TISSUE ENGINEERING OF SMALL DIAMETER VASCULAR GRAFTS BY USING PCL/COLLAGEN BASED SCAFFOLDS

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ABSTRACT

TISSUE ENGINEERING OF SMALL DIAMETER VASCULAR GRAFTS BY USING PCL/COLLAGEN BASED SCAFFOLDS

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Cardiovascular system is composed of the heart, blood vessels and blood. This system consists of five types of blood vessels: arteries, arterioles, veins, venules and capillaries. Cardiovascular diseases (CVDs) including diseases of coronary arteries and blood vessels of the brain are responsible for 17.3 million deaths a year in the world. Atherosclerosis is the main reason for CVDs which is the hardening and thickening of arterial walls with lipid molecules and affects especially the walls of medium and large sized arteries. For the treatment of this disease autologous vessels availabity of which is limited are used. Synthetic blood vessels are successfully used in large diameter vessels (> 6mm). However they are not successful in small diameter vessels (< 6mm) due to early thrombosis formation. Tissue engineering is an interdisciplinary approach which applies the fundamentals of engineering to life sciences to replace, repair, maintain or exchange of damaged tissues or organs. Tissue engineered blood vessels are promising for the treatment of CVDs.

The aim of this study was the production of a tissue engineered blood vessel as a small diameter vascular graft and testing *in vitro*. For this purpose polycaprolactone-collagen based tubular scaffolds were fabricated by electrospinning. These scaffolds were crosslinked by treatment with glutaraldehyde. They were characterized microscopically by using stereomicroscope, and SEM. Thicknesses of scaffolds and fiber dimensions of scaffolds were calculated from the micrographs. Stability was tested in both PBS and collagenase type II. Their mechanical strength was determined by uniaxial tensile testing either in tubular form or in mat form.

Fiber diameter was found to be 289±89 nm in the inner surface of the scaffold while it was 641±206 nm on the outer surface of the scaffold. Thickness of the scaffolds was found 117±23µm. Glutaraldehyde treatment did not change the stability or the mechanical strength of the scaffolds.

In vitro studies were carried out by using human vascular smooth muscle cells (VSMC) on the one side of the mat and human internal thoracic artery endothelial cells (HITAECs) on the other side of the mat. Three types of constructs were tested: single VSMC seeded, single HITAEC seeded and cocultured of VSMC and HITAEC. VSMC seeded scaffolds were cultured for 21 days and it was shown that they supported cell attachment and proliferation. HITAEC seeded scaffolds were cultured for 14 days and it was shown that in the first week there was an increase but in the second week there was a plateau or a decrease in their proliferation. With the 12 days co-cultured scaffold it was observed that the optical density (OD) observed was higher than the individual cells combined indicating synergistic effect. These results were supported by the SEM micrographs. Single VSMC and HITAEC seeded mats and cocultered of VSMC and HITAEC mats increased the mechanical properties of these scaffolds.

Suturability of the tubular scaffolds was tested on these scaffolds without any tear.

This study showed that scaffolds made of electrospun PCL/Collagen supported cell proliferation and had appropriate mechanical properties. It can be said that they have a potential for use as a small vascular substitute.

Keywords: Tissue engineering, Blood vessel, Vascular smooth muscle cells, Endothelial cell, Polycaprolactone.

ÖZ

POLİKAPROLAKTON/KOLAJEN TEMELLİ KÜÇÜK ÇAPLI DAMARLARIN DOKU MÜHENDİSLİĞİ YÖNTEMİYLE ÜRETİLMESİ

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Kardiyovasküler sistem kalp, kan damarları ve kandan oluşmaktadır. Bu sistem arterler, arteriyoller, venalar, venüller ve kapilerler olmak üzere beş tip kan damarını içerir. Kalp-damar hastalıkları koroner arter ve beyine giden kan damarlarından kaynaklanan hastalıkları içermekte olup, bu hastalıklar her yıl dünyada 17.3 milyon insanın ölümüne yol açmaktadır. Atheroskleroz, kalp-damar hastalıklarının temel sebebidir. Atheroskleroz, lipid molekülleri ile arterlerin kalınlaşması, sertleşmesidir ve orta ve büyük çaplı damarları etkiler. Bu hastalığın tedavisinde kullanılan otolog damarlar sınırlı sayıda bulunur. Sentetik damarlar, geniş çaplı damarların (6 mm üzeri) tedavisinde başarılı bir şekilde kullanılır. Ancak erken pıhtı oluşumu nedeniyle küçük çaplı damarların (6 mm altı) başarılı bir şekilde tedavisinde kullanılamamaktadır. Doku mühendisliği disiplinlerarası bir alan olup, mühendislik bilimin temellerini hayat bilimlerine uygulayarak hasar gören doku ve organların yenisiyle değiştirilmesi, tedavi edilmesi ve korunmasını amaçlamaktadır. Doku mühendisliği yöntemiyle üretilen damarlar bu hastalıkların tedavisinde umut vericidir.

Bu çalışmanın amacı, doku mühendisliği yöntemiyle küçük çaplı yapay damarların üretilmesi ve bunların *in vitro* da test edilmesidir. Bu amaçla polikaprolakton/kolajen temelli tüp şeklindeki hücre taşıyıcıları elektroeğirme yöntemiyle üretilmiştir. Bu hücre taşıyıcıları glutaraldehit işlemiyle çapraz bağlarla bağlanmıştır. Taşıyıcılar stereomikroskop ve taramalı elektron mikroskopu kullanılarak karakterize edilmiştir. Bu görüntüler kullanılarak taşıyıcıların kalınlıkları ve fiber çapları hesaplanmıştır. Taşıyıcıların dayanıklıkları fosfat tamponlu tuzlu çözeltisinde ve kolajenaz tip II enzimiyle test edilmiştir. Boru veya hasır şeklindeki taşıyıcların mekanik dayanıklılığın tek eksenli çekme testiyle belirlenmiştir.

Taşıyının iç kısmındaki fiber çapı 289±89 nm olarak bulunurken, dış kısmındaki fiber çapı 641±206 nm olarak bulunmuştur. Taşıyıcıları kalınlığı 117±23µm olarak bulunmuştur. Glutaraldehyde işlemininin hücre dayanıklılığı üzerinde etkisi olmamıştır. Ayrıca bu işlemin taşıyıcıların mekanik dayanımları üzerinde etkisi olmamıştır.

In vitro çalışmalar, taşıyıcının bir tarafına insan damar düz kas hücreleri ve diğer tarafına insan torasik arter endotel hücreleri ekilerek yapılmıştır. Sadece damar düz kas hücresi ekili, sadece torasik arter endotel hücreleri ekili ve iki hücrenin birlikte kültüre edilen taşıyıcı olmak üzere üç tip yapı elde edilmiştir. Sadece damar düz kas hücresi ekili taşıyılar 21 gün kültür uygulanmıştır ve hücre tutunmasını ve çoğalmasını arttırdığı gözlenmiştir. Sadece endotel hücreleri ekili olan taşıyıcılar on dört gün kültür uygulanmıştır ve yedinci günden sonra hücre çoğalmasında düşüş gözlenmiştir. Damar düz kas hücreleri ve endotel hücreleri ekili olan ve on iki gün kokültür uygulanan taşıyıcılarda iki hücre tipi diğerinin çoğalmasında eş etki yapmıştır. Bu sonuç taramalı elektron mikroskobu görüntüleriyle de desteklenmiştir. Damar düz kas hücresi ekili olan, insan torasik arter endotel hücreleri etkili olan ve kokültür uygulanan taşıyıcılarda mekanik dayanım arttığı gözlenmiştir.

Tüp şeklindeki taşıyıcıların dikilebilirliği test edilmiş ve yırtık olmadan yapılabildiği gözlenmiştir.

Bu çalışma polikaprolakton/kolajen temelli taşıyıcıların uygun mekanik özelliklere sahip olduğunu ve hücre çoğalmasını desteklediğini göstermiştir. Bu nedenle bu taşıyıcıların yapay damar olarak kullanılması umut vermektedir.

Anahtar kelimeler: Doku mühendisliği, Damar, İnsan damar düz kas hücresi, Endotel hücresi, Polikaprolakton

Dedicated to my grandfather & nephew

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LIST OF ABBREVIATIONS

3D	Three Dimensional
BSA	Bovine Serum Albumin
b-FGF	Basic Fibroblast Growth Factor
CO_2	Carbon Dioxide
Coll	Collagen
Da	Dalton
DAPI	4', 6-diamidino-2-phenylindole
DHT	Dehydrothermal
DMEM/F12	Dulbecco's Modified Eagle Medium/Ham's F12 Nutrient Mixture
DMSO	Dimethyl Sulfoxide
E	Young's Modulus (upon tension)
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
ELP	Elastin Like Polymer
ELR	Elastin Like Recombinamer
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
g	gram
GAG	Glucosaminoglycan
Gly	Glycine
h	hour
ITT	Inverse Transition Temperature
HAc	Acetic Acid
min	minute
Mg	Magnesium
mg	milligram
mm	millimeter
mL	milliliter
nm	nanometer
K	Potassium
kDa	kilo Dalton
M	Molarity
mM	millimolar
Na	Sodium
NaCl	Sodium Chloride
NaH_2PO_4	Sodium phosphate monobasic
Na_2HPO_4	Sodium phosphate dibasic

PBS	Phosphate Buffer Saline
PCL	Poly(ε-caprolactone)
PDMS	Poly(dimethylsiloxane)
PEG	Polyethylene Glycol
PE	Polyethylene
Pen/Strep	Penicillin/Streptomycin
PGA	Poly(glycolic acid)
PHB	Poly(3-hydroxybutyrate)
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PK	Penetrating Keratoplasty
PLA	Poly(lactic acid)
PLGA	Poly(Lactic Acid-co-Glycolic Acid)
PLLA	Poly(L-Lactic Acid)
SDS-PAGE	Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
Tt	Transition Temperature
UTS	Ultimate Tensile Strength
UV	Ultra Violet
UXL	Uncrosslinked
v/v	volume/volume
WHO	World Health Organization
w/v	weight/volume
μm	micrometer

CHAPTER 1

INTRODUCTION

1.1 An Overview of the Cardiovascular System

The cardiovascular system consists of the heart, blood vessels and blood (Figure 1.1). Blood vessels constitute a network and the blood is pumped by the heart through the organs, tissues and return to the heart. Blood is responsible for carrying oxygen, nutrients and hormones through the tissues. In the cardiovascular system there are five types of blood vessels; these are arteries, arterioles, veins, venules and capillaries. Arteries transport blood away from the heart. Diameters of arteries decrease as they enter through the tissues and organs they are called arterioles. From the arterioles the blood passes to the capillaries where exchange of nutrient, oxygen and metabolic wastes. From the capillaries blood goes back to the heart through venules and veins ending at the heart with the vena cava (Martini, 2012).

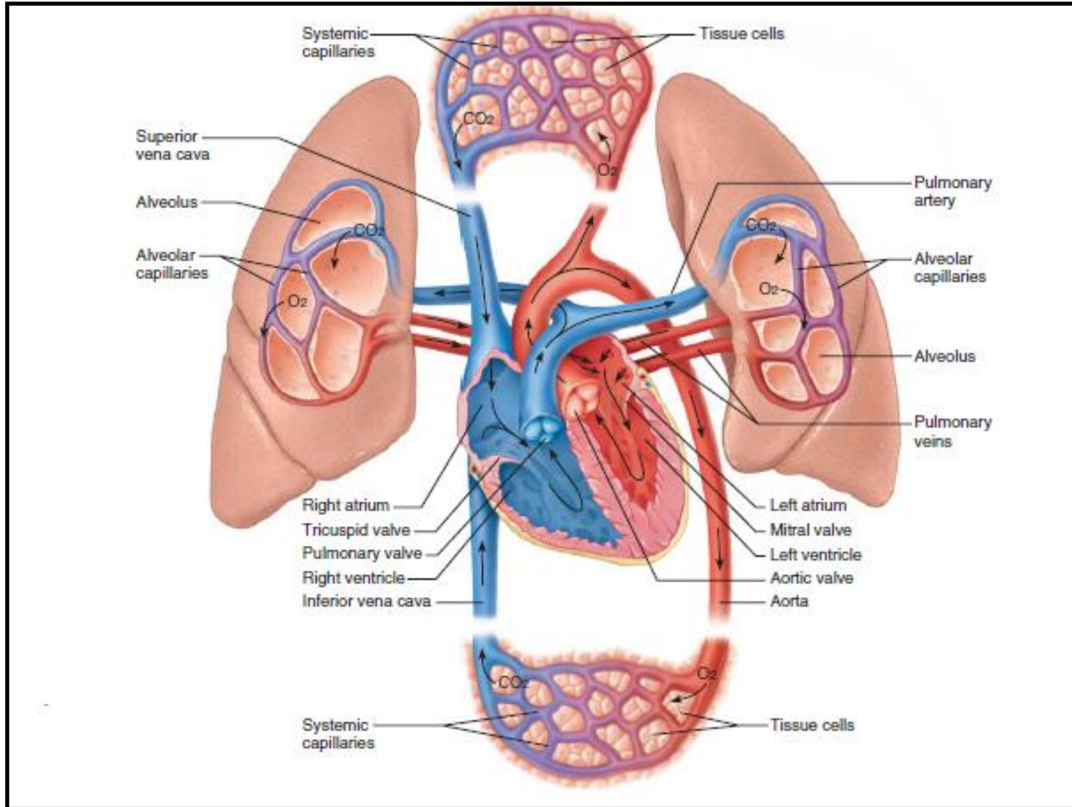

Figure 1.1 Scheme of cardiovascular system (Adapted from Martini 2012).

1.1.1 Structure and Function of Blood Vessels

Arteries

The walls of arteries consist of three layers which are: tunica intima, tunica media and tunica externa (Figure 1.2.a.).

Tunica intima (tunica interna) is the innermost layer of the vessel wall. It is composed of a layer of endothelial cells that form the endothelium and the connective tissue that is constituted of elastic and collagen fibers. Endothelium is the blood interfacing surface which does not allow blood clotting and thus makes the arteries antithrombogenic. An internal elastic lamina separates the layers of tunica intima and tunica media.

Tunica media is the middle layer and includes circumferentially oriented smooth muscle cells and a thick layer of elastic tissue. The tissue gives elasticity to the artery structure, and therefore, the artery can withstand high blood pressures.

Tunica externa (tunica adventitia) is the outermost layer. This layer is mainly composed of ECM matrix, collagen and fibroblasts and it is separated from the tunica media by external elastic lamina.

The arteries can be classified as elastic arteries (also known conducting arteries), and muscular arteries (also known medium size arteries). **Elastic arteries (conducting arteries)** are with a diameter of upto 2.5 cm. These vessels are responsible for transportation of large volumes of blood from the heart through the body. Due to the large amount of elastic fibers and few smooth muscle cells in its tunica media layer, the walls of these arteries are more resilient. **Muscular arteries (medium size arteries)** are responsible of carrying the blood through the internal organs and skeletal muscles. Diameter of muscular arteries is around 4.0 mm and the tunica media layer of these vessels is thick. They have more smooth muscle cells than elastic arteries.

Arterioles are thinner than muscular arteries. The walls of arterioles are similar to that of arteries; however the middle and external layers are thinner. Diameter of these vessels is 30 µm or less and in contrast to other types of arteries, the tunica media layer is composed of only few smooth muscle cells (Shier, 2010).

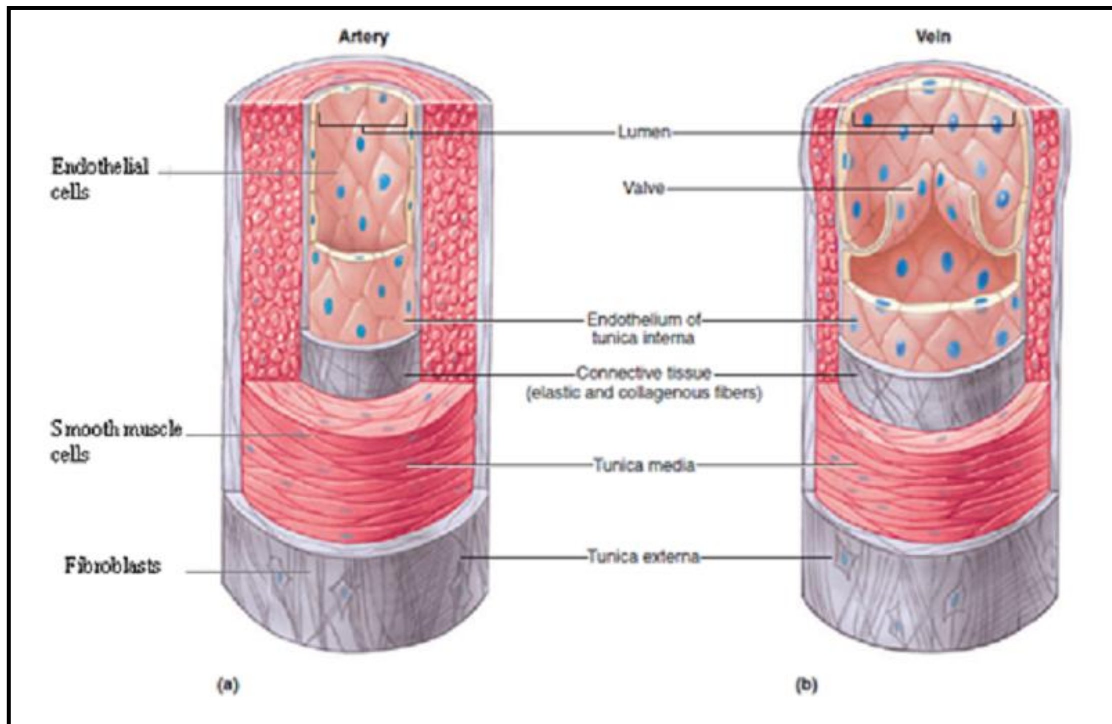

Figure 1.2 Walls of blood vessels a. artery and b. vein (Adopted from Shier 2010)

Veins

Veins are responsible for transportation of blood from tissues and organs to the heart. Walls of veins include three distinct layers similar to arteries (Figure 2.b.). The middle layer of the vein is thinner, and has less smooth muscle cells and less elastic tissue than the arteries. In comparison to arteries their lumens are larger. According to their sizes veins are classified into three groups: large veins, medium sized veins and venules.

