Schulz, C.; Ruesten-Lange, M.von; Krueger, A.; Lendlein, A.; Jung, F.:
Viability and function of primary human endothelial cells on smooth poly(ether imide) films
In: Clinical Hemorheology and Microcirculation (2012) IOS Press

DOI: 10.3233/CH-2012-1604

Viability and function of primary human endothelial cells on smooth poly(ether imide) films

Schulz C, Lange M, Krüger A, Kratz K, Lendlein A, Jung F

Center for Biomaterial Development and Berlin Brandenburg Center for Regenerative Therapies, Institute of Polymer Research, HZG - Helmholtz-Zentrum Geesthacht, Kantstr. 55, 14513 Teltow, Germany

Abstract

Poly(ether imide) (PEI) is being explored as potential biomaterial for cardiovascular applications. Different studies showed that human umbilical venous endothelial cells (HUVEC) are able to adhere and proliferate on PEI membranes (R_q = 13.20 ± 1.58 nm). A recently published study revealed evidence for much lower platelet adhesion on very smooth PEI-films (R_q = 2.37 ± 1.40 nm).

Therefore, we explored whether primary human venous endothelial cells (HUVEC) are able to adhere and proliferate on such very smooth PEI-films compared to tissue-cultured polystyrene (TCP) as reference material.

Cytotoxicity testing revealed that PEI had a slight cytotoxic effect on HUVEC accompanied by a marginal reduced integrity of the plasma membrane and a significant lower mitochondrial activity. However long-term seeding experiments up to eleven days exhibited that HUVEC were able to proliferate on the PEI-films till confluence (TCP 96,190 ± 18,289 cells/cm²; PEI 91,590 ± 19,583 cells/cm²). Further studies are planned to monitor the influence of shear force on the endothelial cell monolayer in a dynamic test system to determine its stability in view of shear resistant endothelialization of PEI for cardiovascular devices.

Introduction

Cardiovascular diseases (CVDs) are one of the major causes of death globally.[23] As therapeutic approaches currently two main therapies are available. Percutaneous transluminal angioplasty (PTA) with bare metal (BMS) or drug eluting stent (DES) implantation, as a procedure to dilate or re-open occluded blood vessels. The application of DES has reduced significantly the frequency of restenosis compared to BMS.[35] Although a marginal rate of late thrombosis (though with a death rate of 30%) and in-stent restenosis still exists.[13,36]

Replacement of blood vessels is the second established (surgical) technique. When stent intervention is not longer possible, bypass surgery is performed by using patient's own vessels as preferential surrogate. However the use of synthetic coronary vessels is becoming progressively more important because the number of autologous vessels is limited. A percentage of patients have no suitable vessels available due to previous operations or in consequence of advanced vascular disease.[22]

An alternative option is the implantation of artificial vascular bypass grafts predominantly consisting of expanded polytetrafluroethylene (ePTFE) or polyethylene terephalate (Dacron).[17] However, these synthetic polymers show significantly lower patency rates than autologous vein or artery grafts due to insufficient hemocompatibility. The contact of blood compounds to body foreign surfaces generally provokes adherence and activation of thrombocytes bearing the risk of gradual or complete occlusion of the artificial graft - especially for small diameter vessels (<6 mm).[15,21] Therefore the improvement of the hemocompatibility of biomaterials is a necessary step towards creating an anti-thrombogenic surface of artificial vascular implants to prolong graft patency.

Since the hemocompatibility of biomaterial devices is strongly influenced by surface properties like topography, charge, wettability and chemical composition, the evaluation of new synthetic polymers with suitable physicochemical properties for the use as potential biomaterials is necessary.[3,30]

However preventing thrombocyte activation by the surface properties is not the only approach for improving hemocompatibility. Covering the biomaterial surface using autologous endothelial cells presents also an encouraging technique for overcoming the limitations of thrombogenicity.[2,5,32] A promising alternative for overcoming current limitations would be a hybrid system of both methods. A biomaterial equipped with a sufficient hemocompatibility preventing thrombus formation prior to

complete endothelialization that also allows a fast formation of a shear resistant and functional confluent endothelial cell layer.

A candidate material for this demand is poly(ether imide) (PEI) which proved to be cyto- and immunocompatible.[27,28,31] Its stability against heat and hydrolysis enables steam sterilization, which is a clear advantage compared to many other biomaterials. Tzoneva *et al.* reported that human umbilical venous endothelial cells (HUVEC) were able to adhere and proliferate on PEI-membranes.[37] In a comparative study between PEI-membranes and -films, Braune *et al.* showed that platelet adherence can strongly be reduced by the processing of PEI (by changing surface roughness).[6]

Therefore, this study was performed to investigate the behavior of HUVEC seeded on smooth PEI-films (R_q = 2.37 ± 1.40 nm). In the framework of the study the cytotoxicity, endothelial cell monolayer formation, cell-substrate as well as cell-cell interaction and the secretion profile of vasoactive as well as inflammatory mediators under *in vitro* conditions were analyzed to evaluate if this low thrombogenic material is suitable as biomaterial for cardiovascular devices.

Material and methods

Preparation of poly(ether imide) films (PEI-films)

PEI-films with a thickness of 300 µm were prepared by solving the commercially available polymer (ULTEM® 1000, General Electric, New York, NY, USA) in dichloromethane (DCM) and pulling out on a cleaned, smooth glass surface as described previously.[6] Drying of the PEI-films was extended up to 27 days under steadily increasing temperature (up to 105 °C) and concurrent decreasing atmospheric pressure (down to 15 mbar) to allow evaporation of the solvent remained from the production process. Prior to use in any experiment, the PEI-films were sterilized by steam sterilization method for 20 min at 121 °C and 200 kPa using a FVA A1 autoclave (FEDEGARI, INTEGRA Biosciences).

Validation of remaining solvent by headspace GC

Steam sterilized PEI-film (1 g, ~150 cm²) was incubated in a sealed sample flask with 9 ml Millipore water under shaking at 37 °C for 96 hours. The supernatant atmosphere was analyzed by gas chromatography (GC) using headspace GC (HP 7694 Headspace-Sampler, HP 5890 Series II GC, DB-624, 75m column).

Surface characterization by contact angle and atomic force measurements (AFM)

Surface contact angle measurements (DSA 100, Krüss, captive bubble, water - air) were carried out at three different areas of each sample (n = 3) analyzing the advancing ($\Theta_{adv.}$) and receding ($\Theta_{rec.}$) contact angle with subsequent calculating of the hysteresis.

Surface topography of the PEI-films was characterized by using atomic force microscopy (AFM, NanoScope, MultiMode V, Bruker Nano GmbH, tapping mode). Prior the samples were pre-wetted in phosphate buffered saline without calcium and magnesium ions (PBS -/-) at pH 7.4. The scanning area for each scan was 50 µm square size. Surface mean roughness (R_a) and mean square roughness (R_q) were determined by analyzing three samples on three different areas.

Endotoxin load

The endotoxin load of the PEI-film (n = 4) was determined by using the Limulus amebocyte lysate (LAL) test (QCL 1000™, Lonza, Cologne, Germany).

Cell culture

L929-fibroblasts

L929-fibroblasts (NCTC clone 929) from the connective tissue of mice were purchased from ATCC (LGC Standards GmbH, Wesel, Germany) and cultured under static cell culture conditions (37 °C, 5 vol% CO_2) in polystyrene-based cell culture flasks (TPP, Techno Plastic Products AG, Switzerland) using standard cell culture medium MEM EAGLE (Biochrom AG, Berlin, Germany).

