NOVEL NANOFIBER-BASED GRAFT FOR HEART VALVE REPLACEMENT

BY

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DEDICATION

Dedicated to my family, teachers, friends, and to the memory of my grandfather
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<th>Description</th>
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<tbody>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>BME</td>
<td>Beta-mercaptoethanol</td>
</tr>
<tr>
<td>DDH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Distilled and deionized water</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>HMDS</td>
<td>Hexamethyldisilazane</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>HFP</td>
<td>1, 1, 1, 3, 3-hexafluoro-2-propanol</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFC mat</td>
<td>Electrospun mat of collagen - Fibroin – Poly (glycerol-sebacate) with 4.5: 4.5: 1 weight ratio</td>
</tr>
<tr>
<td>PGS</td>
<td>Poly (glycerol-sebacate)</td>
</tr>
<tr>
<td>PITC</td>
<td>Phenylisothiocyanate</td>
</tr>
<tr>
<td>PTC</td>
<td>Phenylthiocarbamyl</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N, N' - tetramethylenediamine</td>
</tr>
<tr>
<td>VHD</td>
<td>Valvular heart disease</td>
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</table>
ABSTRACT

Creating a functional heart valve graft that lasts a lifetime is one of the main targets of cardiovascular tissue engineering. While processed conventional collagen-based heart valves have been used for many years, they eventually fail because of inadequate cell infiltration and insufficient replacement of graft material by tissue remodeling. To overcome the graft failure due to inadequate tissue remodeling and growth, an improved graft material was created by incorporating type I collagen protein with mechanical robust silk fibroin, and hemocompatible synthetic elastic polymer poly (glycerol-sebacate) (PGS). The central hypothesis of this project was: Electrospun grafts created from composites of type I collagen, silk fibroin, and PGS are stable, less thrombogenic and more readily available than the conventionally used collagen-based grafts for aortic heart valve replacement. The aim was to fabricate a graft material that mimicked the composition, structure and mechanical properties of native heart valve tissue, and to promote the formation of an intact nonthrombogenic endothelial cell layer. In this study, an improved heart valve material composed of type I collagen, silk fibroin, and PGS was developed. The homogeneity of blended composites was demonstrated using Fourier transform spectroscopy. Tensile stress, strain, and elastic modulus of the electrospun mats were measured by an Instron mechanical tester. The elastic moduli were between 2.3 - 5.0 Mpa, tensile stresses ranged from 0.8 to 1.5 Mpa, and strains were of 30% - 70%. These values were similar to those reported for native heart valves. Suture retention tests showed a highest 0.32N pull-out force at the single-loop suture
site in electrospun mats with 4.5:4.5:1 collagen, silk fibroin and PGS weight ratio (PFC mats). Functional test of PFC mats demonstrated a slow degradation rate as compared to other collagen-based grafts. Cells adhered to and proliferated on PFC mats. Endothelial cells were observed to form tight junctions on the material. Several studies with isolated platelets showed the PFC mats were less thrombogenic in comparison to collagen hydrogels and structurally similar electrospun collagen mats. In summary, this work demonstrated PFC mats provided strong, slowly degradable, and nonthrombogenic grafts that promoted cell adhesion and growth. Therefore, PFC mats could be used as a functional and durable heart valve replacement.
BACKGROUND

I. Valvular Heart Disease

Heart valves play an important role in directing blood flow. Dysfunctional valves are unable to perform the normal physiological requirement to completely open or close. This leads to either restriction of blood flow (stenosis) or allows retrograde backflow of blood (regurgitation). Thus valvular heart disease (VHD) can be fatal. Severe valvular heart disease (VHD) affects 1 out of 40 adults in the United States, and it is responsible for 28,000 deaths per year (Nkomo, Gardin et al. 2006). Among all VHD, aortic valve disease has the highest mortality and is approximately 64% of total. The treatment usually necessitates surgical repair or replacement (Apte 2011). Selecting suitable aortic heart valve prostheses is critical for the long term outcome.