Large veins consist of three layers. They include thinner tunica media layer. **Medium sized veins** include thinner tunica media layer with few smooth muscle cells. Internal diameter of these vessels varies from 2 to 9 mm. **Venules** transport the blood from capillaries to the veins (Martini, 2012).

Capillaries

Capillaries are placed between the arteriols and venules and they are responsible for exchange of the nutrients and waste products between blood and the tissues. A simple capillary is composed of thin basement membrane with a layer of endothelial cells (Figure 1.3.). It contains neither tunica adventitia nor tunica media. Average diameter of a typical capillary is 8 µm. Continuous capillaries and fenestrated capillaries are the main types of capillaries (Martini 2012).

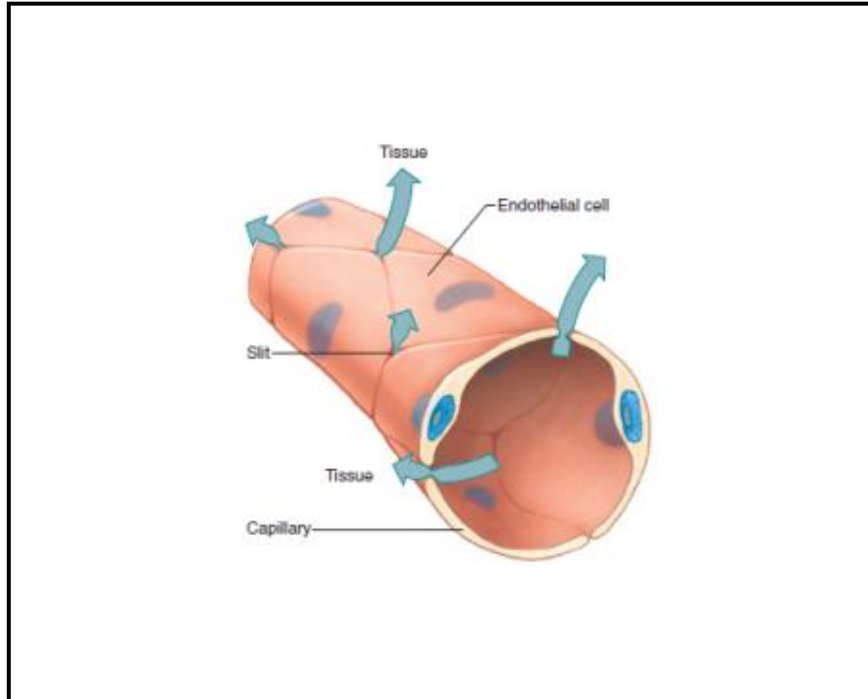

Figure 1.3 Structure of a capillary (Adapted from Shier 2010).

1.2 Cardiovascular Diseases

In the developed countries cardiovascular diseases (CVDs) constitute the major cause of mortality and morbidity. Since both environmental and genetic factors are involved in vascular pathologies it is difficult to prevent or treat all types of CVDs with the same approach (Zaragoza et al., 2011). According to a report of World Health Organization, CVDs are responsible for 17.3 million deaths a year (WHO Global Atlas 2011). CVDs include diseases of coronary arteries and blood vessels of brain. There are different types of CVDs. Coronary artery disease (e.g. heart attack), cerebrovascular disease (e.g. stroke) and diseases related with aorta and arteries containing peripheral vascular disease and hypertension occur due to atherosclerosis. Other types of CVDs are congenital heart disease, cardiomyopathies (disorder of heart muscle), rheumathic heart disease, cardiac arrhythmias and heart valve diseases. Atherosclerosis is the hardening and thickening of arterial walls with lipid molecules and affects especially the walls of medium and large sized arteries. Hypertension, high levels of lipids in blood (cholesterol), diabetes, gender and physiological factors (e.g. stress) are the main factors that induce the formation of atherosclerosis.

1.2.1 Atherosclerosis

Atherosclerosis is the accumulation of lipid molecules in the media layer of the arteries and lead to the dysfunction of the endothelial intima layer of the arteries. Development of atherosclerosis occurs as follows (Figure 1.4.): Due to a variety of factors the endothelium of an artery may be damaged. Lipids, mainly lipoproteins, and circulating cells penetrate the endothelial layer and form a lesion. Increased lipid accumulation and the cell numbers make the endothelial layer thrombogenic where the platelets can now bind. Due to release of growth factors from platelets, macrophages, and endothelial cells and increase of proliferation of SMCs and fibroblasts increase cause plaque formation, narrow the blood vessel and lead to a reduction in blood flow which induces thrombosis in arteries. All these result in the formation of a complex lesion of atherosclerosis. (Schachter 1997, Lefkowitz and Willerson, 2001, Chinta and Abhishek, 2012).

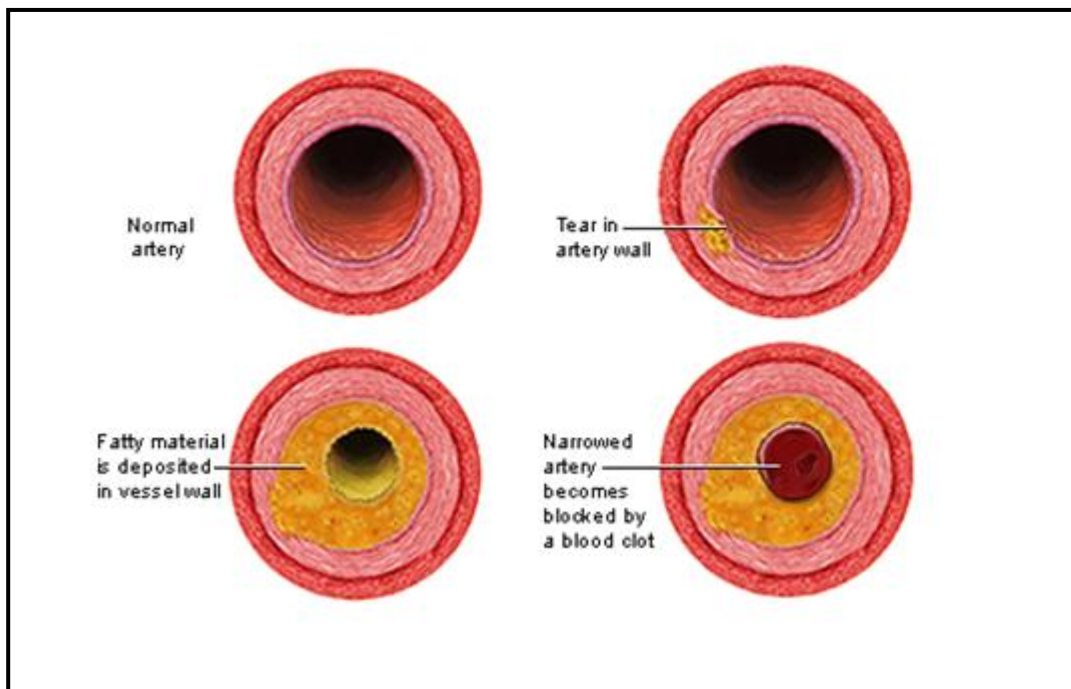

Figure 1.4 Development of atherosclerosis. 1. Normal artery, 2. A tear in the artery, 3. Fatty material deposition, 4. Very narrow artery clogged with a blood clot. (http://www.nlm.nih.gov/medlineplus/ency/imagepages/18020.htm).

1.3 Approaches Towards Treatment of Cardiovascular Diseases

1.3.1 Biological Substitutes

Coronary artery disease (CAD) is a type of cardiovascular disease that occurs due to atherosclerosis. One-third patients with CADs undergo balloon angioplasty and stent. Baloon angioplasty is used to open the occluded arteries (Figure 1.5.). Baloon tipped catheter is placed into narrowing side of the artery. When bloon is inflated in the artery it compresses the plaque and opens the occluded artery. The stent expands and presses against the arterial wall. Stents are small wire-mesh metal tubes that prevents closing the vessel again (restenosis) after bloon angioplasty. Baloon angioplasty is not applied to some patients who have CADs if their coronary arteries are too small or occluded coronary arteries are not completely opened with baloon angioplasty (Michaels and Chatterjee, 2002).

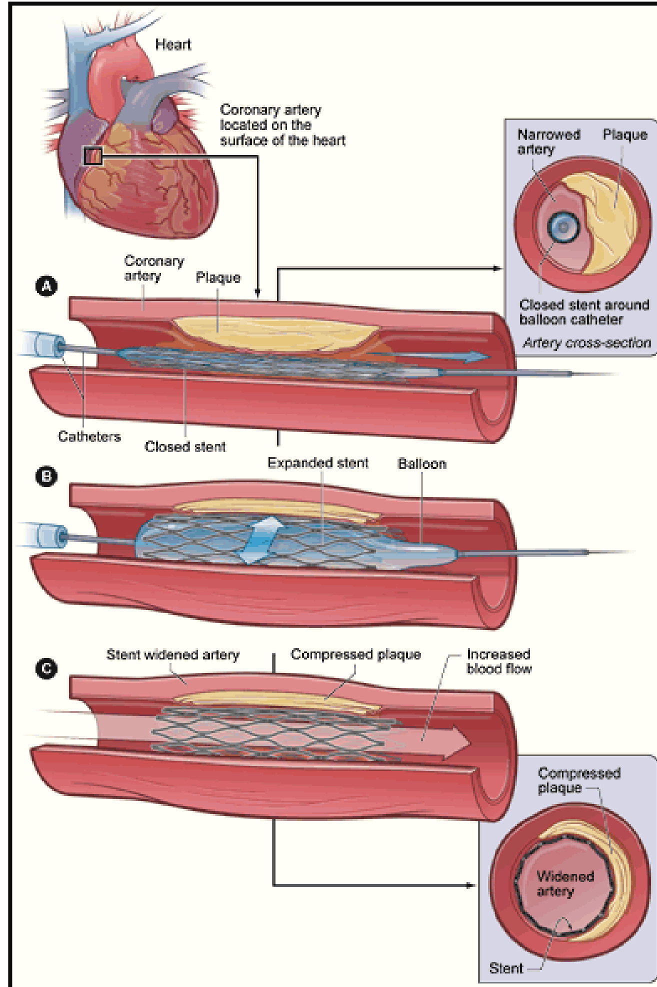

Figure 1.5 Baloon angioplasty and stenting A.Deflated balloon in artery B. Inflated balloon in artery C. Stent keeps artery open (http://healthpages.org/surgical-care/angioplasty-coronary-heart-stents/)

10% of the patients with coronary artery disease will undergo coronary artery bypass graft (CABG) surgery. (Michaels and Chatterjee, 2002). Autologous vessels such as arteries and veins are widely utilized in coronary and peripheral bypass surgeries. The sources for these are internal mammary artery, saphenous vein, internal thoracic artery and radial artery autografts. However, over 60% of the patients do not have suitable vessels due to damage, peripheral vascular diseases or previous surgeries (Moneta and Porter, 1995).

Arterial allografts also called homografts are used for the replacement of occluded arteries. Fresh allografts are susceptible to rapid rejection (Callow, 1996). However cryopreserved once used in bypass surgery has low patency rates due to occlusion (Schmidt and Baier, 2000).

Xenografts are heterografts derived from non-human species; porcine (Teebken et al., 2000, Concklin et al., 2002, McFetridge et al., 2004) and bovine origin (Jacobson and Haimov, 1975) xenografts were widely studied. Their disadvantage is the risk of disease transmission from animal species to the host.

Both the allografts and xenografts need to be subjected to chemical or physical pretreatments for the following reasons: (1) improvement of the mechanical properties of the grafts against enzymatic and hydrolytic degradation (by crosslinking), (2) sterilization of the material, and (3) prevention of immunogenic responses (by decellularization) (Schmidt and Baier 2000).

Biological scaffolds can be obtained by decellularization of tissues and organs. The aim of decullarization process is the removal of all cellular and nuclear materials of the structure while preserving the biological composition and mechanical properties of the ECM (Piterina et al., 2009).

Decellularization might be achieved by physical, chemical and enzymatic approaches. Physical methods used for decellularization are agitation, sonication, and freezing. Mechanical agitation or sonication causes cell lysis and are generally used together with chemical treatments to remove the cell debris. Rapidly freezing of the tissue causes formation of ice crystals in the cells which results in disruption of the cell membrane, and thus cell lysis (Gilbert et al., 2006).

There are several chemical methods for decellularization. Alkaline and acid treatments are utilized to solubilize the cells and remove the DNA and RNA. Peracetic acid (PAA), hydrochloric acid, sulfuric acid and ammonium hydroxide are some examples of this approach. Non-ionic detergents such as (Triton X-100) are used to disrupt lipid-lipid and lipid-protein interactions. Ionic detergents effectively solubilize both the cytoplasmic and nuclear membrane. They can also disrupt protein-protein interactions and denature proteins. Sodium doceyl sulfate (SDS), Triton X-200 and sodium deoxycholate are widely used ionic detergents. Zwitterionic detergents show the properties of both ionic and non-ionic detergents. 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) is an example of Zwitterionic detergents and is used in the decellularization of blood vessels (Dahl et al., 2003). Tri(n-butyl)phosphate (TBP), hypotonic and hypertonic treatments and chelating agents such as EDTA (typically used with typsin) and EGTA are the other chemicals used in decellularization (Gilbert et al., 2006).

Enzymes used in decellularization of tissues and organs include nucleases, trypsin, collagenase, lipase, dispase, thermolysin, and galactosidase. Enzymes are used to remove the cells and ECM components. However, only an enzymatic treatment is not enough to disintegrate the cells completely. Nucleases such as DNases and RNases hydrolyze the nucleic acids and assist removal of the. Trypsin is a serine protease and used with EDTA in decellularization through hydrolysis of protiens (Crapo et al., 2011).

Crosslinking is used to increase the mechanical properties of the allo and xenografts and help to preserve their compliance. Chemical crosslinkers are glutaraldehyde, carboimidies (e.g. 1-ethyl-3-(3-dimethylaminopropyl) carbodimide, EDC), hexamethylene diisocyanate (HMDC), polyepoxy compounds and dye-mediated oxidation (Schmidt and Baier, 2000, Altizer et al., 2010).

1.3.2 Synthetic Blood Vessels

Synthetic blood vessels are utilized successfully in the resconstruction of large diameter (>6 mm) blood vessels (Xue et al., 2003). However, they were not successful in small diameter vessel applications due to small cross section and early thrombosis formation. There are three major commercially available synthetic graft families used in vascular surgery: poly(ethylene terephthalate) (PET, Dacron, Terylene), expanded poly(tetrafluoroethylene) (Teflon, GoreTex) and polyurethane. Knitted, woven and nonwoven forms of these synthetic substitutes are available.

PET

PET is a type of thermoplastic polyester and its vascular grafts are made of multiple filaments in knitted and woven form (Figure 1.6.). Woven forms have smaller pores than the knitted vascular grafts. These large pores of the knitted forms need to be coated with albumin or gelatin to prevent blood leakage and sometimes antibiotics are introduced to prevent infection (Cziperle et al., 2004). Its high tensile strength (40 to 80 MPa) and Young's modulus (up to 3 GPa) values are appropriate for use as blood vessel substitute (Chlupac et al., 2009, Chinta and Abhishek 2012).

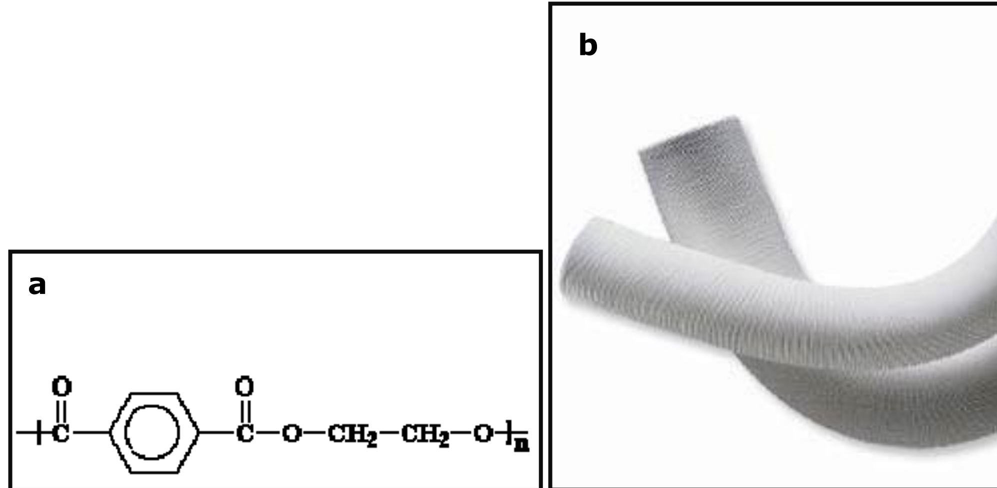

Figure 1.6 a. Chemical structure of PET (Dacron) and b. Texture of Bard® DeBakey® vascular graft (Chinta and Abhishek, 2012, http://www.bardpv.com/vascular/product.php?p=1)

ePTFE

Polytetrafluoroethylene is an inert hydrophobic fluorocarbon and polymer. It generally has a high molecular weight. ePTFE vascular graft is fabricated by heating, stretching and extruding this polymer leading to a non-textile vascular graft (Figure 1.7.). This polymer has a highly crystalline structure (>%90). Its stiffness is about 0.5 GPa, and its tensile strength about 14.0 MPa. The ePTFE vascular graft is composed of solid nodes and a fibrillar structure. Its microporous structure helps tissue healing process. But the large pore sizes can cause bleeding. The graft surface is electronegative and this decreases its reaction with blood making it non-thrombogenic (Kannan et al., 2005, Chlupac et al., 2009, Chinta and Abhishek 2012).

