

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

**IMPROVEMENT OF INADEQUACIES OF SMALL DIAMETER
ARTIFICIAL BLOOD VESSELS**

Ph.D. THESIS

İpek YALÇIN ENİŞ

Textile Engineering Department

Textile Engineering Program

SEPTEMBER 2016

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**KÜÇÜK ÇAPLI YAPAY DAMARLARIN MEVCUT YETERSİZLİKLERİNİN
İYİLEŞTİRİLMESİ**

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Date of Submission : 18 August 2016
Date of Defense : 28 September 2016





To my spouse,



FOREWORD

First of all, I would like to express my gratitude to my advisor, Prof. Dr. Telem GÖK SADIKOĞLU for her guidance during toilsome thesis process while encouraging me to proceed to the next step everytime.

A substantial part of my thesis was shaped in Liberec Technical University, Department of Nonwovens and Nanofibrous Materials. Therefore, I would like to show gratitude to RNDr. David LUKAS for offering me such an opportunity and sharing with me his valuable experiences. Moreover, I would like to thank to Jana HORAKOVA, PhD. for her great assistance and motivation during my stay.

I would like to thank to Erasmus Mobility Programme and Tinçel Cultural Foundation for funding visiting scholarship to abroad.

The research presented in the thesis was supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK; Project no: 214M089) and İTÜ Projects of Scientific Investigation (BAP; Project no: 37678). Further more, I would like to thank to TÜBİTAK 2211, National Scholarship Programme for PhD Students for supplying scholarship during my thesis period.

Owing to the interdisciplinary topic of my thesis, I would like to thank to everybody who contributed to my research and who I benefitted from their experiences and knowledge.

Last but not least thanks are for my colleagues and my best friends as well, who never let me alone during this challenging yet attainable period, my family especially my spouse for keeping my motivation high everytime and supporting me in all aspects of thesis.

August 2016

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ABBREVIATIONS

2D	: Two Dimentional
AA	: Acetic Acid
AI	: Alignment Index
ANOVA	: Analysis of Variance
AR	: Aspect Ratio
AVG	: Average
BSA	: Bovine Serum Albumin
CASMC	: Coronary Artery Smooth Muscle Cells
CD	: Circumferential Direction
CLSM	: Confocal Laser Scanning Microscopy
CVDs	: Cardio Vascular Diseases
DCM	: Dichlormethane
DMEM	: Dulbecco's Modified Eagle Medium
DMF	: Dimethylformamide
DNA	: Deoxyribonucleic Acid
ECM	: Extra Cellular Matrix
ECs	: Endothelial Cells
EDTA	: Ethylenediaminetetraacetic acid
ePTFE	: Expanded Polytetrafluorethylene
FA	: Formic acid
FDA	: Food and Drug Administration
FITC	: Phalloidin-Fluorescein Isothiocyanate
FTIR	: Fourier Transform Infrared Spectroscopy
GACR	: The Czech Science Foundation
H&E	: Hematoxylin Eosin
HFIP	: Hexafluoroisopropanol
IH	: Intimal Hyperplasia
LD	: Longitudinal Direction
Mn	: Number Average Molecular Weight
MTT	: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	: Phosphated Buffer Saline
PCL	: Polycaprolactone
PDLA	: Poly (D-lactide acid)
PDLLA	: Poly (L,D-lactide acid)
PEG	: Poly ethylene glycol
PET	: Polyester
PGA	: Poly glycolic acid
PGS	: Poly (glycerol sebacate)
PLA	: Poly lactic acid
PLCL	: Poly (L-lactide co-ε-caprolactone)
PLGA	: Poly (lactic-co-glycolic acid)

PLLA	: Poly (L-lactic acid)
PU	: Polyurethane
SD	: Standard Deviation
SEM	: Scanning Electron Microscope
SMCs	: Smooth Muscle Cells
TA	: <i>Tunica Adventitia</i>
THF	: Tetrahydrofuran
TI	: <i>Tunica Intima</i>
TM	: <i>Tunica Media</i>
TUBITAK	: The Scientific and Technological Research Council of Turkey
SE	: Sythetic Elastin



SYMBOLS

λ	: Wavelength
A_{avg}	: Average pore area
$^{\circ}C$	: Celcius
D	: Maximum pore diameter
p	: Gas pressure
γ	: Surface tension
$0^{\circ}, 90^{\circ}, 45^{\circ}$	: Testing directions



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IMPROVEMENT OF INADEQUENCIES OF SMALL DIAMETER ARTIFICIAL BLOOD VESSELS

SUMMARY

As the world population is getting older, cardiovascular diseases are increasing correspondingly. Artificial blood vessel implantations are promising operations for bypass patients. Vascular grafts of polytetrafluorethylene (ePTFE), polyethylene terephthalate (PET) and polyurethane (PU) work well in the replacement of large diameter (>6 mm) vessels. However they fail for small diameters due to incompatibility of radial elasticity, insufficient porosity and insufficient cell growth. Considering these requirements with the large number of patients in the need of replacement grafts, the demand for an alternative small diameter graft is enormous.

This thesis aims at improving inadequencies of small diameter artificial blood vessels by determining new design criterions and developing alternative construction designs as well as modification and establishment of custom designed electrospinning technology.

Within this thesis, four supplementary articles were given in sequence taking the topic integrity into consideration. All articles given in separate chapters have novelty in research and discuss remarkable and original outcomes of the thesis topic.

In the first review article, after providing brief information about native blood vessels' histological and structural properties, the requirements for vascular grafts, the technology of electrospinning and the advantages of electrospun vascular grafts were introduced. The vascular graft design is accepted to be successful to the degree that it mimics the target tissue. Therefore, the design steps should be based on mimicking the native blood vessels' structural, mechanical, biological and topographical properties. In this article, the design parameters of biodegradable electrospun vascular grafts, mainly as the fiber diameter, pore size and porosity, fiber orientation, wall thickness and material selection are discussed in detail with recent literature works.

Owing to the defined design criterions, multilayer designs seem to be more advantageous for vascular graft production in order to create a composite structure with differing functions and capabilities in separate layers, as it is in native arteries. Therefore, inner layer of the vascular graft should be available for pre-endothel cell growth in order to achieve non-thrombogenicity and pore size should be limited to blood cell dimensions. On the other hand, the pore size of outer layer should be larger than the dimensions of the smooth muscle cells in order to achieve the diffusion of these cells through the wall thickness of the scaffold. Moreover, insufficient mechanical properties of this layer can be improved by achievement of radial fiber orientation as the radially oriented collagen bundles in the native vessels. Various biodegradable polymers can be used for vascular grafts' production but it is important to find the most suitable one which behaves like a stable backbone with enough mechanical integrity until it degrades and have enough elasticity to overcome radial elasticity mismatch. Therefore, the selection of material type and production

technology is also important in designing a scaffold to overcome inadequacies of small diameter vascular grafts.

It is intended that the blended and enriched context of this review article aims to overcome complex design problems for multilayer vascular grafts while serving a discussed literature for further studies of the thesis topic.

Second article involves the optimization of selected polymer and solvent types and their electrospinning process. Among several design strategies, fiber diameter and pore size of fibrous scaffolds are one of the most determinant parameters for tissue designs; i.e. multilayer vascular grafts. Since blood vessels are composed of layered structures, pore size distribution of layers differ from each other based on cell type content and their functions in related layers. Pore size of the scaffold should enable smooth muscle cell diffusion in the middle and outer layer of native blood vessel while hindering blood leakage in the inner layer. Improved spinnability of biomaterials with differing characteristics according to desired requirements lead up design flexibility in tissue designs. Therefore, the effect of polymer concentration and addition of acids to PCL/chloroform: ethanol solution were investigated based on solution characteristics and surface morphology of the fibrous webs including fiber diameter and pore size distribution. Moreover with Fourier Transform Infrared Spectroscopy (FTIR) analysis, residual solvent risk on produced surfaces were examined.

Thesis continues with the third article on the topic of the production of single layer scaffolds for vascular grafts. Before production, basic electrospinning apparatus was modified with the requirements of vascular graft production such as 6 mm rotating type of collector for tubular scaffold production, high rotational speed motor integration to achieve radial fiber orientation, pneumatic sliding feeding unit for homogeneity of the scaffold and so on. Histology of the native vessels were also observed in the context of this article to prove the discussed design parameters in first article. Taking care of these histological results, pre-mentioned design parameters and modifications of electrospinning unit, single layer scaffolds were produced. PCL in chloroform:ethanol solvent system with 18% weight ratio was chosen and pre-determined electrospinning production parameters were used in the light of our previous works. Radial fiber orientation was achieved by increasing the rotational speed of the mandrel in order to improve the mechanical performance and cell organization. Fiber diameter distribution as well as pore size area and porosity analysis was examined to evaluate its suitability for cell proliferation and diffusion through the *tunica media*. The relationship between the orientation of fibers and cells was confirmed in vitro by seeding fibroblasts. This study aims to constitute a considerable step forward in designing multilayered vascular graft.

Lastly, bilayer scaffolds were produced in the fourth article in the light of first three articles and gained experiences. In this article, PCL (in differing molecular weights) and a copolymer poly(L-lactide) caprolactone (PLC) single layer scaffolds composed of either oriented or randomly distributed fibers were produced in 6 mm tubular forms by electrospinning. Then, bilayer scaffolds from the same polymers in different combinations including randomly distributed inner layers and radially oriented outer layers were produced. Their morphologies before and after mechanical tests were analyzed and discussed within the context of their mechanical performance. The mechanical test results were discussed based on used polymer type composition, fiber orientation and composite effect of each layer of bilayer grafts. Produced fibrous

scaffolds were tested with smooth muscle cells to test biocompatibility of presented scaffolds.

In conclusion, four articles in the context of this thesis reveal the original and updated sides of the thesis topic. When all studies in the thesis are evaluated as a whole, this thesis has unique contribution to the literature with novel and detailed sub-titles. Furthermore, according to the literature survey, no experimental studies were found available on this topic in Turkey. In this regard, besides being an innovative partner to the international literature with its scientific quality, the outcomes of this thesis are thought to be a guiding light to the national researchers.





KÜÇÜK ÇAPLI YAPAY DAMARLARIN MEVCUT YETERSİZLİKLERİNİN İYİLEŞTİRİLMESİ

ÖZET

En yaygın görülen tipleri arasında periferik arter hastalığı, serebrovasküler hastalık, koroner kalp hastalığı, derin ven trombozu, akciğer embolisinin yer aldığı kalp ve damar hastalıkları gün geçtikçe artmakta ve bu durum teşhis ve tedavi olanaklarının geliştirilmesini gerekli ve zorunlu kılmaktadır. Bu hastalıkların tedavi seçenekleri arasında her ne kadar ilaç ile tedavi mevcut olsa da, çoğu hastalıkta, tıkalı veya zarar görmüş atar damarın cerrahi olarak onarımı veya değişimi olarak tanımlanan by-pass uygulamalarına ihtiyaç duyulmaktadır. Hastanın vücudundan alınan otogrefter by-pass cerrahide öncelikli tercihtir. Ancak, önceden geçirilmiş olan rahatsızlıklar ve cerrahi müdahaleler hastada sağlıklı damar yapısının oluşmasına neden olduysa cerrahide yapay damar kullanımı zorunlu hale gelmektedir.

Günümüzde büyük çaplı (>6 mm) damarların ihtiyacı, ticari genişletilmiş politetrafloroetilen (e-PTFE), polietilen tereftalat (PET) ve poliüretan (PU) yapay damarların başarılı şekilde implantasyonu ile giderilebilmektedir. Ancak, küçük çaplı yapay damarların değişimi, bir çok kısıtlamayı beraberinde getirmektedir. Başlıca yetersizlikler, düşük kan pıhtılaşma direnci, yetersiz yapısal gözeneklilik, yetersiz radyal elastisite şeklinde olup, bunlar günümüzdeki küçük çaplı yapay damar uygulamalarındaki başarısızlıkların temel nedenleri arasındadır.

Yapay damar tasarımı, tüm doku tasarımlarında da olduğu gibi, hedef dokuyu taklit edebildiği ölçüde başarılıdır. Bu nedenle, izlenecek tasarım esasları, gerçek damar yapısını esas almalı, onun yapısal, mekanik, biyolojik, topografik özellikleri ile örtüşmelidir. Bu bağlamda, üretilecek yapay damara ait tasarım kriterlerinin önceden belirlenmesi önem taşımaktadır.

Tez kapsamında, konu bütünlüğü dikkate alınarak sıralanmış dört adet makale yer almaktadır. Her bir makale tezin ayrı bölümleri olarak verilmiş olup, literatüre alanında yenilikçi sonuçlar sunmaktadır.

Tezin ilk makalesinde, elektrospinning yöntemi ile biyobozunur polimerlerden üretilen yapay damarlara ait tasarım bileşenlerine yer verilmiştir. Bu makale, gerçek damarın katmanlı yapısını, katmanların bileşenlerini ve hücre dağılımlarını, yapay damarlardan beklentileri, elektrospinning yönteminin genel çalışma prensibini ve yapay damar üretimindeki kullanımına dair bilgileri ve bunlara ek olarak, literatürdeki mevcut çalışmaların taranarak öncelikli olarak belirlenen, lif çapı, gözeneklilik ve gözenek boyutu, lif oryantasyonu, yapay damar duvar kalınlığı ve malzeme seçimi gibi temel tasarım kriterlerini içermektedir. Bu makalenin amacı literatürdeki çalışmaları ham olarak özetlemek yerine özellikle son on yılda yoğunlaşan mevcut çalışmaların sonuçlarının detaylı ve kıyaslamalı olarak tartışılmasını sağlamaktır.

Belirlenen kriterler ışığında, katmanlı bir yapay damar yapısının, tek katlı bir yapıya göre oldukça avantajlı olduğu anlaşılmıştır. Katmanlı bir yapıda birbirinden bağımsız yapısal, morfolojik ve mekanik özelliklere sahip olan katmanların oluşturduğu yapay

damarın tıpkı gerçek damar yapısındaki gibi birbirinden ayrılan görevleri üstlenebileceği düşünülmektedir. Bu bağlamda genel hatları ile yapay damar tasarımında iç katmanın kan pıhtılaşma direnci için ön-endotel hücre büyümesine elverişli olması, ayrıca kan sızdırmazlığı için gözenek boyutunun, kan hücresi boyutu ile sınırlı olması beklenirken, dış katmanın, düz kas hücrelerinin radyal kalınlıktaki difüzyonu için düz kas hücre boyutundan büyük gözenek boyutuna sahip olması gerektiği sonucuna varılmıştır. Bu çalışmada, elektrospinning yöntemi ile üretilen yüzeylerin lif çapı ve gözenek çapı üzerinde etkili olan üretim ve çözelti parametrelerinin yanı sıra büyük gözenek çapına sahip yüzey üretimi için kullanılabilecek çeşitli tekniklere de yer verilmiştir. Ayrıca, dış katmanda, yetersiz mekanik özellikler radyal lif oryantasyonunun arttırılması gibi kimi yöntemlerle iyileştirebilirken, küçük çaplı döner toplayıcı ile yüksek hız limitlerine çıkabilmenin çok kolay olmadığına bu nedenle araştırmacıların farklı teknikler üzerinde çalıştıklarına değinilmiştir. Lif oryantasyon eldesi için gerekli olan çevresel hız limitleri oldukça detaylı olarak irdelenmiş, polimer tipinin de etkisi dikkate alınarak tez kapsamında üretilen 6 mm çaptaki yapay damar üretiminde radyal lif oryantasyonu eldesine izin verecek çevresel ve açısal hız limitleri belirlenmiştir. Duvar kalınlığının radyal elastisite üzerindeki etkisi tartışılmış, gerçek damara ait duvar kalınlığına yakın kalınlıkta yapay damarın duvar kalınlık eldesi için kontrol edilmesi gereken üretim parametrelerine değinilmiştir. Öte yandan, yapay damar üretiminde kullanılan biyobozunur malzeme seçiminde bir çok polimere yer verilmiş, mevcut çalışmalar ışığında mekanik, biyolojik ve morfolojik özelliklere etkisi açısından avantaj ve dezavantajları irdelenmiştir. Gerçek damar yapısının taklit edilebilirliği açısından çeşitli tasarım bileşenlerini tek damarda bir araya getirebilmek için katmanlı yapay damar tasarımının en iyi seçenek olacağı sonucuna varılmıştır.

Yapay damar üretiminde kullanılacak biyopolimerlerin seçiminde başlıca belirleyici özellikler olarak biyoyumluluk, yeterli mekanik dayanım, radyal elastisite sorununu bertaraf edecek ölçüde elastiklik ve implantasyon sonrası hücre tutunması, çoğalması ve hücrelerin kendi proteinlerini sentezleme süresine izin verecek uzunlukta biyobozunurluk süresine sahip olması gelmektedir. Tez çalışması dahilinde, yapay damar tasarımında kullanılmak üzere yeterli mekanik özelliklere ve biyobozunurluk süresine sahip olan polikaprolakton (PCL) ve kopolimeri poli(laktik-ko-kaprolakton) (PLC) seçilmiştir. Üretim tekniği olarak da sunduğu bir çok avantajdan ve istenilen yüzey üretimine yönelik modifikasyona izin vermesinden ötürü elektrospinning tekniği seçilmiştir.

Tezin ikinci makalesinde, polikaprolakton polimeri için medikal uygulamalarda kullanımı kabul gören kloroform çözücüsünün yanı sıra farklı çözücü sistemleri üzerinde çalışılmış, asit ilavesinin ve polimer konsantrasyonunun öncelikle elektrospinning sürecine etkiyen çözelti parametreleri üzerindeki etkisi, akabinde üretilen yüzeylerdeki lif morfolojisi, lif çapı ve gözeneklilik ile gözenek boyutu üzerindeki etkileri incelenmiştir. Böylece tez kapsamındaki polimer-çözücü sistemi ve üretim parametrelerinin optimizasyonu sağlanmıştır. Fourier Transform Kızılötesi Spektroskopisi (FTIR) analizi ile üretilen yüzeylerde artık çözücü olup olmadığı üzerinde yorumlar yapılmıştır. Elde edilen sonuçlar, asit ilavesi ile PCL'in lif çapının küçültülebileceği, üretilirliğinin geliştirebileceğini göstermektedir. Gözenek çapının lif çapı ile doğrudan ilişkili olduğunu, lif çapı küçüldükçe gözenek çapının da küçüldüğü gözlenmiştir. FTIR sonuçlarında özellikle kloroforma ait karakteristik piklerin, üretilen yüzeylerde mevcut bulunmadığı tespit edilmiştir.

Tez, tek katlı tübüler yapıların üretimi ile devam etmektedir. Bu aşamada, belirlenen tasarım kriterleri ve optimize edilmiş üretim parametreleri dikkate alınarak öncelikle üretim teknolojisinin modifikasyonuna başlanmıştır. Öte yandan, yapay damar tasarımı söz konusu olduğunda, öncelikle gerçek damar yapısının incelenmesi, katman içeriklerinin ve bu katmanlardaki hücre dağılımlarının bilinmesi gerekmektedir. Bu nedenle, gerçek damarın katmanlı yapısına ait histolojik analizler gerçekleştirilmiştir.

Tez kapsamındaki üçüncü makale, elektrospinning ünitesinin modifikasyonu üzerine bir çok yenilikçi teknik bileşenin yanı sıra, gerçek damarın histolojik analizlerine, birinci makalede tartışılan tasarım kriterlerinin tek kat yapay damar üretiminde işlenmesine kadar bir çok detayı içeren oldukça kapsamlı bir makedir.

Üretim teknolojisi incelendiğinde, makaleyi özgün kılacak noktalara şu şekilde yer verilebilir. Literatürde radyal oryantasyona sahip yüzeyler büyük çaplı silindir toplayıcılarla düşük dev.dk^{-1} hızlarında elde edilirken, 6 mm'lik toplayıcı için bu değerin yetersizliği dikkat çekicidir. Piyasada yüksek hız değerlerine ulaşabilen motor bulmak kolay değildir. Bu sebeple, mevcut çalışmalarda büyük çaplı döner toplayıcılar üzerinde radyal oryantasyona sahip lifler üretilmektedir. Tez kapsamında 35.000 dev.dk^{-1} hız değerine çıkabilen matkap motoru ile toplayıcı entegrasyonuna sahip bir elektrospinning ünitesi tasarlanmış ve kurulumu gerçekleştirilmiştir. Literatürde tarama yapıldığında bu hızlara çıkabilen motor tahriki ile küçük çaplı döner toplayıcılarda radyal lif oryantasyonu elde eden çalışmaya rastlanmamıştır. Genellikle maksimum 5.000 dev.dk^{-1} hız limitlerine çıkılmış, çoğu zaman büyük toplayıcı çapı tercih edilmiş veya oryantasyon için metal plaka entegrasyonu gibi farklı tekniklere ihtiyaç duyulmuştur. Ayrıca elektrospinning ünitesinin kurulumunda, yatay iğne hareketinin pnömatik bir sistemle sağlanmasıyla homojen yüzey eldesine olanak tanımıştır.

Öte yandan, literatürde yapay damar tasarımına ait kriterlerin belirlenmesinde önemli rol oynayan gerçek damarın histolojik yapısı, tez çalışması kapsamında gerçekleştirilmiş, gerçek damar yapısındaki katmanlar, elastin ve kolajen içerikleri, düz kas ve endotel hücre dağılımları özel boyama teknikleri kullanılarak, net bir şekilde ayırt edilebilmiştir. Bu analiz ile hedef doku taklidi için gerekli parametreler tasdik edilmiştir. Bu kapsamda da, gerçek damara ait histolojik analizlere yer verilmesi, makalenin özgün değerini arttırmıştır. Tasarıma ait, katman kalınlıkları, kolajen fibrillerinin yapıdaki radyal dağılımları ve fibriller çapları taklit edilerek, tek kat yapay damarlar üretilmiş, bu yapıların morfolojik, mekanik ve biyolojik analizleri bir çok test tekniği ile gerçekleştirilmiştir.

Son olarak, çift kat yapay damar üretimlerini içeren dördüncü makalede, farklı molekül ağırlıklarına ait PCL ve kopolimer PLC ile farklı oryantasyon derecelerinde kombinasyonlara gidilmiş, morfolojik, mekanik ve biyolojik analiz sonuçlarına etkileri incelenmiştir. Üretilen çift katlı yapay damarların mekanik özellikleri, bileşenlerini oluşturan ve ayrıca üretilen tek katlı yapay damarlarla kıyaslanmış, kompozit etkisinin mekanik özellikler üzerindeki önemi tartışılmıştır. Duvar kalınlığı, polimer tipi, polimerin hangi katmanda ve hangi oryantasyon derecesinde kullanıldığına bağlı olarak değişen çift kat tasarımların morfolojik analizlerinin yanı sıra, çekme ve patlama test sonuçları detaylı olarak tartışılmıştır. Makalenin özgün değerini arttıran bu çalışmalar, çift kat yapay damar üretiminde katmanlar arası farklı kombinasyonlara gidilmesinin nihai tasarımda ne denli farklı etkilere neden olabileceği sonucunu ortaya koymuştur. Öte yandan bu çalışma kapsamında, dış katmanlardaki radyal oryantasyon eldesi ile gerçek damarın dış katmanındaki kolajen

fibrillerinin radyal oryantasyonu taklit edilmiştir. Üçüncü makalede fibroblast hücreler ile yapılmış olan hücre analizleri bu kez, dış katmanda çoğalması ve yayılması hedeflenen koroner düz kas hücreleri ile tekrarlanmış, biyouyumlulukları ve radyal oryantasyona sahip liflerle aynı yönelimde çoğaldıkları floresan mikroskop çekimleri ile kanıtlanmıştır.

Sonuç olarak, tez kapsamındaki bu dört makale, tez konusunun güncel ve farklı yaklaşımlarını gözler önüne sermektedir. Makalelerin oldukça detaylı ve yenilikçi alt başlıkları ile henüz yeni sayılabilecek bir çalışma alanı içerisinde literatüre özgün katkı sağladıkları düşünülmektedir. Bunun yanı sıra, taranan mevcut çalışmalarda, önerilen tez konusu üzerinde Türkiye’de yürütülen deneysel çalışmalara rastlanmamıştır. Bu bağlamda çalışmanın bilimsel kalitesi ile uluslararası literatür için yenilikçi bir paydaş olmasının yanı sıra, ulusal araştırmacılara da yol gösterici olacağı öngörülmektedir.



1. INTRODUCTION

Advanced cardiovascular diseases (CVD's) make the necessity of the vascular grafts a current issue owing to growing demands of patients. Despite the achievement in usage of large diameter vascular grafts (> 6 mm) in by-pass surgeries, synthetic small diameter vascular grafts still have complications in applications mainly as limited capability of endothelium regeneration and tissue growth, compliance mismatch, thrombogenicity and intimal hyperplasia (IH). Currently used PET, ePTFE and PU grafts have limited capability of endothelium regeneration and tissue ingrowth. Since endothelium is a natural barrier to the platelet activation on the luminal surface of the vessel, in default of it, blood proteins deposit and platelet formations occur on the luminal surface of the bare graft leading to thrombus formation. Moreover, IH narrows the lumen of the vessel and reduces flow rate of blood which can also result in local thrombosis. Compliance mismatch refers to inadaptability in radial elasticity at conjunction points of implanted graft and native vessel, may result in IH.

In order to overcome these complications, many researchers from various interdisciplinary fields bring this topic to a center of attention and define the main goal as designing a vascular graft that mimics the target tissue as much as possible. Electrospun biodegradable scaffolds are the most promising field of studies in this concept allowing variety of opportunities to create endless vascular graft designs.

This thesis is composed of four articles in the order of topic integrity. Roughly these articles involve sieved back ground information; definition and discussion of design parameters for vascular grafts, investigation of current situation, inadequencies and requirements of small diameter vascular grafts; optimization of solution and production parameters for electrospun vascular grafts; modification of electrospinning system based on the requirements of tubular scaffolds production; histological analysis of native vessel as a target tissue; application of discussed design parameters in practice of single layer tubular scaffolds, and lastly bilayer vascular graft designs with pre-determined design parameters, examination of composite effect in mechanical

properties of bilayer scaffolds, in vitro pre-clinical analyses of the scaffolds by seeding fibroblasts and coronary artery smooth muscle cells.

First article involving the summarized back ground information and discussed current studies in the light of literature constitutes the backbone of the thesis. This review includes comprehensive knowledge about whole thesis topic rather than serving the raw data. The objective is to discuss comparatively the current studies on the design parameters of electrospun vascular grafts, defined as fiber orientation, fiber diameter, pore size and porosity, wall thickness, and material selection, based on the structure of native blood vessels, the requirements of vascular grafts, electrospinning technology, and the advantages of electrospun vascular grafts, to give a broad outlook for further studies. The outcomes of the review lead us to decide the selection of production technique and polymer type together with construction variables of multilayer scaffold design.

Polycaprolactone is preferable biodegradable polymer to be used for vascular graft fabrication owing to its good biocompatibility, slow degradation rate with improved tensile and elongation properties. Solvent type is also an important parameter due to the suitability to be used in implantations without leaving any hazardous residues behind. Electrospinning technique which enables ease of modification with regards to the requirements of vascular graft production is chosen as a production technique. The composition, macrostructure and internal morphology of the scaffolds leading to improved mechanical performance and cell – material interactions can be adjusted by electrospinning technology. It also allows wide range of biopolymer spinning with desired properties. Therefore, solvent systems and electrospinning production parameters should be optimized as well, in order to produce continuous homogenous fibers at desired fiber diameters. In this context, second article is composed of electrospinning of polycaprolactone with various solvent systems. In this part of study, physical properties of prepared solutions and morphological analysis of electrospun PCL surfaces were analysed. The effect of polymer concentration and acidic solvent additions to mostly used chloroform solvent was investigated based on fiber morphology. FTIR analysis was done to investigate if there is any hazardous solvent in the surface after production or not.

The thesis continues with the third article including the modification of electrospinning apparatus for requirements of vascular graft productions. 6 mm

diameter mandrel type collector was used for small diameter vascular graft production. Feeding unit was modified with a pneumatic system to make reciprocal movement in order to create homogenous structure. Taking defined design parameters into consideration, radial fiber orientation was found obvious on outer layer of the scaffold design. In general, electrospinning technology is suitable for production of oriented fibers by modifying a collector as a rotating unit. However, it is not easy to reach such high speeds ($>10,000 \text{ min}^{-1}$) by using small diameter ($<6 \text{ mm}$) rotating mandrels. Therefore, a new empowering mechanism was designed for electrospinning apparatus, in order to rotate 6 mm diameter mandrel above limits of peripheral speeds stated in the first article. Rotary tool up to $35,000 \text{ min}^{-1}$ rotational speed capability was modified to the electrospinning apparatus to empower the rotating mandrel which enables fiber orientation in radial direction. All details with regards to the modification of custom designed electrospinning apparatus were given in the third article. Assessment of radial fiber orientation and its effects on mechanical and biological functions of vascular grafts were studied based on varied rotational speeds. Both randomly distributed and radially oriented single layer PCL scaffolds were produced. Fiber diameter distribution as well as pore size area and porosity analysis was examined to evaluate its suitability for cell proliferation and diffusion through the wall thickness of the scaffold. The relationship between the orientation of fibers and cells was confirmed in vitro by seeding 3T3 mouse fibroblasts. Moreover, third article involves the histological analysis of native vessel. To prove the hypothesis of mimicking native extra cellular matrix (ECM) blood vessel components, a histological investigation of the artery having the same internal diameter (6 mm) was also carried out in this article. This part of the thesis aims to constitute a considerable step forward in designing multilayer vascular graft.

To resemble the native vessel, multilayer scaffolds rather than single layer scaffolds are well ahead to gather all functions including morphological, structural, mechanical and biological in one design. Therefore, bilayer scaffolds were designed in the fourth article and last part of the thesis.

In the fourth article, both single layer and bilayer tubular scaffolds were electrospun from PCL with different molecular weights and PLC polymers. In the light of previous articles, bilayer scaffolds were designed by using different combinations of the polymer types in each layer and obtaining fiber orientation in outer layers. Scaffolds

were analysed morphologically and mechanically. Obtained results of mechanical performance were discussed according to the used polymer type composition, fiber orientation and composite effect of both layers in the final graft. Moreover, newly custom designed burst pressure measuring apparatus for vascular grafts' testing was used for the produced vascular grafts in this article. All technical data for the apparatus and testing principle were given in the article. Lastly, coronary artery smooth muscle cells were seeded on the scaffolds to test biocompatibility of presented scaffolds.

To sum up, from definition of design parameters to modification of production and testing technologies, this thesis brings novelty to the literature. Furthermore, with various designs and productions of single and double layer scaffolds with regards to prescribed design parameters and their morphological, structural, dimensional, mechanical and biological analysis, this thesis offers remarkable improvements of inadequencies of small diameter artificial blood vessels.

2. DESIGN PARAMETERS FOR ELECTROSPUN BIODEGRADABLE VASCULAR GRAFTS *

The inadequacies of the currently used small diameter, non-biodegradable synthetic grafts prompt researchers to focus on the design parameters of vascular grafts. Since the purpose is to mimic the native vessel as far as possible, the design parameters are mainly determined by the layout of cell types and proteins in the layers of the vessels and the nano and micro structure of their environments. In consequence of this, the complex structure of native vessels has become a broad source of inspiration for researchers. Electrospun fibrous scaffolds with their well accepted advantages are promising candidates and researchers are able to work with various materials with differing forms, structures, dimensions, and surface modifications according to their requirements. On the other hand, both synthetic biodegradable polymers and natural proteins are the key materials that enable researchers to take one step closer to achieving the goal of creating an autologous vessel at some time after implantation. When the priority and significance of the need for small diameter vascular grafts is considered, the research field to improve vascular grafts is worthy of note.

In this regard, the objective of this review is to discuss comparatively the current studies on the design parameters of electrospun vascular grafts, defined as fiber orientation, fiber diameter, pore size and porosity, wall thickness and material selection, based on the structure of native blood vessels, the requirements of vascular grafts and electrospinning technology, and the advantages of electrospun vascular grafts, to give an outlook for further studies.

Keywords: Vascular graft, electrospinning, biodegradable polymers, pore size, fiber orientation.

* This chapter is based on the paper “Yalcin Enis, I., and Sadikoglu Gök, T., 2016. Design Parameters for Electrospun Biodegradable Vascular Grafts, Journal of Industrial Textiles, 0(00), 1-23. (Published online in June 2016, DOI: 10.1177/1528083716654470)”

2.1 Introduction

As the world population is getting older, cardiovascular diseases are increasing correspondingly. Vascular graft implantations are promising operations for by-pass patients. Vascular grafts of polytetrafluorethylene (ePTFE), polyethylene terephthalate (PET) and polyurethane (PU) work well in the replacement of large diameter (>6 mm), high-flow vessels. However, they fail for small diameters, mainly due to the incompatibility of their radial elasticity, insufficient porosity and insufficient cell growth. Considering these requirements with the large number of patients in need of replacement grafts, the demand for an alternative small diameter graft is enormous and has driven scientists to search for new design ideas [1–3].

