Seda GÜRDAP A BIOMIMETIC APPROACH FOR SMALL DIAMETER VESSELS: BILAYERED VASCULAR GRAFTS MADE OF ALGINATE E AGU 2019

A BIOMIMETIC APPROACH FOR SMALL DIAMETER VESSELS: BILAYERED VASCULAR GRAFTS MADE of ALGINATE and POLY(ε-CAPROLACTONE)

A THESIS
SUBMITTED TO THE DEPARTMENT OF BIOENGINEERING
AND THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF ABDULLAH GUL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER

By
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ACCEPTANCE AND APPROVAL

M.Sc. thesis titled ‘’ A Biomimetic Approach for Small Diameter Vessels: Bilayered Vascular Grafts Made of Alginate and Poly(ε-Caprolactone)’’ and prepared by Seda Gürdap has been accepted by the jury in the Bioengineering Graduate Program at Abdullah Gül University, Graduate School of Engineering & Science.

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(Date)

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ABSTRACT

A BIOMIMETIC APPROACH FOR SMALL DIAMETER VESSELS: BILAYERED VASCULAR GRAFTS MADE OF ALGINATE AND POLY(ε-CAPROLACTONE)

Seda GÜRDAP
MSc in Bioengineering
Supervisor: Prof. Dr. Sevil DİNÇER İŞOĞLU

December, 2019

Cardiovascular diseases (CVDs) still remain one of the leading causes of morbidity and mortality across the world. A typical symptom of CVDs is the vascular occlusion. There are many strategies for treatment such as angioplasty, stent application and bypass grafting. Although synthetic blood vessels are successfully used in bypassing of the medium (>10 and<6) and large sized (≥10 mm) vessels, they have high failure problem for the replacement of small diameter (≤ 6 mm) vessel because of early thrombosis formation. Tissue engineering, mimicking the structural, mechanical and cell growth characteristics of the native vessels is a promising treatment method for CVDs. In this study, it was aimed to fabricate a bilayered vascular scaffold by combining thermally induced phase separation and electrospinning methods. First, alginate porous layer was produced as the inner layer with the average pore diameter of approximately 100 μm to enable endothelial cell attachment and proliferation. Then, the inner layer was covered with electrospun polycaprolactone (PCL) membrane to strength the endurance of vascular graft. The mechanical test showed that the bilayered vascular scaffold has a close mechanical characteristic to native vessels with elastic modulus of 2 .45 ±1.7 MPa and estimate burst pressure of 0,18 MPa. Also, heparin was chemically immobilized to scaffold to elongate the release time, which can result in reduced thrombosis. In addition, cross-linked scaffold lost 21% of its mass for 6 weeks showed the moderate degradation level that can support the neotisue formation via cell migration to the scaffold, while the scaffold is synergistically degraded. According to the results, the materials prepared by biomimetical approach revealed that they have a great potential to be used as a synthetic vascular graft.

Keywords: Tissue engineering, Bilayered vascular grafts, Thermally induced phase separation, Electrospinning

ÖZET

KÜÇÜK ÇAPLI DAMARLAR İÇİN BİYOMİMETİK BİR UYGULAMA: ALJİNAT VE POLİKAPRALAKTON'DAN OLUŞAN ÇİFT KATMANLI DAMAR GREFTİ

Seda GÜRDAP
Biyomühendislik Yüksek Lisans
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Aralık, 2019

Kardiyovasküler hastalıklar halen dünya genelindeki hastalıkların ve ölümlerin önde gelen nedenlerinden biridir. Kalp damar hastalıklarının en tipik semptomu damar tıkanıklığıdır. Tedavide anjiyoplasti, stent kullanımı ve baypas grefti gibi birçok yöntem mevcuttur. Sentetik greftler geniş çaplı damarların baypasında başarılı bir şekilde kullanılmalarına rağmen, küçük çaplı damarlar için kullanımda erken dönemde tromboz oluşturdukları için başarısız olmaktadırlar. Doku mühendisliği, doğal damarın yapısal mekaniksel özelliklerini taklit etme ve hücre büyümesine olanak vermesinden dolayı kardiyovasküler hastalıklar için ümit verici bir tedavi yöntemidir. Bu çalışmada sıcaklıkla indüklenmiş faz ayrımı ve elektro eğirme fabrikasyon yöntemlerinin beraber kullanımı ile çift katmanlı vasküler doku iskelesinin üretimi hedeflenmiştir. Başlangıçta, yaklaşık olarak 100 μm por çapı ile iç katman olarak porlu aljinat tabakası üretilmiştir. Bu özellikleri ile porlu aljinat tabakası endotel hücre yapışmasına ve çoğalmasına olanak sağlamıştır. Daha sonra, vasküler doku iskelesinin dayanımını artırmak için aljinat iç tabaka, elektro-eğrilmiş polikaprolakton ile kaplanmıştır. Çift katmanlı vasküler doku iskelesinin 2.45 MPa elastik modülü ve 0,18 MPa patlama basıncı ile doğal damarın mekanik özelliklerini taklit edebileceği mekanik test ile gösterilmiştir. Ayrıca pıhtılaşma önleyici özelliği olan heparin doku iskelesine kimyasal olarak bağlanmıştır ve bu şekilde heparinin salım süresi uzatılmıştır. Bununla birlikte çapraz bağlanmış doku iskelesi 6 haftada gösterdiği yaklaşık %21'lik kütle kaybı ile uygun bir degradasyon profili sergilemiştir. Bu degradasyon miktarının, bozunma ile eş zamanlı olarak, doku iskelesine hücrelerin yapışması ile yeni oluşumuna olanak vereceği düşünülmektedir. Tüm bu sonuçlara göre, biyomimetik yaklaşımla hazırlanan malzemelerin sentetik damar grefti olarak kullanım potansiyeli oldukça yüksektir.

Anahtar kelimeler: Doku mühendisliği, Çift katmanlı vasküler greftler, Sıcaklıkla indüklenmiş faz ayrımı, Elektro eğirme

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To my dear family,

Chapter 1

1) Introduction

1.1 Cardiovascular System

The cardiovascular system is a multifunctional system where nutrients, gases, electrolytes are carried and waste products of metabolism such as urea and hormones are taken away. Cardiovascular system composed of the heart, blood and blood vessels (Figure1.1.1). The blood is transported to the body through blood vessels that surround like a network system to the whole body [48].

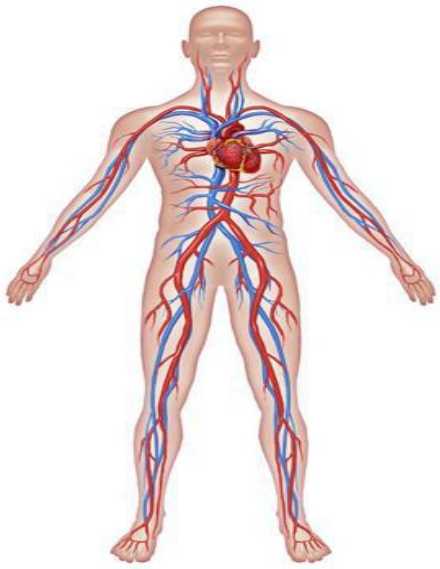


Figure 1.1.1 Scheme of Cardiovascular System [49]

1.1.1 Structure and Types of Blood Vessels

Blood vessels are responsible for the transportation of blood throughout the body. The essential types of vessels are the arteries, the capillaries, and the veins. Arteries generally carry the oxygenated blood away from the heart to the body except pulmonary arteries and umbilical arteries. Capillaries, which are the smallest vessels in size, allow the exchange of water, gases (CO_2, O_2) and chemicals between the blood and the tissues. Veins conduct deoxygenated blood from the tissue to the heart except for pulmonary veins. While capillaries made of a single endothelial layer, the arteries, and veins comprised essentially of three integrated layers which are called tunica intima, tunica media and tunica adventitia (Figure 1.1.1.1).

Tunica Intima

The inner layer of a vessel contacting the blood is called the tunica intima or also called tunica interna. Intima comprised of the specialized simple squamous monolayer of endothelial cells that is termed endothelium, which maintains vascular tone and hemostasis. The primary reason for blood formation is that endothelium gets damaged and then blood contacts with collagenous fibers. The basement membrane is next to the endothelial lining which is also called basal lamina. Basal lamina supports the binding of the endothelial layer and connective tissue effectively. The basement membrane gives strength and contributes to the maintenance of flexibility. Additionally, the basement membrane is permeable, enabling materials to pass through it. The outermost layer of tunica interna comprise of a couple of areolar connective tissue which composed of elastic fibers to give extra flexibility and some collagenous fibers to enable extra strength to vessels.

Tunica Media

The middle layer of the vessel wall is called tunica media consisting of smooth muscle cells that maintain vascular strength with the support of connective tissue which especially consists of collagenous elastic fibers. Collagenous fibers bind the tunica media to the intima layer and outer layer. Tunica media is commonly known as the thickest layer for arteries. When this layer in arteries and veins compares, the layer of arteries is thicker to endure higher pressure in arteries.

Tunica Externa

The tunica externa, tunica adventitia, is the outermost layer which is a substantial connective tissue that is mainly made up of collagenous fibers, a small number of elastic fibers and also groups of smooth muscle fibers. This layer is especially the thickest part of vessel vein and it is possibly thicker than tunica media in some larger arteries [50,51].

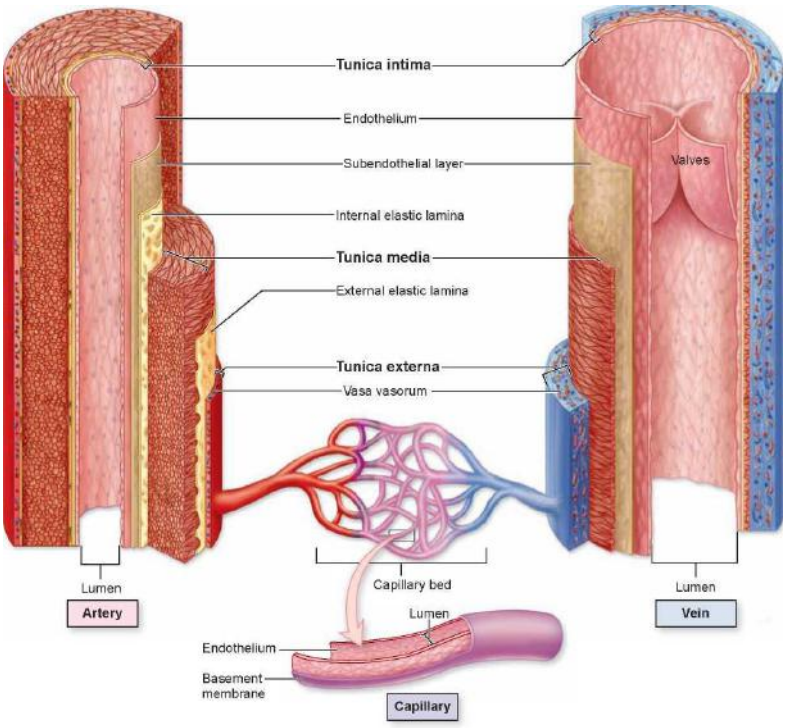


Figure 1.1.1.1 The intima, media and adventia for walls of a human artery, capillary and veins [52]

1.1.2 Types of Vessels

Arteries

Arteries are categorized into three types as elastic arteries (30mm to 5 mm), muscular arteries, (6 mm) and arterioles (>50 μm) depend on their size [2]. Elastic arteries and muscular arteries, which are mainly behave as distributors for transportation of the blood to arterioles. Arterioles also has same function in terms of distributors. However, they have additional function, which supports maintenance of

normal blood pressure and circulation. Therefore, they are sometimes termed as resistance arteries. Elastic arteries with thick wall are large vessels consisting collagen and elastin fibrin and the closest arteries to heart. Diameter of these arteries is larger than 10 mm. The muscular arteries comprise of relatively more smooth muscle and less elastic tissue as compared with elastic arteries. They are medium sized arteries found between elastic arteries and resistance vessels. Their diameter ranges from 0.3 mm to 10 mm. Arterioles are smallest arteries including mainly smooth muscle and a few dispersed elastic fibers in tunica media. Their dimeters are smaller than 0.3 mm [3,4].