II. Anatomy and Physiology of the Heart Valve

To create functional valvular grafts for replacements, one must understand the anatomy and physiology of the heart valves. There are two semilunar valves: aortic and pulmonary valves, and two atrioventricular valves: mitral and tricuspid valves (Hinton and Yutzey 2011).

A. Key Structures and Functions

Anatomically, semilunar valves have 3 film-like cusps and are supported from the coronet-shaped annuli that attach to the outflow vessels. Semilunar valves have an average thickness of 300-700µm (Ratner, 1996). The root of the half-
moon shaped leaflet attached to the aorta is thicker than the middle of the leaflets where they congregate. Different from semilunar valves, atrioventricular valves have irregular ring of membranous tissue that are anchored by chordae tendineae and papillary muscles, which made it geometrically harder to engineer than semilunar valves (Hinton and Yutzey 2011).

Physiologically, heart valves play an important role in coordinating the direction of blood flow during the cardiac cycle. The leaflets of the valves passively open and close to direct unidirectional blood flow and prevent the retrograde backflow. The semilunar valves are the outflow valves that open when ejecting blood from the ventricles. These valves close when the ventricles relax and fill with blood. The inlet or atrioventricular valves are located in between atria and ventricles (RV and LV). These valves open to allow blood filling the ventricle and close when the ventricles contract (Figure 1). Because the defined anatomical feature and physiological requirements of aortic valves, the goal of current study was to create an aortic heart valve grafts.
Figure 1 Schematic drawing illustrating anatomy of the heart and aortic valve. Left: Illustration showing the positions of aortic valve (labeled) and pulmonary valve; Right: Image showing axially sectioned view of aortic outflow vessel (adapted from Gray, 1918).
B. Valvular Extracellular Matrix

The composition and structure of heart valve tissue ensures the physiological valvular function while leaflets open and close. Valvular tissue is a highly organized connective tissue. A mature native valvular tissue primarily consists of an intricate interconnected meshwork of extracellular matrix (ECM). The main compositions of valvular ECM are type I collagen and elastin in a 4:1 dry weight ratio (Mol 2004). Collagen and elastin proteins are the major structural components that absorb stress and ensure a flexible range of motion while leaflets opening and closing during cardiac cycle. To further facilitate the biological function of heart valve, ECM provides protein binding sequences and mechanical cues to mediate cell adhesion and proliferation.

C. Valvular Cells

Valvular cells respond to ECM microenvironment cues during adhesion, proliferation, and deposit ECM composites during tissue remodeling. They are the active functional components in the valvular tissue. Adequate infiltration of viable valvular cells is particularly important in forming a healthy heart valve after replacement. Valvular interstitial cells and endothelial cells are the two main cell populations in valvular tissue (Breuer 2004). The valvular endothelial cells exhibit cuboidal phenotype and form a non-thrombogenic monolayer on the leaflet surface that is in direct contact with blood. These endothelial cells also regulate nutrients transport, biological signaling in interstitial cells, and inflammatory response (Sacks, Schoen et al. 2009; Butcher, Mahler et al. 2011). The valvular
interstitial cells have a fibroblastic phenotype and are distributed within the aortic valve leaflets. They are capable of remodeling ECM proteins as a result of mechanical and chemical signaling (Sacks, Schoen et al. 2009; Butcher, Mahler et al. 2011). Therefore, these interstitial cells influence the long-term durability and functionality of the heart valve tissue. Moreover, the crosstalk between valvular endothelial cells and valvular interstitial cells is important in maintaining stable cell phenotype, cell proliferation, as well as preventing thrombogenesis and calcification (Butcher, Mahler et al. 2011). Thus, screening normal or pathological responses of endothelial cells and interstitial cells on heart valve grafts is of great importance in determining the long-term performance of heart valve grafts.

