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NANOFIBROUS VASCULAR GRAFTS

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SUMMARY OF THE THESIS

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Abstract

There is a pressing need to develop vascular graft since no clinically available appropriate prosthesis with inner diameter less than 6 mm works in a long term after implantation. In the thesis, blood vessel substitutes made from biodegradable polymers were created and characterized as potential candidates for such a medical device. The idea of tissue engineering scaffolds is based on mimicking natural environment - extracellular matrix. Therefore ideal bypass graft was designed as double layered structure with defined morphology of each layer. The proposed structure was created by electrospinning of polycaprolactone (PCL). The morphology of the resulting fibers resembled inner and medial layer of native arteries suggesting that this similarity will help body to regenerate functional tissue after implantation. Besides PCL, novel polymer from the same group of polyester - copolymer polylactide-polycaprolactone (PLC 70/30) was electrospun into a tubular form. Vascular graft made from copolymer PLC created only single layered prosthesis.

Further tests were conducted with both presented electrospun materials in order to compare their bulk and surface properties. Copolymer PLC was slightly more hydrophilic than polycaprolactone. Thermal behavior revealed that copolymer is mostly amorphous with melting temperature about 110°C whereas polycaprolactone is semicrystalline polymer with melting temperature about 57°C. Mechanical strength and elongation at break of electrospun graft made from copolymer PLC was about ten times higher compared to electrospun graft made from polycaprolactone.

Biological tests using fibroblast and endothelial cell line proved the biocompatibility of both tested electrospun polymers. Higher proliferation rate was found when cells were cultured on electrospun copolymer PLC suggesting that higher hydrophilicity contributes to favorable cell adhesion. Hemocompatibility testing of produced samples were carried out using platelets. It was found that fibrous layers are more thrombogenic than smooth surface when compared with foils made from the same materials. Platelets became activated and aggregated after incubation with fibrous materials. The level of activation increased in dynamic conditions.

Electrospun fibers were successfully used as a drug delivery system of nitric oxide (NO) that has many beneficial effects on cardiovascular system. Polycaprolactone fibers were blended with NO donors from the group of S-Nitrosothiols that are capable of long term NO release in physiological levels up to 42 days *in vitro.* After implantation of such grafts as a replacement of rat abdominal aorta, the NO release was found to strongly inhibit cellular infiltration into the medial and luminal regions of the vascular graft. The reduced presence of inflammatory cells within these regions may confer increased protection against neointimal hyperplasia from smooth muscle cells.

Keywords: Vascular grafts, Nanofibers, Electrospinning, *In vitro* tests, Nitric Oxide

Anotace

V současnosti není v klinické praxi cévní náhrada s vnitřním průměrem pod 6 mm, která by spolehlivě fungovala v dlouhodobém horizontu. Disertační práce se zabývá přípravou maloprůměrových cévních náhrad z biodegradabilních polymerů, které jsou testovány jako potenciálně vhodné materiály pro přípravu tkáňových nosičů pro vaskulární cévní systém. Hlavní myšlenkou tkáňového inženýrství je napodobování přirozeného prostředí - mezibuněčné hmoty. Proto byla ideální cévní náhrada navržena jako dvouvrstvá tubulární struktura s definovanou morfologií vláken. Tato struktura napodobující vnitřní a střední vrstvu nativní cévy byla vytvořena elektrostatickým zvlákňováním polykaprolaktonu (PCL). Podobnost morfologie vláken s mezibuněčnou hmotou předpokládá, že po implantaci do organismu proběhne regenerace funkční tkáně. Kromě polymeru polykapronu byl testován polymer ze stejné třídy polyesterů - kopolymer polylatidu a polykaprolaktonu (PLC 70/30). Cévní náhrada připravená z toho polymeru byla tvořena pouze jednou vrstvou.

Pro porovnání vlastností polymerů byla provedena charakterizace obou elektrostaticky zvlákněných materiálů. Kopolymer PLC je mírně hydrofilnější než polykaprolakton. Termické vlastnosti obou polymerů se značně liší. Zatímco kopolymer PLC je převážně amorfní s teplotou tání okolo 110°C, polykaprolakton je semikrystalický polymer s teplotou tání kolem 57°C. Mechanická pevnost a prodloužení je přibližně desetkrát větší u elektrostaticky zvlákněného kopolymeru PLC než u polykaprolaktonu.

Biologické testování elektrostaticky zvlákněných materiálů potvrdilo biokompatibilitu obou testovaných polymerů s fibroblasty i s endotelovými buňkami. Vyšší proliferační stupeň byl pozorován při kultivaci buněk na mírně hydrofilnějším kopolymeru PLC, který zřejmě umožňuje lepší buněčnou adhezi. Vlákenné materiály byly rovněž testovány po interakci s krevními destičkami, které se po inkubaci aktivovaly a agregovaly. Mírnější aktivace byla pozorována po interakci s hladkými foliemi vyrobenými ze stejných materiálů, což dokládá, že na aktivaci destiček má vliv morfologie povrchu. Zvýšená aktivace trombocytů byla pak také pozorována při dynamické inkubaci vlákenných tubulárních vzorků.

Vlákenné tkáňové nosiče byly využity jako systém cíleného uvolňování léčiv, konkrétně oxidu dusnatého (NO), který má mnohé pozitivní účinky na kardiovaskulární systém. Vlákna polykaprolaktonu byla obohacena o donory NO ze skupiny S-Nitrosothiolů, které umožňují uvolňování NO ve fyziologickém rozmezí po 42 dní během testování *in vitro*. Po implantaci cévních náhrad jako náhrada břišní části aorty u potkanů bylo zjištěno, že NO inhibuje buněčnou infiltraci do vnitřní a střední vrstvy cévní náhrady. Tento snížený výskyt zánětlivých buněk může bránit vzniku neointimální hyperplazie způsobenou hladkosvalovými buňkami v pozdějších stadiích implantace.

Klíčová slova: Cévní náhrady, Nanovlákna, Elektrostatické zvlákňování, *In vitro* testování, Oxid dusnatý

Content

1 Introduction

The development of new medical care and treatment lead to the ageing of the population and more tissues are needed to be repaired or restored. Transplantation is considered to be a gold standard of tissue replacement; however it could be limited due to the lack of appropriate donors. Government and other funding institutions are bewaring of this fact therefore a lot of grants and projects dealing with so called tissue engineering are funded nowadays. The development of tissue engineering scaffold, making them off-the shelf available in various sizes is a real challenge in today's world. Especially in the field of vascular tissue engineering there is a demand of an appropriate scaffold since no small diameter synthetic vascular graft successful in a long term after implantation has been successfully translated to clinic yet.

Cardiovascular diseases (CVDs) are the number one cause of death globally. More people die annually from CVDs than from any other cause according to World Health Organization. A large number of patients suffer from vascular damage, resulting in the need for bypass surgery. Blood vessels can be blocked through a process called atherosclerosis. Cholesterol and fibrous tissue make up a plaque and blood vessels become narrow and stiffen. If the vessel is completely occluded, new pathway for blood flow has to be created during a surgery. A graft can be either autologous using patient own vessel or man-made synthetic tube.

Since there are still limitations in the replacement of small diameter vascular grafts, the need and demand for developing more desirable grafts is increasing day by day. The thesis is focused on a contribution to the development of ideal bypass graft scaffolding material. Currently used materials are commercially fabricated from inert polymers such as expanded polytetrafluorethylene or polyethylene terephtalate known as Dacron. In the thesis, the usage of biodegradable materials is preferred since these materials possess many advantages over the inert ones. After implantation of biodegradable material, the body will start the healing response. Ideally, the scaffold structure and composition would be able to promote healing of the injured or damaged tissue. In this case, scaffold material serves as a temporary support that starts self-renewal of the tissue.

2 Purpose and the aims of the thesis

The aim of the dissertation was to create a vascular graft that will fulfill requirements of small diameter vascular graft in terms of morphological structure that resembles native extracellular matrix (1), possess appropriate mechanical properties (2) and surface properties that will facilitate cell adhesion, especially endothelial cell adhesion to prevent further thrombosis (3). Synthetic vascular grafts could be improved by incorporation of nitric oxide releasing substances. The aim of long term nitric oxide release (4) was hypothesized to reach in the last experimental part of the thesis.

3 Overview of the current state of the problem

Tissue engineering is an interdisciplinary field that applies the principles of chemistry, physics, material science, engineering, cell biology and medicine to the development of biological substitutes that restore, maintain or improve tissue/organ functions (*Langer, 1993*). The combination of classical engineering and life sciences is essential. Biomedical engineering requires the cooperation of materials engineers, cell culture biologists, clinicians and many other experts in different fields in order to develop functional scaffold.

Tissue engineering scaffolds are designed as structural and functional analogues

of extracellular matrix (ECM) assuming that cells recognize their natural environment and undergo the regeneration of the damaged tissue. To reach this goal, many scaffold fabrication techniques has been studied, for example rapid prototyping, solvent casting and particulate leaching, electrospinning or decellularization of tissues. Specific requirements are demanded for each application but some of them are generally accepted. Scaffolds have to be fabricated from biocompatible materials that will further promote normal cell growth without any adverse tissue reactions (*Boland, 2004).*

The issue of small diameter blood vessel replacement remains a major challenge yet to be overcome in the production of appropriate vascular grafts. Specific properties of such grafts have to be maintained not only in time of surgery but also in a long term after the implantation. The production of vascular grafts has to be cost effective, environmental friendly with consistent quality. The final product has to withstand selected sterilization technique. The graft should be available in different sizes, various inner diameters, wall thickness and length. During implantation, the graft has to be easily sutured and provided initial mechanical strength to withstand blood pressure with no bleeding. An ideal vascular graft must meet an extended list of criteria including the strength and elasticity of the vessel wall, biocompatibility, blood compatibility and biostability in the long term *(Greenwald, 2000; Arrigoni, 2006).* It also needs to adapt to the hemodynamic conditions. Vascular graft should enable the regeneration of the vessel wall therefore inert materials are replaced by biodegradable ones. The materials have to be non-immunogenic and non-toxic *(Thomas, 2003; Kakisis, 2005).*

Vascular grafts could be classified as small caliber diameter (< 6 mm), medium size (6-8 mm) and large caliber diameter (> 8 mm) *(Chlupac, 2009).* The latter are successfully used in clinical praxis for years but there is still a pressing need to develop small diameter vascular grafts that can replace failed small diameter arteries when there is an absence of endogenous grafting material. Vascular grafts could be classified into two groups based on their material composition as biological and synthetic. **Biological grafts** are usually the first choice in clinical use. Autologous veins are preferred for bypass grafting of arteries. These grafts provide mechanical stability and natural antithrombogenicity *(Angelini, 1989; Cameron, 1996).* However, increase in the indications for the surgical revascularization, elderly patients' population and increased number of re-operations could be limiting for the availability of suitable autologous grafts. Unavailability of the autologous grafts could be an invitation for the usage of prosthetic conduits.

Synthetic grafts are represented by biostable grafts made from expanded polytetrafluorethylene (ePTFE) and polyethylene terephtalate (PET). These inert materials are succesfully used for large and medium size vascular grafts. However, in small diameter locations the grafts became occluded. The major causes of synthetic vascular graft failure have been thrombosis and intimal hyperplasia *(Esquivel, 1986).* In the last years, new biodegradable materials are under development amongst which polyurethane (PUR) and polyesters has been successfully investigated. *Biodegradable polyesters*, such as poly-ε-caprolactone or poly-L-lactic acid have been successfully used in research for tissue engineering applications, including vascular replacement *(Vaz, 2005; Notellet, 2009; Dong, 2008; He, 2008; Wu, 2010; Hu, 2012; Huang, 2012).* The representative candidates, namely PCL and copolymer composed of polylactic acid and polycaprolactone (PLC) were used in experimental part of the thesis. Polymer PCL has been reported for different tissue engineering applications such as bone tissue engineering *(Rampichova, 2013; Erben, 2015).* Based on literature, electrospun vascular grafts made from PCL were reported by several groups to be a promising candidate for vascular replacement *(Pektok, 2008; Notellet, 2009).* PCL possesses intrinsically slow degradation rate, desirable mechanical properties, and general biocompatibility *(Woodruff, 2010).* However, insufficient regeneration

of the vascular wall as well as graft calcification was reported by Valence et al. *(2012).* Therefore novel material besides PCL was tested in order to improve function of such grafts.

Copolymer PLC composed of polylactic acid and polycaprolactone in different ratios has also been reported as a promising candidate for vascular graft replacement. Mo et al. studied electrospinning conditions of copolymer PLLA and PCL in ration 75/25 proving its biocompatibility with endothelial cells and smooth muscle cells *in vitro (Mo, 2004)*. Dong et al. tested copolymer PLLA and PCL in ration 70/30 with endothelial cells for 105 days proving its long-term compatibility with the endothelial cells that is a crucial task for vascular graft function after implantation *(Dong, 2008)*. He et al. rotationally seeded endothelial cells in the lumen of the graft made from copolymer PLLA and PCL in ration 70/30. After 10 days endothelial cells covered the lumen of the prepared graft during culturing *in vitro*. This construct was subsequently implanted in the rabbit showing patency for 7 weeks *(He, 2008)*.

