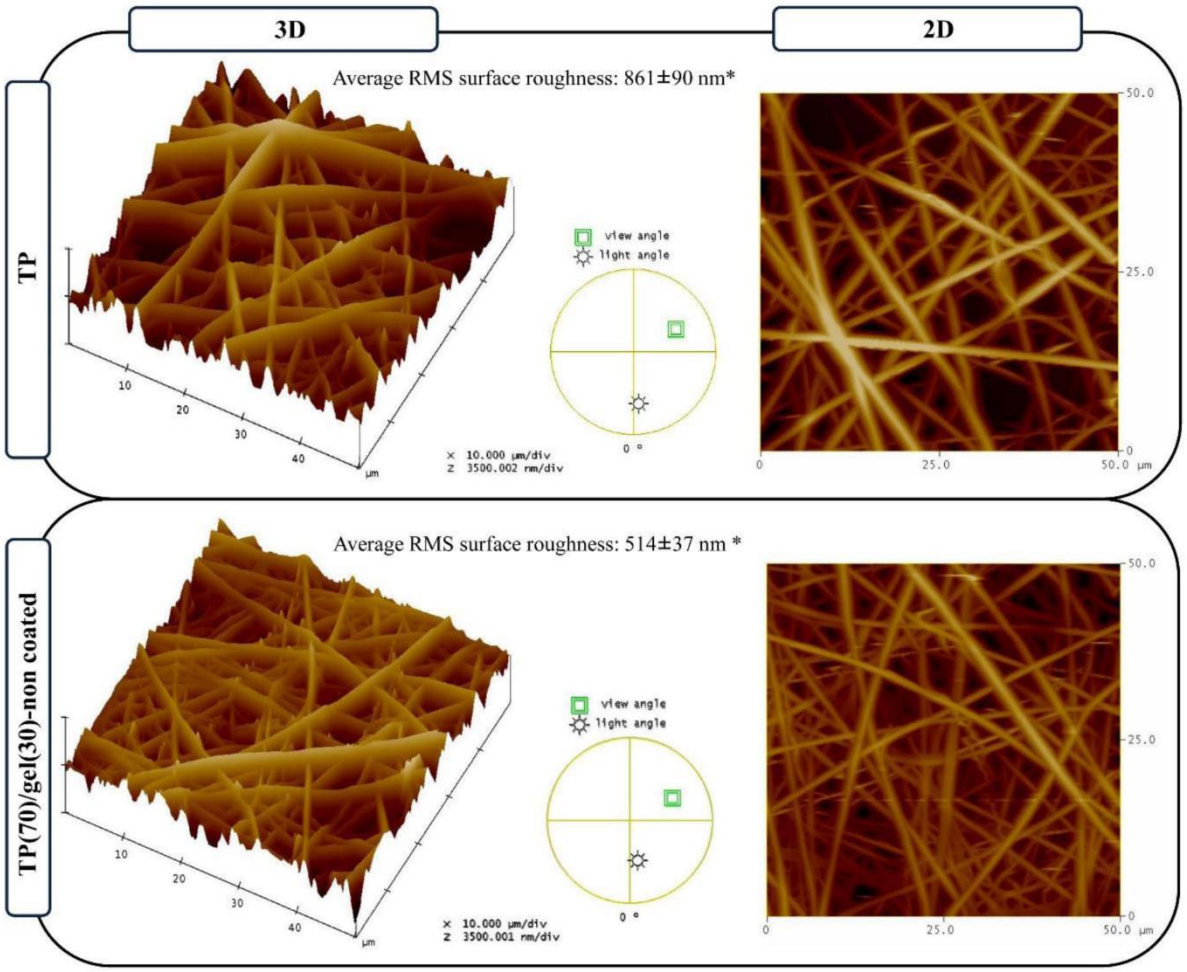

Figure 3. Representative 3D and 2D AFM micrographs of electrospun TP and TP(70)/gel(30) nanofibers. * Significant differences at $p<0.05$