III. Pathology

Pathological changes lead to structural and functional dysfunctions of heart valves. Regurgitation and stenosis are the two major pathological phenomena. In aortic regurgitation, the aortic valve shortens and thickens. Compositionaly, contents of collagen reduces and glycosaminoglycan increases (Yacoub and Cohn 2004; Butcher, Mahler et al. 2011). The geometry changes and results in incompletely closed leaflets, and therefore lead to backward flow of blood from the aorta into the left ventricle during diastole.

In aortic stenosis, blood flow is restricted by a tightened or narrowed opening of the aortic valve (Kidane, Burriesci et al. 2009). Studies suggest that
stenosis is associated with progressive valve calcification along with increased collagen deposition by valvular interstitial cells (Butcher, Mahler et al. 2011).

IV. Conventional Heart Valve Prostheses

Several products for replacing diseased aortic heart valves are currently available. Mechanical aortic heart valves and tissue bioprosthesis are two most commonly used replacements in clinics (Table I).

A. Mechanical Heart Valves

Mechanical heart valves are made from synthetic materials. One of the commercially available products for mechanical prostheses is St. Jude bileaflet tilting disk heart valve. Mechanical valves have good durability and last for around 25 years, however life-long anticoagulant therapy is required for the patient (Jordan, Williams et al. 2012).

B. Tissue Heart Valves

Bioprosthetic collagen-based tissue valves from porcine valves or bovine pericardium also are used widely. But deterioration associated complications due to inadequate cell growth and ECM remodeling of bioprosthetic valves were reported. Inadequate cell infiltration and remodeling eventually lead to valve degeneration and structural failure (Simon, Kasimir et al. 2003). The number accounts for 50% postoperative failure within 12-15 years (Breuer 2004).
These finding suggests successful heart valve grafts should be both durable and functional. The basic anatomy and physiological requirements need to be considered to fabricate structural similar and mechanical robust heart valve graft. Approaches to overcome the pathological failure modes should be taken into consideration in order to select graft composites that are biocompatible, slowly degradable and durable, capable of promoting adequate cell growth, tissue remodeling, and non-thrombogenic.
### Table I Current Mechanical Heart Valve and Tissue Bioprosthesis

<table>
<thead>
<tr>
<th>Types of Heart valve Replacement</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical valves</td>
<td>Durable</td>
<td>Thromboembolic and thrombotic occlusion (lacking endothelial lining), infection; Anticoagulation therapy required</td>
</tr>
<tr>
<td>(Example: St. Jude Medical bileaflet tilting disk heart valve*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue valves</td>
<td>Lower risk of hemolysis and thrombosis</td>
<td>Not as durable, degeneration due to inadequate cell growth and tissue remodeling</td>
</tr>
<tr>
<td>(Example: Carpentier-Edwards PERIMOUNT aortic valve **)</td>
<td></td>
<td></td>
</tr>
</tbody>
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Illustrations and information sources (Yoganathan, He et al. 2004; Nkomo, Gardin et al. 2006)
V. Grafts for Valvular Tissue Replacement

A. Design Criteria

The standard criteria for creating heart valve grafts are to mimic the compositional and structural characteristics of native heart valve tissue. These can be summarized as a favorable surface for cell attachment, high porosity and interconnected network for nutrient transport and cell signaling, and mechanical strength for performing valvular function under physiological stress. Even though most traditional tissue valve grafts are compositionally and structurally similar to native valves, they demonstrate limited cell growth and ECM remodeling (Sacks, Schoen et al. 2009). Therefore, creating heart valve grafts which incorporating active cell binding sites, slow degradability, and can maintain the structural and mechanical integrity during the healing process are needed. This is because first native host cells need to adhere and proliferate in order to deposit proteins such as collagen and elastin which eventually remodel the surrounding environment to form their own ECM. Then, slow degradation and material durability allows tissue remodeling to take place before the graft deteriorates. A balance between the losses of mechanical integrity associated with material degradation and the gains of mechanical strength by ECM deposition from cells needs to be found.