Capillaries

Capillary (< 20 μm) are microscopic vessels which are placed between the small artery and veins [2]. While arteries and veins composed of three layers, capillaries composed of only one layer which made up of a thin basement layer consisting endothelial cells (Table 1.1.2.1).

Table 1. 1.2.1 Structure of Blood Vessels

Type of Vessel	**Tunica Intima (Endothelium)**	**Tunica Media (Smooth Muscle, Connective Tissue)**	**Tunica Externa (Collagenous Fibrous Tissue)**
Arteries	Smooth surfacing	Enables to construction and dilation Thicker than in veins	Allows flexibility that prevents collapse of vessel Thinner than tunica media
Veins	Smooth surfacing with seminular valves	Enables to construction and dilation Thinner than in arteries	Allows flexibility that prevents collapse of vessel Thinner than tunica media
Capillaries	Smooth lining	Absent	Absent

Veins

Veins are also known as collector or reservoir vessels. They not only carry blood back to the heart but they also accommodate the varying amount of blood which don't cause almost no change onto blood pressure because of their excellent potential to stretch. Veins commonly are made up of three walls similar to those of arteries.

Besides, the veins consist of valves that support movement of blood to only towards to heart without any back flowing.

According to their size, they are categorized into three group: large veins, medium sized veins, and venules. Venules support the transportation of blood from capillaries to medium and large sized veins.

Depend on diameter of their lumen, blood vessels are also classified into small diameter vessels (≤ 6 mm), medium-sized and large diameter vessel (≥10 mm) [5].

1.2 Cardiovascular Diseases

Cardiovascular disease (CVD) is used to define the name of disorders regarding heart and blood vessels. Conditions affecting heart include angina, arrhythmia, congenital heart disease, coronary artery disease, heart attack, heart failure, and others. In addition to heart problems, diseases that affect the vascular system especially include peripheral artery disease, aneurysm, atherosclerosis associated with a build-up of fatty deposits inside the arteries (Figure 1.2.1) and blood clotting disorders.

Many reasons such as lifestyle, genetic / family history of heart disease and are thought about the incidence of CVD diseases. As a consequence of lifestyle (smoking, eating unhealthy foods and being physically inactive), family history regarding CVD for genetic susceptibility, aging population, and others, cardiovascular diseases are the leading reason for death globally. World Health Organization announced that they estimated 17.9 million people died because of CVDs in 2016 corresponding to 31% of death worldwide for that year. Additionally, it has been estimated that more than 23.3 million people will annually die due to CVDs by 2030 [6, 7] .

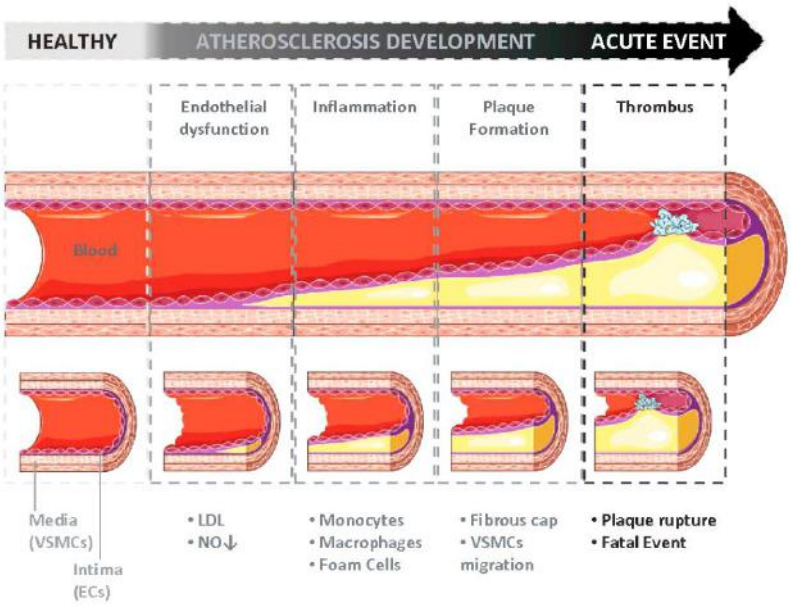


Figure 1.2.1 Illustration of atherosclerosis progressive development inside the artery [53]

1.3 Current Treatment Methods for CVDs

In the current treatment of vascular problems, there are some grafts to bypass blood vessels. These treatment methodologies are mainly categorized as using synthetic grafts and biological grafts [8]. Using artificial grafts fabricated using such polyethylene terephthalate (PET) and expanded polytetrafluoroethylene (ePTFE) materials is a promising treatment method for replacement of large and medium-sized vessel. However, synthetic grafts in bypassing for small diameter vessels are not used successfully because this using causes various complications: aneurysm, second operation necessity for especially pediatric patients, calcification and vascular occlusion because of intimal hyperplasia and thrombosis [9-11]. Biological grafts are the second group which is commonly used in vascular treatment. Biological grafts divide into three types: autologous grafts taken from another area of own patient (such as a saphenous vein, arm vein, mammalian artery, or radial artery), allografts taken the different individual of same species and xenografts taken from the different species. Autologous substitutes show less thrombogenesis and match with mechanical properties of the native vessel after implantation but they have a drawback because of source limitation. Allografts are favorable due to their biocompatibility and supporting with the potential for remodeling

of vascular tissue. Nevertheless, source limitation is a drawback of using these grafts. Xenograft has a huge capacity in greater supply with any sizes but the use of detergent to allowing decellularization and prevent immunogenetic host responses results in the less mechanical properties and the patient needs an afterward second surgical operation [8, 12, 13]. Due to disadvantages of existing methods, tissue engineering is a promising approach for the replacement of diseased small diameter vessels currently.

1.3.1 Tissue Engineering

The term, ‘’Tissue Engineering was introduced in 1993 by Robert S. Langer and Vacanti. Tissue engineering was defined as that it is the application of principles and methods of engineering and life sciences concerning the principal understanding of structure-function relationships in normal and pathologic mammalian tissue and the development of biological substitutes to restore, maintain, or improve function [7, 14]. The essential strategy for tissue engineering includes the design and fabrication of appropriate scaffolds for cell adhesion, proliferation, and differentiation [12]. Additionally, the production of extracellular matrix (ECM) is an expected goal in tissue engineering which supports the formation of the intended tissue via cell migration to tissue engineered scaffold, while the scaffold is synergistically degraded [6, 12].

Vascular Tissue Engineering

Couet et al. were described the vascular tissue engineering as a method which is used the principles of engineering and biology in the creation of a vascular construction with close mechanical and biological characteristics to native vessels [6, 12]. Some requirements must be met in the fabrication of engineered vessel which is summarized in Table 1.3.1.1.

Table 1.3.1.1 Requirements for an ideal vascular scaffold

Biocompatibility	• **Nontoxic** • **Nonimmunogenic** • **Suitable in pediatric patients for long term use** • **Support of functional endothelium to prevent coagulation**
Mechanical Properties	• Close compliance with native vessel • Similar burst pressure and elasticity to native vessels • Good suturability
Processability	• Low cost in fabrication • Maintenance in fabrication of large amount • Sterilizable • Long shelf time

There are extensive applications for creating tissue engineered vascular grafts (TEVGs). However; the first study was performed in the mid- 1980s by Weinberg and Bell. They cocultured bovine endothelial cells (ECs), fibroblast and smooth muscle cells (SMCs) onto the collagen scaffold which was shaped through wrap of the membrane to a tube [15]. Besides, the first FDA approved clinical trial was performed in August 2011 in the US from Yale University which is titled as" A Pilot Study Investigating the Clinical Use of Tissue Engineered Vascular Grafts in Congenital Heart Surgery" [13].

1.3.1.1 Fabrication Techniques for Scaffold Based Tissue Engineering

The great variety of manufacturing techniques have conducted for the fabrication of vascular grafts including molecular self-assembly, the solvent casting–particulate leaching technique, thermally induced phase separation, and the electrospinning.

Thermally Induced Phase Separation

Well defined porous morphology and interconnectivity are expected from tissue engineering scaffold to cell attachment and support the vascularization. The scaffolds produced by thermally induced phase separation (TIPS) meet these expected properties [16]. Additionally, the pore size of a scaffold at the different range and its interconnective characteristics can be changed with adjustment of TIPS parameters such as polymer concentration, quenching temperature, solvent/ nonsolvent ratio and adding to additives to a solution.

TIPS method allows the formation of a two or multi-phase system domain through the de-mixing of polymer solution based on the changing of thermal energy. After phase separation, polymer-rich and polymer-lean phases form. Phase separation commonly happens because the homogenous solution is exposed to an immiscible solvent or this solution is cooled to the temperature which is at the below of the binodal curve. When the solution is heated above the binodal curve, a homogenous solution occurs. However, the solution results in the formation of two phases as polymer-lean and polymer-rich at under the binodal curve There are two curves as called the binodal and spinodal curve which shows temperature and concentration on the phase diagram for polymer- solvent system. (Figure 1.3.1.1.1). The binodal curve expresses the equilibrium between liquid-de-mixing and spinodal curve. The spinodal curve forms a two-phase zone known as the metastable zone and unstable zone by dividing the region at which under the binodal curve. The maximum point where overlaps both binodal and spinodal curves is known as a critical point for temperature- composition phase diagram. While the region at which locates between the binodal and spinodal curve is the metastable region, the area is under the spinodal curve is the unstable zone [17].

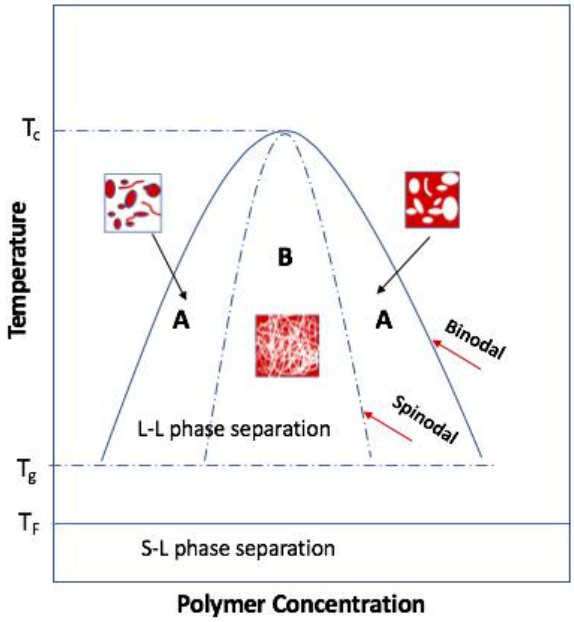


Figure 1.3.1.1.1 Scheme of the temperature- composition phase diagram with variety range polymer concentration where demonstrates that A is metastable region; B is unstable region; Tc is critical temperature of liquid- liquid phase; Tg is glass transition

TIPS is a very common technique for manufacturing of porous materials as scaffold architectures with synthetic polymer such as Poly (L-lactic acid) (PLLA), Poly (ε-caprolactone) (PCL), Polyurethane (PU) or natural biodegradable polymers such as collagen, gelatin and alginate. These scaffolds have been used for variety of application with spinal cord regeneration in spinal cord regeneration (Maquet et al., 2001), bone and cartilage regeneration (Barroca, et al. 2010; Olivas et al., 2009), blood vessel (Hu et al., 2008) [17, 18].

Electrospinning

There are many works regarding the fundamentals of electrospinning and its applications. Electrospinning is described as a flexible method varying the mechanical and biological characteristics of fibers. These fibers can be used versatile fields such as filtration systems, drug delivery agents, wound dressing and sensor applications. The electrospinning method has also huge potential in the manufacturing of fibrous scaffolds in tissue engineering with a controllable fiber diameter ranging from nanoscale to microscale. These fibers can be controlled by varying the electrospinning parameters (applied voltage, collector distance, flow rate, and needle diameter) and parameters of

solution used (conductivity, viscosity, solvent composition and temperature). Figure 1.3.1.1.2 demonstrates the typical most basic setup of electrospinning involving a reservoir such as a syringe which contains the solution, needle nozzle, a pump, a high voltage power source and a grounded conductive collector [19]. Principle of electrospinning is based on production of nano/microfibers using viscoelastic solution under the high electrostatic voltage. Briefly, the polymer solution which is loaded into a syringe is pumped under a high electric field. The voltage results in the formation of repulsive forces within the body of a solution. These repulsive interactions cause counteraction of the surface tension at a specific point for each solution. The solution makes a cone shape which is called Taylor cone at this point of an eruption under high voltage and starts to stretch.

