FABRICATION OF SMALL DIAMETER VASCULAR GRAFT USING STACKED COLLAGEN FILMS

by

SANDEEP SHAH

Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE IN BIOMEDICAL ENGINEERING

THE UNIVERSITY OF TEXAS AT ARLINGTON

December 2012

Copyright © by Sandeep Shah 2012

All Rights Reserved

ACKNOWLEDGEMENTS

I would like to thank Dr. Young-tae Kim for allowing me to work in his lab and assisting me throughout the research work. He was outstanding mentor and advisor to me. He always encouraged me to work hard and assist me in completing my thesis successfully. He was very pleased with my lab work and results. I once again thank his kind support, suggestion and guidance throughout the research work.

I would also like to thank Dr. Cheng-Jen Choung and Dr. Kytai Nguyen for serving on my thesis committee. I would like to thank students of Dr. Kytai Nguyen lab for allowing me to use their lab equipment's when needed for research work. I would also like to thank Michael Palmer, Richard Tran form Dr. Jian Yang Lab for let me use their tools for my research work. I would also like to appreciate Jennifa Ahmed for assisting me on this research project. I would like to appreciate my lab co-workers Sirisha Dhavala, Austin Price, David-Blaha Nelson, Samik Bhattarai, Poorva Abhyanakar, Shahina Ahmed, Farheen Khan, Chetan Bhuwania, and Fatemeh Jelvhei Moghaddam for their support during my research work.

At the end, I would like to dedicate this research work to my wonderful parents Mr. Gopal P. Swarnkar and Mrs. Meena Devi Soni, my Sister Khushbu Shah, and my elder sister Rinkey Shah for their love and support through my entire school career.

November 21, 2012

ABSTRACT

FABRICATION OF SMALL DIAMETER VASCULAR GRAFT FROM STACKED COLLAGEN FILMS

Sandeep Shah, M.S.

University of Texas at Arlington, 2012

Supervising Professor: Young-tae Kim

Collagen I have been widely used in the field of vascular tissue engineering. They are characterized better for their interaction at the cellular level but are often limited as vascular graft due to their weak mechanical strength and thrombic property. The crosslinking chemicals or polymers support are used to overcome weak mechanical property. Crosslinking agents tend to have cytotoxic effects while blend with synthetic polymers have mismatch or compliancy issues inside the body. Here in this set of study, we used stacked collagen films and embedded drug delivery system within the film to construct small tubular conduits to meet the mechanical demands of a successful vascular graft and overcome thrombic nature of collagen material. Later in the studies we also enforce elastin within the collagen film to shows its efficiency of our fabrication design to tune mechanical property to desirable needs. At the end of studies, fibronectin, heparin and aspirin drug have been blended with tubular construct to improve the hemocompatiblity features. Here we report burst pressure of 4259±733 mmHg and suture

retention strength of 293±13 gf of 15 layers collagen tubular construct. We also report burst pressure of 3240±542 mmHg and suture retention strength of 368±40 gf of 10 layers collagen-elastin tubular construct. The burst pressure of both 15 layers collagen and 10 layers collagen-elastin tubular construct was higher than human saphenous veins (4259±733, 3240±542 vs. 1976±419 mmHg) and matched closely with human artery (4259±733, 3240±542 vs. 3128±1551 mmHg). The collagen film supported cell adhesion, differentiation and proliferation well. The collagen tubular construct was successfully coated with fibronectin showing more endothelial cell growth. The toluidine blue staining showed presence of heparin molecules throughout the layer of the tubular structure decreasing the chances of blood clot *in vivo* studies. Finally aspirin drug was embedded within the tubular structure for local release at the site of surgery to avoid platelet adhesion and reduced blood clot. The spectrophotometer analysis showed the behavior of drug release profile over the period of 5 days. The tunable mechanical property and fabrication method free of crosslinking agents makes this design very appealing for the future of vascular tissue engineering of small diameter vascular grafts.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS……..……..….....iii

ABSTRACT……..…….....iv

LIST OF ILLUSTRATIONS……..xi

LIST OF TABLES…….....……....xii

Chapter Page

1. INTRODUCTION…….……1

1.1 Need for Vascular Tissue Engineering…….……1

1.2 Current Strategies for Designing small Diameter vascular graft…....3

1.2.1 Synthetic Biodegradable Polymer…….……..……..3

1.2.2 Decellularized Matrices…….....4

1.2.3 Natural Protein Scaffolds…….....5

1.3 Family of Extracellular Matrix……..……6

1.4 Family of Collagen Molecules…….....7

1.5 Biodegradable Collagen I……...…….…….....11

1.6 Objective of the Research…….....…….….....12

1.6.1 Specific Aims……12

1.6.2 Innovative aspects and outcomes of my research ……13

2. FABRICATION OF PURE COLLAGEN CONSTRUCT AND ITS CHARACTERIZATION STUDIES…….…….14

2.1 Introduction...……..…….....14

2.2 Materials and Methods……15

2.2.1 Materials…….....15

2.2.2 Fabrication of Collagen Film……16

2.2.3 Stacking of Collagen film and designing tubular shape...…….....17

2.3 Mechanical Characterization……..17

2.3.1 Burst Pressure…….....17

2.3.2 Suture Retention Test……18

2.3.3 Effect of Stacks……18

2.4 *In Vitro* Study……...……..19

2.4.1 Endothelial Cell Growth on Films……19

2.4.1.1 Immunostaining for Endothelial Cells…….....19

2.4.2 Endothelial Cell migration……20

2.5 Results and Discussions…….....21

2.5.1 Fabrication of Collagen film and Collagen tube…….....21

2.5.2 Effect of Stacked Collagen Film…….....24

2.5.3 Mechanical Characterization……26

2.5.3.1 Suture Retention……26

2.5.3.2 Burst Pressure……28

2.*6 In vitro* Cell Adhesion Study…….....30

2.6.1 HUVEC cells for evaluating the Efficiency of Collagen Films……...30

2.6.2 HUVEC migration towards collagen film……31

2.7 Conclusions……..33

3. COLLAGEN AND ELASTIN……...……34

3.1 Introduction to Elastin……..34

3.2 Materials and Methods……35

3.2.1 Materials…….....35

3.2.2 Fabrication of Collagen-Elastin Film……36

3.2.3 Preparation of Collagen-Elastin Tube…….....36

3.3 Mechanical Characterization…….....36

3.3.1 Suture Retention Test……36

3.3.2 Burst Pressure…….....37

3.5 Results and Discussions……...37

3.5.1 Fabrication of Collagen-Elastin film and tube…….....37

3.5.2 Mechanical Characterization……39

3.5.2.1 Suture Retention Test……39

3.5.2.2 Burst Pressure…….....41

3.6 Conclusions…….....43

4. IMPROVING THE HEMOCOMPATIBILITY OF THE COLLAGEN BLOOD VESSELS……..……..……44

4.1 Introduction……..44

4.2 Materials and Methods…….45

4.2.1 Materials……..…….45

4.2.2 Fabrication of Fibronectin coated Collagen film……..45

4.2.3 Endothelial cells Growth on Film…….45

4.2.4 Immobilization of Heparin on Collagen Tube……..46

4.2.5 Toluidine Blue Staining……46

4.2.6 Loading of Drugs……..47

4.3 Results and Discussions…….....47

4.3.1 Collagen coated with Fibronectin……47

4.3.2 Immobilization of Heparin on the Collagen tube……...49

4.3.3 Drug Delivery Study……...50

4.4 Conclusions……..53

5. FUTURE GOALS…….……..55

5.1 Current Work……...55

5.2 Ongoing Studies……...55

5.3 Future Studies……..56

5.3.1 Synthesis and Fabrication of Vascular Conduit……56

5.3.2 Mechanical Characterization……56

5.3.3 *In Vitro* Cell studies……..……...56

5.3.4 Drug Delivery Studies……...57

6. DISCUSSIONS…….…….....58

REFERENCES…….…….62

BIOGRAPHICAL INFORMATION…….……68

LIST OF ILLUSTRATIONS

Figure Page

1.1 The three-dimensional arrangement of amino acid throughout the single collagen molecule..... ……9

1.2 The basic assembly of collagen I molecule……..10

2.1 The schematic diagram for endothelial cell migration study……...20

2.2 Fabrication of Collagen film. 22

2.3 The various construct prepared through this fabrication approach..... ……..24

2.4 Average suture retention test readings for three different layers of collagen film…...27

2.5 Average burst pressure readings for three different layers of collagen..... 29

2.6 The growth of endothelial cells on collagen film..... 31

2.7 Migration of endothelial cell towards collagen film ..... ……..32

3.1 Fabrication of Elastin film……....38

3.2 Suture retention strength of collagen-elastin tube and collagen tube itself. 40

3.3 Pressure comparison of collagen and collagen-elastin tube at 5 and 10 layers. 42

4.1 Average number of cells grown on collagen film and fibronectin coated collagen film ..... ……48

4.2 Image of Collagen stained with Heparin molecule……...50

4.3 The cumulative release of aspiring over 24 hours for five days. 51

LIST OF TABLES

Table Page

1.1 The Characteristics of Ideal Vascular Graft ..... …….2

1.2 The collagen type, their chain composition and basic distribution through the body 8

2.1 The thickness profile of the collagen films individually and when stacked too..... 25

4.1 The amount of aspirin release from single and sandwich layers. 52

CHAPTER 1

1. INTRODUCTION

1.1 Need for Vascular Tissue Engineering

Tissue engineering is a classical approach of using the principles of engineering and life sciences towards the development of biological substitutes to restore, and maintain tissue function [1]. Peripheral vascular disease represents a growing concern in United States and other developing country [2]. According to the American Heart Association 3 million procedures are performed in the United States each year. The larger diameter blood vessel has shown 10 years compliance post implantation while diameter less than <5 mm has resulted in failure due to thrombotic occlusion [3]. There is greater need for functional arterial graft, and success has only been limited to larger vessels such as in case of aorta, arch vessels, iliac, and common femoral arteries. However, in case of small diameter less than (<5mm), autologous or arterial vein graft has only been successful. But the quantities and availability of such vessels is limited due to acute thrombogenicity, anastomotic intimal hyperplasia, aneurysm formation and progression of atherosclerotic disease [2,4]. Hence this problem has triggered the research in these areas to find a suitable vascular graft, which can possess all the ideal characteristics of vascular graft. The characteristics of ideal vascular graft are summarized in Table 1.1

Table 1.1 Characteristics of Ideal vascular graft [5]

High Biocompatibility	Compliant	Easy Processing
Non-toxic, non-allergic	Resistant to neointimal Hyperplasia	Adequate Mechanical property
Non-thrombogenic	Flexible, elastic, without kinking	Readily available in all size
Resistant to Infection	No malignancies	Optional local drug delivery

The characteristics of ideal vascular graft can be summarized in four different categories:

a. Biocompatible: The biocompatibility of a material describes its effect on the living tissues or cells when it comes in contact with them. The surface and molecular shape of the material should facilitate the growth and differentiation of cells in to tissues. Its chemical composition should not induce any foreign body response, which usually is the cause of failure of most graft in the body. If the graft being used in acellular, it should form endothelium layer well within its inner structure to avoid any thrombosis or aneurysm.

b. Compliant: The vascular graft should have adequate mechanical properties that can be comparable to native blood vessels. The native artery and human saphenous vein have burst pressure in the range of 3128±1551 mmHg and 1976±419 mmHg and suture retention strength around 200±119 gf [6]. The native blood vessels should have sufficient viscoelastic properties so that it can adapt to changing blood flow conditions at regular intervals [7]. The material should be inert and resistance to abrasion. It should possess desirable tensile stress to bear the loading cycles caused due to strenuous activity like exercise, moving and should not fatigue easily. It should be resistant to kinking.

c. Easy Processing: The material should have adequate physical and chemical properties for their durability. It should have easy fabrication designing process and should not involve very complex storage parameters and expensive materials. It should be easy to suture and be very cost efficient. Any initial cell seeding should be avoided before implantation because cell seeding can take longer time and also involves time consumption and more labor.

d. Optional importance: The polymer for vascular graft should have the advantages of special surface modification to be used for other purpose in biomedical application like drug delivery, protein adsorption studies, and many more.

1.2 Current Strategies for Designing Small Diameter Vascular graft

1.2.1 *Synthetic Biodegradable Polymers*:

To substitute the high demand and need for small diameter vascular graft, a lot of research has been going in to designing vascular graft out of synthetic polymers that possesses biodegradability, bioresorbability, and biocompatibility. Synthetic polymers like PCL, PLA, PLGA and their copolymer have been widely used in vascular tissue engineering. These polymers can be easily modified with certain functional groups to achieve desired biological properties [8]. A graft constructed out of poly(ether)urethane-polydimethylsiloxane (PEtU-PDMS) showed superior handling and compliance characteristic than commonly used standard ePTFE graft in the industry [2]. Synthetic polymers have also served as transplantation device along with delivering drugs over period of time. In a study done by Sheridian et al. porous PLG matrix was loaded with VEGF growth factor and 90% of the bioactivity was also retained [9]. While synthetic polymers have shown great promising approach in constructing a successful

graft for small diameter blood vessels, they have some disadvantage, which need to be overcome in the nearby future.

These synthetic polymers are often made with notion of incorporating cells or growth factors before *in vivo* application. These polymers are synthesized in harsh conditions, which don't make them cell friendly in *vivo*. Sometime the rate of degradation is faster than rate of formation of fully matured ECM protein in at the site of injury, which leads to biological failure of the graft [10]. The compliance mismatch has been a major problem for widely used in synthetic polymers like Dacron, and ePTFE based graft [11]. Some polymers have shown to get dilated and cause aneurysm inside the body after few days of transplantation [11]. Lack of mechanical stability even after reinforcing Dacron mesh has been seen frequently [4].

1.2.2 *Decullarized matrices*:

Decullarized tissue has been reinforced as substrate for replacing disease vascular tissues [12-14]. Researchers usually remove all xenogeneic cellular components leaving behind a non-antigenic ECM for cellular repopulation [14]. In this kind of procedure mechanical integration is maintained by carefully extracting non-structural components. This has great advantage over most methods used to design vascular graft because it by pass fabrication step to design vascular graft and avoids all the complications that is needed to mold a conduit that closely resembles native vessels in term of mechanical property. These scaffolds preserve all the anisotropic mechanical behavior of native vessels [15]. They show property of anti-thrombogenicity, durable, cell viability and most of all overcomes most disadvantages of synthetic polymers like

patency compliance, biodegradability, toxicity due to fixation of polymers, and insufficient mechanical properties.

Despite decellularized matrices showing great promising approach in designing vascular graft, it has certain disadvantages that have not made it popular in clinical trials. Extracellular matrix turnover and their behavior in mechanical strength on long-term durability are poorly understood [14]. The decellularized matrices are usually animal products and its use in human cannot meet the structural similarity and may lead to certain complications and problems [14]. To overcome the mismatch compliance in tissue model, luminal seeding is done which can be lengthy wait for patient and its efficiency trial will need to be performed before implantation [16].