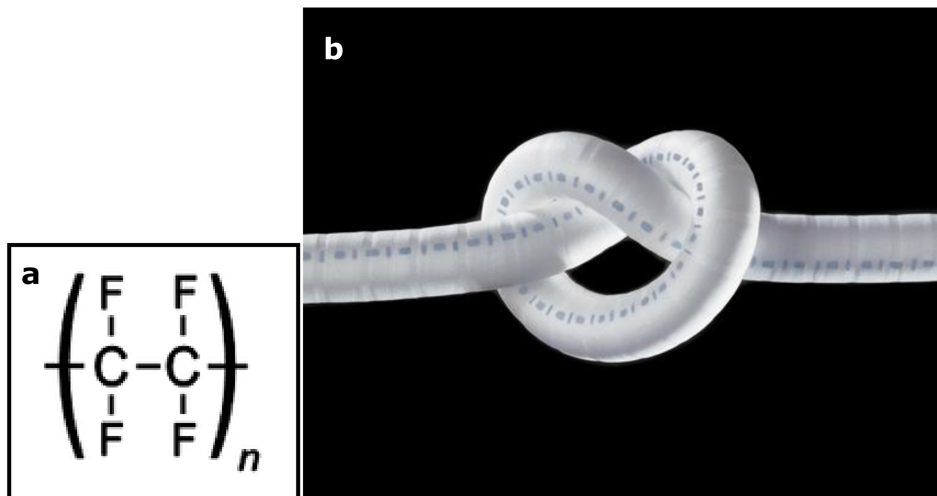

Figure 1.7 a. Chemical structure of PTFE and b. GORE-TEX® vascular graft

(http://www.tiptekmedikal.com.tr/templates/urunview/topresim.php?Id=5957220&ResId=bs196914)

Polyurethane

Polyurethanes (PU) are synthesized from molecules with isocyanate and alcohol groups. They are hemocompatible, when processed into a blood vessel they have smooth, non-thrombogenic surfaces (Figure 1.8.). Polyurethanes have soft segments which give them flexibility and the hard segments, stiffness. By changing the proportion of soft and hard segments in the composition, tensile strength can be changed between 20 MPa and 90 MPa (Kannan et al., 2005). The major obstacle with the first generation PUs was their in vivo degradation. The second generation PUs are carbonate-based and carry no ester linkages, and therefore, their degradation is prevented (Kannan et al., 2005).

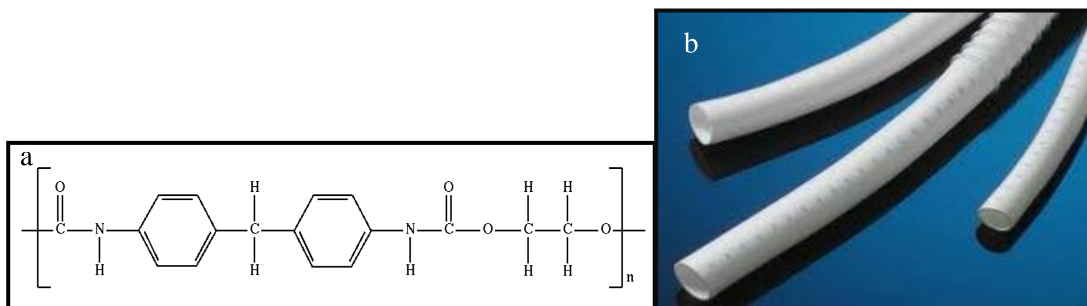

Figure 1.8 a. Chemical structure of polyurethane and b. AVFLO polyurethane vascular graft (http://trade.indiamart.com/details.mp?offer=4724473912)

1.3.3 Tissue Engineering in General

Tissue engineering is an interdisciplinary field which applies the fundamentals of engineering to life sciences to replace, repair, maintain or enhance damaged tissues or organs (Nerem and Sambanis 1995). Tissue engineering is based on three major components: scaffolds, cells and biochemical signals. Scaffolds can be two or three dimensional. They create an appropriate environment for cell growth and development of the new tissue. Cell types are chosen according to the targeted tissue. Sources include autologous cells isolated from the patients, allogeneic cells isolated from a donor and xenogeneic cells isolated from other species. Xenogeneic cells carry the risk of transmittance of pathogens from the animals to the patients.

Scaffolds are fabricated from synthetic (polyglycolic acid, polycaprolactone, polylactide-co-glycolid) and natural polymers (collagen, elastin, silk fibroin) and they should be biocompatible and biodegradable. Many techniques are used to fabricate scaffolds: solvent casting and particulate leaching, electrospinning, freeze drying, phase separation, rapid prototyping of solid freeform fabrication, phase separation, extrusion, gas foaming, fiber bonding, melt molding, peptide self-assembly and polymer/ceramic composite fabrication (Griffith and Naughton, 2007, Liu et al., 2007, Murphy and Mikos, 2007).

The cells used in tissue engineering can be mature (or primary cells) and stem cells. Mature cells can be isolated from the patient or donor by tissue biopsies but they are generally differentiated and their proliferation capacity is therefore low. A stem cell is described as an undifferentiated or immature and primal cell type that are in capable of selfrenewal and differentiattion into several cell types. Stem cells can be embryonic or adult stem cells. According to their plasticity the stem cells can be categorized into three classes: Totipotent cells are capable to differentiate into form all types of cells. Pluripotent stem cells can form most cell types (e.g. ES cells). Multipotent stem cells are capable of forming a tissue (e.g. adult stem cells) (Griffith and Gail Naughton, 2007, Buttery and Shakesheff, 2008).

Biochemical signals such as growth factors and morphogens stimulate the cell response (Griffith and Naughton, 2007, Buttery and Shakesheff, 2008). Growth factors are essential for tissue engineering and regenerative medicine. They are proteins that are known to stimulate cell growth, proliferation, migration and differentiation. They can be secreted from different types of cells and their effects on cells are concentration dependent. They can be used for tissue engineering in two ways. First approach includes combination of growth factors with scaffolds. In second approach growth factors are added cell culture medium during *in vitro* studies (Liao et al., 2008). Basic fibroblast growth factor (bFGF), bone morphogenetic protein-2 (BMP-2), bone morphogenetic protein-7 (BMP-7), epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF),

nerve growth factor (NGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF) are examples of growth factors used in tissue regeneration (Whitaker et al., 2001, Lee et al., 2011).

1.4 Vascular Tissue Engineering

An ideal vascular graft must have (1) compliance, (2) thromboresistance, (3) non-immunogenicity, (4) resistance to infections, (5) ability to heal, (6) longterm mechanical strength, and (7) porosity for cell tissue growth and nutrient transport. A tissue engineered blood vessel consists of a tubular scaffold made of either natural or synthetic polymer, stem or vascular cells and appropriate signal molecules (Kakisis et al., 2005, Couet et al., 2007).

Generally appropriate cells are harvested. Then, isolated cells are cultured to obtain the required cell numbers. Cells are seeded onto porous, tubular scaffolds and incubated in a medium supplemented with growth factors to form a functional tissue. Finally the mature tissue is implanted (Figure 1.9.).

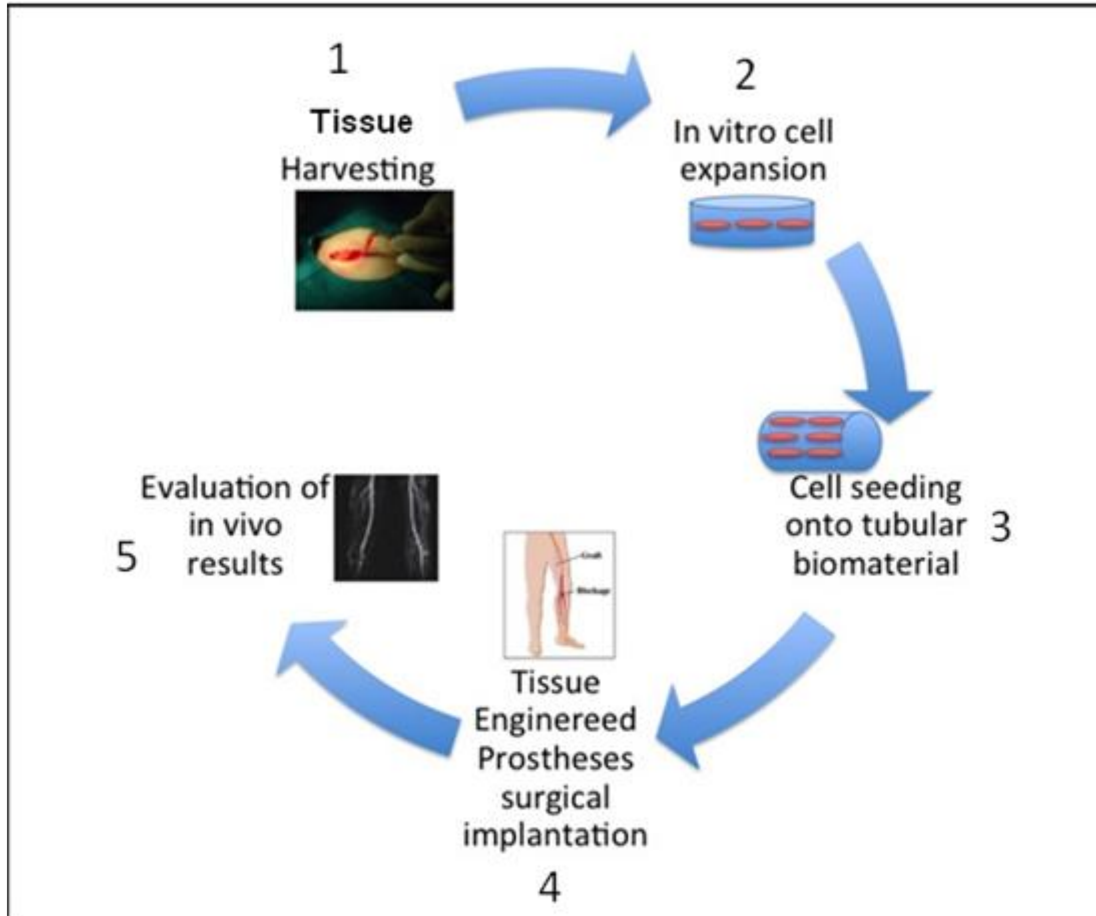

Figure 1.9 General strategy for vascular tissue engineering (http://www.intechopen.com/books/biomaterials-science-and-engineering/new developments-in-tissue-engineering-of-microvascular-prostheses)

1.4.1 Scaffolds Used in Vascular Tissue Engineering

The main function of a tissue engineering scaffold is to provide a temporary support for cells until the new ECM is secreted and tissue is formed. Vascular tissue scaffolds can be fabricated mainly from polymers. According to their origins polymers can be classified into two main groups: natural and synthetic polymers.

1.4.1.1 Natural Polymers

Two major classes of natural polymers used in tissue engineering applications are polypeptides (collagen, elastin, gelatin and silk) and polysaccharides (agarose, alginate and chitosan). Scaffolds from natural polymers might have better cell attachment due to presence of certain groups but are susceptible to enzymatic degradation. Besides like all polymers, natural polymers have low mechanical properties and this should be improved by a variety of approaches including crosslinking (Fisher, 2007).

Chitosan is a natural polysaccharide derived from chitin. Its antimicrobial activity and low cost makes chitosan a good material for tissue engineering applications (Khor and Lim 2003). However, its positive charge has been a cause for concern due to the toxic effect it had on cells. Structural similarity of chitosan to the glucoseaminoglycan (GAG) based native components of ECM is another advantage (Drury and Mooney 2003). Due to low mechanical properties like any other hydrophilic polymer use of chitosan in vascular tissue engineering was limited (Couet et al., 2007). A blend of chitosan with collagen (Chen et al., 2010), with thermoplastic polyurethane (TPU) (Huang et al., 2011), and gelatin (Huang et al., 2005) are used for vascular tissue engineering studies.

Hyaluronic acid (HA) is a nonsulfated GAG. HA is present in many tissues including blood vessels (arteries and veins), skin, and connective tissues (Laurent et al., 1996, Fraser et al., 1997). Limitations related with hyaluronic acid use are fast degradation rates and low mechanical properties (Couet et al., 2007). Since mechanical propertes of materials are very important for an ideal vascular graft, Arrigoni et al (2006) added sodium ascorbate to esterified hyaluronic acid (HYAFF) to increase the mechanical properties of the vascular construct.

Elastin is found especially in the elastic tissues such as the walls of arteries. It is responsible for the elasticity and high strain capacity of arteries (Couet et al., 2007). Elastin blended with collagen (Koens et al., 2010), with polycaprolactone, and silk (McClure et al., 2012), and recombinant human tropoelastin alone (McKenna et al., 2012) were utilized for fabrication of vascular grafts.

Another polypeptide is **fibrin** which is an insoluble protein that is responsible for blood clotting. The main advantage of using fibrin as a scaffold material is the ease of production of fibrin from the patient’s own blood (Couet et al., 2007). As a result fibrin-based scaffolds do not cause any inflammatory reactions (Ye et al., 2000, Jockenhoevel et al., 2001). A fibrin based scaffold combined with a polylactic acid mesh was used to construct small diameter blood vessel as an implantable graft (Koch et al., 2010).

Silk fibroin is a protein widely utilized in biomedical applications. The best known of silk fibroin is the one derived from cocoons of silkworm *Bombyx mori* (Altman et al., 2003). Spiders also produce fibroin and recently researches started to use them. According to their sources the structure and composition of silk fibroins vary. Due to their slow degradation rate in the body silk fibroin is considered as a good candidate for vascular tissue engineering (Altman et al., 2003). Zhang et al (2008), used a coculture of endothelial cells and smooth muscle cells on electrospun silk scaffolds and tested them as a vascular graft.

1.4.1.1.1 Collagen

Collagen is the most abundant protein family found in the native ECM and until now 29 different types of collagen have been identified (Gelse et al., 2003). Collagen is responsible for providing mechanical strength and supporting the tissues and organs. According to their structures, collagen families can be classified into fibril-forming collagen, basement membrane collagens, microfibrillar collagen, anchoring fibrils and other types of collagen. Collagen type III (fibril forming) and collagen type VI (microfibrillar) are found in vessel walls (Gelse et al., 2003). A simplified structure of collagen is a right handed triple helix formed of 3 left handed helices forming a superhelix (Figure 1.10.) and all the members of collagen family share this supramolecular structures in the ECM.

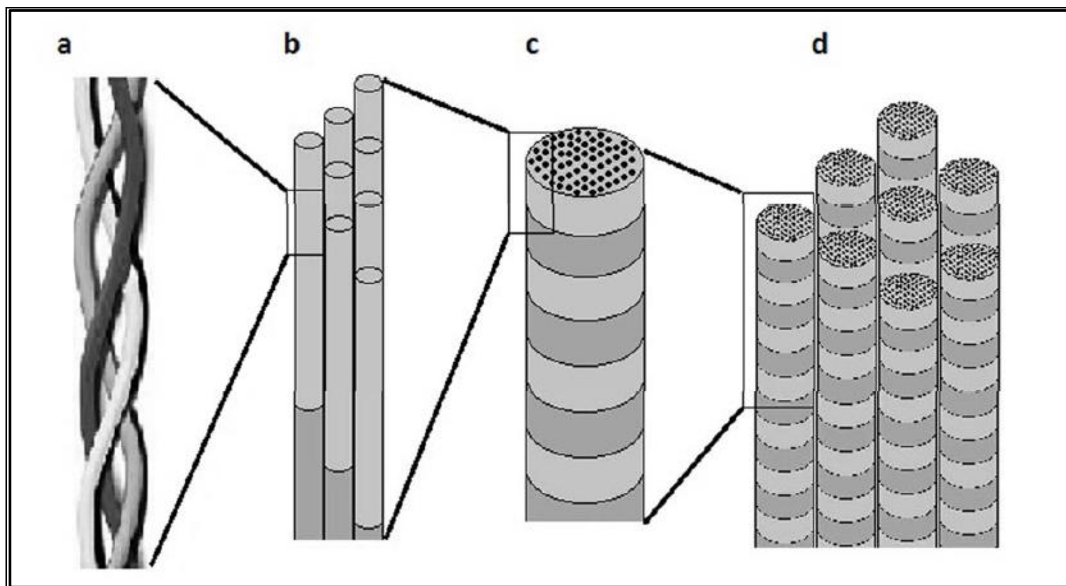

Figure 1.10 Structure of collagen. a. α triple helix segment b. tropocollagen molecule c. collagen fibril (in a diameter of 10 to 300 nm) d. a collagen fiber consists of collagen fibrils (diameter 0.5 to 3 μm) (Parenteau-Bareil et al., 2010).

Its natural origin and biodegradability are the most important properties of collagen. Since collagen is rapidly hydrolyzed by water and the enzymes, in order to control its degradation rate it needs to be crosslinked using physical, chemical and enzymatic methods. The principle of chemical crosslinking of collagen is based on forming a covalent bond between amine and carboxyl groups within the same collagen molecule (Parenteau-Bareil et al., 2010). Physical crosslinking of collagen can be achieved by UV radiation and by dehydrothermal treatment (DHT); both were shown to increase the tensile strength of the collagen (Weadock et al., 1995). UV radiation is simpler than DHT because it might be achieved in only 15 min. Since UV crosslinked collagen scaffolds makes the structure enzymatic degradation resistant this method is more beneficial for load bearing applications (Weadock et al., 1996). There are several chemical crosslinking methods used with collagen based scaffolds. Glutaraldehyde is the most commonly used chemical crosslinker (Lee et al., 2008, Tillman et al., 2009). Genipin is isolated from fruits of *Gardenia jasminoides ELLIS*. It is used as a crosslinking agent for biological tissues. In comparison to glutaraldehyde it is about 5000-10000 times less toxic (Mi et al., 2004). Carbodiimide family is another group of chemical crosslinkers. 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) is a member of carbodiimide family used in combination with N-hydroxysuccinimide (NHS). They are used to crosslink biological tissues. They do not remain in tissues but they release urea molecule (Powell and Boyce, 2006).

Collagen is one of the main proteins located in blood vessel structure. It is mainly secreted by smooth muscle cells in the media and fibroblasts in the adventitia. Therefore collagen is widely used for vascular tissue engineering to mimic the native blood vessel (Bou-Gharios et al., 2004). Collagen type I is hemocompatible since does not induce blood coagulation and it enables adhesion and aggregation of platelets (Boccafoschi et al., 2005).

Weinberg and Bell (1986) developed the first tissue engineered blood vessel. Collagen and bovine SMCs were casted together to make circular mold. Inner surface of the scaffold was seeded with bovine ECs and outer surface of scaffold was seeded with bovine fibroblasts. The Dacron mesh integrated to scaffold to increase the mechanical strength. Without dacron mesh the scaffold resisted low pressure (< 10 mm Hg). Integration of the mesh increased the burst strength (40-70 mm Hg) L'Heureux et al (1993) developed tubular vascular model by seeding SMCs, ECs and fibroblasts on tubular collagen gel. As a result vascular SMCs created three dimensional network on scaffold, fibroblasts were randomly oriented and homogenous endothelium was obtained. It was reported that hybrid tubular vascular graft for low pressuere circulatory system was created by collagen type 1 cultured with canine jugular SMCs and ECs. The mechanical strength of the graft was poor and to increase it, the graft was wrapped with Dacron mesh. (Hirai et al., 1994, 1996).

