Warszawa, 8.03.2024 r.

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Review of the PhD Thesis

of Mr. Moein Zarei, entitled:

"Biodegradable and biomimetic fibrous composites of hierarchical structure"

Supervisor: Professor Mirosława El Fray

1. Scope and significance of the thesis

According to World Health Organization, cardiovascular diseases (CVDs) are the leading cause of death globally, taking an estimated 17.9 million lives each year, representing 32% of all global deaths. This high incidence of CVDs stimulates the research for developing effective treatments methods. For example, new coronary artery metallic stents significantly improved vascular performance by expansion of the narrowed vessels. There is also a need for an ideal vascular graft with adequate biological and mechanical properties in the clinics. Currently, polytetrafluoroethylene, polyethylene terephthalate and polyurethane synthetic implants have been used to replace natural vessels. However, thrombus formation and compliance mismatch are the main reasons for clinical complications.
One of the solutions to the problem with synthetic grafts could be tissue engineering aiming at supporting new vessels formation. The first tissue-engineered blood vessel substitute was proposed by Weinberg and Bell in 1986. They aimed to reproduce native blood vessels made of a concentric three-layered structure: the intima, media, and adventitia layers. They cultured

endothelial cells, smooth muscle cells and fibroblasts in layers of collagen gel supported by a stronger Dacron mesh. They did demonstrate an initial feasibility of a tissue-engineered graft, however, it fails after 6 weeks mostly due lower mechanical stability.

Since then, the researchers are working on appropriate cell sources and optimal biomaterials and fabrication and maturation techniques to create a suitable scaffold for in situ vascular regeneration or for ex vivo formation of a blood vessel substitute. Regarding biomaterials, research is focused on biodegradable synthetic or natural polymers, they degrade and are replaced and remodeled by the extracellular matrix (ECM) secreted by the cells. Polyglycolic acid or poly-l-lactic acid, polyhydroxyalkanoate, polycaprolactone or polycaprolactone-co-polylactic acid and polyethylene glycol have been tried for vascular scaffolds. However, there are still problems with provided suitable bioactivity or biodegradability of the synthetic polymers. Moreover, although the mechanical strength of the materials could be similar to the native vessels, the compliance mismatch limits long-term performance. Therefore, many researchers are looking for new polymers to reduce disadvantages of currently used biomaterials. For instance, Professor El Fray and others have been working on developing of biodegradable materials such as poly(butylene succinate) (PBS) or poly(butylene succinate-co-dilinolene succinate) (PBS-DLS) copolyesters, with interesting thermo-mechanical properties and the proven biodegradability. To enhance mechanical properties the polymeric matrix could be reinforced with nanofiller, for instance nanohydroxyapatite or nanocellulose.

Alternatives to synthetic materials are natural one such as collagen, gelatin, hyaluronic acid, elastin, or fibrin gels and fibers can direct cell fate and enhance formation of new blood vessels. However, their low mechanical strength and stability are the main drawbacks of using these polymers. There is also strategy where decellularized allogeneic or xenogenic tubular tissues have been proposed as vascular conduits. These grafts have been met with some success, but drawbacks include the inability to tailor matrix content and architecture, progressive biodegradation and the risk of viral transmission from animal tissue.

To engineer biocompatible and biomimetic blood vessel different biofabrication methods have emerged in recent years, including sheet rolling, matrix molding, electrospinning and 3D bioprinting. One of the most explored methods is electrospinning. The electrospinning allows to form scaffolds with high porosity as well as high surface area-to-volume ratio, thus simulating the dimensions and structure of native collagen and elastin fibrils of the vessels. The 3D printing is also promising approach to fabricate tissue engineering construct. It allows the generation of blood vessels with maximum cell survival and ideal recapitulation of the three essential vascular layers.

Finally, to obtain functional implantable blood vessels with sufficient mechanical properties to stand stitching during in vivo implantation and to support physiological blood pressure the fabricated cell containing constructs shall be matured within dynamic bioreactors.