The final vascular graft could be designed as multi layered tube that will match the properties of native tissues. Different polymers, fabrication techniques as well as drug delivery systems could be employed in order to produce ideal vascular graft. Such approach has been published for example by Han et al. The scaffold was prepared by electrospinning of poly(ethylene glycol)-*b*-poly(L-lactide-co-ε-caprolactone) (PELCL), copolymer of polyglycolic acid and polylactic acid (PLGA) and PCL to ensure sufficient mechanical properties. Specific growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) were incorporated into the inner and middle layer of the graft. The structure od prepared graft together with drug delivery system supported new blood vessel formation and maturation *in vivo* when sutured as a replacement of rabbit common carotid artery for 8 weeks *(Han, 2013).* Similar study using double layered electrospun scaffold was performed by Zhang et al. The combination of gelatin, elastin, PCL and poliglecapron (PGC) was used to promote endothelialization. Human aortic endothelial cells favored the biomechanics and biochemistry of such scaffold for at least 11 days *in vitro (Zhang, 2010).* A three layered electrospun scaffold made from PCL, collagen and elastin was described by McClure et al. The combination of polymers led to construction of vascular graft with distinct properties for each layer such as fiber diameter, suture retention and compliance. Mathematical modeling was implemented in order to achieve the best mechanical combination of materials and to help the prediction of future graft optimization *(McClure, 2010)*. Fiber orientation in vascular graft was studied by Wu et al. through the combination of regulating the electric field and the rotation of collector leading to tubular scaffolds with different nanofiber orientation (circumferential, axial and its combination). They stated that such a complex nanofiber orientation can be constructed to achieve desirable macroscopic mechanical property and cell responses along specific directions *(Wu, 2010).*

Fabrication of biodegradable synthetic vascular grafts seems to be a promising approach to generate appropriate scaffolds in terms of morphological similarity to the native ECM, appropriate bulk and surface properties. However, limitations in the healing response of electrospun vascular grafts made from PCL were described *(Valence, 2012)*. To overcome the issues of thrombogenicity of the grafts, lack of endothelialization of the graft lumen, intimal hyperplasia development as well as inflammatory reaction, the incorporation of nitric oxide releasing substances was introduced in the experimental part of the thesis. NO is a diatomic free radical, known as the endothelium-derived relaxing factor (EDRF). Endothelial cells produce NO that has many beneficial effects on cardiovascular system. NO is thromboresistant due to the inhibition of platelet aggregation, adhesion and activation *(Radomski, 1987)*. The affects of NO differs for certain cell types in blood vessels. Whereas NO stimulates endothelial cell proliferation *(Ziche, 1994)* and prevents endothelial cells apoptosis *(Tzeng, 1997)*, it also inhibits smooth

muscle cells growth and migration *(Garg, 1989; Mooradian, 1995)*. NO possess anti-inflammatory properties due to the inhibition of leukocyte adhesion and migration *(Lefer, 1997)*. NO has limited solubility in water (2-3 mM), and it is unstable in the presence of various oxidants. This makes it difficult to introduce into biological systems in a controlled manner. Consequently, the development of chemical agents that release NO is important *(Wang, 2005)*. One promising group of NO donors is S-nitrosothiols (RSNO), which can be readily incorporated into a polymeric vascular graft. S-Nitrosothiols are present in biological systems, where they serve as a reservoir and transporter of NO *(Jourd'heuil, 2000)*.

4 Materials and Methods

4.1 Materials used

Synthetic polyesters were used for fabrication of small diameter vascular grafts, namely polycaprolactone (PCL, M_n=45,000, Sigma Aldrich) and copolymer of poly-L-lactide and polycaprolactone (PLC, 70/30, PURASORB). Polymer PCL supplied by Sigma Aldrich has the average number molecular weight of 45,000 (M_n 40,000-50,000) and polydispersity index between 1,2 and 1,8 with the mass average molecular weight of 48,000-90,000. Copolymer PURASORB PLC 7015 is a GMP grade (Good Manufacturing Practice) copolymer of L-lactide and ε-caprolactone in a 70/30 molar ratio. Content of L-lactide is determined by the supplier in the range of 67-73 mol % and caprolactone between 33 and 27 mol %. Instead of molecular weight of the polymer, inherent viscosity is determined by the supplier. The midpoint of inherent viscosity is 1,5 dl/g (ranging between 1,2 and 1,8 dl/g).

4.2 Fabrication of small diameter vascular grafts

Synthetic vascular grafts were prepared by electrospinning with special set up that is depicted in figure 1. Special collector in the form of rotating stainless steel was used for obtaining tubular scaffolds. Electrospinning parameters like speed of polymer dosage, voltage, distance between needle tip and collector, speed of mandrel rotation, relative humidity and temperature were recorded. The parameters are described together with resulting structures in each experiment.

The custom designed electrospinning apparatus consisted of a positive high-voltage power supply (Spellman SL 150, Direct Industry), a syringe pump, a plastic syringe, a hypodermic needle and a grounded stainless steel rotating mandrel (1-6 mm diameter, 20 cm length). The speed of rotation varied between 250 rpm and 15 000 rpm. The data of rotation speed are specified later in each chapter together with the results. Reciprocal movement of the needle spinning electrode was achieved using a linear actuator. Polymeric solution dosage was set to 1,5 ml/h. Time of electrospinning was adjusted to the required thickness of the gatft that was measured during the fabrication using micrometer screw gauge. After electrospinning, the tubular scaffold was dried overnight and then removed from the madrel by manually pushing.

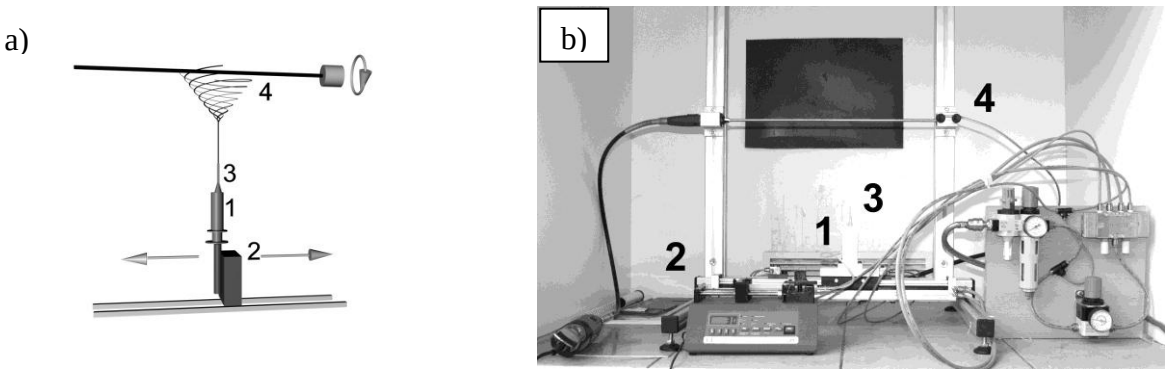


Figure 1: The schema (a) and photo (b) of electrospinning setup used for small diameter vascular grafts. Syringe pump (2) doses polymeric solution in the syringe (1) that is connected to the positively charged needle (3). Forming fibers are collected on the rotating mandrel (4).

4.3 Mechanical properties of tubular scaffolds

Microfibrous samples with similar morphology were prepared by electrospinning of 18 wt% PCL and 10 wt% PLC on rotating mandrel collector having the inner diameter of 6 mm. Mechanical properties were investigated in single layered tubular scaffolds based on the hypothesis that media layer of final vascular graft is responsible for its mechanical properties. These tubular samples were cut into rectangular shapes with a constant width of 10 mm. The thickness of the graft wall was measured using micrometer screw gauge before each experiment. The samples were clamped into jaws and stretched using loading rate of 50 mm/min until break. The active length of measured sample was 50 mm. During the tensile test, force and elongation of the scaffold were recorded to obtain stress-strain curves of each tested material (n=3).

4.4 *In vitro* tests of electrospun layers

Electrospun layers made from PCL and PLC were analysed *in vitro*. Firstly, fibroblasts (3T3 mouse fibroblasts, ATCC) were used to assess the overall cell behavior on prepared layers. Further, these layers were tested with Human umbilical vein endothelial cells (HUVEC, Lonza). Finally, the layers were tested for their thrombogenic potential after incubation with thrombocytes. Specific *in vitro* tests (endothelial cell seeding, thrombogenicity testing) regarding final the usage of scaffolds in vascular tissue engineering was carried out with nanofibrous PCL (PCL nm), microfibrus PCL (PCL μm) and microfibrous PLC (PLC) since the copolymer is not able to create fibers in nanoscale. Therefore material composition influence as well as its morphology was evaluated.

To test cell adhesion and proliferation, colorimetric test using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT test) was used for measurement of cell viability during the cultivation time. Fluorescence microscopy and scanning electron microscopy (SEM) was used for analyses of cellular morphology on the fibrous layers. Fibroblasts/endothelial cell lines were seeded onto ethanol sterilized scaffolds having circular size of 6 mm in diameter. The above mentioned analysis were done after 1, 3, 7 and 14 days of cell culturing.

Thrombogenicity tests were carried out in static conditions by incubation of the scaffolds in 96well plates (6 mm in diameter) with thrombocytes rich solution (TRS). In order to compare different surface morphology, samples made from PCL (foils and nanofibrous electrospun layers) and PLC (foils and microfibrous electrospun layers) were prepared in the same way as in previous experiment and incubated with TRS. The analysis

was carried in a similar way as in culturing method – the viability was measured after 2 hours, 1 day, 4 days and 7 days by MTT assay. Scanning electrone microscopy was done in the same intervals to depict thrombocyte morphology.

Dynamic conditions were simulated using bioreactor that allow the flow of solution. Blood flow is an important aspect that contributes to thrombocyte activation. The first prototype of such a device was designed and constructed in the Technical University of Liberec. Two double layered PCL vascular graft (inner layer interacting with thrombocytes was composed of nanofibers) and two single layered microfibrous PLC grafts were attached in bioreactor followed by thrombocyte rich solution circulation through each tested graft for 2 hours (10 ml/min). The analysis was done by SEM only to observe the change of thrombocyte shape.

4.5 Modification of vascular grafts by nitric oxide donors

This part of the thesis was done in Michigan Technological university (MTU), Department of Biomedical Engineering. The project was focused on the development of a long-term NO-releasing polymeric vascular graft by blending different NO releasing compound from the group of S-Nitrosothiols with PCL by the way of electrospinning. Newly synthesized compound, S-Nitrosoacetyl-D-penicillamine derivatized cyclam (SNAP-cyclam), was studied for long term NO release.

Vascular grafts made from poly-ε-caprolactone (PCL, Sigma Aldrich, M_n=45000) were obtained by electrospinning a 16 wt% solution of PCL dissolved in chloroform/ethanol/acetic acid (8/1/1 v/v/v) using similar device as described in figure 1. Vascular grafts with long-term NO release were prepared by mixing the SNAP-cyclam (0,2734 wt%) into the PCL electrospinning solution. The final concentration of NO releasing compounds was optimized by: the amount of compounds added to the electrospinning solution (a), the way of preparation of electrospinning solution (b), the release kinetics of produced grafts (c). After a series of optimization procedure of electrospinning solution preparation, the electrospinning parameters were set as follows: the solution was charged at 20 kV and ejected through a 22 G needle at a constant rate of 2,54 ml/h. The fibers were collected on a rotating stainless steel mandrel at a rotational speed of 250 rpm. The mandrel was placed 15 cm from the needle tip.

Measurement of NO release was carried out using a Siever's Nitric Oxide Analyzer (NOA) that is based chemiluminiscence reaction of NO with ozone:

$$NO + O_3 \rightarrow NO_2^- + O_2$$
$$NO_2^- \rightarrow NO_2 + h\nu$$

The device offers the most versatile detection system for NO analysis. Samples releasing NO were measured immersed in phosphate-buffered saline (PBS) at 37°C to imitate conditions in the body. S-Nitrosothiols release NO group in the presence of copper ions and ascorbic acid. Therefore when NO release declined, these agents were added to PBS and further NO release was detected.

In order to simulate body conditions, vascular grafts were incubated in PBS and in complete medium between NO release measurements. Vascular grafts made from PCL modified by SNAP-cyclam (length 1 cm) were weighted and incubated in PBS as well as complete medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum and 1% penicillin/streptomycin. The data obtained by incubation in PBS/complete medium should simulate the environment *in vivo* where the same vascular grafts were further implanted for time period of 10 days.

Six Sprague Dawley rats received an abdominal aorta replacement graft with an inside diameter of 1,65 mm and wall thickness of 685,5 ± 53,3 μm, of which three were pure

electrospun PCL controls, and three were experimental NO releasing, electrospun vascular grafts containing SNAP-cyclam.

After 10 days, the grafts were collected with the host distal and proximal ends of the artery. The samples were embedded in freezing medium (Neg 50; Thermo Scientific), snap frozen in liquid nitrogen and cryosectioned. Basic histological assessment (hematoxylin eosin staining, fluorescence staining of cell nuclei) was carried out in order to characterize the scaffodls engraftment.