Zorlutuna et. al (2009) studied the effect of topographical surfaces on mechanical properties of tissue engineered vascular grafts. They showed that nanopatterned collagen scaffolds aligned vascular smooth muscle cells and the alignment increased the mechanical strength of these scaffolds.

Blends of collagen with synthetic polymers are also used to produce tissue engineered blood vessel. In a study electrospun PCL nanofibers coated with collagen was seeded with human coronary artery SMCs, SMCs proliferation and migration, muscle tissue formation were increased the mechanical properties of the matrix (Venugopal et al., 2005).

1.4.1.1.2 Elastin-Like Recombinamers (ELRs)

Elastin-like recombinamers (ELRs) are also known as elastin-like polypeptides (ELPs) and they are artifical proteins produced by recombinant DNA technology. They can be designed to have certain properties as a result of the tailored aminoacid sequence. The stimuli-responsiveness and self-assembly properties of some ELRs make them suitable. The composition of ELRs is based on pentapeptide repeats. Val-Pro-Gly-Xaa-Gly (VPGXG) where X shows natural or modified amino acid without proline. The compostion was used in this study due its attractiveness for endothelial cells which are the essential ingredients of a blood vessel (Rodriguez-Cabello et al., 2009).

Inverse temperature phase transition is a characteristic feature of most ELRs based on VPGXG sequences. As temperatures below their inverse transition temperature (ITT or T_t) these ELRs are soluable in aqueous solution. Above Tt the ELRs aggregate and from an insoluable coacervate or simply precipitate (Urry, 1997).

Among the many applications of ELRs in tissue engineering are cartilage (Betre et al., 2006), ocular (Martinez- Osorio et al., 2009), liver (Janorkar et al., 2008), cell sheet (Mie et al., 2007) and oral mucosa tissue engineering (Kinikoglu et al., 2011).
The REDV sequence (R: Arginine, E:Glutamic acid, D: Aspartic acid and V: Valine) used in this study is an adhesion sequence for endothelial cells (Figure 1.11.) Hubbell at al (1991) showed that this sequence was located in the IIICS domain of human plasma fibronectin and was responsible for endothelial cell attachment.

Figure 1.11 Amino acid sequence of an elastin-like recombinamer (ELR), REDV, designed for its attractiveness towards endothelial cells.

1.4.1.2 Synthetic Polymers

Degradable synthetic polymers are the main materials used in the construction of tissue engineering scaffolds and drug delivery systems. The main advantages of these polymers over the biological ones are their controllable features. These reflect on their thermal and mechanical properties, degradability and biocompatibility such as compostion, molecular weight and crystallinity. Some synthetic polymers undergo degradation and therefore suitable for tissue engineering but their degradation products might evoke adverse reactions such as inflammation. Polyester is the main polymer group used in tissue engineering. Polyanhydrides, polycarbonates, and polyphosphazenes are some other types of polymers used in this field (Fisher 2007, Nair and Laurencin 2007).

Polyglycolic acid (PGA) is an aliphatic and highly crystalline (45-55%) polyester. Due to its high crystallinity it has high tensile strength and stiffness (Nair and Laurencin, 2007). Tubular PGA based scaffold was cocultured with bovine smooth muscle cells and endothelial cells in a bioreactor with pulsatile perfusion system and it was shown that the thickness of vessel wall and suture retention of the engineered vessel were increased (Niklason et al., 1999). In another study unwoven PGA scaffold was seeded with canine carotid smooth muscle cells and this approach was efficient for large vessel engineering applications (Xu et al., 2008). Wang and colleagues (2010) produced tissue engineered blood vessel by smooth muscle cells differentiated from human adipose derived stem cells with induction of transforming growth factor-b1 (TGF-b1) and bone morphogenetic protein-4 (BMP4) seeded on PGA scaffold. They showed that the engineered vessel had appropriate biomechanical strength similar to normal blood vessel.

Polylactic acid (PLA) is crystalline polyester (37% crystallinity). It has two optically active forms which are L-lactide and D-lactide. This polymer exhibits slow degradation depending on crystallinity in comparison to same property of PGA (Nair and Laurencin, 2007). PGA-PLLA based porous scaffold was cocultured human smooth muscle cells and ECs differentiated from endothelial progenitor cells to produce functional tissue engineered microvessels (Wu et al., 2004).

Polyhydroxyalkanoates (PHA) are polyesters produced by microorganisms. PHAs include poly(3-hydroxybutyrate) (PHB), copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), poly(4-hydroxybutyrate) (P4HB), copolymers of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) and poly 3-hydroxyoctanoate (PHO). PHAs and their blends are used to product nerve guide, skin substitutes, wound dressing, and vascular graft (Chen and Wu 2005, Philip and Roy 2007).

1.4.1.2.1 Polycaprolactone (PCL)

PCL is another polyester widely used in drug delivery systems (microspheres, nanospheres), tissue engineering applications (bone, cartilage, and cardiovascular etc) and in medical devices (sutures, wound dressings, contraceptive devices, and in dentistry). PCL (Figure 1.12.) is a hydrophobic, semi-crystalline and biodegradable polymer which has a low degradation rate (2-3 years). When this synthetic polymer undergoes degradation, the degradation products can be removed from the body either by the TCA cycle or by renal filtration (Woodruff and Hutmacher, 2010). Due to lack of appropriate enzymes this polymer cannot be enyzmatically degraded in human and animal bodies (Vert 2009) but, it can be degraded by organisms (bacteria or fungi). PCL has mechanical properties suitable for some biomedical applications. For example, it has low tensile strength (23 MPa) and extremely high elongation break (Gunatillake et al., 2006).

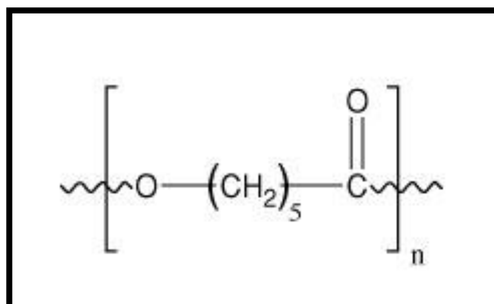

Figure 1.12 Structure of polycaprolactone (PCL)

PCL was used alone or as blends with natural and synthetic polymers in tissue engineering of blood vessels. These are poly(ε-caprolactone)/poly(trimethylene carbonate) (Jiang et al., 2013), polylactic acid (PLA) (Vaz et al., 2005), collagen (Venugopal et al., 2005, Tillman et al., 2009), elastin and collagen (McClure et al., 2010), polyurethane (PU) (Williamson et al., 2006), collagen and chitosan (Yin et al., 2012), elastin, silk and collagen (McClure et al., 2012), human elastin (Wise et al., 2011), chitosan and RGD-recombinant spider silk protein (pNSR32) (Zhao et al., 2013), and RGD protein (Zheng et al., 2012).

1.4.2 Cell Sources Used in Vascular Tissue Engineering

In this study the design of the small vascular graft was based on using endothelial cells (ECs) and smooth muscle cells (SMCs) to mimic the intima and media of a native vessel. ECs and SMCs isolated from harvested blood vessels are used in most of the studies. The most important function of endothelial cells in the blood vessel is improving the thromboresistance (Fisher 2007, Naito et al., 2011). Smooth muscle cells produce collagen, elastin and proteoglycans. Their functions are vasoconstriction and dilation of blood vessel depending on physiological conditions (Isenberg et al., 2008).

Stem cells are undifferentiated cell sources in the field of tissue engineering and regenerative medicine since they have ability to self-renewal and differentiate into mature cells. They can be isolated from bone marrow, muscle adipose and umbilical cord. For vascular graft studies autologous stem cells offer powerful opportunities (Wu et al., 2006). There several types of stem cells used in vascular tissue engineering applications (Table 1.1).

Mesenchymal stem cells (MSCs) and endothelial progenitor stem cells (EPCs) are the most studied stem cell types in vascular tissue engineering (Wu et al., 2006). MSCs can be isolated from adipose tissue, bone marrow and trabecular bone of various tissues. These cells have ability to differentiate into the cells of bone, muscle and cartilage (Barry and Murphy, 2004). Gojo et al (2003) showed that when isolated MSCs were injected directly into adult heart they had the ability to differentiate into endothelial cells, smooth muscle cells, pericytes and cardiomyocytes.

Endothelial progenitor cells (EPCs) are another group of cells that can be isolated from bone marrow (Reyes et al., 2002), cord blood (Murohara et al., 2005) and circulating mononuclear cells (Asahara et al., 1997, Lin et al., 2000). Differentiation of EPCs into endothelial cells depends on the culture conditions (Hristov et al., 2003) It has been demonstrated that vascular endothelial growth factor (VEGF) and fibronectin induce the formation of endothelial cells from differentitation of EPCs (Wijelath et al., 2004).

Embryonic stem cells (ESCs) have the ability to differentiate into all types of human embryonic cell lineages (Odorico et al., 2001). They are a good cell source for vascular tissue engineering because they can differentiate into both endothelial cells and smooth muscle cells. Levenberg and coworkers (2002) showed that embryonic stem cells could differentiate into endothelial cells that can be used in developing vascular like structures. Hu et al (2012) demonstrated that smooth muscle cells were derived from mouse embryonic stem cells were promising cell sources for production of vascular substitutes.

Bone marrow stem cells (BMSc) are another alternative stem cell source for tissue engineered blood vessel. They are capable of differentiation into endothelial-like cells and vascular smooth muscle-like cells (Cho et al., 2005). Adipose derived stem cells (ASCs) are located in the adipose tissue and are capable of differentiating into osteoid, adipose, muscle and cartilaginous cells (Zuk et al., 2001). Human umbilical cord vein endothelial cells (HUVECs) are a cell line used in vascular tissue engineering applications (Schechner et al., 2000, Kelm et al., 2010). Human artery derived fibroblasts (HAFs) is another group of stem cells has been shown to induce the formation and maturation of ECM (Kelm et al., 2010).

Table 1.1 Stem cells used in tissue engineered blood vessels.

Stem Cell Source	Differentiation into	Reference
Mesenchymal Stem Cells (MSCs)	Bone, cartilage, muscle and endothelial cells	Gojo et al., 2003 Barry and Murphy, 2004
Endothelial Progenitor Stem Cells (EPSc)	Endothelial cells	Hristov et al., 2003 Wijelath et al., 2004
Embriyonic Stem Cells (ESCs)	Endothelial cells Smooth muscle cells	Levenberg et al., 2002 Hu et al., 2012
Bone Marrow Stem Cells (BMSc)	Endothelial-like cells Smooth muscle-like cells	Cho et al., 2005
Adipose Derived Stem Cells (ADSc)	Osteoid, adipose, muscle and cartilaginous cells,endothelial cells	Zuk et al., 2001

1.4.3 Growth Factors Used in Vascular Tissue Engineering

Growth factors are used to induce cell proliferation, migration, and differentiation angiogenesis and secreted from different types of cells. They have important role on development of new tissues (Whitaker et al., 1998). Vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGFs), fibroblast growth factor (FGF) and transforming growth factor-beta (TGF- β) are the growth factors used in vascular tissue engineering.

Vascular endothelial growth factor (VEGF) is used to stimulate cell proliferation and migration of endothelial cells, also angiogenic vascular growth and differentiation of stem cells into blood vessel cells. Wang et al., 2012 produced VEGF releasing PCL scaffolds and demonstrated that these scaffolds induced endothelial proliferation with minimum immune response in comparison to unmodified PCL scaffolds. Platelet derived growth factor (PDGF)

is produced from different cell types including endothelial cells, smooth muscle cells, and macrophages (Stegemann and Nerem, 2003). Transforming growth factor-beta (TGF-β) is secreted from both vascular endothelial cells and smooth muscle cells (Stegemann and Nerem, 2003). It is demonstrated that TGF-β induced human ADSc to express smooth muscle related markers (Gimble et al., 2007, Wang et al., 2010). Fibroblast growth factor (FGF) is a growth factor which has an effect on tissue regeneration and repair. (Yun et al., 2010).

1.4.4 Fabrication of Tubular Scaffolds

1.4.4.1 Electrospinning

Electrospinning is widely used in producing fibers from polymers either synthetic or natural with diameters between 2 nm to several micrometers by using electrical forces (Lannutti et al., 2007; Hunley and Long, 2008). Due to the small gaps between fibers and high surface area the electrospun fibers are widely used in scaffold fabrication in tissue engineering, wound healing and drug delivery.

In electrospinning setup has three major components: (1) a syringe with a metal needle, (2) high voltage supply, and (3) grounded collector (metal screen, plate or rotating mandrel) onto which electrospun fibers are collected (Figure 1.13.). Electrospinning process can be controlled using several parameters which have direct effect on both properties and morphology of the electrospun fibers. These are solution viscosity, conductivity, molecular weight and surface tension, process parameters applied electric field, the distance between the tip and the collector, flow rate, humidity of the environment and temperature.

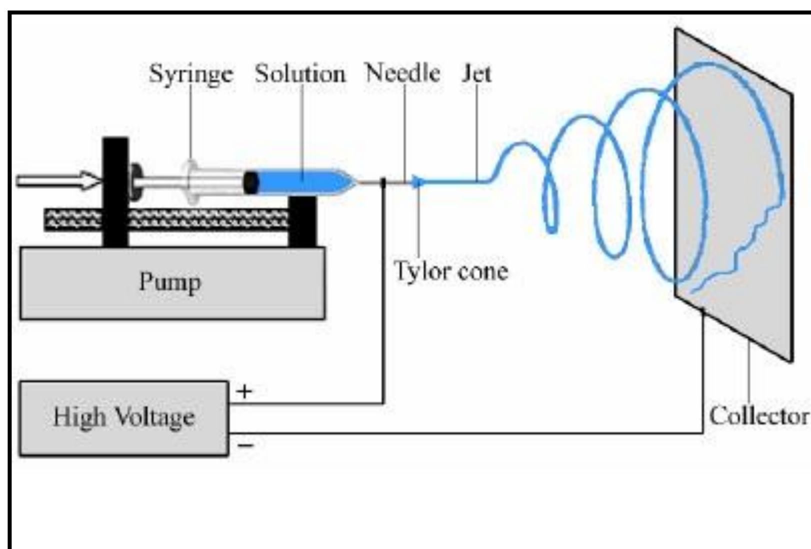

Figure 1.13 Electrospinning setup (Ziabari et al., 2009)

Fibers made by electrospinning are especially suitable to mimic the ECM. Synthetic and natural polymers are successfully used with this technique. Natural polymers are used in electrospinning are silk chitosan, gelatin, hyaluronic acid and collagen and the synthetic polymers are PGA, PCL, PLLA, PU, PS (Jin et al., 2012).

1.5 The Scope of This Study

The aim of the study was the production of a tissue engineered blood vessel as a small vascular graft with appropriate mechanical properties. Blood vessels with diameters smaller than 6 mm are considered small blood vessels but in this study, for convenience of production and characterization a larger (8mm) blood vessel substitute was aimed. For this purpose polycaprolactone-collagen based tubular scaffolds and polycaprolactone-collagen-ELR based fibrous mats were fabricated by electrospinning. The scaffolds were crosslinked by glutaraldehyde treatment and degradation tests (in PBS and collagenase) were performed. These scaffolds were characterized microscopically by using stereomicroscope, SEM. They were also characterized for their mechanical strength. For *in vitro* studies, human vascular smooth muscle cells (VSMCs) were seeded one side of the fibrous mat and human internal thoracic artery endothelial cells (HITAECs) were seeded to other side of the fibrous mat. Cell viability, proliferation and cell alignment on the grafts were tested.

1.6 The Novelty of This Study

In the present study, polycaprolactone and collagen types I was used to mimick natural structure of an artery. Polycaprolactone was used to increase the mechanical strength of the vascular graft since an ideal graft should withstand under physiological pressures. Collagen type I was used since it is native component of an artery and has an effect on cell attachment and proliferation. This study is the first using ELRs including REDV sequence to increase endothelial attachment and form a confluent endothelial layer which makes the vascular graft for the treatment of cardiovascular diseases. To crosslink the scaffolds UV radiation was used which has no toxic effects on cells in comparison to its counterparts such as glutaraldehyde.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

DMEM-Low glucose, fetal bovine serum (FBS), trypsin-EDTA (0.25%), Ham's F12 and SnakeSkin pleated dialysis tubing were purchased from Thermo Scientific (USA).

Human Internal Thoraric Artery Endothelial Cells (HITAECs) and their medium were purchased from European Collection of Cell Cultures (UK).

Sodium phosphate monobasic and dibasic, sodium chloride (NaCl), dimethylformamide (DMF), ethanol, acetic acid (HAc), acetone, and Tween-20 were purchased from Merck Millipore (Germany).

1,1,1,3,3,3-Hexafluoroisopropanol (HFIP), glutaraldehyde, paraformaldehyde (37%), FITC-labeled phalloidin, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), anti-PECAM-1 (CD31), antiactin α smooth muscle, polycaprolactone (M_n= 70,000-90,000), Bovine serum albumin (BSA), amphotericin B, glutaraldehyde, paraformaldehyde (37%), sodium cacodylate trihydrate were purchased from Sigma (USA).

Dimethylsulfoxide (DMSO) and Triton-X 100 were obtained from AppliChem.
Collagenase Type II was obtained from GIBCO (USA).

Penicillin/streptomycin (100 units.mL^{-1}/100 μg.mL^{-1}) was purchased from Lonza.
Tissue-Tek O.C.T Compound (tissue freezing medium) was purchased from Sakura (USA).

Sprague-Dawley rat tails to extract collagen type I were a kind gift of Tayfun Ide from GATA Animal Experiments Laboratory (Ankara, Turkey).

ELPs were a kind gift of Prof. Jose *Carlos Rodríguez-Cabello* from University of Valladoid, Spain.

2.2 Methods

2.2.1 Collagen

2.2.1.1 Isolation of Collagen Type I

Collagen type 1 was isolated from Sprague-Dawley rat tails. The rat tails were dissected and all tendons were removed. Removed tendons were placed in aqueous acetic solution (0.5 M) and stored in 4 °C with continuous stirring for a week until they were completely dissolved. Then, the solution was filtered by using glass wool. The solution was dialyzed against dialysis buffer (5 L; 12.5 mM sodium phosphate dibasic, 11.5 mM sodium phosphate monobasic, pH 7.2) which was changed every day. After dialysis, the solution was centrifuged (Sigma 3K30, Germany) at 4 °C, 16,000 g for 10 min and pellets were placed again in acetic acid solution (0.15 M) and stored at 4°C with continuous stirring until completely dissolved. Then, the solution was precipitated by salting out with addition of NaCl (5% w/v), centrifuged, the pellet dissolved in acetic acid solution (0.15 M) and dialyzed under the same conditions for another week. After centrifugation the pellets were placed in 70% (v/v) ethanol for 2 days. After the centrifugation step the pellet was frozen (-80 ºC, Sanyo MDF-U53865, Japan), lyophilized (FreeZone 6, Labconco Co., USA) and stored at 4 ºC.