A native blood vessel is composed of three layers called the *tunica intima*, *tunica media* and *tunica adventitia*. Each layer has its own cell type and protein content, which facilitate the unprecedented functions of native blood vessels. On the other hand, vascular graft design is accepted as successful to the degree that it mimics the target tissue, as in all tissue designs. Therefore, the design steps should be based on mimicking the native blood vessels' structural, mechanical, biological and topographical properties [3].

In this review, after providing brief information about native blood vessels' histological and structural properties, the requirements for vascular grafts, the technology of electrospinning and the advantages of electrospun vascular grafts are introduced. The design parameters of biodegradable electrospun vascular grafts, mainly such as the fiber diameter, pore size and porosity, fiber orientation, wall thickness and material selection, are discussed in detail with recent literature works. It is intended that the blended and enriched context of this paper will help researchers to overcome the complex design problems of electrospun vascular grafts by providing them with new research areas.

2.2 Components of Blood Vessel

Both the pulmonary and systemic circulatory systems are made up of arteries, capillaries, and veins. Capillaries are the thinnest blood vessels with 5–10 μm diameters. These micro vessels are responsible for the connection of veins and arteries and they are also capable of exchanging water, oxygen, carbon dioxide, and other

chemical substances between the blood and surrounding tissues. Without the need for extra transport mechanisms, the single layer endothelium capillary wall allows both gas and lipophilic molecules to pass through [4]. Veins are the vessels that carry the blood from the capillaries to the heart, while arteries carry the blood from the heart to the body parts. Due to the low pressure in the veins, the transport of blood depends on nearby muscular constructions. Back-flow of blood is prevented by the valves in the veins. Unlike the capillaries, the walls of veins and arteries have three layers; *tunica intima*, *tunica media* and *tunica adventitia* (Figure 2.1) [4,5].

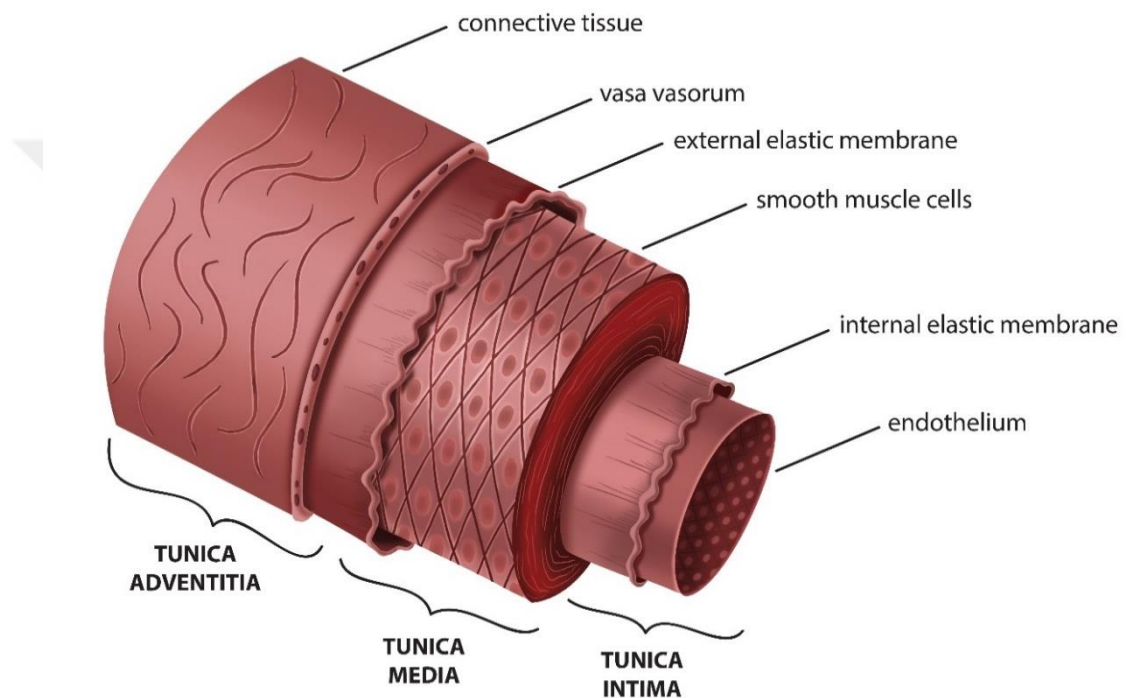


Figure 2.1 : Layers of an artery.

The *tunica intima* is the layer that is in contact with blood. This layer serves as a thrombo-resistant barrier to enable laminar blood flow through the vessel. It controls vessel tone, platelet activation, adhesion and aggregation and leukocyte adhesion [6,7]. It is composed of a single layer of endothelial cells (ECs) and sub-endothelial layers. The sub-endothelial layer is made up of collagen type IV and elastin [5]. An extra-cellular matrix (ECM) in the *tunica intima* also promotes diffusion, proliferation and growth of ECs [8]. The *tunica media* includes many layers of smooth muscle cells (SMCs) in a matrix of collagen types I and III, elastin, and proteoglycans [5]. Collagen bundles in this layer give mechanical resistance to the vessel, while elastin enables an elastic ability. The contracting and relaxing behaviors of arteries and veins are determined by glycoproteins and proteoglycans [9]. The *tunica adventitia* serves as a

protective sheet that consists of soft connective tissue mainly containing collagen type I mixed with elastin, nerves, and fibroblasts [5,6]. The protein structure of collagen and elastin assists the tensile performance and elasticity of the vessel while preventing it from vessel rupture and pulsatile deformation [5]. This layer consists of small vessels called *vasa vasorum* that nurture the arterial wall [10].

2.3 Requirements for Vascular Grafts

The main goal of an engineered tissue is to create living autologous neo tissues that can be used in place of damaged and diseased tissue and it should perform more like the native target tissue and less like a prosthetic material [11]. An ideal scaffold should reflect the biomechanical properties of native blood vessels, while serving as a topographically and structurally ideal backbone for cell attachment, proliferation and diffusion [6].

Mechanical properties are essential for vascular grafts, because the grafts must withstand blood pressure and match arterial compliance to avoid flow disturbance around the anastomosis [3]. Compliance, which is one of the most important mechanical properties for basic blood vessel function, is a measure of radial elasticity. A compliance mismatch between the graft and the native vessels is one of the leading causes of graft failure in both autologous and vascular grafts [12,13]. It can cause inconsistent blood flow profiles and flow stagnation. Unstable flow through the graft can lead to stress concentrations at the suture locations, causing intimal hyperplasia (IH) [14,15], thrombosis and even occlusion of the graft. Besides compliance performance, the graft should withstand in vivo stimuli including cyclic loading, shear stress, longitudinal stretch, circumferential stretch and stress concentrations from sutures as well as internal pressures [2,14,16,17].

Spiral blood flow is documented as a normal flow pattern for medium and large sized vessels, mostly along the inner walls of curved segments and the outer walls of arterial bifurcations. This flow enables washing of the smoother inner surface of the vessel by the blood so that atherosclerotic plaques cannot accumulate on the surface [18–20]. In small diameter vascular grafts, the blood flow rate is low and not enough to prohibit thrombus formation. Therefore, the replication of spiral flow in small diameter vascular grafts is used to solve acute occlusion due to thrombus formation [20].

Topographical features have direct effects on cell adhesion, alignment, migration, cytoskeleton protein production, and differentiation. Nano topography, rather than micro topography, should be ideal for vascular ECs since they naturally live in a nano environment. On the other hand, forming micro porous structures is another design parameter. Micro porous structure is necessary for cell migration and tissue ingrowth since the size of SMCs is larger than 5 μm [3]. In other words, the ideal structure of a vascular graft scaffold should have an extensive network of interconnecting pores so that cells can migrate, multiply and attach deep within the scaffolds [21].

In order to be used in surgery, the vascular grafts should be nonthrombogenic or resistant to blood clotting, nonimmunogenic, noncorrosive at body temperature and pH, and resistant to infection. They should also be biocompatible and hemocompatible [2]. Long term biostability is required for biodegradable grafts. As the biodegradable polymer degrades hydrolytically over time, cells continuously infiltrate the matrix, producing collagen, elastin, and proteoglycans to replace the degrading material. Eventually, a fully functional artery is created composed of autologous SMCs and ECs. Therefore, a long degradation time i.e., long term biostability, is one of the most crucial properties for biodegradable vascular grafts that provides a prolonged mechanical backbone for cells to infiltrate and a low inflammatory response [7,22]. Furthermore, the vascular grafts should have low manufacturing costs, be able to withstand some type of sterilization process and be available in different specifications (diameter, length, etc.) to fit the need for the implant. Other issues to be considered might be availability in urgent cases and ease of suturing [2,16,17,21,23].

2.4 Electrospun Vascular Grafts

Electrospinning has shown extreme potential in the last few decades, which has promoted its usage in tissue engineering applications owing to the great advantages of electrospun fibrous surfaces. In this context, the area of vascular grafts comes to light which enables the use of biopolymers and natural proteins to design biodegradable scaffolds via electrospinning technology [3].

2.4.1 Working principle of electrospinning

Electrospinning apparatus is mainly composed of three components: a high voltage supplier, a capillary tube with a tip and a collector [24]. Here, the capillary tube can

have either one tip or multiple tips. On the other hand, free surfaces can also be used as a feeding unit especially for mass production [25]. The collector can be chosen as a plate shape or rotating mandrels in varying diameters based on the end use [26,27]. Production can be carried out in the vertical or horizontal directions according to the design of the electrospinning establishment [26,28].

In this process, a polymer solution or melt is held by its surface tension at the tip of a capillary tube which is subjected to an electric field. Charge is induced on the solution/melt surface by the electric field. As the intensity of the electric field is increased, the hemispherical surface of the solution/melt at the tip of the capillary tube deforms to form a conical shape, which is named a Taylor cone. When the voltage increases and the electric field reaches a critical value at which the repulsive electrical forces overcome the surface tension forces, a charged jet of the solution/melt is ejected from the tip of the Taylor cone towards the collector. Since this jet is charged, its trajectory can be controlled by an electric field [24,29]. A straight section of the jet which comes out of the Taylor cone turns sideways and forms loops in the horizontal plane under the effect of bending instabilities [28]. The loop diameter increases with time during the motion towards the counter electrode, which is called a whipping action. During this process, the jet is highly stretched and reduced. This procedure is repeated until the solvent evaporates or the melt solidifies, leaving behind a discharged interconnected polymer fiber accumulated on the collector [24]. As a result of these instabilities, fibers with diameters down to a few nanometers can be generated [28].

Since electrospinning is based on a simple working principle, producing fibers with desired properties is more complex. Thus, it is necessary to optimize the solution parameters (viscosity, concentration, conductivity, surface tension, polarity), polymer properties (molecular weight, solubility, glass transition temperature), ambient parameters (temperature, relative humidity, vapor pressure) and production parameters (voltage, feeding rate, distance between needle tip and collector) to improve the spinnability and thus the morphology of the fibers [28,30].

2.4.2 Advantages of electrospun vascular grafts

Electrospun scaffolds are promising candidates for tissue engineered scaffolds i.e. vascular grafts providing a wide range of advantages [2].

Mimicking ECM is one of the advantages of electrospun vascular grafts owing to the nanotopography created by the nanofibers and interconnected pores, which results in a large surface area that promotes endothelium formation to prohibit arterial thrombosis. It is known that attachment and proliferation of ECs is better around fibers with diameters smaller than the cell diameter ($<5\text{ }\mu\text{m}$) [3]. On the other hand, the controllable pore size and porosity of electrospun scaffolds allow SMCs to grow, migrate, and regenerate through the scaffold via creating large spaces ($>5\text{ }\mu\text{m}$ to allow cell diffusion).

Beside designing spiral flow guiders [31,32] for small diameter vascular grafts to replicate spiral flow and avoid thrombus formation, the surfaces of electrospun vascular grafts can be modified with protein lining, heparin incorporation, or polymer surface chemistry, which reduces thrombosis and IH while promoting the endothelium [3].

Moreover, electrospinning technology allows the design of the desired scaffolds either separately or in combinations of one scaffold with multilayers. Therefore, by designing composite scaffolds based on the variable material selection, flexibility in modification of the production technology and adjustment of the production parameters, it is possible to tune the mechanical compliance, material degradation, and surface properties according to the needs of tissue regeneration [3,12].

2.4.3 Design parameters for electrospun vascular grafts

When producing electrospun vascular grafts, the design parameters must be well understood in order to mimic the native blood vessels. In the last decade, there have been many studies about electrospun vascular grafts which focus on these design parameters. Although it is necessary to handle all the design parameters as a whole, it is essential to discuss them separately to understand the current situation of the literature and overcome any deficiencies.

2.4.3.1 Fiber diameter, pore size and porosity

The pore size is one the most important parameters for vascular grafts. The pores must allow the infiltration of SMCs while hindering blood leakage [33,34]. Since nanofibrous surfaces serve as a useful means for cell attachment with high porosity and small pore sizes, they pose an obstacle for SMC diffusion. To overcome this

challenge, several techniques were used to achieve dual porosity in either multilayer [33–36] or single-layer [37] vascular grafts.

One of the best known ways of controlling the pore size of the electrospun scaffold is to change the fiber diameter. In a study by Yalcin Enis et al. [38] the effect of additional acids (acetic acid and formic acid) on the most used polycaprolactone (PCL) (45,000 Mn) /chloroform:ethanol solution was investigated based on fiber diameter and pore size analysis. According to the results, 2.22 μm diameter micro fibers were produced from 18% PCL /chloroform:ethanol solution. With the addition of pre-determined amounts of acetic acid and formic acid to the same solution, fiber diameters were reduced to 158 nm and 256 nm for each acid type. Moreover, the average pore area was also reduced almost 50 times (from 10 μm^2 to 0.2 μm^2) based on fiber diameter decrement, i.e. additions of acidic solvent [38].

In three complementary studies by Bagherzadeh et al. [39–41], PCL (80,000 Mn) was dissolved in chloroform:methanol, (3:1) solvent system at 10–20% polymer concentrations [39,40], and in chloroform:DMF (4:1) solvent system at 10–14% polymer concentrations [41] to examine the pore size and porosity of the scaffolds with several experimental and developed theoretical techniques. Beside a good agreement that was obtained from two experimental techniques, namely the capillary flow porometry and confocal laser scanning microscopy (CLSM) methods [39,40], a theoretical method developed by the authors was validated by these experimental techniques and compared with accepted models to determine the average pore size and pore size distribution of multilayer nanofibrous scaffolds for tissue engineering applications [41]. It was also confirmed by Bagherzadeh et al. [39–41] that with an increasing concentration of polymer solution, the fiber diameter as well as the pore size, porosity, and pore interconnectivity increase significantly. Similar to the outcomes of Yalcin et al. [38], these results also showed that the fiber diameter of fibrous scaffolds is a crucial structural parameter that can determine the pore structure of fibrous scaffolds [39–41].

In parallel with these outcomes, Ju et al. [33] produced an electrospun bilayer vascular graft from PCL/collagen mixture composed of different pore sizes. In order to vary the pore size, the fiber diameter was altered by changing the solution and production parameters. Both SMCs and ECs were seeded to the scaffolds in order to evaluate the cellular interactions based on fiber diameters, i.e. pore sizes. The produced bilayer

vascular graft was composed of a nanofibrous inner layer (0.27 μ m fiber diameter) and microfibrinous outer layer (4.45 μ m fiber diameter). As the outcome of the study, a nanofibrous layer with nano pore sizes served as an improved surface for endothelial cell attachment. On the other hand, the 1 μ m fiber diameter was determined as a sub limit in the outer layer for smooth muscle cells infiltration [33]. Similarly, De Valence et al. [34] produced electrospun bilayer grafts with a fibrous structure of low porosity in either the inner or outer layer. It was stated that the inner layer with lower porosity reduced blood leakage while promoting cell penetration from the outer layer (through higher porosity). On the other hand, cell invasion and neovascularization were significantly reduced in grafts composed of lower porosity structure in the outer layer (Figure 2.2) [34].

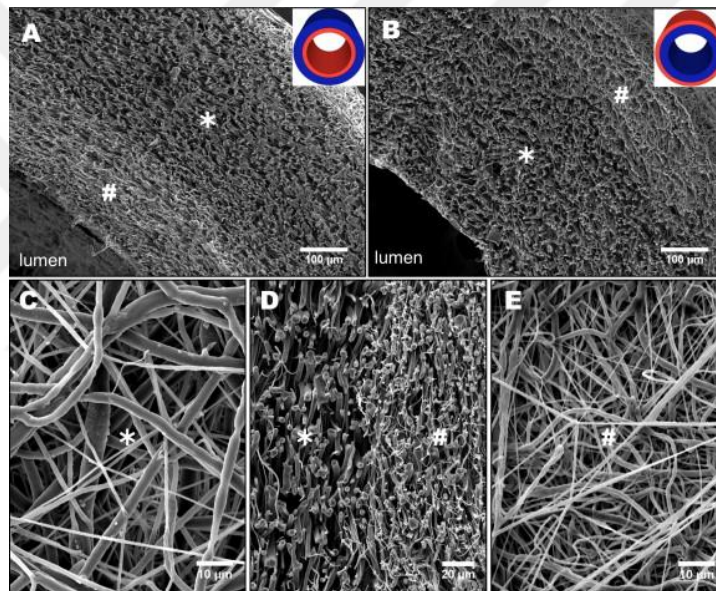


Figure 2.2 : Vascular graft structures. Two scaffolds with different porosities were electrospun sequentially to form bilayered vascular grafts. The inside-barrier graft (A) has a high-porosity layer on the adventitial side (*, C) and a low-porosity layer on the luminal side (#, E). The outside-barrier graft (B) was constructed inversely. The transition between the two layers was continuous, with no delamination (D).
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In a study by Wang et al. [35], 25% and 10% PCL (80,000 Mn) were dissolved in chloroform: methanol (5:1 v:v) to produce nano and micro fibers with dimensions 0.69 μ m and 5.59 μ m. The average pore size and average porosity of these fibrous layers were measured as 4.66 μ m/65% and 40.88 μ m/85% respectively. It is well understood that the thicker fibers lead to a larger pore size and higher porosities. And it is stated that larger pores allow enhanced cell invasion and vascularization in both in vivo and in vitro conditions [35]. It is also supported by Brauker et al. [36] that

neovascularization is promoted by allowing cell penetration even with the pore size of the graft ranges between 0.8 μm and 8 μm . Implantation of grafts with 5 μm pore size under the skin of rats led to almost 100 times more vascular structures compared to 0.02 μm pore sized structures [36]. For the same purpose but from a different point of view, Lee et al. [37] produced three-dimensional montmorillonite-reinforced poly-L-lactic acid (PLLA) scaffolds. Nanofibrous scaffolds were produced by the electrospinning technique and then micro sized pores were achieved in nanofibrous webs by a salt leaching/gas forming technique. For the transportation of metabolic nutrient and wastes nanosized pores (100–200 nm) and for facile cell growth and blood vessel invasion micro sized (50–300 μm) pores were developed in a single layer graft. As a conclusion, dual porosity was found to be essential to meet the demands of vascular grafts [37].

Therefore, all the reported studies [33–37] placed excessive emphasis on micro sized pores especially for SMCs' invasion. Since it is not stated that a nano scaled pore size is a must for endothelialization, its advantages in hindering blood leakage [34] and allowing a fluent monolayer of ECs on lumina [42] make their use worthy of note when designing a multilayer graft.

2.4.3.2 Fiber orientation

Fiber orientation is another important parameter when designing a vascular graft. Especially the radial fiber orientation achieved in tubular scaffolds is required for enhancing the diffusion of SMCs and improving the mechanical strength against radial pulsatile pressure. There are several studies about methods such as increasing the rotational speed of the collector [43–47] or using metal plates [48], which focus on how to improve the radial orientation for tubular scaffolds.

Matsuda et al. [43] produced a tubular electrospun scaffold for small diameter vascular grafts from polyurethane (PU) polymer. In order to obtain the fiber orientation, 150 min^{-1} and 3,400 min^{-1} rotational speeds (1.41 $\text{m}\cdot\text{min}^{-1}$ and 32.02 $\text{m}\cdot\text{min}^{-1}$ peripheral speeds) were used for a 3 mm rotating mandrel. At 3,400 min^{-1} , partial fiber orientation was achieved. The increased fiber orientation in the radial manner improved the circumferential tensile strength while decreasing the longitudinal tensile strength. However, lower compliance was achieved with the improved radial fiber orientation [43]. On the other hand, a small diameter PCL vascular graft was fabricated by Hu et

al. [44]. To investigate the fiber orientation and drum speed relation in detail, five peripheral speeds (240 m.min^{-1} , 320.4 m.min^{-1} , 400.2 m.min^{-1} , 480 m.min^{-1} , 640.8 m.min^{-1}) were used. For their use in small diameter vascular grafts, membranes produced at 80 m.min^{-1} , 320.4 m.min^{-1} and 480 m.min^{-1} peripheral speeds were wrapped on a small diameter rod with varied angles. The final grafts were prepared from randomly oriented, moderately and highly aligned fibrous membranes. The results demonstrated that the alignment index (AI), i.e. fiber orientation, increased with the increased peripheral speeds (from 240 m.min^{-1} to 640 m.min^{-1}). The mechanical behavior of the scaffolds was found to be under the influence of fiber orientation. It was also stated that fibroblasts were aligned with the oriented fibers, indicating the potential to control the microstructure of electrospun scaffolds (Figure 2.3) [44].

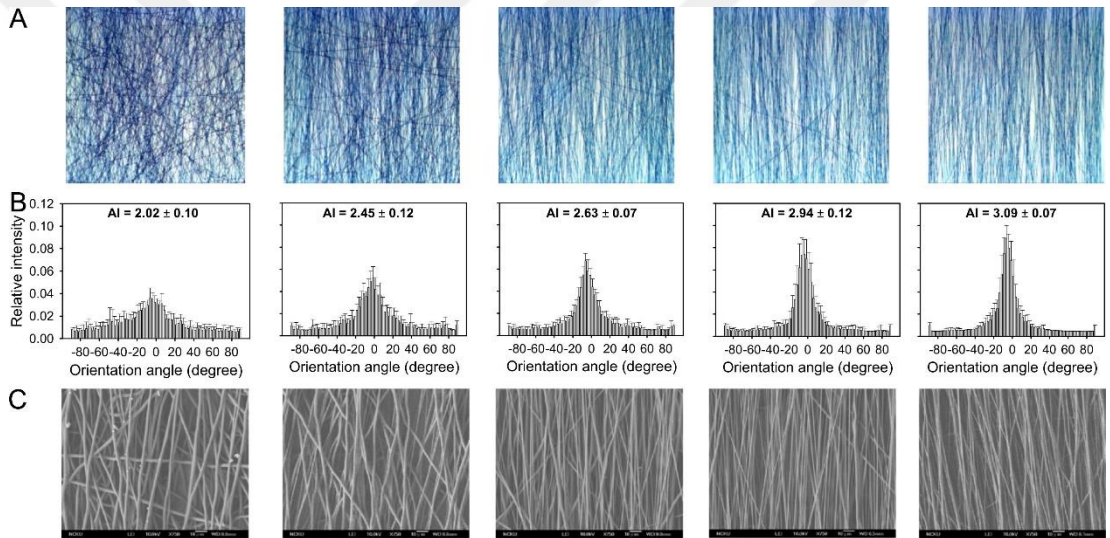


Figure 2.3 : Representative optical microscopy images of electrospun PCL fibers collected at different drum rotation speeds (a), the corresponding distributions of fiber orientations and the alignment indices (peripheral speeds from left to right: 240 m.min^{-1} , 320.4 m.min^{-1} , 400.2 m.min^{-1} , 480 m.min^{-1} , 640.8 m.min^{-1}) (b), and the corresponding SEM images (c). Reproduced from [44] with permission from Elsevier.

By using a rotating mandrel (32 mm in diameter) type collector, a similar study was realized by Edwards et al. [45]. In this study, the peripheral velocity of the collector was varied from 0– 480 m.min^{-1} to investigate PCL fiber orientations. It was reported that there is no fiber orientation up to 120 m.min^{-1} peripheral velocity since the fibers are not exposed to mechanical stress. Above this limit, there is a reduction in fiber diameters that is explained by the existence of mechanical stress. The optimum fiber orientation was achieved at 150 m.min^{-1} , while it rose to a maximum level at 300

m.min⁻¹. Above 300 m.min⁻¹, mechanical deformation was observed on the fibers because of the high mechanical stress and affected the fiber orientation in a negative way [45]. In a study by Chaparro et al. [46], randomly distributed PCL fibers were spun onto a 53 mm diameter steel rod rotated at 9000 min⁻¹ (net peripheral speed of 84.6 m.min⁻¹), while aligned PCL fibers were spun onto a rotating mandrel having a 2.01 m circumference (diameter: 640 mm), rotated at 500 min⁻¹ (providing a peripheral speed of 2000 m.min⁻¹) [46].

The above studies show that, although high rotational speeds improve the fiber orientation, it is not easy to reach such high speeds (>10,000 min⁻¹) by using small diameter (<6 mm) rotating mandrels. Therefore, most of the studies use large diameter collectors (630 mm [42], 102 mm [44], 32 mm [45], and 640 mm [46]) for accessible rotational speeds. Matsuda et al. [43] reported that they achieved partial fiber orientation at 32.02 m.min⁻¹ for a 3 mm rotating mandrel diameter which had been explained by Edwards et al. [45] as having an insufficient peripheral velocity. Moreover, the type of polymer should be taken into consideration when optimizing the speed for fiber orientation.

To overcome the speed limitations, Yalcin et al. [47] produced a PCL (45,000 Mn) vascular graft by a custom-designed electrospinning unit modified with a rotating mandrel powered by a high-speed motor. Radial fiber orientation was achieved by varying the rotational speed (250–5000–10000–15000 min⁻¹) of a 6 mm diameter rotating mandrel. Fiber orientation was achieved at 5000 min⁻¹ (94.2 m.min⁻¹) and 10000 min⁻¹ (188 m.min⁻¹). The prevailing fiber orientation in the radial direction was improved with the increasing rotational speed up to 15000 min⁻¹ (282.6 m.min⁻¹) [47]. In order to obtain radial fiber orientation, peripheral velocities were found in the range of previously confirmed values (150 m.min⁻¹ to 300 m.min⁻¹ by Edwards et al. [45]). No fiber orientation is observed at 250 min⁻¹ (4.71 m.min⁻¹) [47] since the fibers are not exposed to a notable extension force at less than 120 m.min⁻¹ speed [45]. On the other hand, elongated cell shapes were observed, which suggests that cells tend to orient themselves with the fibers in the radial direction. The increase in the nuclei elongation ratios, which were calculated as 1.22, 1.75, 1.87, 1.94 for 250, 5000, 10000, and 15000 min⁻¹, confirmed the cell orientation along the fibers (Figure 2.4) [47].

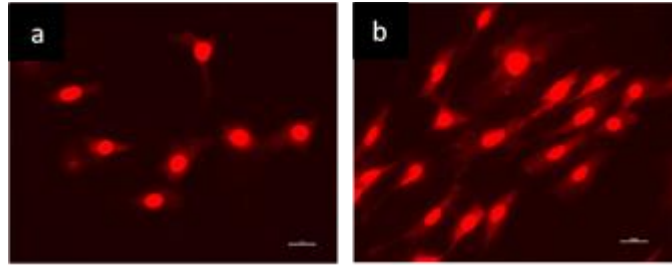


Figure 2.4 : Fluorescence microscopy pictures of 3T3 mouse fibroblasts nuclei stained with propidium iodide after 1 day of culturing anisotropic structure (4.71 m.min^{-1}) (a), and isotropic structure (188 m.min^{-1}) (b). Scale bars $25 \mu\text{m}$. Reprinted from [47].

Modification of the electrospinning unit with metal plates to improve the fiber orientations of electrospun small diameter vascular grafts was studied by Wu et al. [48]. A 4.5 mm rotating mandrel was chosen for small diameter vascular graft production while 50 min^{-1} and 300 min^{-1} rotational speeds (0.7 m.min^{-1} and 4.2 m.min^{-1} peripheral speeds) were used for the longitudinal and radial fiber orientations respectively. For both orientations the electrospinning unit was supported by directionally positioned metal plates. The results demonstrated that by using metal plates it is possible to achieve fiber orientations in both directions. Although cell attachment was observed in both oriented and non-oriented PCL scaffolds, it was noted that the oriented fibers facilitated and regulated the cell attachment. Directional mechanical properties were achieved by using oriented fibers. This study concluded that designing a multilayer vascular graft consisting of both longitudinal and radial oriented fibers was found to be ideal for improved mechanical performance and cell response [48]. As discussed in [44] and [45], 300 min^{-1} is insufficient for a 4.5 mm collector diameter (4.2 m.min^{-1} peripheral speeds) to develop the fiber orientation radially. However, Wu et al. stated that with the modification of the electrospinning with directionally positioned metal plates, excellent radial fiber orientations can be obtained [48].

2.4.3.3 Wall thickness

Beside the mentioned morphological parameters such as fiber diameter, pore size and fiber orientations, the wall thickness of the scaffold as a dimensional property is also deterministic on the mechanical and biological properties of the vascular grafts. If the other production and solution parameters are kept constant, the production time is directly affected by the wall thickness [47]. It is possible to obtain a homogeneous wall

thickness during electrospinning by the reciprocal movement of either the feeding unit [47] or the collector mechanism [49]. On the other hand, the ease of removing the polymeric scaffold in tubular form from the mandrel after production enables the graft's inner diameter to be fixed as the same as the collector diameter.

Yalcin et al. [47] carried out a histological analysis of native artery when designing an electrospun vascular graft. The wall thickness of the analyzed native artery was measured in a range of 400 to 1000 μm along its length. Moreover, detailed information about the structural properties of the native blood vessel was given in the study. Microscopic images reveal that ECs are arranged through a single layer covering the luminal surface, whereas SMCs are organized into numerous layers inside the *tunica media*. Elastin, which gives the vessel elasticity, was found densely in the *lamina elastica interna*, as well as the *tunica media* and the *tunica adventitia*. The experimental results were also proven by the literature [22,50–54]. It was also observed that fibrous collagen bundles oriented in a radial manner appear in micrometer scales (Figure 2.5) [47].

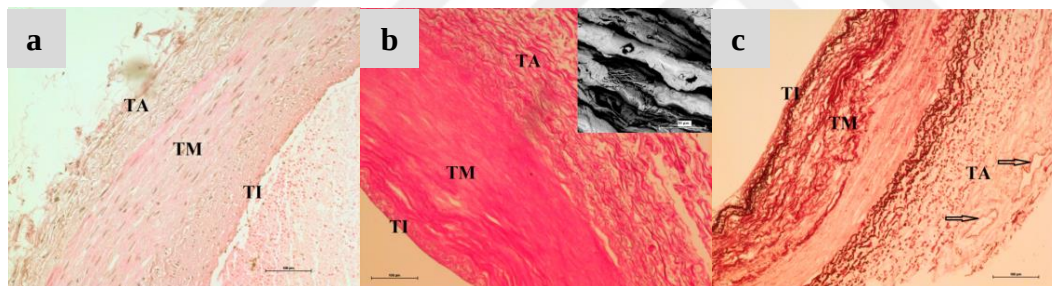


Figure 2.5 : Hematoxylin-eosin staining (a), collagen (b), and elastin (c) in the *tunica intima* (TI), *tunica media* (TM), and *tunica adventitia* (TA). Scale bars: 100 μm . Collagen nanofibers arranged in microfibrillar bundles are depicted in SEM picture intercalated in (b), scale bar: 10 μm . Black arrows in picture (c) indicate *vasa vasorum* in the *tunica adventitia*. Reprinted from [47].

In a study by Inoguchi et al. [55] electrospun poly (L-Lactid-co- ϵ -caprolactone) PLCL vascular grafts with a thinner wall thickness demonstrated improved compliance properties. On the other hand, the outcomes of Johnson et al.'s study [56] indicate that the compliance of PCL tubes remains steady within a range of 2–4% / mmHg when the wall thicknesses range between 400 μm and 1000 μm . However, the thinner PCL grafts exhibit a large variance in compliance measurements. Moreover, when the wall thickness increases from 200 to 1000 μm , the burst pressure values increase in a linear fashion from 0.6 MPa to 2.9 MPa [56]. In the study by Thijssen et al. [57], the impact

of the wall thickness on vascular responsiveness was investigated. The conduit diameter, wall thickness and wall to lumen ratio (W: L) of the arteries taken from the lower and upper limbs of healthy men were examined. The results indicated the regional heterogeneity is present between arteries. Especially small diameter arteries had higher W:L ratios than large diameter arteries. It was concluded that this regional heterogeneity can influence the vasoactive stimuli [57]. Variation of the W:L ratio within a single artery was also demonstrated by Bjarnegard et al. [58].

2.4.3.4 Material selection

Since electrospinning enables a wide range of polymer selection for production, electrospun vascular grafts can be produced from nonbiodegradable synthetic polymers (polyurethane [35], polyethylene terephthalate [59]), biodegradable synthetic polymers (polycaprolactone [47,53,60], polylactic acid (PLA) [53], poly(glycerol sebacate) (PGS) [61, 62], poly glycolic acid (PGA) [63]), structural proteins (collagen [64, 65], elastin [66], fibrin [67], silk fibroin [68,69]) and their mixtures [60,70,71] or copolymers [22,72,73].