5 Summary of the results achieved

5.1 Electrospinning of polycaprolactone

One of the goal of the thesis was mimicking of structure of native blood vessel that is naturally composed of 3 layers. The histological investigation of blood vessel composition is also a part of the thesis and yields in the design of ideal double layered vascular graft that will be mimicked by electrospinning. The idea was to mimic only 2 layers (inner and medial) assuming that the third outer layer will create naturally after implantation into the body. The proposed structure is depicted in figure 2. The inner layer supports the endothelial cells that are crucial for vascular graft function within the body. Endothelialization of the graft lumen will ensure the antithrombotic surface. Medial layer should allow smooth muscle cell penetration into many layers that are naturally found in native vessels and ensure mechanical stability of the vessel.

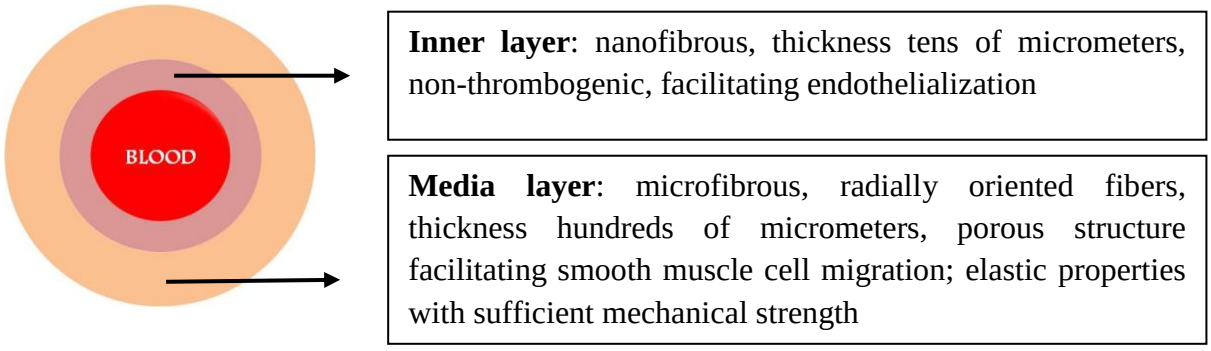


Figure 2: Structural design of double layered vascular grafts.

Electrospinning of PCL solution led to the production of proposed structure designed in the figure 2. The inner layer was composed of nanofibers that were electrospun from 16 wt % PCL. It has been discovered that the addition of acetic acid into electrospinning solution decrease the fiber diameter therefore PCL for inner layer was dissolved in chloroform/ethanol/acetic acid 8/1/1 (v/v/v). The solution led mostly to the fibers having about 150 nm diameter with a few deformed fibers and beads as depicted in figure 3 a. Nanofibrous structure also serves as a barrier for migration of other cell types into this layer that could cause severe complications such as intimal hyperplasia. The thickness of the layer was adjusted by the time of electrospinning that was set to 5-10 minutes for the thin inner layer (giving the thickness of tens of micrometers). There was no requirement for fiber orientation therefore the rotation speed was set between 3 000 and 5 000 rpm. The media layer has to ensure the mechanical strength and support for smooth muscle cells that are radially oriented in many layers. The infiltration of the smooth muslce cells are supported by microfibrous structure allowing cells to penetrate the middle part of the graft. The media layer was prepared by electrospinning of 18 wt % PCL dissolved in chloroform/ethanol 9/1

(v/v) having diameter of around 1 μm (figure 3 b). The layer was thicker (250-300 μm set by the time of electrospinning that was about 1 hour) in order to ensure appropriate mechanical strength of the graft. The speed of collector rotation was adjusted to 10 000 rpm in order to obtain fiber orientation. The electrospinning parameters were the same for both layers: temperature 22-23°C, relative humidity 50-60%, voltage 15 kV, distance 20 cm, feed rate 3 ml/h. Cross section of double layered PCL graft is seen in figure 3 c.

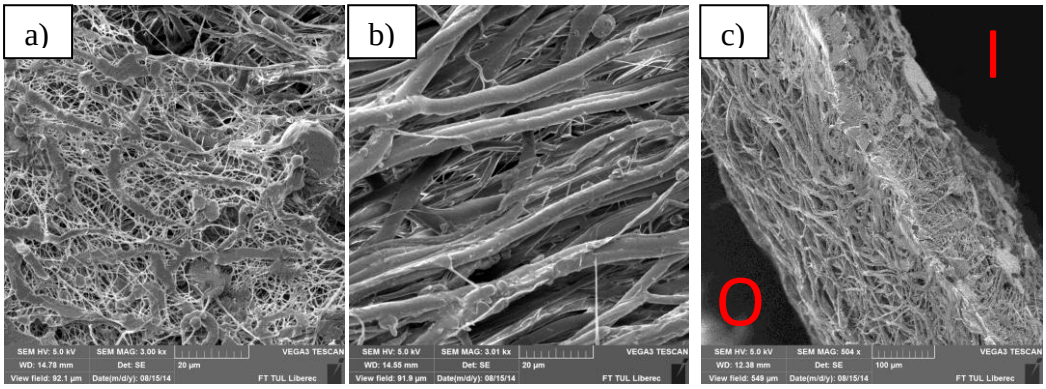


Figure 3: Inner layer of the graft composed of nanofibers with a few deformed fibers and beads (a) and outer layer composed of oriented microfibers (b), scale bars 20 μm. Cross section of double layered PCL graft composed of nanofibers in the inner side (I) and microfibers in the outer side (O). Scale bar100 μm (c).

5.2 Electrospinning of copolymer PLC

Copolymer PLC was succesfully electrospun from electrospinning solution composed of 10 wt % PLC dissolved in chloroform/ethanol/acetic acid 8/1/1 v/v/v for production of tubular scaffolds. The structure of fibers electrospun on rotating mandrel as well as tube cross section is depicted in the figure 4. Electrospinning parameters were set as follows: needle diameter 0,6 mm, volage 15 kV, distance between the tip of the needle and collector 20 cm, speed rotation of the mandrel 5 000 rpm, feed rate 3 ml/h. Copolymer PLC did not enable the creation a double layered vascular graft with morphology resembling native ECM. Electrospinning of PLC on rotating mandrel led to the structure composed of uniform microfibers. Changing of electrospinning solution composition nor electrospinning conditions did not enable the creation of nanofibers from this copolymer.

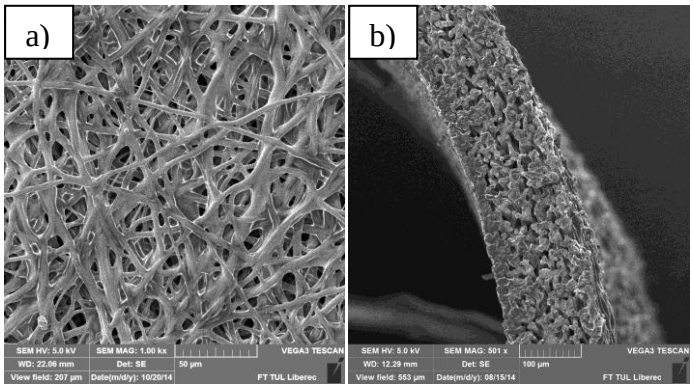


Figure 4: Fiber morphology of electrospun copolymer PLC (a), scale bar 50 μm and cross section of vascular graft made from PLC (b), scale bar 100 μm.

5.3 Mechanical properties

Mechanical behaviour of tubular samples reflected different mechanical behavior between PCL and PLC fibrous layers. The maximum strength of PCL tubular samples reached the values between 2,3 and 4,4 MPa (the average engineering tension of 3,3 ± 1,1 MPa). The elongation of tubular samples made from PCL ranged between 32 and 48% (the average value of 37,5 ± 8,8 %). The scaffolds made from copolymer PLC showed different shape of measured stress-strain curves than in PCL reaching the maximum strength between 26 and 43 MPa (the average of 37,2 ± 9,2 MPa) and elongation at break of 230-450% with average value of 377,4 ± 157,4 %. Representative stress strain curves for each tested tubular sample with similar wall thickness is depicted in figure 5. Electrospun copolymer PLC is capable to withstand higher engineering tension and elongation at break that are considered to be important aspects of functional vascular grafts.

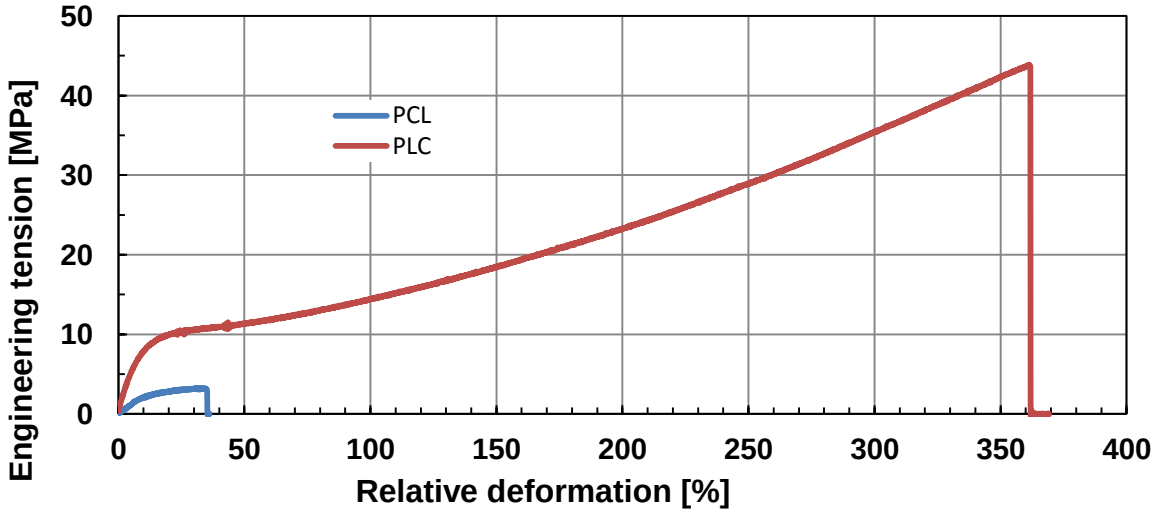


Figure 5: Comparison of stress-strain curves of PCL tubular fibrous layer (blue line, 29 µm thickness) and PLC layer (red line, 29,5 µm thickness).

5.4 Biological performance of fibrous layers

Cell viability seeded on PCL and PLC microfibrous scaffold was measured by MTT test during the time of cultivation in days 1, 3, 7 and 14. Data were expressed herein as mean ± standard deviation of measured absorbance. Tests for significant differences used a two-tailed Student's t-test and required $p<0,05$ to claim significance. After the first and the third day of cultivation, the adhesion of fibroblasts did not show statistically significant difference between both tested materials. After a week of cultivation, higher proliferation rate was found in PLC layer. After 7 and 14 days of cell culture, the viability of fibroblasts on copolymer PLC was significantly higher compared to PCL fibers as depicted in graph in figure 6.

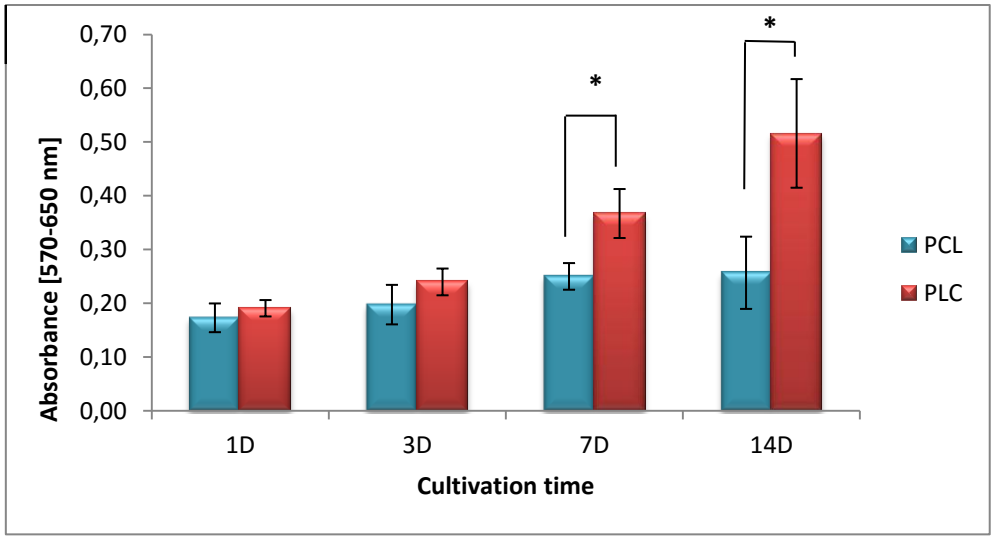


Figure 6: Cell viability measured by MTT test after 1, 3, 7 and 14 days of scaffold cultivation with 3T3 mouse fibroblasts (indicates $p < 0.05$).*

Endothelial cells cultured on the same materials showed similar trend of increasing cellular viability during the period of cultivation time. Cell morpohology is depicted in figure 7 after 1, 3, 7 and 14 days of cultivation after double staining with phalloidin-2-(4-amidinophenyl)-1H -indole-6-carboxamidine (DAPI).

