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***In vitro* biocompatibility testing of polymeric nanofiber scaffolds: fine-tuning for a better prediction of the *in vivo* behaviour**

Abstract: Biomaterial research efforts focus on the development of biomaterials that mimic the natural extracellular environment. In addition, different strategies are applied to render materials for blood-contacting devices nonthrombogenic through surface modifications that would suppress activation of platelets, coagulation and the complement system. A confluent thin layer of endothelial cells lines all blood vessels and produces factors responsible for inhibition of coagulation, thrombosis and neointimal hyperplasia. Thus, the ability to rapidly form a healthy endothelium upon implantation represents a desired property of biomaterials used for cardiovascular devices. In this study we used advanced *in vitro* methods to investigate the biocompatibility of a biodegradable and a permanent electrospun nanofiber fabric, poly-L-lactic acid and polycarbonate-based silicone elastomer respectively, with the focus on endothelialization and hemocompatibility.

Keywords: nonwovens, electrospinning, PLLA, TSPCU, endothelialization, biocompatibility, hemocompatibility.

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1 Introduction

Cardiovascular diseases are the number one cause of death worldwide [1]. Many efforts on the field of biomedical engineering focus on the development of blood-contacting devices with the mechanical compliance and biological

properties similar to the native environment at the tissue-implant interface. Such materials should minimize the activation of platelets, coagulation cascade and tissue overgrowth – well studied mechanisms behind the thrombus formation and neointimal hyperplasia leading to implant failure [2]. A confluent thin layer of endothelial cells lines all blood vessels and produces factors responsible for inhibition of thrombosis and neointimal hyperplasia [3-5]. Thus, the ability to rapidly form a healthy endothelium upon implantation represents a desired property of biomaterials used for cardiovascular devices.

Electrospinning is a versatile process for fabrication of polymeric biomaterials with unique physicochemical properties that allow for an optimal integration of the implant into the inflammatory tissue environment [3].

Biocompatibility testing according to DIN EN ISO 10993 evaluates the safety of a medical device in its final form prior to initiation of clinical studies. The choice of the right polymer for the biomaterial fabrication and its processing to a final biomaterial are crucial steps during the development of new medical devices and require extensive experience in biocompatibility testing. Thus, *in vitro* biocompatibility testing of the sole biomaterial during the early development stages is extremely important and should use cell culture models that resemble the biological environment at the implantation site. In addition, blood contacting devices need to be evaluated with respect to hemocompatibility. Although different methods exist to address blood-implant interactions, the main obstacle is the lack of standardization on this field [6].

In this study we used advanced *in vitro* methods to investigate the biocompatibility of a fully biodegradable [7] and permanent electrospun nanofiber fabric, poly-L-lactic acid (PLLA) and polycarbonate-based silicone elastomer (TSPCU) respectively, with the focus on endothelialization and hemocompatibility.

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2 Methods

2.1 Scaffold fabrication and treatment

Homogenous polymer solutions of 4 wt% PLLA (RESOMER L210, Evonik, Germany) and 7.5 wt% TSPCU (Chronosil 55D, Advansource, USA) were obtained by dissolving commercially available polymers in a mixture of trifluoroethanol/chloroform (4/1) and dimethylformamide/trifluoroethanol/chloroform (1/1/8), respectively. Scaffolds of 100 x 200 mm in size were generated by dynamic electrospinning at 4SPIN C4S LAB2 (Contipro, Czech Republic) at room temperature. Fiber structure and diameter were assessed via scanning electron microscopy (SEM, Quanta 250 FEG, FEI Company, USA).

2.2 Cell culture

All media components were purchased from PAN Biotech (Germany). Human plasma was from Affinity Biologicals Inc (Canada). Human endothelial cells EA.hy926 (ATCC) were maintained in Dulbecco's Modified Eagle's Medium/10% fetal calf serum (FCS)/antibiotics (pen/strep) at 37°C, 5% CO₂ under humidified atmosphere. For experiments 10⁴ cells per well were used. For cytotoxicity testing, material extracts were prepared according to DIN EN ISO 10993 and used for cell treatment, followed by viability testing (CellQuantiBlue, BioAssaySystems, USA). Data was normalized to cells grown on tissue culture-treated polystyrene (TC-polystyrene).