1.2.3 *Natural Polymers*:

Biologically derived polymers have been widely used in vascular tissue engineering. Natural polymers like elastin, collagen, chitin, and silk have been used in designing small diameter vascular graft [17]. These polymers are also used as hybrid scaffold often with synthetic polymer to overcome the limitations of cell friendly substrate for the cell growth [18,19]. Since these components are derivation of living body, these create excellent environment for cell differentiation and growth, provide structural integrity to the healing tissues and avoid any kind of immune rejection. These polymers also undergo chemical and physical modifications to become more bioactive molecules for tissue engineering applications. Natural polymers are completely biodegradable, biocompatible, mechanically strong, less expensive, and

easily available. Hence they are considered to be alternatives for synthetic polymers for small diameter vascular grafts.

1.3 Family of Extracellular matrix

Extracellular matrix components (ECM): In the field of tissue engineering, scaffolds that can allow cells to proliferate at continuous rate becomes very essential for its compliancy. The growing cells from extracellular matrix and then replaces the scaffold with new vessels [20]. Researchers have realized the importance of formation of extracellular matrix at faster rate for the success of designed graft in the clinical trial. Since cells ultimately forms extracellular matrix, they growth is much more visible when one or more components of extracellular matrix is used while building scaffold.

Fibrillar collagen is the load-bearing agent in the arterial walls. Elastin and associated microfibrils provides artery and other tissues with resilience and passive recoil without energy input [21]. Fibronectin helps in cell adhesion, growth, migration and differentiation.

ECM comprises of proteoglycans, heparin sulfate, Chondroitin sulfate, keratin, collagen, elastin, fibronectin, and laminin. These components vary in their composition in the tissue. All these components have been used solely or combined with other polymers in designing vascular graft. ECM has multiple functions. Some of the main functions are providing biomechanical properties of tissue in determining strength and shape, serving as biologically active scaffold for cells to adhere or migrate, and also helping to regulate the phenotype of the cells. Researchers have tried to imitate the ECM matrix while designing the scaffold. Collagen, elastin and PCL construct was designed as hybrid ECM scaffolds and showed favorable mechanical and

biological mechanical properties [22]. The importance of cell cultured in ECM mimicking 3D scaffolds has shown potential to grow and differentiate to express functionality [23]. Hence several studies have been done to design artificial ECM for cell-adhesive substrate, obtaining three dimensional tissue structure and specific mechanical signals [24].

1.4 Family of Collagen molecules

Collagen I is naturally occurring proteins and the most abundant proteins in mammals. It is the main component of connective tissue and found abundantly in cornea, cartilage, bone, blood vessels, and gut. It accounts for the structural integrity of blood vessels and contribute a framework within the tissue functions [25, 26]. The collagenous spectrum ranges from Achilles tendon to the cornea. They exist in various types and each type has its own important functions. The various types of collagen and their primary functions are outlined in Table 1.2

Table 1.2 shows the collagen type, their chain composition and basic distribution through the body [26].

Collagen type	Chain composition	Tissue distribution
I	$(\alpha1(I))_2\alpha2(I)$, trimer $(\alpha1(I))_3$	Skin, tendon, bone, cornea, dentin, fibrocartilage, large vessels, intestine, uterus, dentin, dermis, tendon
II	$(\alpha1(II))_3$	Hyaline cartilage, vitreous, nucleus pulposus, notochord
III	$(\alpha1(III))_3$	Large vessels, uterine wall, dermis, intestine, heart valve, gingiva (usually coexists with type I except in bone, tendon, cornea)
IV	$(\alpha1(IV))_2\alpha2(IV)$	Basement membranes
V	$\alpha1(V)\alpha2(V)(3(V)$ or $(\alpha1(V))_2\alpha2(V)$ or $(\alpha1(V))_3$	Cornea, placental membranes, bone, large vessels, hyaline cartilage, gingiva
VI	$\alpha1(VI)\alpha2(VI)\alpha3(VI)$	Descemet's membrane, skin, nucleus pulposus, heart muscle
VII	$(\alpha1(VII))_3$	Skin, placenta, lung, cartilage, cornea
VIII	$\alpha1(VIII)$ $\alpha2(VIII)$ chain organization of helix unknown	Produced by endothelial cells, Descemet's membrane
IX	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	Cartilage
X	$(\alpha1(X))_3$	Hypertrophic and mineralizing cartilage
XI	$1\alpha2\alpha3\alpha_1$ or $\alpha1(XI)\alpha2(XI)\alpha3(XI)$	Cartilage, intervertebral disc, vitreous humour
XII	$(\alpha1(XII))_3$	Chicken embryo tendon, bovine periodontal ligament
XIII	Unknown	Cetal skin, bone, intestinal mucosa

Since scaffold in this design is made out of collagen I, it is predominant in all the higher animals. It is compound composed of three chains, two of which are identical and its also forms trimer in its structure sometimes. Since all synthetic polymers are composed of different chemistry which gives them different physical and chemical properties. The understanding of

their basic chemistry is crucial in understanding the effect on cells growth and its integrity with body tissue.

Collagen type I is composed primary of amino acids. The arrangement of amino acids occurs in coil fashion and this coil is also responsible for its structural strength and shape. The basic coil arrangement of amino acids in collagen I is given below in Fig. 1.1

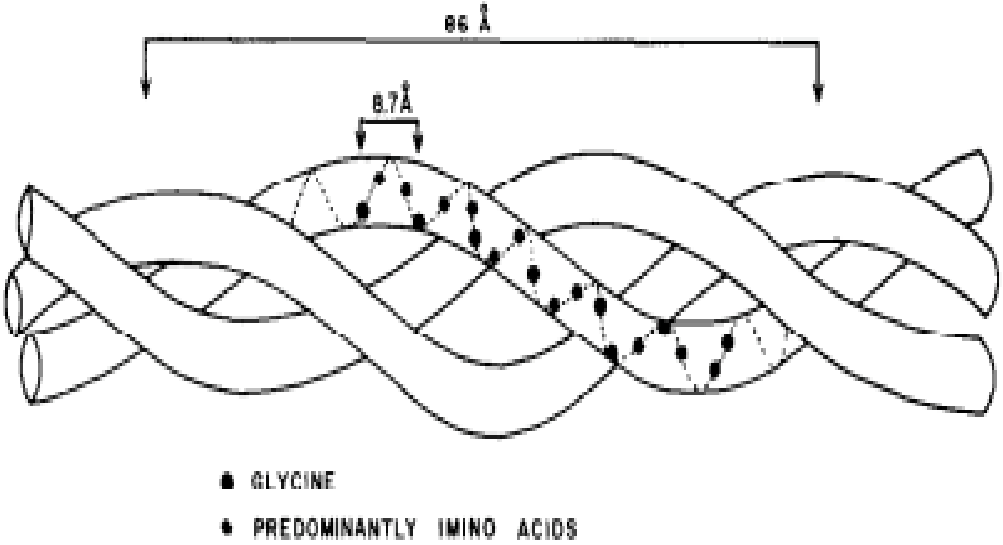


Fig. 1.1 below shows the three-dimensional arrangement of amino acid throughout the single collagen molecule [25].

In each collagen molecule, every third residue is glycine. The triple helical structure generates a symmetrical pattern of three left-handed helical chains with a pitch of approximately 86 A^0. The distance between two amino acids is 2.91 A^0. The uniqueness of this helical structure is that it will not allow intrachain bonds to form and only allows interchain hydrogen bonds formation. The strength of these collagen I molecule can be better understood when looked at their intramolecular configurations rather than just looking at intermolecular patterns. X-ray diffraction studies on collagen I molecule has shown the existence of supermolecular coiling that

shows microfibrils packing within every five collagen molecules [26, 27]. These basic events lead to formation of procollagen molecule when then assemble together to form complete collagen fiber [27]. The Fig. 1.2 below shows the assembly of microfibrils in to procollagen to collagen fiber.

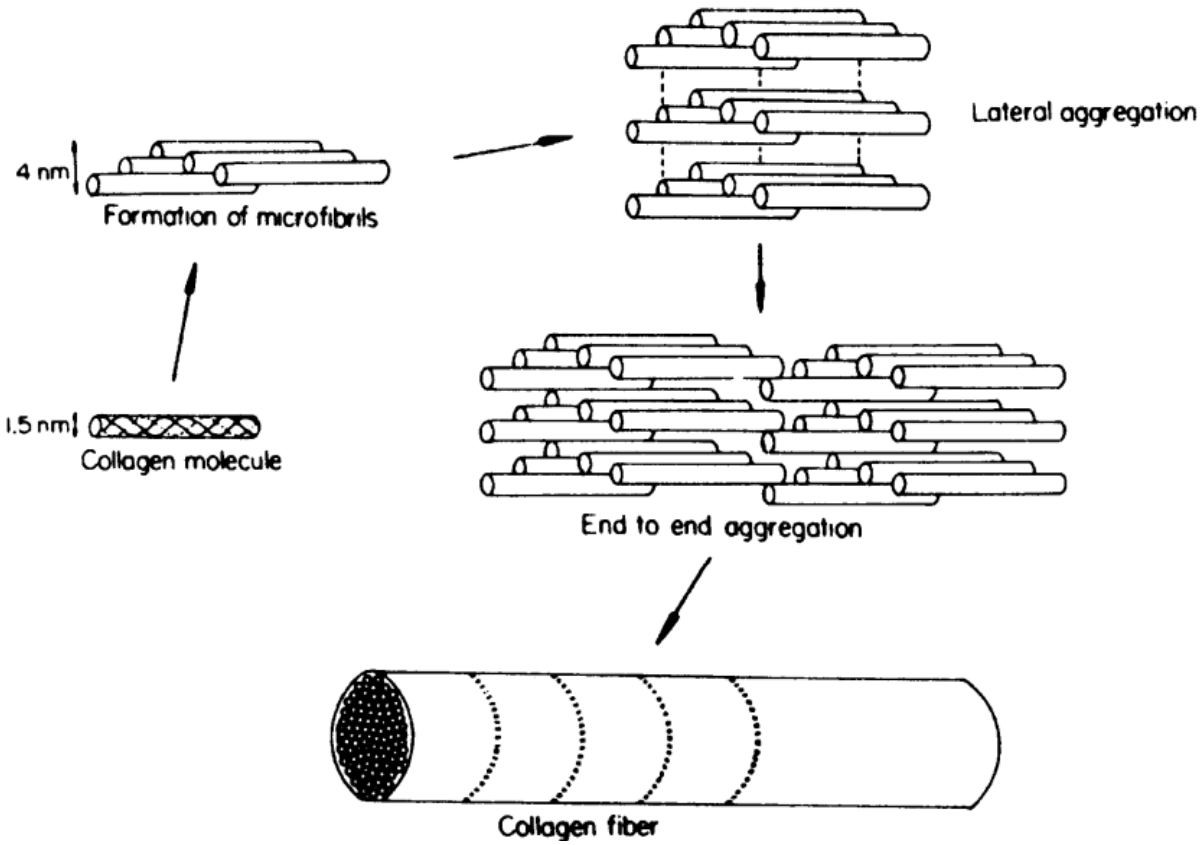


Fig. 1.2 shows the basic assembly of collagen I molecule. [26]

1.5 Biodegradable Natural Polymer Collagen I

Collagen I is abundantly used in various fields like drug delivery, skin tissue engineering, cartilage tissue engineering, bone tissue engineering and also in vascular tissue engineering [28-31]. Owing to its biocompatibility, biodegradability and ease of synthesis has led to its extensive research over many other polymers used in tissue engineering. Collagen I has been preferred over commonly used Dacron grafts, polytetraflouroethylene (ePTFE) vascular prostheses in small diameter vascular graft because those grafts function good in high flow conditions and low resistance and these are not seen in smaller vessels (<3mm) [32].

Chin Wu et al. designed a vascular prosthesis made with pure collagen (2mm OD) with endothelial cells and smooth muscles cells cultured on the inner layer showed no sign of coagulation *in vivo* after 12 weeks, and excellent mechanical property of tensile modulus 1.49±0.19 MPa [32]. In another study conducted by Joseph et al. a hybrid Collagen I with cell seeding on inner layer showed increase in mechanical property when compared with Collagen I itself. In this case, burst pressure was found to be 650 mmHg over 10 mmHg [33]. Hence collagen I has been used frequently in designing vascular graft because it ultimately overshadow many disadvantages of synthetic polymer, few natural polymers by providing extracellular matrix environment for cell differentiation and proliferation.

However, Collagen I is primarily used in conjunction with other polymers or supported with cell seeding for the compliancy in small diameter vascular graft. Collagen I was blended with synthetic polymers PLLA for designing small vascular graft in order to increase mechanical and bioactive properties. Collagen is usually fixed with glutaraldehyde to increase strength,

which often increases risk of toxicity and calcification. Similarly collagen fiber was also used with segmented polyurethanes to create a residual stress environment in native arteries but synthetic polymer have compliancy problem of rigid behavior from polymer [34]. In general collagen construct have shown in adequate mechanical properties to withstand hemodynamic pressure and shear stress [33]. But knowing the nature of collagen molecule, its excellent ability of allowing endothelial cells growth and its supercoiled structure, a vascular construct made from purely collagen without any harmful crosslinking agent and capable of meeting the mechanical features of native vessels would bring a new dimension to the field of vascular tissue engineering.

1.6 Objectives of the Research

1.6.1 *Specific Aims*

Collagen I is known to be the most abundant protein in all mammals. Its interaction with the endothelial cell is well understood [35]. It is completely biodegradable and biocompatible. Despite all these excellent features of collagen I molecule, it has been deprived of necessary mechanical property to design small diameter blood vessels. The main objective of this research was to use collagen I molecules to construct a small diameter blood vessels which can possess adequate mechanical properties, be flexible to design various diameter with simple approach, avoid any kind of chemical crosslinking and shows potential for drug delivery.

AIM I: Fabrication of Collagen construct and its characterization studies through mechanical tests and cell viability.

AIM II: Fabrication of vascular construct from combining collagen and elastin and its characterization differences between collagen construct and collagen-elastin construct.

AIM III: Improving the hemocompatibility of the collagen blood vessels.

Hence, the successful results obtained in all the experiments will pave a new dimension in tissue engineering for designing vascular graft, will be able to establish a very noble approach to fabricate scaffold in large quantities and at low prices.

1.6.2 *Innovative aspects and outcomes of my research work*

In this whole study, easy fabrication technique is introduced which is very efficient. The use of natural polymers like collagen and elastin in pure form without any parts of synthetic polymer is enough to construct various sizes of small vascular grafts. The fabrication technique allows someone to tune mechanical property of the constructs. The collagen films are functional in nature and can be easily modified on the surface for attachment of glycoproteins like fibronectin, and heparin. The drugs can be embedded between the film layers for release at the site of anastomosis.

Here we have obtained appropriate mechanical property of collagen and collagen-elastin vascular construct (see Fig.2.3, Fig.2.4, Fig.3.2, and Fig.3.3). We have also shown the growth of Human Umbilical Vein Endothelial Cells (HUVEC) on the surface of collagen film (see Fig. 2.4). The functionalization of collagen film with fibronectin, heparin and drug to improve the hemocompatibility nature of collagen surface has been successfully achieved (see Fig. 4.1, Fig. 4.2, and Fig. 4.3). Hence the fabrication method used in this study has the potential to construct mechanically strong biocompatible small diameter vascular grafts.