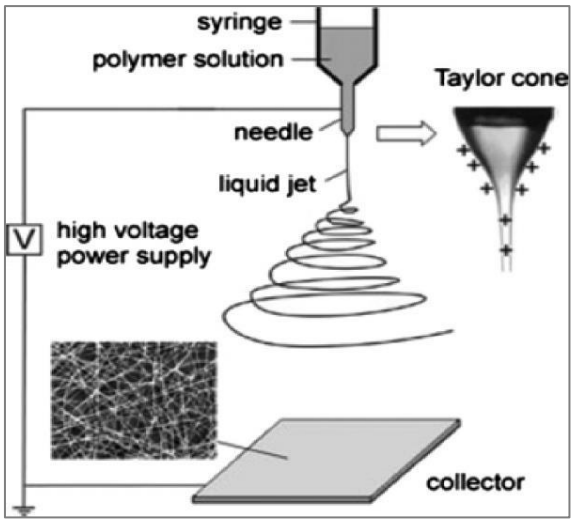


Figure. 1.3.1.1.2 Basic Setup of Electrospinning [54]

Electrospun nanofibers from sub-micrometer to nanometer diameters, have been produced by electrospinning with loosely connected porous mats which have a high surface to volume ratio. In literature, several studies with electrospinning have been reported due to high porosity and good mechanical characteristics for the biomedical applications ranging from the artificial skin to organ regeneration or from neural application to cardiovascular studies. For example, Kim et al. demonstrated the fabrication of thin fibrous scaffolds for promotion of Schwann cell migration and repair of the critically size damage area for nerve gaps. Stitzel and coworkers

produced fibrous conduit using PLGA with collagen and elastin for vascular tissue engineering [20, 21].

1.3.1.2 Materials used for TEVG

The source of scaffold materials includes decellularized matrices, natural and synthetic polymers, and polymer blends. Some natural and synthetic polymers attract attention with their intrinsic characteristics of biological and mechanical, their accessibility and manufacturability**.**

1.3.1.2.1 Natural Polymers

Collagen, gelatin, fibrinogen, elastin, silk- fibroin, chitosan, and alginate have been intensively investigated in tissue engineering due to their in vivo and in vitro biocompatibility that can effectively promote tissue remodeling. However, natural polymers exhibit poor mechanical characteristics and faster degradation.

Alginate

Alginate is a hydrophilic linear biopolymer derived polysaccharide and especially found in brown seaweeds. Alginate composed of two uronic acids called mannuronic acid (M) and glucuronic acid (G) (Figure 1.3.1.2.1.1). Alginate hydrogels are biocompatible, low toxic, nonimmunogenic and relatively low cost. Besides, Alginate forms hydrogels under mild conditions because of high affinity for alkaline metals such as Ca^{+2} and this contributes to the chelation as a result of binding carboxylates in the G-blocks, which form ‘egg-box’ chelate.

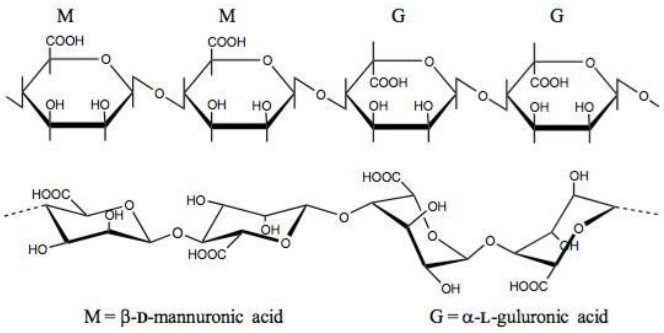


Figure 1.3.1.2.1.1 Alginate chemical structure consisting β-D-mannuronic acid and α-L-glucuronic acid residues [55]

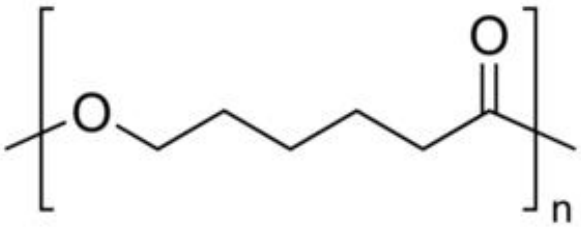
1.3.1.2.2 Synthetic Polymers

Synthetics polymers have been demonstrated better mechanical strength with tunable properties and slow degradation. (PU), poly(ethylene glycol) (PEG), poly(glycolic acid) (PGA), poly(lactic acid–co–glycolic acid) (PLGA), poly(vinyl alcohol) (PVA), poly(lactic acid) (PLA), poly(glycerol-sebacate) (PGS) poly(3–hydroxybutyrate–co–3–hydroxyvalerate) (PHBV), poly(L-lactic acid–co–ε-caprolactone) (PLCL), and poly(ε–caprolactone) (PCL have confirmed to be candidate because of mechanical characteristic.

Many studies were combined synthetic and natural polymers to fabrication of vascular scaffold with better mechanical and biological characteristics. Such as co-electrospinning of synthetic polymer, poly-caprolactone and the natural polymer, gelatin [19, 22].

PCL

Polycaprolactone (PCL) is usually referred to as a biodegradable polymer which is a linear aliphatic polyester for tissue engineering applications (Figure 1.3.1.2.2.1). PCL has widely used polymer because of its biocompatibility, biodegradability, structural stability, and mechanical properties. On the other hand, its low bioactivity and hydrophobic characteristic leading to cell attachment and proliferation limit the use of PCL.



F**igure 1.3.1.2.2.1** PCL chemical structure [56]

1.4 Functionalization of Fibrous Small Diameter Blood Vessel Scaffolds

Scaffolds for tissue engineering without any modification can result in thrombosis formation after transplantation. Thus, the use of anticoagulants such as drugs like dipyridamole (DPA) and aspirin, heparin, and some antithrombogenic polymers for scaffold functionalize might decrease a considerable extent blood clotting formation due to enhancement of endothelialization and further cell proliferation. Functionalization can be performed through physisorption, electrostatic deposition, and covalently binding of specific molecules to scaffold surface [19, 22].

Heparin

Heparin is the first discovered and isolated anticoagulant agent for clinically use and is still used in widespread. Heparin is a linear polysaccharide belonging to the family glycosaminoglycans (GAGs). It composed of repeating D-uronic acids containing sulfated groups (Figure 1.4.1).

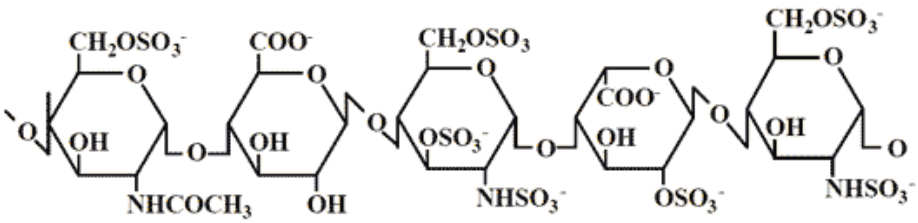


Figure 1.4.1 Heparin, approximate molecular formula [57]

Because of its unique sulfated GAGs structure and surface charge distribution, heparin can inactivate coagulation cascade system [6, 23, 24]. Figure 1.4.2 illustrates the coagulation cascade system with heparin.

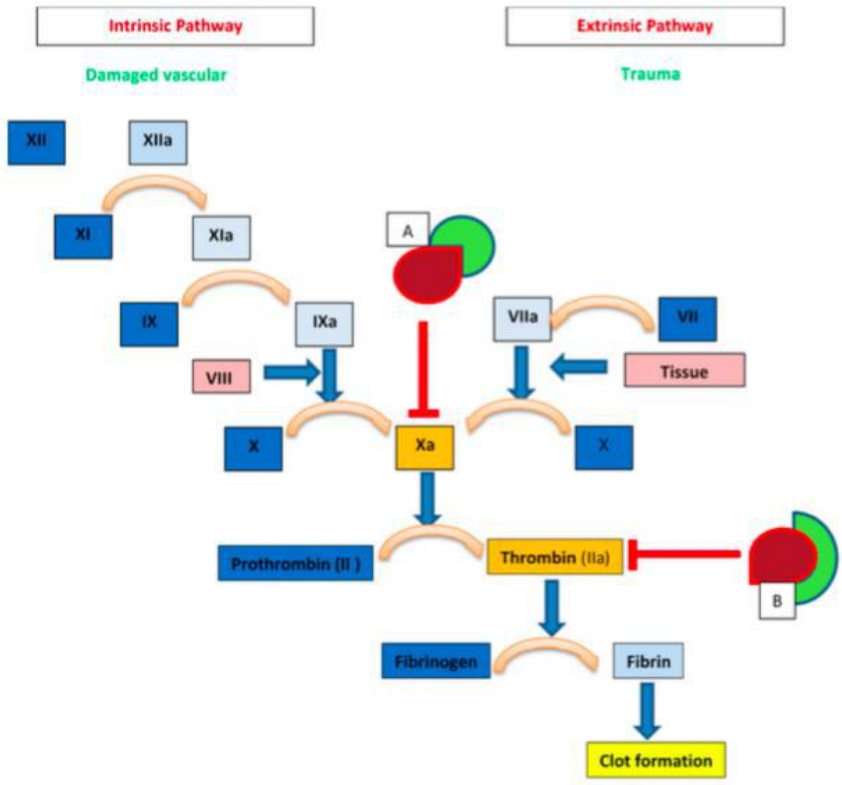


Figure 1.4.2 Heparin mechanism within coagulation cascade system. Box A: AT (red) bound with heparin fragments (green) of any length within pentasaccharide sequence might inhibit factor Xa or Box B: AT (red) bound with heparin fragments (green) with chai [58]

Chapter 2

2 Materials and Methods

2.1 Materials

Alginic Acid Sodium Salt (180947, Sigma), N-(3--Dimethylaminopropyl-N-ethyl carbodiimide hydrochloride, (E7750, Sigma Aldrich), *N*-Hydroxysuccinimide (130672, Sigma Aldrich), Ethylenediamine (240729,Aldrich), heparin (Nevparin 25,000IU/5 ml, Mustafa Nevzat), $CaCl_2$ (1.02391.1000, Merck), Polycaprolactone (440744, Aldrich), chloroform (1.02445.2500, Merck), methanol (1.06012.2500, Merck), sodium chloride (S9888, Sigma Aldrich), sodium bicarbonate (1,06329,1000, Merck), potassium chloride, potassium phosphate dibasic trihydrate, magnesium chloride hexahydrate, hydrochloric acid (1.00317.2501, Merck), calcium chloride (1.02391.1000, Merck), sodium sulfate, tris(hydroxymethyl) aminomethane (154563, Sigma), toluidine blue(CB3340, ChemBio), hexane (208752, , Sigma Aldrich), DMSO (8.02912, Merck), heparin (Nevparin, 25.000IU/5ml, Mustafa Nevzat), glutaraldehyde (G5882, Sigma), Fetal Bovine Serum (FBS) (10270, Gibco), and Human umbilical vein endothelial cell line (HUVEC).

2.2 Methods

2.2.1 Preparation of Inner layer of Tubular Scaffold by Thermally Induced Phase Separation

In this study, in order to facilitate porous structure, inner layer of the scaffold was produced by thermally induced phase separation of alginate solution to support endothelial cell attachment. First, the alginate solution at 60 °C was poured into space of cylindrical molds with 6 mm inner diameter which were designed using syringes with 1 ml and 5 ml volume and they were concentrically aligned. Following this, the molds were rapidly cooled to -20 °C for at least 4 hours. After thawing, tubular scaffolds were taken out from molds and scaffolds were put in 96% ethanol for 1 day at -20 °C to remove the solid solvent crystals, to form an interconnected pore texture. Then, scaffolds were washed with distilled water to remove residual of ethanol and finally lyophilized for 24 hours. Tubular scaffolds were stored in a desiccator until use. To obtain optimal pore morphology, several parameters affecting thermally induced phase separation process such as polymer concentration (1%, 2.5%, 5%, and 10%) and cooling conditions (-20 °C, -80 °C and liquid nitrogen) were changed and resulted materials were examined by SEM (Zeiss LS-10).