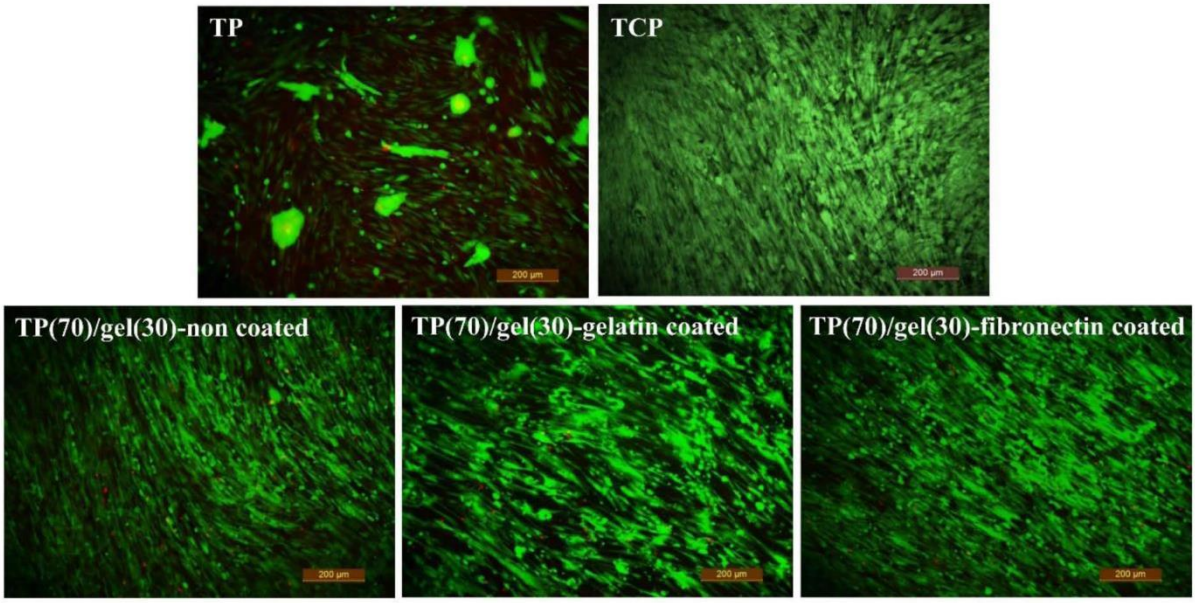

Figure 4. Cell survival and morphology of SMCs grown on electrospun TP, TP(70)/gel(30)-non coated, TP(70)/gel(30)-gelatin coated, TP(70)/gel(30)-fibronectin coated scaffolds and TCP.

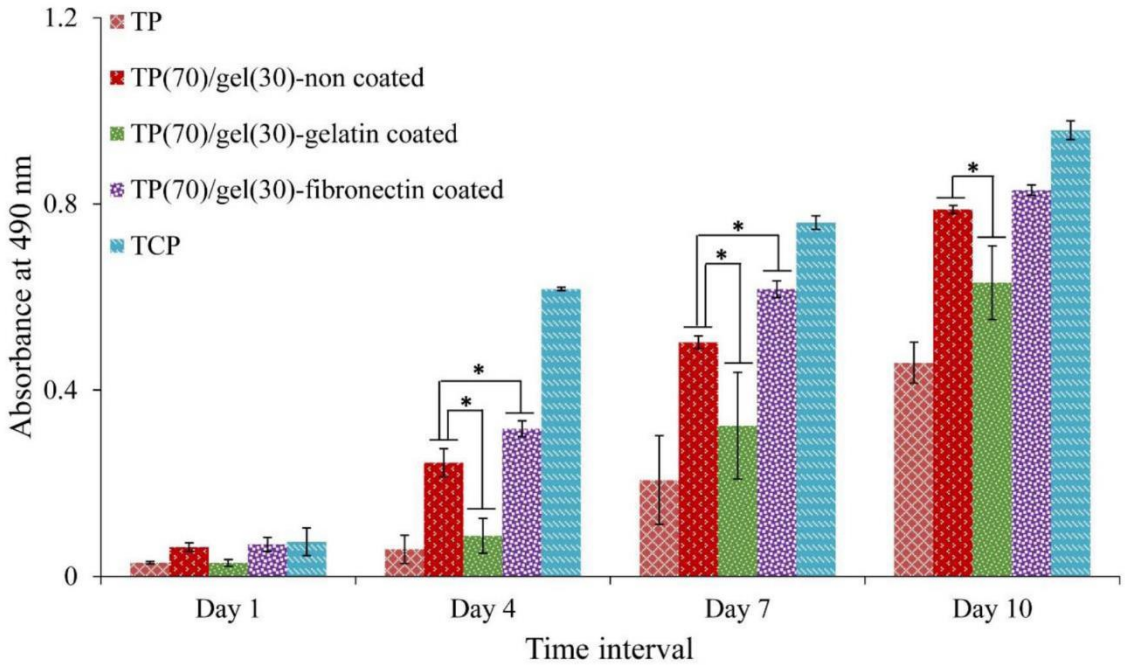

Figure 5. Proliferation of SMCs on electrospun TP, TP(70)/gel(30)-non coated, TP(70)/gel(30)-gelatin coated, TP(70)/gel(30)-fibronectin coated nanofibers and TCP. * Significant differences against cell proliferation on TP(70)/gel(30)-non coated scaffolds at $p<0.05$.

