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Nanofibrous polyamide 6 scaffolds promote adhesion of endothelial cells

Abstract: The usage of synthetic scaffolds is a promising approach in development of implant materials. In this study we fabricated nanofibrous nonwovens of polyamide 6 (PA-6) by means of electrospinning and performed a systematic characterization with regard to the mechanical and biological performance of scaffold materials. Mechanical strength was assessed by uniaxial tensile testing and biological performance was evaluated by measuring cell viability and qualitative analysis of cellular morphology when human umbilical vein endothelial cells (EA.hy926) or human fibroblasts (HT-1080) were grown on polymeric substrates. While all polymeric materials exhibited an excellent biocompatibility with respect to cell viability, their surface topography promoted the adhesion of endothelial but not fibroblast cells. A better understanding of the physico-chemical and morphological material properties with a selective impact on cell adhesion will help to further improve biocompatibility of nonwovens for biomedical applications.

Keywords: nonwovens, electrospinning, cell adhesion, biocompatibility, endothelial cells, fibroblasts.

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1 Introduction

Electrospinning is an emerging field of development of implant materials that aims to produce polymeric scaffolds that could substitute for biological tissue, facilitate cell infiltration and restore tissue function [1,2]. This implicates a three-dimensional scaffold that supports cell attachment, migration, intercellular communication and proliferation.

Thrombus formation upon implantation of artificial cardiovascular grafts is one of the main causes for implant failure. Cardiovascular grafts stand in direct contact with blood and thus antithrombotic properties of the biomaterial are crucial in order to sustain their function [3]. Endothelialization is an essential process that renders the implant surface antithrombotic and also reduces the risk for occurrence of neointimal hyperplasia [4,5]. Despite the huge progress achieved on the field of development of implant materials, there is still a strong need for biocompatible biomaterials that facilitate a rapid endothelialization of the implant surface [6]. In this study we aimed to investigate the potential of electrospun polyamide 6 scaffolds for cardiovascular applications.

2 Materials and methods

2.1 Preparation of fibrous samples by electrospinning

Clear and homogenous polymer solution of 14 wt% to 20 wt% was obtained by dissolving commercially available polyamide-6 in a solvent mixture of acetic acid and formic acid (2:1 v/v) at 37 °C. For the process of electrospinning a device of Contipro (Dolní Dobrouč, Czech Republic) 4Spin C4S LAB2 was used. Fibrous nonwovens were fabricated from polymer solution by the use of static continual collector with needleless rod emitter and rotating continual collector at 80 U/min with multi jet capillary emitter with six cannulas (19G). For all trials nonwoven samples were fabricated at an emitter collector distance of 22 cm. The applied high voltage was 50 to 55 kV at a feed rate of 30 to 50 µl/min for static electrospinning and 150 µl/min for dynamic electrospinning. The ambient conditions were kept constant at a temperature of 22 °C ± 1 °C and a relative humidity of 25 % ± 9 %. Spinning process was performed for about 1 h to reach a layer thickness of 50 to 100 µm on average. In **Table 1** relevant information of the trials are summarized. For quality

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assurance of the produced nonwoven structures scanning electron microscopy scanning electron microscopy (Quanta 250 FEG, FEI Company, USA) (SEM) was used for the analysis of fiber structure and fiber diameter at different areas of the nonwoven.

Table 1: Parameters used for fabrication of PA-6 scaffolds

Sample	Polymer concentration [wt%]	Static/dynamic electrospinning
PA-6-14	14	static
PA-6-18	18	static
PA-6-20	20	dynamic

2.2 Cell culture

All media components were purchased from PAN Biotech (Aidenbach, Germany). Cells were maintained at 37°C and 5% CO_2 under humidified atmosphere. Human endothelial EA.hy926 cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin. Human fibroblasts HT-1080 (DSMZ) were cultured in Eagle's Minimum Essential Medium supplemented with 10% FCS and penicillin-streptomycin. For experiments 10^4 cells per well were seeded on PA-6 scaffolds placed in a 96-well plate 48 h prior to measurement.

2.3 Cell viability assay

Cell viability was determined using the CellQuanti-Blue assay (BioAssaySystems, Hayward, CA, USA) according to the manufacturer's instructions. Briefly, 10% of the total culture medium volume of CellQuanti-Blue reagent was added to each well and allowed to rest for 2 h. Samples were excited at 544 nm and the resulting fluorescence was measured at 590 nm using a microplate reader (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany). Data was normalized to cells grown on tissue culture-treated polystyrene.