In summary, while tissue-engineered vascular grafts have shown promising results, the challenges of creating the ideal vascular substitute persist. Investigators are actively working towards generating multifunctional materials with an optimized release of bioactive molecules. Additionally, they aim to balance polymer degradation rates with extracellular matrix (ECM) production and cellular infiltration to effectively guide in situ vascular regeneration.

Taking into consideration the need for functional durable small-caliber (diameter: <6 mm) biodegradable engineered blood vessels, Mr. Moein Zarei aims in his PhD work to apply combination of 3D printing and electrospinning to fabricate a novel 3-layer vascular graft made of PBS-based biomaterials and gelatin. The scope of the thesis and proposed approache are significantly relevant to current trends and challenges in the field of implants and tissue engineering.

2. Form and scientific content

The thesis is entitled: Biodegradable and biomimetic fibrous composites of hierarchical structure. It is quite general, missing the focus on the main application which is vascular vessels. The dissertation is written in English. It is quite extensive and consists of 148 pages. It consists of 6 main chapters. It begins with introduction providing short motivation and explaining shortly the proposed biomaterial-based approach in reconstruction of blood vessels. The main objective is already given in this part of the thesis: Synthesis, characterization and processing new multiblock copolymers of poly(butylene succinate – dilinoleic succinate - ethylene glycol succinate), PBS-DLS-PEG, and their composition with cellulose nano crystal (CNC), spanning a wide range of tunable properties, to create micro/nano tubular hierarchical structures using 3D printing and electrospinning techniques. It is interestingly assumed that addition of DLS and PEG, soft segments, to the PBS, hard segments, will allow for enhanced elasticity and wettability of the resulting polymers. Additionally, the Author anticipates that an incorporation of nanocellulose in polymeric matrix will results in increase of mechanical properties of composite nanofibers.
The second chapter, consisting of 26 pages presents in a condense way the state-of-the art in the topic of the dissertation. It properly underlines the need for better solutions in the treatment of cardiovascular diseases (CVDs), particularly the need for functional vascular grafts. Utilizing almost hundred relevant publications, the reader is familiarized with anatomy and biomechanics of the vessels as well as current approaches and challenges for their reconstructions. ~~showing~~ Valuable part of this chapter is provided information about polymers used in the thesis. For instance, the PBS-DLS material synthesized in the group of Prof. El Fray is presented as a starting point to further development, which is the one of the aims of the thesis. The composites with nanocrystal cellulose are also discussed as an interesting biomaterial. Finally, the rationale of using in the thesis of two fabrication techniques such as 3D printing and electrospinning is given. When reading this chapter, one would like to have more information about different advanced approaches in vessels tissue engineering, including multimaterials and multicellular biofabrication.

The next chapter is entitled: Aim of the work, and is focused of describing the general approach for development of new polymeric materials, which in the opinion of the Author could be ideal candidate for fabrication of biomimetic scaffolds for vessels regeneration. The aim of the thesis is to fabricate the vascular grafts consisting of three layers. The middle layer will be a 3D printed tubular scaffold with suitable mechanical properties. The external layer will be made using electrospinning technique to mimic natural fibrous structure of tunica externa. The gelatin coating of the inner surface of the tubular scaffold ~~with~~ will reproduce the tunica intima. The PhD candidate assumes that such complex structure results in engineering of the vascular graft with enhanced biocompatibility, mechanical strength, and endothelialization, overcoming limitation of currently use graft. The scope of the thesis is also provided in this chapter.

The important part of the dissertation is the chapter 4 describing experimental tasks performed by PhD candidate to reach the goal. In the first step, the PBS-DLS and novel PBS-DLS-PEG polymers were synthesized. A two-step process (transesterification and polycondensation in melt) was used to obtain multiblock copolymers. The resulting materials were extruded to the water bath and collected on rotating collector in the form of filament. The process was optimized to obtain filament with diameter of 1.75mm suitable for 3D printing. The standard deviation is not given. Finally, four different copolymer compositions were fabricated with following PBS, DLS, PEG ratios: 70-30-0, 70-25-05, 60-40-0, and 60-35-05.