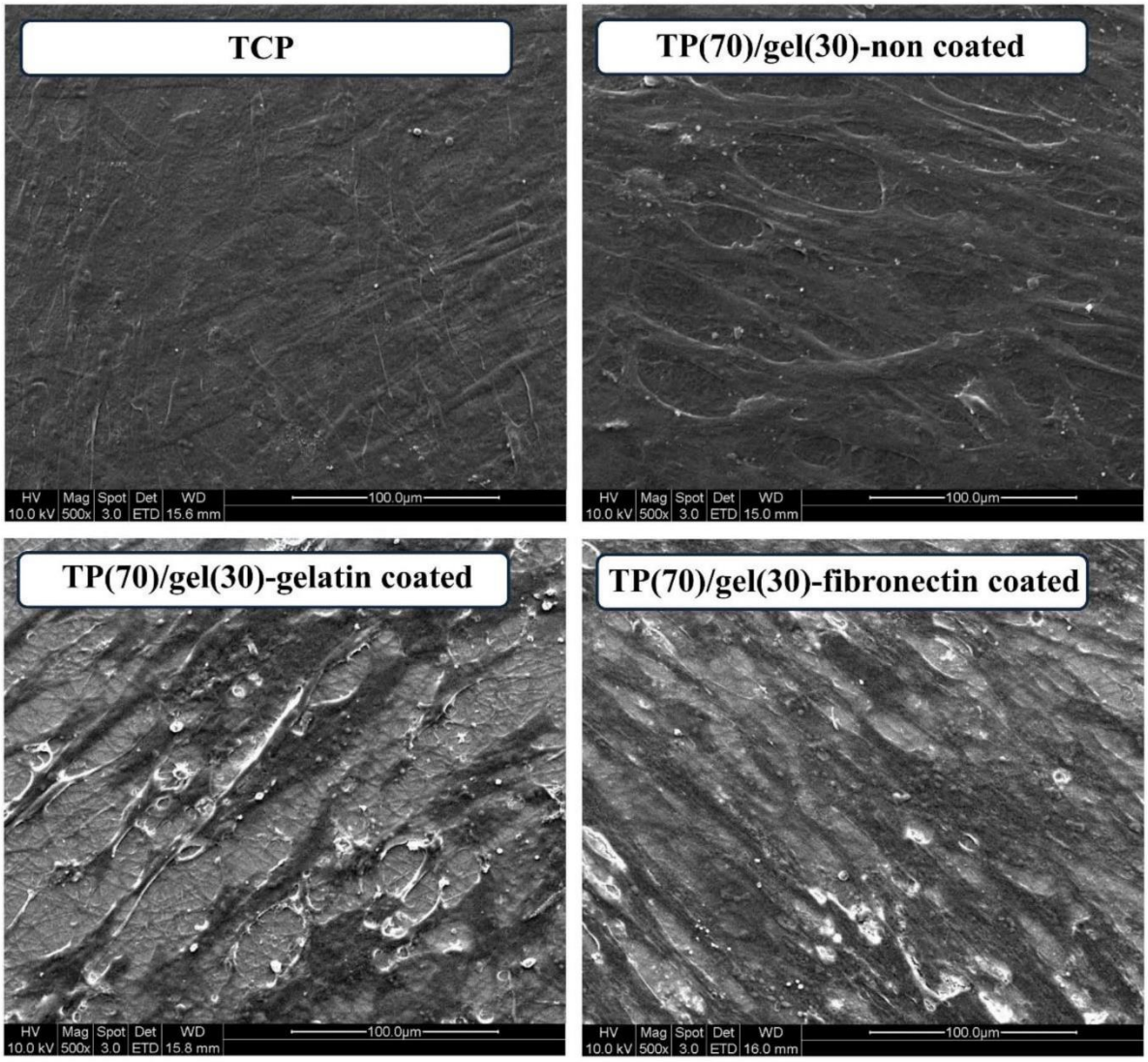

Figure 6. Morphology of SMCs on electrospun TP, TP(70)/gel(30)-non coated, TP(70)/gel(30)-gelatin coated, TP(70)/gel(30)-fibronectin coated scaffolds and TCP.