Compared to natural polymers, synthetic biodegradable polymers generally demonstrate better mechanical properties with high reproducibility. PCL, PLA, PGS and PGA are the polymers approved by the Food and Drug Administration (FDA, USA) for human clinical use [17,21,74].

PGA is a semicrystalline, thermoplastic aliphatic polyester synthesized by the ring-opening polymerization of glycolide, which degrades completely in vivo within 6 months [17]. PLA is a thermoplastic aliphatic polyester synthesized by ring-opening polymerization of lactic acid which exists in two isoforms: poly-L-lactic acid (PLLA) and poly-D-lactic acid (PDLA) [74,75]. The racemic mixture is termed poly-L,D-lactic acid (PDLLA) or (PLA). PLLA is semicrystalline (~37%) while PDLLA is amorphous [74]. The degradation rate for PLLA fibrous scaffold is 42 days, whereas the PLA scaffold is shrunk significantly after only 3 days [75]. PLA shows good biocompatibility and mechanical properties [74]. PLA in scaffolds is mostly in copolymers because of its low degradation rate [75]. PCL is a semicrystalline, aliphatic polyester synthesized by the ring-opening polymerization of ϵ -caprolactone. PCL has improved elasticity that closely matches native tissue. PCL has good biocompatibility and its degradation rate is very slow in vivo (within almost 1 year) [17]. PGS is an

elastomer synthesized by polycondensation of glycerol and sebacic acid. It has good biocompatibility, with elastomeric and tough behavior. PGS degrades in vivo by hydrolysis in 2 months [17,74]. To match the native tissue properties, many researchers focus on improving the inadequate mechanical properties and enhancing the biocompatibility of the scaffolds by either blending these polymers or using their copolymers.

Lee et al. [60] produced small diameter vascular grafts by electrospinning from collagen/elastin alone and a mixture of 45% collagen, 15% elastin and 40% biodegradable synthetic polymers (PLLA, PLGA, PCL and PLCL), which was stated as the real ratio of native blood vessels. According to the results, although the collagen/elastin scaffolds degraded in a few days after production, PCL was found to be the best for 28 day patency levels. On the other hand, the lowest mechanical strength was obtained for scaffolds of PCL mixtures. SMCs were seeded on the scaffolds and in vitro test results indicated that all the scaffolds are biocompatible and promote cell response (Figure 2.6) [60].

Wise et al. [70] produced PCL/elastin vascular grafts by an electrospinning unit. The increase in elastin content improved the cell growth. On the other hand, the increase in the PCL ratio resulted in better mechanical performance [70]. Lee et al. [71] fabricated a vascular graft composed of PCL and collagen by electrospinning. A 520 ± 14 nm fiber diameter and $22.7 \pm 9.6 \mu\text{m}^2$ pore area were achieved from PCL/collagen (1:1) composite scaffold. PCL vascular grafts were produced as control scaffolds. The burst pressure, compliance and yield strength of the PCL/collagen (4915 ± 155 mmHg, 5.6 ± 1.6 % and 4.0 ± 0.4 MPa respectively) were improved as compared to PCL scaffolds (914 ± 130 mmHg, 3.5 ± 1.4 % and 1.5 ± 0.7 MPa respectively). Although there was a decrease in the suture retention strength of the PCL/collagen scaffold, the results were found to be above the sub-limit, which is generally accepted as 2 N. Both ECs and SMCs had good adhesion to the PCL/collagen scaffolds [71].

In a study by He et al. [72], a vascular graft composed of P(LLA-CL70:30) copolymer was fabricated. Collagen coating was done to the surface of the scaffold. The tensile strengths of the scaffolds were found to be 7.0 MPa and 3.9 MPa in the longitudinal (LD) and circumferential directions (CD). The tensile strength of commercial Teflon grafts is stated as 85.2 MPa in LD and 66.7 MPa in CD, while the tensile strength of the abdominal aorta is stated as 1.47 MPa in LD and 5.29 MPa in CD. The in vitro

biodegradation results indicate that the electrospun P(LLA-CL) scaffolds did not lose their structural integrity until 8 months later. After 8 months, most of the nanofibers broke into small segments of fibers. When the scaffold was implanted in a rabbit, 7 weeks patency was observed (Figure 2.7) [72].

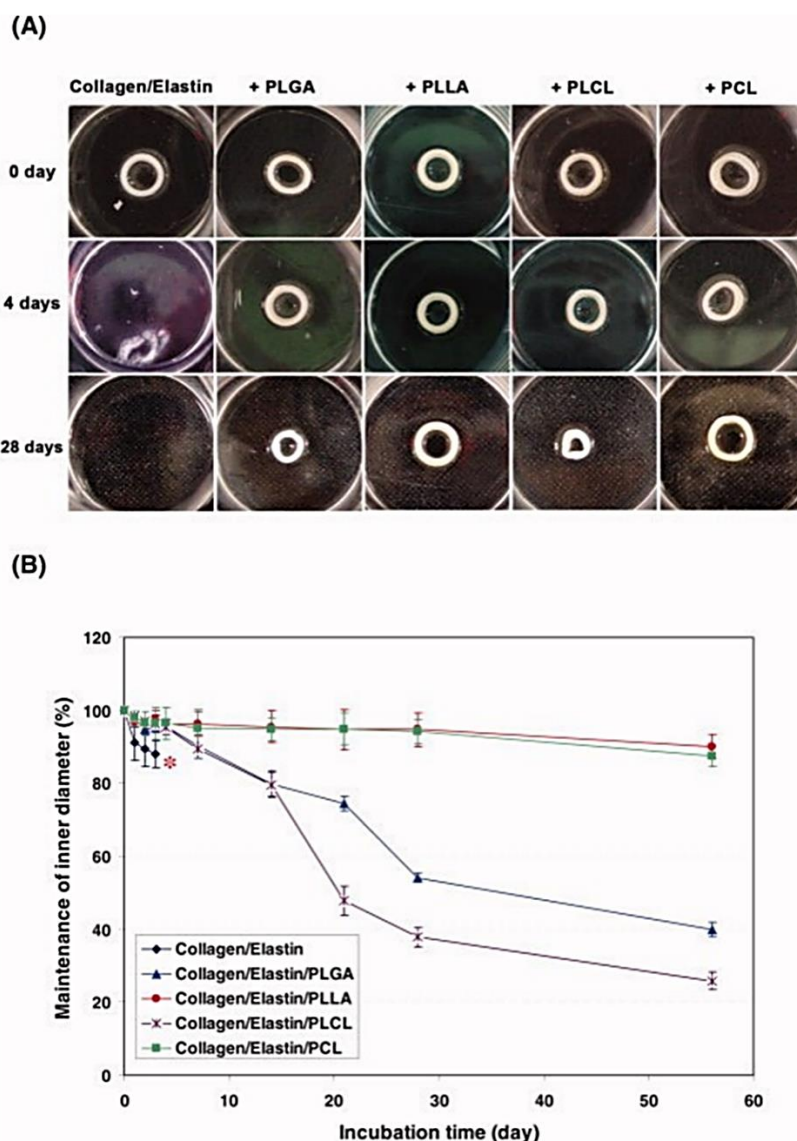


Figure 2.6 : Dimensional stability of the electrospun scaffolds. (A) Gross observation and (B) percentage of remaining diameters of the electrospun nanofiber scaffolds in culture condition. The scaffolds were immersed in culture medium at 37°C for 56 days. *The electrospun collagen/elastin scaffold without synthetic polymer was collapsed after four days incubation. Reprinted from [60] with permission from Wiley Periodicals, Inc.

Similarly, Dong et al. [53] produced a vascular graft from P(LLA-CL 70:30). Mainly, the long term cell viability and degradation of the scaffold were studied. According to the results, the molecular weight of the P(LLA-CL) nanofibers (average fiber diameter

580 nm) decreased sharply after 60 days. The estimation realized in the study showed that after 230 days, P(LLA-CL) nanofibers will completely degrade [53].

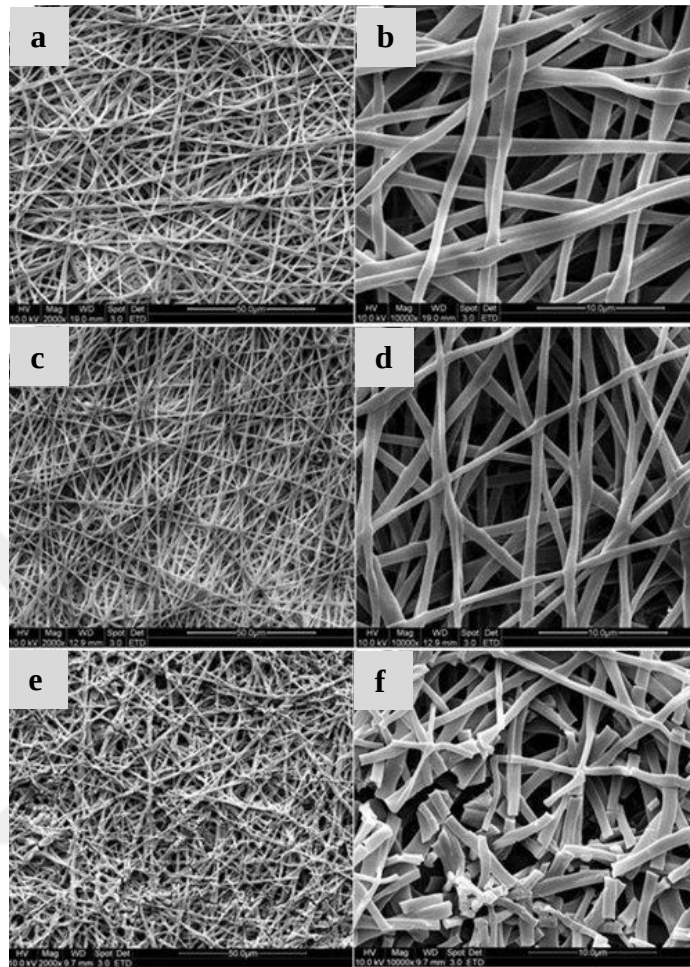


Figure 2.7 : SEM images showing morphology of the P (LLA-CL) nanofiber meshes biodegraded in $1 \times$ PBS at 37°C for (a and b) 0 month, (c and d) 3 months, and (e and f) 8 months. (b, d, and f) are magnified images of (a, c, and e), respectively.
Reprinted from [72] with permission from Wiley Periodicals, Inc.

Kim et al. [73] produced a small diameter vascular graft from PLGA copolymer and modified it with stem cells. Sufficient mechanical properties together with better cell growth were achieved. However, shortly after implanting the graft into a dog, arterial occlusion was observed. The results revealed the difficulty of using PLGA biodegradable polymer in vascular grafts [73]. Del Gaudio et al. [76] fabricated electrospun PCL vascular grafts using both DMF: THF (1:1) and chloroform solvents. The resultant fiber diameters were $0.8 \pm 0.2 \mu\text{m}$ and $3.6 \pm 0.8 \mu\text{m}$ respectively. Human umbilical vein endothelial cells were seeded to the scaffolds. Although sufficient cell adhesion was achieved in both nano and micro scaled fibers, the viability of the cells was better in the micro scaled fibers, with improved cell–fiber interactions [76].

Pfeiffer et al. [42] produced three-layer grafts from different polymer blends. In their first design, the inner and outer layer were produced from a PCL/PLA blend with 1.61–1.43 μm fiber diameter and 5.8–5.3 μm pore size. In the second design, the inner and outer layers were produced from PCL/PLA/polyethylene glycol (PEG) blend with 1.76–1.63 μm fiber diameter and 5.8–6.7 μm pore size. Between the two layers, PCL was used as a barrier layer. Endothelialization was observed in a comparison of ePTFE grafts. The morphological results indicate that only a few cells with a round shape were seen on the native ePTFE grafts, whereas some clusters with partly spreading cells could be observed on the PCL/PLA scaffolds. The best results were obtained on PCL/PLA/PEG grafts where the endothelial cells showed good adherence and many cell-to-cell contacts (Figure 2.8) [42].

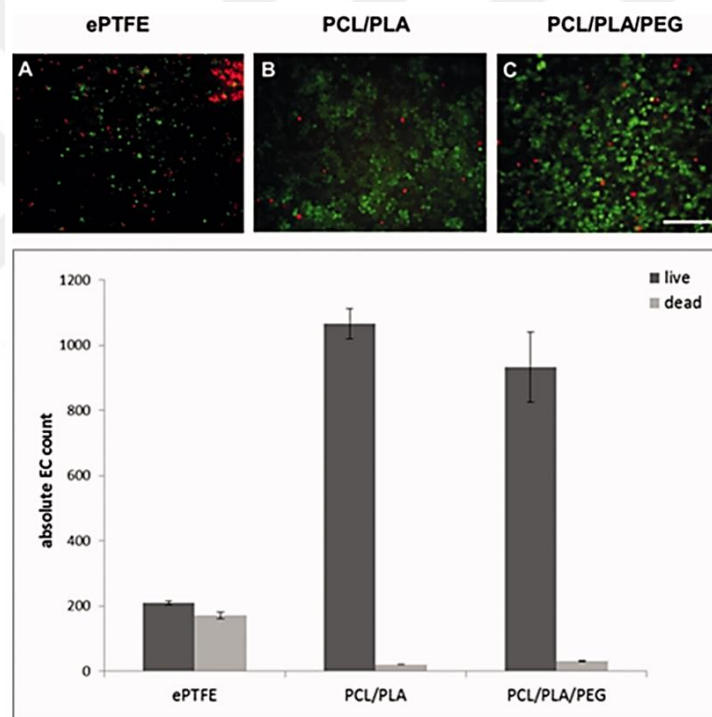


Figure 2.8 : Live/dead analysis of different endothelialized vascular grafts after fibronectin coating: Cells were stained with CalceinAM (green fluorescence) and Annexin V (red fluorescence) to distinguish live (green) from dead cells (red). Numerous dead cells were observed on ePTFE (A) grafts. In contrast, PCL/PLA (B) and PCL/PLA/PEG (C) grafts only sporadically showed apoptotic cells. Scale bar 200 μm . Viability counting of endothelial cells revealed lower attachment and viability of endothelial cells on fibronectin-coated ePTFE compared to PCL/PLA and PCL/PLA/PEG grafts. Reprinted from [42] with permission from Wiley Periodicals, Inc.

Wu et al. [62] designed cell-free biodegradable elastomeric grafts that degrade rapidly to yield neo-artries that are nearly free of foreign materials three months after

interposition grafting in rat abdominal aorta. The bilayer vascular graft is composed of two polymers; a PGS inner and PCL outer layer. After three months of implantation, the neo-arteries composed of PGS and PCL allowed ECs and SMCs substitution *in vivo* respectively, as the polymeric scaffold degraded. It is also stated that elastin, collagen and glycosaminoglycan are synthesized and that tough and compliant mechanical properties were achieved [62].

In a study by Tillman et al. [77], the biomechanical strength of PCL (inherent viscosity: 1.77 dL/g):collagen type I derived from calf skin (1:1) tubular scaffolds fabricated by electrospinning was investigated. The *in vivo* mechanical characterization of the scaffolds indicated that the tensile strength of the grafts measured before implantation was measured at around 1.7 MPa and decreased to 0.9 MPa at one month post-implantation. The resultant tensile strength after implantation was found to be still comparable to native rabbit aorta, which demonstrates that these scaffolds maintain an adequate degree of biomechanical strength in *in vivo* conditions [77]. Similarly, the pre-implant and post-implant mechanical performances of bilayer grafts composed of synthetic elastin inner layer and PCL ($M_n = 70,000 - 90,000$) outer layer were compared with each other by Wise et al. [78]. The results indicated that the outer diameters of the pre-implant and post-implant grafts were unchanged. Improvements of compliance were found in the post-implant grafts, while the burst pressure decreased slightly based on the expected degradation of PCL. All the results were comparable with the control samples, which demonstrates the mechanical integrity of bilayer grafts in *in vivo* conditions [78].

Based on these studies, it is claimed that the long-term patency levels of PCL were found to be better than PLGA, PLLA, and PLCL biodegradable scaffolds, while PCL has the lowest mechanical strength [60], and the compliance of PCL vascular grafts is higher than commercial artificial blood vessels [62]. When PCL was used together with structural proteins it improved the mechanical strength [70], and the use of different solvents resulted in both nano and micro scaled fibers [38,76], whereas better cell growth was achieved in micro scaled fibers [76]. PCL:collagen [77] and SE:PCL [78] vascular grafts demonstrated comparable biomechanical performance in *in vivo* conditions. On the other hand, PLA, PLLA and PLGA polymers with improved mechanical strength are mostly preferred in vascular graft designs [60]. However, these polymers show fast biodegradation after implantation, thus they show poor long-

term patency levels [73], which can therefore cause crucial handicaps when these polymers are used alone in the structure.

2.5 Conclusion

Electrospinning, providing a wide range of polymer selection and allowing various scaffolds in differing shapes and structures, is a promising technique for the production of fibrous vascular grafts. Biodegradable electrospun fibrous scaffolds have many advantages when mimicking the native vessel based on the resemblance to ECM, while behaving like a backbone for the cells during their proliferation and synthesis of their own proteins.

It can be considered from the above discussed studies that researchers tend to focus on electrospun multilayer scaffolds for vascular grafts by either varying the materials or using different pore sizes and fiber orientations in the layers. Thus, the histology of the native blood vessel has a crucial significance when determining these design parameters. To draw general boundaries for future attempts at vascular graft design, it can be said that the *tunica intima* should promote endothelialization to achieve nonthrombogenicity. The pore size of this layer should be limited to blood cell dimensions in order to hinder blood leakage. Nanofibrous layers with nano pores can be preferred for this layer, which can also be modified with heparin bonding to prohibit platelet activation. Against the *tunica intima*, the *tunica media* should have larger pore sizes than smooth muscle cells in order to provide a porous layer for their diffusion through the wall thickness. Therefore, the layer with micro fibers can be electrospun, including larger pores that enable the diffusion of SMC through the *tunica media*. Besides adjusting the fiber diameter to control the pore size, there are other techniques for electrospun fibrous scaffolds, such as the gas forming/salt leaching technique that creates larger pores to allow cell diffusion. Since the *tunica media* is the layer responsible for the mechanical performance of the vessel, improved fiber orientation is required in this layer for sufficient tensile properties in order to withstand the blood pressure after implantation. By adjusting the rotational speeds of the rotating mandrel in the light of the sub-limits determined in the literature, extreme radial fiber orientation can be achieved. Moreover, positioned metal plates can be used to modify the collector system of the electrospinning technology, which improves the radial fiber orientation. However, the reduced compliance properties with improved radial fiber

orientation should also be taken into consideration. In this respect, the wall thickness and the selection of material also become important parameters to obtain adequate tensile strength with improved radial elasticity to overcome compliance mismatch of the scaffold with the sewn vessel. Moreover, the degradation rate of the polymer should be studied for multilayer biodegradable fibrous scaffolds in order to determine the minimum time that is required for biodegradable scaffolds to allow cells to synthesize their own proteins before the biological degradation of the scaffold. Therefore, the final objectives of designing a vascular graft should be the degradation of the scaffold after a sufficient time of implantation by leaving its place to an autologous vessel which is similar to native vessel.

This review compiles knowledge of the electrospinning technology with its flexibility in modifications to create ideal scaffolds for vascular grafts by exploiting the advantages of electrospun biodegradable scaffolds as “*autologous vessels*”. The aim is that the blended and enriched context of this review will enable researchers to overcome the complex design problems of electrospun vascular grafts by encouraging them to explore new research areas.

2.6 Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

2.7 Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

3. ALTERNATIVE SOLVENT SYSTEMS FOR POLYCAPROLACTONE NANOWEBS VIA ELECTROSPINNING *

In this study, polycaprolactone (PCL) was dissolved in 9: 1 chloroform: ethanol mixture at 14%, 16%, 18% and 20% w/v concentrations. Then, acetic acid and formic acid were added at pre-determined amounts to 18% PCL/chloroform: ethanol solution system separately. Before production, viscosity and conductivity of prepared solutions were measured. Electrospinning technique was used for fabrication of fibrous webs. Morphology of produced webs was observed under a scanning electron microscope (SEM) while fiber diameter measurements and pore analysis were realized via SEM images by using Image J Software System. The effect of polymer concentration and acidic solvent additions to mostly used chloroform solvent was investigated based on fiber morphology. Results indicate that the increase in polymer concentration increases the fiber diameter which leads to larger average pore area. Fiber produced from 16% to 20% polymer concentrations in chloroform: ethanol solvent system results in micro fibers. On the other hand, fiber diameter reduce from microscale to nanoscale with the addition of either acetic or formic acid. Fibers produced from PCL/chloroform: ethanol solution at 18% polymer concentration have 2.22 μm average fiber diameter whereas 158 nm and 256 nm diameter fibers were successfully produced without bead like structure by 120 μl of acetic and formic acid additions to the same solution system.

Keywords: polycaprolactone, acetic acid, formic acid, chloroform, electrospinning.

3.1 Introduction

In tissue engineering, it is accepted to be successful to the degree that it mimics the target tissue [3]. Based on the dimensional similarity of natural extracellular matrix

* This chapter is based on the paper “Yalcin Enis, I., Vojtech J., and Sadikoglu Gök, T., 2016. Alternative Solvent Systems For Polycaprolactone Nanowebs via Electrospinning, Journal of Industrial Textiles, 0(00), 1-14. (Published online in February 2016, DOI: 10.1177/1528083716634032)”

[ECM], nanofibrous scaffolds are inevitable environments which promote cell attachment and proliferation [79-81]. A variety of techniques have been proposed to produce scaffolds from biomaterials, one of which is the electrospinning [82,83]. Electrospinning is a technique to create continuous fibrous layers within ranging fiber diameters from few nanometers to some micrometers under electrical field [69,84]. Vascular grafts are the scaffolds where electrospun fibrous webs are nominated as candidates for single layer or multilayer designs. Among several design strategies, fiber diameter and pore size of fibrous scaffolds are one of the most determinant parameters for tissue designs; i.e. multilayer vascular grafts. Since blood vessels are composed of layered structures, pore size distribution of layers differ from each other based on cell type content and their functions in related layers. Pore size of the scaffold should enable smooth muscle cell diffusion in *tunica media* (the middle layer of native blood vessel) while hindering blood leakage in *tunica intima* (the inner layer of native blood vessel) [5,7,8,33]. Therefore, the improved spinnability of biomaterials with differing characteristics according to desired requirements lead up design flexibility in tissue designs. Biocompatibility, long term biostability after implantation and rubbery properties are the preference reasons of polycaprolactone (PCL) to be used in electrospun vascular grafts [85,86]. Both polymer concentration and the corresponding solvent identity can be used to manipulate the final physical properties of electrospun PCL to create vascular scaffolds targeting the specifications such like fiber diameters and pore size of the desired application [87-89]. Since, there are many solvents for PCL such like methylene chloride and dimethyl form amide, chloroform is the one that is mostly preferred for electrospinning of polycaprolactone due to its less hazardous properties especially for medical and tissue engineering applications [90,91]. However, it is known that, only PCL microfibers around 2–5 μm diameters [47,90,91] can be achieved from electrospinning of PCL/chloroform solution. For nanofibrous PCL webs, alternative solvent systems are required. Acetic acid and formic acid were used as solvents for PCL alone or in a mixture, as an alternative solvent systems for chloroform to produce nanofibers [85]. In this study, chloroform was used as main solvent and mixed alternately with acetic and formic acid in various amounts to find an optimum solvent system for PCL nanofibrous webs that can be used in tissue engineered designs, i.e. multilayer vascular grafts.

3.2 Materials and Methods

3.2.1 Solution preparation

Polycaprolactone (PCL, Mn 45000, Sigma-Aldrich, St. Louis, MO) was dissolved in chloroform: ethanol (9: 1 v: v, Sigma-Aldrich) at concentrations of 14-16-18-20% w/v solution. On the other side, 20 μ l, 40 μ l, 80 μ l and 120 μ l of formic acid (FA, 99%, Sigma Aldrich) and acetic acid (AA, 99.98% Sigma Aldrich) were added separately to the prepared 18% w/v PCL/chloroform: ethanol solutions. Based on its optimum viscosity that leads to continuous production in electrospinning process, PCL 18% was chosen for further analysis. All of the solvents are for analytical research and no further purifications are done before usage. Each solution was prepared 10 ml in order to compare the effects of additional acids. Lastly, 18% w/v PCL/AA and PCL/FA solutions were prepared. All solutions were stirred for 8 hours and then immediately electrospun in order to hinder PCL degradation [47]. Solutions with sample codes and details were listed in Table 3.1.

Table 3.1 : Sample codes and details.

Sample codes	PCL/ chloroform: ethanol (9:1 - v:v) [% w/v]	FA addition [μ l]	AA addition [μ l]	FA [% w/v]	AA [% w/v]
C14	14				
C16	16				
C18	18	-	-	-	-
C20	20				
C18FA20	18	20			
C18FA40	18	40			
C18FA80	18	80	-	-	-
C18FA120	18	120			
C18AA20	18		20		
C18AA40	18		40		
C18AA80	18	-	80	-	-
C18AA120	18		120		
FA18	-	-	-	18	-
AA18	-	-	-	-	18

The reason to add ethanol to chloroform is to retard the evaporation rate of chloroform and improve the spinnability of PCL [47]. Components of solvent system used in this work were listed in Table 3.2 with some of their properties.

Table 3.2 : Solvent system components and their properties (Sigma Aldrich).

Components	Chemical Formula	Molecular Weight [g/mol]	Boiling Point [°C]	Density [g/mL] at 25°C	Solubility Parameters [MPa ^{1/2}]	Dielectric Constant [-]	Dipole Moment [D]
Chloroform	CHCl ₃	119.38	60.5-61.5	1.480	19	4.8	1.04
Ethanol	CH ₃ CH ₂ OH	46.07	78	0.789	26.5	25	1.69
Acetic Acid	CH ₃ CO ₂ H	60.05	117-118	1.049	21.4	6.2	1.74
Formic Acid	HCOOH	46.03	100-101	1.220	24.9	58	1.41

3.2.2 Electrospinning

The basic electrospinning apparatus (Inovenso, Turkey, Figure 3.1) was used in horizontal feeding direction. Distance between the needle tip and the collector was adjusted to 20 cm and needle diameter was chosen as 0.8 mm. Feed rate, voltage and production time were varied in ranges of 0.4-1 ml/h, 12-15 kV and 10-20 min respectively based on used solvent systems. All samples were produced under laboratory conditions.



Figure 3.1 : Electrospinning apparatus.

3.2.3 Solution characterization

Solution viscosities were determined by a HAAKE RotoVisco 1 from Thermo Scientific paired with Rheowin 4 Job and Data Manager Software. Measurements were realized at 24°C with a RV1 spindle at 10 rpm to 4000 rpm for 90 second.

Conductivity of each solution was measured by electrical conductivity meter (Chromservis, Eutech Instruments, CON 510) which has a glass probe suitable for solutions used in this work.

3.2.4 Morphological characterization of fibrous webs

Morphology of the fibrous scaffolds was observed under ZEISS EVA, MO10 scanning electron microscope after sputter coating with gold alloy. Average fiber diameters were measured from at least 100 fibers for each of 5 SEM images (standard deviations are added in bar graphs as error bars) while pore areas and porosities were analysed based on thresholding technique by Image J Software System (version 1.48) from 5 SEM images (average values $\pm$ standard deviations are given). Thresholding technique is based on the principle of separating the pixels which fall in defined range from those which do not. A grayscale image is converted to binary Black & White colour by defining a grayscale cut off point. Grayscale values below the cut off become black and those above become white. Thresholding is commonly used when detecting edges, counting particles or measuring pore areas [92,93].

FTIR analysis was realized using Perkin Elmer, Spectrum Two Model FTIR to observe if there is residual solvent in the fibrous webs after production. IR spectrum of C18 and C18AA20 were analysed in fibrous webs and compared with the measured IR spectrum of chloroform solvent and PCL granule.

3.3 Results

3.3.1 Solution characterization

Solution conductivity is mainly determined by the polymer type, solvent sort, and the additives [94]. Conductivity of solvents used in this study was measured as 0.00 $\mu\text{S/m}$, 0.00 $\mu\text{S/m}$, 0.65 $\mu\text{S/m}$ and 230 $\mu\text{S/m}$ respectively for chloroform, acetic acid, ethanol and formic acid. It is known from literature [95] that pure acetic acid has very low polarity thus it is nonconductive unless it is mixed with water or other conductive additives. On the other hand, formic acid is defined as one of the most polar acids [85]. The electrical conductivity and viscosity of 18% PCL dissolved in pure AA and FA solvents were measured as 0.00 $\mu\text{S/m}$ and 88.9 $\mu\text{S/m}$; 0.142 Pa.s and 0.041 Pa.s respectively. The spinnabilities of 18% PCL/AA and PCL/FA solutions were so poor that no fibrous webs were achieved via electrospinning of these solutions.

It is clear from Figure 3.2 that viscosity is under influence of polymer concentration. The increase in polymer concentration results in polymer viscosity increment

[89,94,96]. On the other hand, conductivity of these samples was measured as 0.00 $\mu\text{S/m}$; so do pure chloroform solvent.

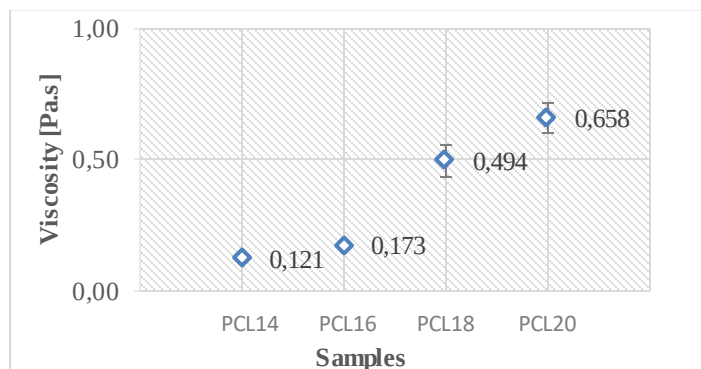


Figure 3.2 : Viscosity change as a function of polymer concentrations.

Since the addition of acetic acid does not vary the conductivity of the chloroform solution (because both their conductivities were measured as 0.00 $\mu\text{S/m}$), formic acid addition increases the conductivity of the chloroform solutions (Figure 3.3 (a)) [94]. On the other hand, there is no clear effect of additional acids on viscosity of PCL/chloroform solutions were observed in this study (Figure 3.3 (b)).

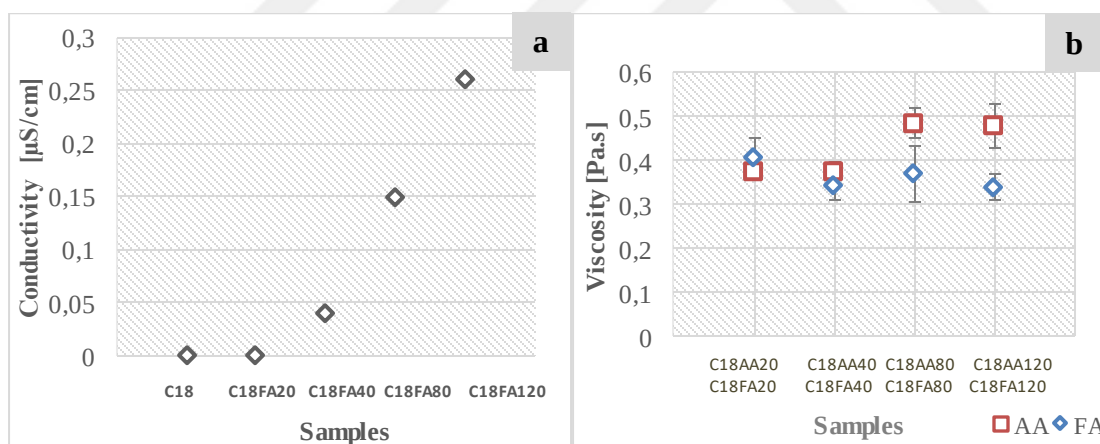


Figure 3.3 : Effects of additional (a) formic acid on conductivity, (b) acetic and formic acids on viscosity of C18 solution.

3.3.2 Morphological analysis

SEM images of fibrous webs including varied polymer concentrations (14-20%) of PCL/chloroform: ethanol solutions are illustrated in Figure 3.4 while Figure 3.5 and 3.6 show the images of AA additions and FA additions respectively to PCL/chloroform: ethanol solutions.