2.2.1.2 SDS-PAGE Analysis of Isolated Collagen Type I

The purity of the isolated type 1 collagen from Sprague-Dawley was examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Isolated collagen type 1 was incubated with mercaptoethanol at 95 ºC for 5 min. Then the 0.5% (w/v) collagen solution (in acetic acid) was loaded on gels (separating gel: acrylamide/bisacrylamide 12%, stacking gel: acrylamide/bisacrylamide 4% acrylamide/bisacrylamide) and run at 3 mA for 2.5 h. Then samples were stained with 0.2% (w/v) Coomassie Brilliant Blue by overnight incubation. Samples were destained with solution of methanol: acetic acid: distilled H_2O (9:2:9) and studied

2.2.2 Preparation of Tubular Scaffolds

2.2.2.1 Electrospinning Process

Collagen type 1 isolated from rat tails and PCL (polycaprolactone, M_n= 70,000-90,000) were used for fabrication of the tubular scaffolds. The blend of PCL: Coll a ratio of 72:28 (10% w/v in HIFP) and PCL: Coll: ELP (65:25:10) was prepared and stirred overnight. Then, the polymer solution was put into a 10 mL plastic syringe with a metal needle with a blunt end (18G x 1.1/2', 1.20 mm inner diameter x 32 mm length). The syringe was placed in the

syringe pump (New Era Pump Systems Inc., UK) and connected to a high voltage-DC power source (Gamma High Voltage Research, USA) and a rotating mandrel (20 cm length, internal diameter 8.5 mm). The tip of the needle was connected to the positive electrode of the power supply and the rotating mandrel was connected to the negative electrode. The voltage was set as 13 kV and the flow rate was 3 μL/min. The rotation rate of the mandrel was 320 rpm. The distance between the rotating mandrel and the tip of the needle was 10 cm. Electrospinning setup used for production of tubular scaffolds is shown at Figure 2.1.

The tubular scaffolds were air dried overnight, and stored in a desiccator at RT.

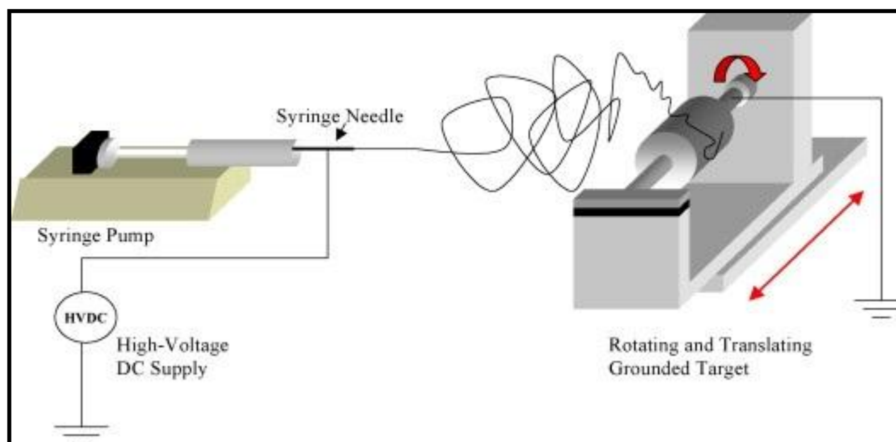

Figure 2.1 Electrospinning setup (Kenawy et al., 2009)

2.2.2.2 Crosslinking of Tubular Scaffolds

For crosslinking, the tubular scaffolds were immersed in 1% (v/v) aqueous glutaraldehyde solution at room temperature (RT) for 40 min, rinsed with distilled water to remove the residual glutaraldehyde, they were immersed in 0.1 M aqueous glycine solution at RT for 40 min (Rho et al., 2006). Then the scaffolds were rinsed once more with distilled water and air dried overnight. Figure 2.2. shows crosslinking of collagen by glutaraldehyde.

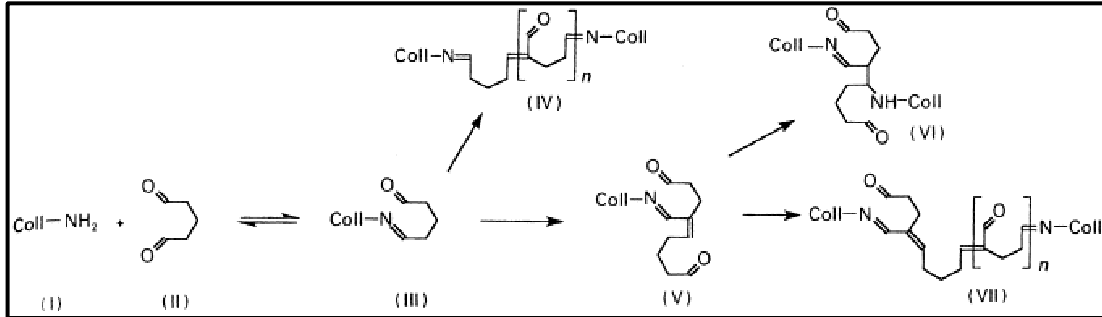

Figure 2.2 Collagen crosslinking mechanism with glutaraldehyde. (I) Collagen, (II) Glutaraldehyde, (III) Schiff base indermediate, (IV) Crosslinked collagen, (V) α-β schiff base indermediate, (VI) and (VII) Crosslinked collagen (Sheu at al., 2001).

2.2.3 Characterization of Scaffolds

2.2.3.1 Microscopy

Electrospun tubular scaffolds were examined by stereomicroscopy (SMZ 1500, Nikon, USA). Also electrospun tubular scaffolds and membranes were coated under vacuum with platinum and gold and examined with a scanning electron microscope (SEM, Mini-SEM, South Korea) to study the surface topography and fiber organization

2.2.3.2 Measurement of Fiber Dimensions

The fiber diameters of electrospun tubular scaffolds were measured using the SEM images and the NIH Image J program (USA). The image of each sample was divided into four equal regions and diameter of fibers was calculated making 30 random measurements in each of the four regions.

2.2.3.3 Measurement of Scaffold Thickness

Electrospun membranes, in dry and wet states, were measured using a standard micrometer (Erste Qualitat, Germany) to a sensitivity of 0.1 µm. Five replicate samples were used for each state and at least 5 measurements were done on each sample.

2.2.3.4 Degradation Test

2.2.3.4.1 In PBS

Uncrosslinked and crosslinked electrospun scaffolds (8 mm length, 0.01 g) were incubated in PBS (10 mM, pH 7.4) at 37 °C for a week. Degradation of the scaffolds was determined by weighing every 2 days.

2.2.3.4.2 In Collagenase

Collagenase test was performed as an accelerated test to determine the stability of the scaffolds in the presence of enzyme collagenase. Electrospun tubular scaffolds were incubated in collagenase type II solution (0.1 mg/mL in PBS pH 7.4) for 2 h, the samples were rinsed with distilled water, frozen at -80°C, lyophilized and weighed to determine the weight loss of the samples.

2.2.3.5 Mechanical Test (Tensile Test)

Electrospun tubular scaffolds (20 mm length, 8.5 mm diameter) were crosslinked with 1% (v/v) glutaraldehyde solution. UXL and XL tubular scaffolds in dry and wet states were mechanically tested. The crosshead speed was set as 30 mm/min and the gauge length was 10 mm. Ultimate tensile strength (UTS), Young's modulus (E) and elongation at break (%) of the scaffolds were calculated. The control set (n=5) was uncrosslinked. For electrospun fibrous mats the crosshead speed was set to 15 mm/min.

Stress-strain curve of viscoelastic materials are represented in Figure 2.3. Ultimate tensile strength (UTS), Young's modulus (E) and elongations at break (%) were calculated as follows;

$$\mathrm{UTS} = \frac{\mathrm{F}}{\mathrm{A}}$$

$$\mathrm{E} = \frac{\Delta\,\mathrm{F}}{\mathrm{A}} \times \frac{\mathrm{L}}{\Delta \mathrm{L}}$$

$$\text{Elongation at break (\%)} = \frac{\mathrm{L_f} - \mathrm{L_i}}{\mathrm{L_i}}$$

where

F: Force (N)
A: Area (mm^2)
E: Elastic Modulus (N/mm^2)
L: Length (mm)
L_i: Initial Length (mm)
L_f: Final Length (mm)

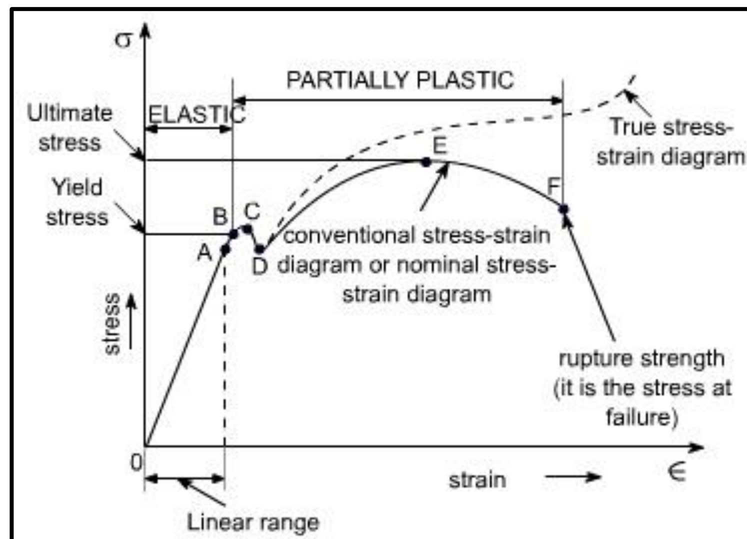

Figure 2.3 Typical stress-strain curve of viscoelastic materials (http://www.nptel.iitm.ac.in/courses/Webcourse-contents/IIT ROORKEE/strength%20of%20materials/lects%20&%20picts/image/lect11/lecture11.htm)

2.2.3.6 . Suturability Test

Suturability test was performed by suturing the unseeded PCL/Col based tubular scaffold with a prolene suture (5/0) with cutting needle.

2.2.4 *In Vitro* Studies

For *in vitro* studies two types of cells were used: VSMCs (vascular smooth muscle cells) and HITAECs (human internal thoracic artery endothelial cells).

2.2.4.1 VSMCs (Vascular Smooth Muscle Cells) Culture

VSMCs of passages 2 to 10 were used. VSMCs were stored in 15% DMSO in their standard medium at -80 °C. After thawing, cells were cultured in their standard medium (3:1 DMEM Low Glucose: HAM F12 supplement with 10% FBS and 1% penicillin-streptomycin, % 0.2 amphotericin B) until they reached confluence. The medium was changed every two days

2.2.4.2 HITAECs (Human Internal Thoracic Artery Endothelial Cells) Culture

HITAECs of passage 2 to 10 were used. They were stored in 15% DMSO in their standard medium at -80 °C. After thawing, cells were cultured in HITAEC growth medium, MesoEndocell growth medium that contains MesoEndocell Basal Medium, 6% MesoEndocell Growth Supplement, 1% penicillin-streptomycin and % 0.2 amphotericin B under standard cell culture conditions until they reached confluency. The medium was changed every two days.

2.2.4.3 Co-culture

2.2.4.3.1 Cell Seeding on Fibrous Mats

Elelctrospun fibrous mats were placed in 12 well plates. Each side of mats were exposed to UV sterilization for 1 h. After sterilization, VSMCs were detached from tissue culture flask by using Trypsin-EDTA (0.25%) for 5 min at 37°C. Then they were centrifuged for 5 min at 3000 rpm and the pellet was resuspended in the standard medium of VSMCs. Cell numbers were determined with hemocytometer. VSMCs were seeded on the outer layer of the mat with a density of $3.2x10^4$ cell/µL (in 40 µL) and incubated at 37 °C for 7 days in carbon dioxide incubator to reach confluency. Medium was changed every day. After 7 days of incubation, HITAECs were detached from the tissue culture flask by using Trypsin-EDTA (0.25 %) for 5 min at 37 °C, centrifuged for 5 min at 3000 rpm and pellet was resuspended in the standard medium. Cell numbers were determined with a Nucleocounter (ChemoMetec, Denmark). HITAECs were seeded other side of the fibrous mats with a density of 4.0 $x10^4$ cell/ µL (in 40 µL) and incubated at 37 °C for 12 days in a carbon dioxide incubator until confluency. Medium was changed every two days.

Only VSMCs seeded fibrous mats were cultured 21 days and only HITAECs seeded membranes were cultured 14 days.

2.2.4.3.2 Cell Proliferation

MTT cell viability tests were performed to determine the cell proliferation profiles. For only VSMCs seeded membranes Day 1, 7, 14, 21, only HITAECs seeded membranes Day 1, 7, 14 and cocultered membranes Day 1, 7, 12 were the time points. At the each time point, membranes were removed from the medium and washed with colorless medium (DMEM High Modified) two times. MTT solution prepared in colorless medium (in a concentration of 1 mg/mL) was added onto the membranes and incubated for 3 h at 37 °C. The purple formazan crystals formed were dissolved in 1 mL acidified isopropanol (4% v/v). The absorbances were measured at 570 nm with an Elisa plate reader (Molecular Devices, USA). The absorbance value was converted to cell numbers by using the calibration curve's of each cell types (Calibration curves are shown Appendix A).

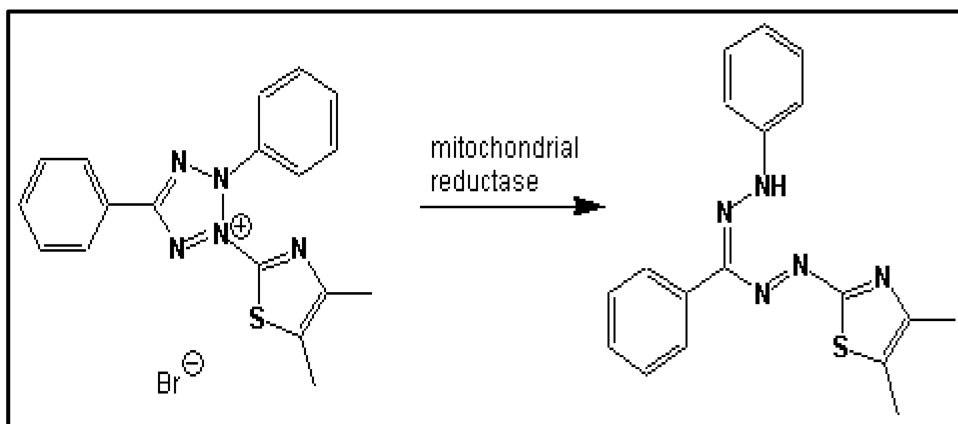

Figure 2.4 Scheme of MTT cell viability test; reduction of (yellow) (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to (purple) formazan crystals by the mitochondrial reductase of the cells.

2.2.4.3.3 SEM

Cell seeded electrospun tubular scaffolds were washed with PBS and with cacodylate buffer (0.1 M sodium cacodylate, pH 7.4). They were fixed with glutaraldehyde solution (2.5 % in cacodylate solution) for 2 h at room temperature. After fixation the scaffolds were washed with cacodylate buffer and freeze dried for 3 h. They were coated with gold under vacuum before examination.

2.2.4.3.4 Mechanical Test(Tensile Test)

Tensile test was performed on cell seeded membranes. The tensile testing rate was set as 15 mm/min and the gauge length was 10 mm. ultimate tensile strength (UTS), Young's modulus (E) and elongation at break (%) were calculated.

2.2.4.3.5 Fluorescence Microscopy Analysis

2.2.4.3.5.1 Phalloidin DAPI staining

VSMCs (p11) and HITAEC (p8) were seeded onto cover slips and fixed with 4% (v/v) parafomaldehyde (PFA) at room temperature (RT) for 15 min. Cells were permeabilized with Triton-X (0.1%, v/v) for 5 min at RT and washed with PBS (10 mM, pH 7.4). They were incubated in blocking solution (1% BSA in PBS) for 30 min at 37 °C. After this step they were incubated in FITC-labelled Phalloidin (1:1000 dilution in 0.1% BSA) for 1 h at 37 °C. After washing with PBS they were incubated in DAPI (1:1000 in 0.1% BSA) for 10 min at RT. After washing with PBS, cells were examined with a fluorescence microscope (Olympus IX70, Japan).

2.2.5 Statistical Analysis

Statistical analysis was performed using a Student's t-test with a minimum confidence level of 95% (p value lower than 0.05) for statistical significance. All values were reported as the mean ± standard deviation of the mean (s.d.m).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 SDS PAGE Analysis of Isolated Collagen Type 1

The purity of isolated collagen type 1 was determined by SDS-PAGE analysis. The results are shown in Figure 3.1. Column I shows the isolated collagen, column II shows the protein ladder and column III shows the commercial collagen. Protein marker ladder has 4 different bands: 260 kDa, 140 kDa, 100 kDa and 70 kDa. Isolated collagen type I and commercial collagen had only 2 doublet bands; around 260 kDa and 100 kDa. According to this result isolated collagen (from Sprague-Dawley rat tails) was pure collagen type I.

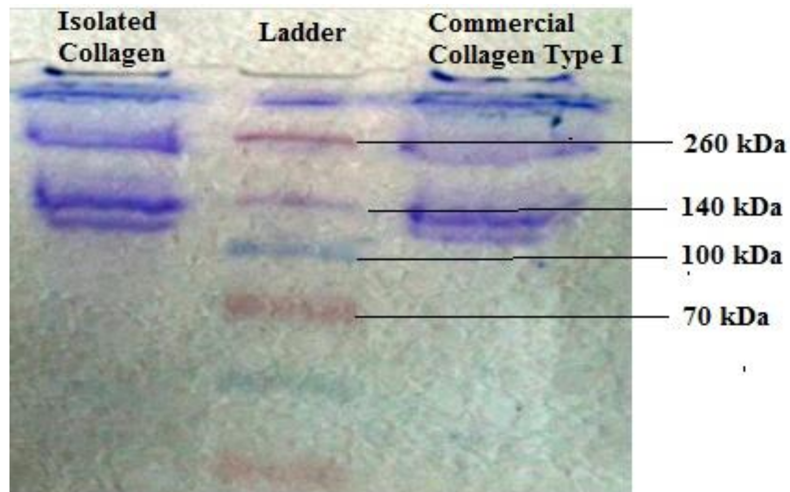

Figure 3.1 SDS-PAGE of collagen type 1 isolated from Sprague-Dawley rat tails (I) isolated collagen type I, (II) ladder (protein marker), (III) commercial collagen.