CHAPTER 2

FABRICATION OF PURE COLLAGEN CONSTRUCT AND ITS CHARACTERIZATION STUDIES

2.1 Introduction

There are several methods of fabricating scaffold for small diameter vessels. Some of those techniques include Electrospinning, freeze and dry methods to create porous collagen scaffold, solvent casting, dip coating and few more. While all these techniques have shown success in designing scaffold on other polymers in vascular tissue engineering, using collagen has not found to be tremendous success [33]. The chemical nature of collagen is exposed to deterioration from Electrospinning while other methods include crosslinking agents to create a strong tubular shape out of collagen. Collagen is protein and its handling in harsh environment can help denature its content. Collagen prepared as film, thin sheets or thin tube are mechanically very weak and cannot be solely used to design a material that needs mechanical stability. Rather it is enforced with certain synthetic polymers, and other natural polymers to create a tubular structure of desired mechanical behavior. Moreover, it is difficult to precisely control the material properties of the collagen based construct due to the lack of means to tune its properties. Having a method to precisely control the properties of collagen would render it more useful as a biomaterial and enable the creation of numerous collagen enabled tissue-engineered constructs

However from basic concept in which stacked objects of any sheet or film is able to gain

valuable mechanical strength if it is able to hold its stack effect when compared to its single sheet or film. In this set of fabrication procedure, that very concept is used. Collagen film is prepared through solvent casting method on Polydimethylsiloxane (PDMS) mold. The individual films are then wrapped around the Teflon tube of desired diameter using water and air to create bonding between collagen films avoiding any use of chemical crosslinking agent. Then mechanical test is performed to evaluate their effectiveness in comparison with native artery and veins. Following that cell viability is studied using Human Umbilical Vein Endothelial Cells (HUVEC). The coating behavior of the film is also studied because collagen itself being a favorable clotting agent will need coating to overcome the effect for *in vivo* study. Fibronectin, another extracellular matrix was coated and its coating effect was done with the help of cell study. In addition to it, heparin molecule was coated and toluidine blue staining was done to evaluate the effectiveness of coating. All the results from above mentioned procedure have shown positive impact of the fabrication approach in designing small vascular graft.

2.2 Materials and Methods

2.2.1 *Materials*

All the materials desired for the completion of this experiment was obtained from Sigma Aldrich unless otherwise mentioned. All the materials were properly used as suggested and disposed properly if needed.

2.2.2 *Fabrication of collagen film*

The collagen film was made using PDMS mold which was designed as 1mm x 2.5 mm and 100 micron depth. The collagen solution was prepared from rat-tail as process described below. Briefly, the rate tails were used as source to obtain tendons and ultimately extract collagen I. The tendons were then poured in cold 1XPBS buffer solution to remove any kind of blood residues present to get maximum purity. Following to that, it was moved to cold acetone for five minutes, which would make those tendons brittle in nature. Those brittle tendons were then placed in cold 0.02 N acetic acid and allowed to mix over night or more if needed at 4^0 C overnight to dissolve the collagen I in acetic acid solution. The pH was maintained low to control viscosity of solution. The mixed solution was then transferred to wide petri dish with mixture of crushed ice for 24 hours to keep collagen I protein in its active form. Finally the ice-collagen I mixture cube were placed in bottle and lyophilize for 48-72 hours to obtained collagen I sponge.

This collagen I sponge was used to with dilute acetic acid (0.02N). The concentration of solution was 6mg/ml in cold acetone. To make each film, 300 μL collagen solution was used to fill the mold and placed in air station for several hours (3-4hrs) for drying purpose. After the air-dry, the film was moved to UV light (about 25 min) for crosslinking purpose. With the help of clean forceps, the film was carefully peeled of the mold and placed in 35mm beaker. The film was placed under bio-safety cabinet for extra UV treatment for about 30 minutes to make cross-linking stronger which is used later to designing scaffold.

2.2.3 *Stacking of collagen film and designing tubular shape*

The individual collagen film once pilled is placed on the non-sterilized 1xPBS. The Teflon rod is carefully placed on the one end of the film and rolled slowly to avoid any crumbling or damage to film. The process is repeated with five films first, followed by 10 films and 15 films separately. The rolled film is dried with air nitrogen before rolling another film on the top of it. The rolled stacked films around the tube are then dried at room temperature over night. The stacked films around Teflon mandrel are removed with the help of a pair of tweezers.

2.3 Mechanical Characterization

2.3.1 *Burst Pressure*

An easy setup is done to carry our burst pressure readings. In briefly, the collagen tube (4.5 mm inner diameter) is connected to plastic diffuser at the both ends of the tube. The film is firmly attached with the help of UV glue in order to avoid any air or liquid leakage. One end of the tube is connected to the injection carrying solution and other end is connected to manometer to record the pressure in unit mmHg. The whole tube is prefilled with milk of magnesia before connecting to pressure gauge. An injection filled with magnesium hydroxide (milk of magnesia) is allowed to inject solution continuously at constant rate until the scaffold bursts out. The pressure readings are taken at the instant when the burst takes place. This pressure is recorded as maximum pressure that the scaffold can withhold. The value observed is being compared with burst pressure values obtained for human saphenous vein and human artery shown by Nicolas et al [6].

2.3.2 *Suture Retention Test*

The suture retention test was done with the help of measuring device named Instron Mechanical Tensile machine. The suture material 6-0 prolene (thread length 45cm-60cm, needle length 13-60mm) was used to connect the tube to mechanical tester device. One end of the specimen (n=3) was fixed by stage clamp while the other end of the specimen was connected to another clamp by the prolene thread. The measurement was performed using 10 Newton maximum load cell. The tensile force was applied until the grafts were completely torn off and the maximum force was recorded in units' gram force (gf). The values of suture retention strength obtained were compared with suture retention values of human saphenous vein and human artery shown by Nicolas et al [6].

2.3.3 *Effect of stacks*

In order to measure the effect of stack collagen films, the thickness of the film made out of 100 μm depth PDMS mold was measured. The thickness of the film was measured with the help of KLA-Tencor Alpha-Step IQ Profilometer (400 micron vertical range, 10 mm max. scan length, sun-angstrom electronic bit-wise vertical resolution) at the UTA Nano fab center. The thickness of individual single film (n=3) was measured followed by thickness of two stacked films (n=3) and thickness of three stacked films (n=3).

2.4 *In Vitro* cell adhesion study

2.4.1 *Endothelial cell growth on films*

To measure the adhesive and proliferative ability of collagen film; Human Umbilical Vein Endothelial Cells (HUVEC) was used. HUVEC was cultured in 25 ml polystyrene flask in with 10% FBS medium, 1ml LSGS, antibiotic gentamycin (25µL). The cells were cultured for one-week period. The cultured cells were then placed in Petri dish containing collagen film.

A well plate was taken in which three wells were used to place collagen film. Before the films were placed inside well plate, the individual collagen films were attached on the 25x25 cover glass with the help of DI water and air nitrogen. The attached glass-film was slowly placed inside well plate and covered with PBS for overnight. After 24 hours, the PBS was replaced with HUVEC culture medium for few hours. After few hours, equal amount of cells was placed in three wells and placed inside the incubator for their attachment and growth. The cells were allowed to culture for a week period. During the period medium were changed every 48 hours to keep cell healthy and avoid any contaminations.

2.4.1.*1Immunostaining for endothelial cells*:

After a week period, the endothelial cells were fixed with 4% formaldehyde and immuno staining was performed to perform quantitative and qualitative analysis of endothelial cells. Briefly, the fixed sample was covered with primary solution. The primary solution was made 4% goat serum in 1X Triton solution. It was then followed by treatment with secondary solution, which comprises of antibody to endothelial marker. The cells were stained for Von Willebrand

Factor (VWF) glycoprotein because it acts as a marker for endothelial cells. DAPI staining was also performed to stain nucleus.

2.4.2 *Endothelial cell migration*

A device was made out with the help of PDMS and 35 ml Petri dish to carry out cell migration movement. Initially, two circular PDMS mold was taken that could be easily placed inside Petri dish. The small circular hole was made on the PDMS using 6 mm biopsy punch to fit film easily inside its circular circumference easily. The collagen film was attached on the glass fitted within the circular circumference. The PDMS with circular hole was placed on the top of film with circular center on the top of film directly without touching any part with PDMS mold. The next PDMS mold was cut accordingly in circular shape to fit around the first mold with slight gap in between for initial cell seeding and culture placement.

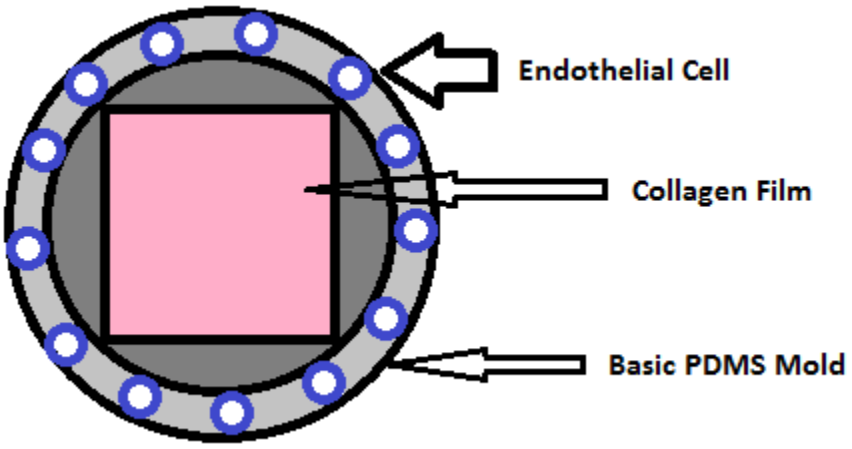


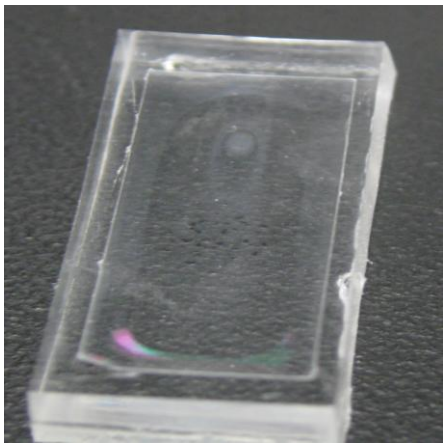
Fig. 2.1 below shows the schematic diagram for endothelial cell migration study. Endothelial cells are seeded around the outer circular area and were allowed to migrate to the surface of the films to grow and adhere. The device was placed inside the incubator (37^0 C, 5% CO_2) and cell growth observed on the film at day 1, day 3 and day 16.

After the design of device, the HUVEC medium is placed for several hours before initial seeding of cells in the narrow gap between two molds. The cells were then allowed to attach on the petridis. The image was taken to see if those cells were firmly attached on the gap region. Now, the first PDMS mold surrounding film was slightly taken out and allows few days to migrate HUVEC cells on the film. The images were then taken after 1,3 and 16 days to see the migration and further quantification analysis.

2.5 Results and Discussions

2.5.1 *Fabrication of collagen film and collagen tube*

The characterization of collagen film was done after the collagen solutions were dried on mold. The film was carefully seen to not have any signs of tear or damage before and after peeling from the mold. All the incorrectly dried films were discarded away. The films obtained were very thin and fragile in nature. The shape of the film obtained after solvent casting on PDMS mold is shown in Fig. 2.2.

a. 

b.

Fig. 2.2 Fabrication of collagen film.a) below shows the dried collagen film on PDMS mold after solvent casting for several hours, and b) shows the peeled film.

Form the above Fig. 2.2 it can be clearly seen that film is nice, dried and has taken the pattern shape. In order to evaluate the strength its thickness was measured with help of surface profilometry. Surface profilometry of film showed thickness of 1.64± 0.32 μm. With this low thickness, constructing a tubular diameter almost doesn't seem convincible but these films thickness can be added up to increase strength. There is relationship between the concentration of collagen solution and thickness of film obtained. The higher the concentration of collagen solution the thicker the film is obtained. In a study conducted Brandon et al. 0.636%

concentration of type I bovine collagen fibrils from 4ml and 6ml produced a transparent and thicker film [36].

While the collagen film have been made previously for skin tissue engineering [36]. Collagen have also been mixed with other substances like hydroxyapatite (HA) and hyaluronic acid to make films for coating on titanium based scaffold in bone tissue engineering, and as surgical adhesive respectively. Similarly our methods allow to control the transparency and thickness of the film. Because of this ease modification, this method can be exploited to obtain various size and shape of films for application in cornea tissue engineering, blood oxygenator membrane, and soft tissue engineering like cartilage.

These films rolled on various diameter Teflon mandrel gave very specific inner diameter (ID) tube and these tube are shown in Fig. 2.3. The ease of modification that allows making different diameter blood vessels gives the medical industry flexibility of preparing large quantities with fewer expenses. Of the shelf can be easily made available. These tubes don’t possess any kind of mechanical damage while being fabricated. The advantages of this fabrication allow catering to all kind of cardiovascular diseases that is related to damage in arteries or veins.

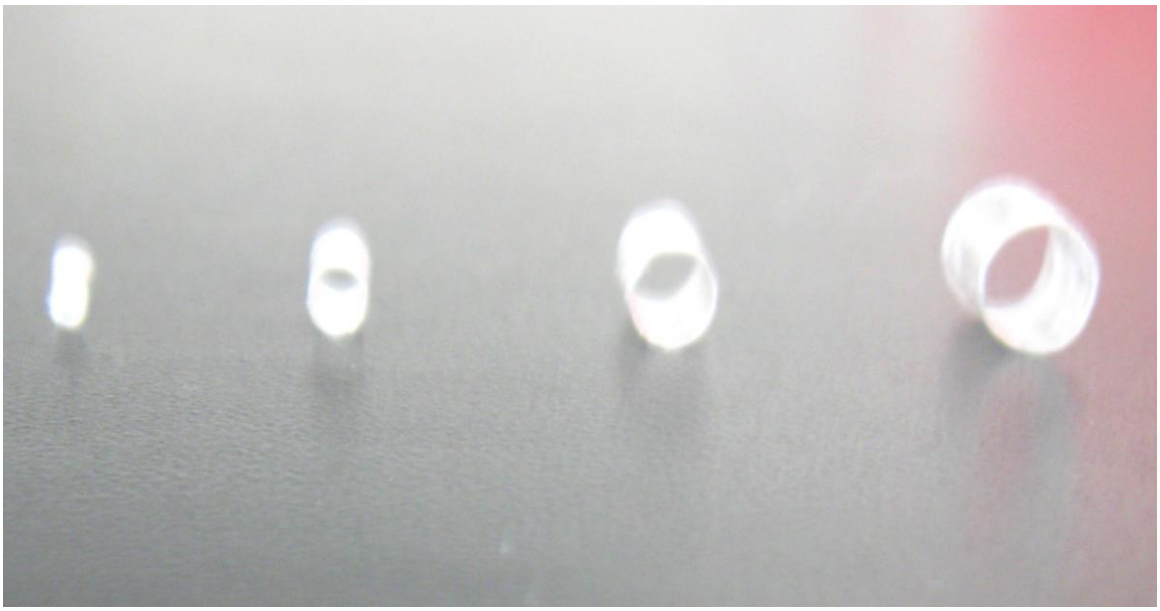


Fig. 2.3 Variable diameter construct prepared through this fabrication approach. Sizes are 1.5 mm; 3 mm, 4.85 mm and 6mm tube respectively when seen from left to right.