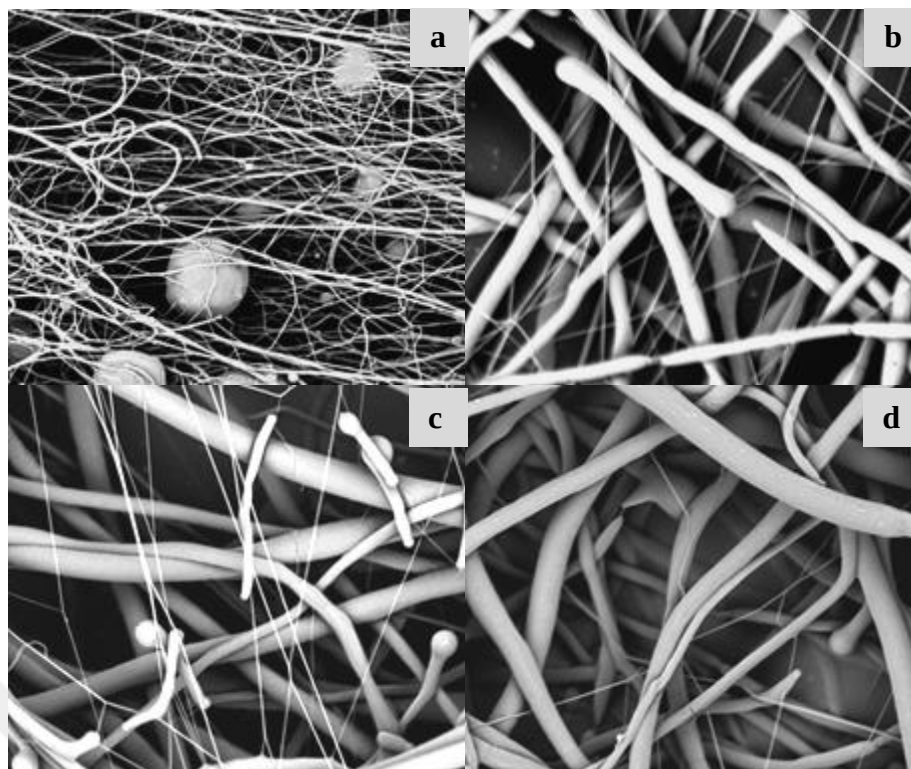


Figure 3.4 : SEM images of (a) C14, (b) C16, (c) C18, (d) C20 (Magnification 5000X).

Although C14 has continuous nano fibrous structure, large beads were observed in SEM images (Figure 3.4 (a)). In all other samples, micro fibrous structures were achieved (Figure 3.4 (b) to (d)). In Figure 3.5 (a), (b) and Figure 3.6 (a), (b) nanofibers and microfibers were observed together. However, Figure 3.5 (c), (d) and Figure 3.6 (c), (d) indicate confluent nanofibrous structures.

No fibrous web formation was achieved from 18% PCL/AA and PCL/FA solutions. It can be resulted from too low viscosities of the solutions [89]. It is also studied in the literature that there is no steady state conditions for PCL/pure formic acid or PCL/pure acetic acid solutions which may result in PCL degradation [85]. The reason is explained with highly polar formic acid and poorly polar acetic acid, both of which lead to unstable electrospinning process. Therefore mixture of these two acids [85] or addition of conductive additives like salt [97, 98] to acetic acid is suggested as good solutions to improve the spinnability of PCL in acids.

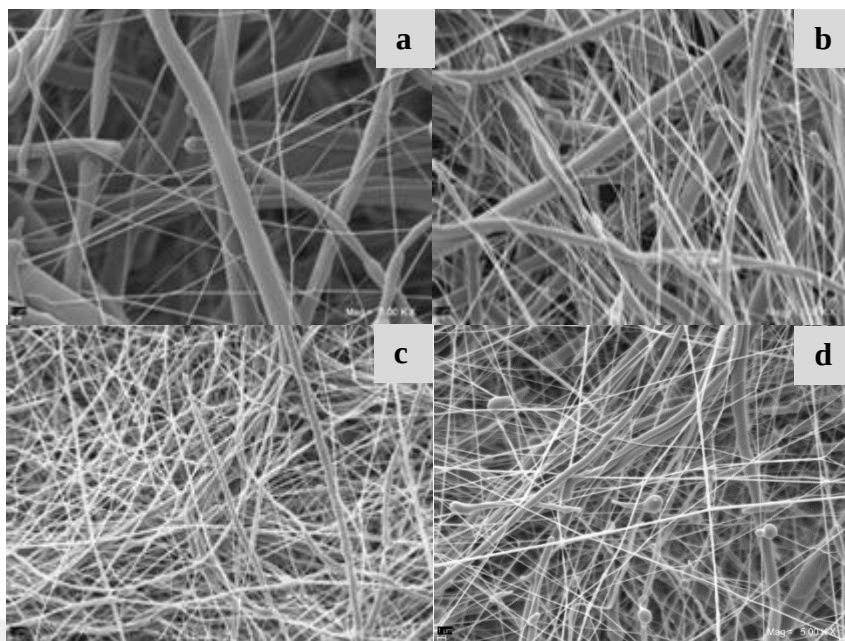


Figure 3.5 : SEM images of (a) C18AA20, (b) C18AA40, (c) C18AA80, (d) C18AA120 (Magnification 5000X).

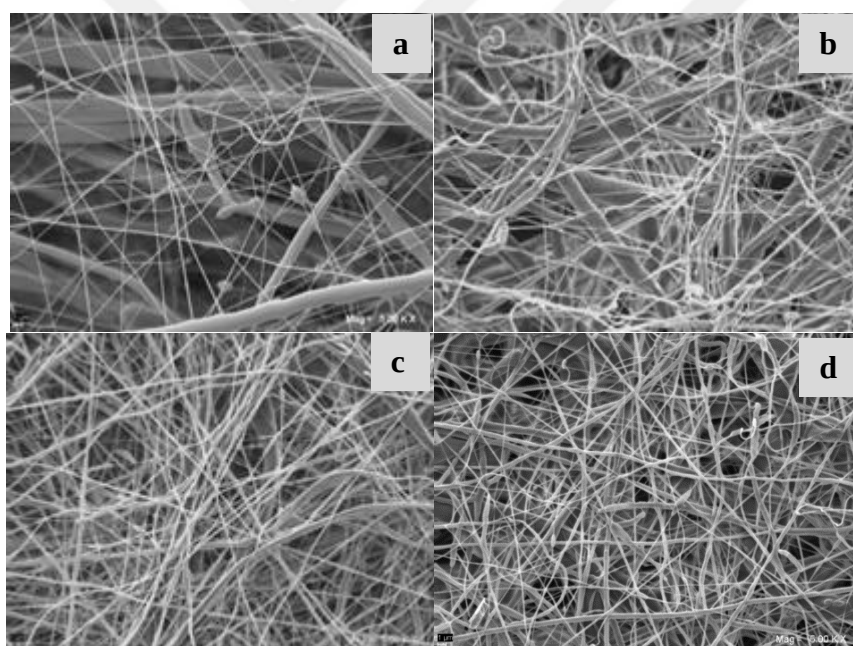


Figure 3.6 : SEM images of (a) C18FA20, (b) C18FA40, (c) C18FA80, (d) C18FA120 (Magnification 5000X).

3.3.3 Image J analysis

3.3.3.1 Fiber diameter

Fibrous webs produced from chloroform: ethanol solvent system (Figure 3.4) are composed of micro fibers except C14 (Figure 3.7). Although C14 has nano scale fibers, large beads were observed in morphological analysis (Figure 3.4 (a)). Figure 3.4 also

indicates that, average fiber diameter increases with increasing PCL concentration [96,99]. Moreover high standard deviation was observed which states there are different fiber diameter groups in the samples.

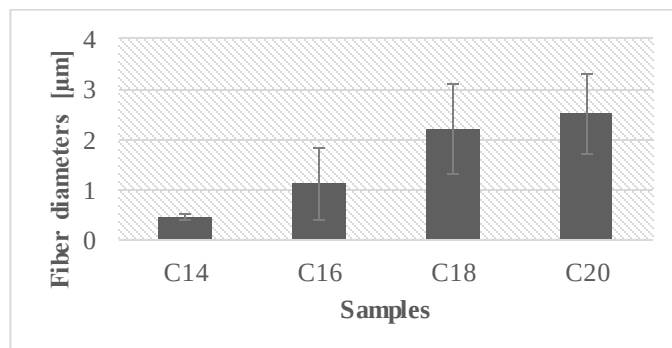


Figure 3.7 : Fiber diameters of C14, C16, C18 and C20.

Figure 3.8 shows the fiber diameters of C18 and its acidic solvent added samples. Results indicate that, the addition of both acetic and formic acid causes a decrease in fiber diameter. Although 20 μ l and 40 μ l acid addition have appreciable but not so respectable effects on fiber diameter due to its high standard deviations, 80 μ l and 120 μ l result in distinct drop of fiber diameters with lower standard deviations. Samples of C18AA80 and C18AA120 show the smallest average fiber diameters. (196 nm; 158 nm). Since there is no clear change in viscosities of the solution after acid additions were observed (Figure 3.3(b)), increasing conductivity by adding formic acid (Figure 3.3(a)) and decreasing surface tension with acetic acid concentration as reported in the literature [100] might be the reason for reducing fiber diameters.

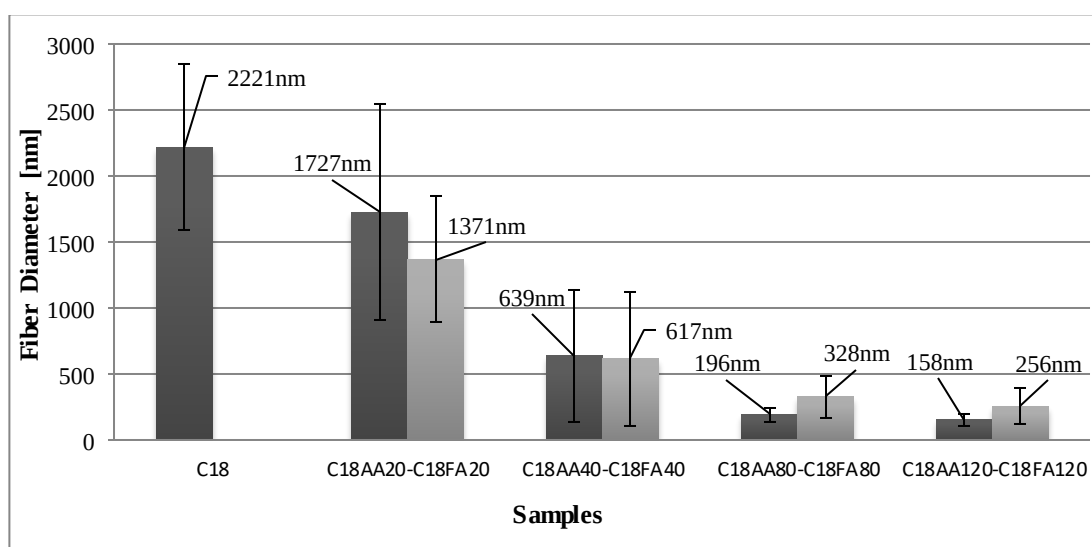


Figure 3.8 : Fiber diameters of the samples.

On the other hand, it can be noted from SEM images of C18AA20, C18FA20, C18AA40 and C18FA40 that there are different fiber diameter groups in the structure (Figure 3.5(a) and (b); Figure 3.6(a) and (b)). Image J analysis results also prove these visuals. Average fiber diameters were measured as 1.72 μm and 1.37 μm respectively for C18AA20 and C18FA20. On the other hand, it is clear from SEM images that these samples are composed of both nano and micro fibers which results in high deviation of average values. So that, while discussing the average fiber diameters mentioned in Figure 8, this must be taken into account.

According to Figure 3.9, C18AA20 has both nano and micro fibers with an average of 196 nm and 2.05 μm diameters while C18FA20 contents both nano and micro fibers with an average of 153 nm and 1.92 μm diameters. Similar results were achieved for samples C18AA40 and C18FA40 which are composed of 246 nm/1.57 μm and 160 nm/1.16 μm diameter fibers. Reason of these two grouped fiber diameter distribution can be the poor effect of acid solvent.

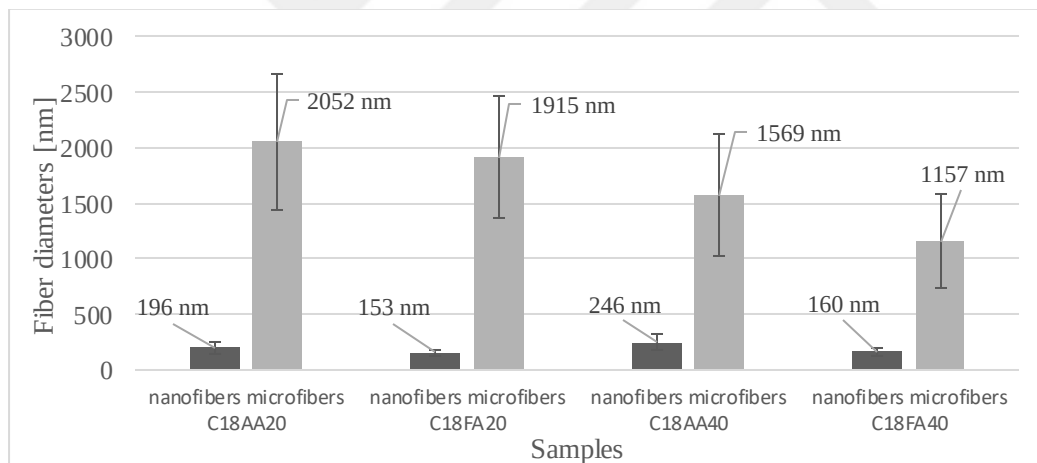


Figure 3.9 : Fiber diameter distribution for C18AA20, C18FA20, C18AA40, C18FA40.

3.3.3.2 Pore area and porosity

SEM images and their thresholdings of samples used for Image J pore area and porosity analysis are illustrated in Figure 3.10 and 3.11. Average pore area and porosity test results can be found at Table 3.3.

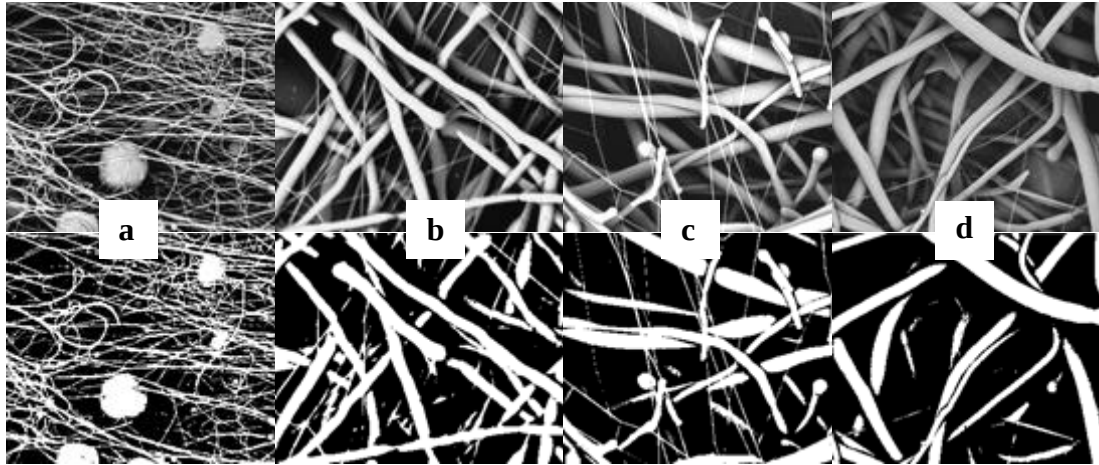


Figure 3.10 : SEM images (Magnification 5000X) and thresholdings of (a) C14, (b) C16, (c) C18, (d) C20 for Image J Analysis.

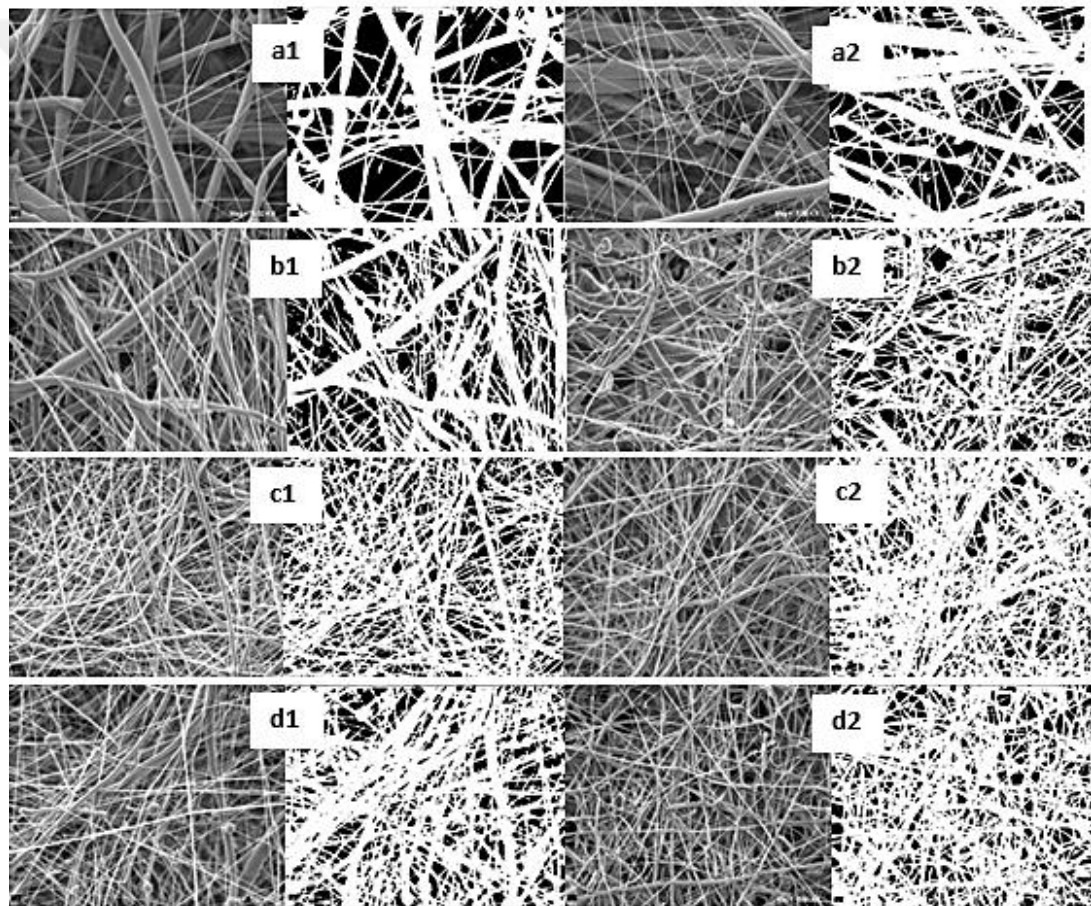


Figure 3.11 : SEM images (Magnification 5000X) and thresholdings of (a1) to (d1): C18AA20, C18AA40, C18AA80, C18AA120; (a2) to (d2): C18FA20, C18FA40, C18FA80, C18FA120 for Image J Analysis.

According to the data given in Table 3.3, it can be said that, pore area is mostly under the influence of fiber diameters [33]. The evaluated pore area for sample C18 is around $10\mu\text{m}^2$ but it reduce almost 50 times for samples C18AA80, C18AA120, C18FA80 and C18FA120 to around $0.2\mu\text{m}^2$ based on fiber diameter decrement i.e. acidic solvent

additions. Porosity has a tendency of increase in a similar way of pore area. Since this is a 2D software analysis based on thresholding technique, porosity values are calculated from surface of the SEM image which results in higher porosities in larger diameter fibers.

Table 3.3 : Average pore area and porosity results and their standard deviations.

Sample codes	Average pore area $\pm$ SD [μm^2]	Porosity $\pm$ SD [%]
C14	0.38 $\pm$ 0.02	33.82 $\pm$ 2.09
C16	5.74 $\pm$ 1.56	44.20 $\pm$ 3.04
C18	10.21 $\pm$ 5.12	56.69 $\pm$ 2.63
C20	20.38 $\pm$ 4.01	56.82 $\pm$ 4.74
C18FA20	1.21 $\pm$ 0.19	33.84 $\pm$ 2.01
C18FA40	0.34 $\pm$ 0.02	31.52 $\pm$ 0.05
C18FA80	0.23 $\pm$ 0.03	20.60 $\pm$ 1.69
C18FA120	0.26 $\pm$ 0.03	23.49 $\pm$ 1.18
C18AA20	2.58 $\pm$ 0.35	42.52 $\pm$ 6.05
C18AA40	0.38 $\pm$ 0.01	23.83 $\pm$ 1.02
C18AA80	0.21 $\pm$ 0.04	24.20 $\pm$ 0.09
C18AA120	0.29 $\pm$ 0.02	24.34 $\pm$ 0.02

3.3.4 FTIR analysis

FTIR analysis graphs are given in Figure 3.12.

According to Figure 3.12(a), C18 shows very similar peak characteristic with PCL granules differing from chloroform solvent. In Figure 3.12(b), C18 and C18AA120 show similar behaviours which can be accepted as there is no notable residual acetic acid solvent in C18AA120. When Figure 3.12(a) and (b) are compared to each other, none characteristic peaks of chloroform (3034 cm^{-1} /CH stretch, 1220 cm^{-1} / CH wag, 774 cm^{-1} and 680 cm^{-1} / CCl_3 stretches) were found in both C18 and C18AA120 fibrous webs [101].

3.4 Conclusion

In this study, electrospun webs including microfibers were produced from PCL/chloroform: ethanol solution the average fiber diameter of which is $2.22\text{ }\mu\text{m}$. On the other hand, since no fibrous webs were achieved when PCL was dissolved in pure acids, the addition of any acetic or formic acid solvents to PCL/chloroform: ethanol solution caused a notable drop in fiber diameters (for AA up to 158 nm , for FA up to 256 nm).

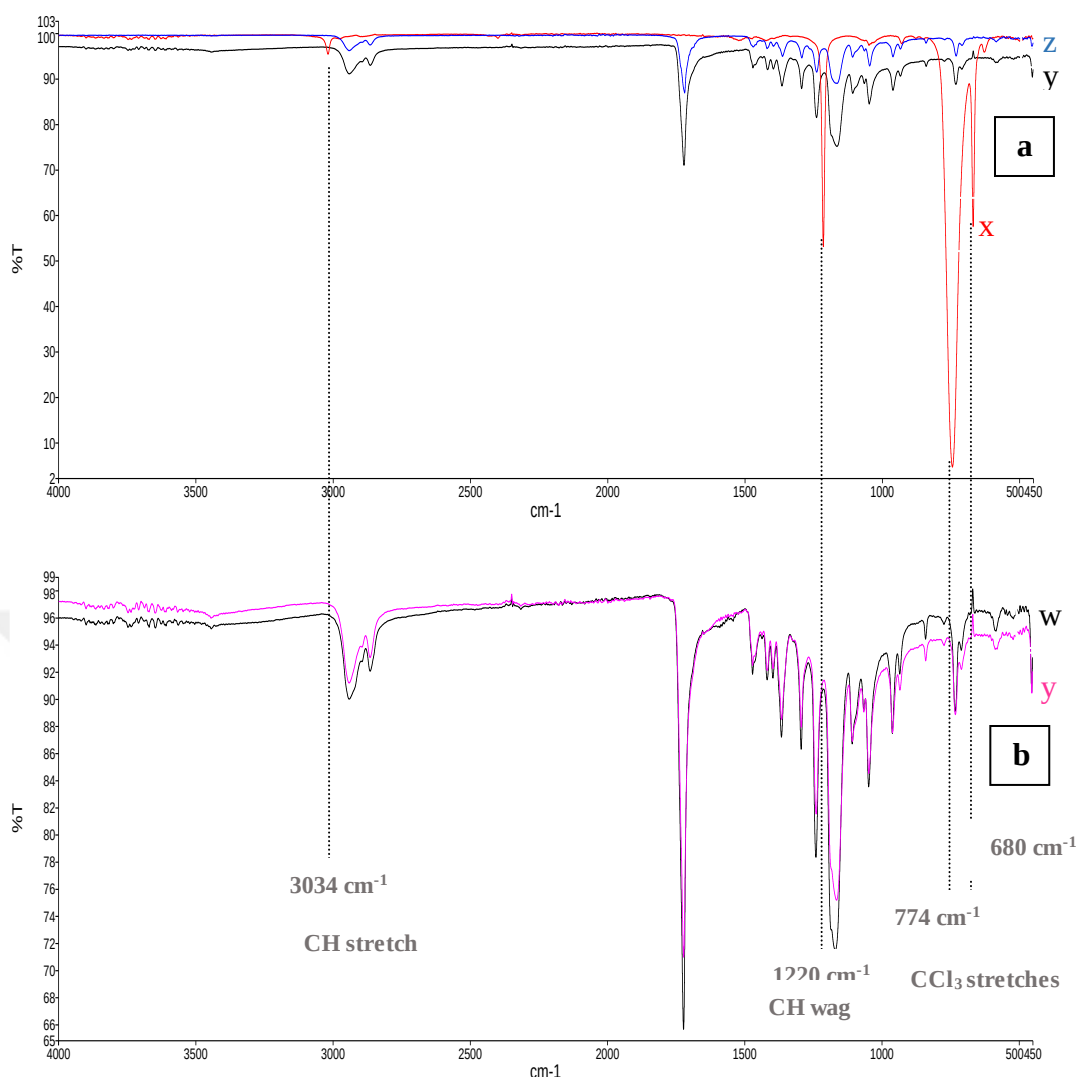


Figure 3.12 : FTIR analysis of (a) chloroform solvent (x), C18 fibrous web (y) and PCL granule (z) and (b) C18AA120 fibrous web (w) and C18 fibrous web (y).

Although chloroform has already accepted solvent for medical use of PCL with its less harmful properties, producing microfibers limits its use where mimicking ECM is a necessity. Vascular grafts are one of the potential application areas for nanofibrous PCL webs that ECM is required to be mimicked for endothelial cells' attachment and proliferation. Therefore addition of acetic acid or formic acid to PCL/chloroform: ethanol solutions can be used where nanofibrous PCL webs are required especially for tissue designs or specifically multilayer vascular grafts due to its less hazardous properties.

3.5 Funding

This study was supported by TUBITAK (The Scientific and Technological Research Council of Turkey) (Project Number 214M089) and Istanbul Technical University, Scientific Research Projects. (Grant Number BAP37678).

3.6 Acknowledgement

Authors are thankful to Technical University of Liberec, Department of Nonwovens and Nanofibrous Materials for assisting solution characterization equipments.



4. DESIGN OF POLYCAPROLACTONE VASCULAR GRAFTS *

A large number of patients suffer from vascular diseases, resulting in the need for bypass surgery. 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. In this study, polycaprolactone small-diameter (6 mm) vascular grafts were produced successfully using custom-designed electrospinning apparatus. Radial fiber orientation was achieved by increasing the rotational speed of the collector. The morphological, structural, mechanical, and biological properties were examined. The results show that oriented scaffolds with 2 μm average fiber diameter provide 1MPa ultimate tensile strength in the radial direction. The pore size area was found to be adequate in the oriented samples required for cell proliferation and diffusion through the *tunica media*. In vitro biocompatibility of the grafts was proven with 3T3 mouse fibroblasts. After cell seeding, the oriented fibers serve as a cue for radial cell alignment. An understanding of electrospun material parameters together with the knowledge of native blood vessel structures and properties is a considerable part in designing small-diameter vascular grafts.

Keywords: vascular graft, polycaprolactone, electrospinning.

4.1 Introduction

Cardiovascular diseases (CVDs) are the number one cause of death globally: more people die annually from CVDs than from any other cause [102]. Conventional therapeutic strategies include angioplasty, endarterectomy, or bypass grafting [103]. Currently available synthetic grafts work well in the replacement of large diameter (>6 mm), high-flow vessels. However, they fail for small diameters due to acute thrombogenicity of the graft, anastomotic intimal hyperplasia, aneurysm formation,

* This chapter is based on the paper “Yalcin, I., Horakova, J., Mikes, P., Gok Sadikoglu, T., Domin, R., and Lukas, D., 2016. Design of Polycaprolactone Vascular Grafts, *Journal of Industrial Textiles*, 45(5), 813-833.

infection, and progression of atherosclerotic disease [104]. Autologous arterial and venous grafts seem to be the best choices for small-diameter bypass grafts, but in many patients their usage is limited for reasons such as vasospasm, limited length, poor quality, and prior use [33,71]. Considering these requirements with the large number of patients in need of replacement grafts, the demand for an alternative small-diameter graft is enormous and has driven scientists to search for new materials. The ideal vascular graft has to possess mechanical strength, compliance, biocompatibility, nonthrombogenicity, nontoxicity, nonimmunogenicity, and off the-shelf availability [105,106]. The issue of small-diameter blood vessel graft remains a major challenge yet to be overcome in the production of appropriate substitutes. To develop a suitable scaffold for small diameter blood vessel replacement, the native structure and properties has to be considered. The native artery is an extremely complex multilayered tissue composed of a number of different extracellular matrix (ECM) proteins and cell types. In order to withstand the high flow rate, high pressure, and pulsating nature of blood flow, an artery is comprised of three distinct layers called *the tunica intima*, *tunica media*, and *tunica adventitia*. Each of these layers has a different composition and plays a different physiological role [5]. The composition varies in cell types as well as in the morphology of ECM. The intimal layer of the blood vessels consists of a single layer of endothelial cells (ECs) lining the vessels internal surface [107]. This layer is in contact with the bloodstream therefore it provides a critical barrier to platelet activation. The endothelium has many functions. It provides the vessel with a dynamic layer of cells which, moreover, displays antithrombotic properties in their resting state. This is achieved by physically preventing elements in the blood to come into contact with prothrombotic elements in the subendothelium and by active synthesis of various mediators [7]. ECs are comprised of a laminin-rich basement membrane which lines under the endothelial interior surface of the blood vessel. The ECM in the *tunica intima* provides critical support for vascular endothelium and it influences ECs migration, invasion, survival, and organization. ECs are attached to ECM by cell-surface integrins. Cell adhesion can be supported by interstitial fibrin and collagen I [8]. The *tunica media* begins in the internal elastic lamina that separates the *tunica intima* and the *tunica media*. The middle layer is composed of smooth muscle cells (SMCs) with many functions, including vasoconstriction and dilatation; synthesis of various types of collagen, elastin, and proteoglycans; and elaboration of growth factors and cytokines [7]. The *tunica media*

is organized into concentric lamellar units composed of elastic fibers and SMCs, separated by an interlamellar matrix containing collagens, proteoglycans, and glycoproteins [9]. Collagen fibers provide tensile stiffness whereas elastin gives the tube the required elastic properties. Compressibility of the vessel and the irreversible deformation against pulsating blood flow are provided by proteoglycans and glycoproteins. The composition of ECM in the *tunica media* regulates the activity and phenotype of SMCs [50]. In vitro studies confirm the involvement of ECM–SMC signaling in establishing and maintaining the mature tubular structure [9]. Finally, the adventitia extends beyond the external elastic lamina and is composed mainly of randomly arranged collagen fibers and fibroblasts [5,51,52]. This outermost layer is nourished by vasa vasorum, thin capillaries providing an important source of nutrition [10]. When designing a multilayer structure for vascular grafts, it is important to mimic the topographical, morphological, and mechanical properties of each layer as much as possible [48]. For this purpose, an electrospinning technique was utilized as the main technology. An electrospun nanofibrous structure provides a biomimetic cellular environment which resembles the ECM of native blood vessels. Moreover, the high surface-to-volume ratio of these structures provides potential anchoring points for cell attachment [33,53]. To achieve the different structures of the abovementioned layers, various modifications to the electrospinning process were employed. The AVflo™ Vascular Access Graft is the first commercial vascular access graft to exploit the unique properties of electrospun nanofabrics. Since it is made of polycarbonate urethane which is a biocompatible synthetic polymer, it has improved patency levels. On the other hand, the use of multilayered nanofibrous structure provides many advantages one of which is easy suturing that offers a solution for hemodialysis patients [108].

The choice of material is another crucial task that has to be considered. Biodegradable materials provide a key advantage. As the material degrades hydrolytically over time, cells continuously infiltrate the matrix, producing collagen, elastin, and proteoglycans to replace the degrading material. Eventually, a fully functional artery is created composed of autologous smooth muscle and ECs. Therefore, long degradation time is one of the most important properties for the biopolymer that provides prolonged mechanical backbone for cells to infiltrate and low inflammatory response [22].

Polycaprolactone (PCL) is a promising candidate for vascular grafts due to its bioactivity for both ECs and SMCs, nontoxicity, and comparatively high elastic behavior. In addition to that, hydrophobic nature and the high level of crystallinity of PCL scaffolds result in a long degradation time (more than 18 months in vivo [109]) which provides a prolonged mechanical support for cell infiltration [7,22,45].