2.4 Cell morphology analysis

For cell morphology analysis cells were rinsed with PBS and fixed in 10% formalin for 30 min. Cells were stained with 0.2% solution of Coomassie brilliant blue G-250 (Applichem, Darmstadt, Germany) in methanol, acetic acid and water (46.5 / 7 / 46,5 (v/v/v)) for 1 h. Samples were washed three times with PBS and analyzed at Olympus BX53 (Olympus, Tokio, Japan) using 20x objective.

2.5 Uniaxial tensile tests

Uniaxial tensile testing was performed on a Zwicki ZN 2.5 (Zwick, Ulm, Germany) with a 10 N load cell and a crosshead speed of 20 mm/min. During the tensile tests the samples were immersed in physiological saline solution at a temperature of 37°C. The tensile force was measured as a function of elongation. The elastic modulus E was determined in the linear elastic region via linear regression. Furthermore, the elongation at break ε_B and the ultimate tensile strength σ_M were extracted.

3 Results

3.1 Characterization of nanofibrous scaffolds

Analysis of polymeric nanofibrous scaffolds by SEM revealed increasing fiber diameter with increasing polymer concentrations (Figure 1, Table 2). All three polymer concentrations resulted in nanofibrous nonwovens with fiber diameters ranging from 114 nm for 14 wt% to 465 nm for 20 wt%. Noteworthy, when dynamic electrospinning was applied using a 20 wt% PA-6 solution, higher fluctuations in fiber diameter were observed as compared to the static electrospinning procedure.

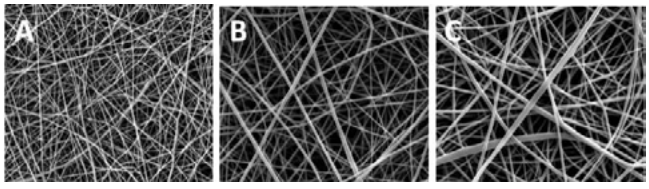

Figure 1: SEM micrographs of the electrospun fibers. (A) PA-6-14, (B) PA-6-18, (C) PA-6-20

The elastic modulus E and elongation at break ε_B show a substantial increase with increasing fiber diameter (Tab. 3). For the ultimate tensile strength σ_M there is no trend noticeable. Thus, mechanical properties increase with higher fiber diameter.

Table 2: Fiber diameter of PA-6 scaffolds

Sample	Fibre diameter [nm]	Static/dynamic electrospinning
PA-6-14	114 ± 47	static
PA-6-18	385 ± 164	static
PA-6-20	465 ± 218	dynamic

Table 3: Mechanic properties of PA-6 scaffolds

Sample	Elastic modulus E [MPa]	Elongation at break σ_M [MPa]	Ultimate tensile strength ε_B [%]
PA-6-14	16.7 ± 0.5	4,9 ± 0.4	53.6 ± 2.7
PA-6-18	25.8 ± 1.3	3,8 ± 0.1	66 ± 3
PA-6-20	40.5 ± 2.9	4,5 ± 0.1	70 ± 6

3.2 Evaluation of cell viability and morphology on nanofibrous scaffolds

Cell viability of endothelial cells EA.hy926 and fibroblasts HT-1080 seeded on different PA-6 scaffolds showed no significant differences for different scaffolds and cell types (Fig. 2). Relative viability of endothelial cells was comparable for all three polymer concentrations ranging from 83% (PA-6-18) to 87% (PA-6-14 and PA-6-20). For human fibroblasts the highest relative viability was observed on PA-6-14 (90%) and slightly lower values for PA-6-18 (80%) and PA-6-20 (83%). Thus, concentration of the polymer solution used for scaffold manufacturing did not have any impact on cell viability of either cell type.

Next, morphology of cells growing on the scaffolds was analyzed by light microscopy. On all PA-6 scaffolds

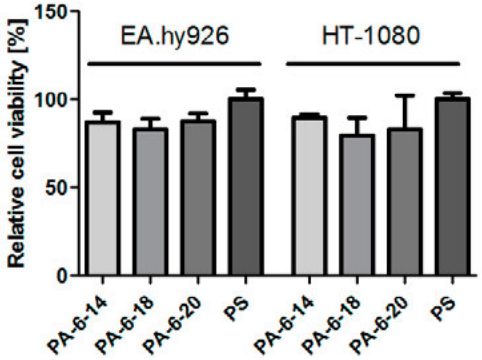

Figure 2: Relative cell viability of human endothelial EA.hy926 cells and human fibroblasts HT-1080 after 48 h growth on PA-6 scaffolds (PS: reference material tissue culture-treated polystyrene, n = 3, mean + SD)