HUVEC

Commercially available primary human umbilical vein endothelial cells (HUVEC, Lonza, Cologne, Germany) were cultured under static cell culture conditions (37 °C, 5 vol% CO_2) in polystyrene-based cell culture flasks. Cells were used for no longer than 4 passages cultivating them with endothelial basal medium EBM-2 supplemented with EGM-2 Single Quots® kit and 2 vol% FCS (Lonza, Cologne, Germany).

Indirect cytotoxicity testing (L929-fibroblasts)

To investigate influences of eluting components from the polymeric material on the cells, 27 cm² of the PEI-film (3 cm² per 1 ml eluate corresponding to EN ISO 10993-5 and -12)[14] were incubated for 72 hours under standard cell culture conditions (37 °C, 5 vol% CO_2) in 9 ml L929 cell culture medium. Subsequently undiluted and 10-fold diluted extracts from the PEI-film were used to seed out L929-fibroblasts in polystyrene tissue culture plates (TCP). Morphological changes of the L929 cells were determined 48 hours after eluate exposure by phase contrast microscopy in transmission (Axiovert 40 C, Zeiss, Oberkochen, Germany). After the same timeframe cell membrane integrity was examined by lactate dehydrogenase (LDH) activity in the extracellular fluid (Cytotoxicity Detection Kit LDH, Roche, Germany). The metabolic activity of the cells was determined by quantifying the mitochondrial activity using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS assay, Promega, Mannheim, Germany).

Direct cytotoxicity testing (HUVEC)

Evaluation of HUVEC compatibility was carried out by seeding 3 x 10^4 cells directly on PEI-films (15 mm discs; n = 8) with TCP as the control. Morphological changes of the cells were determined after 48 hours of culturing by phase contrast microscopy in transmission (Axiovert 40 C, Zeiss, Oberkochen, Germany). After the same timeframe cell membrane integrity was examined by lactate dehydrogenase (LDH) activity in the extracellular fluid (Cytotoxicity Detection Kit LDH, Roche, Grenzach, Germany). The metabolic activity of the HUVEC was determined by quantifying the mitochondrial activity using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS assay, Promega, Mannheim, Germany).

Cell viability assessment

For long term cell culture experiments 3 x 10^4 HUVEC were seeded out per well on PEI-films (15 mm discs; n = 8) with TCP as the control. Viability and monolayer formation of HUVEC were examined after two, seven and eleven days by fluorescence staining. Viable cells were stained with fluorescein diacetate (FDA, 25 µg/ml, Invitrogen, Carlsbad, CA, USA) whereas propidium iodide (PI, 2 µg/ml, Sigma, Taufkirchen, Germany) staining indicates dead cells. Downstream documentation was realized by taking three pictures from different areas of each sample by using a confocal laser scanning microscope (cLSM) with a 10x primary magnification (LSM 510 META, Zeiss, Oberkochen, Germany).

Cytoskeleton and focal adhesions evaluation

Actin cytoskeleton and the focal adhesion complex protein vinculin were stained by specific fluorescence labeled antibodies after seeding 3 x 10^4 HUVEC out on PEI-films (15 mm discs, n = 3) for 48 hours, with glass (15 mm discs, Gerhard Menzel GmbH, Braunschweig, Germany) as the control. The cells were fixed with paraformadehyde (4%, v/v in 0.9% NaCl, 30 min, 4 °C) and pre-treated with Triton X-100 (0.5% v/v). F-actin was fluorescently stained with Phalloidin-Alexa555 (1 : 40, Molecular Probes®, Invitrogen, Germany) whereas for vinculin staining the polyclonal mouse anti-human vinculin IgG (Sigma, Taufkirchen, Germany) 1 : 50 and the Cy2 conjugated polyclonal goat anti-mouse IgG (Jackson ImmunoResearch, Hamburg, Germany) 1 : 200 was used. The genomic DNA/nuclei was stained by using 4',6-diamidino-2-phenylindole (DAPI, 1 : 5000, Roth, Germany). Afterwards samples were documented by taking three different fields of view in various primary magnifications (20x, 40x and 100x) with the cLSM (LSM 510 META, Zeiss, Oberkochen, Germany).

Secretion profile screening

The Prostacyclin (PGI_2) and Thromboxane A_2 (TXA_2) concentrations from PEI-film and TCP seeded HUVEC were determined by using a competitive enzyme immuno assay for the stable hydrolysis products of PGI_2, 6-keto-prostaglandin $F_{1\alpha}$ and TXA_2, thromboxane B_2 (TXB_2) after two, seven and eleven days of culturing (6-keto-Prostaglandin $F_{1\alpha}$ EIA Kit and Thromboxane B_2 EIA Kit from Cayman Chemical Company, Ann Arbor; MI, USA). Similarly the concentrations of prominent secreted inflammatory cytokines (IL-6 and IL-8) were quantified from the supernatant by a Bioplex™ Cytokine Assay (Bio-Rad Laboratories Inc., Munich, Germany) whereas cell culture medium EGM-2 was always used as a negative control. As the positive control recombinant human IL-1β (10 ng/ml, R&D Systems Inc., MN, USA) was added to TCP seeded cells 24 hours prior analyzing the supernatant.

Statistics

Data are reported as mean values ± standard deviation for continuous variables and were analyzed by a two-sided Student's t-test for paired samples. A p value of less than 0.05 was considered significant.

Results

Validation of remaining solvent and surface characterization

Since evaporation of remaining solvent from the PEI-films into the cell culture medium can influence the behavior of the cells, release of dichloromethane (DCM) was determined using headspace GC. After 96 hours of incubation a very low amount of DCM (3.55 ppm) was found in the supernatant atmosphere.

Contact angles of θ_{adv} = 85.3° ± 2.1° and θ_{rec} = 61.2° ± 6.1° with a calculated hysterese of 24.1° as well as a surface roughness of R_a = 1.05 ± 0.44 nm and R_q = 2.37 ± 1.40 nm confirmed the smooth and homogenous surface of the films.

Indirect cytotoxicity testing with L929 fibroblasts

The morphology of L929 cells 48 hours after seeding with an extract of the PEI-film, obtained by stirring in cell culture medium at 37 °C for 72 hours (72h-extract), was slightly different to the morphology of the cells seeded with pure cell culture medium (control). (Figure 1) There was a higher cell density as well as a higher amount of feeder cells visible.

pure culture medium (control) **undiluted 72h-extract of PEI-film**

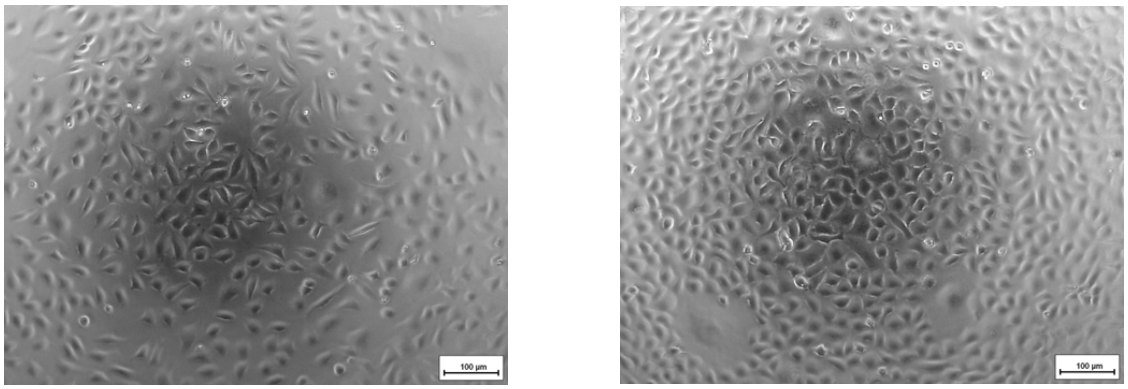

Figure 1. L929 fibroblasts 48 hours after seeding with pure culture medium and an undiluted 72h-extract of the PEI-film. Pictures were taken by phase contrast microscopy in transmission with 10x primary magnification. (scale bar: 100 µm)

