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Nanofiber diameter as a critical parameter affecting skin cell response

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ABSTRACT

Electrospun polymer nanofibers have opened new opportunities in the rapidly evolving field of tissue engineering, particularly due to their topography and variability of available biomaterials. In order to better understand nanofiber influence on cell growth, the impact of their diameter was systematically examined. In this study homogenous, randomly oriented poly(vinyl alcohol) nanofibers with five different average diameters, ranging from 70 nm to 1120 nm, were produced, characterized and their impact on morphology, proliferation and mobility of keratinocytes and skin fibroblasts was evaluated. The results have shown that nanofiber diameter affects cell response and that this response is cell line specific. Nanofiber thickness affected size, morphology and actine organization of keratinocytes much more than fibroblasts. Specifically, the keratinocyte grown on nanofibers were more spherical and smaller compared to the control cells, while the fibroblasts were much less affect. They stayed almost unchanged and spread across growth surface. The cell proliferation determined based on their metabolic activity was the highest, when keratinocytes were grown on 305 nm thick nanofibers, whereas proliferation of fibroblasts grown similar nanofibers was decreased. Finally, fibroblasts exerted higher mobility than keratinocytes. Both tested cell lines on nanofiber diameters of 300 nm resulted in decreased cell mobility. These findings suggest that the control over nanofiber diameter offers promising possibility to better design the tissue scaffolds, since cells distinguish between differently sized nanofibers and respond accordingly.

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1. Introduction

Tissues are complex structures composed of highly organized populations of individual cells. The basic principle of coordinated cellular events is cell communication with the environment; meaning interactions between cells as well as interactions of cells with the natural extracellular matrix (ECM) (Frantz et al., 2010). ECM is protein-based structure mostly composed of collagen fibers that extend in length over tens of micrometers, have thickness between 260 and 410 nm, as well as topography and structural features specific for cell response (Bettinger et al., 2009; Huang et al., 2012). When natural ECM is disturbed, either by illness or injury, regeneration process should be established to restore the tissue. Regeneration of tissues can be achieved by the combination of living cells, which provide biological functionality, and materials, which support the cell proliferation. Since the cell response *in vivo* depends on biological signals received from the surrounding environment, it is of crucial importance for particular tissue regeneration to resemble those signals (Zani and Edelman, 2010; Kim et al., 2013). Therefore, the biocompatible materials used in tissue regeneration should simulate natural behavior of treated cells.

Polymer nanofibers can be used for temporal replacement of natural ECM and its functions, since they can improve regenerative process (Engel et al., 2008; Pelipenko et al., 2013a). Therefore, nanofibers are intensively explored in the field of chronic wounds, where organism is, due to the imbalance between synthesis and degradation of ECM, incapable of producing functional ECM and orchestrating the cell response leading to tissue restoration (Alves et al., 2010). Understanding interactions between cells and engineered nanostructured biomaterials is crucial for successful tissue healing. Most of these biomaterials have been studied in terms of nanofiber production abilities (Thompson et al., 2007; Yördem et al., 2008; Rošic et al., 2012; Cramariuc et al., 2013; Casasola et al., 2014) and much less has been investigated about their nanotopography and role in the cell response (Beachley and Wen, 2010; Bacakova et al., 2011).

Abbreviations: ECM, extracellular matrix; PVA, poly(vinyl alcohol); MEM, minimum essential medium; SEM, scanning electron microscopy; PBS, phosphate buffered saline.

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The cell cytoskeleton is known to have an important role in mechanosensing and mechanotransduction. Recent research has shown that filopodia first probe the environment, then long-lasting focal adhesions are established and, finally the extension of cell protrusions occurs (Albuschies and Vogel, 2013). The focal contact cell structures are mostly composed of integrins, which have size in nanometer range (Anselme et al., 2010; Huang et al., 2012). Therefore, it is assumed that they can be differently influenced by nanofibers with different diameters. The interactions between integrins and the extracellular environment (ECM or nanofibers) can result in change of cell adhesion, morphology, proliferation and even cell orientation and mobility. Nanotopography seems to have an important impact on cell behavior; therefore, it is now being explored with the aim to optimize scaffolds for tissue regeneration.

The aim of our study was to investigate how to select proper nanofiber diameter to promote cell proliferation *in vitro*, which would probably *in vivo* result in healing and tissue regeneration. Firstly, electrospun poly(vinyl alcohol) (PVA) nanofibers were produced and characterized. Secondly, the impact of nanofiber diameter on the response of keratinocytes and skin fibroblasts, namely cell morphology, proliferation, and migration, was investigated in turn for further development of an effective nanofibrillar scaffolds.