2.2.2 Crosslinking of Inner Alginate Layer

To overcome degradation problem of alginate scaffold, crosslinking was performed by using $CaCl_2$. Chelate forms between Ca^{2+} and guluronic acid (G) residues on the alginate chain which is called 'egg-box' crosslinking model (60). Dried alginate tubular scaffold was soaked into 15 ml $CaCl_2$ crosslinking solution in different concentrations (0,5M, 1M and 2M). The samples were named as ''Alg(0.5M), Alg(1M) and Alg(2M)''. The alginate scaffold was subjected to crosslinking solution for 15 minutes with gently mixing. Then, alginates were taken out and washed to

remove excess crosslinking solution. Scaffolds were freeze-dried and stored at desiccator until use.

2.2.3 Preparation of Heparinized Alginate Scaffold

Conjugated heparin-alginate scaffolds named Hep-Alg(0.5M), Hep-Alg(1M), and Hep-Alg(2M) were synthesized by using of carbodiimide reaction at two steps for Alg (0.5M), Alg (1M) and Alg (2M), respectively. In the figure 2.2.3.1, the mechanism of carbodiimide reactions for conjugation of heparin and alginate was illustrated.

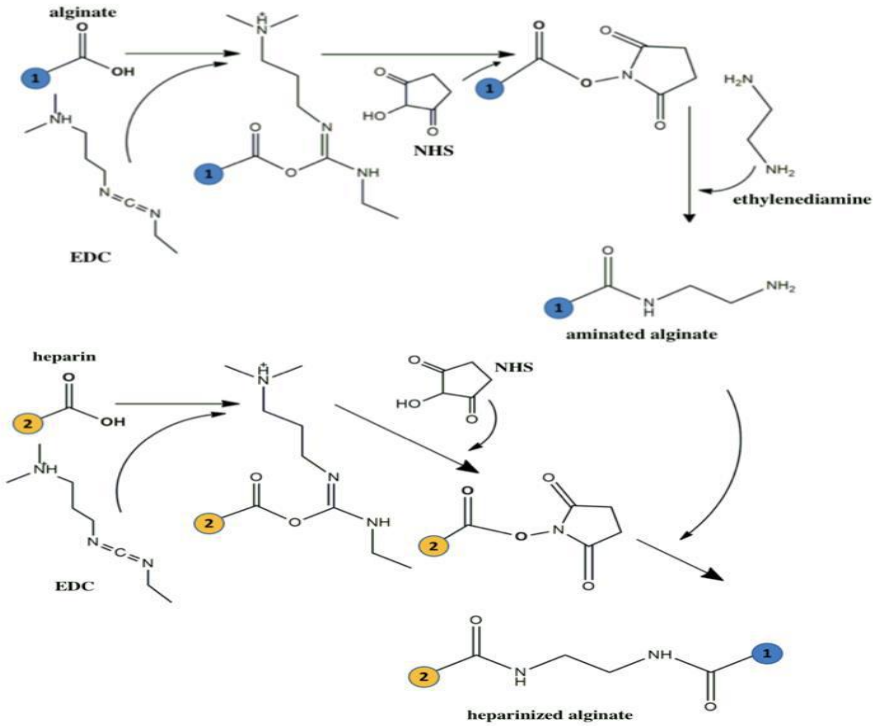


Figure 2.2.3.1 The mechanism of carbodiimide reactions for synthesize heparinized alginate

First modification was performed to alginate tubular scaffolds with ethylenediamine to obtain aminated alginate scaffold. Next, heparin was conjugated with modified alginate scaffold. At first step, 33,3 mg alginate scaffold was soaked into 2 ml PBS buffer containing EDC (2,74mg), NHS (4,84mg), and mixed for 1 hour. Then

ethylenediamine (6 µl) was added to PBS solution and mixed vigorously overnight at room temperature. Once the reaction had completed, the scaffold was placed into a dialysis bag and dialyzed against distilled water for 1 day. After dialysis, the solution was lyophilized. In the second step carbodiimide reaction was performed without ethylenediamine by following procedure. Firstly, heparin was activated in PBS solution consisting EDC and NHS. Then, aminated alginate scaffold was put in PBS buffer and was mixed for overnight at room conditions. After reaction was accomplished, dialysis was applied for 1 day against distilled water and the solution lyophilized. Confirmation of heparin conjugation was examined by Fourier transform infrared spectroscopy (Thermo Nicolet 6700).

2.2.4 Fabrication of The Bilayered Scaffold by Electrospinning

To strengthen the mechanical endurance of the porous alginate layer, crosslinked alginate scaffolds, named Alg (0.5M), Alg (1M) and Alg (2M) were covered with PCL electrospun layer and obtained bilayered scaffolds as labeled bilayered (0.5M), bilayered (1M) and bilayered (2M) respectively. First, PCL solution was prepared in methanol: chloroform (1:4) mixture with a concentration of 20% (w/v). Solution was stirred for overnight at room temperature for complete homogenization. In order to get a tubular scaffold at electrospinning, controlled rotating steel mandrel was used. The mandrel was inserted into the cylindrical TIPS conduit with 6 mm diameter and fixed to the collector where placed 12 cm from the needle tip was. The rotation rate of steel mandrel was set at 250 rpm. Then, PCL solution was put into a 10 ml plastic syringe was pumped out at a flow rate of 2 ml/h with 20 kV voltage. All the processes were performed at room temperature. Finally, bilayered scaffolds were dried under room temperature. Experimental setup used for fabrication of bilayered vascular scaffold is demonstrated in Figure.2.2.4.1.

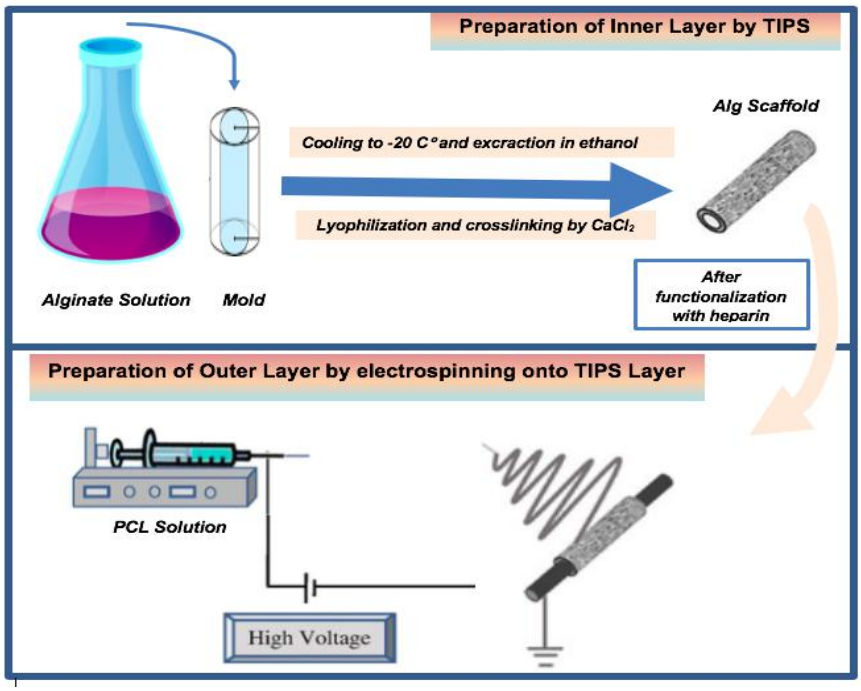


Figure 2.2.4.1 Schematic illustration of fabrication for alginate/PCL bilayered scaffold

2.2.5 Degradation Study

In vitro degradation of scaffolds was assessed over 1 month. In order to degradation study, Alg(1M) was weighed and immersed in simulated body fluid solution at 37 °C for different time intervals for 1, 2, 4 and 6 weeks. Firstly, simulated body fluid (SBF) was prepared according to literature [4]. In beginning, three samples were placed into a centrifuge tube with 5ml of this solution for each time point. The solutions were replenished for each week. After scaffolds were taken out for each time point, they were washed with distilled water and freeze-dried. Then the dry weight of the sample was measured. The weight loss of samples was calculated by using Equation 2.2.5.1 (where W_0 is the initial weigh of dried scaffold, W_t is the dry weight of sample after degradation). The average value of three scaffolds was used for each time interval.

$$Weight\ Loss = \frac{Wo-Wt}{Wo} X100 \qquad (2.2.5.1)$$

2.2.6 Mechanical Characterization of Scaffolds

The mechanical features of the scaffolds were characterized using an electronic universal testing machine (Shimadzu AG-XD). Mechanical tests were performed for alginate TIPS scaffold, crosslinked alginate TIPS scaffolds, heparinized alginate, PCL and bilayered scaffold. For each sample, tests were done 3 times and samples were soaked into PBS solution for at least 30 min before test to enable mimicking in transplantation environment. However; alginate TIPS scaffolds weren't put in PBS because alginate degrades readily. For the tensile test, first, scaffolds cut into 1 cm x 3 cm rectangular pieces. Then, specimens were fixed with the clamps of a testing machine under max 100 N load and pulled at 0.1 mm/min until sample fracture. Stress-strain curves were created using load displacement data and their elastic modulus, ultimate tensile strength and strain at failure were computed. Additionally, burst pressures (Bp) were estimated using Equation 2.2.6.1 by rearranging Laplace's law (37) for pressured thin walled hollow cylinder where UTS is ultimate tensile strength, t is the thickness of tubular scaffold and ID is initial inner diameter of the sample before mechanical test:

$$\boldsymbol{Bp = 2x\frac{UTS.t}{ID}} \qquad \textbf{(2.2.6.1)}$$

2.2.7 Water Contact Angle

The hydrophilicity/hydrophobicity of the bilayered scaffolds was measured by contact angle analyzer (Biolin Scienific, Theta Lite). To determine contact angle, samples were cut into 1 cm X 1 cm rectangular pieces and 3 uL distilled water was drop onto the surfaces. Contact angle measurement was performed at three different areas of scaffold and average values were taken.

2.2.8 Heparin Release

To follow heparin release from heparin conjugated scaffold, 30-50 mg scaffold for (Hep-Alg (1M), and Hep-Alg (2M)). Hep-Alg (0.5M), was not assessed because of its weaker mechanical properties. To release protocol, firstly, scaffold was immersed into a plastic tube with 5ml SBF and shaken at 37 °C for 28 days. 1 ml solution was taken out from the tube for different time intervals and replenished by the addition of fresh SBF to the system. Following this, toluidine blue assay was performed. Amount of released heparin was detected by using a standard curve prepared with heparin solutions in varying concentrations (0.075%, 0.1%, 0.25%, 0.5% and %1).

Toluidine Blue Assay

Toluidine blue solution was prepared in aqueous media consisting of 0.01 M HCl with 0.005% NaCl (w/v). 1 ml of heparin solution was mixed with 1,5 ml toluidine blue solution and waited for 2 hours. Then, 1 ml hexane was added to heparin/toluidine blue mixture and agitated to allow interaction between dye and heparin. The amount of released heparin from scaffold was detected by using UV/VIS spectrophotometer (Thermo Scientific, Genesys 10S) at 632 nm absorbance.

2.2.9 Cell Studies,

Human Umbilical Vein Endothelial Cells (HUVECs) Culture

HUVECs (Human Umbilical Vessel Endothelial Cells) were stored in a medium consisting of 5% DMSO and 10% FBS in their stand medium with 85% concentration. HUVECs of passage 13 were used in vitro studies. After thawing of cells, HUVECs were cultured in their growth medium that contains DMEM/F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin following standard cell culture requirements to reach confluence. The medium was refreshed for 2-3 days.

Cell Seeding

HUVECs (Human Umbilical Vessel Endothelial Cells) cultured in DMEM/F-12 medium containing 10% FBS and 1% penicillin/streptomycin with 5% CO_2 in a humidified incubator. After reaching 80–90% confluence, 2 ml of Trypsin-EDTA was added cell culture flask and rocked to enable that solution to cover the whole surface of the culture flask. Flask was transferred to an incubator for 3-5 min at 37 °C for detachment of cells from the surface of culture flask. 6 ml medium was added to flask and centrifugation was performed for 5 min at 125g. The supernatant was removed and the pellet was resuspended with 1 ml medium. Thoma cell counting chamber was used to determination of cell number. HUVECs were seeded onto each scaffold with 10^4 cells (in 100 µl).

