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

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\textbf{Abstract.} Nanofibrous composite scaffolds based on Tecophilic (TP) and gelatin (gel) were electrospun, and further modification of their surfaces was performed by the 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; thus, our study provides a better insight into the fabrication of functional constructs for vascular regeneration.

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1. Introduction

Extracellular matrix (ECM) is an intricate network of fibrillar and nonfibrillar macromolecules surrounding the cells consisting of collagen and elastin fibers along with proteoglycans and glycoproteins, providing both structural and regulatory functions to the cells. ECM provides mechanical support for 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 an electrospun composite scaffold 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 considered as thromboreistance and possesses inherent elasticity [6], suggesting its application as a vascular tissue-engineered graft. It was found that the addition of gelatin containing many integrin binding sites to TP could 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 such as 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 have found that 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 physical adsorption of biomolecules 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 Phosphat Buffer 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 blunt 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 the 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 37°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 was pipetted onto the upper surface of each well and allowed to be attached 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) containing 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 also were 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 were expressed as mean ± Standard Deviation (SD) of the mean. One-way ANOVA combined with Turkey post hoc tests was carried out for multiple comparisons 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 provides SEM micrographs of porous

![Figure 1. Morphology and fiber diameter distribution histogram of electrospun TP and TP(70)/gel(30) scaffolds.](image)

*Significant differences at p < 0.05.
and uniform interconnected structures of electrospun scaffolds along with fiber diameter and size distribution.

SEM images clearly demonstrated that the fiber diameter was significantly decreased from 881 ± 228 nm to 409 ± 150 nm, and the diameter distribution was 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 decrease the viscosity of the composite solution [9].

The arterial wall tissue possesses 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 the results reported by Miller et al. [14], surface roughness increased with increasing constituent fiber diameter (Figure 2). The average RMS roughnesses of the electrospun TP and TP(70)/gel(30) surfaces were 861 ± 90 nm and 514 ± 37 nm, respectively, which corroborated a significantly 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 were attributed to asymmetric and symmetric stretching vibrations 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 appearing 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 to 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. Vasactivity 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 the preservation of SMCs contractility on electrospun TP(70)/gel(30) scaffolds [4,5]. While the proliferation of SMCs seeded on scaffolds is generally essential to regenerate a vascular tissue, uncontrollable proliferation in the implanted graft can cause the thickening of the vessel wall and narrowing of the vessel lumen [7]. In this study, we have focused on the surface modification of electrospun TP(70)/gel(30) scaffolds with the physical adsorption of either fibronectin or gelatin onto the surface to investigate their effects on SMCs proliferation.

The 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 calcine AM to the intensely fluorescent calcine. The polyanionic dye calcine 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 were 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 scaf-
folds 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 are 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 levels, respectively, compared to TP(70)/gel(30)-noncoated scaffold 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 on 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. These findings suggest that TP(70)/gel(30) scaffolds can provide a suitable substrate for SMCs, and physical adsorption of various biomolecules on their surface enables researchers to obtain a desirable proliferation rate. This finding, in accordance with those 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 the initial interaction of SMCs with the scaffold can influence the long-term behavior of the cells 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 to composite...
scaffolds after 7 days of cell culture, 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 appearing on TP and TCP, the cell proliferation rate on these composite constructs (covered area by cells) was their main difference.

The proliferation of CMCs on composite scaffolds increased in the following order: TP(70)/gel(30)-fibronectin coated > TP(70)/gel(30)-noncoated > TP(70)/gel(30)-gelatin coated, all suggest 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 the appropriate regeneration of vascular tissue-engineered grafts.

4. Conclusion

This study demonstrated the important role of the specific cell adhesion biomolecules, including gelatin and fibronectin, coupled with the surface of electropun 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 might promote the surface to enhance cell growth, while the presence of gelatin biomolecules on the surface of the scaffold leads to a decreasing propensity in the proliferation of SMCs. Therefore, TP(70)/gel(30)-gelatin coated scaffolds can be a more attractive candidate for the development of a functional vascular graft with low proliferated SMCs in the wall.

References


Biographies

Elham Vatankhah joined the Department of New Technologies Engineering at Shahid Beheshti University as an Assistant Professor in 2016. She completed her PhD in Textile Engineering at Isfahan University of Technology. Her research interests include biomedical textiles and nanofibrous biomaterials for tissue engineering and drug delivery.

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