2.3 Microscopy and image analysis

For the vacuum treatment scaffolds were covered with water and briefly incubated at 20 mbar (Memmert, Germany). Vacuum treated or untreated samples were incubated with FCS, human plasma or serum at 37°C overnight. Scaffolds were rinsed with PBS and incubated with 10⁴ cells/well for 48 h. Cells were rinsed with phosphate buffered saline (PBS) and fixed in formafix (PathoMed, Logistik GmbH, Germany) for 10 min. Cells were washed with PBS and permeabilized with 0.5% Triton X-100/PBS. Nuclei and actin were stained with NucBlue Fixed Cell Stain and ActinRed555 ReadyProbes (Invitrogen, USA) followed by confocal microscopy (Olympus FV-1000, Japan) at 200x. Initial image processing (background subtraction, contrast adjustment, maximum intensity projections) of the acquired z-stacks was performed with Fiji (NIH, USA). Quantitative analysis of at

least three representative images for each sample was performed with CellProfiler and cellular parameters such as cell morphology and area covered by cells have been measured.

2.4 Hemocompatibility testing

Platelet rich plasma (PRP) was isolated from fresh blood of healthy d. 13 mm polymer punches were incubated with 1.5 ml of PRP in a 24 well plate at room temperature. TC-polystyrene was used as reference material. At time point 0 and 1 h 1 ml of each sample was transferred into CTAD tubes (Sarstedt, Germany) on ice. PF4 release was assayed by ELISA (Asserachrom, France). For SEM analysis scaffolds were fixed with 2.5% buffered glutaraldehyde, followed by dehydration in an ascending alcohol series and hexamethyldisilazane. Samples were mounted on carriers and sputter coated with gold prior to measurement at Quanta FEG 250 (FEI Company, USA). For the complement activation assay scaffolds were incubated for 2 h with human normal serum (Quidel, USA) in an overhead mixer at 37°C. Supernatants were harvested by the addition of the specimen stabilizer (Quidel) and stored at -20°C. Formation of the terminal complement complex was assayed by enzyme immunoassay (MicroVue SC5b-9, Quidel).

3 Results

3.1 Fiber diameter and cytotoxicity

SEM analysis of polymeric nanofibrous scaffolds fabricated either from PLLA or TSPCU revealed fiber diameters mainly ranging from 500-1000 nm for both polymers. (Figure 1, Table 1). Next, nonwovens were tested for cytotoxicity according to DIN EN ISO 10993 (Figure 2). While none of the nanofiber scaffolds tested showed a negative impact on cell viability, cells treated with TSPCU extracts showed reproducible increased metabolic activity as compared to TC-polystyrene with values ~130%.

Figure 1: SEM analysis of fabricated scaffolds. Scale bar: 10 µm.

Table 1: Fiber diameters of PLLA and PCU- Si nonwovens

Sample	Polymer concentration [wt%]	Fiber diameter [nm]
PLLA	4	500 - 900
TSPCU	7,5	600 - 900

Figure 2: Viability of endothelial cells on TC-polystyrene (100%) or material extracts. HDPE: high density polyethylene, biocompatible reference material (mean $\pm$ SD, n=3).

3.2 *in vitro* assessment of the endothelialization of nonwovens

In a typical approach to the assessment of the endothelialization potential, cells are seeded on top of a scaffold. However, cell morphology is frequently affected and cell adhesion is poor due to the hydrophobic nature of polymers and the air trapped between the nanofibers [8, 9]. In addition, this *in vitro* approach only partly resembles the situation *in vivo*, where proteins from blood are adsorbed at the biomaterial surface immediately upon implantation, also known as the Vroman effect [10]. In order to challenge and further improve the classical *in vitro* system, nonwovens were subjected to low vacuum and/or preconditioned with FCS, human plasma or human serum overnight at 37°C prior to cell seeding. Microscopic analysis revealed that some samples showed huge differences in cell morphology (round vs. elongated) and cell density at the centre and border of the polymeric discs. Despite this fact, area covered by cells and cell morphology was bigger when nonwovens were preconditioned with plasma (Figure 3). In addition, quantitative analysis of representative images showed that data sets, where no vacuum was applied, had higher fluctuations with respect to biomaterial-cell-interactions as indicated by standard deviations (Figure 4). The highest value for the occupied area was in the sample treated by vacuum and plasma (~60%). Quantification of round cells in the sample resulted in the lowest value for the plasma treated scaffolds (~10%) and the highest for no treatment (~45%) or treatment with water (~30-60%), respectively (data not shown). Also, preconditioning with FCS seems to support cell adhesion to scaffolds (Figure 3, 4). Noteworthy, no

obvious differences were observed between cell adhesion to PLLA and TSPCU nonwovens.

Figure 3: Cell morphology and scaffold coverage by endothelial cells. Cytoskeleton (actin) is shown in grays (scale bar 50 μ m).

Figure 4: Quantitative image analysis of the scaffold area covered by cells upon treatment (mean $\pm$ SD, n=3).