2. Materials and methods

2.1. Materials

PVA (Mowiol® 20–98, Mw 125,000 g/mol) was supplied by Sigma–Aldrich (Germany), ethanol (96%) from Kefo (Slovenia). Minimum essential medium (MEM), non-essential amino acids, L-glutamin, antibiotic/antimycotic solution, fetal bovine serum, phalloidin rhodamine, trypan blue solution and Triton X-100® were all supplied by Sigma (Germany). Hoechst 33342 was obtained from Riedel de Haen (Germany), Cell Titer 96® Aqueous One Solution Cell Proliferation Assay was from Promega (Madison, WI). All other chemicals used were of analytical grade. 6, 24 and 96 well plates were from TPP (Switzerland) and custom-made inserts used in cell mobility experiments were provided by Denis Štraus, s.p. (Slovenia).

2.2. Methods

2.2.1. Nanofiber preparation and characterization

Aqueous PVA solution was placed in a plastic 20 ml syringe fitted with a metal needle with an inner diameter of 0.8 mm. A steady polymer solution flow rate was ensured by a syringe pump (model R-99E, Razel™ Scientific Instruments, USA) and the high voltage by a generator (model HVG-P60-R-EU, Linari Engineering s.rl. Italy). A planar stand covered with aluminum foil was used as a collector. Electrospinning parameters were as follows: polymer concentration 8–15%, w/w, relative humidity 2–60%, applied voltage 10–30 kV, and needle to collector distance 10–20 cm. In order to produce nanofibrillar supports with comparable thicknesses, the electrospinning process was performed for 10 min for each sample. After electrospinning nanofibers were thermally stabilized against dissolution in aqueous environment by dry heat at 160 °C for 30 min.

The morphology of the stabilized nanofibers was determined using a scanning electron microscopy (SEM, Supra 35 VP, Carl Zeiss, Oberkochen, Germany) at an accelerating voltage of 1 kV and a secondary electron detector. Based on SEM images the average nanofiber diameter and average interfibrillar pore size were determined by measuring 40 randomly selected nanofibers or interfibrillar pores using ImageJ 1.44p software (NIH, USA).

2.2.2. Cell culture and treatment

Immortalized human keratinocytes (cell line NCTC2544, ICLC, University of Genoa, Italy) or human skin fibroblasts (cell line 149BR, Public Health England, United Kingdom) were cultured as adherent monolayers at 37 °C in a humidified atmosphere of 5% CO_2 in air. They were grown in MEM supplemented with 1% (v/v) non-essential amino acids, 2 mM L-glutamin, and 100 U/ml antibiotic/antimycotic and 10% (v/v) and 15% (v/v) FBS for keratinocytes and fibroblasts, respectively. The cells were regularly subcultured when reaching 80% confluency. Their viability was tested using trypan blue exclusion assay. The experiments were performed using cell seeding density 2×10^4 cells/cm^2 and the final cell confluency reached in the experiments did not exceed 80%.

2.2.2.1. Examination of cell morphology. The cells were grown for 3 days on nanofibrillar supports, then the cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) for 10 min and then permeabilized with 0.1% Triton X-100® in PBS (pH 7.4) for 10 min. The cell nuclei were stained with the DNA intercalating dye Hoechst 33342 (5 μg/ml) for 30 min protected from light. The dye used for cell nuclei staining simultaneously stained the polymer nanofibers. Actin filaments were stained with the red-fluorescent dye phalloidin rhodamine, according to the manufacturer's procedure. Afterwards, coverslips were fixed on the slides and analyzed under fluorescence microscope using 360/420 nm (Hoechst 33342) and 535/635 nm (Phalloidin rhodamine) excitation/emission filter sets (Olympus IX81, Tokyo, Japan). Average size of cells was determined based on analysis of 40 randomly chosen cells. Based on SEM images the average nanofiber diameter and average interfibrillar pore size were determined by measuring 40 randomly selected nanofibers or interfibrillar pores using ImageJ 1.44p software (NIH, USA).

2.2.2.2. Assessment of cell mobility. Nanofibers, electrospun on glass coverslip (ϕ = 15 mm), were transferred to the bottom of a 24-well plate and then custom-made insert with diameter of 1.5 mm was mounted onto nanofibrillar support, representing a model "wounded field" with a defined area of 1.77 mm^2. Afterwards, cells (2.5×10^4 cells/well) were seeded and incubated at 37 °C and 5% CO_2 in air for 24 h to attach on nanofibrillar support, then the insert was removed and cell mobility was evaluated after 3 and 5 days using an inverted light microscope (CKX31, Olympus, USA).

2.2.2.3. Cell proliferation. Nanofibers, electrospun on round glass cover slips (ϕ = 5 mm), were transferred to the bottom of a 96-well plate, then cells were seeded on them and incubated at 37 °C and 5% CO_2 in air for 3 or 5 days. Their proliferation was determined by MTS assay according to the manufacturer's procedure. The sample absorbance was measured at 490 nm using a Synergy H4 microplate reader (BioTec, USA).

2.2.3. Statistical analysis

The results are expressed as means ± standard deviations. The statistical analysis was carried out using an independent samples Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

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