2.5.2 *Effect of Stacked Collagen films*

After collecting all the good films, these films were attached on a glass for thickness measurement. The films were attached to clean cover glass with the help of DI water and dried with air Nitrogen. The thickness of the collagen film was determined using surface profilometery. We determined the thickness of a single film and also went and measure the thickness of two and three stacked collagen films to see how our scaffold designing will improve the mechanical property of tissue graft. The thickness of one film was 1.64μm while thicknesses of two and three stacked films were 3.7 μm and 5.4 μm respectively. After the measurements, it could be clearly seen that the effect of stack would increase the thickness progressively. This trend makes it very clear that stacking collagen films will give ultimate thickness desired and

will significantly improve mechanical property of the vascular graft designed. The Table 2.1 below shows the thickness profile of our stacked films.

Table 2.1 The thickness profile of the collagen films individually and when stacked too. The thickness was measured for 1 layer film, 2 layer films, and 3 layers film respectively. Each sample was measured three times to get better average and standard deviation.

Sample No.	100 μm depth mold		
Dimensions	1 layer	2 layer	3 layer
1	1.52	3.78	6.18
2	2.01	3.79	4.76
3	1.39	3.55	5.26
Average	1.64	3.71	5.40
Standard dev.	0.33	0.14	0.72

Our stacking design could ultimately help prepared vascular graft of different diameter. The different tube sizes can be clearly seen below in Fig. 2.0. The number of collagen film was reduced when making smaller diameter tube because we use the number of revolution that was required to reach the thickness of 5mm tube when preparing smaller tube like 3mm or 2mm. These stack method enabled us to prepared tube as small as 0.7mm for our animal study design if needed for *in vivo* characterization. Until now, this stacked behavior of collagen has not been performed elsewhere. This stacked collagen gives us the idea how building tubular shape with

various number of collagen films will behave different during mechanical testing of suture retention and burst pressure measurements.

2.5.3 Mechanical Characterization

2.5.3.1 *Suture Retention*

Suture retention test is particularly important when designing vascular graft that has potential for human use. To investigate the strength our suture retention strength, the values were compared with normal human artery and human saphenous vein values that were obtained from the study conducted by Nicolas et al. [6]. The value obtained for 10 layers matched closely to human artery value (216±11.3 vs. 200±119). The value obtained for 15 layers exceeded the human artery value (293±9.2 vs. 200±119). Suture strength is very important because it holds the two tissues together after anastomosis. The scaffold should be able to withstand the force that is created by suture material over the course of time in holding two tissues together. While we did not want our suture design be able to hold only maximum suture retention of native human artery or vein because failure can happen within few days of surgery from stress and strain caused due to blood flow and animal movement. So we made scaffold design that could have significantly higher value that native artery and vein.

From the Fig. 2.4 below, it can be seen that 10-layer tube has suture retention value comparable to human artery and vein but our 15-layer tube has significantly higher strength. The suture retention values are significantly different in case of 5, 10 and 15 layers respectively. The suture retention values in case of 5 and 10 layers were significant (84±11.8 vs. 216±11.3 gf, $P=0.004<0.05$) and it was also significant in case of 10 and 15 layers (216±11.3 vs. 293±9.2 gf,

P=0.02<0.05) respectively. These tube could be a better fit for long term *in vivo* study. This design gives flexibility to researcher to increase suture strength to their desired value.

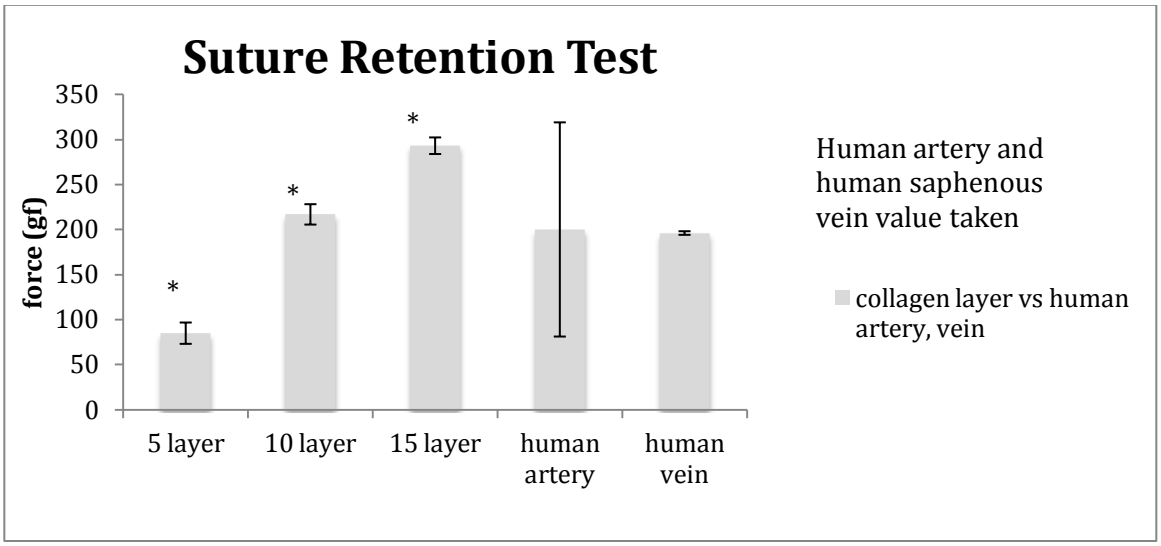


Fig. 2.4 Average suture retention test readings for three different layers of collagen film. It is compared with all the possible values of human saphenous vein and human artery taken from Nicolas et al [6]. Values were recorded in gram force (gf). * Corresponds to P<0.005 for comparison between 5 and 10 layers, and 10 and 15 layers too.

The importance of stability is suture retention strength in compliancy of vascular graft is very important. Recently all the polymers used in designing vascular graft have met the minimum requirement of suture retention values. In case of tissue engineered graft (4.5 mm) obtained from culturing human fibroblast showed suture retention values of 162±15 gf [6]. In another study to create vascular graft from sheet of fibroblast showed maximum suture retention values of 359 gf [37]. Chiara et al. calculated the suture retention value of their collagen tubular construct by wrapping collagen I sheet around cylindrical support between 116-151 gf, which is still lower than native artery and veins [38]. In above studies, cell sheets were used to reinforce mechanical property or simply pure collage I construct was used. While cell sheets did show

required mechanical properties for suture retention values, it is still not ideal case to prepare a graft in case of surgery. Here our design has potential to meet characteristics and replace of all type of possible polymer used to obtain significant suture retention strength. Our design does not incorporate any cell or mechanical support with other polymer but still can achieve all the desired values.

2.5.3.2 *Burst Pressure*

The burst pressure obtained for each of three 5, 10 and 15 layer tubular construct was significantly different from each other (5 layers vs. 10 layers, $P=0.00002< 0.05$, and 10 layers vs. 15 layers, $P=0.0003 <0.05$). The values obtained for 5, 10 and 15 layers were 474±111, 1804±801, and 4259±733 mmHg respectively. There was direct co-relation between pressure values with number of layers used to obtain construct. The increased layer numbers showed more burst pressure readings. Reinforced bonding due to thickness of the compile film on top of each other can be the main contribution for such values recorded. The burst pressure for 15 layers was greater than human artery but was not significant (4259±733 vs. 3128±1551 mmHg).

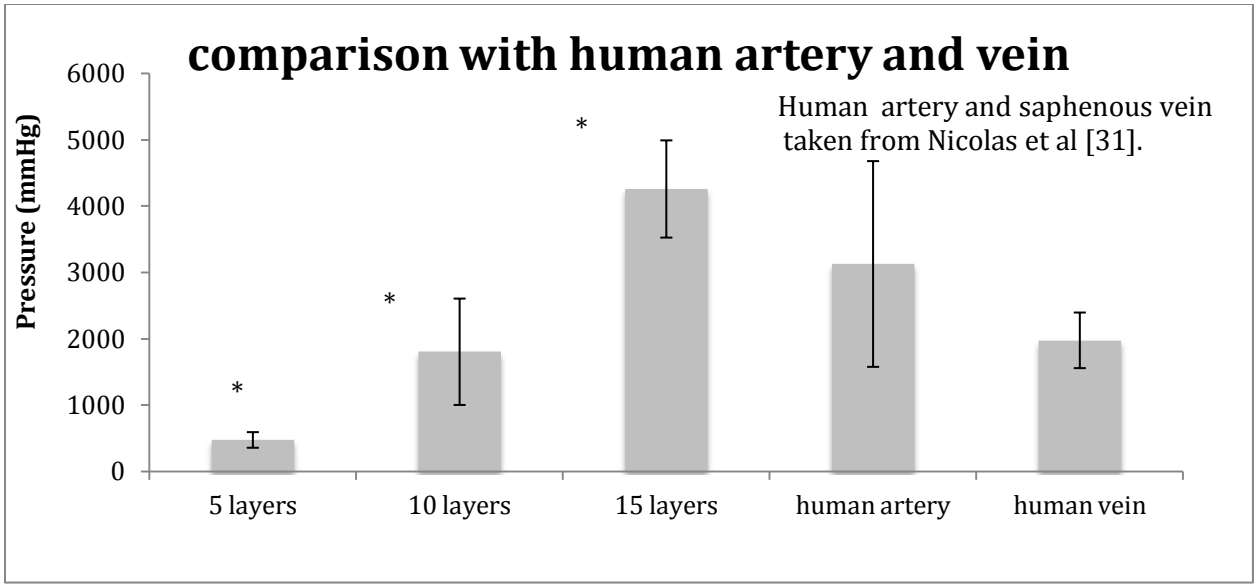


Fig. 2.5 Average burst pressure readings for three different layers of collagen film. It is compared with both human artery and saphenous vein values. Values were recorded in mmHg. * Corresponds to P<0.005 for comparison between 5 and 10 layers, and 10 and 15 layers too.

Burst pressure measurement is key parameter for all type of vessels suitability for implantation. Several groups have reported bust pressure for TEBVs greater than 2000 mmHg [37]. Chiara et al. reported burst pressure measurements in their tubular dense collagen construct around 1225-1574 mmHg [38]. Similarly Joseph et al. showed significantly low burst pressure of 650 mmHg on its cell seeded collagen hybrid construct [33]. While burst pressure is just not dependence on thickness of the tubular construct but also dependent upon the diameter of the construct [37]. Smaller diameter with same thickness of larger vessels will tend to show double the value of burst pressure recorded for same vessels. In this study, the burst pressure measurement was taken for 4.5 mm diameter tube for all three types of specimens.

All the higher burst pressure reported from many studies have either used synthetic polymers or use chemical crosslinking with natural soft polymer to increase burst pressure

values. The toxicity of chemical crosslinking agent has well been reported and incompliancy of synthetic polymers well understood despite strong burst pressure values. With all these implications, our design has great advantages over such scaffold in small diameter vascular grafts.

2.6 *In Vitro* cell adhesion study

2.6.1 *HUVEC cells for evaluating the efficiency of Collagen film*

In order to see effect of cell adhesion and differentiation on collagen I surface, HUVEC cells were seeded. Immunostaining was done to see the quantitative analysis. Cells were stained for endothelial marker vWF and nucleus with DAPI staining. The images were taken with the help of fluorescence microscope.

The Figure 2.6 below clearly shows the cells attaching and growing on the surface of collagen film. Researchers have already shown the growth of endothelial cells on the collagen surface in previous studies [39, 40].

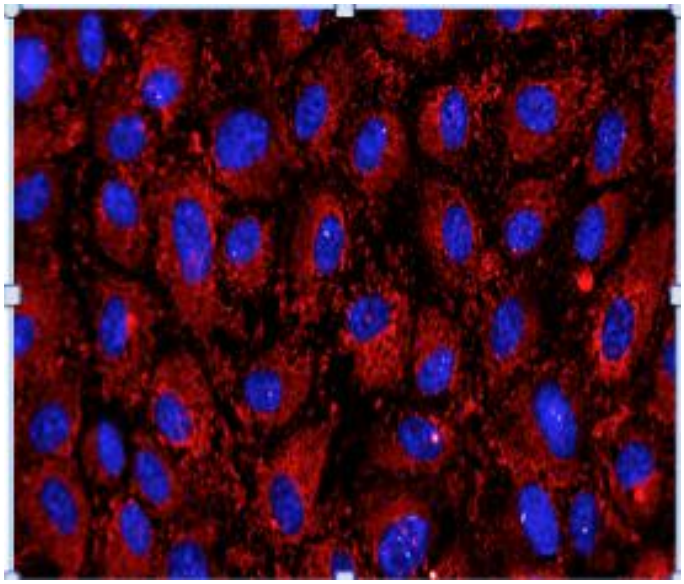


Fig. 2.6. Growth of endothelial cells on collagen film. Blue stained shows nucleus staining with DAPI and red staining shows endothelial marker VWF

Here collagen film shows very cell friendly substrate for endothelial cell growth and indicates that upon implantation *in vivo* show help the faster population of endothelial cells to avoid blood clot.

2.6.2 *HUVEC migration towards collagen film*

In order to evaluate the efficiency of collagen I scaffold, artificial cell migration device was built which would allow cell to migrate after culture to grow and proliferate. The Fig. 2.7 below shows the different images taken at different period of times to see the migration of endothelial cells to the surface of the film attached on the glass plate.

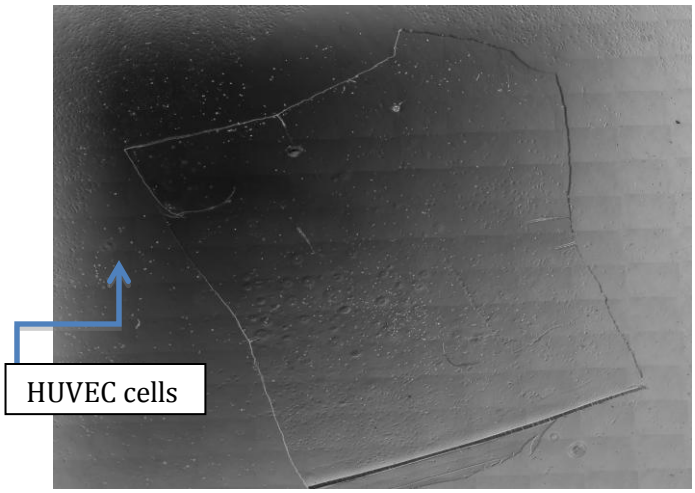


Fig. 2.7 Migration of endothelial cells towards collagen film a. Cell around the film just before the barrier was peeled off to allow cell to migrate

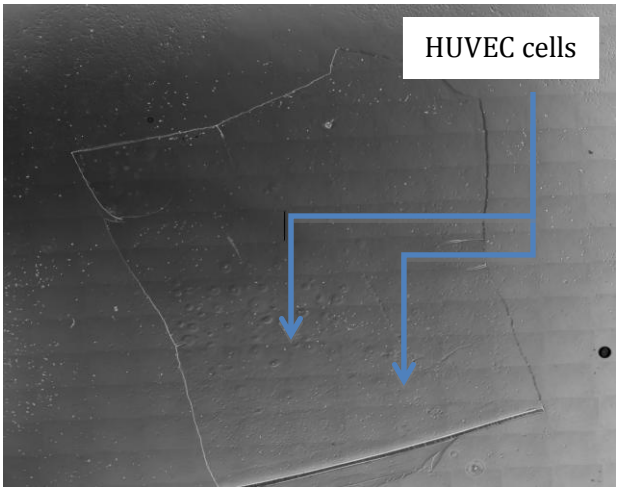


Fig. 2.7 b. Cell migrated on the film after three days of initial seeding. It is about two-third covered with HUVEC cells.