3.2 Characterization of the Electrospun Blood Vessel Scaffolds

3.2.1 Scaffold Thickness

Thickness of scaffolds was measured in order to characterize the blood vessel scaffolds and to observe the effect of crosslinking with glutaraldehyde. Two types of scaffolds, uncrosslinked (UXL) and crosslinked (XL) electrospun tubular scaffolds were tested in both dry and wet states (Table 3.1.). It was observed that the thickness of UXL scaffolds in dry and wet states were the same. Upon crosslinking with glutaraldehyde, the thicknesses appear to be larger but not significantly different. It can be therefore said that crosslinking or wetting did not affect the thickness. This is to be expected because the scaffolds carry a significant amount of hydrophobic polyester PCL in their structure.

Table 3.1 Thickness of scaffolds

Type of Scaffold	State of Scaffold	Thickness (μm)
UXL	Dry	117±23
	Wet	117±27
XL	Dry	130±33
	Wet	122±34

3.2.2 Fiber Diameter

Fiber diameters of tubular scaffold were determined by using the SEMs and the program NIH Image J. Interestingly, the average fiber diameter of the inner surface was 289 ± 89 nm, while for the outer surface it was 641 ± 206 nm, 2-fold thicker than that of inner surface. The fiber diameters of the scaffold were similar to extracellular matrix of natural tissues which were between 300-700 nm (Xu et al., 2004, Venugopal et al., 2005)

3.2.3 Degradation Test

Stability of the scaffolds in *in vitro* cell culture conditions is important since the ideal scaffold should maintain its structural properties in the culture media for cell attachment and proliferation until the natural vascular tissue forms (Lee et al., 2007).

To study the effect of crosslinking with glutaraldehyde on scaffold stability, degradation tests were performed (in PBS and collagenase).

3.2.3.1 In PBS

The stability of the UXL and XL PCL: Coll tubular scaffolds were studied by incubation in PBS (pH 7.4, 10 mM at 37 °C) for a week (days 0, 1, 3, and 6) and the results are represented in Figure 3.2. XL fibers showed slightly higher stability at four time points in comparison to UXL sample but the difference is not statistically different.

In this study a blend of polycaprolactone with collagen (2:1) was used to fabricate the tubular scaffolds by electrospinning. PCL is synthetic, biodegradable polyester which is very hydrophobic and has a low degradation rate (1-2 years) depending on properties and form. These results are therefore expected. Thus, the only loss that would be observed in a week would be that of collagen, and this collagen was blended with hydrophobic polymer that hindered water ingredients.

In a similar study electrospun polydioxanone (PDO)-elastin fibers, mixed in different ratios, were tested in PBS and it was observed that as the fraction of PDO increased in the blend, the amount of scaffold remained in the degradation test also increased and this result supported our observation (McClure et al., 2008).

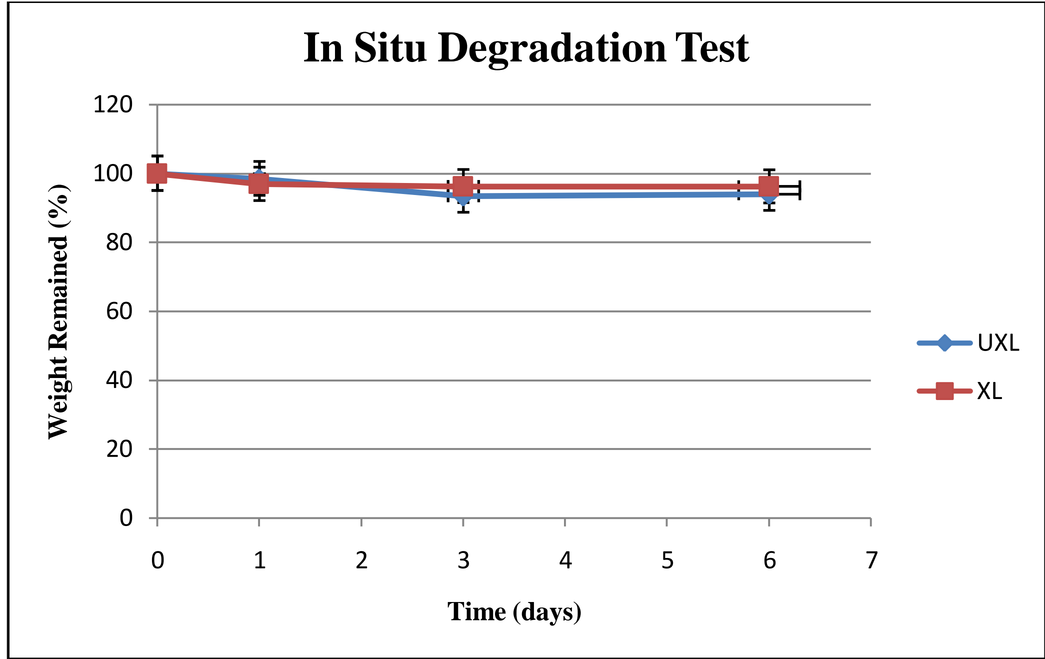

Figure 3.2 Scaffold degradation in situ (PBS, pH 7.4, 10 mM, 37 °C)

3.2.3.2 In Collagenase

Matrix metalloproteinases (MMPs) have an important role in vascular matrix remodeling since they are involved in cell migration and formation of new tissues. Collagenases, gelatinases, stromolysins, metalloelastases, membrane type-matrix metalloproteinases (MT-MMP) are examples of MMPs and they degrade vessel ECM components (Bou-Gharios et al., 2004). Therefore, testing the stability of the blood vessel substitutes with collagenase is important. In addition, collagenase test is a well accepted test for the stability of the protein (especially collagen based scaffolds).

The degradation profile of electrospun fibers were obtained by incubating the scaffolds in collagenase type II (1 mg/mL) at 37 °C for 2 h (Table 3.3.). According to the gravimetric results, 96.23% of the weight of UXL PCL: Coll and 98.27% of the XL PCL: Coll remained after 2 h incubation in collagenase. The difference between UXL and XL scaffolds after collagenase test was not significant which demonstrated that crosslinking with glutaraldehyde solution did not improve the stability of the tubular blood vessel substitutes.

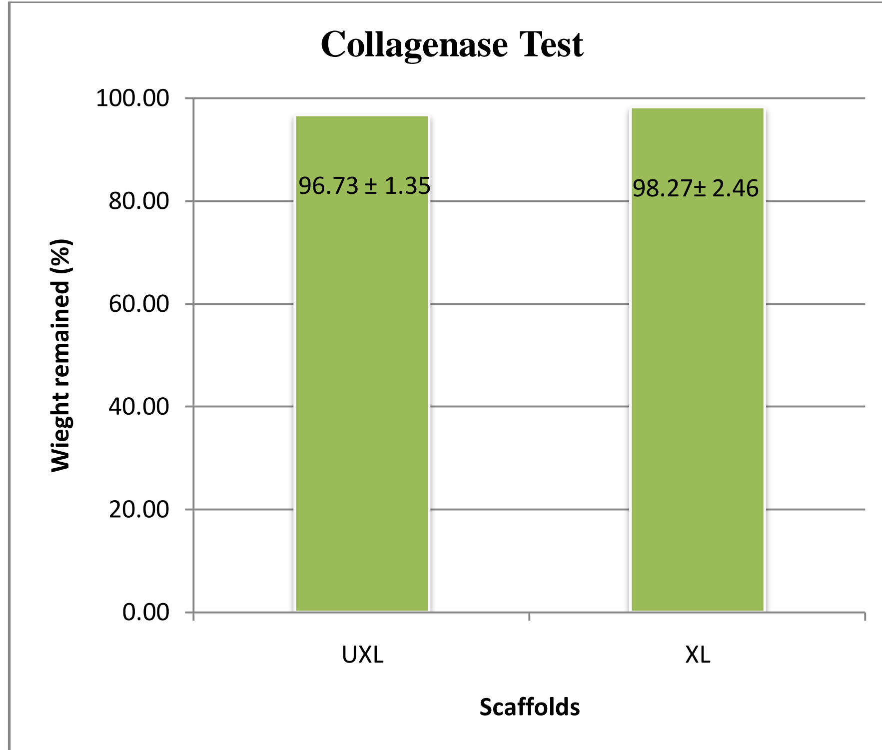

Figure 3.3 Degradation of PCL: Coll scaffolds by collagenase (1 mg/mL, at 37 °C for 2 h)

Even though a 1 week in PBS or 2 h collagenase study could be considered too short still with the deposition of natural ECM in the actual use (in vivo and in the clinic) the level of stability obtained in this study is very acceptable. The absence of improvement upon crosslinking was also due to the excess hydrophobic polymer presence.

3.2.4 SEM

The structures of tubular scaffolds were examined with SEM and the results are shown in Figure 3.4. As reported earlier average fiber diameter inside the scaffold was higher than the fibers on the outside of the scaffold. Figure 3.4a shows a proper scaffold without any discontinuities, tears or holes. It appears quite non-porous but when looked at higher magnifications both the inner and outer surfaces and highly porous. Even though the tubular construct was produced on a rotating mandrel a perfect alignment normal to the axis of the tube is not observed. The outer surface was less aligned than the inner surface probably because the underlying (inner) layer is not aligned.

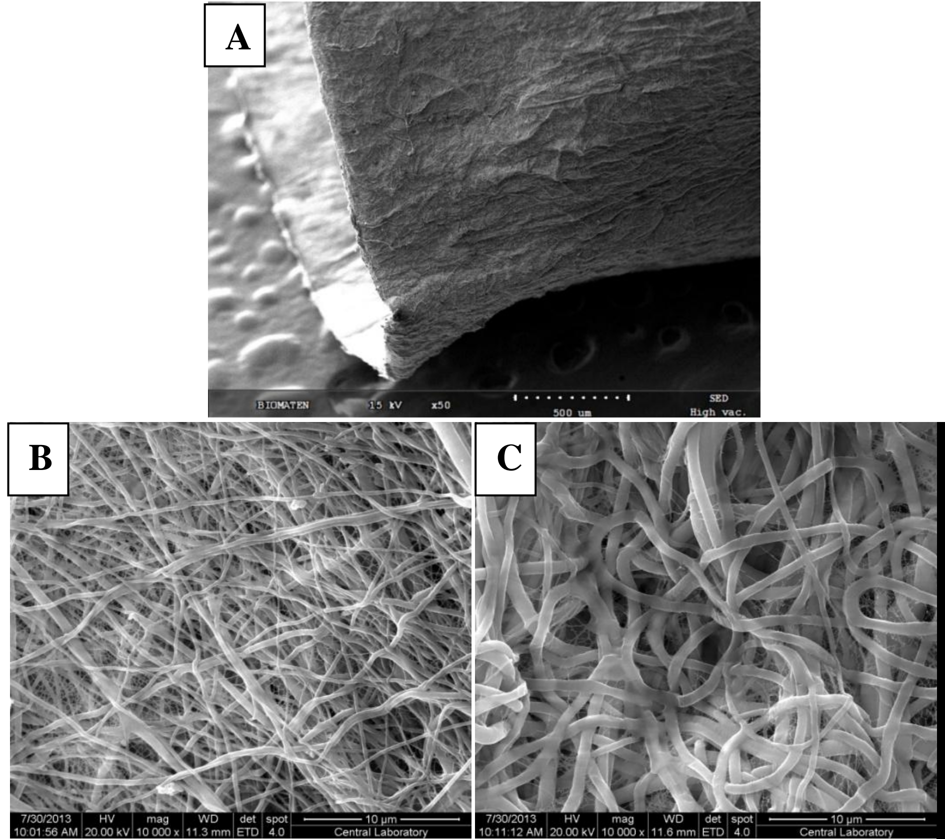

Figure 3.4 SEM images: (A) Tubular scaffold (x50), (B) Inside of the tubular scaffold (x10000), (C) Outside of the tubular scaffold (x10000).

3.2.5 Mechanical Test (Tensile Test)

Electrospun PCL: Coll based tubular scaffolds were crosslinked with glutaraldehyde and mechanically tested in wet and dry conditions. The tensile test results are shown in Table 3.4.

Ultimate tensile strengths (UTS) of dry UXL and XL tubular scaffolds in dry states are 17.52 ± 2.91 and 15.40 ± 2.64, respectively. Also, UTS values of UXL and XL tubular scaffolds in wet states are 15.41 ± 3.84 and 12.03 ± 2.05, respectively, show that upon crosslinking both these properties are decreased. The same result was observed in the elongation at break. For two groups, crosslinking with glutaraldehyde causes a decrease in UTS. For the Young's Modulus value (E). there was a statistically significant difference in the E of UXL and XL in dry state (161.00 ± 22.73 and 112.22 ± 7.63) ($p < 0.05$). However, in the wet state Young's Modulus values significantly of UXL and XL scaffolds in wet state, there was no statistically significant difference.

The wetting also decreased the mechanical properties, and is understandable because of the lubricating effect of the water that enters between the molecules and fibers.

Crosslinking caused substantial decreases in the UTS and E values in most samples. Statistical treatments show significant changes only in the the Young's Modulus value change for the dry sample. It can be concluded that glutaraldehyde treatment in general deteriorates the properties of collagen of the PCL: Coll composite scaffold.

In a study where PCL: Coll based tubular scaffolds were fabricated by electrospinning and crosslinked with glutaraldehyde vapor they also observed that wetting decreases the UTS and the Young's Modulus as was observed in the present study (Lee et al., 2008). In addition, the UTS and Young's Modulus data reported were much lower than obtained in the present study (UTS dry state was 8.3 ± 1.2 MPa, and UTS wet state was 4.0 ± 0.4 MPa). Young's Modulus values in dry and wet state were 5.6 ± 3.8 MPa and 2.7 ± 1.2 MPa, respectively.

Table 3.2 Tensile testing results of uncrosslinked (UXL) and crosslinked (XL) PCL: Coll tubular scaffolds (crosslinking agent: glutaraldehyde).

	Mechanical Properties		
Type and State	Ultimate tensile strength, (UTS) MPa	Young's Modulus (E), MPa	Elongation at break %
UXL DRY	17.52 ± 2.91	161.00 ± 22.73[a]	150.33 ± 41.50
UXL WET	15.41 ± 3.84	27.88 ± 8.10	175.76 ± 30.25[b]
XL DRY	15.40 ± 2.64	112.22 ± 7.63[a]	130.80 ± 24.41
XL WET	12.03 ± 2.05	26.7 ± 6.39	118.20 ± 18.53[b]

* Test sample length: 20 mm, i.d. : 8 mm (n=5).

a. The difference between Young's Modulus values of UXL and XL scaffolds is significant (in dry state) ($P < 0.05$).
b. The difference between Elongation at break values of UXL and XL scaffolds is significant (in wet state) ($P < 0.05$).

Additionally, the electrospun PCL:Coll based fibrous mats were crosslinked via UV and mechanically tested in both wet and dry states (Table 3.3). UTS increased upon crosslinking with UV from 7.22 ± 0.77 to 8.17 ± 0.76 in dry state and from 5.25 ± 0.74 to 5.80 ± 0.81 in wet state.

While Young's Modulus values of dry samples significantly increased from 36.99 ± 8.89 to 65.50 ± 11.54 ($P < 0.05$), there was no significant difference in Young's Modulus values of the mats in wet state.

UV crosslinking caused substantial decreases in elongation at break values from 82.22 ± 16.39 to 80.76 ± 6.01 in dry state and from 125.78 ± 12.56 to 124.59 ± 20.00 in wet state results that were expected. These decreases were, however, statistically not significant.

Table 3.3 Tensile mechanical testing results of UXL and XL PCL: Coll fibrous mats (Crosslinking agent: UV)

Type and State	Mechanical Properties		
	Ultimate tensile strength (UTS), MPa	Young's Modulus (E), MPa	Elongation at break (%)
UXL DRY	7.22 ± 0.77	36.99 ± 8.89[a]	82.22 ± 16.39
UXL WET	5.25 ± 0.74	9.92 ± 1.15	125.78 ± 12.56
UV XL DRY	8.17 ± 0.76	65.50 ± 11.54[a]	80.76 ± 6.01
UV XL WET	5.80 ± 0.81	8.57 ± 2.23	124.59 ± 20.00

a. The difference between Young's Modulus values of UXL and XL mats is significant (in dry state) ($P < 0.05$).

3.2.6 Suturability Test

Suturability of the tubular scaffolds were successfully perfomed with 5/0 prolene suture with a cutting needle. Figure 3.5.A shows the sutured tear within the scaffold and Figure 3.5.B shows the two tubular scaffolds connected to each other with suture. As a results any tear was not observed during this test.

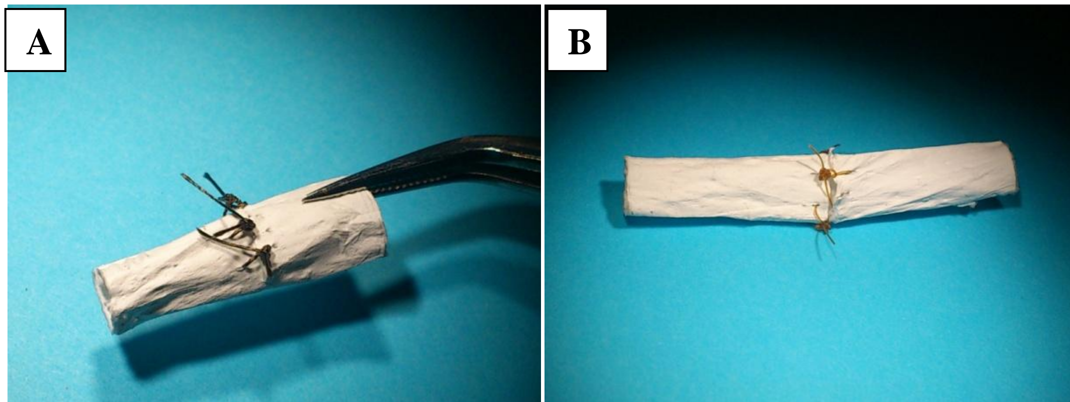

Figure 3.5 Proof of suturability of the unseeded tubular scaffolds (A) Sutured within the scaffolds (B) Two scaffolds sutured to each other.