In the next step, the extended characterization of the obtained material was conducted. Several techniques were employed to describe the chemical structure (^{1}H NMR and FTIR), molecular weight (GPC), viscosity, thermal properties (DSC, DMTA), wettability (CA), melt crystallization, melt flow index, and mechanical properties. The SEMs were used to micro/nano structure and morphology characterization of the fabricated samples. The copolymers biodegradation was evaluated by expose the materials to enzymatic degradation. The mass loss and water uptake were studied. Additionally, influence of degradation on the chemical structure and thermal properties was investigated. It is not clear why bioactivity in SBF to CaP formation was tested in vessel tissue engineering. Biocompatibility tests were performed according to ISO10993-5. The attachment of the L929 cell line to copolymer's film was also assessed. The attachment of the cell to final products could be more valuable from the point of view of future application. In summary, generally, the proposed characterization methods are properly selected and planned.

The fabrication of hierarchical structure is described in chapter 4.4. In the first stage, the parameters of fused filament fabrication (FFF) and solution electrospinning processes were optimized to fabricate testing samples using synthesized copolymers and composite. The information about tested materials is missing in the methodology of the FFF process. Then, the FFF was utilized to print a tubular scaffold with diameter of 5mm and wall thickness of 0.5mm. The hexagonal pores (with size of 0.8mm) were generated within tubular structure. The rationale for the size and shape of the proper are related only to technological difficulties. The length and porosity of the tube are not given. Interestingly, the printed porous tube was used as a template in a template-assisted electrospinning what resulted in covering of the tubular scaffolds with electrospun mat of nanofibers. The final stage for vascular graft fabrication was coating inner surface of the tubular scaffold with gelatin using perfusion technique.

The mechanical performance of the vascular graft hierarchical structure we tested according to the methods described in chapter 4.5. The planned tests such as tensile strength measurement, burst pressure and leaked tests, and compliance test are often used in evaluation of vascular grafts in the literature. However, more detailed information about methodology of the tensile strength determination should be provided.

The most valuable part of the dissertation is chapter 5, where results and discussion of the results are provided. ^{1}H NMR and FTIR spectra (adapted from Zarei et al. 2023) confirm the synthesis of the PBS-DLS and PBS-DLS-PEG copolymers. The obtained compositions are similar to the theoretical ones. The DSC study reveals low glass transition temperature (Tg) of about -50°C and high melting temperature (Tm) of about 90°C for all copolymers. This is typical behavior of thermoplastic elastomers. The interesting discussion about polymer crystallization from the melt is provided which depends on polymer composition. Water contact angle measurements confirm that addition of 5 wt% PEG to PBS-DLS decrease contact angle of about 20 degrees. Quasi-static tensile tests revealed that the synthesized copolymers show stress-strain curves typical for thermoplastic elastomers with yield point and rubber-like elasticity. Increase of hard segment content in copolymer resulted in increase of stiffness and strength of the fabricated materials. It is still unclear why elongation to break for PBS-DLS-PEG (60-35-05) is significantly lower than PBS-DLS (60-40). The melt flow behavior of synthesized copolymers was also evaluated. The MFI value increased with incorporation PEG to PBS-DLS. It is important to study biodegradability of the materials for tissue engineering application. The enzymatical degradation was performed to study change of chemical and molecular structure, mass, thermal properties, and surface morphology of the different copolymers. It was noticed that presence of hydrophilic building block, PEG, has accelerated degradation process of the polymers. The molecular weight of the copolymers containing or not PEG decreased after 20 days of degradation. The reduction of Mn was higher in the case of the PBS-DLS-PEG materials. Similarly, the introducing PEG in the copolymer structure boosted its mass loss. The Author also discussed the change of thermal properties of the degraded materials. The SEM examination of the sample revealed surface erosion mechanism during exposure to enzymes. Additional microscopy observations of the sample cross-sections could confirm or not that the bulk erosion is not present. Given that the form of the material can influence degradation, one might wonder why films were used in the degradation tests instead of fibers and nanofibers.