Advances in synthetic polymers and natural proteins provide new avenues to tailor biomaterials for accommodating the functionality of a specific tissue type. Selection in both composites and structures should be taken into consideration based on anatomical specifications and physiological functions of a healthy heart.
valve. Moreover, it is important for the selected materials to be durable and be able to uphold the hemodynamic stress (Table II).

**B. Material Selection**

In order to create a heart valve graft that meets these criteria, a strategy of incorporating natural proteins with an elastic polymer was applied. Type I collagen, silk fibroin and PGS have been respectively reported to promote adhesion and proliferation of endothelial cells (Bondar, Fuchs et al. 2008; Sell, McClure et al. 2009; Sant, Hwang et al. 2011). Each of these compositions has their own characteristics useful in constructing a graft material for potential use as a heart valve replacement.

\textit{a. Type I Collagen}

Specifically, among the natural proteins, collagen is the most abundant and load-bearing component of aortic valve cusp while elastin provides the flexibility of the soft tissue. The fundamental unit of the fibrillar collagen is the triple helix. The triple helix is made up of 3 polypeptide chains that each are 1000 amino acid long with glycine-X-Y (Gly-X-Y) repeats (Alberts, Johnson et al. 2002; Malafaya, Silva et al. 2007). The amino acid sequences, such as RGD (Arg-Gly-Asp), DGEA (Asp-Gly-Glu-Ala) or GFOGER (Gly-Phe-Hyp-Gly-Glu-Arg) in type I collagen motifs specially binds to \( \alpha_2 \beta_1 \) integrin to regulate cell adhesion (Gu and Masters 2010; Shekaran and Garcia 2011). As the major structural protein, type I collagen absorbs most of the stress during the closing of the aortic valve in diastole when the ventricle filled with blood (Table II).
Table II Pressure gradients to be considered in designing aortic valve grafts

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td><strong>Diastolic Pressure</strong> (Chobanian, Bakris et al. 2003)</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>60-79mmHg</td>
</tr>
<tr>
<td>Prehypertension</td>
<td>80-89mmHg</td>
</tr>
<tr>
<td>Stage I Hypertension</td>
<td>90-99mmHg</td>
</tr>
<tr>
<td>Stage II Hypertension</td>
<td>≥100mmHg</td>
</tr>
</tbody>
</table>
b. Poly (glycerol-sebacate) (PGS)

The polymer poly (glycerol-sebacate) (PGS) mimics the mechanical behavior of the ECM protein, elastin (Pomerantseva, Krebs et al. 2009). It has low elastic modulus and large capacity in elongation that is similar to elastin in valvular ECM (Alberts, Johnson et al. 2002; Sant and Khademhosseini 2010). Most importantly PGS has been reported to promote synthesis of mature and organized elastin, as well as having a superior hemocompatibility over other synthetic polymers such as poly (l-lactide-co-glycolide) (PLGA) (Motlagh, Yang et al. 2006; Lee, Stolz et al. 2011). PGS was also reported to be biocompatible in vivo and in vitro. Endothelial cells and fibroblasts were viable when cultured with PGS (Wang, Ameer et al. 2002; Yi and LaVan 2008). Minimal inflammatory response and no fibrous collagen capsule were observed for PGS (Wang, Ameer et al. 2002). The elastic property and biocompatibility of PGS make it a potent biomaterial for cardiovascular tissue grafts.

c. Silk Fibroin

To improve the strength of graft material and incorporate slow degradability, silk fibroin was selected (Horan, Antle et al. 2005). The adjacent – (Ala-Gly)- repeated sequence forms polypeptide chains with molecular weights of 390kDa (heavy chain) and 25kDa (light chain). Serving as the structural and major protein in the silk, fibroin protein polypeptide chains have interchain hydrogen bonds that contribute to the special highly crystalline β-sheet conformation. Degradation is defined as the breakdown of the materials and leads to changes in physical properties. Degradation rate of silk fibroin was
reported to be controllable for lasting from hours to years (Rockwood, Preda et al. 2011). By incorporating these properties of silk fibroin, the material is expected to provide sufficient mechanical support and perform the physiological function of valve tissue under a range of blood pressures (Table II). The extracellular matrix remodeling takes up to 20-week. The slow degradation rate allows maintenance of a durable functional graft before cell infiltration and growth can take place (Horan, Antle et al. 2005). This is particularly important for heart valve graft due to their special requirements in functionality and durability.