Viability and Cytotoxicity Assay

The cell proliferation profile was specified by MTS (CellTiter 96® Aq_{ueous} One Solution Cell Proliferation Assay) cell viability assay for HUVECs seeded onto the scaffolds three groups of scaffolds: Alg(1M), Hep-Alg(1M), Hep-Alg(2M) and PCL for 1, 2, 3 days. Additionally, cells seeded to plate without scaffold as control group. Firstly, the scaffold was sterilized with at 70% percentage ethanol and exposed the UV for 1h. After sterilization, scaffold was saturated with DMEM/F-12 medium and was placed into 96 well plates. 10^4 cells were seeded to each well in the presence of the scaffold. Then, the plate was incubated at 37 ˚C, 5% CO2 humidified atmosphere. Incubation for the MTS experiment was performed for 1 day, 2 days and 3 days. Briefly, 20 µl MTS reagent solution was added each well and cells were incubated for 4 h, at 37 ˚C, in a 5% CO2 atmosphere. After incubation, the scaffold removed from well and the absorbance of each well was determined at 490 nm using a microplate reader (Thermo Scientific, Varioscan Lux).

Cell Attachment Assay

The scaffold was soaked into 24 well plates and cell seeding were performed using $5x10^4$ cells for each well. The scaffold was taken from the plate for the time intervals (2 and 3 days) and was washed with PBS to ensure that nonattached cells were removed. Then, %2.5 glutaraldehyde was used for the fixation of cells to scaffold. After the fixation process, the scaffold was taken into gradient alcohol solution and dried under the vacuum.. Cell attachment was investigated for Alg(1M), Hep-Alg(1M), and Hep-Alg(2M), by SEM analysis.

2.2.10 Hemocompatibility Assay

To prove that heparinized scaffolds are good biocompatible materials, the hemolysis test was carried out for Hep-Alg (0.5M), Hep-Alg (1M) and Hep-Alg(2M) scaffolds. Human blood was collected from healthy volunteers by venipuncture into tubes containing ethylendiaminetetraacetic acid (EDTA) which shows anticoagulant properties. Then, scaffold cut into pieces with 50 mm^2 area and placed into 5 ml of 0,9 % NaCl solution to determine the hemolysis ratio. After, 100 µl fresh blood was added into tubes. Following the incubation time at 37°C for 30 minutes for erythrocyte disruption, each tube was centrifuged at 1000 g for 10 minutes. Supernatants were collected and transferred into a 96 well plate to measure at 545 nm using spectrophotometric plate reader (Thermo Scientific, Varioscan Lux). The hemolysis ratio was calculated with Equation 2.2.10.1

$$\boldsymbol{HR = 100x\frac{As - An}{Ap - An}} \qquad (\mathbf{2.2.10.1})$$

Where A_s, A_p, and A_n indicate the absorbance value of hemoglobin in the sample, in the positive control and in the negative control, respectively.

Chapter 3

3 Results and Discussion

3.1 Characterization of Inner Layer of Tubular Scaffold by TIPS

As we aimed to prepare a bilayered scaffold by the combination of TIPS and electrospinning techniques, first we started with the fabrication of alginate tubular scaffold by TIPS. According to the many reports in the literature, the interconnectivity of scaffold with large pores supports the gas exchange and can promote cell penetration[25, 26]. In our case, the TIPS method, which we apply for inner layer preparation, enables the creation of interconnected porous texture and result in better cell migration compared with electrospun fibers. However, the TIPS scaffold commonly exhibits poor mechanical characteristic due to its high volume to mass ratio. The combination of TIPS and electrospinning methods to produce multiple layered scaffold can allow the higher cell affinity because of inner alginate porous layer and the better mechanical properties with electrospun PCL outer layer [27]. As seen in Figure 3.1.1 (a), the prepared alginate tubes show a smooth and symmetrical form with 6 mm in diameter and 5 cm in length. The crosslinked bilayered vascular grafts didn't lose their smooth surface and protect the integrity of tubular shape without any defect after crosslinking process (Fig 3.1.1 (b)). The internal texture of alginate scaffold was examined by using SEM (Zeiss LS-10) images. As shown in Fig. 3.1.1 (c), alginate layer was obtained with an interconnected porous structure at nano-scale, which will allow the endothelial cell attachment, growth and nutrient/gases flow. Thickness of inner layer was measured approximately 1.7 mm and its pore size was 175± 25μm.

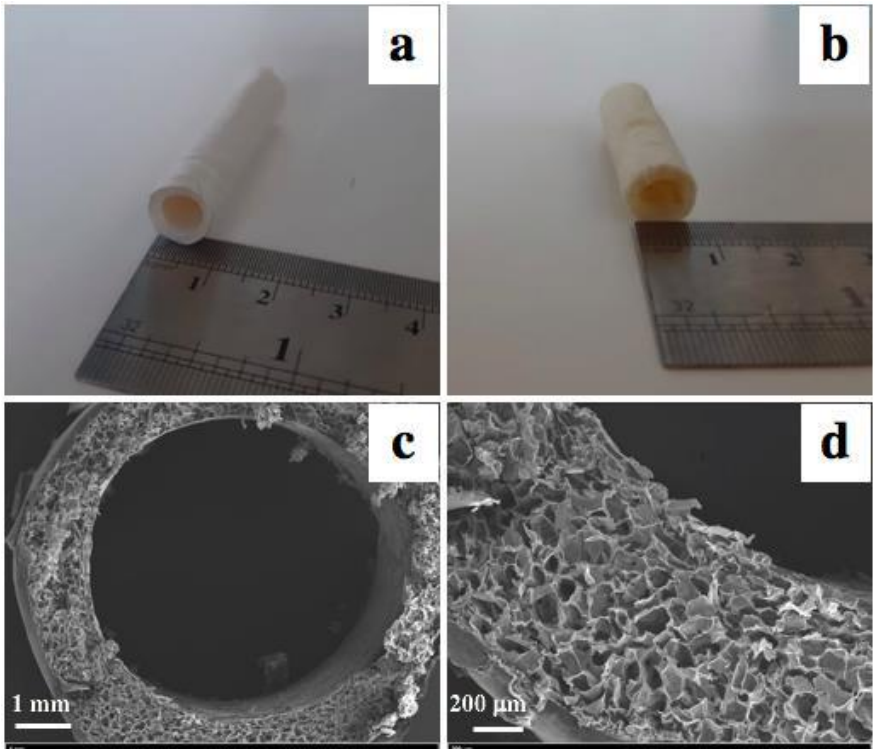


Figure 3.1.1 Digital photographs and SEM images of for internal characteristics of alginate and crosslinked alginate scaffold: physical appearance of (a) alginate scaffold, (b) crosslinked alginate scaffold; SEM images of (c) alginate scaffold, (d) alginate in high magnification

The scaffold morphology might be controlled by changing TIPS process parameters: polymer types and concentration, solvent/nonsolvent composition and quenching conditions (temperature and time). In this work, process parameters including polymer concentration and quenching temperature were changed to control the pore size of the scaffold and its interconnectivity. When polymer concentration is determined, it should be above a critical concentration otherwise the scaffold can be collapse upon solvent removal because the polymer-lean phase is more than the polymer-rich phase [16]. For example, in our study, alginate scaffold with 1% (w/v) concentration was prepared but it was collapsed in fabrication process because it is below critical concentration. Thus, in our study, higher concentrations of alginate solution at 1% were prepared to investigate concentration affect onto scaffold morphology (2.5%, 5%, and 10%). Zhang et al. reported that decreasing the polymer concentration causes an increase in scaffold porosity and the decrease in scaffold density [16, 28]. However, separation in solid-liquid phase with high polymer concentration might result in the formation of anisotropic ladderlike forms because orientation is preferentially induced by the solvent

crystallization [16]. As presented in Fig 3.1.2, while 2.5% (w/v) alginate scaffold had the largest pore, 10% (w/v) alginate scaffold had the ladder like structure with the smallest pore size because increasing in polymer concentration causes decreasing of freeze point which enables the formation of long ice crystal before solvent removal. Besides, the 5% (w/v) alginate scaffold (Fig 3.1.2 (b)) is a better candidate to fabricate vascular graft with porous structure and pore diameter of approximately 150 μm, which might enable cell attachment and growth.

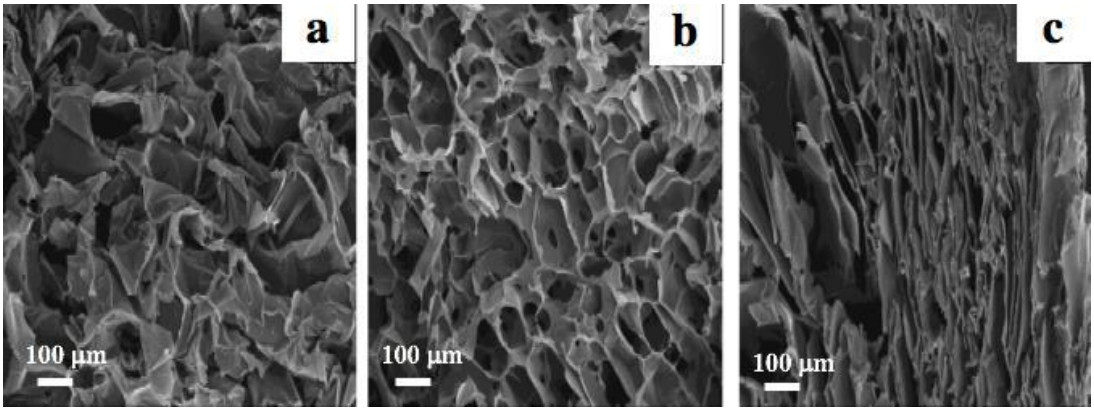


Figure 3.1.2 SEM micrographs of alginate scaffold fabricated by different concentration (a) at 2.5% (b) at 5% and (c) at 10% (w/v)

Quenching time is another essential parameter to design pore size and its connectivity of scaffold. Quenching of the solution to low temperature causes the faster cooling and creates smaller pores in the scaffold because TIPS processes including solvent nucleation, crystal growth and phase separation perform in a short time. However, quenching to the high temperature supports scaffold with larger pores [29]. In order to optimize the conditions for alginate tubular scaffold preparation at 5% concentration by TIPS, we also changed quenching temperature by applying -20 °C, -80 °C and liquid nitrogen. Alginate scaffold fabricated at the quenching temperature of -20 °C (Fig 3.1.3 (a)) had larger pore sizes within the scaffold up to 200 μm with its interconnectivity because cooling degree of distilled water at this temperature with low nucleation rate and high growth rate, allows large solvent crystal formation. However, scaffolds prepared by cooling to -80 °C (Fig 3.1.3 (b)) and using liquid nitrogen (Fig 3.1.3 (c)) for quenching resulted in scaffolds which had the smaller pores with insufficient interconnectivity with ladder like forms.

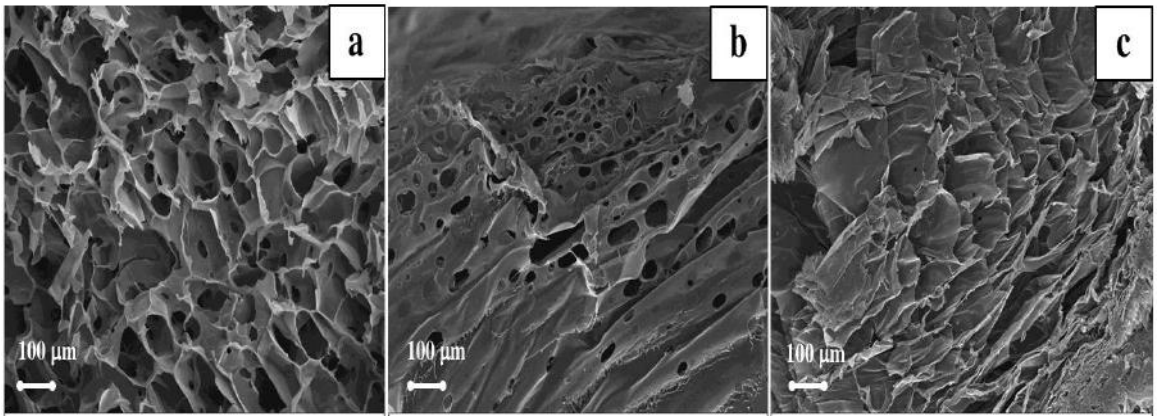


Figure 3.1.3 SEM micrographs of alginate scaffold at 5% (w/v) fabricated by different quenching temperatures (a) at -20 ℃ (b) at -80 ℃ and (c) with liquid nitrogen

3.2 Crosslinking of Alginate Inner Layer

Alginate scaffolds prepared by TIPS support cell attachment and proliferation but their rapid collapse in solution restricts their use in long-term for in vivo studies. To enhance the mechanical stability of alginate, we crosslinked alginate scaffold by $CaCl_2$ solution in different concentrations [30]. $CaCl_2$, which is a type of divalent ions provides an extension in the dissolution period of the scaffold with better mechanical characteristics [31]. Calcium ions binds to -OH group of the carboxylate in guluronic acid and forms ‘‘egg-box’’ structure. In literature, crosslinking of alginate by $CaCl_2$ was successfully performed to improve mechanical stability for many studies at varying applications. For example, Matyas et al. used crosslinked alginate hydrogels to form the robust neurite without a rapid disintegration [30]. In another study, Vo and coworkers have been developed calcium alginate hydrogel beads as a candidate matrix for the delivery of nutrient and pharmaceutical compounds [31].