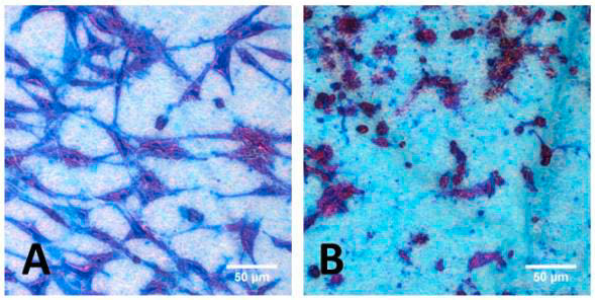

Figure 3: Micrographs of human endothelial EA.hy926 cells (A) and human fibroblasts HT-1080 (B) growing for 48 h on PA-6-18 scaffold

endothelial cells spread nicely forming networks, while fibroblasts had spherical shape and formed less intercellular contacts. No obvious differences in cell morphology could be detected when cells were grown on scaffolds produced from different polymer concentrations. **Figure 3** shows cell morphology growing on nanofibrous nonwoven PA-6-18 being representative for the other two nonwovens PA-6-14 and PA-6-20. Thus, nanofibrous PA-6 mats are biocompatible and possess unique properties that selectively promote growth of endothelial cells but not fibroblasts independently of the fiber diameter of nonwovens within the range of 114-465 nm.

4 Discussion

Electrospun nanofibrous scaffolds are promising candidate biomaterials for development of implant materials. The dense fiber network of electrospun mats can mimic extracellular matrix components and provides thereby an optimal three dimensional growth surrounding for cells [1,7]. Rapid coverage of the graft by a layer of endothelial cells is a prerequisite for the reduction of thrombotic events at the implant surface, one of the major causes for implant failure upon initial blood contact [8].

In this study we have fabricated nanofibrous polymer mats of polyamide 6 with fiber diameter ranging from 100-500 nm. This was achieved by modifying polymer concentrations and switching from static to dynamic electrospinning process. Proteins of the extracellular matrix create a network of fibers in the same size range (50-500 nm) [9]. Thus, nanofibrous scaffolds used in this study have the potential to mimic the architecture of the native tissue environment that can be found *in vivo*. The effects in the mechanical behaviour correlate with the morphology of the PA-6 nanofiber matrices. Higher polymer concentration increases thickness of PA-6 nanofiber matrices.

Consequently, the elastic modulus also increases and elongation at break decreases.

All materials were biocompatible as reflected by relative cell viability values of endothelial cells as well as fibroblasts growing on scaffolds. Strikingly, the scaffolds selectively promoted adhesion of endothelial cells. While human endothelial cells EA.hy926 could nicely spread on the scaffold, human fibroblasts HT-1080 exhibited spherical phenotype and showed only few contacts to scaffold surface. Nearly identical cell viability values for endothelial cells and fibroblasts are inconsistent with the microscopic analysis of cells growing on scaffolds. This can be explained by poor adhesion of fibroblasts on PA-6 scaffolds and removal of cells by subsequent washing steps during the staining procedure. Further experiments are needed to address this discrepancy.

Matschegewski et al. have investigated the morphology and viability of EA.hy926 growing on PA-6 and poly(L-lactide) (PLLA) nanofibrous scaffolds [10]. Electrospun PA-6 scaffolds were fabricated using a 16 %wt polymer solution, which is similar to polymer concentrations used in our study. However, they reported a moderate and nearly identical cytotoxic effect for both, PA-6 and PLLA nonwovens and poor spreading of EA.hy926 cells on the material surface, concluding that topography of the material surface is superior over its chemical composition. The data presented in our study suggests that for PA-6 nonwovens the fiber diameter within a range of 114-465 nm does not have any significant impact on spreading of endothelial cells and fibroblasts.

Fabrication of scaffolds for cardiovascular devices with a low risk for neointimal hyperplasia and good antithrombotic properties is of great interest for the field of development of implant materials [3]. Thus, a systematic screening of the growth behaviour of endothelial cells and fibroblasts on nanofibrous scaffolds manufactured from different polymers with distinct fiber diameters is a promising approach for the improvement of biomaterials for cardiovascular applications.

5 Conclusion

PA-6 nanofibrous nonwovens were prepared and their physico-chemical properties as well as cytocompatibility were investigated. Different polymer concentrations resulted in changes regarding morphological and mechanical properties of electrospun PA-6 nanofiber matrices. All nonwovens exhibited low cytotoxicity and supported spreading and network formation of endothelial cells, but not fibroblasts. Further work will address the potential of PA-6 nanofibrous scaffolds as platform for a rapid endothelialization of the implant surface.

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