C. Material Fabrication

Besides the composition, the structural feature is another important design specification. Large surface area and sufficient porosity allow cell adhesion, nutrient transport, and signal transmission to enhance cell response for tissue remodeling. Electrospinning is a fabrication technique that is widely applied to rapidly create an ECM analogous graft that meets these criteria. Moreover, production of fiber diameters and alignments can be altered by changing electrospinning parameters such as solution viscosity, voltage, environmental humidity, and collector orientation (Sell, Wolfe et al. 2010). Electrospinning requires a simple set up of equipment as illustrated below (Figure 2). Polymer composites are dissolved in appropriate solvent such as hexafluoroisopropanol (HFP) to form the electrospinning solution. The electrospinning solution usually is loaded into a syringe that is placed on a pump to inject the solution at a constant rate. A high voltage power source is connected to a conductive syringe tip. A conductive collector is grounded on the opposite side to the syringe to create an
electric field. Polymer solution at the syringe tip forms a small droplet called a Taylor cone. When the electrostatic force overcomes the surface tension of the polymer solution, jets of polymer solution travel toward the collector and form an interconnected fiber matrix. In the current study using this technique, type I collagen, fibroin protein along with PGS were electrospun into an interconnected nanofiber matrix graft.

Figure 2 Picture illustrating the set-up of electrospinning equipments
D. Cell Adhesion and Proliferation

Heart valve grafts are directly in contact with blood. Whole blood is composed of red blood cells, white blood cells, platelets, and plasma proteins. In the body, platelets adhere to sites of vascular lesions and participate in one of the initial events of thrombosis (blood clot). A monolayer of endothelial cells which lines both surface of valvular tissue provide further mechanical strength to valvular ECM and a protective layer to prevent thrombosis. The endothelial cells prevent platelets from coming into contact with subendothelial collagen which lead to platelet activation and thrombosis (Zhu, Negri et al. 2010). Therefore, a graft material that promotes the formation of an endothelial cell monolayer on the graft in a continuous manner is important for ensuring the functionality and hemocompatibility of the implant.

E. Hemocompatibility

In the current study the interaction of platelets with electrospun mats was studied. This is because platelet activation plays an important role in thrombosis which will directly affect with the hemocompatibility and success of a heart valve graft. An amino acid sequence -Arg-Gly-Asp (RGD) on proteins such as collagen can activate platelet aggregation by induce high binding affinity of the platelets. The mechanism is through binding of the glycoprotein GPIb and GPIIb/IIIa receptor on the platelet surface to the proteolytic factor von Willebrand factor that recognizes and binds to the binding domains in thrombogenic materials such as collagen. This signaling will change the conformation of integrin on the platelet
surface and lead to thrombus formation (Zhu, Negri et al. 2010; Mendelboum Raviv, Szekeres-Csiki et al. 2011). To test the hemocompatibility of graft materials, platelet activation can be studied by the number of adhered platelets and their morphologies on various substrates.
**HYPOTHESIS AND SPECIFIC AIMS**

The central hypothesis of this project is: Electrospun mats created from composites of type I collagen, silk fibroin, and poly (glycerol-sebacate) (PGS) are stable, less thrombogenic and more readily available than the conventionally used collagen-based graft for aortic heart valve replacement. To test this hypothesis, two specific aims are proposed:

**Aim 1: To characterize the physical and mechanical properties of the electrospun material with different weight ratios of type I collagen, silk fibroin and poly (glycerol-sebacate) (PGS) composites.** The aim was accomplished by: (a) chemically identifying the major functional groups in the electrospun mats using Fourier transform infrared (FTIR) spectroscopy; (b) characterizing the physical properties of the mats by measuring the nanofiber diameters, porosities and thermal transition temperatures; (c) measuring the elastic modulus, tensile stress and stain by uniaxial tensile testing by using Instron mechanical tester and suture pull-up strength test using BOSE-Electroforce mechanical tester.