The release of the extracellular lactate dehydrogenase (LDH) with an absorption of 0.87 ± 0.01 absorption units (AU) for the control and 0.84 ± 0.12 AU for the undiluted (p = 0.5524) or 0.79 ± 0.17 AU (p = 0.2651) for the 10-fold diluted PEI-extract did not differ. (Figure 2) However, the MTS assay

showed a significantly increased mitochondrial activity of the L929 cells treated with the PEI-extract. Absorption values for the PEI-extracts were 0.98 ± 0.09 AU (p = 0.0023) for the undiluted and 0.92 ± 0.10 AU (p = 0.0471) for the diluted extract and thereby significantly increased compared to the control (0.80 ± 0.07 AU). This indicates a mild impact of the PEI-extracts on the cell activity in a concentration dependent manner.

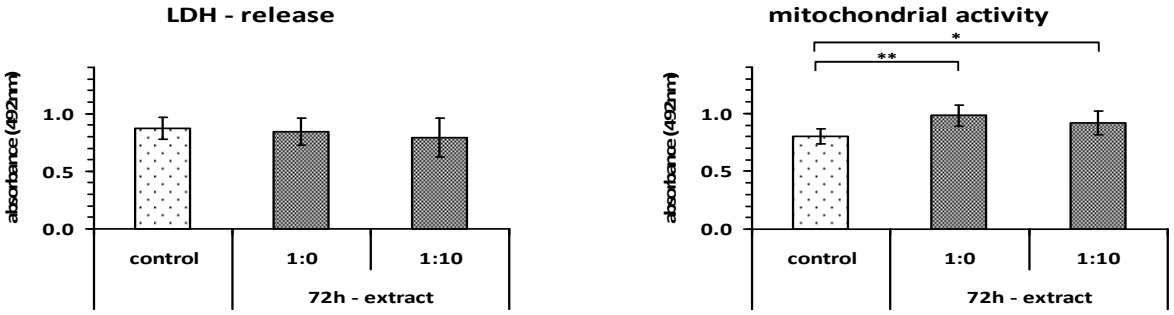

Figure 2. Lactate dehydrogenase (LDH) activity in the extracellular fluid and the mitochondrial activity of the cells 48 hours after seeding L929 cells with pure cell culture medium (control) and with a 72h-extract of the PEI-films. (Data analyzed by student's t-test; * $p < 0.05$; ** $p < 0.005$; n = 8)

PEI-films (n = 3) showed an endotoxin content of 0.077 ± 0.001 EU/ml, which is less than the critical endotoxin burden of 0.5 EU/ml for biomaterials and consistent with the demands from the FDA.[10]

Direct cytotoxicity testing (HUVEC)

The visual evaluation of HUVEC growing on PEI-films suggested no obvious changes in the morphology. The HUVEC were able to adhere and spread on the PEI-films in the same manner as on the control (TCP). The only visible difference was a lower cell density on PEI-films. (see Figure 3 and cell viability assessment section for cell density) This suggests an effect of the polymer on the activity state of the endothelial cells.

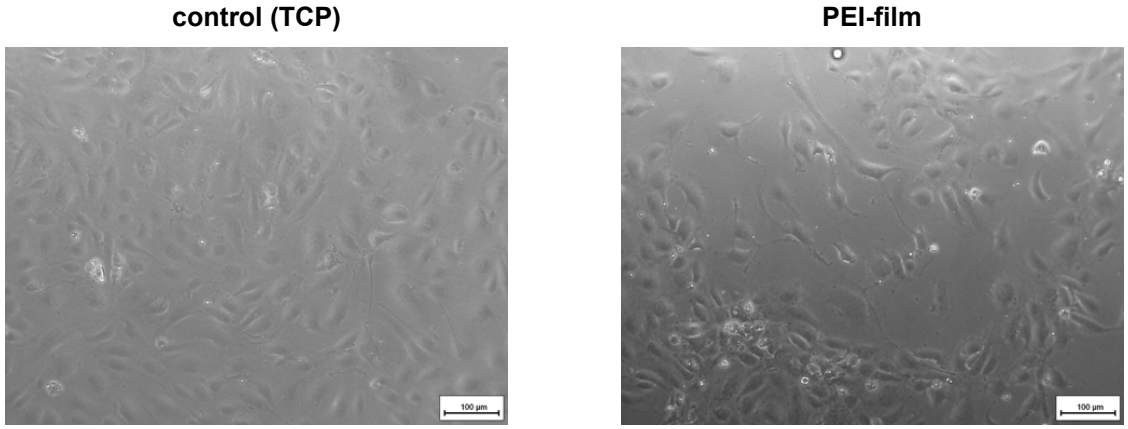

Figure 3. Primary HUVEC 48 hours after seeding on PEI-films and TCP. Pictures were taken by phase contrast microscopy in transmission with 10x primary magnification. (scale bar: 100 µm)

There was a slight but not significant increase in the activity of extracellular LDH between TCP (0.10 ± 0.02 AU) and the PEI-films (0.11 ± 0.02 AU; p = 0.2041) detectable, point towards a low impact on the integrity of the HUVEC plasma membranes when seeded on the polymeric material. Nevertheless the mitochondrial activity of the HUVEC was significantly reduced on PEI-films (0.40 ± 0.06 AU; p = 0.0002) compared to TCP (0.54 ± 0.04 AU), indicating a slower metabolic activity and confirmed the previously made morphological observation.

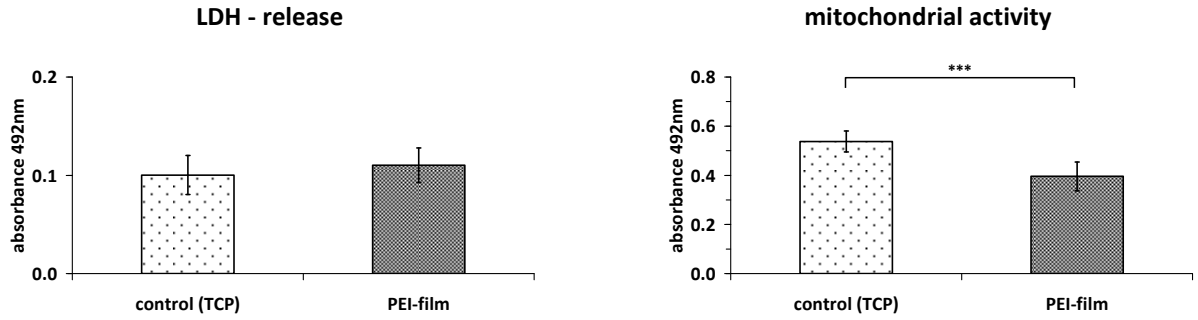

Figure 4. Lactate dehydrogenase (LDH) activity in the extracellular fluid and the mitochondrial activity of the cells 48 hours after seeding HUVEC on different material surfaces. (Data analyzed by student's t-test; *** p < 0.0005; n = 8)