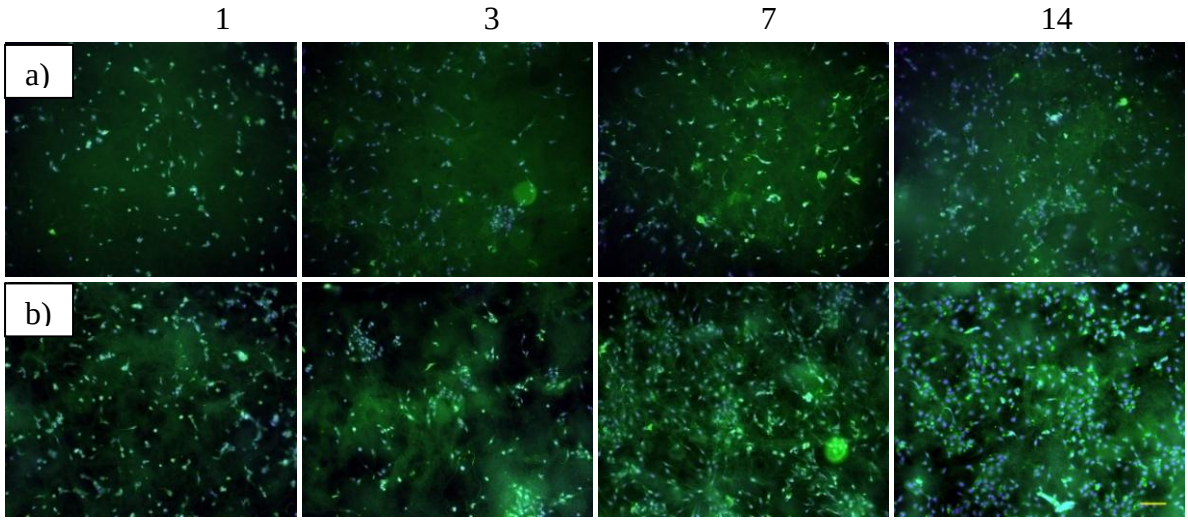


Figure 7: Fluorescence microscopy pictures of human umbilical vein endothelial cells stained with phalloidin-FITC (green) and DAPI (blue) during cell culture (1, 3, 7 and 14 days): a) PCL, b) PLC. Scale bar 100 µm.

When different morphology of PCL fibers was compared (microsized and nanosized), surprisingly microfibers supported endothelial cell attachment and proliferation more than nanofibers. Viability measured by MTT test was higher in microfibrous layers. After 14 days of culturing, more microfibrous surface scaffold was covered by endothelial cells compared to nanofibrous one as depicted in figure 8.

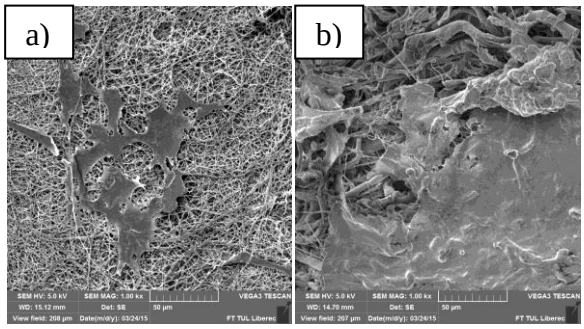


Figure 8: Scanning electrone microscopy pictures of human umbilical vein endothelial cells after14 days of culturing on: a) PCL nm, b) PCL µm. Scale bars 50 µm.

The main failure of vascular grafts after implantation is the acute thrombogenicity that is affected by chemical composition of surface, morphology of exposed surface and other factors. In order to characterize an extent of thrombogenicity of the electrospun layers, samples were tested with thrombocyte rich solution in static and dynamic conditions. The highest metabolic activity of adhered thrombocytes after 2 hours of static incubation measured by MTT test was found in nanofibrous layer from PCL but the difference did not claim significance (see figure 9). Platelets lost their viability during the incubation time. After 4 and 7 days of incubation the metabolic activity was very low that is in agreement with the life-time of thrombocytes.

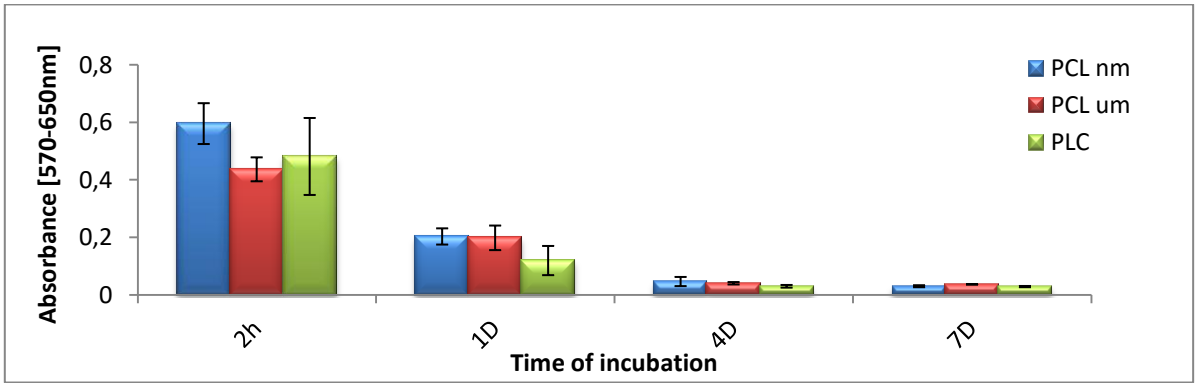
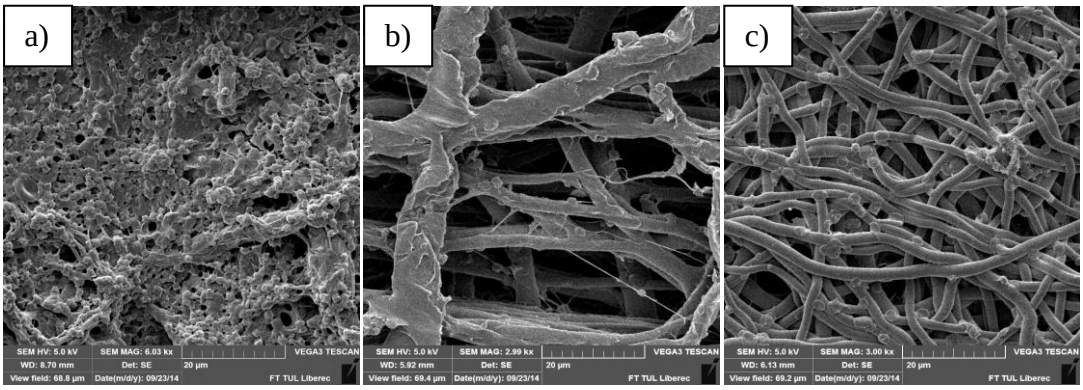


Figure 9: Metabolic activity of thrombocytes adhered to electrospun layers after 2 hours, 1,4 and 7 days.

After 2 hours of static incubation with TRS the nanofibrous PCL layer (PCL nm) contained the highest number of thrombocytes that corresponds with the result of MTT test. The surface of nanofibrous layer was fully covered with adhered thrombocytes (figure 10a). On the other hand, microfibrous structures allow the platelets to penetrate the layer inside (figure 10 b, c) and they were found not only on the surface as in case of nanofibrous structure. This could be an explanation of similar metabolic activity measured by MTT test.



Fiure 10: SEM pictures of adhered thrombocytes after 2 hours of incubation in thrombocyte rich solution on PCL nm (a), PCL µm (b) and PLC (c). Scale bars 20 µm.

In order to examine the influence of surface roughness, foils and electrospun layers made from PCL and copolymer PLC were tested in similar way. Viability measurement showed that fibrous layers activated more thrombocytes than foils prepared from the same materials. The rate of platelet activation was also visible in scanning electron microscopic pictures. While fibrous layers were fully covered with spread thrombocytes as shown in previous figure 10, smooth surfaces of foils were covered by individual platelets in different stages of their activation. Circular resting platelets (indicated by blue arrows) as well as irregular shapes of thrombocytes with pseudopodia (indicated by red arrows) were found as seen in figure 11.

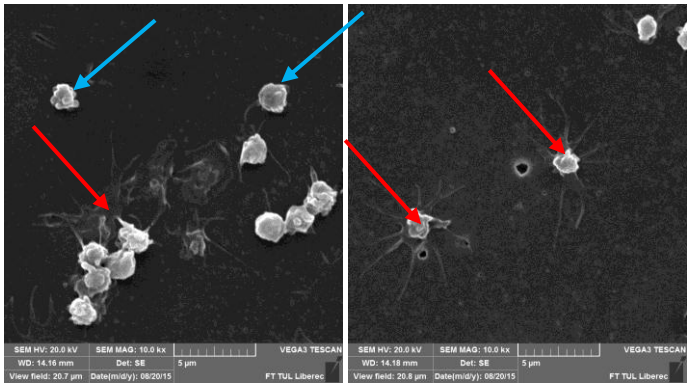


Figure 11: SEM pictures of platelets incubated in PLC foils. Red arrows mark activated spread platelets, blue arrows sign resting circular platelets. Scale bars 5 µm.

Thrombogenicity of the tubular scaffolds were tested in dynamic conditions. After 2 hours of thrombocytes rich solution flow through the vascular grafts made from double layered PCL and microfibrous PLC, different morphology of adhered thrombocytes were found in SEM pictures. Thrombocytes adhered to nanofibrous structures were activated and almost no circular platelet was found such as in the picture 10a after static incubation. The fibrous structure was completely covered by spread thrombocytes. In case of microfibrous PLC graft, the platelets were also spread but some of the circular platelets with pseudopodia were found. Nanofibers have high surface to volume ratio therefore it was assumed that the activation of thrombocytes will be higher. No quantitative data were obtained from this experiment but different morphology of platelets was found as depicted in figure 12. The flow of thrombocytes rich solution contributed to higher platelet activation compared to static conditions.

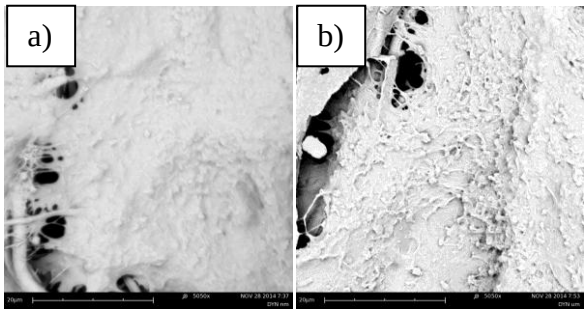


Figure 12: SEM pictures of vascular graft throbogenicity tests under flow conditions: inner nanofibrous layer of PCL vascular graft (a) and microfibrous PLC vascular graft (b). Scale bars 20 μm.

5.5 Modification of vascular grafts by nitric oxide donors

Vascular grafts made from PCL were blended with NO releasing substances in order to improve their functions. Control PCL scaffolds and NO modified grafts were about 5 cm in length, 1,65 mm inner diameter with a wall thickness between 500 and 700 μm. Tubular scaffold made from PCL had average fiber diameter of 143 ± 80 nm, PCL modified by SNAP-cyclam 179 ± 182 nm. In addition to fibers, polymeric beads were present in the structures of both control and NO-releasing PCL grafts.

Nitric oxide releasing PCL grafts were evaluated for NO release after 1 hour incubation period bathed in PBS (figure 13, blue line) and in complete DMEM (figure 13, red line) which are conditions that are known to promote NO release from the polymer composite. The grafts display an initial burst of NO immediately after soaking in PBS between $2x10^{-10}$ moles/(min*cm^2) and $4{,}5x10^{-10}$ moles/(min*cm^2) that is similar to physiological levels released from endothelial cells *(Vaughn, 1998)*. NO release decreased with time until $CuCl_2$ and ascorbic acid were added after 60 minutes. Addition of exogenous solutions caused an increased NO release to comparable levels as measured during the initial burst in PBS.