The application of the materials in medicine requires assessment of their biocompatibility. The indirect and direct cytotoxicity tests conducted according to ISO 10993-5 have shown high L929 cells viability confirming initial biocompatibility of the synthesized copolymers. Analyzing SEM images of L929 cells on surfaces made of different materials, it is intriguing that varying levels of the wetness did not affect cell adhesion.

The most interesting part of the thesis for the application point of view is the chapter 5.3 Fabrication of multilayered tubular graft. In the first step, the Author had optimized process parameters to 3D print tubular structure and to electrospun nanofiber mats, what was confirmed by SEM observations. For instance, the mats without CNC and with 1wt% CNC had fibers with average diameter of about 600 nm and 1000 nm, respectively. It was also confirmed that addition of 1wt% CNC resulted in increase of stiffness and tensile strength of nanocomposite mats. The Author should avoid using the term of Young's modulus for description of the mat stiffness. The tubular structure, scaffold, with hexagonal pores was fabricated, but accuracy of the printed was not evaluated. Then, the scaffold was used as a template for electrospinning to obtain hybrid two-layer construct mimicking two layers of the natural vessels. Subsequently, the lumen of the tubular structure had been coated by gelatin through perfusion. The SEM investigation revealed good attachment between printed, electrospun and coated materials. Better, quantitative characterization of the connections between different layers of the hybrid construct would be useful in evaluation of the applied fabrication method.

Chapter 5.4 describes the mechanical and functional properties of the fabricated micro/tubular structures using two different materials: PBS-DLS 70-30 and PBS-DLS-PEG 70-25-5. It is not clear if the composite mats had been used or not in the final constructs. The ultimate strength of the scaffolds ranges from 15 MPa up to 18 MPa, for 70-30 and 70-25-5 copolymers, respectively. Interestingly, both constructs show high, about 300 % elongation at break. Although the mechanism of the scaffold deformation and fracture presented at the stress-strain curve is not well described. Important results are shown in Figure 60. The higher burst pressure and lower leakage was observed for the constructs with gelatin coating. Moreover, the measured burst pressure was similar to the one found in human veins. The biomimetic was also mechanical compliance of the fabricated graft. The obtained compliance of about 12%/100 mmHg was very close to a compliance range reported for the native arteries (11.5+/-3.9%/100 mmHg). However, it is not clear how many loading cycles had been applied in the test. The last tests concerned the evaluation of the cytotoxicity of the tubular structure. The methodology of the tests has not been described in the materials and methods part of the thesis. The information about method of samples sterilization is also not provided. The results of cytotoxicity assessment in the indirect test revealed high viability of the L929 cells.

The conclusions of the PhD work are presented in the Chapter **6**. The main scientific achievements and overall conclusions are listed in this part of the dissertation. In this chapter, the Author proposes future research directions. The last chapter is the bibliography consisting of 202 scientific articles, closely related to the topic of the thesis. The last two pages is about scientifical records of the PhD candidate.

The overall quality of the dissertation is excellent. It is clearly structured and easy to read. The style and English language are satisfactory. The Author comprehensively presented findings using several tables (9) and many figures (61). There are some editorial errors, but it doesn't detract from the value of the content. For instance, Fig. 44 and Fig.45 present low quality SEM images. There are some errors in presented references. Some figures like Figs. 11, 20, 21, 22, 25, 40 are messing information that they are adapted from one of the Author’s publications. There are also some similarities between the text of the thesis and the published articles by the PhD candidate, what should be avoided. In summary, the thesis is generally well prepared.

3. Main scientific achievements and substantive aspects

Mr. Moein Zarei has submitted a thesis that is well suited to current research trends in the field of cardiovascular tissue engineering. After an extensive analysis of current state-of-the art in the field of vascular grafts and polymeric or composite, Mr. Zarei performed well-organized and high-quality research to investigate:

- new potential copolymers and composites for graft fabrications;

- novel multilayer hybrid structure mimicking native vessels organization for future vascular graft tissue engineering.