Crosslinking of alginate by calcium chloride was confirmed by FTIR (Figure 3.2.1). The band peak at 1407 cm^{-1} represents the C–O bond symmetric stretching of the carboxyl group [31]. As seen in Figure 3.2.1, the shift from 1407 cm^{-1} to 1431 cm^{-1} was formed because of the replacement of the Na^{+1} ion with Ca^{+2} and, thus, changes their atomic weight and charge density. Crosslinking of alginate was also confirmed with change of

hydroxyl peak. Hydroxyl peak shifted to a higher value of wavenumber from 3269 cm^{-1} to 3356 cm^{-1}.

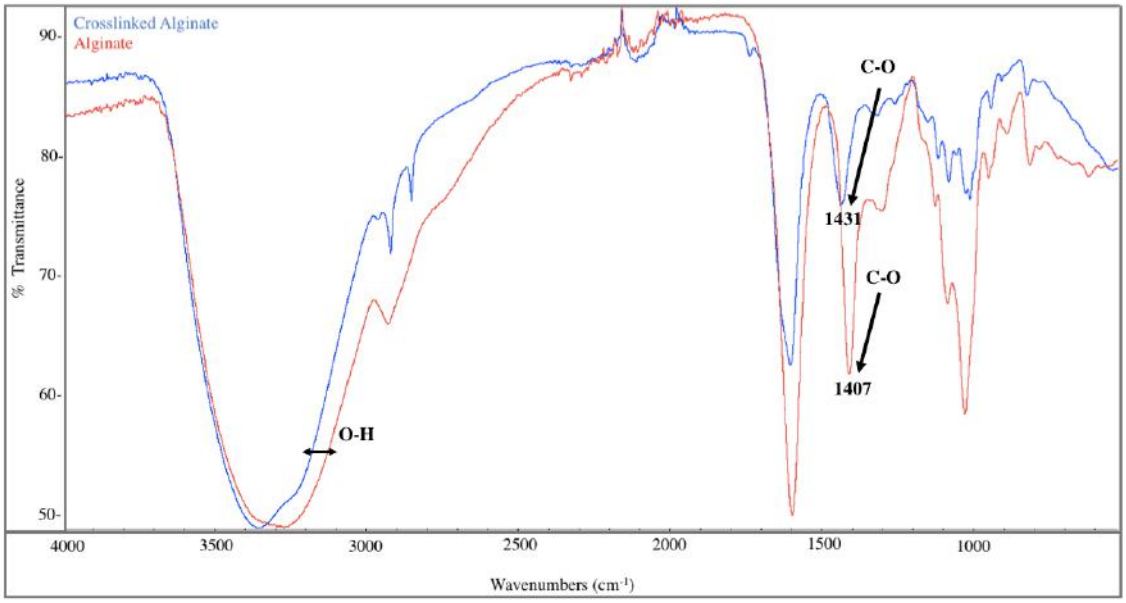


Figure 3.2.1 FTIR spectrum of sodium alginate scaffold (a) and crosslinked alginate scaffold with 1 M CaCl2 (b)

3.3 Heparin Conjugation with Alginate

The variety of applications for blood contacting materials have been developed but they still need to be improved at their surfaces with use of anticoagulants such as heparin and prostaglandin and chemical modifications to prevent thrombosis [32-34]. Heparin is a natural polysaccharide with highly negative charge. It enables prevention of the clotting and formation of thrombus which constitute of fibrin clots and blood cells. Heparin binds to antithrombin III, which is the major inhibitor protein of the coagulation cascade in plasma. Thus, heparin has been studied to functionalize the surface of biomaterials such as porous scaffold [35] and electrospun fibers [36]. Among the functionalization method, the incorporation of heparin to the material surface by ionic binding or by covalent immobilization is an efficient method. In literature, it was

indicated that the covalent immobilization of carboxylic acid group to the biological agent onto biomaterial consisting aminated surface has been recognized as a successful strategy. This strategy allows heparin to tightly bind on biomaterials and it extends heparin release period after the implantation. Lee et al showed that heparin was chemically conjugated to electrospun PCL/gelatin scaffold preserved well under physiologic conditions for 10 days [36] . As chemical conjugation, carbodiimide reaction is a good alternative method due to existence of carboxylic acid groups in the heparin structure. Mi et al. performed heparin conjugation into chitosan- alginate PEC scaffold by the carbodiimide reaction using EDC. Besides, Zuo and coworkers studied onto heparin-conjugated alginate multilayered microspheres prepared by EDC/NHS reaction [3]. In our study, heparin conjugation was performed by using the EDC/NHS reaction twice. In first step, alginate scaffolds were modified with ethylenediamine (EDA). In second step of the synthesis, amine groups of modified alginate scaffold were coupled with carboxyl groups of the heparin. To verify the conjugation of heparin to alginate scaffold, FTIR analysis was used. As demonstrated in Figure 3.3.1, first step reaction has been shown that it was accomplished successfully as indicated by N-H stretching absorption peak at 2849 cm^{-1} and N-H bending peak at 1558 cm^{-1}. Besides, in the second reaction, the characteristic absorption band at in the range between 1160 cm^{-1} 1260 cm^{-1} that originates from the S=O asymmetric stretching was also appeared. Therefore, the conjugation of heparin to the alginate surfaces has been confirmed.

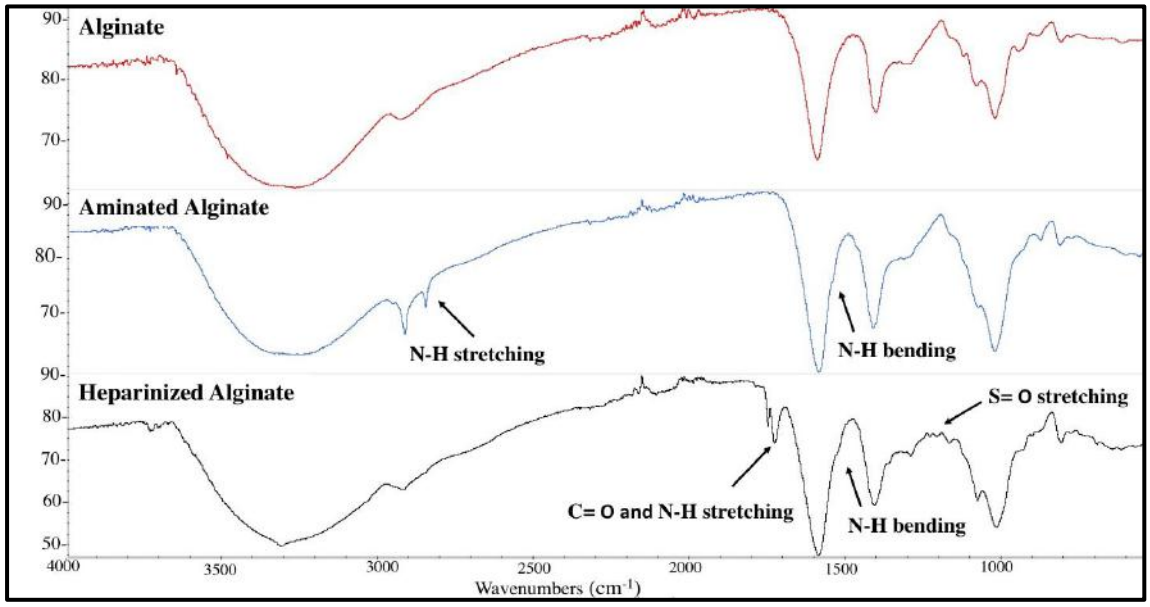


Figure 3. 3.1 FTIR spectra of alginate, aminated alginate and heparinized alginate

To characterize of the internal structure of alginate scaffold after the functionalization with heparin, SEM analysis was performed. Figure 3.3.2 showed that scaffold was maintained the porous structure with pores with larger diameter.

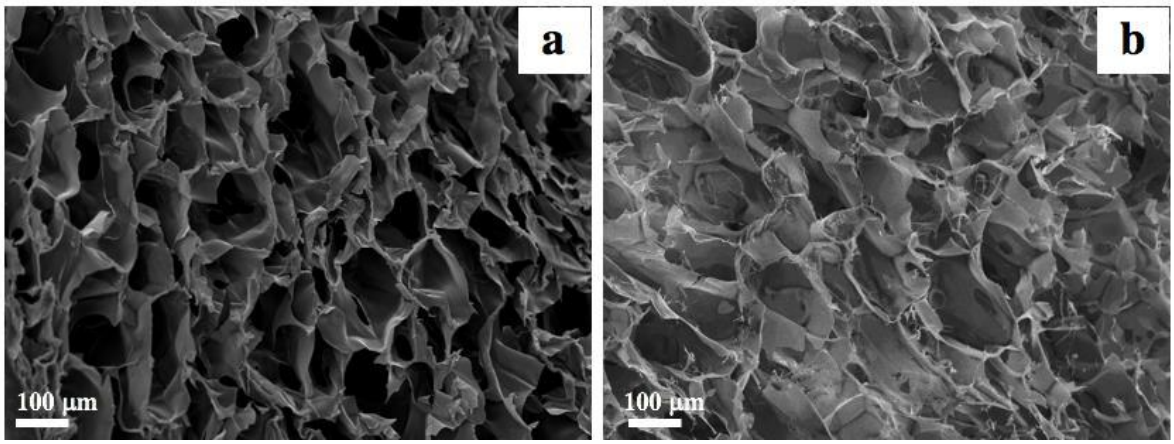


Figure 3.3.2 SEM images of alginate scaffold Alg (1M) (a) and heparinized alginate (b)

3.4 Characterization of Bilayered Scaffold

Using bilayered or multilayered scaffolds as vascular graft are promoted for mimicking of the layered structure of native blood vessel [37]. In literature, there are various applications regarding with fabrication of the multilayered scaffolds for vascular graft applications. For example, the bilayered scaffolds with elastic PCL inner layer and fibrous PLA outer layer was fabricated by Vaz and coworkers. In another study, Soletti et al. produced a PEUU-based scaffold for small diameter VTE application which support the desirable microstructural and mechanical properties through the combining of TIPS and electrospinning methods [38]. Similarly, Mi and Coworkers fabricated the Multiple-Layered Vascular Scaffolds combining TIPS and electrospinning methods using Poly(propylene carbonate) (PPC) and Thermo- plastic polyurethane (TPU) because highly porous inner layer support the cell penetration and the fibrous layer reinforce the scaffold strength [6].