3.3 *In Vitro* Studies

3.3.1 VSMC Culture

3.3.1.1 Cell Proliferation

VSMCs proliferation on the fibrous mats of PCl: Coll, the MTT cell viability test was performed on Days 1, 7, 14, and 21 and results are shown in Figure 3.6. On Day 1 there was 17% less cells on the mats since all the cells may not have attached to the mat. Between Days 1 and 7 the cell number significantly increased (2-fold). However, the cell number increase rate between Days 7 and 21 was lower in comparison to first week. This could be because the cells might have covered the mat and may not have space left to proliferate (Xu et al., 2004).

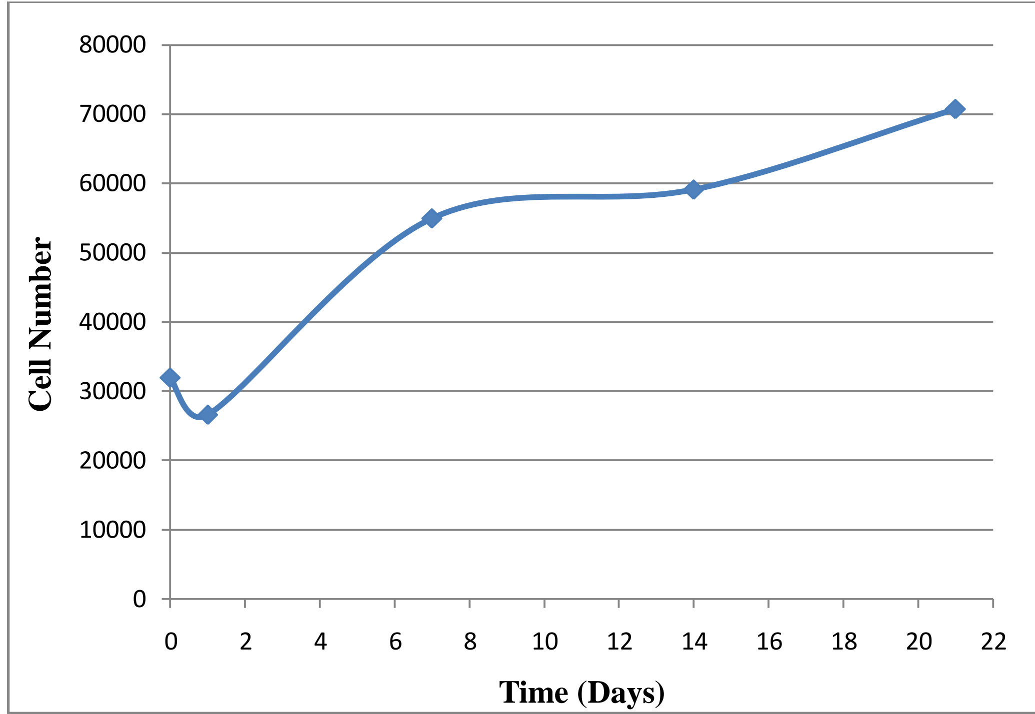

Figure 3.6 VSMC proliferation PCL: Coll mats (initial cell seeding density per sample: $3.2x10^4$).

3.3.1.2 SEM

SEM of VSMC seeded mats on Day 21 show that VSMCs attached well, spread and proliferated on the scaffolds (Figure 3.7.).

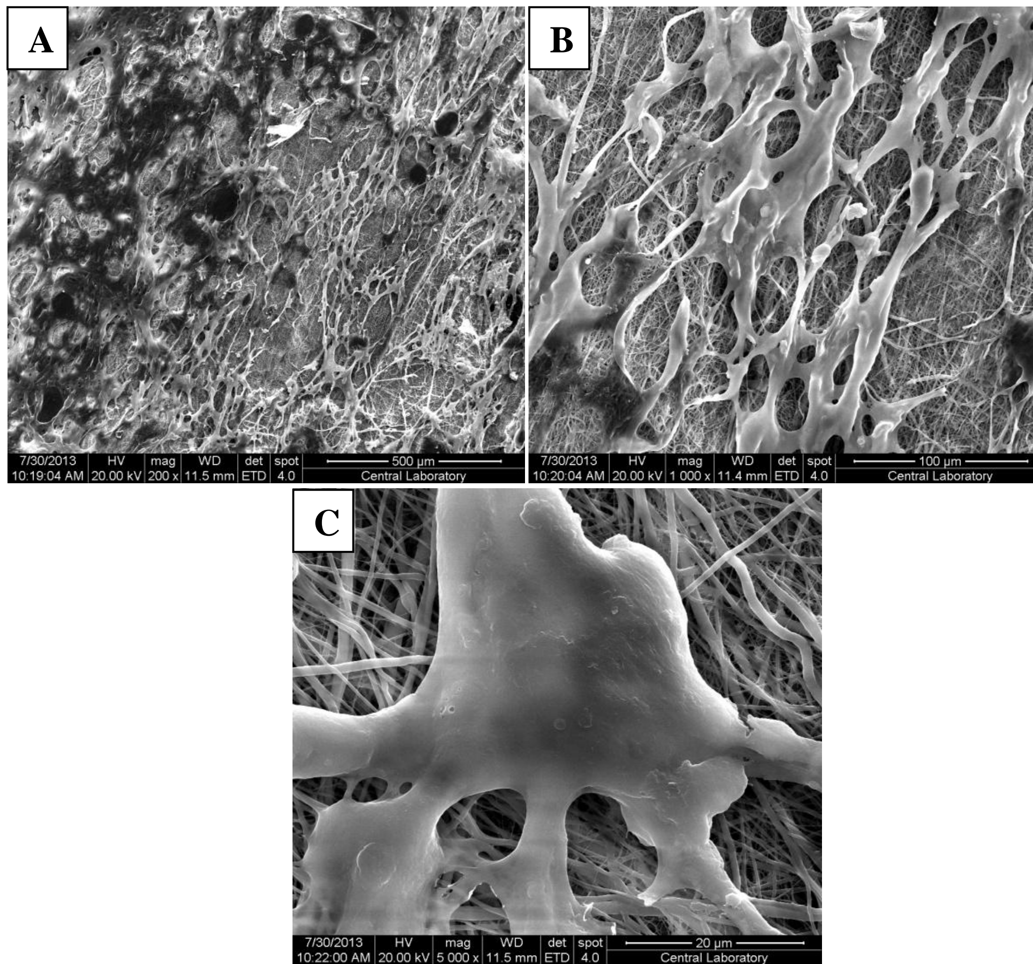

Figure 3.7 SEM of fibrous mats seeded with VSCM on Day 21 (A) x200, (B) x1000, (C) x5000.

3.3.1.3 Fluorescence Microscopy Analysis

In order to visualize a typical VSMC under fluorescence microscopy the VSMCs (p11) were seeded onto cover slip, the cytoskeleton was stained with FITC labeled phalloidin and cell nuclei was stained with DAPI. The size of the cells appear to be around 50-200 µm, and is consistent with the literature and the cell are healty with proper conformation.

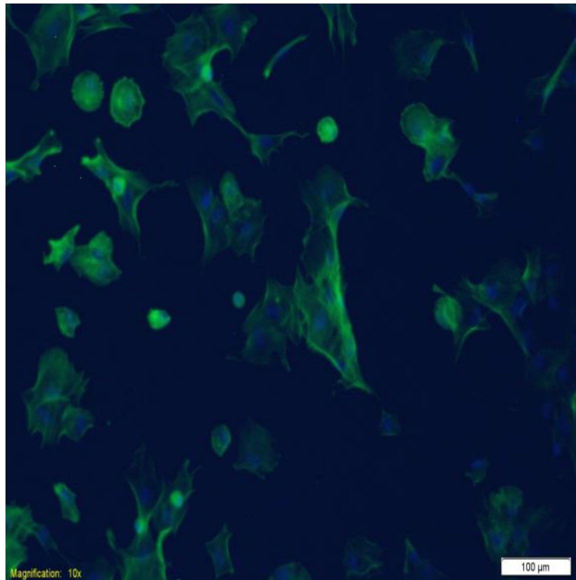

Figure 3.8 Fluorescence micrographs of FITC-labelled Phalloidin and DAPI staining of VSMCs (x10).

3.3.2 HITAEC Culture

3.3.2.1 Cell Proliferation

HITAECs proliferation on the mat determined by MTT cell viability test performed on Days 1, 7, and 14 (Figure 3.9.). On Day 1 75% of the cells seeded were attached. In the first week HITAECs numbers increased followed by a decrease between Days 7 and 14. This decrease could be interpreted as reaching confluency (Figure 3.10.).

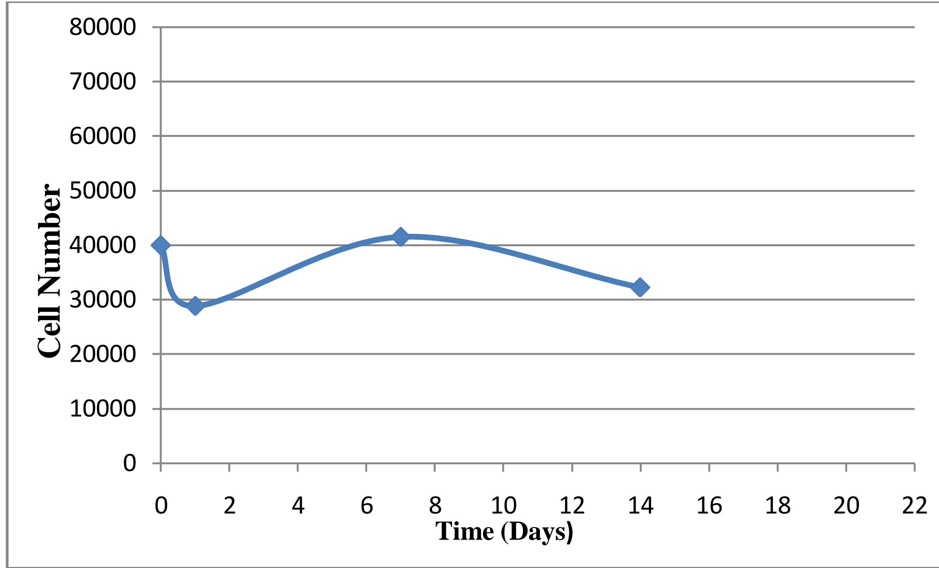

Figure 3.9 HITAEC proliferation on PCL: Coll membranes after 14 days of incubation (initial cell seeding density per sample: $4x10^4$).

HITAECs attachment on PCL: Coll and PCL: Coll: ELP mat was determined using the MTT cell viability test on Day 1 and results were shown in Figure 3.10. On Day 1, 95% of the seeded cells were attached on PCL: Coll: ELP mats. However 75% of the seeded cells were attached on PCL: Coll mats on Day 1. Due to presence of ELP in mats may increased attachment of HITAECs.

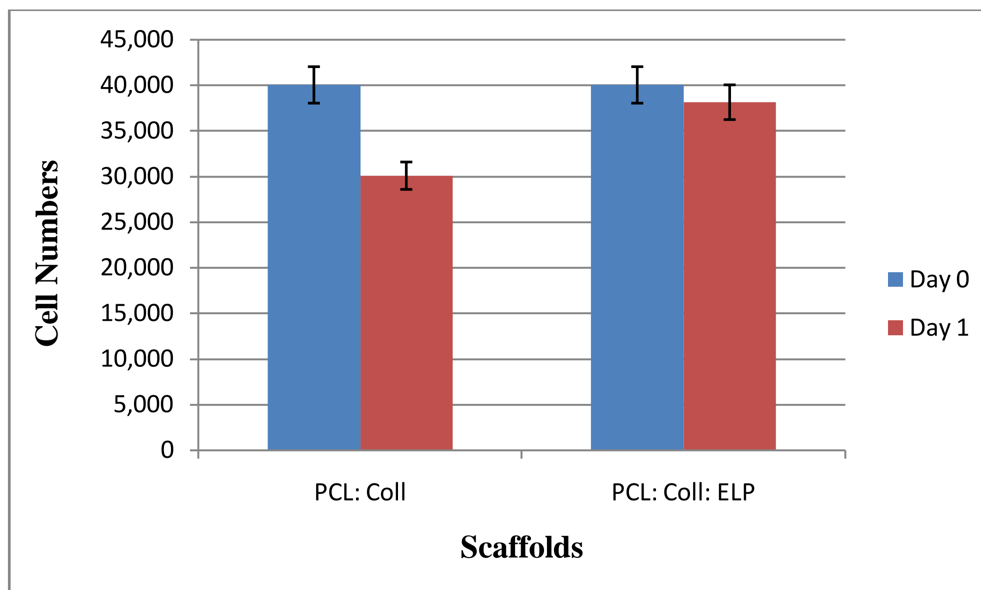

Figure 3.10 HITAEC attachment on PCL:Coll and PCL: Coll: ELP on Day 1 (initial cell seeding density per sample: $4x10^4$).

3.3.2.2 SEM

The SEM of HITAEC on Day 14 (Figure 3.11.). HITAECs attached and proliferated on the scaffolds covering the whole surface. The micrograph shows that the gaps between the fibers are too small for the cells to penetrate into the structure, and therefore, when the surface is covered it becomes like a mat surface covered and contact inhibition stents. This is supported by the MTT results.

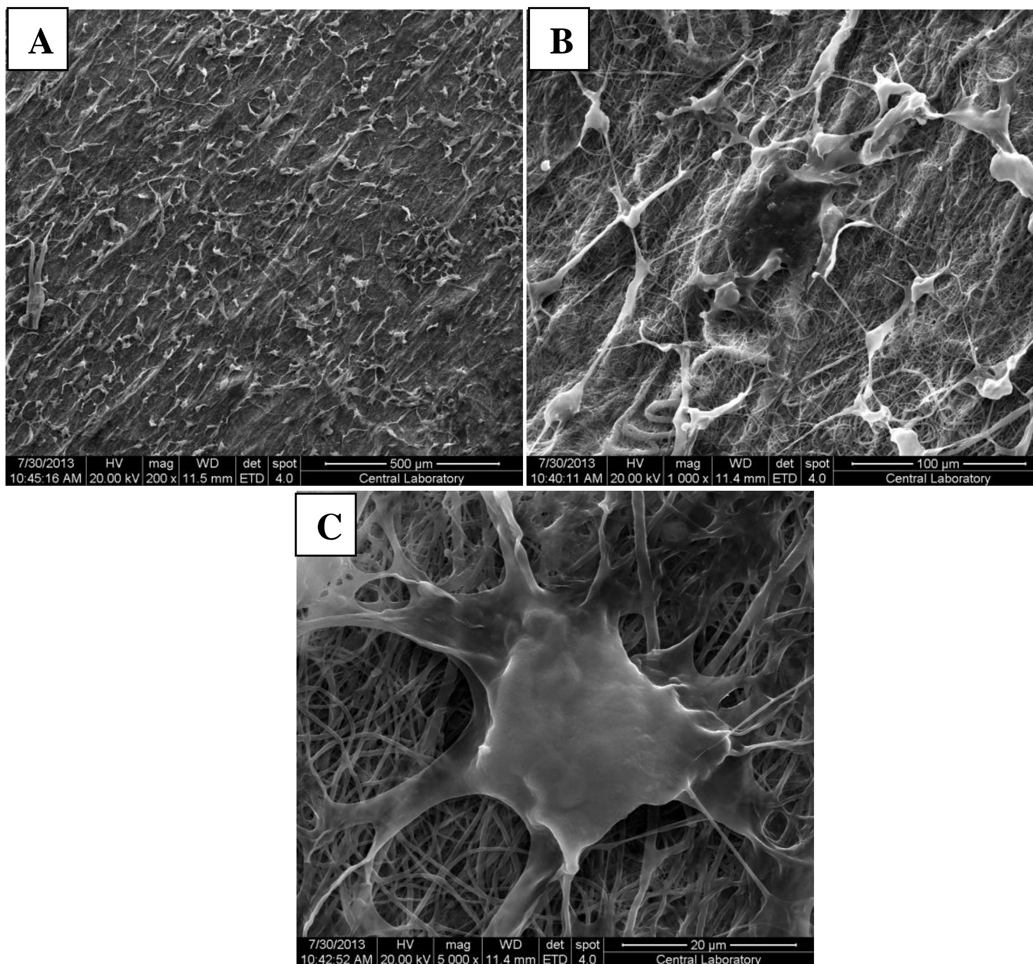

Figure 3.11.SEM of mats seeded with HITAEC on Day 12 (A) x 200, (B) x 1000, (C) x 5000.

3.3.2.3 Fluorescence Microscopy Analysis

In order to visualize a typical HITAEC under fluorescence microscopy the HITAECs (p8) were seeded onto cover slip, the cytoskeleton was stained with FITC labeled phalloidin and cell nuclei was stained with DAPI. The cells appear to be healty with proper conformation.

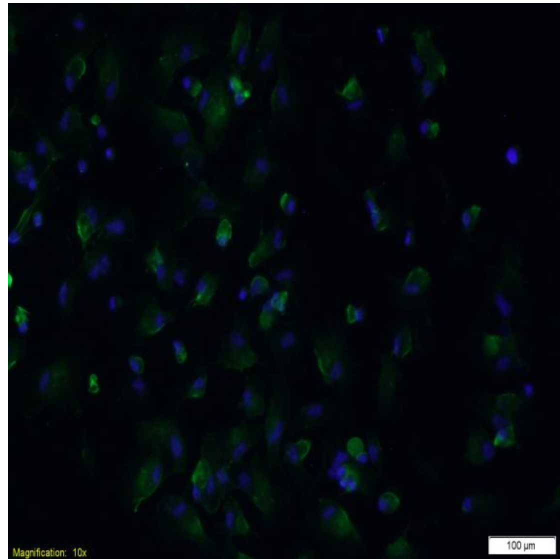

Figure 3.12 Fluorescence micrographs of FITC-labelled Phalloidin and DAPI staining of HITAECs (x10).