Cell viability assessment

Long term seeding experiments up to eleven days confirmed that HUVEC were able to grow until confluence on the PEI-films. Seven days after seeding confluence was reached on both materials with no visible gaps in the endothelial cell layer. Immunofluorescence staining and counting of viable as well as adhering dead cells showed a significant lower cell density of viable HUVEC on PEI-films two days after seeding (TCP 45,667 ± 8,106 cells/cm² and PEI 32,116 ± 6,909 cells/cm² ($p = 0.0012$)). (see Figures 5 and 6) Over time HUVEC reached on PEI-films a comparable cell density as on TCP. However, the amount of adhering dead cells was significantly different between TCP and PEI-films seven (TCP 10,396 ± 2,421 cells/cm² and PEI 7,769 ± 1,845 cells/cm² ($p = 0.0263$)) and eleven days after seeding (TCP 6,245 ± 2,195 cells/cm² and PEI 13,200 ± 5,410 cells/cm² ($p = 0.0030$)).

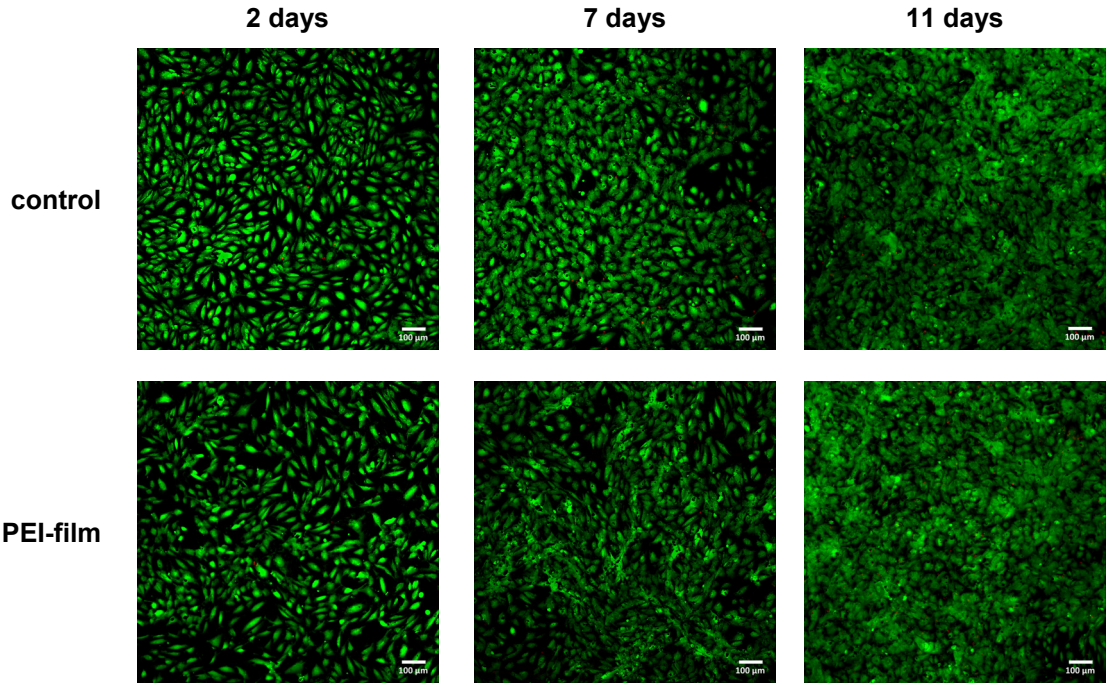

Figure 5. Viability and layer formation of primary HUVEC different time points after seeding. 3 x 10^4 cells were seeded out on PEI-films (15 mm discs) as well as on TCP as the control. Afterwards viable cells were stained with fluorescein diacetate (FDA, green) whereas propidium iodide (PI, red) staining indicates dead cells. Pictures were taken using the cLSM with a 10x primary magnification. (scale bar: 100 µm)

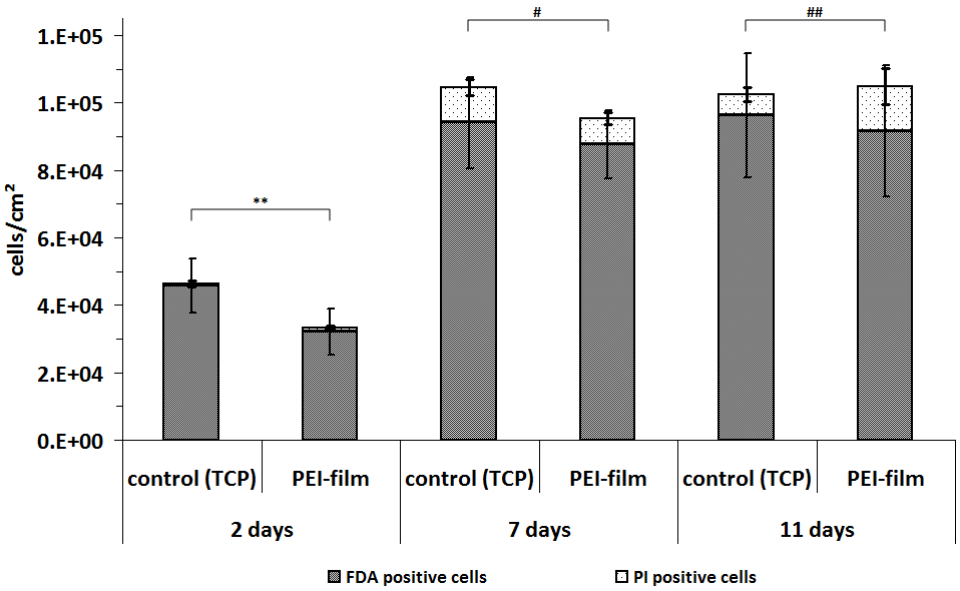

Figure 6. Cell density of HUVEC seeded out on PEI-films and TCP as the control for up to eleven days. (Data analyzed by student's t-test; $^{\#}$ $p < 0.05$, $^{**,\#\#}$ $p < 0.005$; n = 8)

Evaluation of cytoskeleton and focal adhesions

Cytoskeleton and focal adhesion complex formation were evaluated through immunofluorescence staining of actin filaments and vinculin. (Figure 7) Two days after seeding, HUVEC showed on both materials the typical net-like pattern of a sub confluent monolayer. Moreover they exhibit a distinct formation of central actin stress fibers, which are usually formed during the adhesion process. The termini of these cytoskeleton fibers are in most cases co-localized with vinculin, which is an essential structural protein of focal adhesion complexes. Staining cell genomic DNA/nuclei with DAPI was only visible on PEI-films at higher primary magnifications and is caused by the strong background fluorescence of this highly aromatic polymer.