We fabricated the bilayered scaffold which can be a good candidate through structural characteristics that are close the mechanical and biological characteristics of native vessel. Alginate inner layer was covered with PCL electrospun membrane because PCL has a great potential within synthetic biodegradable polymers with the better stability in vivo compared other polymers especially natural polymers for using implantable material. Additionally, PCL can be processed easily due to its low melting temperature and outstanding viscoelastic characteristics [39]. Electrospinning can also be used for inner layer production with better mechanical characteristic but the main issue associate with the electrospun scaffolds with small pore size in thick scaffold, especially for tissue engineering vascular grafts still limit use of them due to lack of adequate cell penetration. As seen Figure 3.4.1 alginate tube was covered with PCL named bilayered alginate scaffold. (Fig 3.4.1 (a)) and it prolonged its smooth surface without any deformation and protected its structural integrity compared to the form before electrospinning (Fig 3.4.1 (b)). The internal structure of scaffold was characterized by using SEM (Zeiss LS-10). The bilayered scaffold consisted of two different layers with interconnected inner porous layer with alginate, which can enable endothelial cell attachment, growth and nutrient/gases flow with approximate diameter of 150 μm and PCL nanofibrous outer layer with the nanofiber diameters of 4±2 μm which allows

significantly better mechanical characteristics compared alginate inner layer (Fig 3.4.1 (b)). While thickness of inner layer was measured approximately 1.7 mm and its size of pore diameter was 150± 25μm, outer layer has thickness of 210±80μm.

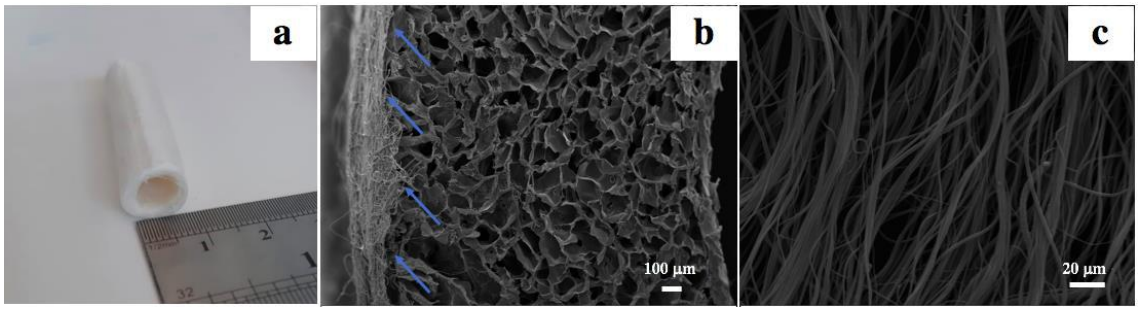


Figure 3.4.1 Digital photograph and SEM images of for internal characteristics of bilayered scaffold and outer layer of bilayered scaffold: (a) physical appearance of the bilayered scaffold (b) SEM images of the bilayered scaffold (c) outer electrospun

3.5 Degradation Study

The degradation of scaffold is one of the major problems that must be overcome because it affects cell adhesion and proliferation. However, scaffold degradation must be supporting the enough stability for neo-tissue organization with their integration. Therefore, when a tissue engineered scaffold is implanted to the host body, slow degradation and scaffold integrity are desired which promotes replacement of implanted biomaterial with native vessel over an extended period of time. For example, to obtain desired degradation profile, Zhou et al. was worked on development of hybrid small diameter vascular grafts using chitosan with high biocompatibility and PCL with better mechanical characteristic in dog as model organism [40]. Thus, they described that degradation profile at moderate level for weight loos was preferable for electrospun PCL/CS vascular scaffold because it has supported endothelization and deposition of elastin and collagen that may result in the close burst pressures that is comparable with that of native CA. In another study, Mi et. al. stated that moderate degradation, taking > 6 months with 30% weight loss can be preferable for vascular scaffolds because it allows for vascular cell proliferation through scaffold and maintain available mechanical endurance. However, Norouzi and coworker showed that mass degradation with 52% during 6 weeks has been supported long enough time to cell proliferation for vascular

engineering application [38]. While, so slow degradation is not desired characteristic for scaffolds because it occupies the space and restricts cell growth, fast degradation profile is not also desired for scaffolds which result in insufficient period to enable cell proliferation and blood leakage. In order to obtain scaffold which has moderate degradation profile, fast degraded polymer can be combined with slow degraded polymer to enhance the endurance of scaffold [27]. In our study alginate was chosen due to high biocompatibility and non-antigenicity with advantageous characteristics but it degrades instantly within minutes. So, in order to prevent collapse of alginate in fluids, crosslinking was performed. Then it was covered with PCL to enhance mechanical characteristic and mimic layered vessel structure. Degradation profile assessed for 6 weeks in SBF for Alg(1M) which has better mechanical and biological characteristics. As shown in Figure 3.5.1, moderate degradation level of Alg(1M) scaffold with 25% weight loos after 6 weeks show that alginate/PCL scaffold can be a promising candidate as vascular scaffold which enables time for endothelization, deposition of elastin and collagen and neo tissue formation.

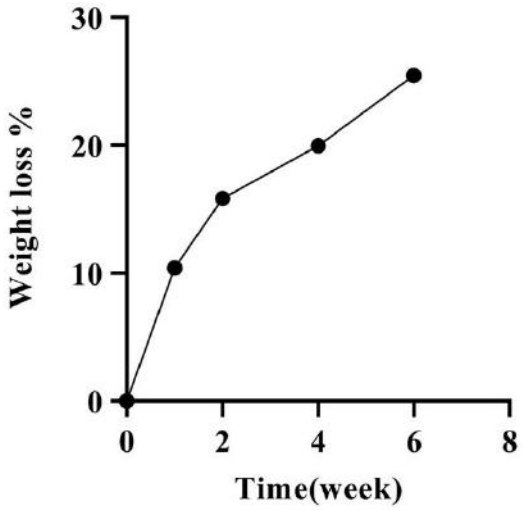


Fig.3.5.1 Degradation profile of crosslinked alginate scaffold (Alg (1M))

To illustrate final alginate texture after degradation process, it was assessed by SEM analysis for week 1 and week 6 degradation scaffolds. SEM results in Figure 3.5.2 showed that increased in degradation time caused the disruption of scaffold and thus increased its the pore diameter which confirmed the degradation of the material.

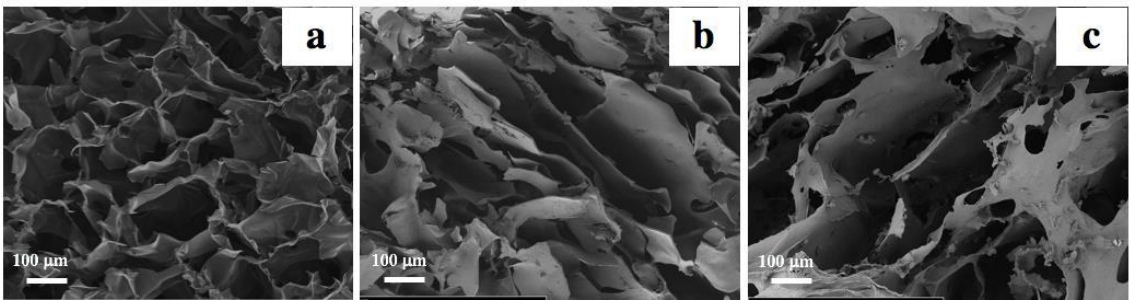


Figure 3.5.2 SEM images of scaffold (Alg (1) (a) before degradation, after degradation for (b) 1 week and (c) 6 weeks

3.6 Mechanical Characterization

One of the major issues for fabrication of vascular scaffold is achieving mechanical properties with close the native vessels and enabling continuity between native vessels and vascular scaffold. In figure 3.6.1, modelling of blood vessel is shown from biomechanical perspective to define dynamic forces onto vessel wall [6]. When the scaffolds are designed, mechanical properties of scaffold must encounter to mechanical forces.

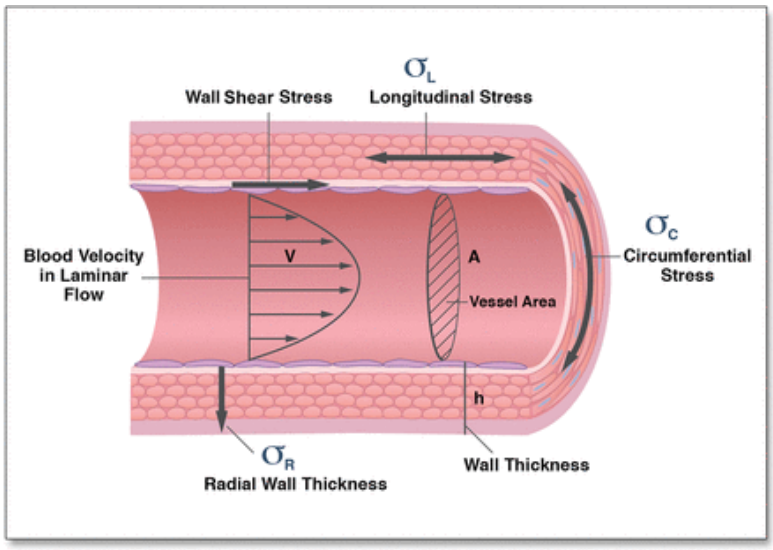


Figure 3.6.1 Schematic illustration of mechanical forces for blood vessels [59]

In our study, the bilayered scaffold was fabricated by combination of crosslinked alginate and PCL prepared by using TIPS and electrospinning, respectively. Because of alginate has less mechanical stability and can even disrupt with so small force, crosslinked alginate prepared different concentration of crosslinker was used for the fabrication of vascular graft. As seen in Table 3.1, elastic modulus of Alg(1M) scaffold increased to 0.92 MPa from elastic modulus of 0.45 MPa for Alg(0,5M). Besides, Alg(2M) showed the better mechanical characteristic. These results showed that in increase the crosslinking concentration resulted in the better scaffold fabrication respect to mechanical characteristics. Besides, heparin functionalization result in the increase of hydrophilicity of Hep-Alg(1M) scaffold and so caused the decrease in mechanical endurance with 0.66 MPa elastic modulus. Additionally, burst pressure (BP) was calculated the equation obtained by rearranging Laplace's law for pressured thin walled hollow cylinder and BP values were showed in Table 3.6.1.

Table 3.6.1 Mechanical Properties of Prepared Scaffolds and Natural Coronary Artery

	Young Modulus (MPa)	**UTS (MPa)**	**Burst Pressure (MPa)**
Alg (0.5)	0,44 ± 0.5	0.1 ± 0.04	
Alg (1)	0,92 ± 0.7	0.304 ±0.07	
Alg (2)	1,14 ±0.6	0.25 ± 0.06	
PCL	7 ± 1.2	3.44	0.25
Hep-Alg (1)	0.66 ± 0.11	0.21 ± 005	
Bilayered Scaffold	2 .45 ±1.7	0.9	0.18
Coronary Artery	1.41 ± 0.72 (17-51)	-	-
Saphenous Vein	-	-	0.16 ± 0.06 (2)

In our work, the bilayered scaffold can show better resistance characteristic to pressure toward scaffold wall compared natural blood vessel. Moreover; mechanical strength can be improved varying process parameters as increasing thickness of wall of vascular scaffold or decreasing of inner diameter of scaffold. Pressure endurance of blood vessel varies between 80 mm Hg and 140 mm Hg. In our work, the bilayered scaffold can show 4 times better resistance characteristic to pressure toward scaffold wall compared natural blood vessel.

3.7 Water Contact Angle

Hydrophilicity of biomaterials is defined as one of the important factors affecting cell attachment and following cell behaviors because it is effective to adsorption of protein onto scaffold surface and water contact angle (WCA) test are used to characterize hydrophilicity of biomaterial surface. Most of animal cell generally has better cell adhesion and proliferation capacity with moderate hydrophilicity rather than superhydrophilicity (WCA<5) or superhydrophobicity (WCA>150) [41]. Goriainoov et al. also showed that material surface with moderate hydrophilicity causes good cell attachment [42].

In our study; PCL scaffold, crosslinked alginate scaffold and heparinized alginate scaffold were examined. Our results showed that while PCL scaffold had water contact angle of 114° with hydrophobic character, alginate tube had water contact angle of 55°, with hydrophilic feature. Kharazi et al. stated that scaffold with 60° WCA can be used ideally which is moderate hydrophilicity to promote the highest vascular cell attachment and proliferation [43]. Thus, using the alginate scaffold for inner layer of vascular graft contacting with blood, can supports the endothelial cell attachment because of its moderate hydrophilicity. Besides, in our study, alginate scaffold functionalized with heparin conjugation was used to improve anticoagulant characteristic of the scaffold. However, contact angle test couldn't apply the heparinized scaffold because it absorbed the water. So that result showed that the heparinization process caused an increase in hydrophilicity. In literature it has also been stated from Chen and coworkers that heparinized tissues had more hydrophilicity

than non-heparinized and suppressed the adsorbed fibrinogen which has key role in thrombogenesis [40].