There are many studies which call for the usage of electrospun PCL for vascular grafts. Lee et al. [60] produced scaffolds from the blends of biodegradable polymers (PCL, poly-L-lactic acid (PLLA), poly(lactic-co-glycolic acid) (PLGA), poly(Llactide-co-caprolactone) (PLCL)) with structural proteins (elastin and collagen). Based on the results it can be said that long-term patencies of the scaffolds consisting of PCL are better in comparison with other scaffolds produced from biodegradable polymers (PLLA, PLGA, PLCL) [60]. On the other hand, Wise et al. [70] indicates that the mixture of PCL with structural proteins improves the mechanical strength [70]. In a study of Gaudio et al. [76], the use of chloroform as a solvent for PCL results in micro-scaled fibers (3.6 ± 0.8 mm) which leads to better SMCs viability, improved interfacial surface between cells and fibers, and promoted cell colonization in comparison with nanofibrous web produced by PCL in DMF:tetrahydrofurane (1:1) solvent system [76]. Beside in vitro studies, the in vivo implantation of PCL vascular grafts in rats was successfully realized by Wang et al. [35], Valence et al. [109], Pektok et al. [110], and Nottelet et al. [111] with the results of adequate long-term patency levels against to thrombosis risk.

This study aims to design the best copy of native vessel ECM. We presume that the internal morphology of such a graft, mimicking the native ECM, will facilitate cell ingrowth and maintain all its essential functions. In this respect, pore size and fiber orientation become crucial design parameters for vascular grafts. Ju et al. produced two layered scaffolds with differing pore sizes from PCL/collagen solution. Both SMCs and ECs were seeded on the scaffold and results indicate that different pore sizes can be produced by varying fiber diameters. Moreover it is stated that the increase in fiber diameter enables improved SMCs diffusion whereas less than 1 μm pore size was found insufficient for SMCs infiltration [33]. On the other hand, Lee et al. [37] defined suitable pore sizes for SMCs infiltration in the range of 100–400 μm . Radial fiber orientation is essential for SMCs infiltration while mimicking *tunica media* [37]. Hu et al. [44] produced tubular scaffolds from PCL in Dichloromethane

(DCM)/Dimethylformamide (DMF) solution by using differing rotational speeds (250, 1000, and 1500 min^{-1}) for 10 cm diameter cylindrical collector. For radial fiber orientation, minimum 200 m.min^{-1} peripheral speed was found to be required [44]. Edwards et al. [45] similarly studied on radial fiber orientations for PCL/dichloroethane solution. For 3.2 cm collector diameter, peripheral speed of collector was varied from 0 to 480 m.min^{-1} . Results show that fibers are not exposed to a notable extension force when less than 120 m.min^{-1} speed was used. 150 m.min^{-1} was defined as an optimum speed for fiber orientation achievement whereas mechanical fiber deformation was observed at 300 m.min^{-1} [45].

In this study, tubular scaffolds were produced using custom-designed electrospinning apparatus with a needle spinning electrode and modified rotating mandrel type collector. PCL in chloroform:ethanol solvent system with 18% weight ratio was chosen and predetermined electrospinning production parameters were used in the light of our previous works [112,113]. Radial fiber orientation was achieved by varying the rotational speed of the mandrel in order to improve the mechanical performance and cell organization. Fiber diameter distribution as well as pore size area and porosity analysis was examined to evaluate its suitability for cell proliferation and diffusion through the *tunica media*. The relationship between the orientation of fibers and cells was confirmed in vitro by seeding 3T3 mouse fibroblasts.

This study aims to constitute a considerable step forward in designing multilayered vascular graft.

4.2 Materials and Methods

4.2.1 Histological preparation of native blood vessels

Small-diameter blood vessels were obtained from Liberec Regional Hospital. The carotid artery was fixed in formaldehyde followed by gradual dehydration with alcohol and soaking in toluene. Samples were embedded in paraffin and 3 μm sections were prepared for histological analysis. After removal of the embedded resin, the samples were stained with hematoxylin eosin (H&E) in order to see the cells (a), Van Gieson staining in order to see the collagen (b), and Acetic orcein in order to see the elastin fibers (c). The stained samples were placed on slides and analyzed through a Nikon ECLIPSE Ti-E/B light microscope. A thin section of the embedded tissue was

prepared directly on the target for further scanning electron microscope (SEM) analysis.

4.2.2 Polymers

Polycaprolactone (PCL, Mn 45000, Sigma-Aldrich, St. Louis, MO) was dissolved in chloroform/ethanol (9/1v/v, Sigma-Aldrich) at a concentration of 18 w/v % solution. Since chloroform evaporates very fast, polymeric solution could get concentrated quickly. The addition of ethanol slows down the evaporation rate of chloroform and improves the spinnability of PCL. The solution was stirred for 8h and then immediately electrospun.

4.2.3 Electrospinning

The custom-designed electrospinning apparatus consisted of a positive high voltage power supply (Matsusada Precision Inc., Japan), a syringe pump (Model NE-1000x; New Era Pump Systems Inc., USA), a 10 ml plastic syringe, a hypodermic needle with 0.6 mm inner diameter, and a grounded stainless steel rotating mandrel (6 mm diameter, 20 cm length). Reciprocal movement of the needle spinning electrode was achieved using a sliding holder powered by a step electromotor or alternatively by a drilling machine. The length of the slide was limited to 10 cm while its peripheral speed was fixed at 0.6 m/min during production. A diagram and a photograph of the electrospinning apparatus is shown in Figure 4.1.

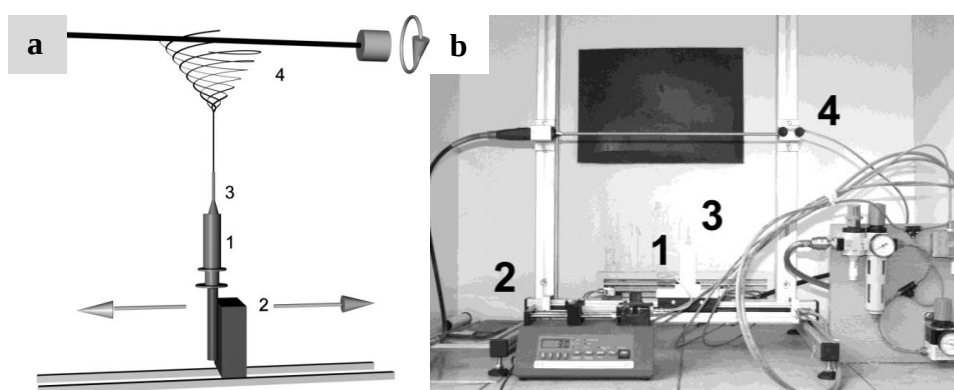


Figure 4.1 : Diagram (a) and picture (b) of the electrospinning apparatus: A high voltage is applied between the needle tip (3) and the mandrel type collector (4). The polymer solution is delivered to the needle tip at a constant feed rate using a syringe pump (1) attached to the sliding unit (5). An electric field is generated by the high voltage power supply (2). Finally a continuous web is collected on the rotating mandrel (4) in a tubular form.

4.2.4 Scaffold fabrication

The tubular PCL scaffolds were produced using custom-designed electrospinning apparatus. The production parameters were set as follows: applied voltage was 7.5 kV and the feed rate was 2.0 ± 0.5 ml/h. The temperature and relative humidity were 20 ± 3 °C and $34 \pm 4\%$, respectively. The following rotational speeds of the mandrel were used: 250, 5000, 10,000, and 15,000 min^{-1} (e.g. peripheral speeds were 4.71, 94.2, 188.4, and 282.6 $\text{m} \cdot \text{min}^{-1}$). The collector was driven by a step motor up to 250 r/min. For higher rotational speeds (5000, 10000, 15000 min^{-1}), a drilling machine was integrated to electrospinning apparatus to empower the collector.

Finally, the tubular graft was left to dry overnight at room temperature (20 ± 3 °C). Then PCL fibrous tube could be obtained from the rod by pushing manually. After removal from the mandrel, the specimens were cut up for the various analyses as described in the following sections.

4.2.5 Morphological analysis

The tubes were cut to obtain $0.5 \text{ cm} \times 0.5 \text{ cm}$ flat samples and both inner and outer sides of the tubes were analyzed. Specimens were placed in a holder, coated with gold, and analyzed using a SEM from Phenom-World (FEI Company, USA). The average fiber diameter, fiber orientation distribution, pore size area, and porosity analysis were carried out using NIS Elements Software System (Nikon Instruments Inc.). Pore sizes were also evaluated using the Bubble method. These analysis techniques are based on different measuring principles therefore their results were compared and the differences were discussed.

4.2.5.1 NIS Elements software

The average fiber diameter, the orientation of the fibers, porosity, and pore size area were measured using NIS Elements Software for all rotational speeds (250, 5000, 10,000, and 15,000 min^{-1}). The average fiber diameter was measured from 100 randomly selected fibers from three different SEM images. Fiber orientation was analyzed manually by drawing lines in the directions of 100 randomly chosen fiber elements. The reference line, that is the zero degree direction, is identified as the radial direction of fibers produced. The angle between the reference line and the line drawn tangentially to a fiber axis was recorded. Finally, the fiber orientation distribution in

the range from -90° to $+90^\circ$ was plotted in the graph. Porosity and pore size area were evaluated by automatic pore detection from six SEM photos. The average pore size, the minimum and the maximum values, the standard deviation, and also the porosity were recorded. All measurements were realized by SEM images of 1000x magnification.

4.2.5.2 The Bubble method

The maximum and average pore sizes were determined using the Bubble method testing apparatus (ASTM F316 – A3, CR.). The Bubble method is a nondestructive way to determine the diameter of the largest pore size of a fibrous membrane and the mean pore size. The testing range is from 0.2 to 200 μm . For this study, the sample size was reduced from a standard value of 19.61 cm^2 in order to adapt the testing conditions for the tubular scaffolds. This technique involved two steps: dry and wet measurements. In dry measurement, samples were placed in the housing of the testing unit and exposed to gradually increasing gas pressure (pressure gradient range from 0 to 0.6 MPa). The data were recorded by a software system. Then, for wet measurement, samples were impregnated with a wetting liquid (in this case, mineral oil with a surface tension of 49 mN/m) and the increasing gas pressure was applied again. When a constant flow of rising bubbles was observed on the top side of the sample, the gas pressure p [Pa] was recorded. The maximum pore size diameter D [m] was determined from the equation (4.1).

$$D = \frac{2\gamma}{p} \quad (4.1)$$

where γ is the surface tension [N/m] and p is the applied gas pressure. The mean pore size was estimated from the equilibrium point of wet and dry measurements. The pores are considered to be circular so the final area of pore size was calculated. Each sample was measured twice and the average diameter $\pm$ standard deviation was recorded. More details about the principles of this technique can be found in Yu et al. [114] and Agarwal et al. [115].

4.2.6 Mechanical characterization

Mechanical characterization of the tubular scaffolds was carried out using a tensile tester (Labor-Tech s.r.o., CR). The thickness of the samples was measured using a digital thickness gauge (Nova Technology s.r.o., CR). The tubular scaffolds were cut

and unfolded into a planar form. Four specimens were prepared in 18 mm x 10 mm dimensions in axial (90°), radial (0°), and 45° directions. The stroke rate and the gauge length were set to 3 mm/min and 10 mm, respectively.

4.2.7 Cell sources and seeding

Prior to cell seeding, the scaffolds were cut into round segments of 6mm in diameter and sterilized by immersion in 70% ethanol for 30 min followed by double washing in a phosphated buffer saline (PBS, Lonza). Mouse 3T3 fibroblasts (ATCC, USA) were cultivated in Dulbecco's Modified Eagle Medium (DMEM, Lonza) supplemented by 10% fetal bovine serum (Lonza) and 1% penicillin/ streptomycin/amphotericin B (Lonza). The cells were placed in a humidified incubator with an atmosphere of 5% CO₂ at 37°C. When the cells became confluent, they were suspended using trypsin–Ethylenediaminetetraacetic acid (EDTA) solution (Lonza). The number of cells was determined using a Luna™ cell counter (Logos Biosystems). The fibroblasts were seeded on the scaffolds placed in 96-well plate at a rate of 5x10³ per well plate. The experiment took 14 days and the medium was changed three times a week during this period.

4.2.8 (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) (MTT) assay

The viability of the cells seeded on the scaffolds was analyzed using an MTT test after 1, 4, 7, and 14 days of the culture period. MTT had been reduced to the purple formazan by mitochondrial dehydrogenase in the cells indicating normal metabolism. MTT solution (50 µl) was added to 150µl of DMEM and the samples were incubated at 37 °C for 4 h. Violet crystals of formazan were formed and were dissolved using acidic isopropanol. The optical density was measured using an ELx808 Absorbance Microplate Reader (Biotec) at the following sample and reference wavelengths (λ_{sample} 570 nm, $\lambda_{\text{reference}}$ 690 nm). Each measurement was taken in quadruplicates; finally the average wavelength (λ_{sample} 570 nm - $\lambda_{\text{reference}}$ 690 nm) and its standard deviation were calculated and depicted in the graph.

4.2.9 Fluorescence microscopy analysis

After 1 day of cultivation, the samples were washed with PBS and fixed in frozen methanol for 30 min. Then, PBS washing followed staining with propidium iodide for

10min in the dark. Images of stained cell nuclei were captured using a Nikon ECLIPSE Ti-E/B fluorescence microscope (magnification 400x) and analyzed using NIS Elements. Twenty cell nuclei from each sample were chosen and their maximum and minimum Feret's diameters were measured. The elongation of the cell nuclei was determined from the ratio of their maximum and minimum Feret's diameters. The ratio close to 1 indicates no cell elongation, while a ratio greater than 1.5 indicates cell nuclei elongation along the fiber axes. To support the data, cell elongation was also investigated with Image J's function Aspect Ratio (AR).

4.2.10 SEM

After 7 days of cultivation, the samples were washed with PBS and fixed in 2.5% glutaraldehyde in PBS for 30min (4 °C). Then, the samples were dehydrated by treating with a series of graded ethanol solutions (60, 70, 80, 90, 96, and 100%). After water removal, the scaffolds were transferred to an SEM holder, coated with gold, and analyzed using an SEM Tescan Vega SB EasyProbe (Czech Republic).

4.2.11 Statistical analysis

The differences between vascular scaffolds specific to fiber diameter, MTT analysis, and cell elongation ratios were evaluated by Analysis of variance (ANOVA) while tensile strength and elongation at break results were analyzed by Student's t-test with GraphPad Prism 6.0 Software. Differences were considered significant at $P < 0.05$ and shown in the graphs with *.

4.3 Results

4.3.1 Histology of native blood vessel

To prove the hypothesis of mimicking native ECM blood vessel components, a histological investigation of the artery having the same internal diameter (6 mm) was carried out. The main components of ECM such as collagen and elastin were studied as well as the arrangement of cells in the different layers (Figure 4.2). The wall thickness of the analyzed samples varies from 400 to 1000 μm along its length. H&E staining provides information about the cell arrangement in the different layers (Figure 4.2(a)). ECs create a single layer covering the luminal surface, whereas SMCs are organized into numerous layers inside the *tunica media*. The cross section of the SMC

nuclei shows radial elongation, which corresponds with the organization of collagen and elastin fibers inside the middle layer. The outermost layer, *tunica adventitia*, is composed of fluffy fibers with a few fibroblast cells. Collagen frequently occurs in all three layers of native blood vessel as shown in Figure 4.2(b). The collagen nanofibers are organized in micrometer scale bundles as depicted in the top right corner of the same picture. Elastin forms an elastic layer between the *tunica intima* and the *tunica media* called the *lamina elastica interna*. Elastin fibers are also present in the *tunica media* and *tunica adventitia* and give the blood vessel the desired elastic properties. In Figure 4.2(c), the *vasa vasorum* can be seen in the *tunica adventitia* which nourishes the outermost layer [10].

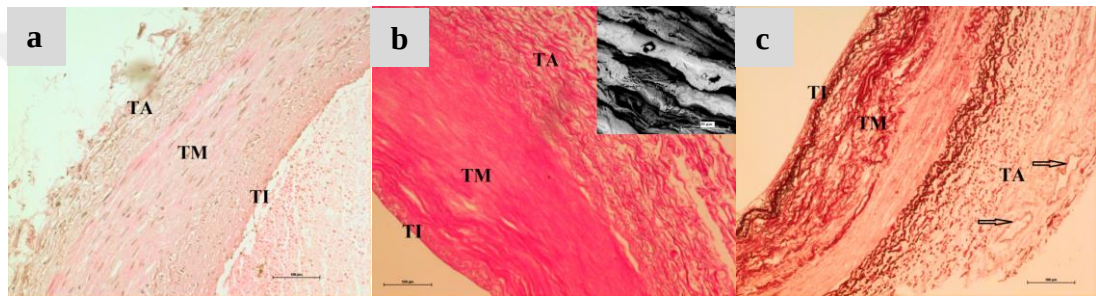


Figure 4.2 : Hematoxylin eosin staining (a), collagen (b), and elastin (c) in the *tunica intima* (TI), *tunica media* (TM), and *tunica adventitia* (TA). Scale bars: 100µm. Collagen nanofibers arranged in microfibrillar bundles are depicted in SEM picture intercalated in (b), scale bar: 10 µm. Black arrows in picture (c) indicate *vasa vasorum* in the *tunica adventitia*.

4.3.2 Scaffold fabrication

The tubular PCL scaffolds of length up to 10cm, inner diameter of 6 mm, and width about 250 µm wall thickness were successfully fabricated using the custom designed electrospinning apparatus. The length and diameter of the scaffolds were adjusted by choosing mandrels with various sizes and by adjusting the length of the reciprocal movement of the spinning electrode. The wall thickness of the tubular nanofibrous scaffolds was controlled by the duration of the electrospinning (up to 90 min).

4.3.3 Morphology

Both sides of the tubular scaffold are composed of fibrous network. The inner and outer surface of the tube can be seen from the SEM images in Figure 4.3. Different fibrous structures can be observed in the inner and outer side of the tube. In the inner layer (Figure 4.3(a)), PCL fibers adhere to the rotating mandrel during production resulting

in a distinct morphology. In contrast, the observed fibers are smooth and continuous without bead like formation. In order not to be affected by the adherence effect of PCL fibers, fiber diameters were measured from outer surface images of the scaffolds.

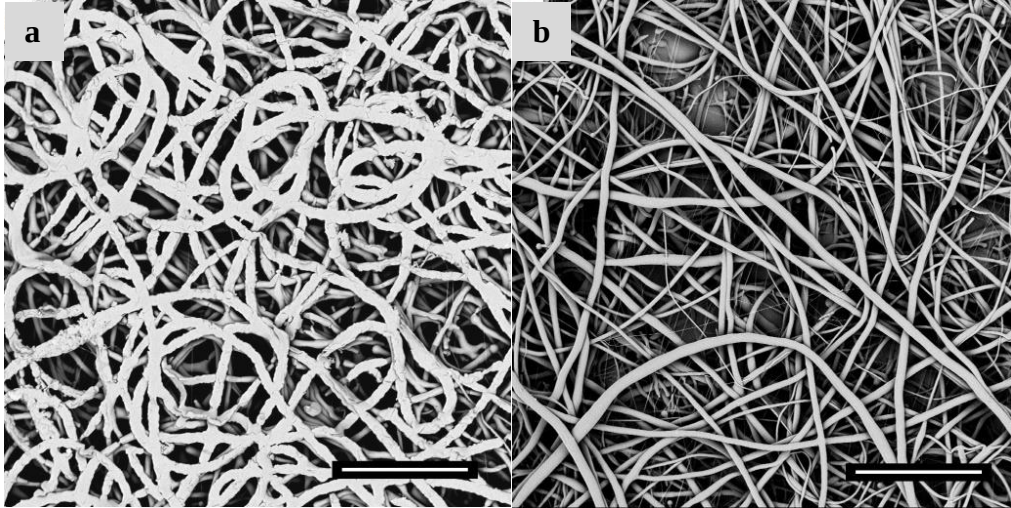


Figure 4.3 : Inner (a) and outer (b) layers of the tubular scaffold consist of different fiber morphology. Scale bars 60 μ m.

Figure 4.4(a) shows average fiber diameters measured at speeds of 250, 5000, 10,000, and 15,000 min^{-1} . From these data, it can be said that there is no significant difference among average fiber diameters of varying rotational speeds ($P > 0.05$). On the other hand, Figure 4.4(b) indicates fiber diameter histograms for all rotational speeds. All histograms include two peaks indicating that the scaffold structure is composed of two types of fiber diameters: thinner fibers and thicker ones. The first peaks are for the diameter range of 0.25–0.75 μm , whereas the second peaks appear at the diameter range of 1.75–2.25 μm .

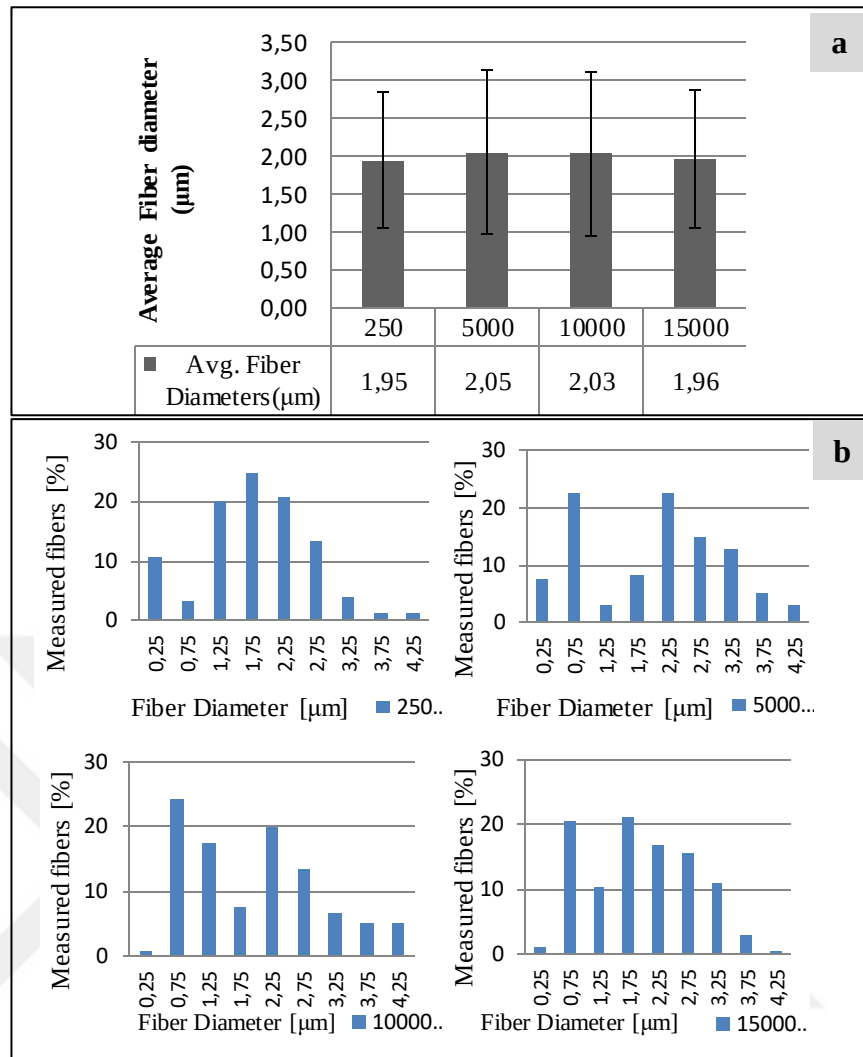


Figure 4.4 : (a) Average fiber diameters measured at speeds of 250, 5000, 10,000, and 15,000 min^{-1} . There is no significant difference among fiber diameters ($P = 0.189 > 0.05$) (b) Distribution of fiber diameter in the samples obtained at varying rotational speeds.

4.3.3.1 Fiber orientation analysis

Planar anisotropy of fibers can be seen in both the graph produced using the NIS Elements Software (Figure 4.5(A)) and SEM pictures (Figure 4.5(B)). Orientation of the fibers in the radial direction tends to improve with increasing rotational speed. In the samples produced at 250 min^{-1} , the fibers are randomly oriented. The most parallel fiber alignment appears at 15,000 min^{-1} . This nearly perfect alignment is reflected in the sharpest peak around 0° of the directionality histograms among others (Figure 5(A)).

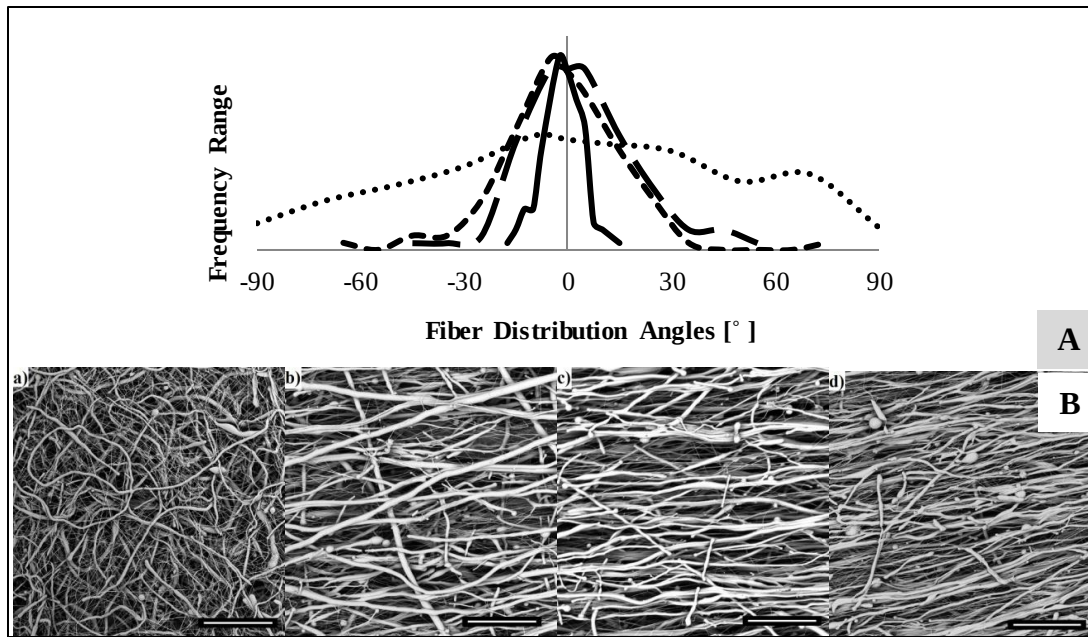


Figure 4.5 : (A) Fiber orientation distribution between -90° to $+90^{\circ}$ using the NIS Elements SW for different rpm of rotating mandrel: dotted line (250 min^{-1}); cut line (5000 min^{-1}); long cut line (10000 min^{-1}); continuous line (15000 min^{-1}). The zero direction is the radial one. (B) SEM pictures of PCL scaffolds produced at different rotational speeds (a) 250 min^{-1} (b) 5000 min^{-1} (c) 10000 min^{-1} (d) 15000 min^{-1} . Scale bars $60 \mu\text{m}$.

4.3.3.2 Pore size and porosity analysis

Pore size and porosity analysis were carried out using the NIS Elements Software and the Bubble method. Data are given for comparison in Table 4.1.

Table 4.1 : Summary of pore characterization obtained from NIS Elements and the Bubble method: Average (A_{Avg}), maximum (A_{Max}) and minimum (A_{Min}) pore size with standard deviations (SD) are recorded as well as the porosity of the nanofibrous layers produced at different min^{-1} values: 250, 5,000, 10,000 and 15,000.

min^{-1}		250	5000	10000	15000
Bubble method	$A_{\text{Avg}} [\mu\text{m}^2]$	49.27	93.83	62.35	65.33
	SD	1.41	14.67	5.01	4.25
	$A_{\text{Max}} [\mu\text{m}^2]$	506.71	1206.87	838.28	1164.16
	SD	50.76	123.65	259.27	438.13
	$A_{\text{Avg}} [\mu\text{m}^2]$	8.60	29.40	29.00	28.30
	$A_{\text{Min}} [\mu\text{m}^2]$	0.11	0.22	0.22	0.22
NIS Elements	$A_{\text{Max}} [\mu\text{m}^2]$	673.60	574.70	713.10	667.70
	SD	25.00	52.18	14.42	8.27
	Porosity [%]	36.39	29.44	28.97	28.25

NIS Elements software results for average pore size area and porosity

The effect of the rotational speed on the average pore size area and porosity was investigated using SEM and NIS Elements Software. Nonoriented fiber samples obtained at 250 min^{-1} with an average pore size area (A_{Avg}) of $8.60 \mu\text{m}^2$ and porosity of 36.39 % are significantly different from the oriented fiber samples produced at 5000, 10,000, and 15,000 min^{-1} with A_{Avg} of $29 \mu\text{m}^2$ and porosity of 29% ($P < 0.05$). High values of standard deviation denote very small pores ($0.11 \mu\text{m}^2$) as well as big ones (hundreds of μm^2) are present in the structure. Nonoriented fibers show higher porosity (36.39 %) compared to oriented ones (around 29 %), but the average pore size area is too small ($8.60 \mu\text{m}^2$) for cells to grow into the layer. Oriented fibers with larger pore size areas seem to be more appropriate for SMCs infiltration through the middle layer of vascular grafts.

Pore size determination using the Bubble method

The nonoriented fiber samples obtained at 250 min^{-1} have $49.7 \mu\text{m}^2$ A_{Avg} pore size, while the oriented fibers obtained at 5,000, 10,000, and 15,000 min^{-1} have average pore sizes of 93.83, 62.35, and $65.33 \mu\text{m}^2$, respectively. Similarly, the maximum pore size areas are higher in the oriented samples than in the nonoriented ones ($250 \text{ m} \cdot \text{min}^{-1}$). Although no significant differences were noted among oriented samples, nonoriented fiber samples were statistically different from oriented fiber samples regarding to average and maximum pore sizes ($P < 0.05$). Both techniques indicate that oriented samples show better structures with higher pore sizes which are beneficial for SMC infiltration. On the other hand, there is no clear relation between the pore size areas and increasing rotational speeds in the oriented samples.

4.3.4 Mechanical performance

The relation between the rotational speed and both the tensile strength and the elongation at break are shown in Figure 4.6(a) and (b). The mechanical test results (Figure 4.6) verify the outcomes of the orientation analysis test results (Figure 4.5). A significant difference in ultimate tensile strength values in the radial (0°) and axial (90°) directions was achieved, except at 250 min^{-1} ($P < 0.05$). The increased ultimate tensile strength in the radial direction indicates parallel fiber orientation in the scaffold prepared at 5000, 10,000, and $15,000 \text{ min}^{-1}$ (Figure 4.6(a)). This difference is most notable at 10,000 and $15,000 \text{ min}^{-1}$. The higher tensile strength values in 0° direction

(1 MPa) clearly result from the increased amount of radial oriented fibers that contribute to the maximum tensile strength. Similarly, higher elongation at break values is achieved in the axial directions of 5000 and 10,000 min^{-1} such as 324 and 386%, respectively (Figure 4.6(b)). At 15,000 min^{-1} , the comparatively low elongation value (224%) in the 90° direction can be explained by the notable decrease in the number of interconnecting fibers that can resist loading.

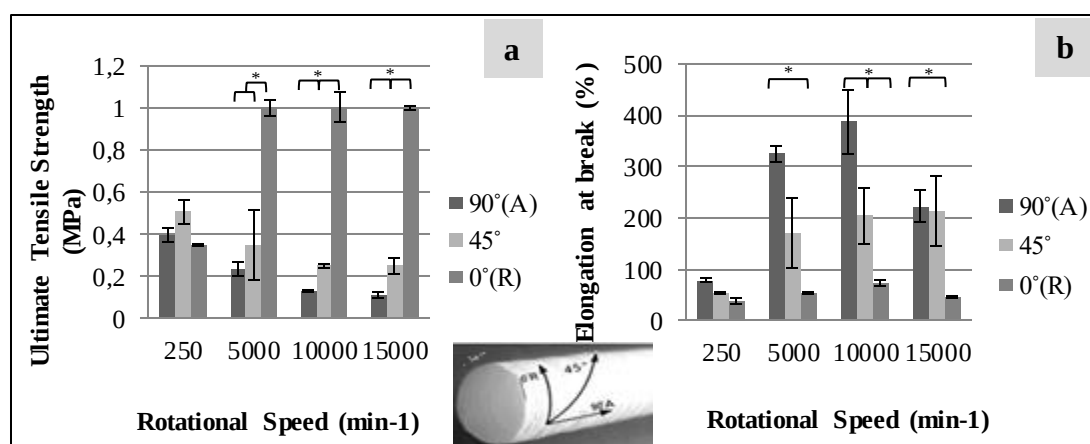


Figure 4.6 : The effect of rotational speed on ultimate tensile strength (a) and elongation at break (b) ($n=4$; mean $\pm$ standard deviations are plotted). * indicates a significant difference ($P < 0.05$).

Similar to tensile strength test results, a significant difference in elongation at break values in the radial (0°) and axial (90°) directions was achieved, except at 250 r/min ($P < 0.05$).

4.3.5 MTT test

An MTT test was carried out to observe the extent of cell proliferation within 14 days of cultivation. The data depicted in Figure 4.7 show continual increase of cell numbers during experiment period in all scaffold structures with a significant difference ($P < 0.05$). However, no difference was noted among samples produced from different rotational speeds (250, 5000, 10,000, and 15,000 min^{-1} , $P < 0.05$). This trend could signify that fiber orientation is not critical for 3T3 mouse fibroblasts during static cultivation. All these PCL samples indicate very good biocompatibility.