The thesis demonstrates an original work which makes an important contribution to knowledge in the field of polymeric biomaterials for cardiovascular application. The series of new copolymers was obtained by combination of poly(butylene succinate), dimer linoleic acid and poly(ethylene glycol).

For the first time, the PBS-DLS-PEG has been investigated as a material for vascular graft fabrication. The two fabrication techniques, 3D printing, Fused Filament Fabrication, and template-assisted electrospinning, have been applied to produce two-layer tubular scaffold for vessels tissue engineering. The developed materials and scaffolds have been extensively investigated using numerous experimental techniques including: ^{1}H NMR, FTIR, GPC, DSC, DMTA, goniometer, mechanical testing machines, and SEM. It should also underline that some of the experimental setups have been designed and developed by PhD candidate. The preliminary results from the characterization of the proposed materials and the hybrid scaffold evaluation indicate the high application potential of the developed solutions.

When reading the PhD thesis, the following comments and questions arise:

1. The title of the thesis is too general considering that the main focus of the work was on vascular grafts.
2. The more explanation is required to the statement that "the integration of hexagonal pores not only enhances the mechanical properties of the tunica media-like structure but also should offer a biologically relevant environment for cells, promoting tissue integration and functionality within the graft."
3. The using gelatin for coating of the internal surface of the scaffolds is an interesting proposition. However, the stability of this coating in vitro or in vivo maybe limitation of this solution.
4. Maturation techniques to create a suitable scaffold for in situ vascular regeneration or for ex vivo formation of a blood vessel substitute is not discussed in this thesis.
5. If the concept of ready-to-use vascular grafts will be considered such device will be in direct contact with blood before a native endothelium covers its surface, and consequently, the first requirement that the base material must be hemocompatible. Why only cytotoxicity of L929 cells was investigated?
6. The evaluation of the materials/scaffolds long-term degradation is needed. The degradation of the scaffold should be "synchronized" with new tissue formation. How will be in the case of the proposed solution in this thesis?
7. Why a suture retention strength was not studied. It is an essential surgical parameter in transplantation of artificial vascular grafts into the human body. Therefore, the graft should have enough strength to withstand the tensile load of the sutures without failure.
8. Why has bioactivity for CaP formation been investigated in the thesis? Is it truly advantageous to have the formation of bioceramics in the vascular grafts?
9. The cell attachment strongly depends on morphology of the surface. The film and final scaffold morphology may significantly differ. Therefore, cell attachment should be also tested on the surface with final morphology.

10. A more extensive description of future research directions would be helpful in better understanding the current limitations of the work and planning the next steps to advance the development of materials and the idea of biomimicking vascular grafts closer to clinical applications.

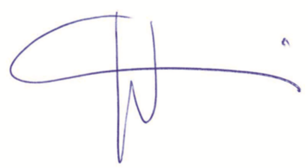
In summary, the reviewed doctoral dissertation has many cognitive aspects and is an original contribution to the development of the field of vascular graft. The greatest achievements of the dissertation are the development of new PBS-DLS-PEG copolymers with interesting properties and perspective for future medical applications. Additionally, the fabrication of multilayer construct mimicking natural vessel's structure is also a significant achievement of the PhD candidate. Also noteworthy is the application of numerous research methods for the characterization of materials and scaffolds, particularly the custom-made research facilities developed by the candidate for assessing the functional properties of the grafts.

4. Final conclusions

Considering the interesting results obtained by Mr. Moein Zarei, good mastery of the research technique and correct interpretation of the research results, herein it can be concluded that his doctoral dissertation meets all conditions specified in Article 187 of Act of July 20, 2019 Law on Higher Education and Science (Journal of Lawa of 2018, item 1668, as amended), and thus I am asking the Scientific Council of Materials Science and Engineering of West Pomeranian University of Technology in Szczecin for admission Mr. Moein Zarei to the next stages of the doctoral process.

Given the high quality and significance of the results presented in the dissertation, as well as the outstanding scientific achievements of the doctoral candidate published in several leading scientific journals, the reviewed PhD thesis deserves distinction.

Sincerely yours,



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