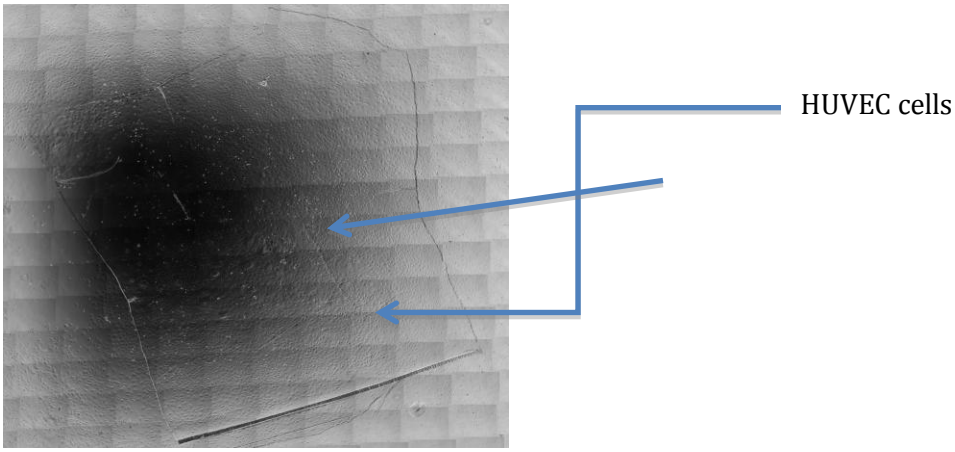


Figure 2.7 c. HUVEC cells fully occupied the surface of collagen film after 16 days of initial seeding.

As it can be clearly seen that there were no endothelial cells on the first day of cell culture but after 72 hours following cell culture, significant migration of cells were seen on the

surface of film. The image was also taken after 16 days and the film was fully covered with the HUVEC cells showing that collagen is good substrate for cell attachment.

The importance of cell migration is very important especially when considering the fact that scaffolds being used *in vivo* is acellular. The cells from the neighboring artery or vein where the graft is used should be able to attach and proliferate on the graft material at faster rate to avoid the rejection of graft due to blood clot, a common problem for collagen based vascular graft. Here we have shown that our scaffold is capable of allowing cells to adhere, proliferate and grow at faster rate.

2.7 Conclusion

In this set of studies, collagen scaffold have been fabricated and design for various small diameter size allowing the researcher to manufacture any size regarding the use for clinical trials. There has been no additional use of crosslinkers or fixatives to enhance the strength of stacked collagen films. The mechanical characterization of the scaffold has shown outstanding burst pressure of 4000 mmHg and suture retention values 350 gf approximately to exceed 2 times the value of normal artery and veins. The effect of stack has summarized the importance of fabrication method and how stacking procedure can be tuned to match desired mechanical properties of small diameter vascular conduits. The in *vitro* cell study has proved the collagen as substrate for endothelial cells attachment and also allows moving endothelial cells to adhere, proliferate and grow collectively.

CHAPTER 3

COLLAGEN AND ELASTIN

3.1 Introduction to Elastin

Elastin belongs to the family of extracellular matrix protein. Elastin is an extremely durable polymer. It is well known to provide elasticity in tissue and organs. In case of vascular tissue engineering, elastin is of major importance because blood vessels relax and stretch more than a billion times in a life span [41, 42]. Elastin is biocompatible, biodegradable, and non-immunogenic. The strength of elastin fibres has made it popular with its application in vascular tissue engineering. Elastin fibres are rope like structures present in the media of elastic arteries and veins. Elastin is composed of two elements: amorphous elastin and microfibrils, which are 10-12 nm in diameter [41]. With increasing importance of the elastin biomaterial property, elastin like polypeptides is being synthesized to tune the properties of vascular constructs and other tissue engineering applications as well [43].

In order to recapitulate the biochemical and biomechanical features of native ECM incorporation of elastin and collagen with various polymers for different tissue engineering applications has already been reported [44, 22]. The Young Modulus of elastic fibres have been reported around 300-600kPa whereas for collagen is 10^6 Pa. This is indication that elastic fibres can undergo billions of cycles of extension and recoil without mechanical failure [42]. The use of elastin and collagen has already been previously studied. Jefferey M et al. used microfibers of

elastin like protein with synthetic collagen and obtained greater compliance, burst pressure of 1483±143mm Hg and suture retention of 173±4 gf [6]. Steven G Wise et al. incorporate tropoelastin on to synthetic elastin/polycaprolactone conduit mimic human internal mammary artery (IMA) mechanical properties and found out that it responded elastically at low pressures and produce burst pressure equivalent to IMA [46]. Elastin is not only used for enforcing mechanical property but improving cellular interaction to increase compliancy. In another study conducted by M.J.W. Koens et al. they used elastin and collagen for triple layered vascular grafts to mimic native vessels with elastin as inner layer to reduce platelet aggregation and increase elastic features [47].

In this set of studies, soluble form of elastin obtained from bovine neck ligament is mixed with collagen I to see the changes in mechanical properties. A set of similar mechanical test done for collagen only construct was repeated with new collagen-elastin vascular conduit to characterize the significance of elastin fibres within collage fibres and whether our method of fabrication will allow doing it.

3.2 Materials and Methods

3.2.1 *Materials*

Soluble form of Elastin and all the materials was bought from Sigma Aldrich unless otherwise specified. Collagen soluble form was extracted from rat-tails in the lab and also briefly explained in fabrications method for collagen film.

3.2.2 *Fabrication of Collagen-Elastin Film*

The process was very similarly done when compared to preparing individual collagen film itself. The ratio of collagen-elastin ratio made was 1:9 because this ratio has shown significant improvement in terms of mechanical property when compared to different ratio [48]. Elastin powder and collagen sponge was dissolved in cold 0.02 N Acetic Acid in specified ratio above. The solution was allowed to mix overnight in 4^0 C with the help of magnetic stir bar. After the solution was prepared, 300 μL of the solution was casted on PDMS. The casted film was air dried at room temperature for several hours before they are peeled off. The dried film was then placed under UV light of for 30 min. The film was slowly then removed for further processing and design of tube for blood vessels.

3.2.3 *Preparation of Collagen-Elastin Tube*

The film fabricated from the previous method was then placed under UV for additional 30 minutes. The film was then taken out and individual film was suspended in 1XPBS liquid minimizing any kind of folding on the film. The films were then rolled around 4.5mm Teflon rods. Three types of construct of same diameter were prepared by this method. First construct was prepared by rolling 5 films, followed by 10 films and last one from 15 films separately.

3.3 Mechanical Characterization

3.3.1 Suture Retention test

All three tubes tested for their suture strength. The suture material was 6.0-inch prolene suture material. The suture thread was then inserted on each side of the tube. The suture thread

was tied at one end of the specimen and the other end of the thread was connected to head on the mechanical device. The device was allowed to extend which would then measure the tensile force acting on the suture materials while it tears the film at its peak stress value. This value is then recorded as suture retention strength of the tube.

3.3.2 Burst Pressure

The maximum burst pressure was recorded with the help of manometer. The maximum pressure that could be recorded by the manometer was 200 psi. The collagen-elastin tube was connected to two plastic tubes that would act as flow medium for viscous fluid. The fluid used in this study was milk of magnesia because of its relative viscosity, which is slightly higher than 1XPBS and close to the human blood plasma. One side of the tube is connected to the manometer and other end to the syringe. The fluid is then allowed to flow until the film tears apart and the maximum burst pressure was recorded.

3.5 Results and Discussion

3.5.1 *Fabrication of Collagen-Elastin film and tube*

Collagen-Elastin film was firm and thick enough to be peeled out of PDMS mold without causing any mechanical damage to the film. The collagen-elastin film was less transparent and slightly soft after the fabrication process. The dimensions of the collagen-elastin film were 2.5 mm x 1mm. The image of the film can be seen below in Fig. 3.1. The thickness of the film was not measured because the effect of stack was obvious from previous study of collagen film itself. Similar behavior was predicted from this film and hence it was avoided. The individual tube obtained from 5 layers, 10 layers and 15 layers of collagen-elastin tube has

different amount of stiffness, thickness and roughness. It was very obvious from through feel on the hand and it was supported when mechanical test were completed. These same diameter tube but different layer was designed to achieve different mechanical properties to be compatible with the dynamic stress produced within the body during physical and mechanical workout. There was no induction of any chemical agents for the crosslinking or fixation of each layer with other at any instant. Air-dried and hydrophobic interaction between collagen-elastin films was enough to create stronger binding forces between the films. These films when dried over night creates strong enough bond that it does not fall apart when the tube is placed inside the DI water or PBS solution itself. The tube does not swell at higher rate indicating that their pores are interconnected with less penetration of water molecule and ultimately increasing the degradation time of the collagen tube.

Fig. 3.1 Fabrication of elastin film. The film can be seen with some roughness and smoother surface when compared to collagen film in the Fig. 2.2.

The degradation rate however was not calculated in this study because its degradation is well known mechanism as it been studies by many researchers [49]. The degradation rate of collagen I is not major issues with collagen vascular graft and its degradation rate is very specific with type of collagen it is but not collagenase present in the body [50]. The rate of synthesis of collagen in heart tissue is about 5.2 % per day and its degradation rate is 52 %, which leaves half of new collagen formed, and this accumulation of collagen helps in quick replacement of vascular graft *in vivo* [51]. Similar collagen-elastin film has been prepared previously by Skopinska et al. Collagen-elastin film prepared was found to be around 0.015-0.030 mm thick. The average roughness of the collagen-elastin film was increased compared to collagen film. The polarity component of the collagen-elastin film is also increased when compared to collagen itself [52]. Collagen film itself is a hydrophobic material and when blended with elastin decreases its hydrophobic character.

3.5.2 Mechanical Characterization

3.5.2.1Suture retention test

The peak forces were calculated from this study. The forces value was recorded in units' gram force. The Fig. 3.2 below shows the value of suture retention strength for each 5, 10 and 15 layer tube. The values for 5, 10, and 15 were 80±18.6, 197±21.9, and 368±40 gf respectively. There were significant differences in the suture retention ability between 15 layers tube constructed when compared with collagen 15 layers tube (368±40 vs. 293±13, P=0.012). However there was no significant difference for the 5 layers and 10 layers tube (80±18.6 vs. 84±111.8, 197±21.9 vs. 216±11.3). While these values are significantly greater than the normal

blood vessels level, these tubes can be suited to the replacement of natural blood arteries without any issue of suture breakdown after implantation. Form the value of native human artery suture retention strength obtained from Nicolas et al [6], it can be clearly seen that tube with 10 layer matches closely to human artery and veins (216±11.3 vs. 200±119). The suture retention test is very important for study of compliance of blood vessels. The thickness of suture material and the number of suture materials can have significant impact on the compliance of the tissue-engineered blood vessels [53, 37]. Hence Suture retention strength represents the compliance behavior.

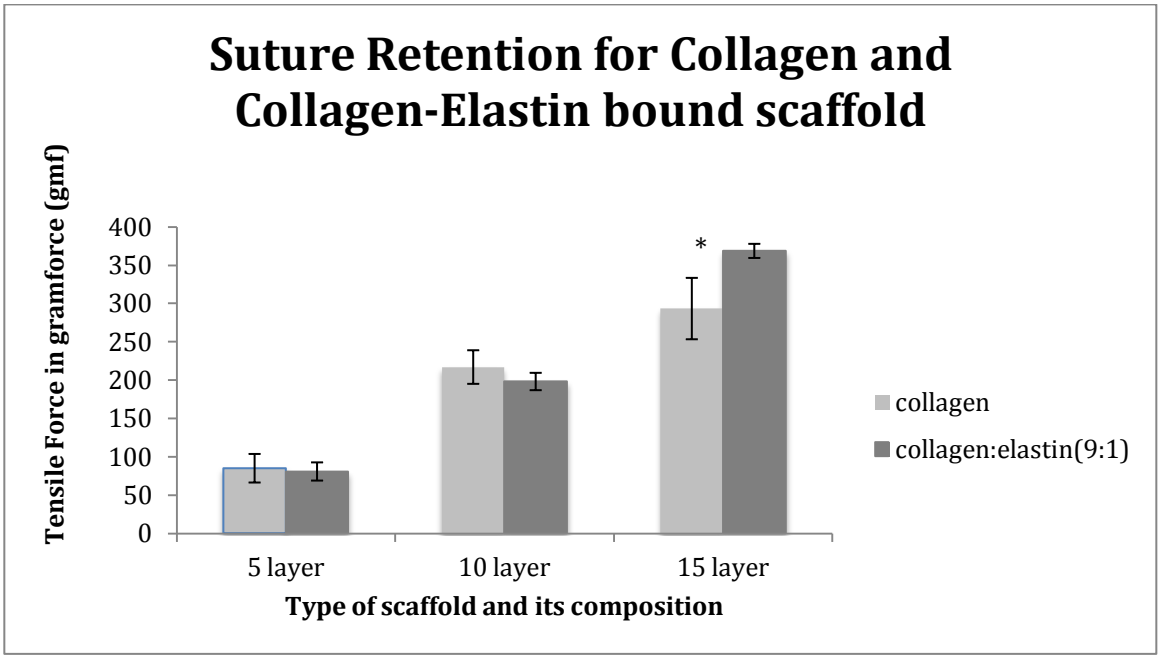


Fig. 3.2 Suture retention strength of collagen-elastin tube and collagen tube itself. The asterisk sign (*) shows the significant difference in the suture retention values between collagen and collagen-elastin 15 layers tube. The * sign indicates $p<0.05$.

This might have shown indication that elastin fibers alignment within collagen fibrils increases its elastic tensile property and hence increasing its stretching capability which ultimately increases the suture retention values.

The suture retention strength and burst pressure of collagen-elastin scaffold was reported around 173±4 gf and 1483±143 mmHg respectively [45]. Here in this study, suture retention has clearly been more than shown previously and shows the potential to replace current vascular graft material.

3.5.2.2 Burst Pressure

The burst pressure is great characterization test for small diameter blood vessels prior to implantation. Many materials have shown pressure recorded up to 6000 mmHg but the elastin-collagen brings adequate flexibility and elastic property to support tissue information. The average burst pressure recorded in the 10-layer tube was 4500 mmHg. The burst pressure of the 15-layer tube was not recorded because the device could only read pressure up to 200 psi, which is 5100 mmHg. The 15-layer tube was expected to have burst pressure greater than 5100 mmHg because of its elasticity created due to presence of elastin protein. Burst pressure is directly dependent upon the thickness of the film and the diameter of the tube. If the two tubes are of same thickness but different in diameter, then according to Laplace law the tube with small diameter will show higher burst pressure [54]. Sheet based tissue engineered blood vessels (TEBV) has burst pressure about 435 mmHg. However other people have used ringlet or strip tensile testing to replace burst pressure but this test has shown to overestimate burst pressure by

more than 50% which is not suitable for determining the actual property of the engineered tube [55, 47].

Many of the researchers have performed the burst pressure under dynamic mechanical conditioning [56]. However in our case it was done in static condition but it is assumed that it would be able to maintain minimum burst pressure conditions if it was done under dynamic conditions.