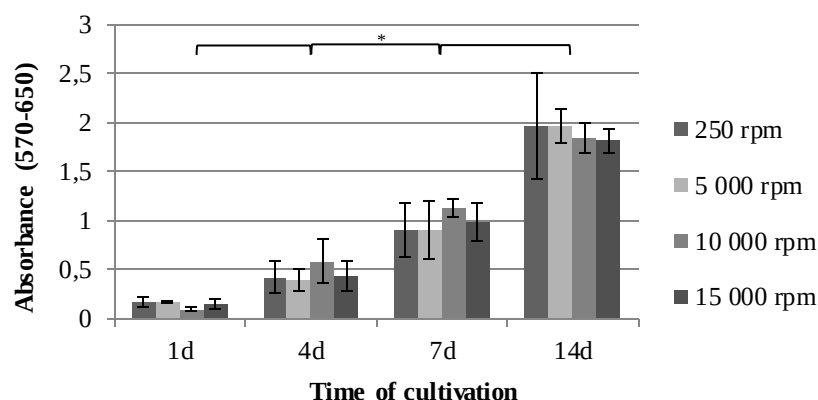


Figure 4.7 : Results of MTT test after 1, 4, 7 and 14 days of cultivation (the main vertical axis). Absorbance of the samples was measured at 570 nm and 650 nm wavelength, these values were finally subtracted. (n=4; mean $\pm$ standard deviations are plotted) * indicates a significant difference ($P < 0.05$).

4.3.6 Fluorescence microscopy

The fluorescence microscopy results confirm the results of the MTT test. Conversely, it is clear from the microscopic pictures that cells became oriented in the same direction as fibers in the oriented samples obtained at 5000, 10,000, and 15,000 min^{-1} (Figure 4.8).

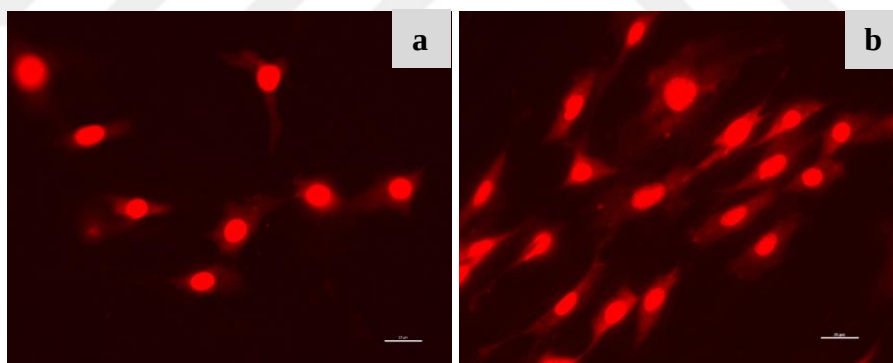


Figure 4.8 : Fluorescence microscopy pictures of 3T3 mouse fibroblasts nuclei stained with propidium iodide after 1 day of culturing anisotropic structure (250 min^{-1}) (a), and isotropic structure (10000 min^{-1}) (b). Scale bars 25 μm .

To evaluate cell elongation, the ratio of maximum and minimum cell nuclei diameter was calculated. The results are summarized in Table 4.2. In the nonoriented fiber samples obtained at 250 r/min , the measured ratio is 1.22. In the oriented fiber samples, the ratios are increased with increasing rotational speed (1.75 at 5000 min^{-1} , 1.87 at 10,000 min^{-1} , and 1.94 at 15,000 min^{-1}) which indicates that cells are also oriented in the direction of the fiber alignments (Figure 4.8). Image J test results show similar trend as described by manual measurement. In nonoriented structure (250 min^{-1}) the

cell nuclei had the smallest AR (1.26 ± 0.19) and the highest degree of circularity (0.76 ± 0.11) compared to oriented structures (AR in range of 1.52 – 1.69 and circularity in range of 0.57 – 0.66). Although no significant differences were achieved among oriented samples of both measurements, non-oriented fiber samples were statistically different from oriented fiber samples regarding to cell elongation ($P < 0.05$).

Table 4.2 : Summary of cell nuclei orientation (means $\pm$ SD of cell elongation ratio, aspect ratio, circularity)

Rotational speed [min ⁻¹]	Cell nuclei elongation ratio	Aspect Ratio [μ m]	Circularity [μ m]
250	1.22 ± 0.22	1.26 ± 0.19	0.76 ± 0.11
5,000	1.75 ± 0.40	1.54 ± 0.37	0.61 ± 0.12
10,000	1.87 ± 0.40	1.69 ± 0.55	0.66 ± 0.16
15,000	1.94 ± 0.32	1.52 ± 0.32	0.57 ± 0.16

4.3.7 SEM

From the SEM images, it can be seen how cells spread across the PCL fibrous layer (Figure 4.9). In the anisotropic structures, the cells become oriented as was confirmed by fluorescence microscopy. In the isotropic structures, the cells are randomly distributed.

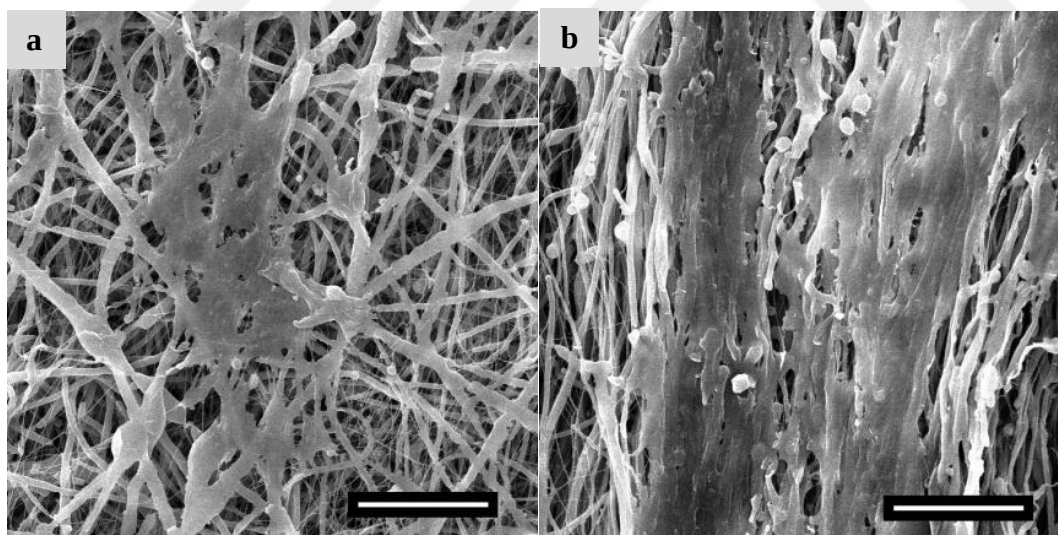


Figure 4.9 : SEM pictures of isotropic structure (250 min⁻¹) with 3T3 mouse fibroblasts after 7 days of culturing (a) and anisotropic structure (15000 min⁻¹) after 7 days of cell culture. Scale bars 50 μ m.

4.4 Discussion

To study structure of blood vessel, histological analysis of native artery was carried out. Microscopic images (Figure 4.2) reveal ECs are arranged through a single layer

covering the luminal surface, whereas SMCs are organized into numerous layers inside the *tunica media*. From the structural proteins, elastin, which gives elasticity of the vessel, was found densely in the lamina elastica interna, as well as the *tunica media* and the *tunica adventitia*. Our results have already been mentioned in the literature [22,50-53,108]. It was also observed that fibrous collagen bundles oriented in a radial manner appear in micrometer scales (Figure 4.2(b)). The electrospinning apparatus (Figure 4.1) was adjusted in order to produce the tubular scaffolds with the desired structural properties. Because of the pliable nature of PCL, the detachment of the tubular scaffold was possible [51]. Fibrous layers were produced in range of fiber diameters approximately 2 μm similar to that of ECM fibers such as collagen bundles as documented by the SEM images (Figure 4.2(b)). The use of microfibers instead of nanofibers for improved SMCs viability and promoted cell colonization was confirmed by Gaudio et al. [76]. The wall thickness of analyzed native artery varied from 400 to 1000 μm along its length. Scaffolds produced by electrospinning for 90 min resulted in graft wall thickness of around 250 μm . The idea is to produce multilayer structure that will facilitate ingrowth of ECs as well as SMCs that are critical for maintaining all blood vessel physiological functions. In the light of the histological analysis of native vessel and literary survey, fiber orientation was studied as one of the crucial parameters in designing vascular scaffolds, i.e. the *tunica media*. In order to investigate fiber orientation in the radial direction, the rotational speed of the collector was varied from 250 to 15,000 min^{-1} . Oriented electrospun fibers were achieved at 5000 min^{-1} (94.2 $\text{m} \cdot \text{min}^{-1}$) and 10,000 min^{-1} (188 $\text{m} \cdot \text{min}^{-1}$) by using a 6 mm diameter mandrel type collector. The prevailing fiber orientation in the radial direction was improved with the increasing rotational speed up to 15,000 min^{-1} (282.6 $\text{m} \cdot \text{min}^{-1}$). In order to obtain radial fiber orientation, linear velocities were found in the range of previously confirmed values 200 $\text{m} \cdot \text{min}^{-1}$ by Hu et al. [44] and 150 $\text{m} \cdot \text{min}^{-1}$ by Edward et al. [45]. There is no fiber orientation observed at 250 min^{-1} (4.71 $\text{m} \cdot \text{min}^{-1}$) since fibers are not exposed to a notable extension force less than 120 $\text{m} \cdot \text{min}^{-1}$ speed [45]. Pore size area and porosity are other important parameters for cell infiltration through the vascular grafts' layers. Based on the findings of the histological analysis, it is observed that SMCs are present in the *tunica media* (Figure 2(a)). To enable SMCs growth into the structure, pore sizes have to be sufficient. Ju et al. [33] suggest that pore sizes higher than 10 μm (for circular pore estimation it corresponds to more than 78.5 μm^2) would permit adequate cellular infiltration. Therefore, oriented layers with higher pore size

area could enable cell growth into the multilayer structure. This is another reason why oriented layers seemed to be more appropriate for the *tunica media*. The results for the lowest pore size areas at 250 min⁻¹ sample are joint results of two analysis techniques. However, when the values are compared, some differences can be noted between the pore size areas and porosities. This can be explained by the difference in the measuring principles of the Bubble method mentioned earlier. For the oriented samples in particular, it is noticeable that there are less interconnecting fibers that can bind the sample together against the air pressure during the test. Therefore, the samples extend more during airflow which results in bigger pore size. Mechanical performance tests of the scaffolds were carried out. Tensile strength of the scaffolds at 250 min⁻¹ was found in the range of 0.35–0.5 MPa which shows no significant difference with direction ($P < 0.05$). On the other hand, tensile strength of the scaffold in the radial direction was improved (around 1 MPa) by obtaining radial fiber orientation. However, it caused a distinct drop in its elongation at break (50–70%). The results show a common mechanical behavior of fibrous structures. Vascular grafts are exposed to four forces (i) shear stress, (ii) luminal pressure (a cyclic normal force attributable to blood pressure), (iii) mechanical stretch (a cyclic circumferential stress caused by blood pressure), and (iv) tension in longitudinal direction as described via Isenberg et al. [104] Although radial tensile strength is improved by the resulting fiber orientation, its elasticity, i.e. the elongation at break, decreased in the radial direction. In a study of Ju et al. [33] 0.71 MPa tensile strength was achieved for PCL–collagen (1:1) in Hexafluoroisopropanol (HFIP) solution (2.39 μm fiber diameter) whereas 1.2 MPa tensile strength for PCL–chloroform solution (fiber diameters in the range of 1.5–6 mm) was achieved by Vaz et al. [51] regardless of direction. Tensile strength of natural abdominal aorta was stated as 1.47 MPa in longitudinal and 5.29 MPa in radial direction by He et al. [72] which prove radially oriented fibrous collagen bundles in the structure. Although mechanical test results of this study match with literature, it must be improved to meet the requirements of native vessels. Therefore, design of a multilayer structure seems to be appropriate to mimic mechanical as well as morphological properties of native vessel. Biocompatibility of PCL fibers was evaluated using mouse 3T3 fibroblasts. Cell alignment and viability was measured. MTT assay showed no significant difference in cell numbers between samples of various planar anisotropies during 14 days of culturing. However, elongated cell shapes were observed which suggests that cells tend to orient themselves with the

fibers in the radial direction. The increase in nuclei elongation ratio, which were calculated as 1.22, 1.75, 1.87, 1.94 for 250, 5000, 10,000, and 15,000 min^{-1} , confirmed cell orientation along the fibers. Since all these mechanisms contribute to normal cell behavior, specialized cell types (ECs and SMCs) need to be tested in a dynamic bioreactor cultivation system [72] to provide more realistic simulations.

4.5 Conclusion

This study provides a new perspective for designing small-diameter vascular grafts. Based on the hypothesis of tissue engineering, mimicking cellular environment of natural tissue is beneficial for cell infiltration. Therefore, an understanding of the structure and properties of the target tissue has to be attained. With this in mind, histological and morphological analyses of the native blood vessel were carried out, the results of which provided an insight into designing vascular grafts. Cell organizations as well as structural proteins were observed to enlighten our design parameters for vascular grafts. Tubular scaffolds used as vascular grafts were produced under optimized conditions by a custom-designed electrospinning apparatus. Fiber orientation was obtained by increasing the rotational speed of the collector and besides mechanical performance structural properties mainly, fiber orientation, pore sizes, and porosity were investigated in order to test its adequacies for cell attachment and cell diffusion ability. Biocompatibility of PCL was found sufficient by MTT test using 3T3 fibroblasts, while morphological analysis of seeded samples under fluorescence microscope and SEM confirms that cells are aligned along the fibers. Although many design parameters were successfully examined in this study, future attempts should mainly be directed at: (i) design of a multilayer scaffold to mimic native vessel, (ii) improving the radial elasticity, and (iii) seeding the graft with specialized cell types using dynamic cultivation.

4.6 Acknowledgement

The authors are thankful to Ondrej Novak, Department of Nonwovens and Nanofibrous Materials, Technical University of Liberec for providing material's mechanical characterization and to Jakub Hruza, Department of Nanotechnology and Informatics, Technical University of Liberec for providing the Bubble method testing apparatus. D.L. acknowledges the support of GACR, Grant no. P208/12/0105.

4.7 Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

4.8 Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.



5. MECHANICAL INVESTIGATION OF BILAYER VASCULAR GRAFTS ELECTROSPUN FROM ALIPHATIC POLYESTERS*

Electrospun biodegradable vascular grafts provide a wide range of design components from the selection of materials to the modification of fiber structure. In this study, both single layer and bilayer tubular scaffolds with inner diameter of 6 mm were electrospun from polycaprolactone with different molecular weights and poly(L-lactide) caprolactone polymers. Bilayer scaffolds were designed by using different combinations of the polymer types in each layer and obtaining fiber orientation in outer layers. Scaffolds were analyzed morphologically and mechanically. Obtained results of mechanical performance were discussed according to the used polymer-type composition, fiber orientation, and composite effect of both layer in the final graft. Smooth muscle cells were seeded on the scaffolds to test biocompatibility of presented scaffolds. Results indicate that the use of different biodegradable polymers in different combinations in each layer causes notable differences in fiber morphology and mechanical performance of the scaffolds. Moreover, fiber orientation in outer layer improves tensile strength and burst pressures in radial directions while creating a suitable fibrous layer for smooth muscle cells by mimicking the extracellular matrix of *tunica media* in native vessels.

Keywords: vascular graft, biodegradable polymers, electrospinning, fiber orientation, burst pressure.

5.1 Introduction

Development of tubular scaffolds for various tissue engineering applications became very attractive and required field. The necessity of functional small diameter vascular graft discovery is in the top level of interest of surgeons since older patients lack appropriate biological substitutes. The vascular graft should be composed of

* This chapter is based on “Yalcin Enis, I., Horakova J., Novak O., Gok Sadikoglu T. and Lukas D., 2016. Mechanical Investigation of Vascular Grafts Electrospun from Aliphatic Polyesters, Polymers for Advanced Technologies, 0(00), 1-13. (Published online in June 2016, DOI:10.1002/pat.3875)”

biomaterials that have adequate degradation rate which provides mechanical integrity and creates an elastic and durable backbone until mature tissue forms *in vivo* [33]. Therefore from the selection of material to the usage of suitable production technology, there are many determinant steps in the creation of such vessel that resembles a native one in its mechanical properties. Polycaprolactone (PCL) is preferable biodegradable polymer to be used for vascular graft fabrication owing to its good biocompatibility, slow degradation rate with improved tensile and elongation properties [71]. Another polymer used in vascular tissue engineering from the same group of aliphatic polyesters is a copolymer poly L-lactide caprolactone (PLC) with its controllable degradation rate and mechanical properties [72]. Electrospinning is one of the most used technology easily modified owing to the requirements of vascular graft production. Via electrospinning, it is possible to control the composition, macrostructure and internal morphology of the scaffolds leading to improved mechanical performance and cell – material interactions [60].

Radial fiber orientation is an essential design parameter for enhancing smooth muscle cells proliferation resulted in improved strength against radial pulsatile pressure. Amongst the most common failure of small diameter vascular grafts belong compliance mismatch between adjacent native vessel and creation of thrombus. This problem mainly raised from the mismatch of used material composition and inner morphology [111]. In native vessel, collagen and elastin fibers are radially oriented in outermost layer which provide concentric dilation and contraction during the diastolic and systolic cycles. [22]. The histology of the native vessel was analyzed by Yalcin et al. [47] in which radially oriented collagen bundles with micro-scaled diameters were presented. In general, electrospinning technology is suitable for production of oriented fibers by modifying a collector as a rotating unit. However, it is not easy to reach such high speeds ($>10,000 \text{ min}^{-1}$) by using small diameter ($<6 \text{ mm}$) rotating mandrels. Therefore most of the studies use large diameter rotating collectors (630 mm, [42] 100 mm, [44] 32 mm, [45] and 640 mm [46]) for accessible rotational speeds without a risk of resonance frequencies. Matsuda et al. [43] expressed that they achieved only partial fiber orientation at $3,400 \text{ min}^{-1}$ for 3 mm rotating mandrel diameter which had been explained by Hu et al. [44] with an insufficient linear velocity. To achieve orientation at rotating mandrels with less than 6 mm diameter, it is a must to increase the rotational speed based on used polymer type. Yalcin et al. [47]

used a drilling machine to empower the collector for higher rotational speeds (up to $20,000 \text{ min}^{-1}$). Moreover, the type of polymer should be taken into consideration while optimizing rotational speed for fiber orientation. In a study of Chaparro et al. [46] at 84.6 m.min^{-1} peripheral velocity, only randomly distributed PCL (80,000 Mn) fibers were electrospun. Supportively, Edwards et al. [45] indicates that there is no fiber orientation of PCL (80,000 Mn) up to 120 m.min^{-1} peripheral velocity since fibers are not exposed to mechanical stress. Orientation was achieved between the peripheral velocities of 150 and 300 m.min^{-1} [45]. On the other hand, Yalcin et al. [47] expressed that PCL (45,000 Mn) fibers are almost oriented radially by using 6 mm diameter rotating mandrel type collector with $5,000 \text{ min}^{-1}$ rotational speed which is equal to 94.2 m.min^{-1} peripheral velocity. From another point of view, Wu et al. [48] stated that by modification of electrospinning with directionally positioned metal plates, excellent radial fiber orientations can be obtained.

Vascular grafts are exposed to shear stress, luminal pressure, mechanical circumferential stretch, and tension in longitudinal direction as described by Isenberg et al. [104]. Beside many other parameters such as fiber diameter, polymer type, wall thickness of the final vascular prosthesis etc., radial fiber orientation is one of the most determinant design parameter influencing further mechanical performance of the tubular scaffolds. In a study of Yalcin et al. [47] tensile strength of the electrospun PCL (45,000 Mn) tubular scaffolds produced at 4.71 m.min^{-1} was found in the range of 0.35–0.5 MPa which shows no significant difference with change of measurement axis ($p < 0.05$). On the other hand, tensile strength of the scaffolds in the radial direction was improved (around 1 MPa) by obtaining radial fiber orientation (achieved at 94.2 m.min^{-1} to 288.2 m.min^{-1} linear velocities). However, it is stated that radial fiber orientation caused a distinct drop in its elongation at break (50–70%) [47]. On the other hand, in a study of Lee et al. [71] ultimate tensile strength and elongation at breaks of randomly distributed electrospun PCL (1.77 dl/g viscosity) scaffolds were measured as $5.1 \pm 0.9 \text{ MPa}$ and $417 \pm 58\%$. The burst pressure of the PCL scaffolds was given as $914 \pm 130 \text{ mmHg}$ [71]. In a study of Nottelet et al. [111], mechanical properties of electrospun PCL (80,000 Mn) grafts were optimized by changing solvent systems and solution parameters and results indicate that it is possible to produce PCL fibers with diameters in a range of 500 nm to 2,500 nm, tensile strength in a range of 2 MPa to 7.4 MPa and strain values in a range of 200% to 1,200% [111]. Tubular

scaffold with 3 mm inner diameter PLC (70/30 molar ratio) were produced at 150 min⁻¹ rotational speed (1.41 m.min⁻¹) having tensile strength of 7.0 MPa in axial and 3.9 MPa in radial direction as published by He et al. [72]. The reason for aligned fibers in axial direction was expressed by the effects of electrostatic forces and air flow on fibers in axial direction which overcomes the effect of rotational speed [72]. Generally, middle layer of native vessel called the *tunica media* bears the majority of the tension under in vivo conditions while the outermost layer called the *tunica adventitia* serves to protect the vessel from outer stretch. Based on the model created by Lu et al. [116] it was found out that in radial direction, the *tunica media* and the *tunica adventitia* of coronary artery bear 62 ± 13 % and 38 ± 13 % of tension, respectively [116]. Therefore, multilayer designs were created to overcome requirements of the vascular grafts in separate layers by varying fiber diameter, fiber orientation, fiber tensile properties, polymer type or their combinations. In a study of Vaz et al. [51], mechanical characterization test results indicate that PCL/PLA bilayer graft has 4 times higher maximum stress (4.3 ± 0.2 MPa) with lower elongation at break values (47 ± 6.3%) owing to the presence of PLA oriented outer layer compared to PCL single layer scaffolds composed of randomly distributed fibers (1.2 ± 0.1 MPa, 260%) [51].

In this study, PCL (45,000 Mn and 80,000 Mn) and PLC (70/30 molar ratio) single layer scaffolds composed of either oriented or randomly distributed fibers were produced in 6 mm tubular forms by electrospinning. Then, bilayer scaffolds from the same polymers in different combinations including randomly distributed inner layers and radially oriented outer layers were produced. Their morphologies before and after mechanical tests were analyzed and discussed within the context of their mechanical performance. The mechanical test results were discussed based on used polymer type composition, fiber orientation and composite effect of each layer of bilayer grafts. Produced fibrous scaffolds were tested with smooth muscle cells to test biocompatibility of presented scaffolds.

5.2 Experimental

5.2.1 Polymers

Polycaprolactone (PCL, Mn 45,000, Sigma-Aldrich) was dissolved in chloroform/ethanol (C/E: 9/1: v/v, Penta) at a concentration of 18% w/v solution, while Polycaprolactone (PCL, Mn 80,000, Sigma-Aldrich) was dissolved in

chloroform/ethanol/acetic acid (C/E/AA: 8/1/1: v/v/v, Penta) at a concentration of 10% w/v solution. A copolymer, Poly L-lactide Caprolactone (PLC, molar ratio 70/30 PLA/PCL, PURASORB) was dissolved in chloroform/ethanol/acetic acid (C/E/AA: 8/1/1: v/v/v,) at a concentration of 10% w/v. Since chloroform evaporates very fast, polymeric solution could get concentrated quickly. The addition of ethanol into the electrospinning solution slows down the evaporation rate of chloroform and improves the spinnability of polymers [47]. Acetic acid was used to improve the spinnability of highly viscose polymers (PCL Mn 80,000 and PLC). Avoiding polymer degradation, all solutions were stirred for 3h and then immediately electrospun [38].

5.2.2 Electrospinning

The custom-designed electrospinning apparatus consists of a positive high voltage power supply (Matsusada Precision Inc., Kusatsu, Shiga, Japan), a syringe pump (Model NE-1000x; New Era Pump Systems Inc., Farmingdale, NY, USA), a 10 ml plastic syringe, a hypodermic needle with 0.8 mm and 1.2 mm inner diameters and length 30 mm, and a grounded stainless steel rotating mandrel (6 mm diameter, 200 mm length). The distance between the needle tip and the mandrel was set to 200 mm. Reciprocal movement of the needle spinning electrode was achieved using a pneumatic linear actuator while rotating mandrel was empowered by a rotary toll with a flexible shaft (with a rotational speed range 5,000 to 35,000 min⁻¹). A scheme and a photograph illustrating the electrospinning apparatus is shown in Figure 5.1.

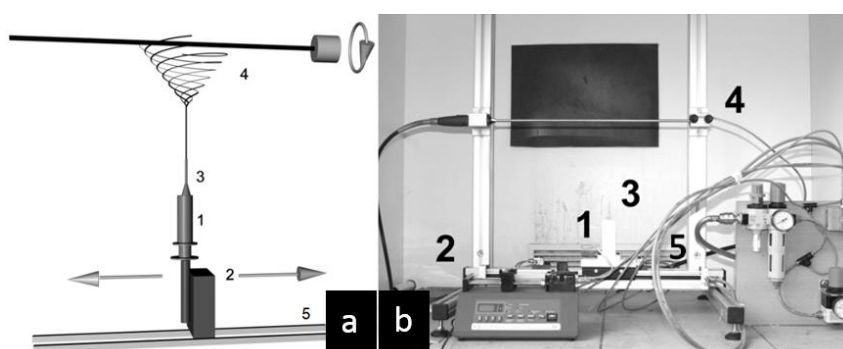


Figure 5.1 : Diagram (a) and picture (b) of the electrospinning apparatus: A high voltage is applied between the needle tip (3) and the mandrel type collector (4). The polymer solution is delivered to the needle tip at a constant feed rate using a syringe pump (1) attached to the sliding unit (5). An electric field is generated by the high voltage power supply (2). Finally a continuous web is collected on the rotating mandrel (4) in a tubular form.

5.2.3 Tubular scaffold fabrication

The scaffolds were produced using custom-designed electrospinning apparatus. The solution components and production parameters for each scaffolds are listed in Table 5.1. Finally, the tubular graft was left to dry overnight at room temperature (20 ± 3 °C). Fibrous tubes could be removed, took off from the mandrel by sliding manually. After removal from the mandrel, the specimens were cut up for various analyses as described in the following sections.

Table 5.1 : The solution components and production parameters.

Codes		Layers	Polymer Type	Solvent system [v/v ratio]	Concentration [%]	Feed rate [ml/h]	Voltage [kV]	Distance [cm]	Collector diameter [mm]	Rotational speed [min ⁻¹]	Needle Diameter[mm]	Production time [min]
SINGLE LAYER	PCL45_R	-	PCL (45,000Mn)	C:E (9:1)	18	1	10	20	6	5,000	0.8	10
	PCL45_O	-	PCL (45,000Mn)	C:E (9:1)	18	1	10	20	6	15,000	0.8	10
	PCL80_R	-	PCL (80,000Mn)	C:E:AA (8:1:1)	10	2	15	20	6	5,000	0.8	10
	PCL80_O	-	PCL (80,000Mn)	C:E:AA (8:1:1)	10	2	15	20	6	15,000	0.8	10
	PLC_R	-	PLC	C:E:AA (8:1:1)	10	2	10	20	6	5,000	1.2	10
	PLC_O	-	PLC	C:E:AA (8:1:1)	10	2	10	20	6	15,000	1.2	10
BILAYER	PCL45+45	Inner Layer	PCL (45,000Mn)	C:E (9:1)	18	1	10	20	6	5,000	0.8	5
		Outer Layer	PCL (45,000Mn)	C:E (9:1)	18	1	10	20	6	15,000	0.8	10
	PCL45+80	Inner Layer	PCL (45,000Mn)	C:E (9:1)	18	1	10	20	6	5,000	0.8	5
		Outer Layer	PCL (80,000Mn)	C:E:AA (8:1:1)	10	2	15	20	6	15,000	0.8	10
	PCL45+PLC	Inner Layer	PCL (45,000Mn)	C:E (9:1)	18	1	10	20	6	5,000	0.8	5
		Outer Layer	PLC	C:E:AA (8:1:1)	10	2	10	20	6	15,000	1.2	10
	PLC+PCL45	Inner Layer	PLC	C:E:AA (8:1:1)	10	2	10	20	6	5,000	1.2	5
		Outer Layer	PCL (45,000Mn)	C:E (9:1)	18	1	10	20	6	15,000	0.8	10
	PLC+PCL80	Inner Layer	PLC	C:E:AA (8:1:1)	10	2	10	20	6	5,000	1.2	5
		Outer Layer	PCL (80,000Mn)	C:E:AA (8:1:1)	10	2	15	20	6	15,000	0.8	10

Fistly, single layer tubular scaffolds were produced from all above mentioned polymeric solutions. Then, bilayered grafts were fabricated by various combinations of polymers and electrospinning conditions. Inner layers were produced for a shorter

time of electrospinning (5 minutes) and lower rotational speeds ($5,000 \text{ min}^{-1}$) were used for fabrication in order to obtain thin layer of non-oriented fibers. The outer layers were electrospun for 10 minutes using higher rotational speed of $15,000 \text{ min}^{-1}$ that provide thicker layer of oriented fibrous structure.

5.2.4 Morphological analysis

Morphology of the scaffolds was observed under scanning electron microscope (TESCAN Vega 3SB Easy Probe, Brno, Czech Republic) after sputter coating with gold alloy. For sample preparation, tubular scaffolds were cut and unfolded into a planar form for assessment of each of layer (inner and outer). Moreover, 5 mm length tubular samples were cut to observe cross-sections of tubular scaffolds. Average fiber diameters of single layer scaffolds were measured by Image J Software from 100 randomly selected fibers of 3 different scanning electron microscope (SEM) images. Fiber orientation of single layer scaffolds was analyzed by Image J Software, Fiji Directionality plugins. Fiber orientation distribution was fit and plotted as line graphs from -90° to $+90^\circ$.

5.2.5 Mechanical characterization

Mechanical characterization of the tubular scaffolds was carried out using a tensile tester LabTest 4.050 (LaborTech, Opava, Czech Republic) and custom designed bursting pressure apparatus. The thickness of the samples was measured using a digital thickness gauge (NT – Nove Technologie, Prague, Czech Republic). Tensile testing was performed on scaffolds in both planar and tubular forms. For planar samples, the tubular scaffolds were cut and unfolded into a planar form. Five specimens were prepared in $18 \text{ mm} \times 10 \text{ mm}$ dimensions and tested in both axial and radial directions. For tubular samples, three tubes were cut in 10 mm length and tested in axial direction. The strain rate and distance between gauges were set to 50 mm.min^{-1} and 8 mm, respectively.

For textile materials, the burst strength is usually applied in a planar form. However, this embodiment is not suitable for the testing of tubular samples due to their limited dimensions. For this reason, a custom method and a device that creates an internal pressure by the inflation of the flexible thin wall sleeve were developed. The sleeve has a diameter lower than the inner diameter of the vascular graft. Therefore the vessel can be slipped over the sleeve. Both are put on the hollow mandrel that has a perforated

part located in the central part. The ends of the mandrel are closed, in one of them is located an air inlet. Vascular graft samples are fixed by fixation members located on closed ends. It secures that the sleeve is inflated only in central part of the mandrel and it prevents to air leakage between the mandrel and sleeve. Due to this the sleeve expands only in the tested sample and not in their open ends. The assembly of testing setup is seen in Figure 5.2.

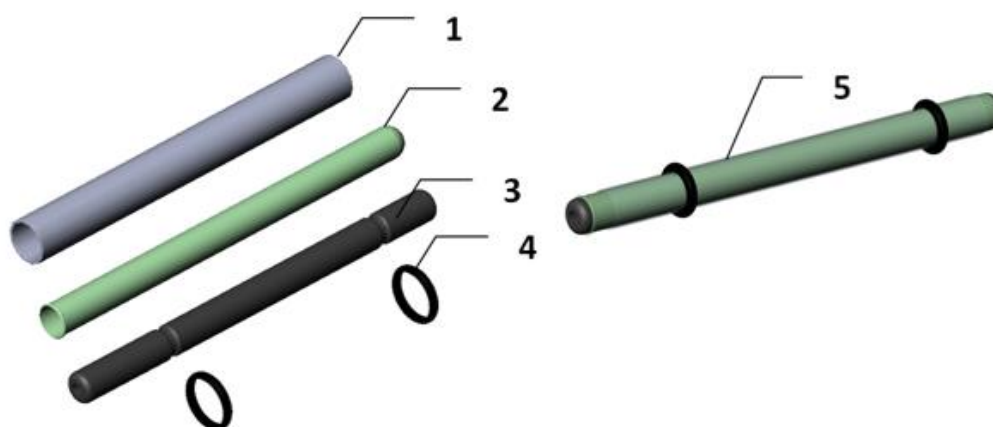


Figure 5.2 : Assembly of measuring section (1) Vascular graft, (2) rubber inflatable sleeve, (3) hollow mandrel, (4) fixation members, (5) assembly.