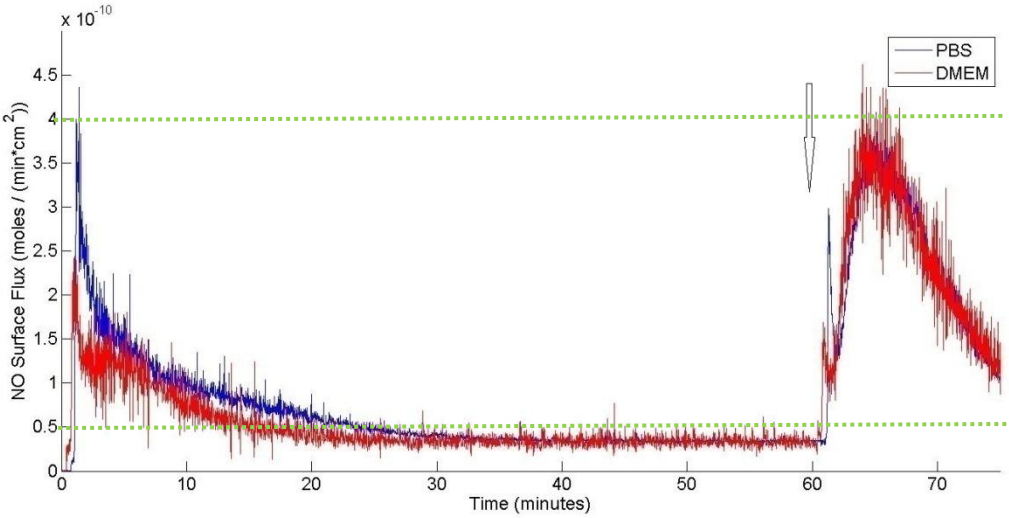


Figure13: NO release from a SNAP-cyclam PCL graft after 1 hour bathing in PBS (blue line) and in complete DMEM (red line). After 60 minutes, $CuCl_2$ and ascorbic acid were added to the PBS solution, stimulating the further release of NO (indicated by an arrow). Green lines displays physiological NO release by endothelial cells.

Both PCL and NO-releasing PCL vascular grafts exhibited excellent surgical handling and suture retention properties during implantation as a replacement of rat abdominal aorta. No significant blood leakage was observed after restoration of blood flow and pressure. After 10 days *in vivo,* none of the six implanted grafts demonstrated thrombosis or aneurysm formation. Longitudinal cross sections revealed extensive cellular infiltration within the wall of the grafts (figure 14, 15). However, the presence and distribution of cells in the control graft differed from that of the NO-releasing grafts. The PCL control graft was homogeneously infiltrated with cells. In contrast, a high density of cells was present in the luminal and adventitial margins of the NO-releasing graft, but cells were nearly absent from the central region. Cells had penetrated the NO-releasing graft largely from the adventitial side, with only a relatively thin band of cells observed along the luminal side of the graft. No evidence of cell injury or necrosis was detected in the NO-releasing graft cross sections (figure 15 D, E, and F).

Quantification of the cells present in each graft confirmed that the PCL control was homogeneously infiltrated by cells. The mean cellular density for the PCL control graft was similar throughout the entire graft (23,5 ± 0,8 cells/100×100 μm) relative to the middle region (24,2 ± 0,6 cells/100×100 μm). In contrast, the NO-releasing graft exhibited an average cell density of 18,1± 1,0 cells/100×100 μm compared to 6,5 ± 0,5 cells/100×100 μm in the middle region of the graft. Both the average cell density and the density of cells from the middle of the graft were significantly reduced in the NO-releasing graft relative to the control PCL grafts. Specific cell staining in order to distinguish different cell phenotype has not been carried out. However, after 10 days of implantation it is expected that most of the cells were immune cells. This suggestion was also noticeable from morphology of cells when observed with higher magnification objective. The majority of the cells belonged to macrophages and neutrophils.

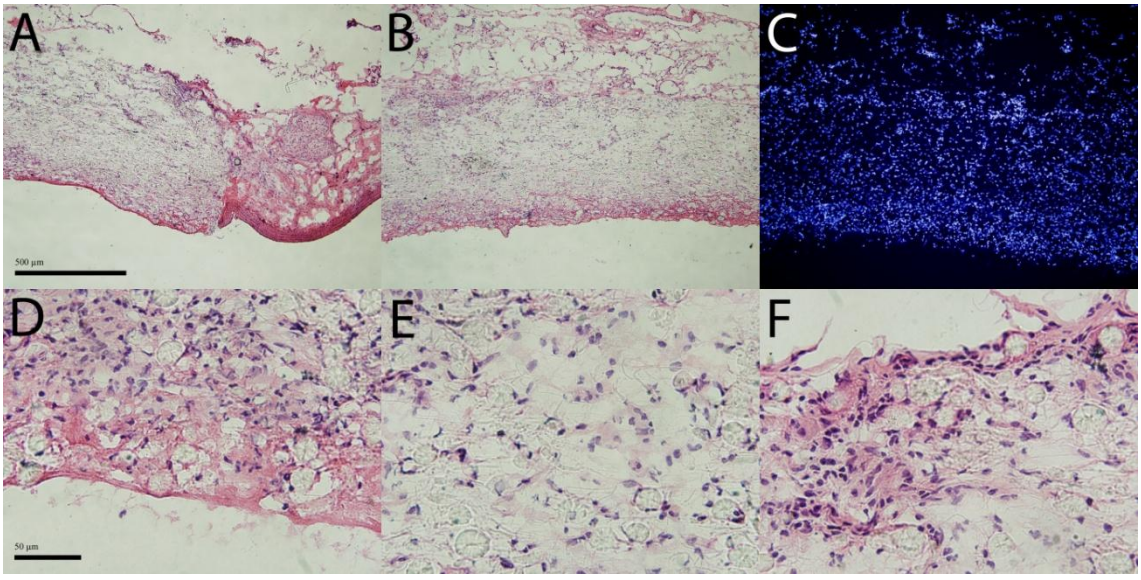


Figure 14: H&E and DAPI staining of PCL vascular graft after 10 days of implantation: Graft-artery junction showing host artery to the right (A), transverse cross section of graft's wall H&E stained (B) and DAPI stained (C); scale bars 500 μm. Detailed images of luminal side of the graft (D), middle part (E) and adventitial part (F); scale bars 50 μm.

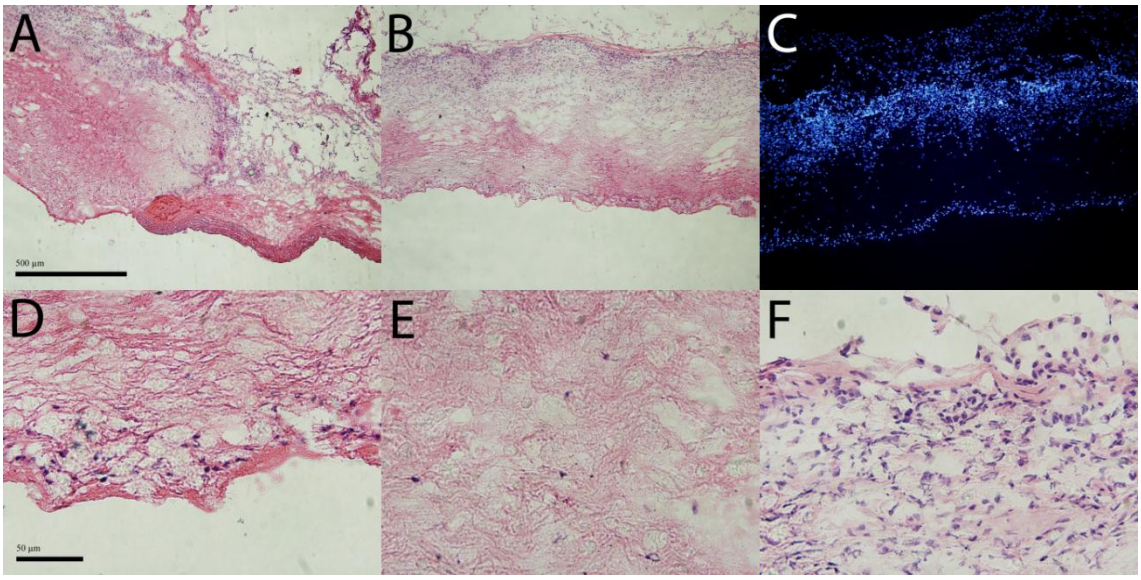


Figure 15: H&E and DAPI staining of a NO-releasing vascular graft after 10 days of implantation: Graft-artery junction showing host artery to the right (A), transverse cross section of graft's wall H&E stained (B) and DAPI stained (C); scale bars 500 µm. Detailed images of luminal side of the graft (D), middle part (E), and adventitial part (F); scale bars 50 µm.

6 Evaluation of results and new finding

The first experimental part of the thesis was focused on appropriate scaffold material development. The idea was based on mimicking the structure of native blood vessel morphology by electrospinning of 2 chosen biodegradable polymers: PCL and copolymer PLC. An ideal model of vascular graft morphology was designed as double layered graft with defined morphologies of certain layers. In case of PCL, the proposed model was created and the double layered graft was produced. When PLC was used for production of tubular scaffolds, only single layer graft was prepared.

The second tested hypothesis was the mechanical performance of vascular grafts. Copolymer PLC created tubular scaffolds possessing excellent elongation properties of 377,4 ± 157,4 % of elongation until break compared to only 37,5 ± 8,8 % achieved with tubular scaffolds made from PCL. Mechanical strength of electrospun PLC was also higher than PCL (37,2 ± 9,2 MPa in case of PLC compared to 3,3 ± 1,1 MPa for PCL). Based on the mechanical behavior, copolymer PLC seems to be more appropriate candidate for production of vascular grafts even if double layered structure has not been achieved.

Biocompatibility of the electrospun layers was tested *in vitro.* The first test compared PCL and PLC having similar fibrous structure. Copolymer PLC significantly supported fibroblast proliferation on those scaffolds compared to PCL one probably due to the lower hydrophobicity of the surface. Similar results were obtained by using endothelial cells. Copolymer PLC was endothelialized faster than PCL. Another tested hypothesis was whether nanofibers support endothelial cell adhesion but this condition has not been proved. In case of PCL, nanofibers did not fasten the endothelialization of the scaffold surface. The effect seemed to be in the opposite way - microfibers were more beneficial for endothelialization than nanofibers. Based on these results, endothelialization is dependent on fiber diameter and chemical structure; however nanofibers did not show the enhanced endothelialization as expected when cultured under static conditions.

Incubation of nanofibrous and microfibrous scaffolds with thrombocytes did not show significant difference between PCL and PLC nor between nanofibrous and microfibrous layer

made from PCL in terms of measured thrombocyte viability. There was a difference in platelet colonization of the scaffold. Nanofibrous layers did not allow the penetration to the inner structure therefore platelets were abundantly found on the surface. On the contrary, platelets incubated with microfibrous structures made from PCL and PLC were poorly spread over the surface but the platelets reached the inner parts of the scaffolds due to bigger pore sizes. Thrombocyte aggregate formation was typical in these pores.

Roughness of fibrous structure contributes to thrombocytes activation that was manifested by testing the same materials in 2 forms - electrospun fibers and smooth foils. It was found that smooth surface with the same chemical composition is less thrombogenic than corresponding fibrous surface. Electrospun layers were fully covered with thrombocytes whereas foils were covered by single platelets in different stage of their activation that was clearly visible from SEM pictures in figure 11.

Taken together, even if copolymer PLC was not able to create previously designed double layered graft, other materials properties in general became more important when considering ideal material for usage in vascular tissue engineering applications. Copolymer PLC has excellent mechanical properties and it supports cell adhesion and proliferation. Even if PLC was not able to create the designed double layered structure, copolymer PLC is appropriate in terms of better surface wettability properties, higher elongation, mechanical strength, cytocompatibility with both tested cell lines (fibroblasts and endothelial cells) and lower thrombogenicity compared to PCL. Another important property of polyesters is their degradation rate that strongly influenced *in vivo* performance. The degradation studies will be carried out as well in order to fully characterize suitability of presented materials.

The last experimental part was focused on modification of polymeric vascular grafts made from PCL. Nitric oxide donors were added to electrospinning solution and release kinetics was studied *in vitro*. Newly synthesized compound, SNAP-cyclam, was able to release NO in a long term that was proved in *in vitro* as well as *in vivo* conditions. Newly synthesized compound, SNAP-cyclam, was able to release NO at physiological levels up to 42 days measured in PBS after blending with PCL by the way of electrospinning. Similar results were achieved when vascular grafts were incubated in complete medium that more closely simulate conditions within the body. After implantation *in vivo*, vascular grafts were patent after 10 days of implantation. There were differences between control PCL grafts and NO releasing grafts, especially in the way of cellular distribution within the graft thickness. The NO release strongly inhibits the harmful infiltration of inflammatory cells into the middle and inner regions of the vascular grafts. Additional long-term studies should be conducted to confirm a reduced intimal hyperplasia relative to control grafts and measure the rate of re-endothelialization.

7 References

Angelini GD, Newby AC. The future of saphenous vein as a coronary artery bypass conduit, Eur Heart J 1989;10(3):273-80.

Arrigoni C, Camozzi D, Remuzzi A. Vascular Tissue Engineering, Cell Transplantation 2006;15:119-125.

Boland ED, Espy PG, Bowlin GL. Tissue Engineering Scaffolds. In *Encyclopedia of Biomaterials and Biomedical Engineering*; Wnek G, Bowlin GL. ISBN 0-8247-4798-4. p.1630-1638.

Cameron A, Davis KB, Green G, Schaff HV. Coronary bypass surgery with internal-thoracic-artery grafts - effects on survival over a 15-year period, N Engl J Med 1996;334(4):216-9.