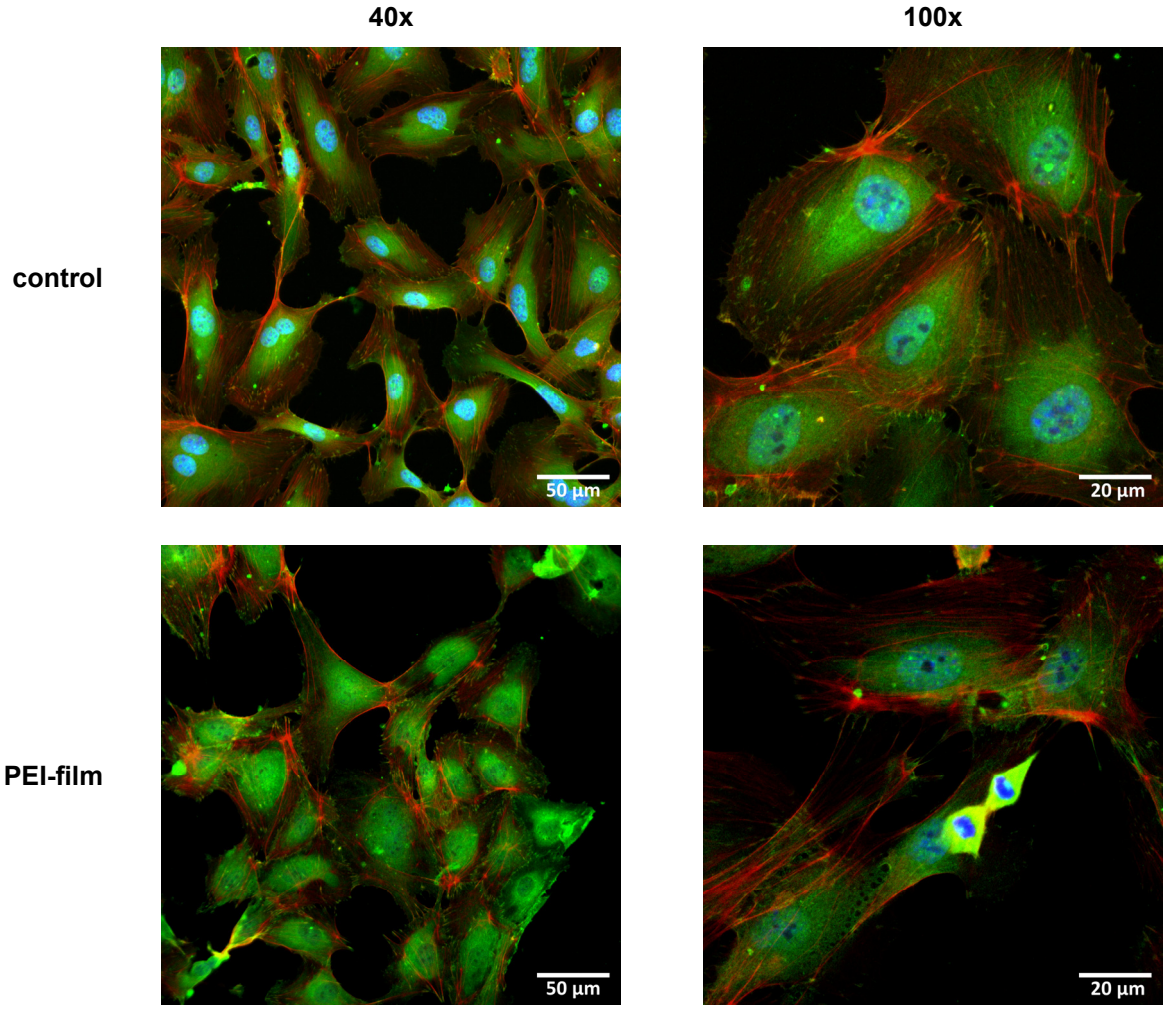

Figure 7. HUVEC cytoskeleton and focal adhesions 48 hours after seeding 3 x 10^4 HUVEC on PEI-films (15 mm discs) with glass as the control. Actin cytoskeleton (red), vinculin (green) and genomic DNA (blue) were fluorescently stained and pictures taken by using the cLSM with a 40x (left) and 100x (right) primary magnification.

Secretion profile screening

Vasoactive mediators like Prostacyclin (PGI_2) and his physiological antagonist Thromboxane A_2 (TXA_2) as well as prominent inflammatory cytokines (IL-6 and IL-8) were quantified from the supernatant by competitive ELISA assay to assess the activation state of the HUVEC on the polymeric material in comparison to TCP. (Figure 8)

There was only a significant material dependent difference between PEI-films (91.5 ± 8.2 pg/ml) and TCP (100.8 ± 2.9 pg/ml) ($p = 0.021$) in the secretion of vasoactive PGI_2 seven days after seeding. The TXA_2 secretion did not differ between PEI and TCP during the whole experiment (for PEI-film 1.1 ± 0.6 pg/ml and TCP 1.4 ± 1.2 pg/ml after two days up to 7.3 ± 1.3 pg/ml for PEI-film and 4.8 ± 1.7 pg/ml for TCP after eleven days).

Secretion of the pro-inflammatory cytokine interleukin 6 (IL-6) in the pg-range was significantly reduced on PEI-films compared to the control (TCP) during the whole experiment (after two days: PEI 40.9 ± 12.2 pg/ml; TCP 62.4 ± 9.2 pg/ml; $p = 0.0002$). Over time the secreted amount of IL-6 raised during the first seven days for each material, but was still significantly reduced for the HUVEC seeded on PEI (after seven days: PEI-film 91.3 ± 13.5 pg/ml; TCP 127.8 ± 33.2 pg/ml; $p = 0.0322$ and after eleven days: PEI-film 94.7 ± 12.5 pg/ml; TCP 128.8 ± 13.4 pg/ml; $p = 0.0011$).

The concentration of secreted interleukin 8 (IL-8) was always in the ng-range. There was a time dependent increase of IL-8 secretion during the experiment visible. Comparison between both materials showed a reduced IL-8 secretion on PEI-films two days after seeding (TCP 615.5 ± 313.6 pg/ml and PEI 445.5 ± 226.8 pg/ml ($p = 0.0106$)). After seven days we observed the opposite with a significant reduced IL-8 concentration on TCP (1,109.9 ± 198.3 pg/ml) compared to PEI-films (1,377.6 ± 372.4 pg/ml ($p = 0.0021$)). However, these differences ranged in the low pg-area which had no influence on the behavior of the HUVEC.