3.8 Heparin Releasing

There are many applications to immobilization of heparin within the scaffold. In our study, chemically conjugation method was used. For the determination of heparin conjugation stability, colorimetric method was used in our study because it is easy and fast method compared other methods such as biological (APTT test and antithrombin-III method) or radiochemical (isotopic substitution with 35S) [44]. To assess the heparin amount, standard curve was firstly established for ranging concentration heparin solution between 0.075 mg/ml and 1 mg/ml. Figure 3.8.1 demonstrates the standard curve of heparin with a good linear relationship.

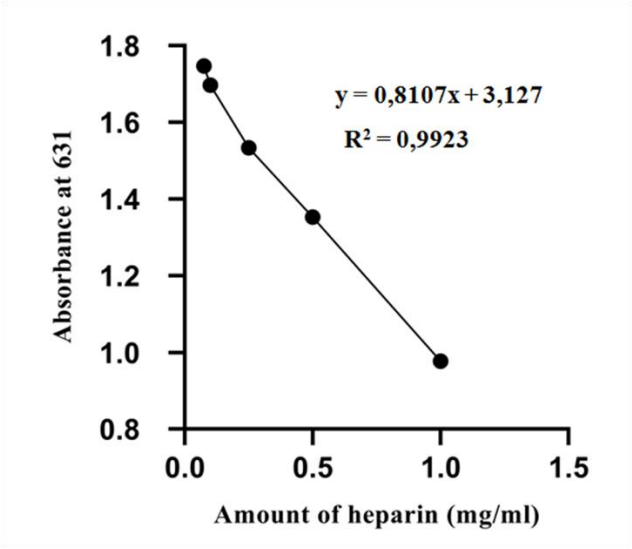


Figure 3.8.1 Standard calibration curve for heparin showing the decrease in a toluidine blue solution at 631 nm with increasing of heparin concentration

Then, heparin release profile was evaluated during a period of days to weeks incubating the PBS for Alg(1M) and Alg(2M) scaffolds. As can be seen in Figure 3.8.2, the high release of heparin in first 3 days was seen for both scaffolds. The results also

showed that scaffolds could be preserved under physiologic conditions for 7 days and it approved that heparin conjugation allowed to remain heparin stable for extended periods. Besides, Alg (1M) showed higher heparin release with 0,37 mg/ml compared to Alg (2M) of 0,31 mg/ml. Thus, these results showed that Alg (2M) consisted the lower heparin content because it could be tightly crosslinked compared with Alg (1M) and that crosslinking decreased to conjugation of heparin with alginate.

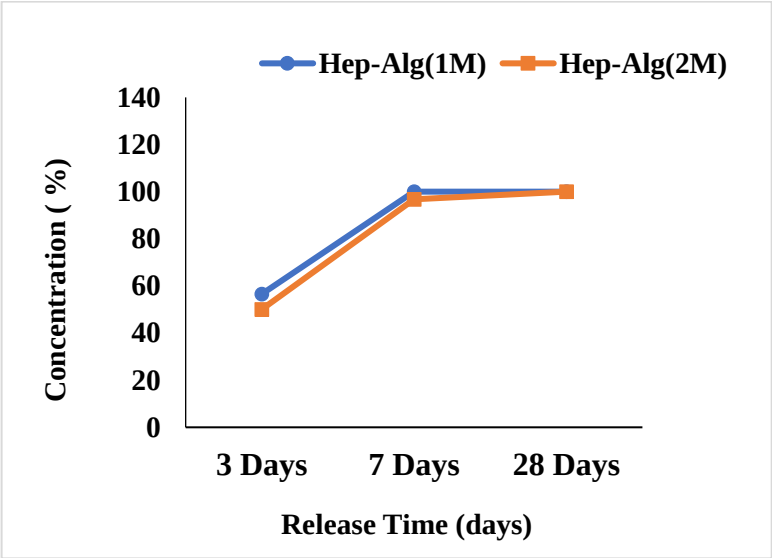


Figure 3.8.2 Quantification of heparin amount for alginate scaffold for Alg (1M), and Alg (2M)

3.9 Hemolysis Assay

Blood compatibility (hemocompatibility) is one of the most important criteria for artificial implants and materials contacting with blood. Non-hemocompatible materials cause hemolysis due to membrane disruption by surface tension of materials affecting the cell and protein attachment. Material interaction sites and topology affect the interaction of biomaterials with biological components especially with blood [45]. Although hemolysis ratio is not only criteria for evaluating, it is an important test as supplementary indicator for hemocompatibility [46]. According to standard, which has been specified by ISO (ISO 10993.4:2002), hemolysis ratio should be less than 5% for safe medical use. Thus, the blood compatibility of vascular scaffold was evaluated for heparinized alginate scaffold which were crosslinked with different concentration of $CaCl_2$ through 1 M and

2 M. As represented in Figure 3.9.1, each heparinized scaffolds were passed the hemolysis test with hemolysis ratio of 1,86% and 1,12% for Hep-Alg (1M) and Hep-Alg(2M), respectively.

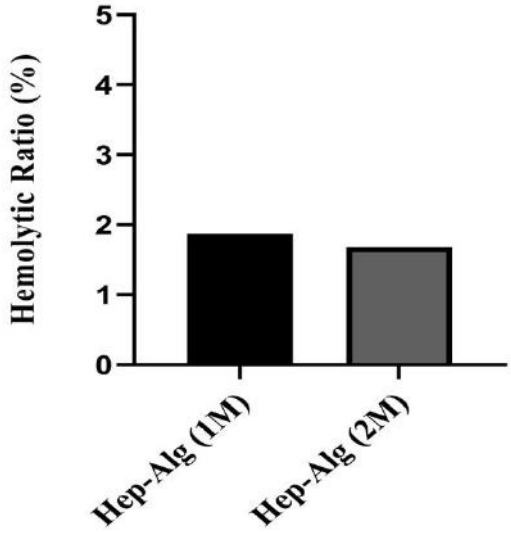


Figure 3.9.1 Hemocompatibility ratio for heparinized alginates

3.10 Cell Studies

In vitro cell viability and cytotoxicity with cultured cells are commonly used because they give rapid result and are inexpensive methods. Additionally, they decrease the use of animal. (155) In our study, cell viability of HUVECs grown on Alg(1M), Hep-Alg (1M), Hep-Alg (2M), and PCL scaffolds for 72 hours was assessed by using MTS procedure. As seen in Figure 3.10.1, all materials had cell viability with higher 95%. Besides, absorbance value is increasing over time for each scaffold that mean they don't have toxicity affect onto HUVECs and they are biocompatible.

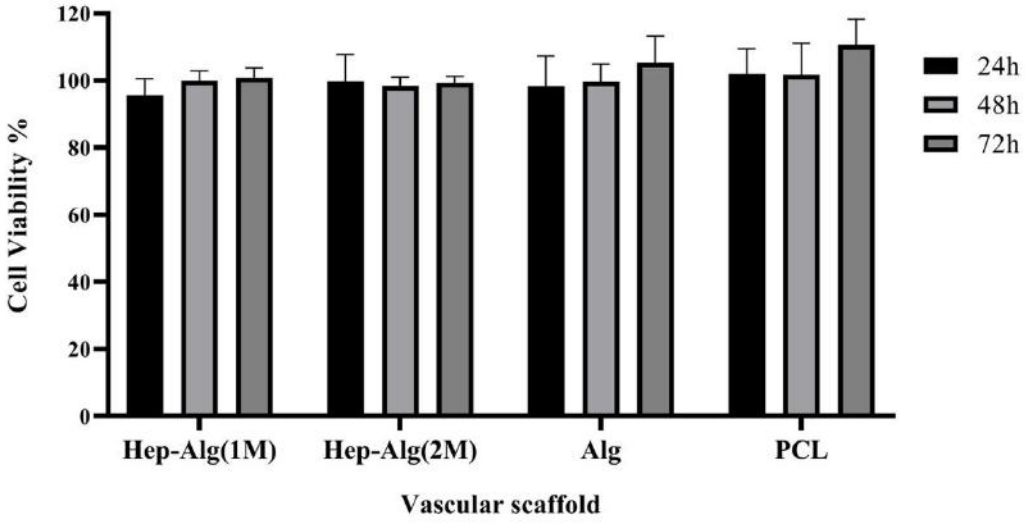


Figure 3.10.1 Biocompatibility evaluation of alginate scaffold, bilayered scaffold consisting alginate and PCL and PCL scaffold by using MTS

Alginate has a great potential in tissue engineering because of biocompatibility, non-immunogenicity and gelation characteristic with divalent cations like Ca^{+2} [47] . As shown in Figure 3.10.1, our case also supports the biocompatibility of the alginate scaffold which forms inner layer of vascular graft because of providing the cell attachment and proliferation. After evaluation of cytotoxicity of scaffolds, cell attachment studies were performed by Alg(1M), Hep-Alg (1M), and Hep-Alg (2M) scaffolds. As seen in Figure 3.10.2, alginate scaffolds supported cell attachment with hydrophilic character. Besides, cell attachment onto inner surface of scaffold increased through heparin functionalization.

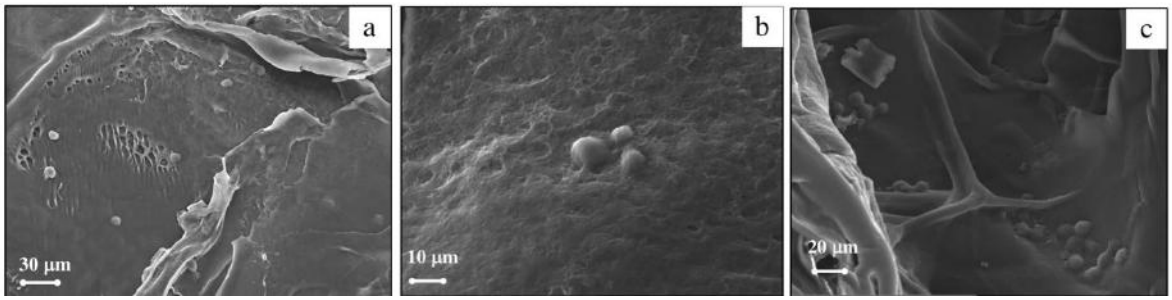


Figure 3.10.2 SEM morphology of endothelial cells seeded on alginate (a), Hep-Alg (1M) (b), Hep-Alg (2M) (c) and after 2 days of incubation

Chapter 4

4 Conclusions and Future Prospects

4.1 Conclusion

It has been projected that cardiovascular diseases including diseases of hearth and blood vessels, will lead to the death of >23.3 million people by the year 2030. Although autologous vessels are used in the replacement of diseased vessels, its resource shortage limits the use of them. In addition to autologous vessels, synthetic vessels are commonly used in treatment. However, while they are successfully used in large diameter vessels, they are not suitable in small diameter vessels due to early thrombosis formation. Tissue engineering vascular grafts seem as good candidates in the treatment of cardiovascular disease.

The aim of this study is to develop artificial vascular grafts for small diameter vessels. Thus, the bilayered scaffold was fabricated with both TIPS and electrospinning methods. The inner layer consisted of porous alginate and the outer layer of scaffold consisted of electrospun PCL. Additionally, heparin as an anticoagulant agent was immobilized to scaffold to improve endothelial cell attachment and prevent the risk of thrombosis. Mechanical characteristics are another essential factor for vascular grafts, which withstand blood pressure. Mechanical characteristic of the bilayered scaffold showed close characteristics with native vessels. The bilayered scaffolds supported endothelial cell attachment and showed the moderate degradation level that can enable to formation neotisue formation. These properties make the heparinized vascular scaffold produced in our study can be a good candidate in the bypassing of small diameter vessels for clinical application. This study is of a great importance in terms of societal impact due to its ultimate goal for the increase life duration and quality of the people suffering from cardiovascular diseases

4.2 Future Prospects

For the future studies, bioreactor systems have to be designed to allow the endothelial cells from inner side and smooth muscle cells form outer to determine the behave of scaffold under dynamic conditions. Moreover, culture duration should be elongated and mechanical tests should be determined for cultured vascular grafts. In vivo test should also be performed in order to understand its potential to be used as a product.

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