Chlupac J, Filova E, Bacakova L. Blood Vessel Replacement: 50 years of Development and Tissue Engineering Paradigms in Vascular Surgery, Physiol Res 2009;58:119-139.

Dong Y, Yong T, Liao S, Chan CK, Ramakrishna S. Long-term viability of coronary artery smooth muscel cells on poly(l-lactide-co-caprolactone) nanofibrous scaffold indicates its potential for blood vessel tissue engineering, J R Soc Interface 2008;5:1109-1118.

Erben J, Pilarova K, Sanetrnik F, Chvojka J, Jencova V, Blazkova L, Havlicek J, Novak O, Mikes P, Prosecka E, Lukas D, Kuzelova Kostakova E. The combination of meltblown and electrospinning for bone tissue engineering, Materials Letters 2015;143:172-176.

Esquivel CO, Blaisdell FW. Why small caliber vascular grafts fail: a review of clinical and experimental experience and the significance of the interaction of blood at the interface, J Surg Res 1986;41(1):1-15.

Garg UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells, J Clin Invest 1989;83:1774-7.

Greenwald SE, Berry CL. Improving vascular grafts: the importance of mechanical and haemodynamic properties, J Pathol 2000;190(3):292-9.

Han F, Jia X, Dai D, Yang X, Zhao J, Zhao Y, Fan Y, Yuan X. Performance of a multilayered small-diameter vascular scaffold dual-loaded with VEGF and PDGF. Biomaterials 2013;34:7302-13.

He W, Ma Z, Teo WE, Dong YX, Robless PA, Lim TC, Ramakrishna S. Tubular nanofiber scaffolds for tissue engineered small-diameter vascular grafts, J Biomed Mater Res A 2008;90:205-16.

Hu JJ, Chao WC, Lee PY, Huang CH. Construction and characterization of an electrospun tubular scaffold for small-diameter tissue-engineered vascular grafts: A scaffold membrane approach, Journal of the Mechanical behavior of Biomedical Materials 2012;13:140-155.

Huang Ch, Geng X, Qinfei K, Xiumei M, Al-Deyab S, El-Newehy M. Preparation of composite tubular grafts for vascular repair via electrospinning, Progress in Natural Science: Materials International 2012;22:108-114.

Jourd'heuil D, Hallen K, Feelisch M, Grisham MB. Dynamic state of S-nitrosothiols in human plasma and whole blood, Free Radical Biol Med 2000;28(3):409−417.

Kakisis JD, Liapis CD, Breuer C, Sumpio BE. Artificial blood vessel: the Holy Grail of peripheral vascular surgery, J Vasc Surg 2005;41:349-354.

Langer R, Vacanti JP. Tissue engineering, Science 1993;260:920-6.

Lefer AM. Nitric oxide: nature's naturally occurring leukocyte inhibitor, Circulation 1997;95:553-4.

McClure MJ, Sell SA, Simpson DG, Walpoth BH, Bowlin GL. A three-layered electrospun matrix to mimic native arterial architecture using polycaprolactone, elastin, and collagen: A preliminary study, Acta Biomaterialia 2010;6:2422-2433.

Mo XM, Xu CY, Kotaki M, Ramakrishna S. Electrospun P(LLA-CL) nanofiber: a biomimetic extracellular matrix for smooth muscle cell and endothelial cell proliferation, Biomat 2004;25:1883-1890.

Mooradian DL, Hutsell TC, Keefer LK. Nitric-oxide (NO) donor molecules: effect of NO release rate on vascular smooth muscle cell proliferation in vitro, J Cardiovasc Pharmacol 1995;25:674-8.

Nottelet B, Pektok E, Mandracchia D, Tille JC, Walpoth B, Gurny R, Moller M. Factorial design optimization and in vivo feasibility of poly(ε-caprolactone)-micro- and nanofiber-based small diameter vascular grafts, J Biomed Mater Res A 2009;89:865-75.

Pektok E, Nottelet B, Tille JC, Gurny R, Kalangos A, Moeller M, Walpoth BH. Vascular grafts in the rat systemic arterial circulation degradation and healing characteristics of small-diameter poly(e-caprolactone), Circulation 2008;118:2563-2570.

Radomski MW, Palmer RMJ, Moncada S. The role of nitric oxide and CGMP in platelet adhesion to vascular endothelium, Biochem Biophys Res Commun 1987;148:1482-9.

Rampichova M, Chvojka J, Buzgo M, Prosecka E, Mikes P, Vyslouzilova L, Tvrdik D, Kochova P, Gregor T, Lukas D, Amler E. An elastic three dimensional poly (ε-caprolactone) nanofibre scaffold enhanced the migration, proliferation, and osteogenic differentiation of mesenchymal stem cells, Cell Proliferation 2013;46:23-37.

Thomas AC, Campbell GR, Campbell JH. Advances in vascular tissue engineering, Cardiovascular Pathology 2003;12:271-276.

Tzeng E, Kim YM, Pitt BR, Litonova A, Kovesdi I, Billiar TR. Adenoviral transfer of the inducible nitric oxide synthase gene blocks endothelial cell apoptosis, Surgery 1997;122:255-63.

Valence S, Tille JC, Mugnai D, Mrowczynski W, Gurny R, Moller M, Walpoth BH. Long term performance of polycaprolactone vascular grafts in a rat abdominal aorta replacement model, Biomaterials 2012;33:38-47.

Vaughn MW, Kuo L, Liao JC. Estimation of nitric oxide production and reaction rates in tissue by use of a mathematical model, Am J Physiol Heart C 1998;274:H2163-76.

Vaz CM, Tuij S, Bouten CVC, Baaijens FPT. Design of scaffolds for blood vessel tissue engineering using a multi-layering electrospinning technique, Acta Biomaterialia 2005;1: 575–582.

Wang TG, Cai TB, Taniguchi N. Nitric Oxide Donors: For Pharmaceutical and Biological Applications, WILEY-VCH, 2005, ISBN: 3-527-31015-0.

Woodruff MA, Hutmacher DW. The Return of a Forgotten Polymer - Polycaprolactone in the 21st Century. Prog Polym Sci 2010;35:1217-1256.

Wu H, Fan J, Chu CC, Wu J. Electrospinning of small diameter 3-D nanofibrous tubular scaffolds with controllable nanofiber orientations for vascular grafts, J. Mater Sci: Mater Med 2010;21:3207–3215.

Zhang X, Thomas V, Xu Y, Bellis SL, Vohra YK. An *in vitro* regenerated functional human endothelium on a nanofibrous electrospun scaffold, Biomaterials 2010;31:4376-4381.

Ziche M, Morbidelli L, Masini E, Amerini S, Granger HJ, Maggi CA, Geppetti P, Ledda F. Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P, J Clin Invest 1994;94:2036-44.

8 List of papers published by the author

8.1 Publications in journals

Horakova J., Strnadova K., Goldman J., Kubikova T., Tonar Z. Healing characteristics of PCL vascular grafts. *Workshop for Ph.D. students of Faculty of Textile Engineering and Faculty of Mechanical Engineering TUL*, 2015, 49-51, ISBN 978-80-7494-229-7.

Horáková J., Procházková R., Jenčová V., Mikeš P., Cudlínová M. Vliv trombocytárních růstových faktorů na proliferaci fibroblastů na nanovlákenném tkáňové nosiči. *Transfuze a Hematologie dnes*, 2014;20:53-58.

Horakova J., McCarthy C., Frost M., Goldman J. Nanofibrous Vascular Graft Releasing Nitric Oxide. *Workshop for Ph.D. students of Faculty of Textile Engineering and Faculty of Mechanical Engineering TUL,* 2014, 33-36, ISBN 978-80-7494-100-9.

Krchová S., Dzan L., Lukáš D., Mikeš P., Jenčová V., Horáková J., Pilařová K. Nanovlákna v hojení kožních ran. *Česká Dermatovenerologie,* 2014; 4:234-240.

Yalcin I., Horakova J., Mikes P., Gok Sadikoglu T., Domin R., Lukas D. Design of Polycaprolactone Vascular Grafts. *Journal of Industrial Textiles*, 2014, 1-21.

Cheng, T., Hund R.D., Cherif Ch., Aibibu D., Horakova J., Cherif Ch. Pure Chitosan and Chitosan/Chitosan Lactate Blended Nanofibres Made by Single Step Electrospinning. *Autex Research Journal,* 2013; 13:128-33.

Horakova J., Yalcin I. Structure optimization of small diameter nanofibrous vascular tube. *Workshop pro doktorandy FS a FT TUL – sborník*, Technická univerzita v Liberci, 2013, 46-49, ISBN 978-80-7372-987-5.

Voriskova J. Testování nanovlákenných tkáňových nosičů *in vitro*. *Workshop pro doktorandy FS a FT TUL – sborník*, Technická univerzita v Liberci, 2012, 124-128, ISBN 978-80-7372-891-5.

8.2 Contriburion in conference proceedings

8.2.1 Poster presentation

Horakova J., Mikes P., Saman A., Ackermann M. Electrospun Polylactide-Polycaprolactone Copolymer for Vascular Tissue Engineering, European Society for Biomaterials (ESB) 2015, Krakow, Poland.

Horakova J., Yalcin I., Telem G.S., Mikes P. Nanofiber Morfology Optimalization for Using as a Tubular Artificial Blood Vessels, Tissue Engineering and Regenrative Medicine Society (TERMIS) 2013, Istanbul, Turkey.

Soukupova J., Horakova J., Mitura K. Nanofibrous Layer Containing Diamonds for Tissue Engineering Applications, Nanomed International Conference on Nanotechnology in Medicine 2012, London, United Kingdom.

8.2.2 Oral presentations

Horakova J., McCarthy C., Frost M., Goldman J., Lukas D., Mikes P. Nanofibrous Vascular Grafts Releasing Nitric Oxide, European Society for Biomaterials (ESB) 2014, Liverpool, United Kingdom.

Horakova J., Prochazkova R., Jencova V., Mikes P., Cudlinova M. Polycaprolactone Nanofibrous Layer Functionalized by Thrombocyte Rich Solution, Human Skin Engineering and Reconstructive Surgery (HUSKIEN) 2013, Praque, Czech Republic.

Horakova J., Soukupova J., Rockova K. Cell Adhesion and Proliferation on Modified Nanofibrous Layers, Strutex 2012, Liberec, Czech Republic.

Laourinne E., Voriskova J., Al-Rez M.F., Cherif Ch. Nanofiber Tubes as Vascular Grafts, NANO2012 XI International Conference on Nanostructured Materials, Rhodes, Greece.

Curriculum Vitae

Personal details:

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Address: Na Strani 258, 471 25 Jablonné v Podještědí

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Education and Training:

- *From 2011* **Technical University of Liberec, Czech Republic**
  Faculty of Textile
  Doctoral studies Textile Material Engineering
- *2009-2011* **Charles University in Prague, Czech Republic**
  Faculty of Pharmacy in Hradec Králové
  Specialist in Medical Instrumental Methods (Master Degree)
  Diploma Master of Science with distinction
- *2006-2009* **Charles University in Prague, Czech Republic**
  Faculty of Pharmacy in Hradec Králové
  Laboratory technician (Bachelor Degree)
  Diploma Bachelor of Science with distinction
- *2002-2006* **Gymnazium Ceska Lipa, Czech Republic**
  Grammar school

Work experience:

- *2013-2014* **Michigan Technological University** (10 months)
  Biomedical Engineering
  Multilayered Nanofibrous Scaffolds for Vascular Tissue Engineering
- *2011-2012* **Technical University Dresden** (4 months)
  Institute of Textile Machinery and High Performance Material Technology
  Electrospinning of pure chitosan, Modification of nanofibrous tubes for vascular tissue engineering
- *2011* **Hospital in Mlada Boleslav** (3 weeks)
  Department of Clinical Biochemistry
- *2009-2011* **University Hospital in Hradec Králové**
  Department of Gerontology and Metabolism
  Research of DNA damage in patients lymphocytes with lung carcinoma
- *2010* **Contipro Group s.r.o.** Dolní Dobrouč (4 weeks)
  Molecular biology, fermentation and analytical chemistry
- *2007-2009* **University of Defence in Hradec Králové**
  Department of Toxicology

Research of DNA damage in cells, effects of antioxidants

- *2009* **University Hospital in Hradec Králové** (3 weeks)
  Institute for Clinical Immunology and Alergology
- *2008* **Generi Biotech s.r.o.** Hradec Králové (2 weeks), molecular biology

Professional awards:

- *2013-2014* Fulbright-Masaryk stipendium (10 months)
- *2009, 2011* Roche´s company award for excellent study results
- *2008, 2009, 2010* Scholarship for excellent study results

Community Service:

- A member of Academic Senate of Technical university of Liberec, October 2012-May 2013
- A member of Filharmonic Choir (Severečeský filharmonický sbor), since January 2010 a member of artistic board of the Choir

Additional information:

- Interested in music (playing the musical instruments, singing in a choir), sport (voleyball, badminton, tennis, swimming), culture (visiting theatre) and reading

Language Skills:

- English – advanced
- German – basic
- Czech – mother tongue

Other details:

- Driving licence category B
- Computer Literate - MS Windows, MS Office (Word, Excell, PowerPoint), Internet

Brief description of the current expertise, research and scientific activities

Doctoral studies

Studies	Textile Engineering
	Textile Technics and Materials Engineering
	Full time
Exams	Specialization in the field (Stereology), 11.5.2012
	Basic of Applied Subjects (Macromolecular Chemistry), 20.8.2012
	Main Subjects of the Field (Tissue Engineering), 20.3.2013
	Bascis of Natural Science (Mathematical Statistics and Data Analysis), 26.4.2013
SDE	State Doctoral Exam completed on 23.2.2015 with the overall result passed.