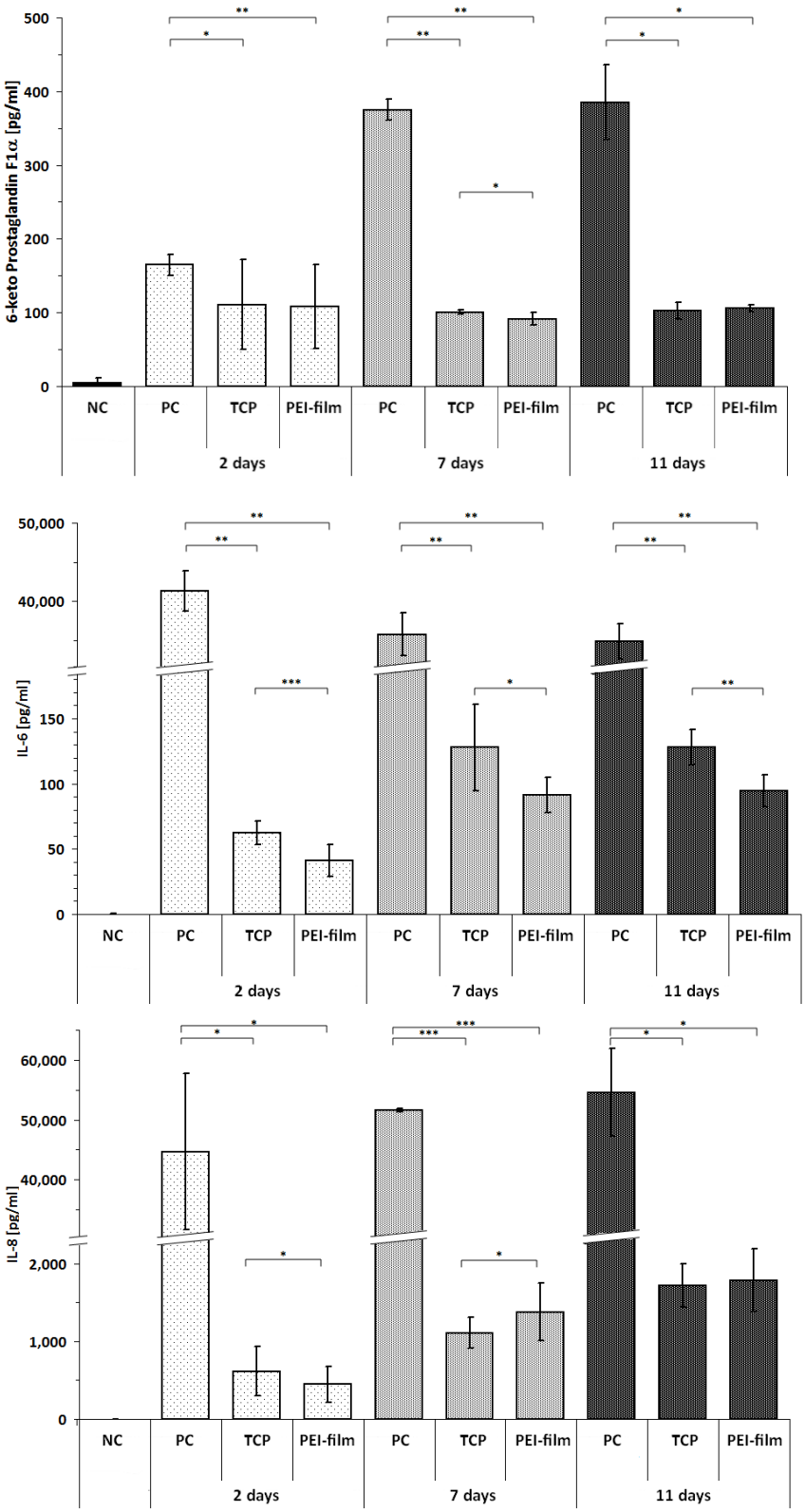

Figure 8. Vasoactive and inflammatory mediators were quantified from the supernatant by ELISA after culturing HUVEC up to eleven days on PEI-film with TCP as the control. (Data analyzed by student's t-test; * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; n = 6; NC: negative control (EGM-2); PC: positive control (IL-1β, 10 ng/ml) n = 3)

Discussion

In the present study the viability and vascular function of primary human umbilical vein endothelial cells (HUVEC) seeded out on smooth PEI-films was evaluated.

Solvent eluting from the PEI-films was found after 96 hours of incubation at 37 °C in water. The quantity of 3.55 ppm detected for approximately 150 cm² of the polymeric material should not influence the biological testing, because the amount of PEI usually used for cell experiments in 24 well plates per sample (~1.8 cm²) is nearly 10 times less. Furthermore remnants of evaporated DCM should immediately be removed from the system, because of the low solubility in water and the high vapor pressure in case of this solvent.

Generally, first biocompatibility testing of a potential biomaterial starts by treating a suitable cell line with eluates of the polymeric material to exclude an influence of eluting components (like solvents and monomers remained from the manufacturing process or degradation products) on the cell behavior. In the present study this indirect cytotoxicity testing was carried out with L929 cells.[14] The higher cell density and the increased occurrence of feeder cells as well as the increased mitochondrial activity can pass for a slight cytotoxic effect of the 72h-extract made from PEI-films.

Seeding of HUVEC on smooth and hydrophobic poly(ether imide) films resulted in a slightly reduced integrity of the plasma membrane. Furthermore a significant lower mitochondrial activity and cell density was observed 48 hours after seeding compared to TCP. However, after seven days the HUVEC monolayer reached confluence on PEI with a comparable cell density as on TCP. On both materials no cell-free areas were visible. The reason for the delayed confluence might be the smooth and hydrophobic surface of the PEI-films, which influences the adhesion process of the HUVEC. The same observations had been made while comparing polymeric surfaces with different wettability in case of biocompatibility. Various cell types exhibit poorer interaction with hydrophobic in comparison to hydrophilic biomaterials resulting in a decreased attachment and proliferation.[42] Initial interaction of biomaterials with the organism is usually the passive adsorption of proteins creating an amorphous layer on the polymeric surface. The composition of this protein layer is stochastic and sometimes leading to conformational changes of the adsorbed proteins.[11,42] This process is not at least dependent on the physicochemical properties of the polymer such as, hydrophobicity and surface topography, chemical composition and surface charges as well as on the composition of the ambient medium (e.g. blood, serum, culture medium, etc.).[4,26] The more hydrophobic a material surface is the more

unspecific is the adsorption because most proteins are denatured, exposing the inner hydrophobic residues to the outside and thereby changing their biological function. The accruing protein layer aggravates the specific interaction between adsorbed proteins and specific surface receptors like integrins exposed by the endothelial cells, which are mediating cell adhesion and extracellular matrix (ECM) formation.[11,19] Therefore the lower cell growing observed in the current study on PEI-films might be accounted for by differences in the biological activity of the adsorbed proteins. The proteins adsorbed on the TCP surface probably had a more biological active conformation and thereby supporting cell adhesion more than compared to those adsorbed to the hydrophobic PEI-films. Adhesion proteins actively produced from HUVEC also seems to play an important role. Endothelial cells are able to secrete their own ECM.[29,42] By creating the ECM, endothelial cells produce predominantly fibronectin and collagen. Synthesis and deposition of these attachment compounds by the cells after seeding requires occasionally several hours to days.[34] This might have been responsible for the delay in the initial phase of HUVEC monolayer formation observed on PEI-films in this study.

As a potential implant biomaterial PEI-films should not alter the morphological, adhesional and functional behaviour of the cells. *In vivo* endothelial cells lining ordinarily the lumen of blood vessels and form thereby a highly selective permeable barrier. For maintaining the functional integrity of this frontier between vascular system and tissue, intercellular as well as cell contacts to the underlying substrate (e.g. ECM) are the crucial factor. Therefore, the formation of the actin cytoskeleton was assessed, which is vital for the stabilization of cell contacts, especially through indirectly linkage to the ECM and the cytoskeleton of neighbouring cells. The actin cytoskeleton of HUVEC is organized in a netlike pattern around the cell cortex with increased filament strength at the extracellular contact points to the ECM and cells junctions, whereby ECM linkage is further stabilized by actin filament bundles in central parts of the cells called stress fibres.[12] Adhesion strength and thereby the interaction of the cells with the underlying substrate can be evaluated by the formation of focal adhesions (FA) involving the actin cytoskeleton and certain adaptor proteins such as vinculin, which is an essential structural protein of focal adhesion complexes. FA are key indicators for cell adhesion and persistence while regulating various vital cell processes (e.g. migration, proliferation, mechano-sensing, etc.).[43] We therefore compared the distribution and co-localization of actin and vinculin by immunofluorescence staining following exposure of HUVEC to smooth PEI surfaces. The HUVEC adhering either on PEI-

films or control (glass) showed the typical netlike pattern of a sub confluent endothelial cell monolayer 48 hours after seeding. Moreover HUVEC exhibited a distinct organization of actin stress fibers in the central parts of the cells, which are usually formed during the adhesion process. The termini of these actin fibers were mainly co-localized with vinculin, indicating a strong interaction of the HUVEC with the substrate. Furthermore the vinculin stained margin around the cells suggests a strong intercellular interaction. In conclusion there was no sign of a reduced interaction of the HUVEC with the underlying substrate based on PEI visible compared to the control.