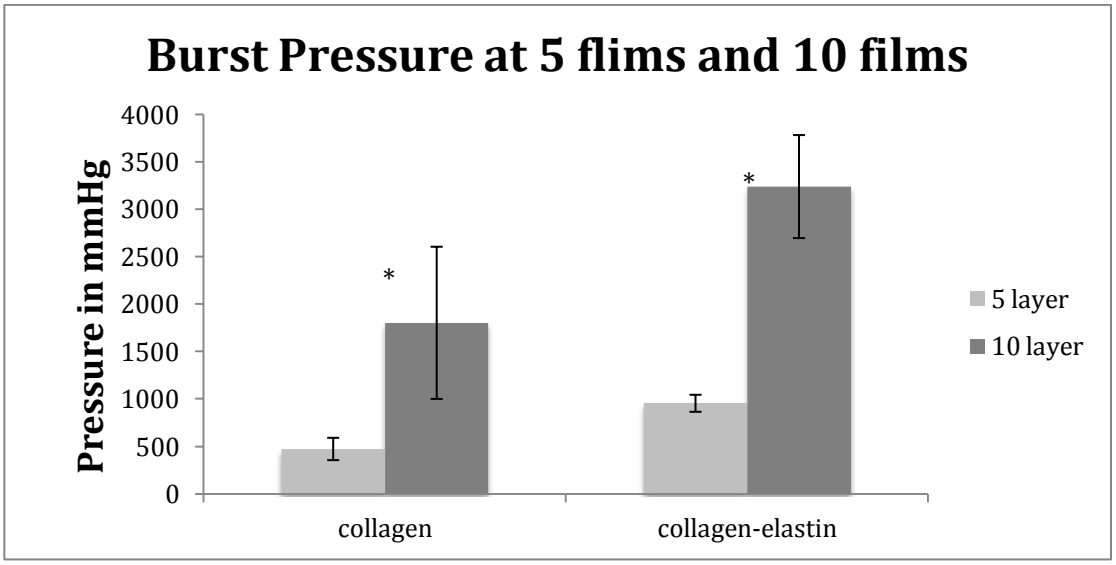


Fig.3.3. Pressure comparison of collagen and collagen-elastin tube at 5 and 10 layers respectively. The asterisk sign (*) shows the significant difference between collagen and collagen-elastin conduits. T-test distribution showed value of $p < 0.05$ for both 5 and 10 layers comparison of burst pressure values.

The burst pressure recorded for 5 layers and 10 layers were 956±174 and 3240±543 mmHg respectively. The burst pressure was very significant in case of comparison of 5 and 10 layers tube with collage tube (956±174 vs. 474±111 mmHg, P= 0.02, 3240±543vs. 1804±801 mmHg, P=0.01) indicating the effect of elastin fibers within the collagen assembly. Burst

pressure was seen to elevate by 400 mmHg when elastin was enforced with collagen to design small vascular graft [47]. Burst pressure is very critical measure for vascular graft. However the blood pressure usually doesn't go beyond 240 mmHg in native arteries but it is constant cyclic fatigue that tells the strength of the blood vessels. So the maximum burst pressure of native artery has to be matched in good amount with artificial conduit for longer compliancy. In this case, the vascular conduit out of collage-elastin has been able to achieve on top of their elastic features as well.

3.6 Conclusions

In this set of studies, soluble form of elastin obtained from bovine neck ligament was used with collagen in 1:9 ratios by weight to fabricate new collagen-elastin film for designing vascular conduit. The roughness and textures of the film was slightly different when compared to collagen film. However, no SEM images were taken but this type of similar study of collagen-elastin in different ration has been done previously. The mechanical characterization showed significant increase in burst pressure values in terms of 5 and 10 layers compared to collagen 5 and 10 layers only. The suture retention strength was found to be significant in case of 15 layers tube compared to collagen 15 layers tube. Both studies showed the importance of mixing elastin in the collagen to increase elastic and mechanical property while closely mimicking the extracellular matrix. In the end, it gives the fabrication design used in the study can be combined easily with certain proteins or polymers to design small vascular grafts.

CHAPTER 4

IMPROVING THE HEMOCOMPATIBILITY OF THE COLLAGEN BLOOD VESSELS

4.1 Introduction

Collagen is known to respond very positively to blood clot formation. It is a good clotting agent. It initiates activation and adhesion of platelets [57,58]. Despite collagen is extracellular matrix; preventing blood clotting at the time of graft is essential for the compliance. The use of collagen as biomaterial for drug delivery has been already in progress [15]. In this set of studies, various methods are being used to show how the hemocompatibility of the collagen-based conduit can be improved. The idea was not to limit the potential of collagen based small diameter vascular graft in clinical industries. However there are several methods that can be done to improve the hemocompatability like seeding of endothelial cells, coating various types of antithrombic agents, and using various types of drugs to prevent blood clot. Here three different types of coating or methods will be discussed to decrease blood clotting in the animal or clinical trial in the future. Firstly, fibronectin will be coated on the collagen film and its potential will be discussed through endothelial cells seeding. Secondly, heparin will be immobilized on the surface of collagen tube and its efficiency will be evaluated through toluidine blue staining. Lastly, aspirin, antithrombic drug, will be embedded in two types of collagen construct to see the drug release behavior and its efficiency will be evaluated using absorption spectroscopy.

4.2 Materials and Methods

4.2.1 *Materials*

Fibronectin, heparin salt, toluidine blue and aspirin (acetylsalicylic acid) were purchased from Sigma Aldrich.

4.2.2 *Fabrication of fibronectin coated collagen film*

The coating of collagen film was done with fibronectin to see if the collagen film would support coating of fibronectin and it was tested with the help of culturing endothelial cells on it. In order to prepare collagen coated with fibronectin, about 200 μL solution of fibronectin was spread out on the already dried collagen film on PDMS mold. The mold then was placed inside the incubator (maintained at 37 C) over night to provide fibronectin enough time to bond well with collagen crosslinking. After 24 hours the dried fibronectin on collagen film is peeled carefully with similar approach done to take collagen film out of the PDMS mold.

4.2.3 *Endothelial Cells growth on film:*

To measure the adhesive and proliferative ability of collagen film and fibronectin coated collagen film; Human Umbilical Vein Endothelial Cells (HUVEC) was used. HUVEC was cultured in 25 ml polystyrene flask in with 10% FBS medium, 1ml LSGS, antibiotic gentamycin (25μL). The cells were cultured for one-week period. The cultured cells were then placed in Petri dish containing collagen film and fibronectin coated collagen film.

A well plate was taken in which three wells were used to place collagen film and the other three contained fibronectin coated collagen film. Before the film were placed inside well

plate, the individual collagen film and fibronectin coated collagen film were attached on the 25x25 cover glass with the help of DI water and air nitrogen. The attached glass-film was slowly placed inside well plate and covered with PBS for overnight. After 24 hours, the PBS was replaced with HUVEC culture medium for few hours. After few hours, equal amount of cells was placed in six wells and placed inside the incubator for their attachment and growth. The cells were allowed to culture for a week period. During the period medium were changed every 48 hours to keep cell healthy and avoid any contaminations.

4.2.4 *Immobilization of Heparin on collagen tube*

All the materials were brought from sigma Aldrich. The heparin immobilization on the surface of the scaffold was done in similar way done by Bastian Sebum [59]. Briefly these films were crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). Heparin was activated with the help of MES Buffer solution. The scaffolds were first incubated in 2-morpholinoethane sulfonic acid (MES) buffer (0.05 M, pH 5.5) for 30 min at room temperature. The samples were then transferred to the crosslinking solution containing 2.3 g EDC and 0.56 g NHS in 215 ml MES-buffer per gm. of collagen (molar ratio of EDC/NHS = 2.5). After 2 h incubation under gentle shaking, the films were placed in a 0.1M Na2HPO4 solution for 2 h to stop the reaction and then rinsed with demineralized water for three times and the scaffold was kept for dry overnight.

4.2.5 *Toluidine Blue Staining*

Toluidine stock solution was prepared from using 1g of toluidine blue in 100 ml of 70% alcohol along with 0.5g sodium chloride and distilled water. The stained solution was then used

to cover the entire non-coated collage scaffold (control) and heparin coated collagen tube. After allowing staining for 5 minutes, the solution was discarded and the tubes were washed with DI water. Images were then taken for further analysis on the binding affinity of heparin molecule on collagen surface.

4.2.6 *Loading of Drugs*

Aspirin was used a drug agent to be embedded within the collagen tube. 5 mg/ml of aspirin solution in DI water was made. Single collagen film was wrapped around 2 mm Teflon tube. The wrapped film was then dipped inside the solution for about 7 min before it was pulled out. After then another film was wrapped around the tube and it was dried over night. Following the procedure, similar step was done but in this case no film was wrapped around after the dip was recovered.

4.3 Results and Discussion

4.3.1 *Collagen coated with Fibronectin*

In order to evaluate the efficiency of fibronectin coated collagen film, human umbilical vein endothelial cells (HUVEC) were cultured to see the adhesive and proliferative ability. Collagen uncoated film was taken as control parameter to observe the differences with collagen-fibronectin coated film. Immunostaining was performed to mark the endothelial cells for quantification analysis. The images were taken with the help of Zeiss Microscope and used for analysis purpose. Images of the whole film were then analyzed for the amount of cell present with the help of Image J software. The Fig. 4.1 below shows the differences in the number of cell on collagen coated with fibronectin and collagen film itself. However the difference was not

significant between collagen film and collagen coated fibronectin film ($1.4x10^6 \pm 17.01$ vs. $1.55x10^6 \pm 16.6$ cells/m^2). The cells density calculated on the surface of collagen film was $1.40x10^6 \pm 17.01$ cells/m^2 and $1.55x10^6 \pm 16.6$ cells/m^2 for collagen-fibronectin film.

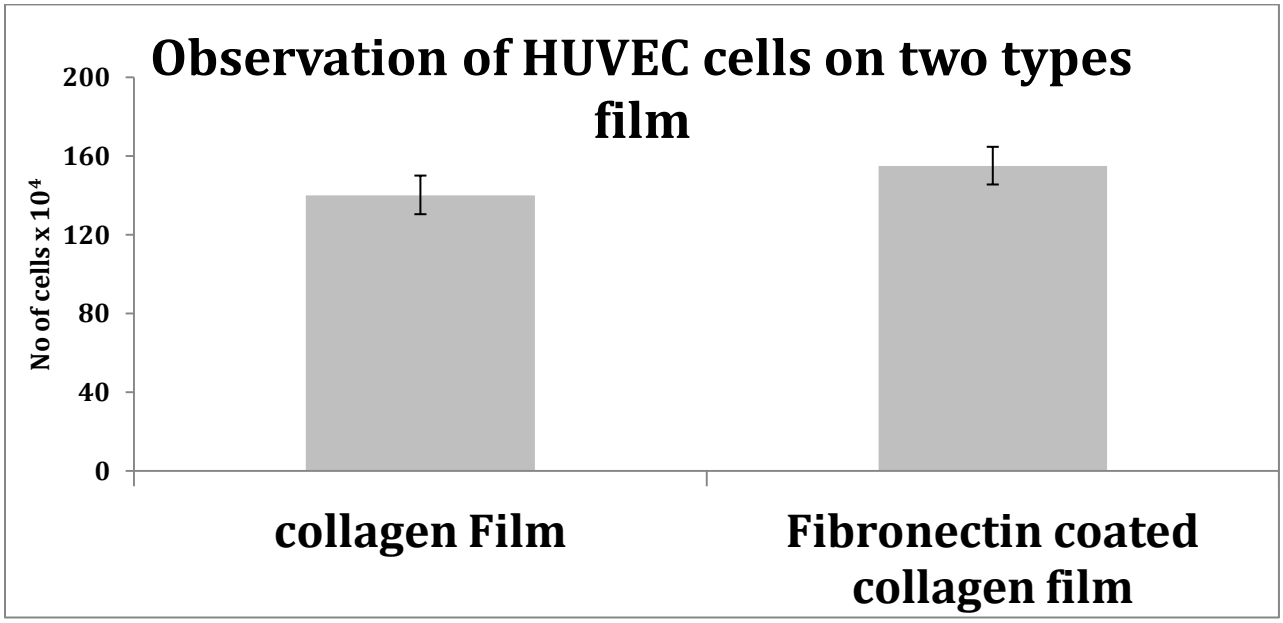


Fig. 4.1 Average number of cells grown on collagen film and fibronectin coated collagen film. The values were slightly greater for fibronectin coated film but was not very significant ($1.40x10^6 \pm 17.01$ vs. $1.55x10^6 \pm 16.6$ cells/m^2).

People have reported significant effect on the growth of endothelial cells when grown on fibronectin surface compared to collagen surface [60]. Collagen I when combined with fibronectin provide a better support for endothelialization. Melissa et al. showed that coating with fibronectin not only supported growth of endothelial cells but growth of the cells was significant when compared with collagen only film and also mimic the extracellular matrix closely [60]. In addition to supporting endothelial cells growth, it is also useful in reducing platelet cell adhesion. M. Ahiara et al. showed that human washed fixed platelets adhesion was

inhibited on collagen device coated with fibronectin and fibronectin was confirm as platelet adhesion agent [61]. Here in our study, endothelial cell growth was slightly higher in number than compared to growth on collagen film but it was not significantly different than each other. However, it gives a sign that fibronectin does help growth and adhesion of endothelial cells and supports all the other studies that have shown significant differences in endothelial cell growth [60].

4.3.2 *Immobilization of Heparin on the collagen tube*

Here heparin was immobilized on the surface of the collagen tube both on the inner and outer surface. In order to know to evaluate the qualitative analysis of heparin attachment toluidine blue staining was performed. Toluidine blue staining is primarily used to stain mast cells that stain histamines and heparin granules. And toluidine blue staining was used to stain heparin molecule on the surface of Decullarized xenograft by Concklin et al. [53]. Fig. 4.2 shows the collagen tube stained toluidine blue and collagen-heparin tube stained with toluidine blue.

This heparin coating now can reduce thrombogenicity of the graft material and increases its efficiency in the *in vivo* studies. Apart from heparin acting as anti-thrombogenic material, it also provides substrate for heparin binding growth factors like basic fibroblast growth factor (bFGF) and vascular endothelial growth factor. Heparin was also able to reduced thrombus formation in in *vivo* studies [53]. Heparin is been standard coating procedure for all types of scaffold being designed for all small vascular graft [62,63]. In clinical trials heparin coating on Dacron graft has been successful with patients as well [63]. Here heparin is covalently bound on

the surface of the collagen tube and it can easily reduce thrombic property of collagen based vascular construct.