3.3 Hemocompatibility testing

Biomaterials used for blood contacting medical devices should prevent thrombus formation and inflammation. Thus, in the next step we evaluated the blood-material interactions with respect to platelet and complement activation. First, platelet rich plasma was incubated with nonwovens or TC-polystyrene under static conditions. SEM analysis showed greater coverage of PLLA-nanofibers as compared to TSPCU indicating stronger platelet activation at the PLLA surface (Figure 5). Slightly more platelet factor 4 (PF4) was released from platelets when incubated with PLLA supporting this finding (Figure 6). Finally we tested the activation of the complement system in an *in vitro* system based on standardized human serum. Importantly, neither PLLA nor

TSPCU increased complement activation as compared serum in absence of scaffolds (Figure 7).

Figure 5: SEM analysis of nonwovens upon contact with platelet-rich plasma (scale bar: 10 µm).

Figure 6: PF4 release upon platelet-scaffold interaction for 1 h at room temperature (mean ± SD, n=2).

Figure 7: Complement activation upon contact of serum with scaffolds (mean ± SD, n=2). (NHS: serum without biomaterial, iNHS: complement inhibition by EDTA, aNHS: strong complement activation by zymosan).

4 Conclusions

Formation of a functional endothelial layer is crucial for the performance of blood-contacting implants. In this study, a biodegradable and a permanent material was successfully fabricated by electrospinning and tested for biocompatibility. Both biomaterials tested here (PLLA and TSPCU) were not cytotoxic and did not activate the complement system. Although cells were rounded on untreated materials, cell morphology improved dramatically upon treatment with plasma. While platelet activation by nonwovens was slightly more pronounced for PLLA as compared to TSPCU, a clearly bigger area of PLLA scaffolds was covered by platelets upon contact with PRP. Our data shows that fine-tuning (i.e. plasma/vacuum treatment) of commonly used *in vitro* testing systems can have a huge impact on the performance of biomaterials. Further work needs to be done in order to reveal the mechanisms behind the enhanced

endothelialization of biomaterials upon plasma treatment and its relevance for the assessment of biocompatibility according to DIN EN 10993. Moreover, the *in vitro* complement activation assay presented here provides a valuable and robust testing system for the initial screening and optimization of biomaterials with respect to hemocompatibility.

Author Statement

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References

- [1] <https://www.who.int/health-topics/cardiovascular-diseases/>, accessed 1 July 2021.
- [2] Li J, Zhang K, Huang N. Engineering Cardiovascular Implant Surfaces to Create a Vascular Endothelial Growth Microenvironment. *Biotechnol. J.* 2017;12:1600401.
- [3] Bruna BBJ, Wakabayashi N, Oyama K, Kamiya H, Braghirolli DI, Pranke P. Vascular Tissue Engineering: Polymers and Methodologies for Small Caliber Vascular Grafts. *Frontiers in Cardiovascular Medicine* 2021;7:376.
- [4] Ren X, Feng Y, Guo J, Wang H, Li Q, Yang J, Hao X, Lv J, Ma N, Li W. Surface modification and endothelialization of biomaterials as potential scaffolds for vascular tissue engineering applications. *Chem Soc Rev.* 2015;44(15):5680–5742.
- [5] Strohbach A, Busch R. Polymers for Cardiovascular Stent Coatings. *Int J Polym Sci.* 2015;2:1–11.
- [6] Sperling C, Maitz MF, Werner C. Test methods for hemocompatibility of biomaterials. *Hemocompatibility of Biomaterials for Clinical Applications.* 2018:77-104.
- [7] Bergstrom, JS; Hayman D. An Overview of Mechanical Properties and Material Modeling of Polylactide (PLA) for Medical Applications. *Ann. Biomed. Eng.* 2016, 44, 330-340.
- [8] Matschegewski C, Matthies JB, Grabow N und Schmitz KP. Cell adhesion and viability of human endothelial cells on electrospun polymer scaffolds. *Current Directions in Biomedical Engineering* 2016;2(1):11-14.
- [9] Szewczyk PK, Ura DP, Metwally S, Knapczyk-Korczak J, Gajek M, Marzec MM, Bernasik A, Stachewicz U. Roughness and Fiber Fraction Dominated Wetting of Electrospun Fiber-Based Porous Meshes. *Polymers (Basel).* 2018; 27;11(1):34.
- [10] Christo SN, Diener KR, Bachhuka A, Vasilev K, Hayball JD. Innate Immunity and Biomaterials at the Nexus: Friends or Foes, *BioMed Research International*, 2015: 342304.