The cellular secretion of the vasoactive mediators Prostacyclin (PGI_2) and Thromboxane A_2 (TXA_2) was always in the low pg-range and thereby for both materials in the range of biological efficacy. Physiological effects of the potent vasodilator and inhibitor of platelet aggregation PGI_2 were reported for concentrations down to 50 pg/ml (~10^{-10} M) whereas TXA_2 as a platelet aggregation inducing factor is four orders of magnitude more potent than his antagonist.[1,24] The concentration of PGI_2 (~100 pg/ml) was nearly constant during the whole experiment and approximately similar to those found in the plasma of healthy donors.[16,33] Measured amount of TXA_2 in the range between 2 - 7 pg/ml was up to 10 times lower than reported for *in vivo* plasma concentrations of healthy volunteers[9,33,40] and showed at no time point significant differences between PEI-films and the control. Similar levels of IL-6 in the area between 50 - 150 pg/ml were found in the supernatant of HUVEC for both materials during the whole timeframe of the study. These findings are in accordance with previous *in vitro* studies quantifying the IL-6 secretion of resting human endothelial cells in static and dynamic test systems.[7,20,38,41] IL-6 has been reported to develop no influence on the endothelial cell proliferation but it increases the permeability of the HUVEC monolayer by inducing a shape change of the cells (spindle form). Furthermore elevated levels of IL-6 are associated with chemotactic recruitment and transmigration of T-lymphocytes into inflammatory lesions.[7,8,25,39] The most important finding from this experiment was that during the whole timeframe of eleven days the amount of IL-6, detected in the supernatant of HUVEC seeded out on PEI-films, was always significantly lower than compared with TCP. An explanation for this finding during the first 48 hours after seeding might be the significantly lower cell density on PEI-films. However, at the following measurements the density of HUVEC on both materials was comparable. Therefore the results from this experiment suggest an anti-inflammatory influence of the polymeric material PEI on the HUVEC. There is no generally accepted view about the impact of IL-8 secretion on the HUVEC monolayer. On the one hand IL-8 secreted by

endothelial cells acts as a mediator of inflammation by chemotactic recruitment of leukocytes into inflamed tissue and enhancement of the adhesion molecule expression. On the other hand the same cytokine is an angiogenic factor, which is involved in the (neo)formation of blood vessels. IL-8 acts on HUVEC anti-apoptotic, promotes proliferation and migration as well as posses anti-inflammatory activities, while it inhibits the adhesion of leukocytes to activated endothelial cells.[7,18] For both materials the level of IL-8 in the supernatant was always consistent with previous *in vitro* studies analyzing the IL-8 secretion of resting human endothelial cells.[7,25] In this study a time dependent increase of the IL-8 amount was observed, which was independent from the underlying substrate. This increase might be accounted for by the increasing cell density over time. During the first seven days after seeding, there was a fluctuating difference in the IL-8 secretion between PEI-films and TCP visible. However, the variations in the IL-8 amount were always in the low pg-range and support no conclusions about a notable material dependent influence on the cell behaviour.

CONCLUSION

Poly(ether imide) in the form of smooth films showed slight cytotoxic effects on seeded HUVEC. The mitochondrial activity was significantly reduced (-25.9%) during the adherence process, but seven days after seeding confluence of the monolayer was reached. There was a strong interaction between the HUVEC and the underlying substrate. Secretion profile of vasoactive and inflammatory mediators did not differ to the control (TCP), except of a significantly reduced IL-6 amount when cells were seeded on PEI-films, which hints to an anti-inflammatory effect of PEI. However, additional studies are needed to clarify this putative effect on HUVEC as well as the monolayer stability under shear force in view of shear resistant endothelialization of PEI for the potential application as cardiovascular devices.

ACKNOWLEDGEMENTS

We would like to thank Susanne Reinhold, Ruth Hesse, Angelika Ritschel, Anja Müller-Heyn, Manuela Keller, Heike Schmidt and Regine Apostel for their technical assistance and measurements.

The authors comply with the Ethical Guidelines for Publication in *Clinical Hemorheology and Microcirculation* as published on the IOS Press website and in Volume 44, 2010, pp. 1–2 of this journal.

LITERATURE

[1] A.I. Ally, D.F. Horrobin, Thromboxane A2 in blood vessel walls and its physiological significance: Relevance to thrombosis and hypertension, *Prostaglandins and Medicine* 4(6), (1980), 431-38

[2] N. Alobaid, H. J. Salacinski, K. M. Sales, G. Hamilton, A. M. Seifalian, Single stage cell seeding of small diameter prosthetic cardiovascular grafts, *Clinical Hemorheology and Microcirculation* 33, (2005), 209-226

[3] J. M. Anderson, Biological responses to materials, *Annual Review of Material Research* 31, (2001), 81-110

[4] J. D. Andrade, V. Hlady, Protein adsorption and materials biocompatibility: A tutorial review and suggested hypotheses, *Advances in Polymer Science* 79, (1986), 1-63

[5] H. P. Arbogast, Antithrombogenicity of human endothelial cells, *Hämostaseologie* 25, (2005), 394–400

[6] S. Braune, M. Lange, K. Richau, K. Lützow, T. Weigel, F. Jung, A. Lendlein, Interaction of thrombocytes with poly(ether imide): The influence of processing, *Clinical Hemorheology and Microcirculation* 46, (2010), 239-250

[7] C. C. Chen, A. M. Manning, TGF-β1, IL-10 and IL-4 differentially modulate the cytokine-induced expression of IL-6 and IL-8 in human endothelial cells, *Cytokine* 8(1), (1996), 58-65

[8] C. P. Fischer, Interleukin-6 in acute exercise and training: what is the biological relevance? *Exercise Immunology Review Band* 12, (2006), 6–33

[9] S. Fuse, T. Kamiya, Plasma thromboxane B2 concentration in pulmonary hypertension associated with congenital heart disease, *Circulation* 90, (1994), 2952-2955

[10] M. B. Gorbet, M. V. Sefton, Endotoxin: The uninvited guest, *Biomaterials* 26(34), (2005), 6811-6817

[11] F. Grinnel, M.K. Feld, Fibronectin adsorption on hydrophilic and hydrophobic surfaces detected by antibody binding and analyzed during cell adhesion in serum-containing medium, *Journal of Biological Chemistry* 57, (1982), 4888–4893

[12] B. Hiebl, J. Cui, K. Kratz, O. Frank, M. Schossig, K. Richau, S. Lee, F. Jung, A. Lendlein, Viability, Morphology and Function of Primary Endothelial Cells on Poly(n-Butyl Acrylate) Networks Having Elastic Moduli Comparable to Arteries, *Journal of Biomaterial Science Polymer Edition* 23(7), (2012), 901-15