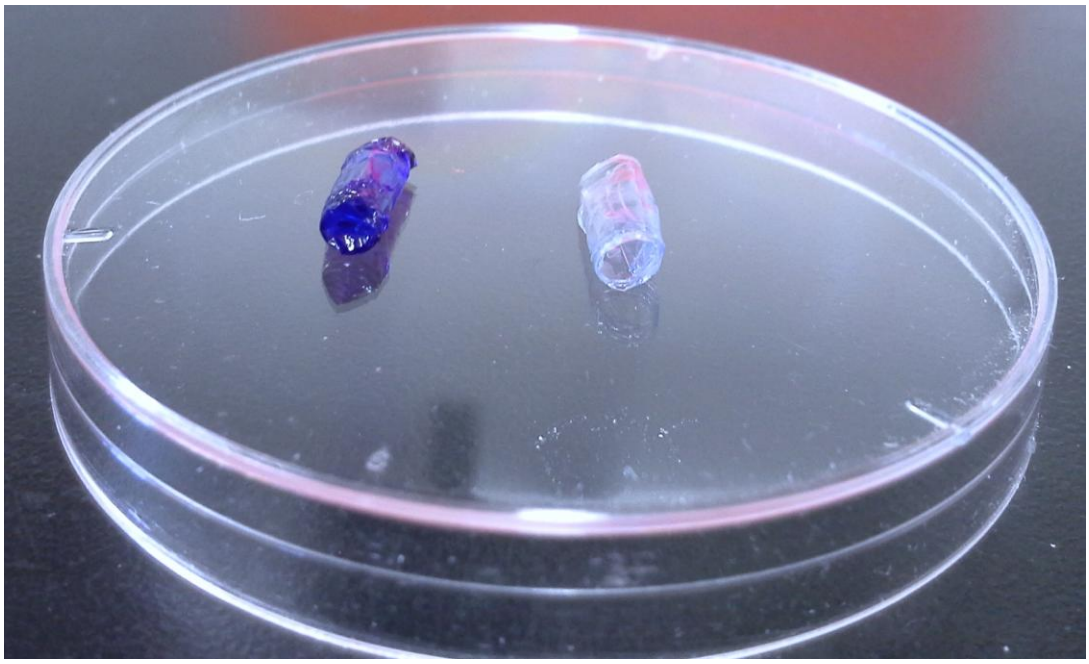


Fig. 4.2 Images of collagen tube stained with heparin molecule. Collagen tube coated with heparin dark purple color (on the left side) and collage tube with clear transparent color.

In the above Figure 4.2 dark blue color of collagen tube shows that heparin was evenly concentrated on the regions of collagen surface throughout the tubular structure and in case of no heparin treatment, there was no effect of toluidine blue staining.

4.3.3 Drug Delivery Study

In this case, aspirin was included as drug agent for the study. Two types of device was used to see if there was any difference in the amount of drug release over period of 5 days in *in vitro* studies. Absorption spectrometer was done for quantification and qualitative analysis each

day after incubation. The standard absorption graph of aspirin solution in DI water used as standard template to compare the amount of aspirin released from the collagen tube.

The Fig. 4.3 shows the cumulative drug release over 5 days from single and sandwich layer device. Over the period of 5 days there was decrease in the amount of aspirin present in the solution. From the Table 4.1 it was clearly seen that there was higher release of drug on the first 24 hours after incubation for both scaffold. There was then slow release of drugs for next 4 days and eventually it ran out of the drugs stored in the scaffold.

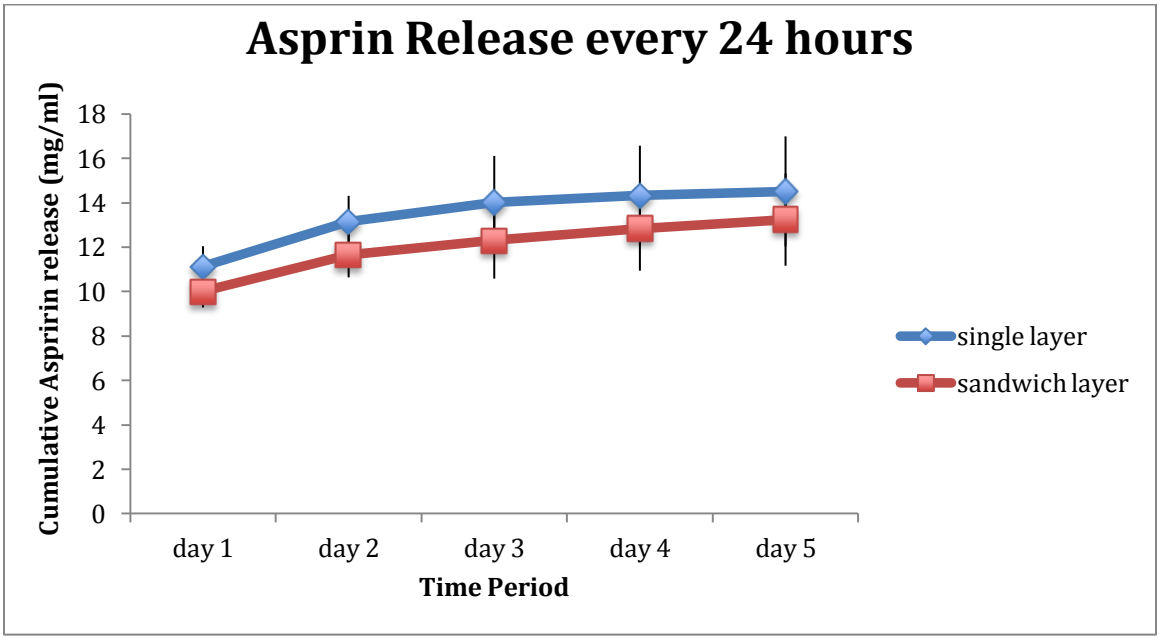


Fig. 4.3 The cumulative amount of aspirin release over 24 hours for successive 5 days. It was done for both single and sandwich loaded aspirin device.

Table 4.1 The amount of aspirin released from both single and sandwich tubular device over 5 days period.

Time period	Concentration of aspirin in the solution (mg/ml)	
	Single layer (mg/ml)	Sandwich layer (mg/ml)
Day 1	11.13±0.014	9.998±0.01
Day 2	2.015±0.002	1.66±0.003
Day 3	0.88±0.014	0.645±0.01
Day 4	0.295±0.001	0.531±0.001
Day 5	0.191±0.002	0.412±0.001

Three samples were used for each type of samples to average the amount of drug released over 5 day's period. The absorbance number is direct indication of the amount of aspirin present in the solution after each day. Higher absorbance values relate to higher amount of aspirin released after incubation. The absorbance value decreases steeply on second day and then decrease is slow and gradual for next three days. As expected single layer was supposed to release more drugs on first day compared to sandwich layer after incubation with DI water (Table 4.1 and Fig 4.3). During the period of 48, 72, and 96 hours, the release of drug from sandwich is more compared to single layer (0.645±0.01 vs. 0.88±0.014, 0.531±0.0007 vs. 0.295±0.0006, and 0.412±0.001 vs. 0.191±0.0017) suggesting sandwich layer holds more drug and is better indication of loading drugs within the tube. The Sandwich layer usually creates barrier with the bonding between the films. Aspirin drugs is embedded between two bonded layers and water penetration over the time period will influence the release of drugs whereas in

case of single layer water penetration to the surface of collagen film is easily and hence the concentration of drug release will be higher.

Aspirin is group as non-steroidal anti-inflammatory drugs that have multiple uses. It is been used for treating pain-associated rheumatoid arthritis, headache and other inflammation problems. Apart from its analgesic effects, aspirin is well known to have antiplatelet effect by inhibiting the formation of thromboxane, which is responsible for accumulating platelets together over damaged surface or injury site in the body [64]. It is just one of the anti-platelet drugs that have been discussed here but it can be used with other drugs desired.

Drug delivery using scaffold in tissue engineering has caught a lot of researchers in the field of tissue engineering approach. Researchers have already been started designing vascular prosthesis, which can deliver drugs or growth factors [65-67]. The vascular graft now can be combined with efficient drug for reducing blood clot on the target site at faster rate with coating of heparin giving another dimension to our design scaffold for smaller vascular tissue engineering.

4.4 Conclusions

In this set of studies, various methods were employed to improve the hemocompatibility of collagen scaffold because collagen is well known to clot blood on its contact with the blood. Three sets of studies were done. First fibronectin coating the top of collagen film was done and its efficiency was evaluating from the cell adhesion study of HUVEC cells seeding. Secondly the heparin was immobilized on the surface of collagen tubular structure and its qualitative analysis was done by use of toluidine blue staining. Lastly aspirin was embedded as drug agent in the

collagen tube to deliver drug over time period. Fist study showed increased endothelial cell growth, second showed distribution of heparin molecule on the surface of collagen tube and lastly aspirin was delivered for consecutively over period of 5 days and difference in the concentration of aspirin delivered was observed using two approaches of drug embedding procedure on the scaffold. Hence drug-carrying behavior can be evaluated in detail for future studies.

CHAPTER 5

FUTURE GOALS

5.1 Current Work

In this set of research studies, the potential and the importance of collagen I in tissue engineering was brought to attention. Collagen considered to be the most important extracellular matrix component has been used in several tissue engineering fields for its diverse nature like thrombogenic agent in skin tissue engineering, coatings in synthetic scaffold for cellular seeding and many more. Still collagen was limited in its purely use because of its mechanically weak nature. However we have overcome that challenge for now and are working to exploit its more properties. We are still working on with vascular conduit project and have some objectives outlined for the future studies.

5.2 Ongoing Studies

Currently the vascular conduit out of pure collagen I has been designed with aspirin embedded in slight higher concentrations than one used in preliminary studies *in vitro* conditions. Five such 0.7 mm outer diameter (OD) tubes has been designed and implanted under the carotid artery in rats. In addition to it, five 0.7 mm tubes have been designed with immobilized heparin molecule on the inner surface of the tube for *in vivo* implantation

5.3 Future studies

5.3.1 *Synthesis and Fabrication of vascular conduit*

The Collagen I and Collagen I: Elastin solution was prepared with concentration of 6 μg/ml. The thickness of collagen film and elastin film is already exploited in this case. The thickness of the film was around 1.64 μm approximately. The solution concentration should be increased to obtain slightly thicker film about 3 μm and be used to construct same layer conduits and see the effect on mechanical property. In case of elastin, the ratio might be altered to different ratio to fabricate film and evaluate the mechanical property as well.

5.3.2 *Mechanical Characterization*

The effect of stacked films on burst pressure and suture retention strength is already been evaluated using collagen and collagen-elastin film separately. Going in the future, the mechanical studies would include all the burst pressure and suture retention in wet conditions. Dynamic mechanical test will also be done by to see the effect fatigue on the collagen tube similar to done by Seliktar et al [56]. Cyclic strain test will also be performed to see the elastic feature of collagen and collagen-elastic scaffold.

5.3.3 *In vitro Cell studies*

Different methods of cell seeding will be evaluated on the top of the collagen conduit. Few examples may include use of bioreactor to observe the cellular growth on the collagen scaffold over specific time period. Study of cell growth on heparin coated collagen tube and

aspirin coated collagen tube *in vitro* conditions will also be evaluate to strengthen the compliancy of the tubular conduits *in vivo*.

5.3.4 *Drug delivery studies*

Drug delivery has been increasingly important in tissue engineering recently. Most small diameter based vascular graft have been able to achieve mechanical property of native arteries and veins, showed positive signs of cell viability studies but the use of the scaffold are controlling drug carrying agent is still under area of active research. In the future, drugs molecule will be embedded in different ways within the scaffold to see the rate of delivery. For example a better sandwich layer can be used to embed drugs within the design to see the effect of drug release. Two or more multiple drugs could be embedded on the scaffold tube within various layers of tubular structure to see the combined release of drugs molecule. The combine heparin and drug agent within the collagen film will also be exploited to see the effect on *in vivo* studies.

CHAPTER 6

DISCUSSIONS

The main objective of this whole study was to use stacked collagen films technique to design acellular scaffold for smaller diameter based vascular graft from Collagen I that is capable of matching mechanical property of native blood vessels, and be easily modified to coatings, and shows potential as drug carrying agent. There were three specific aims designed for the study that would be used to assess the strength of designed vascular conduits in terms of mechanical property and hemocompatibility property. All the specific aims were successfully accomplished.

The fabrication of collagen I tube used stacked individual collagen film of to design 5, 10 and 15 layers tube. In all these procedures, 1XPBS (pH 7.0) was used as solvent system to roll the tube uniformly on a Teflon mandrel. The use of water and dry method was used to induce binding between films layer. This method was essentially free of any crosslinkers and also commonly used like Glutaraldehyde, NDC, and EDC for covalent strengthening. Fabrication of tubular scaffold out of collagen without any crosslinking chemicals is the main critical aspect of this design. Use of Glutaraldehyde or chemical crosslinkers has resulted in cytotoxicity [68]. This design avoids any cytotoxic issues that would have resulted from use of fixing agent inside the body. Another advantage of this fabrication is its ease of access to control mechanical properties. Three scaffolds out of pure collagen were tested for mechanical property like burst pressure, and suture retention strength. The burst pressure obtained for 5, 10, and 15 layer films

were 474±111, 1804±801, and 4259±733 mmHg respectively. The suture retention obtained was 84.71±11.1, 216±11.3, and 293±9 gf respectively. As it can be seen an increase in value of burst pressure with increase in number of layers used. This is the indication that depending upon the desired mechanical property, films number can be regulated and so will adjust the mechanical property of tubular structure. The most important aspect of this fabrication procedure, which make it very important in designing vascular graft is that it is very inexpensive, requires very less time and easy off shelf availability. It does not involve in mechanical device to fabricate, avoid use of any kind of additional chemical except collagen solution and is done manually. Common techniques like Electrospinning, and decellularization technique is avoided because it has potential of denaturing collagen before turning in to fibers and destroying the mechanical integrity of tissue with detergent solution respectively [69,70].

In the second part of the study, use of elastin is enforced. The use of elastin powder with collagen did not result in any solubility problem and desired concentration was prepared easily. In addition to it, films of collagen-elastin film were also easily prepared and difference in the roughness and surface was clearly distinguishable with naked eyes. The mechanical characterization of collagen-elastin tubing showed that this fabrication method could also be combined with other important extracellular matrix component in designing vascular conduit that resembles in mechanical integrity with natural arteries and veins. The ratio of elastin: collagen (1:9) was exploited in these studies but further studies with different ratio can be part of future research. It also allows people to combine mixture of ECM components that is compatible with collagen solution in designing vascular conduit with stacked films method. The importance of elastin with collagen in vascular graft has already been studied. Incorporation of reinforcing

collagen microfibers, recombinant elastomeric protein-based biomaterials played a significant role in load bearing tissue substitutes [45]. The stacked collagen method now not allows to tunable mechanical properties from collagen scaffold but from collagen/other compatible collagen components as well. An example by use of elastin is already illustrated when looking at mechanical comparison between collagen and collagen-elastin scaffold.

In third part of the study the efficiency of coating collagen film was evaluated as part of improving hemocompatibility studies through use of fibronectin, heparin and aspirin drugs. Heparinized collagen tube and drug embedded collagen tube can now be used *in vivo* for compliancy and histological studies. Collagen tube can be efficiently modified in to non-thrombogenic material and also drug delivery agent at the site of vascular surgery. The importance of controlled and local drug delivery is greater topic for research in vascular tissue engineering than conventional covalent attachment of non-specific protein or other biomolecules. The important features of localized and temporal presentation of drugs and growth factors in vascular tissue engineering have been discussed by Zhang et al. [67]. The use of biodegradable perivascular wrap for controlled, local and directed drug delivery has been used to treat chronic hemodialysis [71]. Here in this study, drug delivery has been shown through embedding aspirin within the films. The release profile between two types of scaffold design used for drug study showed signs of controlled drug delivery within the graft. But better controlled release amount for over longer period of times still need to be evaluated and that will be part of the future studies.