Teaching activities

Teaching	Medical Textiles, 2011-2012
	Stereology, 2012-2015
	Materials for Tissue Engineering, 2013-2015
Leading Bachelors/Master Students	Aneta Hniličková, *Development and biodegradability testing of nanofibrous scaffoldfor tissue engineering*, 2012.
	Bc. Pavla Sykáčková, *The influence of hydrophilic/hydrophobic properties of nanofibrous scaffolds on cell adhesion*, 2014.
	Bc. Tereza Pavlíková, *In vitro testing of small-diameter biodegradable vascular grafts*, 2015.
	Bc. Petra Kryšková, *Biological testing of degradable polyesters used for small diameter vascular grafts*, 2015/2016.

Research projects

Nanofibrous Biodegradable Small-Diameter Vascular Bypass Graft (Ministry of the Health of the Czech Republic), co-investigator, 2015.

Nanofiber materials for tissue engineering (VaVpI, Ministry of Education Youth and Sports of the Czech Republic), co-investigator, 2013-2015.

The relationship between nanofibrous structure and cell distribution (Student Grant Competition), investigator, 2013.

Development of nanofibrous scaffolds for tissue engineering and cell proliferation testing (Student Grant Competition), investigator, 2012.

Research of Nanomaterials and Progressive Technologies Applications for Protection against CBRN Agents (Ministry of the Interior of the Czech Republic), co-investigator, 2012.

Polymer Solution in External Electrical Field: Molecular Understanding of Electrospinning Process (Czech Science Foundation), co-investigator, 2012.

Record of the state doctoral exam

TECHNICKÁ UNIVERZITA V LIBERCI
Fakulta textilní

ZÁPIS O VYKONÁNÍ STÁTNÍ DOKTORSKÉ ZKOUŠKY (SDZ)

Jméno a příjmení doktorandky: **Mgr. Jana Horáková**

Datum narození: **10. 2. 1987**

Doktorský studijní program: **Textilní inženýrství**

Studijní obor: **Textilní technika a materiálové inženýrství**

Termín konání SDZ: **23. 2. 2015**

prospěla **neprospěla**

Komise pro SDZ:		*Podpis*
Předseda:	prof. RNDr. Oldřich Jirsák, CSc.	
Místopředseda:	doc. Ing. Lukáš Čapek, Ph.D.	
Členové:	prof. RNDr. Evžen Amler, CSc.	OMLUVEN
	prof. Ing. Ivan Stibor, CSc.	
	doc. Mgr. Irena Lovětinská-Šlamborová, Ph.D.	
	doc. RNDr. Miroslav Šulc, Ph.D.	

V Liberci dne 23. 2. 2015

O průběhu SDZ je veden protokol.

Reccomendation of the supervisor

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Liberec 17. 2. 2016

Disertační práce: **NANOVLÁKENNÉ CÉVNÍ NÁHRADY**
Autorka: **Mgr. Jana Horáková**

Hodnocení školitele

Disertační práce Mgr. Jany Horákové „Nanovlákenné cévní náhrady" se zabývá výzkumem a aplikací vlákenných materiálů pro tkáňové inženýrství. Zejména se věnuje přípravě a testování nanovlákenných cévních náhrad s malým průměrem (menším než 6 mm). Aktuální téma této disertační práce je postaveno na jedinečné vlastnosti textilních materiálů strukturovaných na rozměrech stovek nanometrů, které jsou schopny napodobovat morfologii mezibuněčné hmoty. Pro přípravu cévních náhrad použila doktorandka biodegradabilní polyestery, konkrétně polykaprolakton (PCL) a jeho kopolymer PLC. Laboratorně vyrobené cévní náhrady analyzovala z hlediska jejich fyzikálně-chemických vlastností, morfologie a testovala je *in vitro* v laboratořích tkáňového inženýrství na Technické univerzitě v Liberci a na Michigan Technological University. S podporou obou pracovišť provedla také *in vivo* testy na laboratorních potkanech.

Od samého zadání disertační práce projevovala Mgr. Horáková nejenom hluboký zájem o zvolenou problematiku, ale také nevšední pracovní nadšení, nasazení a invenci. Při prvotním upřesňování tématu se během stáže na Technische Universität Dresden rozhodla věnovat maloprůměrovým cévním náhradám. Zkušenosti s elektrostatickým zvlákňování chitosanu na speciální kolektor učiněné v Německu dále rozvíjela na naší univerzitě. Problematika kontaktu textilního materiálu maloprůměrové cévní náhrady s komplexně a disipativně se chovajícími buněčnými strukturami organismů představuje gigantickou problematiku ve srovnání s nasazením technických textilií pro průmyslové aplikace. S vědomím této složitosti se doktorandka soustředila na vybrané materiály a testy. Za polymery pro umělé cévy zvolila biodegradabilní polyestery. Z počátku pracoval s PCL a poté objevila na trhu kopolymer PLC, který výrazně zlepšil mechanické vlastnosti nanovlákenného materiálu náhrady. Při pobytu v USA se naučila nanovlákenné materiály funkcionalizovat prekurzory kysličníku dusnatého, který má mnohé účinky na kardiovaskulární systém. Ve své práci hledá souvislosti mezi morfologií, fyzikálně chemickými vlastnostmi graftů a reakcí chování náhrady při *in vitro* a *in vivo* testech.

Doktorandka přispěla podstatnou měrou k pochopení vzájemných souvislostí mezi materiálovými a procesními parametry výroby nanovlákenných cévních náhrad a odezvou při jejich interakci s tkáněmi modelových zvířat a laboratorních buněčných populací. O kvalitě její práce svědčí i to, že v roce 2013 získala Fulbrightovo stipendium. Pozitivním výsledkem práce jsou testy *in vitro*, které prokázali cytokompatibilitu zvolených materiálů. Doktorandka prokázala, že nanovlákenná cévní náhrada plní svoji funkci v těle potkana po dobu 10 dnů, je-li použita jako náhrada části břišní aorty.
Jako skvělou hodnotím publikační činnost Mgr. Jany Horákové, která k dnešnímu dni čítá 7 konferenčních příspěvků a 8 časopiseckých publikací. Její h-index je 1. Podtrhuji i tu skutečnost, že se doktorandka velmi aktivně podílela na přípravě 6 projektů.

Navrhuji, aby práce Mgr. Jany Horákové byla přijata k obhajobě.

Školitel: prof. RNDr. David Lukáš, CSc.
Školitel specialista: Ing. Petr Mikeš, CSc.

Opponents' reviews

Oponentský posudek

doktorandské dizertační práce Mgr. Jany Horákové:

Nanovlákenné cévní náhrady

Doktorandská dizertační práce má celkem 119 stran vlastního textu včetně velmi přehledných tabulek, grafů a vyobrazení. Velmi rozsáhlý je i seznam literárních odkazů. Práce má klasické členění dizertační práce se všemi náležitostmi. Po formální stránce je práce velmi pečlivě zpracována. Svým rozsahem i hloubkou informací, které jsou v ní obsaženy, práce, podle mého názoru, vysoce převyšuje nároky, kladené na tento typ prací. Svědčí to o autorčiných rozsáhlých znalostech zpracovávané problematiky.

Téma práce je z klinického hlediska vysoce aktuální a je zvoleno správně. Jak autorka konstatuje, doposud nebyla vyvinuta taková umělá cévní náhrada o malém průměru, která by se dala použít ke kontrukci bypassu na věnčitých tepnách. Kardiochirurgie je tak stále odkázána pouze na použití vhodných autologních cévních materiálů pacienta, jejichž množství je pochopitelně omezené a jsou i situace, kdy už u nemocného vhodnou cévní náhradu nemáme k dispozici.
Kdyby se tedy podařilo použitelnou cévní náhradu vyvinout, znamenalo by to zcela nepochybně průlom v této oblasti klinické medicíny.

Myšlenka, vyvinout biodegradabilní protézu, která by sloužila jako matrix pro endoteliální buňky, které by vytvořily v průběhu vhojování jakousi neocévu, která by, díky přítomnosti endotelu, byla odolná proti tromboze, je velmi originální.

Práce má velmi podrobně rozpracovanou teoretickou část, ve které se čtenář seznamuje s teoretickými základy, ze kterých autorka projektu vycházela. V další části autorka popisuje, jakým způsobem postupovala při tvorbě vlastní cévní náhrady. Analyzovala stěnu nativní cévy a na tomto základě zvolila materiál a rozměrové parametry cévní náhrady. Dále je podrobně popsán proces přípravy cévní protézy, která byla následně podrobená různým testům (mechanickým a následně i biologickým). Vše je v práci velmi podrobně dokumentováno obrazově i graficky.
První, takto připravené protézy byly použity (chirurgicky implantovány) i in vivo v experimentálním modelu na krysách. Výsledky, byť jen krátkodobé, jsou velmi povzbuzující (nedošlo k trombóze protézy). Následně byly vzorky zpracovány i histologicky a výsledky této analýzy jsou také součástí práce. Závěrem tedy autorka může konstatovat, že cíl práce splnila a že byl úspěšně učiněn první krok k vývoji nového typu cévní náhrady.

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FAKULTNÍ NEMOCNICE
OLOMOUC

Je nezbytné zdůraznit, že se jedná o rozsáhlou experimentální práci, která dále pokračuje.

Závěr:

Téma práce je vysoce aktuální a její cíle byly splněny. Práce je čtivá, velmi zajímavá a přináší nové poznatky. Autorka prokazuje velmi hluboké znalosti dané problematiky.

Dizertace přinesla nové poznatky a má význam nejen pro současnou kardiochirurgii, ale i pro celou řadu oborů, které bylo nutné do experimentu zapojit. Metody práce byly správně zvoleny a výsledky jsou zajímavé a originální a byly publikovány.

Mohu konstatovat, že Mgr. Jana Horáková předložila nadstandardně kvalitní dizertační práci. Prokázala v ní, že má hluboké a velmi rozsáhlé znalosti dané problematiky, že umí vědecky pracovat, že je schopna pojednat o řešeném problému, že dovede logicky a exaktně formulovat zjištěné výsledky. Dizertační práci doporučuji přijmout v předložené formě k obhajobě (podle § 47 VŠ zákona 111/98 Sb.) a po jejím úspěšném absolvování doporučuji, aby byl Mgr. Janě Horákové přiznán akademický titul doktor ve zkratce Ph.D.

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Olomouc, 17.2. 2016

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Oponentní posudek

na dizertační práci paní **Mgr. Jany Horákové**, doktorandky Fakulty textilní Technické univerzity v Liberci, na téma **„Nanovlákenné cévní náhrady".**

Doktorandka předložila anglicky psanou dizertační práci o 130 stranách, v níž se zabývá vytvářením a testováním maloprůměrových cévních náhrad z biodegradabilních polymerů. Vlastní text práce je rozdělen do sedmi kapitol, dále je připojen obsahuje soupis 130 citovaných literárních pramenů, seznam použitých zkratek a CD s elektronickou verzí práce.

Význam dizertační práce pro obor: Potřeba vývoje nových typů cévních náhrad je v práci velice dobře zdůvodněna. Téma považuji za vhodně zvolené a jednoznačně aktuální. Jedním z hlavních přínosů práce je výběr a šíře použitých metod, které pokrývají návaznost od vývoje materiálu protéz až k jejich *in vivo* testování. Strategii přizpůsobení nově vyvíjených náhrad vlastnostem a mikroskopické stavbě skutečných cév považuji za prozíravě zvolenou. Kromě využití v evoluci osvědčených principů totiž umožňuje přizpůsobit cévní náhradu tomu, že konkrétní tepna či její segment vzniká a během růstu jedince se remodeluje a přizpůsobuje místním anatomickým a fyziologickým podmínkám dané části řečiště. Takto vyvíjené cévní náhrady jeví slibnou šanci na adaptaci při implantaci do cílové tepny.

Postup řešení problému: Uchazečka formulovala hlavní otázky řešené svou doktorskou prací jako porovnání fyzikálně-chemických vlastností jednovrstvých a dvouvrstvých cévních náhrad připravených ze dvou různých polymerů. Dále náhrady charakterizuje z hlediska mechanických vlastností, mikrostruktury, cytotoxicity, trombogenicity a interakce s buňkami při kultivačních testech *in vitro*. Další částí práce je *in vivo* studie s obohacením tkáňového nosiče o látku uvolňující oxid dusnatý. V úvodu práce formuluje jednotlivé problémy jednoznačně a na základě podrobného rozboru současného stavu poznání, který podrobně rozebírá v druhé, teoreticky zaměřené kapitole. Ve třetí kapitole podává popis výroby a testování náhrad, na což navazuje ve čtvrté kapitole popisem a provedením *in vitro testů.* Pátá kapitola je věnována vývoji a *in vivo* testování náhrad uvolňujících NO. V šesté kapitole jsou jednotlivé okruhy diskutovány ve světle již publikovaných výsledků, v sedmé kapitole jsou pak shrnuty do závěrů.