The device allows testing the pressure to a maximal pressure of 12,000 Torr. In the mode of lower pressures it is maximally 4,500 Torr. This range was used after trials with the samples of artificial vessels and it seems to be sufficient.

For this test, five tubular samples were cut in 60 mm length. Each of the sample was placed to the sleeve and then hollow mandrel as mentioned above which allow sleeve inflating and pressure transfer directly to the sample. The pressure is gradually increased by the precise pressure regulator until rupture of vascular graft. Average values with standard deviations were given.

5.2.6 Smooth muscle cell culture

Single layer tubes (PCL45_R, PCL45_O, PCL80_R, PCL80_O, PLC_R and PLC_O) were tested *in vitro* using smooth muscle cells. Tubular samples were unfolded and cut into 6 mm circular discs that were placed in the 96-well plate. Samples were sterilized by immersion in 70% ethanol for 30 minutes followed by double washing in phosphated buffer saline (PBS; Lonza, Basel, Switzerland)). Coronary artery smooth muscle cells (CASMC; Lonza) in passage 7 were seeded on the samples in

concentration of 2×10^4 cells/well. The cells were cultured in SmGMTM-2 BulletKitTM (Lonza). Materials were incubated for 7 days at 37 °C and 5 % CO₂.

For observation of cellular shape, indirect immunostaining with phalloidin-fluorescein isothiocyanate (FITC) conjugated antibody was performed. Phalloidin binds to F-actin therefore it enables the visualization of actin filaments within the cells. Briefly, scaffolds seeded with cells were washed twice with PBS and fixed using 2.5% glutaraldehyde (Sigma Aldrich) in PBS. Then, scaffolds were washed twice in PBS and incubated in 0.2% Triton X-100 (Sigma Aldrich) in PBS at room temperature for 30 minutes. Antigen blocking was done by incubation with 1% bovine serum albumin (Prolabo, Radnor, Pennsylvania, USA) in Triton X-100 solution for 30 minutes at room temperature. Then, incubation with phalloidin-FITC antibody (Sigma Aldrich, dilution 1:1000) was done for 30 minutes in the dark. After double washing in PBS, counterstaining of cell nuclei was done with 2-(4-amidinophenyl)-1H-indole-6-carboxamide (DAPI 1 mg/ml, dilution 1:1000, Sigma Aldrich) that binds to double stranded DNA in cell nuclei. Stained cells cultured on fibrous scaffolds were observed by inverted microscope Nikon ECLIPSE Ti-E/B (Minato, Tokyo, Japan).

5.3 Results and Discussion

5.3.1 Scaffold fabrication

Both single layer (PCL45_R, PCL45_O, PCL80_R, PCL80_O, PLC_R and PLC_O) and bilayer (PCL45+45, PCL45+80, PCL45+PLC, PLC+PCL45, PLC+PCL80) tubular scaffolds with lengths up to 100 mm, inner diameters of 6 mm, and wall thicknesses in a range of 200-300 μ m were successfully fabricated. The length of the scaffolds was controlled by adjusting the length of the reciprocal movement of the spinning electrode. The wall thickness of the scaffolds was controlled by the duration of the electrospinning. Fiber orientation was controlled with rotational speed of the mandrel.

5.3.2 Morphological analysis

In Figure 5.3, SEM images of single layer scaffolds together with fiber orientation distributions are illustrated. According to SEM images, all scaffolds are free of beads and composed of continuous micro fibers. Moreover, it is clearly observed that

randomly distributed fibers were produced at $5,000 \text{ min}^{-1}$ while radially oriented fibers were produced at $15,000 \text{ min}^{-1}$.

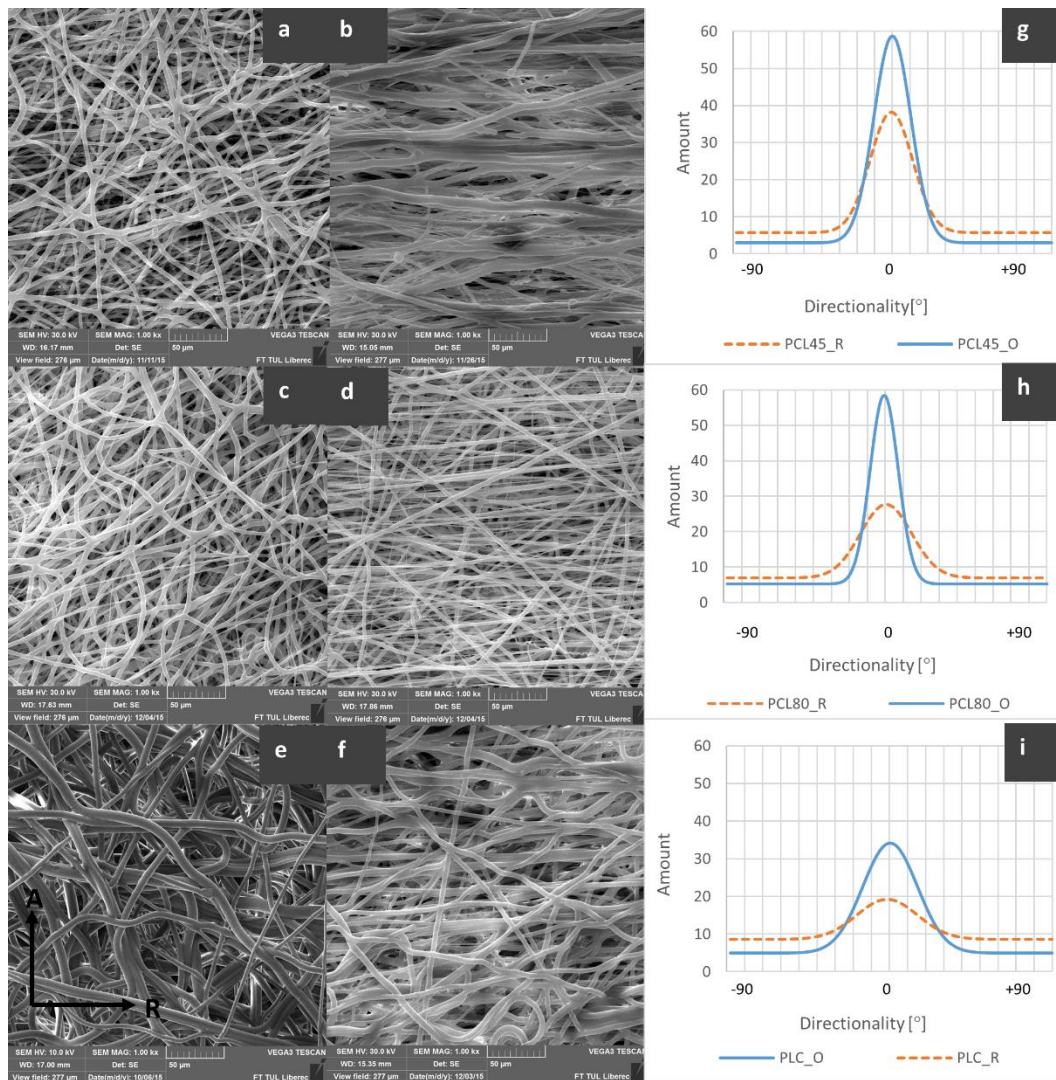


Figure 5.3 : SEM images of single layer scaffolds: (a) PCL45_R, (b) PCL45_O, (c) PCL80_R, (d) PCL80_O (e) PLC_R, (f) PLC_O (scale bar a-f: $50 \mu\text{m}$) with coordinate system indicating R: radial direction; A: axial direction; fiber directionality distributions of (g) PCL45, (h) PCL80, (i) PLC plotted between -90° to $+90^\circ$ whereas 0° indicates the direction of radially oriented fibers. Dotted line represents production at $5,000 \text{ min}^{-1}$ while continuous line represents production at $15,000 \text{ min}^{-1}$. PCL, polycaprolactone; PLC, poly(l-lactide) caprolactone.

The rate of fiber orientation depends also on polymer type, for example PCL45_R is slightly oriented also when $5,000 \text{ min}^{-1}$ are used for fabrication while PLC displays wide range of orientation even with high rotational speeds of $15,000 \text{ min}^{-1}$ are used. Moreover, it is seen in Figure 5.3 (g-i) that either at low rotational speeds, fibers are slightly oriented for all samples. Especially for sample PCL45_R, the fibers possess preferable orientation at $5,000 \text{ min}^{-1}$ (Figure 5.3(g)). In a study of Yalcin et al. [47] it

was also stated that PCL (45,000 Mn) fibers are able to be oriented at $5,000 \text{ min}^{-1}$ by using 6 mm diameter collector [47]. In contrast to PCL45, the most remarkable fiber orientation difference between $5,000$ and $15,000 \text{ min}^{-1}$ was measured for PCL80_R and PCL80_O (Figure 5.3(h)). This can be explained by higher molecular weight of PCL. In a study of Edwards et al. [45], 120 m.min^{-1} linear velocity was defined as sub-limit (since fibers are not exposed to mechanical stress) for getting radial fiber orientation for PCL (80,000 Mn). It was also expressed that optimum fiber orientation was achieved at 150 m.min^{-1} while it rised to maximum level at 300 m.min^{-1} [45]. In this study, $5,000 \text{ min}^{-1}$ (94.2 m.min^{-1}) is below the defined limit (120 m.min^{-1}) that is required for fiber orientation while $15,000 \text{ min}^{-1}$ (282.6 m.min^{-1}) is found very close to the linear velocity defined as maximum orientation level (300 m.min^{-1}). For PLC samples, less fiber orientation in comparison to PCL45 and PCL80 samples at $15,000 \text{ min}^{-1}$ was achieved which may be resulted from thicker fiber diameters.

As seen in Table 5.2, average fiber diameters of PCL45 and PCL80 decreased with increasing rotational speed i.e., improved fiber orientation. The reduction in fiber diameters (from $4.02 \pm 1.22 \text{ }\mu\text{m}$ to $3.47 \pm 0.88 \text{ }\mu\text{m}$ for PCL45 and from $2.96 \pm 0.88 \text{ }\mu\text{m}$ to $2.50 \pm 0.85 \text{ }\mu\text{m}$ for PCL80) can be explained by the existence of mechanical stress [45]. On the other hand, average fiber diameter of PLC_O ($4.59 \pm 1.42 \text{ }\mu\text{m}$) is slightly higher than PLC_R ($4.01 \pm 1.01 \text{ }\mu\text{m}$).

Table 5.2 : Fiber diameters of single layer scaffolds.

Codes	Fiber Diameters $\pm$ SD [μm]
PCL45_R	4.02 ± 1.22
PCL45_O	3.47 ± 0.88
PCL80_R	2.96 ± 0.88
PCL80_O	2.50 ± 0.85
PLC_R	4.01 ± 1.01
PLC_O	4.59 ± 1.42

The reason can be explained by elastic behaviour of PLC polymer as shown later. PLC fibers are oriented at $15,000 \text{ min}^{-1}$ radially but since the fibers are very elastic they recover themselves after production which results in a wavy structure (Figure 5.4). The recovery of the PLC fibers after production can lead to thicker fiber diameters.

Bilayered scaffolds were produced in order to mimic the native structure of blood vessel. Therefore inner layer was composed of randomly distributed fibers and the thickness was lower compared to the outer layer adjusting by the electrospinning time

(5 min). Outer layer was made from oriented fibers such as collagen and elastin fibers in native vessel responsible for mechanical performance.

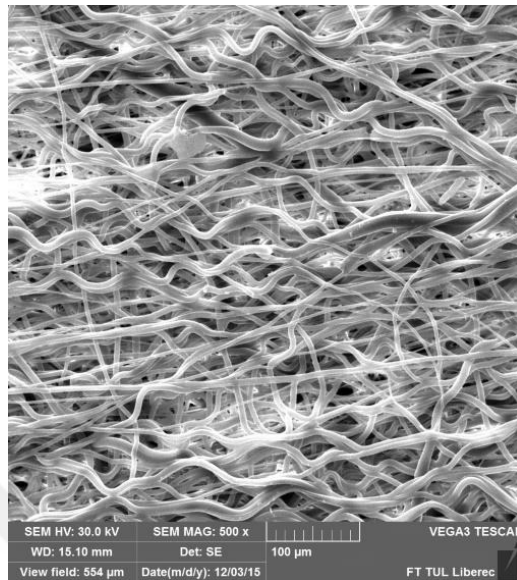


Figure 5.4 : Wavy continuous PLC fibers oriented in radial direction (Scale bar: 100 μm).

The outer layer was electrospun for longer time leading to higher thickness of the final bilayered graft. The total thickness of resulting bilayered grafts was about 200-300 μm depending on the spinnability of the polymeric solutions. SEM images of bilayer scaffolds are illustrated in Figure 5.5. According to the images, inner layers composed of randomly distributed fibers and outer layers composed of radially oriented fibers were successfully produced with different polymer type combinations. From cross-sectional views, it is also possible to distinguish two separate layers of related scaffolds. Only in Figure 5.5(a3) it is difficult to note two different layers since this scaffold was composed of the same polymer PCL45+45 with various fiber orientations. Moreover, for electrospun PCL, it is difficult to obtain cross-section since the fibers are very weak and became easily destroyed when the force is applied. Therefore the cross sectional views of PCL are not clear such as in case of PLC.

On the other hand, two separate layers can be seen in Figure 5.5(c3) which indicates that the usage of PLC as an outer layer caused adherence problem with inner layer. During the production it was observed that PLC detached the mandrel easier than PCL. Thus, it shows similar behavior and do not hold on the inner layer like mandrel as well.

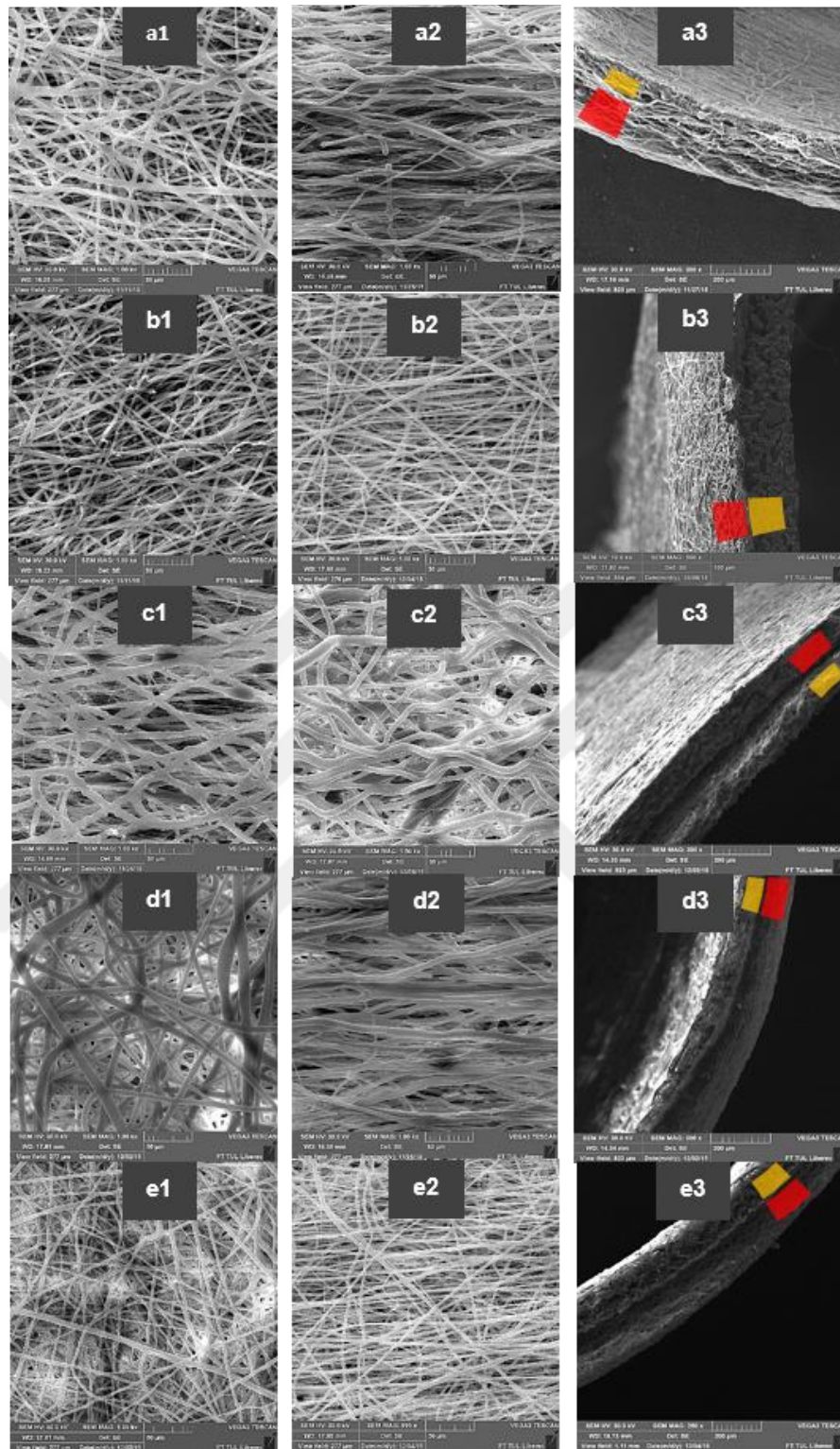


Figure 5.5 : Bilayer scaffolds: PCL45+45 (a1) inner, (a2) outer, (a3) cross-sectional; PCL45+80 (b1) inner, (b2) outer (b3) cross-sectional; PCL45+PLC (c1) inner (c2) outer (c3) cross-sectional; PLC+PCL45 (d1) inner, (d2) outer, (d3) cross-sectional; PLC+PCL80 (e1) inner, (e2) outer, (e3) cross-sectional. (Scale bars of inner and outer layers 50 μm , cross-sectional views 200 μm). In crosssectional views, inner layers were highlighted with yellow color while outer layers were highlighted with red color. PCL, polycaprolactone; PLC, poly(l-lactide)caprolactone.

5.3.3 Mechanical characterization

Ultimate tensile strength and elongation at break test results are given in Figure 5.6. PCL45_O and PCL45_R have similar tensile behaviour due to the fiber orientation analysis results (Figure 5.6(a)). Because the PCL45 fibers are also oriented at low rotational speeds, both PCL45_O and PCL45_R planar samples have higher ultimate tensile strength in radial direction than the planar and tubular samples tested in axial direction. Both tubular and planar samples of PCL80_R have around 3 MPa ultimate tensile strength which assists the fiber orientation test results (Figure 5.6(b)). Similarly, PLC_R samples have around 2.5 MPa ultimate tensile strength in both planar (axial and radial direction) and tubular samples owing to randomly distributed fibers (Figure 5.6(c)). On the other hand, PCL80_O has the highest ultimate tensile strength (6.7 ± 2.8 MPa) tested in radial direction among all other scaffolds with comparatively lower (80%) elongation at break (Figure 5.6(b, e)). Beside polymer type, fiber orientation has crucial effect on tensile strength and elongation at break results. When the fibers are aligned well in the direction of test, they resist against to the applied force all together which result in higher tensile strength. Elongation at break depends on the polymer type and elasticity of the fibrous structure under stress. When the fibers are aligned well in test direction, the elasticity of the fibrous structure is mostly determined with polymer type. On the other hand, when fibers are randomly oriented, there are many joints which holds the structure under stress thus results in higher values of elongation at break [47]. The highest elongation at break were achieved for PLC samples in all sample forms and testing directions due to its improved elastic properties (Figure 5.6(f)). Especially tubular and planar samples of PLC_O possess above 900% elongation at break.

All planar and tubular samples of bilayer scaffolds tested in axial direction had higher elongation at break and lower tensile strength than all planar samples tested in radial direction which indicates that well oriented outer layers achieved in bilayered scaffolds determine the mechanical performance of final graft (Figure 5.7).

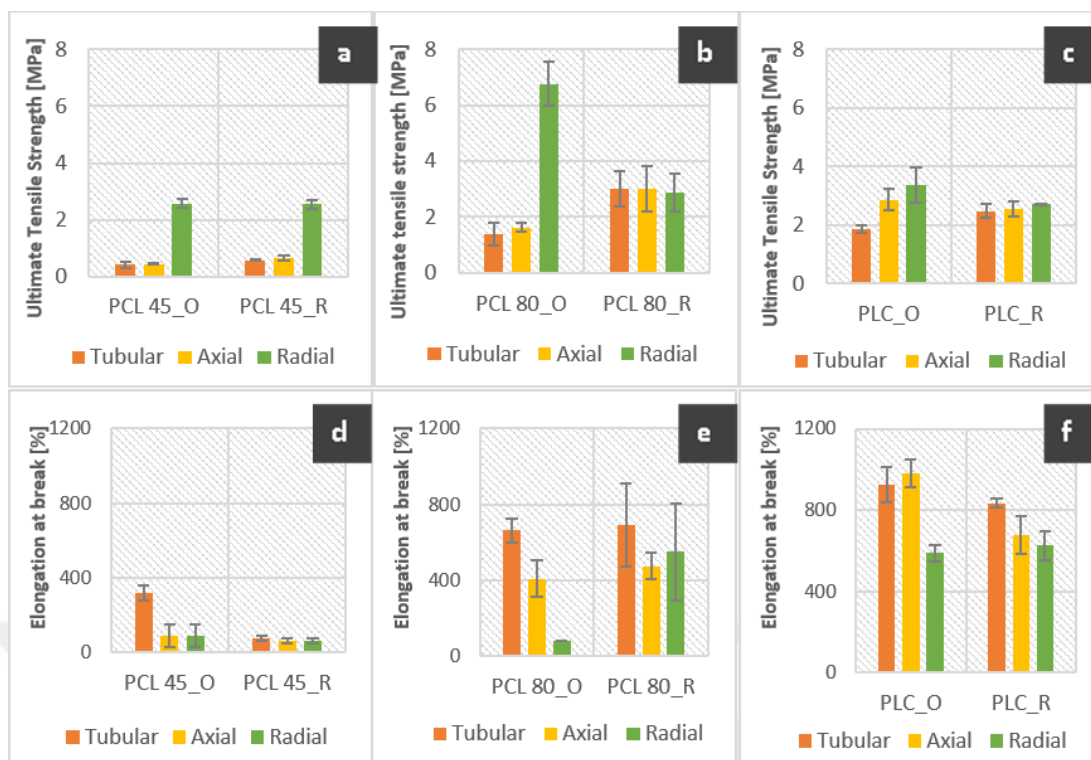


Figure 5.6 : (a) Ultimate tensile strength [MPa] of (a) PCL45, (b) PCL80, (c) PLC; and elongation at break [%] of (a)PCL45, (b)PCL80, (c) PLC single layer grafts. PCL, polycaprolactone; PLC, poly(l-lactide)caprolactone.

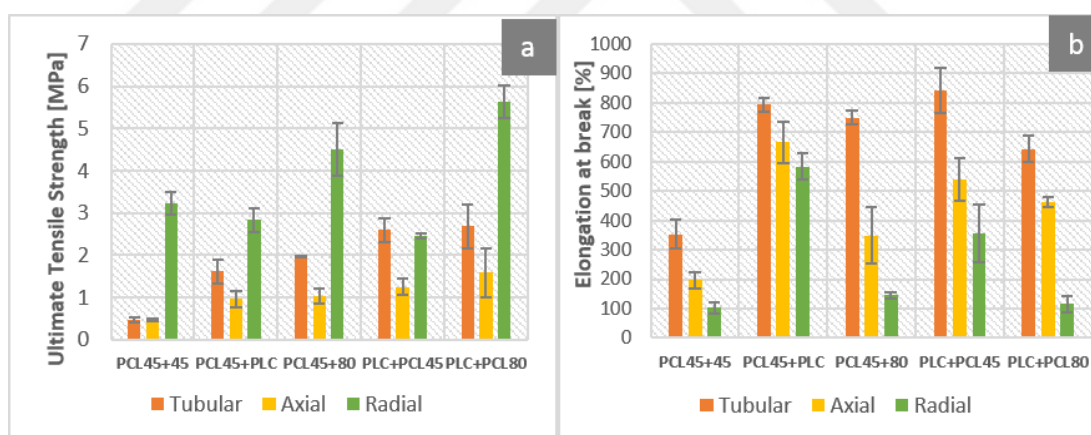


Figure 5.7 : (a) Ultimate tensile strength [MPa], (b) Elongation at break [%] of bilayered grafts. PCL, polycaprolactone; PLC, poly(l-lactide)caprolactone.

Planar and tubular samples of PCL45+45 tested in axial direction had the lowest tensile strength (around 0.5 MPa) among other scaffolds. This outcome is probably related to the lower molecular weight of electrospun polymer that determines the mechanical properties. On the other hand, PCL45+80 had remarkably higher tensile strength in all samples/tested directions than PCL45+45 which expressed that the thicker PCL80_O outer layer influenced the role in tensile performance in a positive way towards higher values of tensile strength and elongation at break (Figure 5.7 (a)). PCL45+PLC tested

in radial direction had the highest strain value due to its well radially oriented outer PLC layer. Although fibers well oriented in test direction results in poor elasticity, wavy continuous PLC fibers allow improved elongation under low tension in radial direction (Figure 5.7 (b) and 5.8 (b)).

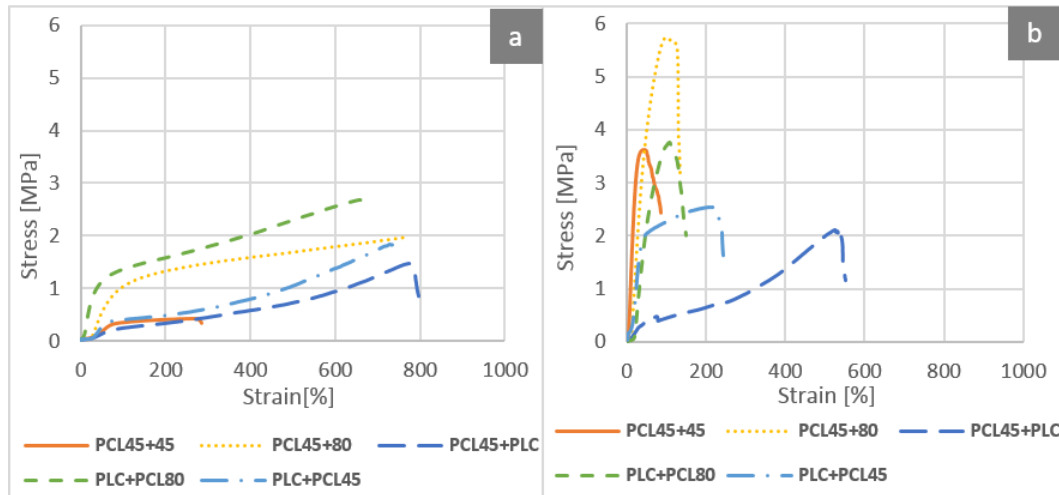


Figure 5.8 : Stress-Strain curves of (a) tubular samples tested in axial direction (b) planar samples tested in radial direction. PCL, polycaprolactone; PLC, poly(l-lactide)caprolactone.

Bilayer scaffolds with PLC inner layers have limited strain values tested in radial direction with the presence of PCL45 and PCL80 outer layers (Figure 5.8 (b)). When PCL45+45, PLC+PCL45 and PCL45+PLC were taken into consideration, the presence of PLC can be noticed in mechanical properties as without PLC, PLC inner layer (thin layer) and PLC outer layer (thick layer). With the increase of PLC amount, the mechanical behaviour of bilayered scaffolds come closer to the mechanical behaviour of pure PLC polymer; higher strain under lower stress (Figure 5.8 (b)). When PLC+PCL80 and PLC+PCL45 are compared, it is seen that the strain value of PLC is limited with outer layers (Figure 5.8 (a, b)). On the other hand, higher tensile strength of PCL80 can be noticed in PLC+PCL80 (5.7 MPa in radial test direction) in comparison with PLC+PCL45 (2.5 MPa in radial test direction) (Figure 5.7(a)). In the literature, it is possible to find mechanical characterization data for several types of native vessels. For instance, in a study of Wei et al. [117] it is stated that abdominal aorta has 1.47 MPa tensile strength in axial direction and 5.29 MPa in radial direction which indicate the oriented fibrous collagen bundles in the tunica media. Lee et al. [71] stated that porcine coronary artery has 2.7 MPa tensile strength with 100% elongation at break. Similarly, 1-2 MPa peak stress with 63-76% elongation at break

was stated for femoral arteries by Sell et al. [5]. It is possible to say that except PCL45+45 bilayer scaffolds based on its poor mechanical performance, all scaffolds have tensile strength in range of 1–2 MPa tested in planar form / axial direction, 1.5–2.7 MPa tested in tubular form / axial direction and 2.5 –5.7 MPa tested in planar / radial direction, which meet the expectations of native vessels. Moreover, all elongation at break data of produced bilayered scaffolds are similar (around 100%) or many times higher (up to 900%) than the requirements.

In Figure 5.9, by comparing the inner layer, outer layer and bilayer scaffolds, composite effect was examined. According to the results, the behaviour of the bilayer scaffolds except PLC+PCL45 was found very close to the behaviour of oriented outer layers. Especially, PCL45+45 bilayer scaffold has very similar behaviour with PCL45_O. On the other hand, although thinner PLC_R is used in inner layer composed of randomly distributed fibers, it has notable effect on mechanical performance of PLC+PCL45. The reason is that PCL45 has very poor mechanical properties either with randomly distributed or oriented fibers thus the stress-strain curve of PLC+PCL45 is closer to the PLC_R rather than PCL45_O.

When the morphology of the tubular sample tested in axial direction is observed under SEM after tensile testing, it is seen that the radially oriented outer layer with less joints in test direction caused separations leading to creation of gaps in outer layer while the inner PLC_R layer is still supporting the outer layer and sharing the applied stress with its higher mechanical strength and elasticity properties (Figure 5.10(a)). Within the use of high strength PCL80_O in outer layer, PLC+PCL80 shows improved stress (2.7 MPa) with 650% adequate strain at breakage as combination of two polymers.

The morphologies at the broken edges of scaffolds after tensile testing are illustrated in Figure 5.10(b-d). As a result of an improved elasticity, the broken edge of PLC recovered itself well like cut by a scissor in contrast to PCL45 and PCL80. The deformation of PCL45 and PCL80 edge with reduced fiber diameter can be observed in Figure 5.10(c,d).

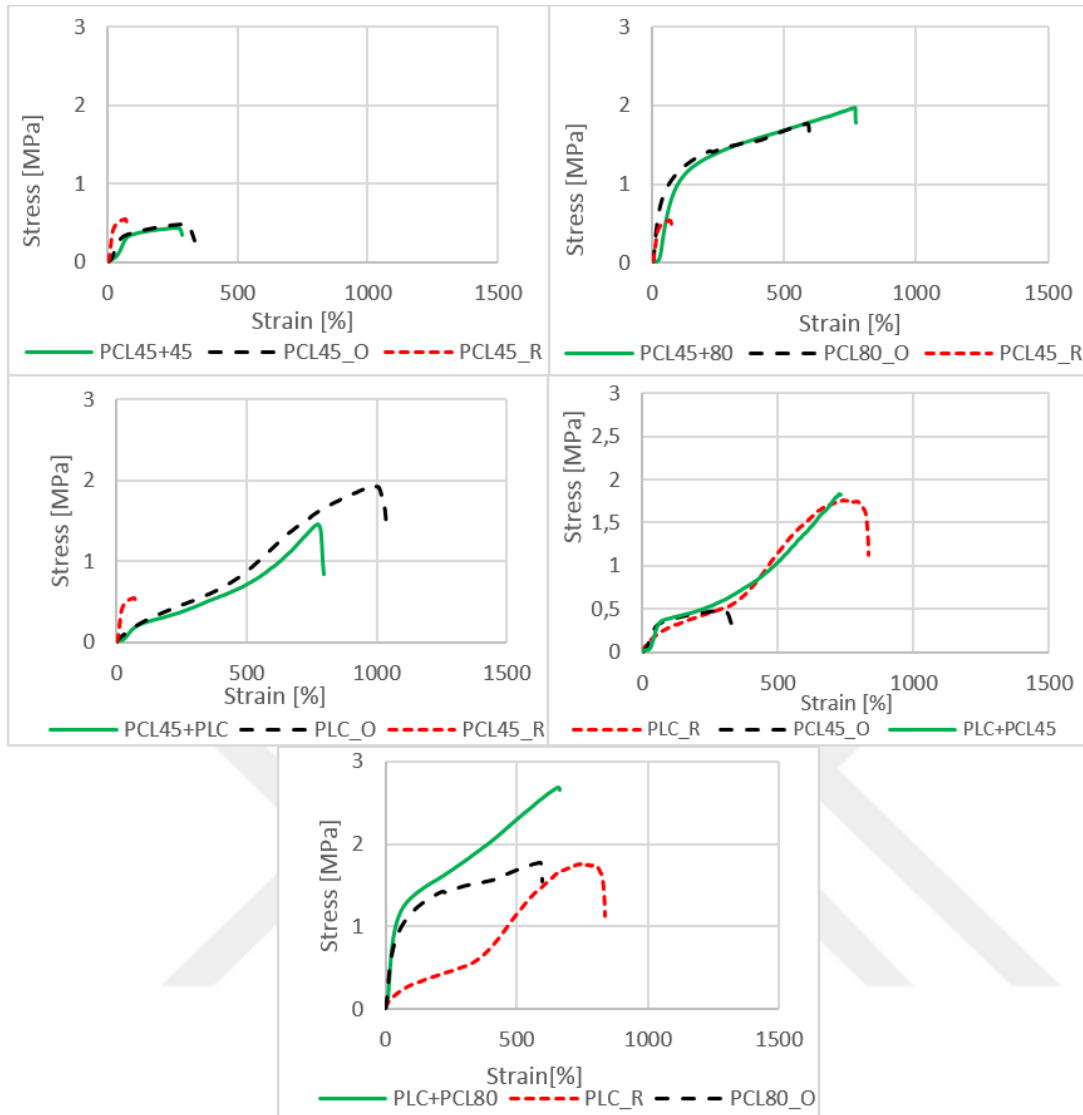


Figure 5.9 : Stress-Strain curves of bilayer scaffolds and their single layer components (pure polymer random, inner layer and pure polymer oriented, outer layer) PCL, polycaprolactone; PLC, poly(l-lactide)caprolactone.