Finally pure collagen can be used to construct small diameter vascular graft with the help of stacked films method. The weak nature of collagen film is now overcome through this

fabrication method discussed in this study. Vascular conduit designed in this study shows potential in replacing diseased vessels in future because its computability, strength and drug delivery capabilities.

REFERENCES

1. Edelman ER. Vascular tissue engineering. *Circulation Research*. –1999;85(12):1115-1117.

2. Soldani G, Losi P, Bernabei M, et al. Long term performance of small-diameter vascular grafts made of a poly(ether)urethane–polydimethylsiloxane semi-interpenetrating polymeric network. *Biomaterials*. 2010;31(9):2592-2605.

3. Heydarkhan-Hagvall S, Schenke-Layland K, Dhanasopon AP, et al. Three-dimensional electrospun ECM-based hybrid scaffolds for cardiovascular tissue engineering. *Biomaterials*. 2008;29(19):2907-2914.

4. Hoerstrup SP, Zünd G, Sodian R, Schnell AM, Grünenfelder J, Turina MI. Tissue engineering of small caliber vascular grafts. *European Journal of Cardio-Thoracic Surgery*. 2001;20(1):164-169.

5. Teebken OE, Haverich A. Tissue engineering of small diameter vascular grafts. *European Journal of Vascular and Endovascular Surgery*. 2002;23(6):475-485.

6. L'Heureux, N, Nathalie D, Gerhardt K, et al. "Human Tissue-engineered Blood Vessels for Adult Arterial Revascularization." *Nature Medicine* 12.3 (2006): 361-65.

7. Nerem RM. Critical issues in vascular tissue engineering. *Int Congr Ser*. 2004;1262(0):122-125.

8. Tian H, Tang Z, Zhuang X, Chen X, Jing X. Biodegradable synthetic polymers: Preparation, functionalization and biomedical application. *Progress in Polymer Science*. 2012;37(2):237-280.

9. Sheridan MH, Shea LD, Peters MC, Mooney DJ. Bioabsorbable polymer scaffolds for tissue engineering capable of sustained growth factor delivery. *J Controlled Release*. 2000;64(1–3):91-102.

10. Isenberg BC, Williams C, Tranquillo RT. Small-diameter artificial arteries engineered in vitro. *Circulation Research*. January 6/20, 2006;98(1):25-35.

11. Venkatraman S, Boey F, Lao LL. Implanted cardiovascular polymers: Natural, synthetic and bio-inspired. *Progress in Polymer Science*. 2008;33(9):853-874.

12. Shimizu K, Ito A, Arinobe M, et al. Effective cell-seeding technique using magnetite nanoparticles and magnetic force onto decellularized blood vessels for vascular tissue engineering. *Journal of Bioscience and Bioengineering*. 2007;103(5):472-478.

13. Shirakigawa N, Ijima H, Takei T. Decellularized liver as a practical scaffold with a vascular network template for liver tissue engineering. *Journal of Bioscience and Bioengineering*. 2012;114(5):546-551.

14. Zhao Y, Zhang S, Zhou J, et al. The development of a tissue-engineered artery using decellularized scaffold and autologous ovine mesenchymal stem cells. *Biomaterials*. 2010;31(2):296-307.

15. Schaner PJ, Martin ND, Tulenko TN, et al. Decellularized vein as a potential scaffold for vascular tissue engineering. *Journal of Vascular Surgery*. 2004;40(1):146-153.

16. Martin ND, Schaner PJ, Tulenko TN, et al. In vivo behavior of decellularized vein Allograft1,2. *J Surg Res*. 2005;129(1):17-23.

17. Gomes S, Leonor IB, Mano JF, Reis RL, Kaplan DL. Natural and genetically engineered proteins for tissue engineering. *Progress in Polymer Science*. 2012;37(1):1-17.

18. McClure MJ, Simpson DG, Bowlin GL. Tri-layered vascular grafts composed of polycaprolactone, elastin, collagen, and silk: Optimization of graft properties. *Journal of the Mechanical Behavior of Biomedical Materials*. 2012;10(0):48-61.

19. Zhang X, Reagan MR, Kaplan DL. Electrospun silk biomaterial scaffolds for regenerative medicine. *Adv Drug Deliv Rev*. 2009;61(12):988-1006.

20. Naito Y, Shinoka T, Duncan D, et al. Vascular tissue engineering: Towards the next generation vascular grafts. *Adv Drug Deliv Rev*. 2011;63(4–5):312-323.

21. Donald G Wallace, Joel Rosenblatt, Collagen gel systems for sustained delivery and tissue engineering, Advanced Drug Delivery Reviews, Volume 55, Issue 12, 28 November 2003: 1631-1649

22. Heydarkhan-Hagvall S, Schenke-Layland K, Dhanasopon AP, et al. Three-dimensional electrospun ECM-based hybrid scaffolds for cardiovascular tissue engineering. *Biomaterials*. 2008;29(19):2907-2914.

23. Dutta RC, Dutta AK. Comprehension of ECM-cell dynamics: A prerequisite for tissue regeneration. *Biotechnol Adv*. 2010;28(6):764-769.

24. Kim B, Park I, Hoshiba T, et al. Design of artificial extracellular matrices for tissue engineering. *Progress in Polymer Science*. 2011;36(2):238-268.

25. Friess W. Collagen – biomaterial for drug delivery. *European Journal of Pharmaceutics and Biopharmaceutics*. 1998;45(2):113-136.

26. Nimni ME. Collagen: Structure, function, and metabolism in normal and fibrotic tissues. *Semin Arthritis Rheum*. 1983;13(1):1-86.

27. Cameron GJ, Cairns DE, Wess TJ. The variability in type I collagen helical pitch is reflected in the D periodic fibrillar structure. *J Mol Biol*. 2007;372(4):1097-1107.

28. Olsen D, Yang C, Bodo M, et al. Recombinant collagen and gelatin for drug delivery. *Adv Drug Deliv Rev*. 2003;55(12):1547-1567.

29. Smith M, McFetridge P, Bodamyali T, et al. Porcine-derived collagen as a scaffold for tissue engineering. *Food Bioprod Process*. 2000;78(1):19-24.

30. Sochynsky RA, Boughton BJ, Burns J, Sykes BC, O'd McGee J. The effect of human fibronectin on platelet - collagen adhesion. *Thromb Res*. 1980;18(3–4):521-533.

31. Köse GT, Korkusuz F, Özkul A, et al. Tissue engineered cartilage on collagen and PHBV matrices. *Biomaterials*. 2005;26(25):5187-5197.

32. Wu H, Wang T, Kang P, Tsuang Y, Sun J, Lin F. Coculture of endothelial and smooth muscle cells on a collagen membrane in the development of a small-diameter vascular graft. *Biomaterials*. 2007;28(7):1385-1392.

33. Berglund JD, Mohseni MM, Nerem RM, Sambanis A. A biological hybrid model for collagen-based tissue engineered vascular constructs. *Biomaterials*. 2003;24(7):1241-1254.

34. Sell SA, McClure MJ, Garg K, Wolfe PS, Bowlin GL. Electrospinning of collagen/biopolymers for regenerative medicine and cardiovascular tissue engineering. *Adv Drug Deliv Rev*. 2009;61(12):1007-1019.

35. Whelan MC, Senger DR. Collagen I initiates endothelial cell morphogenesis by inducing actin polymerization through suppression of cyclic AMP and protein kinase A. *Journal of Biological Chemistry*. 2003;278(1):327-334.

36. Henry, B.G, bhansali, S et al. Optimization of Collagen film to be used in the development of a Biolocompatible Interface 22

37. Konig G, McAllister TN, Dusserre N, et al. Mechanical properties of completely autologous human tissue engineered blood vessels compared to human saphenous vein and mammary artery. *Biomaterials*. 2009;30(8):1542-1550.

38. Ghezzi CE, Marelli B, Muja N, Nazhat SN. Immediate production of a tubular dense collagen construct with bioinspired mechanical properties. *Acta Biomaterialia*. 2012;8(5):1813-1825.

39. He W, Ma Z, Yong T, Teo WE, Ramakrishna S. Fabrication of collagen-coated biodegradable polymer nanofiber mesh and its potential for endothelial cells growth. *Biomaterials*. 2005;26(36):7606-7615.

40. Baker KS, Williams SK, Jarrell BE, Koolpe EA, Levine E. Endothelialization of human collagen surfaces with human adult endothelial cells. *The American Journal of Surgery*. 1985;150(2):197-200.

41. Daamen WF, Veerkamp JH, van Hest JCM, van Kuppevelt TH. Elastin as a biomaterial for tissue engineering. *Biomaterials*. 2007;28(30):4378-4398.

42. Mithieux SM, Weiss AS. Elastin. In: *Advances in protein chemistry*. Vol Volume 70. Academic Press:437-461.

43. Nettles DL, Chilkoti A, Setton LA. Applications of elastin-like polypeptides in tissue engineering. *Adv Drug Deliv Rev*. 2010;62(15):1479-1485.

44. Buttafoco L, Kolkman NG, Engbers-Buijtenhuijs P, et al. Electrospinning of collagen and elastin for tissue engineering applications. *Biomaterials*. 2006;27(5):724-734.

45. Caves JM, Kumar VA, Martinez AW, et al. The use of microfiber composites of elastin-like protein matrix reinforced with synthetic collagen in the design of vascular grafts. *Biomaterials*. 2010;31(27):7175-7182.

46. Wise SG, Byrom MJ, Waterhouse A, Bannon PG, Ng MKC, Weiss AS. A multilayered synthetic human elastin/polycaprolactone hybrid vascular graft with tailored mechanical properties. *Acta Biomaterialia*. 2011;7(1):295-303.

47. Koens MJW, Faraj KA, Wismans RG, et al. Controlled fabrication of triple layered and molecularly defined collagen/elastin vascular grafts resembling the native blood vessel. *Acta Biomaterialia*. 2010;6(12):4666-4674.

48. Daamen WF, van Moerkerk HTB, Hafmans T, et al. Preparation and evaluation of molecularly-defined collagen–elastin–glycosaminoglycan scaffolds for tissue engineering. *Biomaterials*. 2003;24(22):4001-4009.

49. Tamayo RP, Montfort I, Pardo A. What controls collagen resorption in vivo? *Med Hypotheses*. 1980;6(7):711-726.

50. Burleigh MC, Werb Z, Reynolds JJ. Evidence that species specificity and rate of collagen degradation are properties of collagen, not collagenase. *Biochimica et Biophysica Acta (BBA) - Protein Structure*. 1977;494(1):198-208.

51. . McAnulty RJ, Laurent GJ. Collagen synthesis and degradation in vivo. evidence for rapid rates of collagen turnover with extensive degradation of newly synthesized collagen in tissues of the adult rat. *Coll Relat Res*. 1987;7(2):93-104.

52. Skopinska-Wisniewska J, Sionkowska A, Kaminska A, Kaznica A, Jachimiak R, Drewa T. Surface characterization of collagen/elastin based biomaterials for tissue regeneration. *Appl Surf Sci*. 2009;255(19):8286-8292.

53. Conklin BS, Richter ER, Kreutziger KL, Zhong D-, Chen C. Development and evaluation of a novel decellularized vascular xenograft. *Med Eng Phys*. 2002; 24(3):173-183.

54. Prang H D. Laplace Law and the alveolus: A Misconception of Anatomy and Misapplication of Physics. Advan in Physiol Edu. 2003,: 27:34-40.

55. Michael Lee J, J. Wilson G. Anisotropic tensile viscoelastic properties of vascular graft materials tested at low strain rates. *Biomaterials*. 1986;7(6):423-431.

56. Seliktar D, Black R, Vito R, et al. Dynamic Mechanical Conditioning of Collagen-Gel Blood Vessel Constructs Induces Remodeling In Vitro. Annals of Biomedical Endineering. 2000;28(4):351-362.

57. Miyata T, Taira T, Noishiki Y. Collagen engineering for biomaterial use. *Clin Mater*. 1992;9(3–4):139-148.

58. Wissink MJB, Beernink R, Pieper JS, et al. Immobilization of heparin to EDC/NHS-crosslinked collagen. characterization and in vitro evaluation. *Biomaterials*. 2001;22(2):151-163.

59. Sebum B. Heparinized crosslinked collagen structures for the expansion and differentiation of hematopoietic stem cells. PhD thesis. 2007: 97-112.

60. Sgarioto M, Vigneron P, Patterson J, Malherbe F, Nagel M, Egles C. Collagen type I together with fibronectin provide a better support for endothelialization. *Comptes Rendus Biologies*. 2012;335(8):520-528.

61. Aihara M, Takami H, Sawada Y, et al. Effect of fibronectin and von willebrand factor on the adhesion of human fixed washed platelets to collagen immobilized beads. *Thromb Res*. 1986;44(5): 661-672

62. Freischlag JA, Moore WS. Clinical experience with a collagen-impregnated knitted dacron vascular graft. *Ann Vasc Surg*. 1990;4(5):449-454.

63. Lin PH, Bush RL, Yao Q, Lumsden AB, Chen C. Evaluation of platelet deposition and neointimal hyperplasia of heparin-coated small-caliber ePTFE grafts in a canine femoral artery bypass model1. *J Surg Res*. 2004; 118(1):45-52

64. Henry G Watson, Yen Lin Chee, Aspirin and other antiplatelet drugs in the prevention of venous thromboembolism, Blood Reviews, Volume 22, Issue 2, March 2008, 107-116.

65. Blanchemain N, Haulon S, Martel B, Traisnel M, Morcellet M, Hildebrand HF. Vascular PET prostheses surface modification with cyclodextrin coating: Development of a new drug delivery system. *European Journal of Vascular and Endovascular Surgery*. 2005;29(6):628-632.

66. Greisler HP. Growth factor release from vascular grafts. *J Controlled Release*. 1996;39(2–3):267-280.

67. Zhang G, Suggs LJ. Matrices and scaffolds for drug delivery in vascular tissue engineering. *Adv Drug Deliv Rev*. 2007;59(4–5):360-373.

68. Jayakrishnan A, Jameela SR. Glutaraldehyde as a fixative in bioprostheses and drug delivery matrices. *Biomaterials*. 1996;17(5):471-484.

69. Sill TJ, von Recum HA. Electrospinning: Applications in drug delivery and tissue engineering. *Biomaterials*. 2008; 29(13):1989-2006.

70. Gilbert TW, Sellaro TL, Badylak SF. Decellularization of tissues and organs. *Biomaterials*. 2006;27(19):3675-3683.

71. Sanders WG, Hogrebe PC, Grainger DW, Cheung AK, Terry CM. A biodegradable perivascular wrap for controlled, local and directed drug delivery. *J Controlled Release*. 2012;161(1):81-89.

BIOGRAPHICAL INFORMATION

Sandeep Shah was born in Birgunj, Nepal on August 22, 1987. He earned his high school diploma from Rani Public School, Kerala, India. He completed his Bachelor of Science in Biology at the University of Texas at Arlington in 2011. He was enrolled in 5 year Master in Biomedical Engineering Program at UT Arlington. Since joining research lab in Spring 2011, Sandeep Shah has been actively involved in fabrication of small diameter blood vessels with collagen and elastin. His research interest includes drug delivery, tissue engineering, and polymer engineering.