Použité metody: Ke splnění vytčených cílů byly použity úpravy a optimalizace výrobních postupů nanovlákenných náhrad, mikroskopické sledování jejich struktury, mechanické zkoušky, testy biokompatibility s fibroblasty, testy kultivace s endotelovými buňkami, testy trombogenicity a morfoglociké (histologické) vyhodnocení náhrad použitých v *in vivo* experimentu u potkana. Volbu metod pro jednotlivé cíle a zejména jejich vzájemnou návaznost považuji za velmi silnou stránku práce. Metody jsou řádně dokumentovány, jejich použití je vzhledem k cílům vhodně

zvolené a vyvážené, popis provedené práce umožňuje případnou reprodukovatelnost a takto získané výsledky lze považovat za validní.

Splnění cílů práce: Jak vyplývá z kapitol 3-5, dizertační práce jednoznačně splnila v úvodu formulované cíle. Jejich shrnutí je přehledně podáno v kapitole 7.

Výsledky práce a přínos doktoranda: Doktorandka prokázala svoje hluboké znalosti při formulace vědeckého problému z oblasti vývoje a technologie přípravy cévních náhrad, z oblasti *in vitro* i *in vivo* testování, to vše na základě kritického rozboru současné úrovně vědeckého poznání. Prokázala dále schopnost plánovat, provést a vyhodnotit vhodně navržené experimenty v dosti netriviálním uspořádání. Doktorandka je schopna mezinárodní spolupráce s konkrétními výstupy, je schopna výsledky kriticky interpretovat a z těchto výsledků vyvodit odpovídající závěry. Celou práci je schopna shrnout do srozumitelně psaného, přehledně uspořádaného a logicky provázaného vědeckého textu. Vysoce hodnotím skutečnost, že naprostou většinu prací prováděla doktorandka osobně a osvojila si širokou škálu dovedností překračujících hranice několika oborů. Autorka v diskusi navíc nastiňuje další směřování práce ve svém oboru, ke které má dle předložené dizertace vynikající předpoklady.

Formální zpracování a jazyková úroveň: Práce je logicky a přehledně členěna, jednotlivé oddíly jsou dobře logicky provázány, typografické a grafické zpracování je na vysoké úrovni, text je dobře čitelný, popisky obrázků jsou samovysvětlující, všechny zkratky jsou vysvětleny. Práce je psána čtivou, srozumitelnou a gramaticky zcela správnou americkou angličtinou na vysoké stylistické úrovni.

Připomínky k formálnímu zpracování: V anglicky psaném textu je třeba používat i příslušnou formu oddělení desetinných míst od jednotek, tj. nikoliv českou verzi desetinné čárky „1, 47 MPa“, ale tzv. decimal point “1.47 MPa”. V textu jsou naprosto ojedinělé překlepy (str. 11 possess--> possesses, podmětem je „graft“ v jednotném čísle). Na některých místech autorka používá dvě různé konvence zápisu čísel v exponenciálním tvaru ($4x10^{-9}$ vs. $6*10^{-12}$). Na ojedinělých místech textu jsou navíc mezery mezi číslem a znakem procent. Většina zdrojů pro angličtinu (včetně vědecké angličtiny) doporučuje vypisovat číslovky < 10 slovně, nikoliv číslicemi, tj. např. „three pillars“ namísto autorkou používaného „3 pillars“.

Vyjádření k publikacím studentky: Doktorandka dokládá publikaci osmi článků ve sbornících a časopisech. U pěti z nich je první autorkou, tři jsou uvedeny v databázi Web of Science, u dvou se jedná o periodika s faktorem impaktu dle Thomson Reuters Journal Citation Reports. Dále jsou doložena tři posterová sdělení z mezinárodních konferencí a přednesení čtyř přednášek. Schopnost autorky publikovat a obhájit vědecké práce v mezinárodním měřítku tak považuji za přesvědčivě doloženou. I na vysoké úrovni vlastní disertační práce je patrné, že dosavadní zkušenosti s psaním vědeckých sdělení autorka zde bohatě zúročila.

Připomínky a dotazy:

Na str. 19 je uvedeno, že „proliferation rate could be estimated by the area occupied by the cells“ – k tomu bych podotkl, že plocha zarostlá buňkami nezávisí pouze na jejich proliferaci, tj. počtu, ale i na velikostní distribuci, která se může v čase měnit. K vyhodnocení proliferace by možná byl vhodnější dobře definovaný a standardizovaný proliferační index.

Na str. 21 je jako typický rozměr krevních destiček uveden 1-2 μm. Jednalo se o rozměry nativních trombocytů před aktivací nebo o rozměry až po aktivaci? Jednalo se o měření v izotonickém prostředí? U lidských destiček totiž uvádí většina zdrojů obvyklé velikostní rozpětí 2-3 μm.

Na str. 22 byla při charakteristice tunica media uvedena popis typický pro medii tepen elastického typu, tj. největších a srdci nejbližších tepen. Odstavec je však součástí oddílu 2.2. s názvem „Small diameter vascular grafts". Patrně by bylo vhodnější podat zvlášť charakteristiku medie u tepen elastického typu a tepen svalového typu, protože právě stavba medie je u těchto skupin tepen dosti rozdílná.

Díky obsažnosti a mezioborovému přesahu práce je otázek, které se nabízejí, velmi mnoho a doktorandka si zcela správně některých z nich sám všímá v kapitole věnované diskuzi a výhledům do budoucna. K diskuzi při obhajobě si dovolím položit uchazeči následující dotazy:

- Berete při vývoji materiálů pro cévní protézy požadavky cévních chirurgů? Jak je např. okraj textilních štěpů náchylný k otřepu z hlediska retence cévního stehu v protéze? Vyskytují se požadavky na kompatibilitu s tkáňovými lepidly? Lze s nanovlákennými cévními náhradami manipulovat nejen při otevřených chirurgických operacích, ale i při endovaskulárních zákrocích?
- Je pro Vámi využívané materiály znám časový průběh jeho rozkladu v organizmu vyjádřitelný např. jako poločas degradace po implantaci do některého často využívaného standardizovaného místa v cévním řečišti?
- Jaká je naopak požadovaná celistvost a neporušenost cévní náhrady tak, aby bylo možné předejít riziku jejích předčasného rozpadu?
- Je vstřebatelnost výhodou u všech aplikací cévních náhrad nebo naopak existují aplikace, kdy je při náhradě vhodnější nevstřebatelný materiál?
- Jak se liší se vývoj cévní náhrady pro elastické tepny a tepny svalového typu? Je třeba toto zohlednit nebo lze vycházet z totožného materiálu náhrady a jeho uspořádání?
- Na str. 25 popisujete oblast cévy, která je dlouhodobě bez endotelu. Udržuje si povrch náhrady potřebné antitrombogenní vlastnosti i dlouhodobě?
- Můžete se vyjádřit k riziku infekce cévních náhrad? Lze při vývoji vhodného materiálu toto riziko nějak snížit?
- V jakém rozsahu průměrů a tloušťky stěny lze z Vámi testovaných materiálu vyrábět cévní náhrady?
- Na str. 26 zmiňujete diferenciaci monocytů v makrofágy typu M1 a M2. Na čem závisí, do kterého fenotypu se monocyty v cévní náhradě diferencují?
- Složky cévní stěny mají *in vivo* předpětí. Je nutno při vývoji náhrady brát v potaz předpětí materiálu? Mikroskopická vyšetření včetně morfometrie se vztahují na klidový (relaxovaný) stav náhrady, avšak po implantaci do různých částí tepenného řečiště je rozpínána krevním tlakem. Považujete tento rozdíl za významný či zanedbatelný?
- Jsou studované náhrady odolné proti zalomení?
- Mají Vámi vyvíjené náhrady srovnatelnou poddajnost (compliance) jako nativní cévy? Jak ovlivňuje přítomnost cévní náhrady šíření pulzové vlny?

- Na str. 32 je uvedeno, že „thrombocytes preferentially attach to hydrophilic surfaces" a hned dále, že „hydrophilic materials could contribute to prevent acute thrombosis". Není v tom rozpor?
- Na str. 38 by bylo vhodné doplnit, s jakými typy kolagenu se v cévní stěně setkáváme.
- Na str. 40 (Fig. 8) je popisován radiální průběh vláken v medii. Přitom na mikroskopických snímcích medií se s radiálním průběhem nesetkáváme, ten je spirálovitý. Má radiální průběh vláken nahradit kohezi, mezibuněčné spoje a propojení přes matrix mezi jednotlivými vrstvami spirály?
- Skenovací elektronová mikroskopie (např. str. 44 a jinde) má poměrně vysokou hloubku ostrosti, což přináší i problémy s perspektivou. Může to významně ovlivnit měření rozměrů vláken bližších a vzdálenějších vůči pozorovateli?
- Str. 57: dochází při měření mechanických vlastností k tam výrazným deformacím, že by bylo třeba průřez/tloušťku vzorku uvedenou ve vzorci na str. 57 korigovat během samotného měření?
- Str. 73: Čím si vysvětlujete nerovnoměrný růst endotelových buněk při *in vitro* kultivaci v porovnání s rovnoměrnějším růstem fibroblastů?
- Str. 73: Hovoříte o adhezi endotelu – lze tuto nějak objektivizovat ve fyzikálním smyslu pomocí mikromanipulačních technik (např. jako sílu nutnou k odtržení od povrchu) či šlo pouze o vyhodnocení nasedání buněk na testovaný podklad bez vyhodnocení míry adheze?
- Lze nějak kvantifikovat povrch vláken? Event. jej vypočítat při znalosti délek a distribuce průměrů vláken?
- Jsou známky nějaké kritické rozměry vláken, při nichž se některé z testovaných vlastností skokově mění?

Závěr a doporučení: Z předložené práce vyplývá, že doktorandka prokázala schopnost a připravenost k samostatné činnosti v oblasti výzkumu a vývoje a k samostatné teoretické i tvůrčí činnosti. Dizertační práce splňuje požadavky kladené na doktorskou dizertaci zákonem č. 111/1998 Sb. o vysokých školách v platném znění. Proto **jednoznačně doporučuji** její přijetí v předložené podobě jako podklad k obhajobě a za předpokladu úspěšné obhajoby **doporučuji**, aby paní **Mgr. Janě Horákové** byl udělen akademický titul „doktor" (Ph.D.) v příslušném oboru podle § 47 Zákona o vysokých školách č. 111/98 Sb.

V Plzni dne 29.1.2016

Tonar

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February 15th, 2016

PhD Defense Committee:

I have read and carefully considered Jana Horakova's PhD dissertation. Prior to reading this work, I was already very familiar with Jana's accomplishments, as she worked in my lab during her Fulbright Scholarship. Over a relatively short time, she made a strongly favorable impression on me. She demonstrated an existing expertise in electrospinning methodology, and she learned sophisticated NO chemistry as well as histological characterization techniques while she was here. From numerous first-hand interactions, I developed a high confidence in her intellectual ability, her command of the scientific knowledge of her field, her high level of motivation, her fluency in English, and her collegiality.

Presently, there is no suitable non-autologous vessel for bypassing diseased small diameter arteries. Therefore the scientific and industrial community is still searching for a solution. Jana's work furthers the development of synthetic vascular graft materials in an effort to meet a pressing need in human health. Specifically, her contributions relate to polymer processing and characterization, graft design, incorporation of NO-releasing compounds into polymers, and the biological response to materials.

The methodology that Jana used is appropriate for achieving the aims of the study. Jana developed expertise in electrospinning methodology, polymer processing, and materials characterization, which was leveraged to fabricate an experimental vascular graft for in vivo implantation. She provides an extensive characterization of PCL and PLC mechanical properties and in vitro cell response. The work includes many beautiful SEM and histological images and their interpretations, demonstrating her broad expertise with various imaging modalities and her intellectual ability. A sophisticated NO release functionality was successfully incorporated and used to increase the biocompatibility of the polymers. The work opens up broad avenues for future endeavors.

A major contribution of her work relates to the biological response to the NO releasing graft vs. the control graft. Jana discovered that the NO release strongly inhibited the migration of harmful inflammatory cells from the blood circulation into the graft wall. This effect may be exploited to protect a vascular graft from harmful inflammation and intimal hyperplasia.

The layout and organization of the dissertation is effective from the standpoint of clarity. The English language level is very good. Although there are many minor mistakes, none of them detract from the overall clarity of the work. The English quality is on par with or generally exceeds publications in leading scientific journals penned by non-native English speakers.

I strongly recommend the PhD thesis for approval by the defense committee.

Sincerely,
Jeremy Goldman, PhD
Associate Professor
Biomedical Engineering Department
Michigan Technological University