Burst pressure test results of single layer scaffolds are given in Figure 5.11 (a). All samples of PCL 45_R bursted at average 675 mmHg while all samples of PCL45_O bursted at average 795 mmHg.

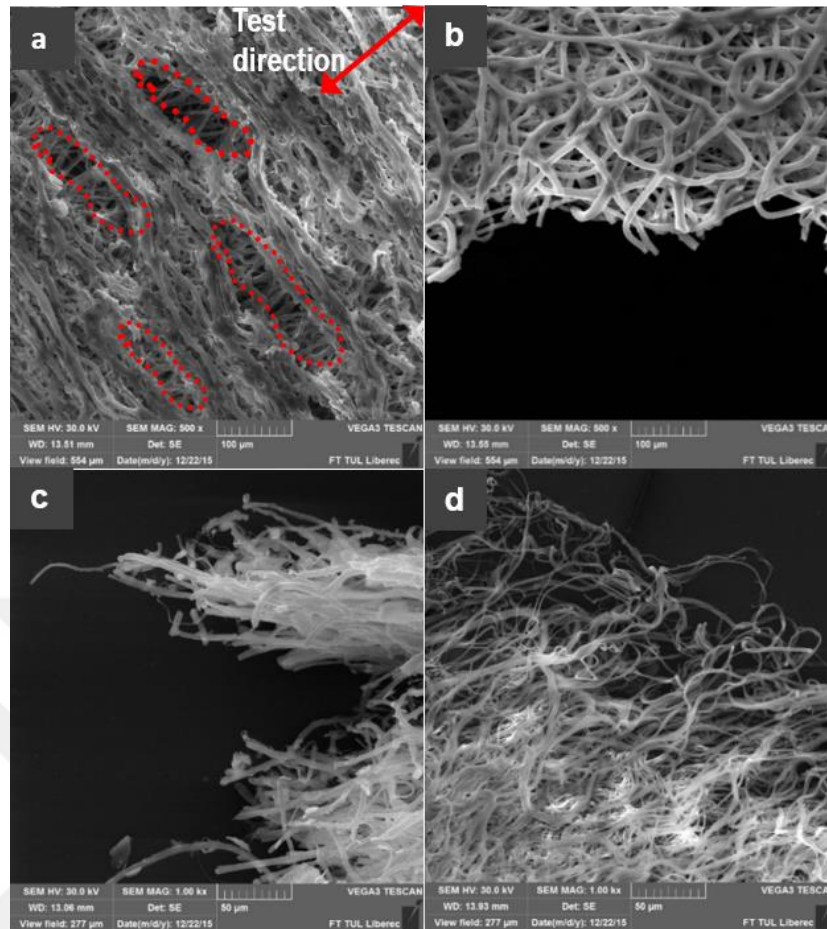


Figure 5.10 : (a) Fracture patterns of PLC+PCL45 tubes: Outer oriented PCL45 fibers separate in parts and then break while inner PLC elongates without any damage, (b) broken edge of randomly distributed PLC inner layer (tested in axial direction; Scale bar: 100 μm). Broken edges of oriented (c) PCL45 and (d) PCL80 layers (tested in radial direction, Scale bar: 50 μm). PCL, polycaprolactone; PLC, poly(l-lactide)caprolactone.

The result can be explained by radially oriented fibers resistance to burst at lower pressures. For PCL 80_R, although the average burst pressure was measured as 975 mmHg, some of the samples showed aneurysm at lower pressures (525 mmHg). The reason for forming an aneurysm could be non-uniform wall thickness of the tubular scaffolds. On the other hand, the inner layer could be slightly destroyed by pushing from the mandrel or during placing on a sleeve in sample preparation of burst pressure test which result in aneurysm. It is thought that aneurysm starts from thinner wall part of the scaffold. Since the burst pressure of PCL80 is not low, randomly distributed fibers resist to the applied pressure as they can. During this, fibers get thinner in diameter and cause a thin layer which results in aneurysm followed by graft bursting [22].

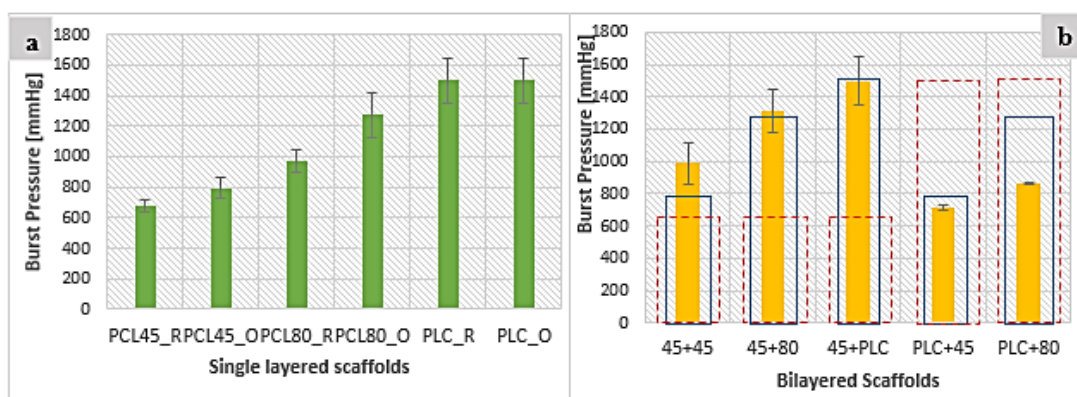


Figure 5.11 : Burst pressures [mmHg] of (a) single layer scaffolds, (b) bilayer grafts [■] in comparison with single layered grafts produced by the same pure polymer of outer layer of relevant bilayered grafts [□] at 15000 min^{-1} and inner layer of the relevant bilayered grafts [□] at 5000 min^{-1} . PCL, polycaprolactone; PLC, poly(l-lactide)caprolactone.

PCL80_R sample before and after burst pressure test is illustrated in Figure 5.12(a,b). According to the SEM image, the reduction in fiber diameter after burst pressure test was observed. Since the elasticity of PCL80 is not as high as PLC it can not recover itself and results in non-uniform scaffold diameter after burst pressure test (Figure 5.12(c)).

On the other hand, PCL 80_O samples bursted at pressures at average 1,275 mmHg. Higher burst pressures of PCL80 in comparison to PCL45 supporting the results of tensile strength analysis. PLC_R and PLC_O samples formed a bulge at lower pressures (562 mmHg) based on its highly elastic behaviour but bursted at average 1,500 mmHg. Figure 5.11 (b) shows burst pressures of bilayer scaffolds and their single layer components (related random inner layer and oriented outer layer). For PCL45+45, higher burst pressure was recorded than PCL45_R and PCL45_O. For PCL45+80 and PCL45+PLC, burst pressures of the bilayer scaffolds are determined by relatively high strength outer layers.

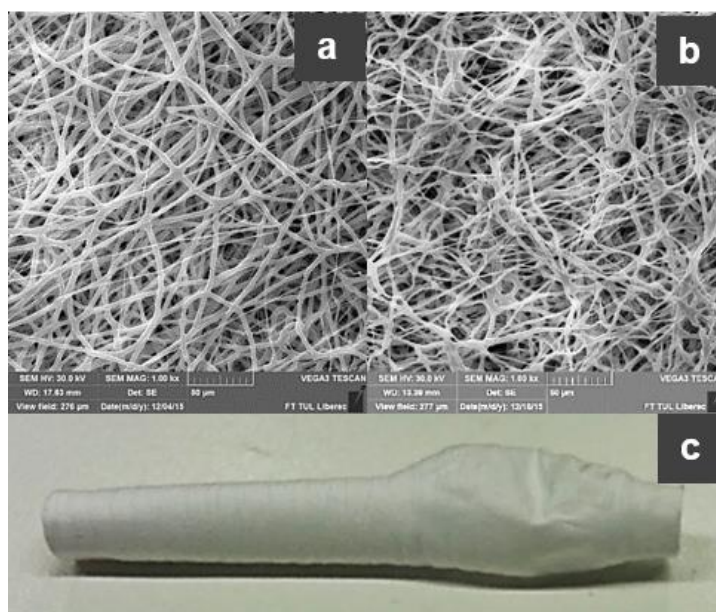


Figure 5.12 : SEM images of PCL80_R: (a)before burst pressure testing (b)after testing (aneurysm part) (Scale bar: 50 μ m) (c) Macro images of aneurysm part along the length of scaffold.

5.3.4 Smooth muscle cell culture

Smooth muscle cells cultured on single layered scaffolds adhered to the fibrous structure and proliferated through the surface of all the tested microfibrillar layers. The cellular morphology was influenced by scaffold structure as depicted in Figure 5.13.

In randomly oriented fibrous structure, the cells were also randomly distributed. In case of oriented fibers, cells became elongated in the same direction as fibers. In agreement with fiber orientation results, PCL 45 was oriented in both tested samples so as the smooth muscle cells (Figure 5.13 (a, b)). The most remarkable difference in cell orientation was seen in the samples made from electrospun PCL_80. In randomly distributed structure, cells were spread in all directions while in oriented sample the cells maintain elongated shape in the fiber direction (see Figure 5.13 (c, d)). Cells cultured on electrospun copolymer PLC were randomly distributed with slight orientation in the sample PLC_O as depicted in Figure 5.13 (e, f).

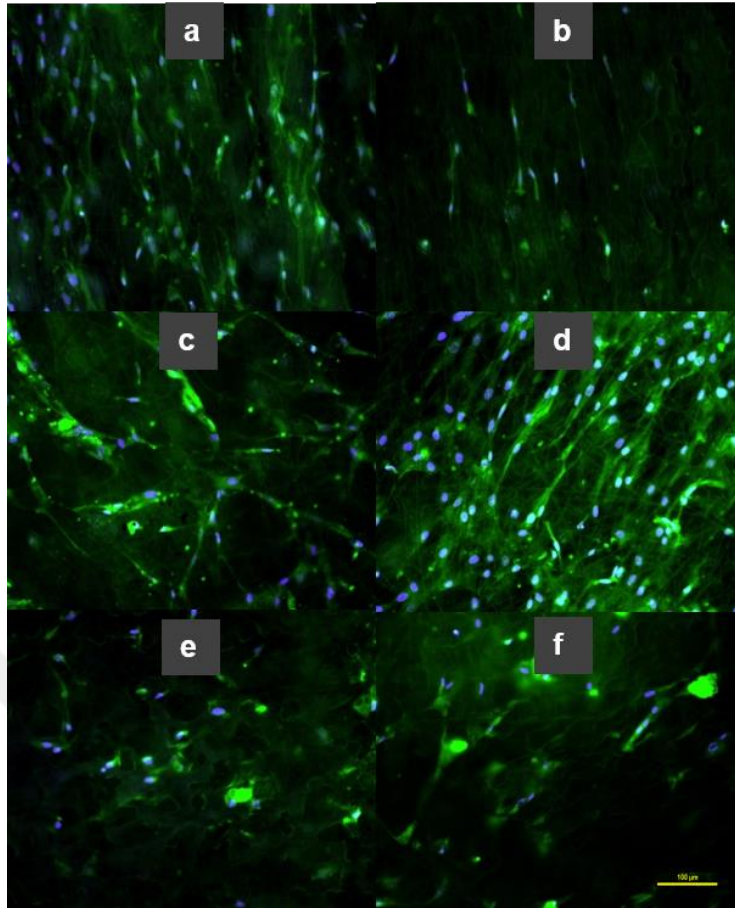


Figure 5.13 : Fluorescence microscopy pictures of coronary artery smooth muscle cells stained with phalloidin-FITC (green) and DAPI (blue) after 7 days of incubation with randomly distributed fibers (a) PCL45_R, (c) PCL 80_R, (e) PLC_R) and oriented fibers (b) PCL45_O, (d) PCL 80_O, (f) PLC_O), scale bar 100μm. PCL, polycaprolactone; PLC, poly(l-lactide)caprolactone.

5.4 Conclusion

In this study, 6 mm diameter single layer and bilayer tubular scaffolds were successfully produced from biodegradable polymers by electrospinning. For each polymer type, fiber orientation was achieved at $15,000 \text{ min}^{-1}$ (282.6 m.min^{-1}) of which was found in an expected range via literature survey. At $5,000 \text{ min}^{-1}$ (92.4 m.min^{-1}), except PCL45, randomly oriented fibers were obtained. Fiber diameter was reduced with increased fiber orientation for PCL45 and PCL80 based on extra stress applied on fibers while PLC reacts in opposite. Since PLC fibers are extremely elastic, they cover themselves easily in oriented samples after production which results in fiber diameter increase. On the other hand, as stated design parameters for vascular grafts in the literature [118], polymer type and fiber orientation have crucial effect on tensile strength and elongation at break results. Tensile strength of PCL45_O, PCL80_O and

PLC_O samples (2.2 MPa, 6.7 MPa and 3.1 MPa) tested in radial direction reach the maximum values of the same polymer type. On the other hand, mechanical characteristics of bilayer scaffolds were found under the influence of outer oriented layer in especially PCL45+45, PCL45+80 and PCL45+PLC scaffolds. For PLC+PCL45 and PLC+PCL80, the dominant PLC characteristics were observed in mechanical evaluations. Similarly, outer oriented layers improves the burst pressures of the scaffolds. For PCL45+PLC scaffolds, 1500 mmHg burst pressure was achieved due to its well oriented outer PLC layer. According to the cell culture test results, smooth muscle cells seeded onto the scaffolds show cell orientation through radially oriented fibers which means the cells copy the scaffold structure. In native vessels, smooth muscle cells are radially oriented therefore oriented fibrous structures serves as ideal scaffold for vascular tissue engineering applications. In conclusion, bilayer scaffolds rather than single layer scaffolds enable to play with the adjustments of design parameters in its layers from material selection to structural modifications and more closely allow the creation of the most appropriate scaffold comply with adjacent native vessel functionalities.

5.5 Acknowledgements

Authors are thankful to Technical University of Liberec, Department of Nonwovens and Nanofibrous Materials for laboratory opportunities. Authors also would like to thank Jakub Vojtech for assisting vascular graft production and analysis; Ondrej Nemecek for designing burst pressure testing apparatus and realizing burst pressure tests.

5.6 Fundings

This work was supported by the [Ministry of Health of the Czech Republic] under Grant [number 15-29241A]; [TUBITAK (The Scientific and Technological Research Council of Turkey)] under Grant [number 214M089]; and [Istanbul Technical University, Scientific Research Projects] under Grant [number BAP-37678].



6. CONCLUSIONS AND RECOMMENDATIONS

This thesis is composed of four supplementary articles. All of these articles bring novelty to the literature by offering innovations in assessment of design parameters, establishment and modification of production or testing technologies and scaffold designs for vascular grafts.

In the first review article, design parameters for electrospun biodegradable vascular grafts were discussed in details. Although, it is obvious to mimic the target tissue, the histology of the native blood vessel has a crucial significance when determining these design parameters for vascular grafts. Therefore, to draw general boundaries for future attempts at vascular graft design, it can be said that the *tunica intima* should promote endothelialization to achieve nonthrombogenicity. Since it is not stated that a nano scaled pore size is a must for endothelialization, its advantages in hindering blood leakage and allowing a fluent monolayer of endothelial cells on lumina make its use worthy of note when designing a multilayer graft. Therefore limited size pores can be preferred for this layer, which can also be modified with heparin bonding to prohibit platelet activation. Against the *tunica intima*, the *tunica media* should have larger pore sizes than smooth muscle cells in order to provide a porous layer for their diffusion through the wall thickness. Therefore, the layer with micro fibers can be electrospun, including larger pores that enable the diffusion of smooth muscle cells through the *tunica media*. Besides adjusting the fiber diameter to control the pore size, there are other techniques for electrospun fibrous scaffolds, such as the gas forming/salt leaching technique that creates larger pores to allow cell diffusion. Since the *tunica media* is the layer responsible for the mechanical performance of the vessel, improved fiber orientation is required in this layer for sufficient tensile properties in order to withstand the blood pressure after implantation. By adjusting the rotational speeds of the rotating mandrel in the light of the sub-limits determined in the literature, extreme radial fiber orientation can be achieved. Moreover, positioned metal plates can be used to modify the collector system of the electrospinning technology, which improves the radial fiber orientation. However, the reduced compliance properties with improved radial fiber orientation should also be taken into consideration. In this respect, the wall

thickness and the selection of materials in each layer also become important parameters to obtain adequate tensile strength with improved radial elasticity to overcome compliance mismatch of the scaffold with the sewn vessel. Moreover, the degradation rate of the polymer should be studied for multilayer biodegradable fibrous scaffolds in order to determine the minimum time that is required for biodegradable scaffolds to allow cells to synthesize their own proteins before the biological degradation of the scaffold. Therefore, the final objectives of designing a vascular graft should be the degradation of the scaffold after a sufficient time of implantation by leaving its place to an autologous vessel which is similar to native vessel.

Owing to these defined and discussed design parameters (fiber diameter, pore size and porosity, fiber orientation, wall thickness and material selection), multilayer designs seem to be more advantageous for vascular graft production in order to create a composite structure with differing functions and capabilities in separate layers, as it is in native arteries. Therefore the outcomes of this review lead us to decide the selection of production technique and polymer type together with other construction variables of multilayer scaffold design in the following parts of the thesis.

Second article involves mainly the optimization process of electrospun PCL (45,000 Mn) fibrous webs and analysis of its morphology based on described design parameters: fiber diameters, pore size and porosity. In this article, PCL fibrous webs were produced by basic electrospinning technique from different solvent systems and polymer concentrations. Mainly chloroform:ethanol solvent system was used for 14-20 % PCL concentrations. Next, acetic acid and formic acid were added in increased amounts to selected 18% PCL/ chloroform: ethanol solution system separately to examine their effects on morphology of the produced fibrous webs. Viscosity and electrical conductivity of the solutions with different polymer concentrations and acid additions were also investigated before production. According to the results, viscosity was found under influence of polymer concentration. The increase in polymer concentration results in solution viscosity increment. On the other hand, with the addition of highly polar formic acid to the chloroform:ethanol solvent system, the electrical conductivity of the solution was increased.

Planar fibrous webs were produced from 18% PCL/chloroform: ethanol solution the average fiber diameter of which is 2.22 μm . On the other hand, the addition of any acetic or formic acid solvents to PCL/chloroform: ethanol solution caused a notable

drop in fiber diameters (for acetic acid up to 158 nm, for formic acid up to 256 nm). Pore size and porosity of the samples were examined by using Image J Software. According to the results, it was seen that fiber diameter is an effective parameter on pore area and porosity of the fibrous webs. The evaluated pore area for the sample produced from 18% PCL/chloroform: ethanol solution was $10.21 \mu\text{m}^2$ but it reduced almost 50 times and reached around $0.2 \mu\text{m}^2$ for samples where 80 μl and 120 μl acetic and formic acids were added to the same solution system. It was explained by fiber diameter decrement, i.e. acidic solvent additions as also mentioned under design parameters title of the first review article. According to pore area analysis results, calculated pore area of micro fibrous webs is still not sufficient ($10.21 \mu\text{m}^2$) to allow smooth muscle cells' diffusion for vascular grafts. As stated in the literature, the pore size must be larger than $10 \mu\text{m}$ to let cell diffusion which corresponds to $78.5 \mu\text{m}^2$ pore area that is required according to the circular pore size estimation. However, it should be considered that Image J software is based on thresholding technique in which pore area and porosity values are calculated from surface of the SEM images rather than whole thickness of the fibrous webs. Other pore size evaluation techniques will also be studied in following parts of the thesis to give different point of views on pore size analysis.

In addition to all, FTIR analysis was done in the second article to observe if there is any residual solvent on the web after production. According to the FTIR data, fibrous webs produced from %18 PCL/chloroform:ethanol solution shows very similar peak characteristic with PCL granules differing from pure chloroform solvent. None characteristic peaks of chloroform (3034 cm^{-1} /CH stretch, 1220 cm^{-1} / CH wag, 774 cm^{-1} and 680 cm^{-1} /CCl₃ stretches) were found in any of tested fibrous webs.

This article let us focus on how to control fiber diameter, pore size and porosity of PCL (45,000 Mn.) fibrous webs based on solution variables (polymer concentration, solution viscosity and solution conductivity) and production parameters. Moreover it serves an alternative solvent systems for PCL (45,000 Mn) in order to achieve nanofibers which can be used in various medical applications such as vascular grafts where ECM is required to be mimicked for endothelial cells' attachment and proliferation. Therefore addition of acetic acid or formic acid to PCL/chloroform: ethanol solutions can be used where nanofibrous PCL webs are required especially for tissue designs or specifically multilayer vascular grafts due to its less hazardous

properties. However, next studies of the thesis does not include the use of nano PCL layer as tunica intima since it has difficulties especially in tubular scaffold production. Since the nano fibrous surfaces to be used in tunica intima should be very thin (according to the histological analysis of native artery stated in either first or third articles), it creates problems while removing it from the rod of the rotating type collector without damage. Moreover, it is not easy to achieve the same morphological structure with using custom designed modified electrospinning instead of basic electrospinning system. However, 18% PCL/chloroform:ethanol solution system was chosen for single layer designs in the third article while the insufficient mechanical property problems of PCL (45,000 Mn) were tried to be solved in the fourth article with multilayer designs with using different polymers (PCL 80,000 Mn and PLC).

The third article involves unique results on investigation of histological analysis of native artery, modification of electrospinning technology, achievement of radial fiber orientation for 6 mm diameter tubular scaffolds, comparison of two techniques for measuring pore size and porosity of the fibrous scaffolds and productions of single layer designs for vascular grafts.

According to the results, microscopic images of histological analysis of native artery reveal endothelial cells are arranged through a single layer covering the luminal surface, whereas smooth muscle cells are organized into numerous layers inside the *tunica media*. From the structural proteins, elastin, which gives elasticity of the vessel, was found densely in the lamina elastica interna, as well as the *tunica media* and the *tunica adventitia*. It was also observed that fibrous collagen bundles oriented in a radial manner appear in micrometer scale. Since it is obvious to mimic the target tissue as much as possible, this part of the article is pioneer in innovation thus it enlightens many of design parameters for vascular grafts discussed in the first review article such like, multilayer artery structure, cell composition and distribution through these layers, presence of structural proteins in these layers, morphology of oriented collagen bundles and wall thickness and so on. Therefore, it takes the first step on the way to build a multilayer structure for vascular grafts.

On the other hand, the electrospinning apparatus was modified in order to produce the tubular scaffolds with the desired structural properties. Since it is not possible to reach high rotational speeds with standard motors, a rotary tool was adapted to the apparatus to empower the 6 mm rotating type of collector. To lower down the vibration in high

speeds, damping matings were located to the connecting pieces of the apparatus. Moreover homogenous scaffolds were produced by moving feeding unit in reciprocal direction by the help of pneumatic system. With this custom designed electrospinning, radial fiber orientation was achieved for small diameter tubular scaffolds without need for further production steps like winding from large diameter unfolded sheets or using metal plates stated as alternative techniques for radial fiber achievements in the first article.

After completing histological analysis and establishment of custom designed electrospinning unit, 6 mm diameter polycaprolactone (45,000 Mn) single layer scaffolds were produced composed of 2 μm diameter fibers similar to that of ECM fibers such as collagen bundles as documented by the SEM images of histological analysis. In the light of the histological analysis of native vessel in this article and defined design parameters in the first article, fiber orientation was studied as one of the crucial parameters in designing vascular scaffolds, i.e. the *tunica media*. In order to investigate fiber orientation in the radial direction, the rotational speed of the collector was varied from 250 to 15,000 min^{-1} . Oriented electrospun fibers were achieved at 5000 min^{-1} (94.2 $\text{m} \cdot \text{min}^{-1}$) and 10,000 min^{-1} (188.4 $\text{m} \cdot \text{min}^{-1}$) by using a 6 mm diameter mandrel type of collector. The prevailing fiber orientation in the radial direction was improved with the increasing rotational speed up to 15,000 min^{-1} (282.6 $\text{m} \cdot \text{min}^{-1}$). The other discussed design parameters were pore area and porosity. Based on the findings of the histological analysis, it was observed that smooth muscle cells are present in the *tunica media*. To enable smooth muscle cells growth into the structure, pore sizes should have sufficient sizes ($> 10 \mu\text{m}$; for circular pore estimation it corresponds to more than $78.5 \mu\text{m}^2$ pore area) that would permit adequate cellular infiltration. In this article, pore area was examined by using two different techniques: NIS Elements Software and Bubble Method. According to evaluated results, lowest pore size area was achieved at 250 min^{-1} for both techniques (NIS Elements: $8.60 \mu\text{m}^2$; Bubble Method: $49.27 \mu\text{m}^2$) which seems to be appropriate to be used in tunica intima to prevent blood leakage while oriented layers with higher pore area (NIS Elements: $29.40 \mu\text{m}^2$; Bubble Method: $93.83 \mu\text{m}^2$) could enable cell growth into the multilayer structure. The difference of results produced at the same rotational speeds is resultant from the measuring principles of these two techniques explained in this article in details. This is another reason why oriented layers seemed to be more appropriate for

the *tunica media*. Moreover, the NIS Elements Software pore area analysis results ($8.60 \pm 2.98 \mu\text{m}^2$ at 250min^{-1}) support the results of Image J Software pore area analysis results ($10.21 \pm 5.12 \mu\text{m}^2$ with static collector) in the second article.

According to the mechanical performance test results, tensile strength of the scaffolds at 250 min^{-1} was found in the range of 0.35–0.5 MPa which shows no significant difference with direction. On the other hand, tensile strength of the scaffold in the radial direction was improved (around 1 MPa) by obtaining radial fiber orientation. However, it caused a distinct drop in its elongation at break (50–70%). Tensile strength of natural abdominal aorta stated in the literature was 1.47 MPa in longitudinal and 5.29 MPa which prove radially oriented fibrous collagen bundles in the structure. It is understood that to match the requirements for native vessel, mechanical properties of the scaffolds must be improved. Therefore, design of a multilayer structure seems to be appropriate to mimic mechanical as well as morphological properties of native vessel.

Biocompatibility of PCL was found sufficient by MTT test using 3T3 fibroblasts, while morphological analysis of seeded samples under fluorescence microscope and SEM confirms that cells are aligned along the fibers. Since all these mechanisms contribute to normal cell behavior, specialized cell types (ECs and SMCs) need to be tested in provide more realistic simulations.

Although many design parameters were successfully examined in this third article, future attempts was directed at design of a multilayer scaffold to mimic native vessel, improving the radial elasticity, and seeding the graft with specialized cell types.

The context of the fourth article was mainly defined based on the future attempts of third article. To design a bilayer scaffold, previously discussed design parameters were studied together. Beside PCL (45,000 Mn), new polymers such as PCL (80,000 Mn) and PLC were introduced in this article to overcome inadequate mechanical properties of PCL 45,000 Mn stated in the third article. Moreover, in histological analysis of a native artery, it was found that radially oriented collagen bundles with micro sized diameters were observed in *tunica media*. To mimic the native artery, bilayer designs were produced with randomly distributed inner layers produced at $5,000 \text{ min}^{-1}$ (92.4 m.min^{-1}) and radially distributed outer layers produced at $15,000 \text{ min}^{-1}$ (282.6 m.min^{-1}). Here, polymer types were varied in layers for several combinations in order to use the exact polymer characteristics in appropriate layer. Similar to the results of the third

article, PCL (45000 Mn) was also oriented in radial direction either at 5000 min^{-1} . However, owing to the use of higher molecular weight PCL and copolymer PLC were used in this study, the sub-limits for rotational speeds to achieve fiber orientations was increased. This subject was also discussed in the first review article under design parameters title. It was stated that, polymer type affects on fiber orientation rates produced at the same rotational speeds. Therefore in the fourth article fiber orientation analysis was done to all produced scaffolds to understand the effect of polymer type.

On the other hand, as stated design parameters for vascular grafts in the literature, polymer type and fiber orientation have crucial effect on tensile strength and elongation at break results. Tensile strength of oriented single layer PCL (45,000 Mn), PCL(80,000 Mn) and PLC samples (2.2 MPa, 6.7 MPa and 3.1 MPa) tested in radial direction reach the maximum values of the same polymer type. On the other hand, mechanical characteristics of bilayer scaffolds were found under the influence of outer oriented layer in especially PCL45+45, PCL45+80 and PCL45+PLC scaffolds. For PLC+PCL45 and PLC+PCL80, the dominant PLC characteristics were observed in mechanical evaluations. Due to the given literature results, porcine coronary artery has 2.7 MPa tensile strength with 100% elongation at break values, femoral arteries have 1–2 MPa peak stress with 63–76% elongation at break values; abdominal aorta has 1.47MPa tensile strength in axial direction and 5.29 MPa in radial direction. It is possible to say that except PCL45 + 45 bilayer scaffolds based on its poor mechanical performance, all scaffolds have tensile strength in range of 1–2 MPa tested in planar form/axial direction, 1.5–2.7 MPa tested in tubular form/axial direction, and 2.5–5.7 MPa tested in planar/radial direction, which can be considered that it meets the expectations of native vessels. Moreover, all elongation at break data of produced bilayered scaffolds is similar (around 100%) or many times higher (up to 900%) than the requirements. Similarly, outer oriented layers improves the burst pressures of the scaffolds. For PCL45+PLC scaffolds, 1500 mmHg burst pressure was achieved due to its well oriented outer PLC layer. In this article, a new custom design burst pressure testing apparatus for vascular grafts was used to test for produced single layer and bilayer scaffolds. On the other hand, the investigation of “composite effect” of bilayer scaffolds in comparison to related single layer components on mechanical properties is the fundamental originality of the research.

With regards to the histological analysis of native artery and previously stated attempts, cell culture of the scaffolds were realized via smooth muscle cells. Cells seeded onto the scaffolds show cell orientation through radially oriented fibers which means the cells copy the scaffold structure. In native vessels, smooth muscle cells are radially oriented therefore oriented fibrous structures serve as ideal scaffold for vascular tissue engineering applications.

To sum up, bilayer scaffolds rather than single layer scaffolds enable us to play with the adjustments of design parameters in its layers from material selection to structural modifications and more closely allow the creation of the most appropriate scaffold that complies with adjacent native vessel functionalities. Up to now, various bilayer scaffolds were successfully produced taking design parameters into consideration and their morphological, structural, mechanical, biological properties were tested in order to compare its performance with the native vessels. Furthermore, the genuine outcomes were presented to the literature via discussed articles in this thesis. With regards to the outcomes of these four articles, significant improvements of inadequacies of small diameter vascular grafts were achieved in this thesis while serving a considerable step in pre-clinical applications of vascular grafts and providing a chance for uncured patients in the need of small diameter vascular graft implantation.

For the next studies, a custom designed bioreactor which allows related cell types seeding from both sides (endothelial cells from *luminal* side and smooth muscle cells from outer side) under dynamic conditions, should be established in order to stimulate the conditions of native blood vessels. Therefore, besides attachment and proliferation, the diffusion of smooth muscle cells through *tunica media* can be promoted under dynamic conditions as it is in native vessels. The diffusion of smooth muscle cells through the wall thickness can be observed in cross sectional views of produced vascular grafts under fluorescence microscope to compare it with the real cell types distribution through layers of a native artery. In order to approve pre-clinical applications, next steps of this study should involve *in vivo* tests.

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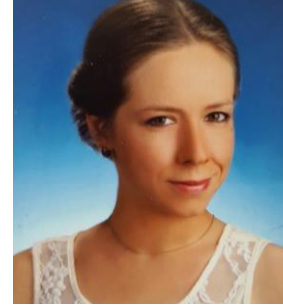
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