3.3.3 Co-culture of VSMC and HITAEC

3.3.3.1 Cell Proliferation

In order to study the coculture behavior of the VSMC and HITAEC cells on the mats MTT cell viability assay was conducted (Figure 3.13.). VMSCs were seeded on the mats, and 1 week later HITAECs were seeded. MTT cell viability test was done after HITAECs were seeded on Days 1, 7, and 12 and corresponding to Days 8, 14, and 19 for VSMC. The values presented in Figure 3.12 are in optical density. Presentation in the form of OD was preferred because of the presence of two different cells with two different growth rates. In one week there was 100% increase in the optical density and the next 5 days there was also another 100%. It shows that both of cells did not affect each other’s metabolic activities and growth rates in the coculture.

Smooth muscle cells have higher growth than that of endothelial cells have under *in vitro* culture conditions (in their culture medium). ECs seeded on quiescent SMCs had higher growth rate than that of on proliferative SMCs. It was demonstrated that quiescent SMCs did not affect the ECs growth rate under cocultured conditions (Lavender et al., 2005).

Zorlutuna et al (2009) produced nanopatterned tubular collagen scaffolds and seeded with HITAECs and VSMC. VSMCs seeded outside of the tubular scaffolds and cultured for two weeks. HITAECs were then seeded on the other side and cultured for another week. Cell proliferations results on Day 21 were similar to our results.

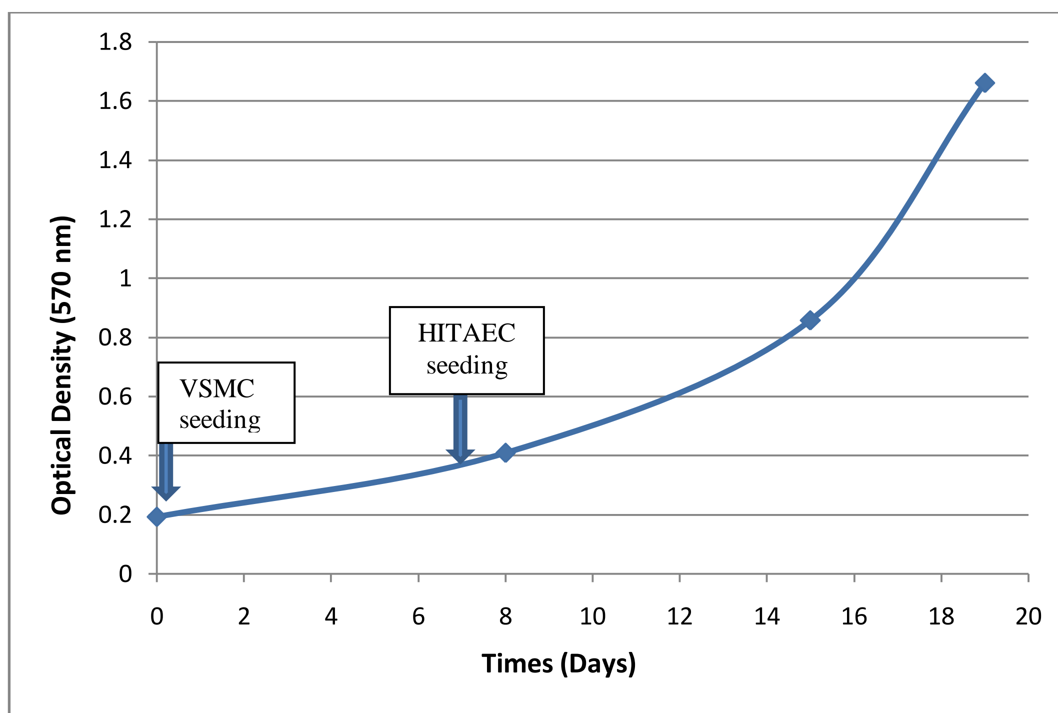

Figure 3.13 Co-culture of VSMC and HITAEC on PCL: Coll membranes after 19 days of incubation (On Day 0 initial VSMC seeding density for per sample: 3.2 x10^4, on Day 7 initial HITAEC seeding density per sample: 4x 10^4).

3.3.3.2 SEM

Figure 3.14. and 3.15. show SEM of the VSMC and HITAEC seeded sides, respectively, of the cocultured mats on Day 19 (Day 12 for HITAEC).VSMC appear to have proliferated and formed a cell sheet on the mat. Figure 3.14. shows HITAEC have also proliferated on the other side of the same cocultured mat on Day 12. Probably due to lower proliferation rate, shorter incubation time and less favorable medium or mix media of VSMC and HITAEC were used, the HITAEC do not seem to have covered the mat as intensely as the VSMC.

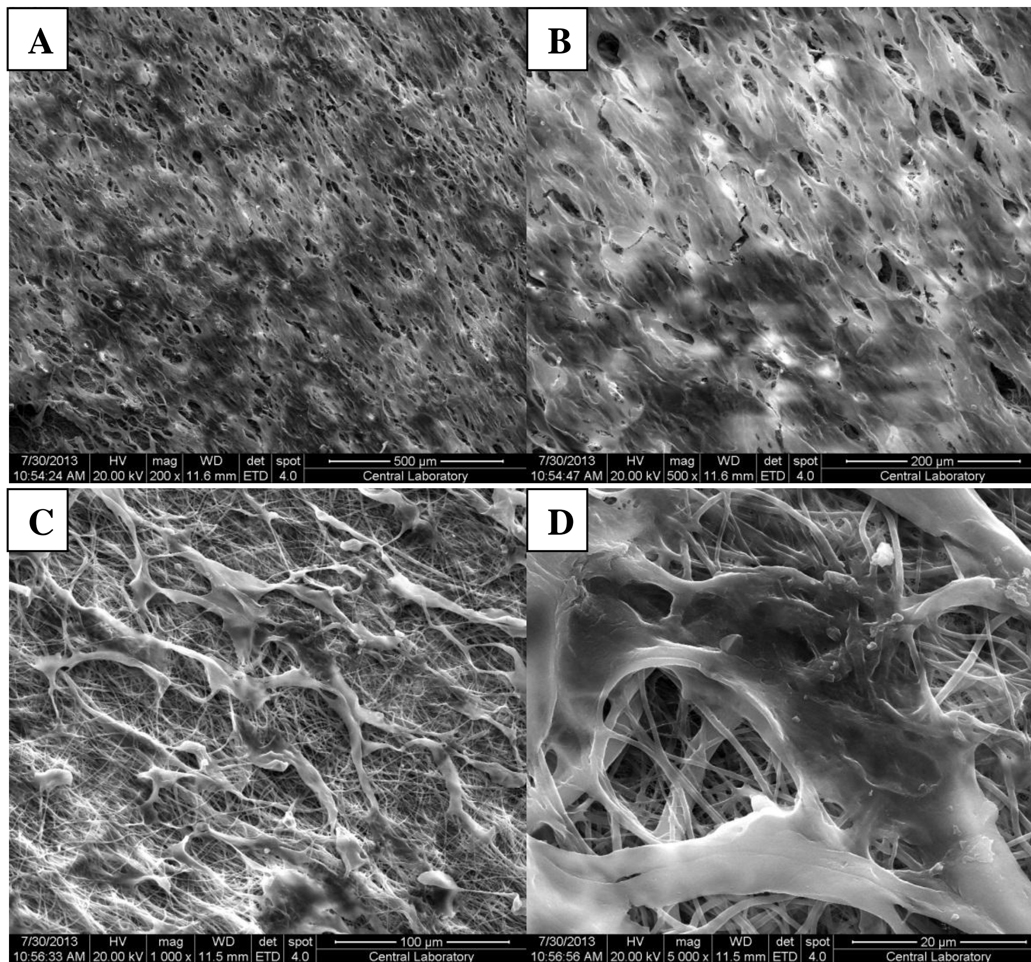

Figure 3.14 Cocultured mat seeded with VSMC and HITAEC showing the VSMC seeded side on Day 19 (Day 12 for HITAEC) (A) x 200, (B) x 500, (C) x 1000, (D) x 5000.

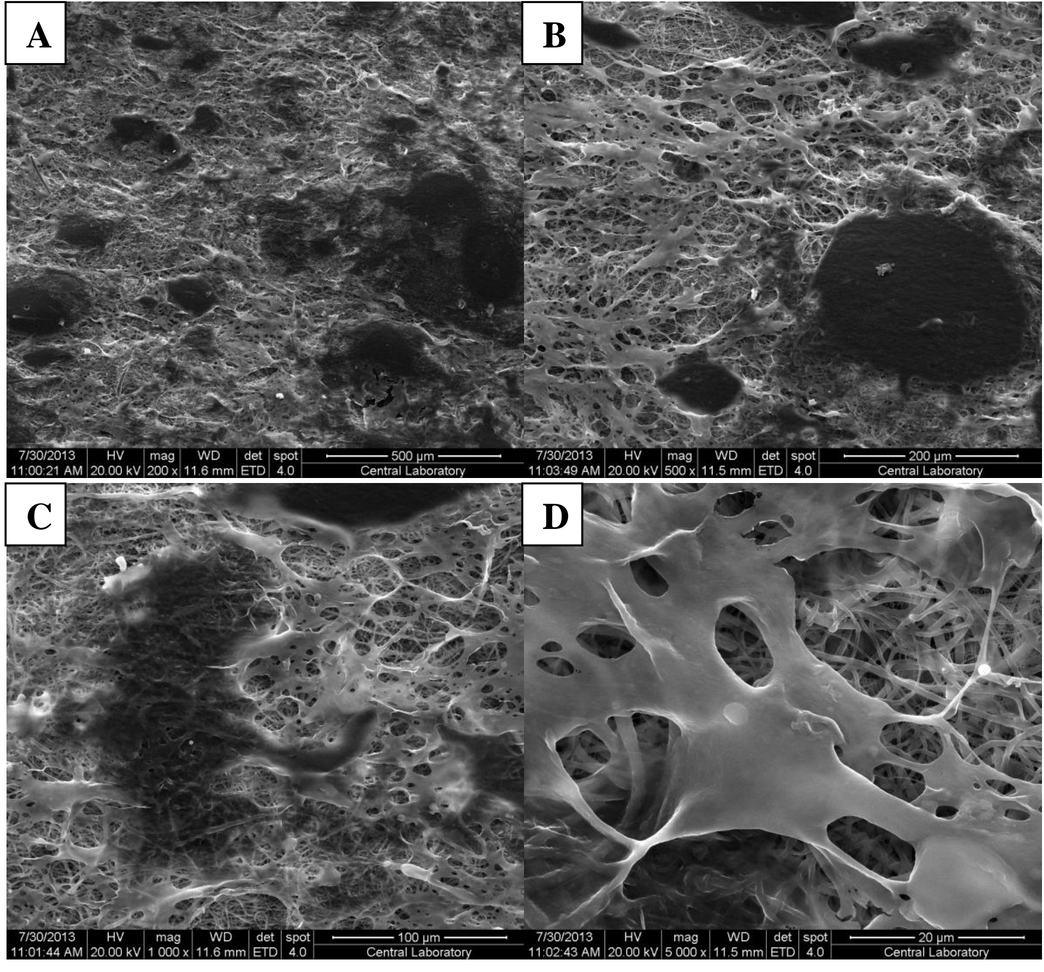

Figure 3.15 Co-cultured mat seeded with VSMC and HITAEC showing the HITAEC seeded side on Day 19 (Day 12 for HITAEC) A) x 200, (B) x 500, (C) x 1000, (D) x 5000.

3.3.4 Mechanical Test

Mechanical properties of vascular grafts are very important because vascular grafts should withstand high blood pressures and flow rates. Collagen provides the mechanical strength of the blood vessels. Circumferentially oriented smooth muscle cells secrete collagen type I in the media and fibroblasts do the same in adventitia.

The mat seeded only with HITAECs, only VSMCs seeded mat or cocultered mat seeded with VSMC and HITAEC seeding with were tensile tested on Days 14, 21 and 19, respectively and the results were compared with unseeded XL mat. The results are presented in Table 3.4 .

The UTS of HITAEC seeded mat (6.63 ± 3.19) was higher than that of unseeded membrane (5.80±0.81). In addition, the UTS value of VSMC seeded mat was higher than the unseeded mat. The UTS of mat was increased probably due to production of collagen type I by VSMC. However, UTS of cocultured mat (9.31 ± 1.32) was significantly higher than that of unseeded mat (5.80±0.81) indicating a statistic effect created by the presence of two different cells.

All seeded mats had higher values of Young's Modulus than those of the unseeded mats, and, the cocultured system had the highest Young's Modulus of all.

Also, cell seeding increased the elongation at break values of mats. The coculture system had the highest elongation at break value.

Table 3.4 Tensile mechanical testing results of unseeded, only HITAEC seeded, only VSMC seedeed and cocultured VSMC and HITAEC mats.

	Mechanical Properties		
Type and State	Ultimate tensile strength (UTS), MPa	Young's Modulus (E), MPa	Elongation at break, %
XL (No cells)	5.80±0.81[a]	8.57±2.23	124.59 ± 20.00
XL HITAEC, day 14	8.63 ± 3.11	10.29 ± 2.88	132.50±32.94
XL VSMC, day 21	6.86 ± 0.47	9.75 ± 1.02	122.47±12.37
XL Coculture VSMC-HITAEC, day 12	9.31 ± 1.32[a]	11.32 ± 1.64	141.34 ± 13.37

a. The difference between UTS values of unseeded and cocultured mats is significant ($P < 0.05$).

CHAPTER 4

CONCLUSION AND FUTURE STUDIES

Cardiovascular diseases (CVDs) including diseases of coronary arteries and blood vessels of the brain are responsible for 17.3 million deaths a year. For the treatment of this disease autologous vessels are used availabity of which is limited. Synthetic blood vessels are successfully used in large diameter vessels (> 6mm). However they are not successful in small diameter vessels (< 6mm) due to early thrombosis formation. Tissue engineered blood vessels are promising for the treatment of CVDs.

The aim of this study was the production of a PCL: Coll tissue engineered blood vessel as a small diameter vascular graft and testing *in vitro*. For this purpose, scaffolds were seeded with vascular smooth muscle cells and endothelial cells.

The unseeded PCL: Coll based mats had appropriate mechanical poperties and they were successfully sutured without any tear. These mats were shown to support cell attachment and proliferation. Interestingly, for cocultered mats two types of cells did not affect each other's growth rate. Presence of the two cells caused synergitic effect and this effect increased the mechanical strength of the mat.

For future studies, PCL: Coll: ELP based mats should be seeded with HITAECs and cultured for 14 days to for continous endothelial layer which is vital for ideal vascular graft. With increasing culture duration, seeded mats also mechanically test should be determined. Moreover, suturability test should be also perfomed on cocultured mats.

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APPENDIX

CALIBRATION CURVES FOR CELL NUMBER DETERMINATION

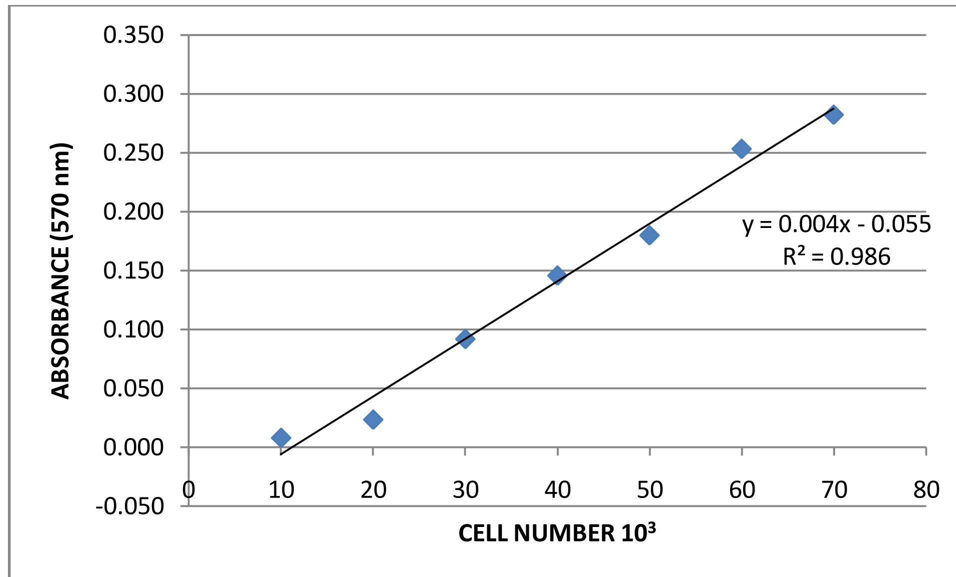

Figure A.1. Calibration curve of HITAECs (p11) for MTT cell viability test

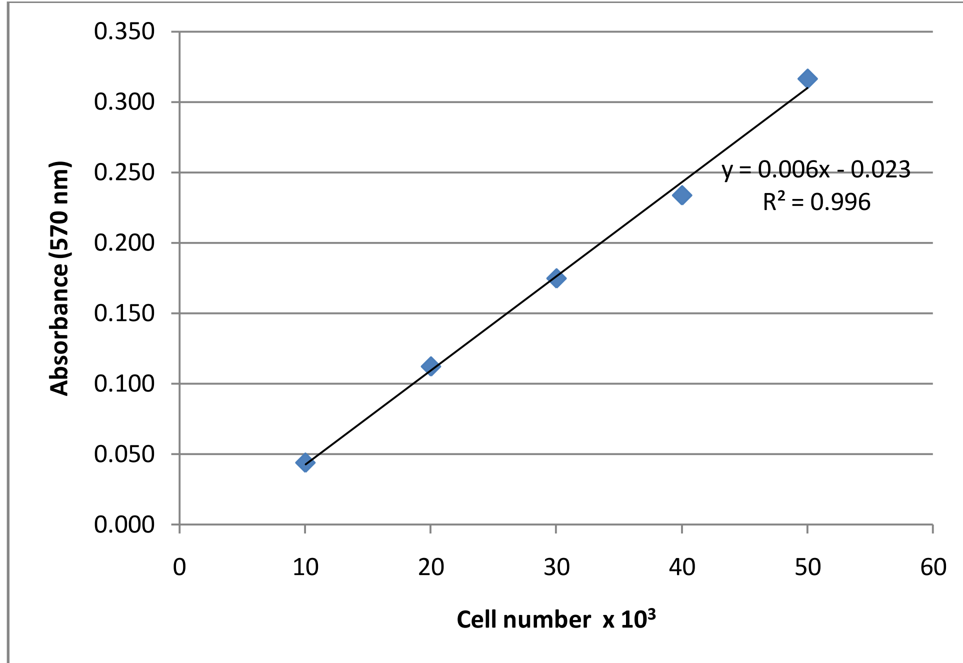

Figure A.2. Calibration curve of VSMCs (p7) for MTT cell viability test