[13] W. L. Hunter, Drug-eluting stents: A multidisciplinary success story, *Advanced Drug Delivery Reviews* 58, (2006), 347–349

[14] International Standards Organization, ISO10993-5:biological evaluation of medical devices- part 5.Geneva:ISO, (1999)

[15] S. W. Jordan, E. L. Chaikof, Novel thromboresistant materials, *Journal of Vascular Surgery* 45, (2007), 104A-115A

[16] P. Kääpä, L. Viinikka, O. Ylikorkala, Plasma prostacyclin from birth to adolescence, *Archives of disease in childhood* 57, (1982), 459-461

[17] R. Y. Kannan, H. J. Salacinski, P. E. Butler, G Hamilton, A. M. Seifalian, Current status of prosthetic bypass grafts: a review, *Journal of Biomedicine Material Research* 74B, (2005), 570–581

[18] A. Li, S. Dubey, M. L. Varney, B. J. Dave, R. K. Singh, IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis, *Journal of Immunology* 170(6), (2003), 3369–3376

[19] D.R. Lu, K. Park, Effect of surface hydrophobicity on the conformational changes of adsorbed fibrinogen, *Journal of Colloid Interface Science* 144, (1991), 271–291

[20] N. Maruo, I. Morita, M. Shirao, S. Murota, IL-6 increases endothelial permeability *in vitro*, *Endocrinology* 131(2), (1992), 710-714

[21] A. J. Mclarty, M. R. Phillips, D. R. Jr. Holmes, H. V. Schaff, Aortocoronary bypass grafting with expanded polytetrafluoroethylene: 12-year patency, *The Annals of Thoracic Surgery* 65(5), (1998), 1442–1444

[22] A. De Mel, G. Jell, M. M. Stevens, A. M. Seifalian, Biofunctionalization of Biomaterials for Accelerated in Situ Endothelialization: A Review, *Biomacromolecules* 9(11), (2008), 2969-2979

[23] C. J. Murray, A.D. Lopez, The Global Burden of Disease: A Comprehensive Assessment of Mortality and Disability From Diseases, Injuries and Risk Factors in 1990 and Projected to 2020. Cambridge, Mass: Harvard University, (1996)

[24] S. Muxel, F. Fasola, M. C. Radmacher, A. Jabs, T. Münzel, T. Gori, Endothelial functions: Translating theory into clinical application, *Clinical Hemorheology and Microcirculation* 45(2-4), (2010), 109-115

[25] E. M. Nilsen, F.-E. Johansen, F. L. Jahnsen, K. E. A. Lundin, T. Scholz, P. Brandtzaeg, G. Haraldsen, Cytokine profiles of cultured microvascular endothelial cells from the human intestine, *Gut* 42, (1998), 635–642

[26] W. Norde, J. Lyklema, Why proteins prefer interfaces, *Journal of Biomaterial Science Polymer Edition: The Vroman Effect*, (1991), 1-20

[27] G. Peluso, O. Petillo, L. Ambrosio, L. Nicolais, Polyetherimide as biomaterial - preliminary *in vitro* and *in vivo* biocompatibility testing, *Journal of Material Science: Materials in Medicine* **5**, (1994), 738–742

[28] 0. Petillo, G. Peluso, L. Ambrosia, L. Nicolais, W. J. Kao, J. M. Anderson, *In vivo* induction of macrophage Ia antigen (MHC class 11) expression by biomedical polymers in the cage implant system, *Journal of Biomedical Materials Research* **28**, (1994), 635-646

[29] S. Rajabalian, Z. S. Bahrami, V. Farahat, F. Shokri, Supportive Effects of Human Embryonic Fibroblast Cell Lines on Growth and Proliferation of EBV-Transformed Lymphoblastoid Cells, *Iranian Biomedical Journal* 7(4), (1993), 147-53

[30] B. D. Ratner, S. J. Bryant, Biomaterials: where we have been and where we are going, *Annual Review of Biomedicine Engineering* 6, (2004), 41–75

[31] R.R. Richardson Jr, J.A. Miller, W.M. Reichert, Polyimides as biomaterials - preliminary biocompatibility testing, *Biomaterials* 14, (1993), 627–635

[32] S. Schopka, F. X. Schmid, S. Hirt, D.E. Birnbaum, C. Schmid, K. Lehle, Recellularization of biological heart valves with human vascular cells - *in vitro* hemocompatibility assessment, *Journal of Biomedicine Material Research* 88, (2009), 130-138

[33] G. J. Slotman, Plasma thromboxane A2 and prostacyclin concentrations in squamous cell carcinoma of the head and neck, *Journal of Surgical Oncology* 38(1), (1988), 33-37

[34] H. S. Smith, J. L. Riggs, M. W. Mosesson, Production of Fibronectin by Human Epithelial Cells in Culture, *Cancer Research* 39, (1979), 4138-4144

[35] C. Stettler, S. Wandel, S. Allemann, Outcomes associated with drug-eluting and bare-metal stents: a collaborative network meta-analysis, *Lancet* 370, (2007), 937-948

[36] G. W. Stone, A. Rizvi, W. Newman, K. Mastali, J. C. Wang, R. Caputo, J. Doostzadeh, S. Cao, C. A. Simonton, K. Sudhir, A. J. Lansky, D. E. Cutlip, D. J. Kereiakes Everolimus-Eluting versus Paclitaxel-Eluting Stents in Coronary Artery Disease, The New England Journal of Medicine 362, (2010), 1663-1674

[37] R. Tzoneva, B. Seifert, W. Albrecht, K. Richau, A. Lendlein, T. Groth, Poly(ether imide) membranes: studies on the effect of surface modification an protein pre-absorption on endothelial cell adhesion, growth and function, *Journal of Biomaterial Science Polymer Edition* 19(7), (2008), 837–852

[38] K. Urschel, I. Cicha, W. G. Daniel, C. D. Garlichs, Shear stress patterns affect the secreted chemokine profile in endothelial cells, *Clinical Hemorheology and Microcirculation* 50(1-2), (2012), 143-152

[39] K. Urschel, A. Wörner, W. G. Daniel, C. D. Garlichs, I. Cicha, Role of shear stress patterns in the TNF-α-induced atherogenic protein expression and monocytic cell adhesion to endothelium, *Clinical Hemorheology and Microcirculation* 46(2-3), (2010), 203-210

[40] L. Viinikkaa, O. Ylikorkala, Measurement of thromboxane B2 in human plasma or serum by radioimmunoassay, *Prostaglandins* 20(4), (1980), 759-766

[41] A. Waage, G. Slupphaug, R. Shalaby, Glucocorticoids inhibit the production of IL 6 from monocytes, endothelial cells and fibroblasts, *European Journal of Immunology* 20(11), (1990), 2439–2443

[42] P. B. van Wachem, T. Beugeling, J. Feijen, A. Bantjes, J. P. Detmers, W. G. van Aken, Interaction of cultured human endothelial cells with polymeric surfaces of different wettabilities, Biomaterials 6, (1985), 403-408

[43] M. A. Wozniak, K. Modzelewska, L. Kwong, P. J. Keely, Focal adhesion regulation of cell behavior, *Biochimica et Biophysica Acta* 1692, (2004), 103– 19