**Aim 2: To test the functionality of type I collagen, silk fibroin and poly (glycerol-sebacate) (PGS) electrospun mats by:** (a) measuring weight loss of electrospun mat to assess material degradation; (b) observing the formation of an endothelial cell layer on the mats using fluorescence staining; (c) quantifying the amount of platelet adhesion on the mats with and without endothelial cell co-incubation using confocal and scanning electron microscopy.
By characterizing material composition, structure, and cell response, the goal is to create a graft which mimics a functional native aortic valve tissue. Ideally, the graft will stimulate synthesis of mature ECM protein produced by cells and ultimately form a functional valvular tissue that last a lifetime.
Figure 3 Diagram illustrating the specific aims and approaches to assessing the characteristics and functionality of electrospun mats. By achieving these goals, an electrospun mat with optimal weight ratio of type I collagen, silk fibroin and PGS could be selected to be potentially used for constructing a functional heart valve replacement.
In summary, the focus of this study was to create a resorbable heart valve graft material which imitated the native aortic valve tissue based on protein compositions, elastic modulus, stress, and strain. The graft materials were tested for degradation, endothelium formation and platelet adhesion. Ultimately, after implantation in the body, the graft is expected to facilitate cell proliferation and ECM remodeling in order to generate a healthy heart valve capable of growing and lasting a life-time.
I. Extraction and Characterization of Silk Fibroin

A. Silk Fibroin Extraction

Silk fibroin protein was extracted according to the published procedure with modifications (Rockwood, Preda et al. 2011). Raw silk (*Bombyx mori*) was commercially purchased from a local vendor in Beijing, China. Figure 4 illustrates the silk fibroin extraction procedure. The extraction of fibroin was performed by first boiling the raw silk in 2L of 0.02 M Na$_2$CO$_3$ solution at 100°C for 30 minutes in a vented container covered with aluminum foil. After the boiling, silk was squeezed to dry and rinsed twice with DDH$_2$O. The degummed silk was then washed in boiling DDH$_2$O (100°C) for 20 minutes to remove glue-like sericine. Once completed, the degummed silk was spread out on dry paper towels to air dry overnight (approximately 15 hours) in the fume hood at 22°C room temperature.

On day 2, dried degummed silk was weighed to calculate the yield after degumming by washing. It was then placed in a clean flask. Calcium chloride solution (5.0M) was added to the degummed silk (with 10ml solution for every 1 gram of degummed silk) so that the material was fully hydrated. The solution was brought to boil for 3 hours until a clear amber color solution was obtained. The solution was centrifuged at 2000g to remove the precipitant and floating contaminants. The clear solution in the middle layer was transferred into a cellulose membrane tube for dialysis (Spectra/por 2 dialysis membrane tube of
12,000-14,000 molecular weight cut-off). The fibroin solution was dialyzed against DDH$_2$O for 48 hours at 4°C. The efficiency of dialysis was checked by adding the dialysis solution to AgNO$_3$ solution. The fibroin solution was transferred from dialysis tubes into 50 ml conical tubes (at most 20ml each tube) for lyophilization. After lyophilization, the silk fibroin powder was stored at 4°C in a sealed container.
Figure 4 Schematic of the silk fibroin extraction procedure
B. Amino Acid Composition

The quality of the extracted silk fibroin was identified for amino acid composition using amino acid assay (Bioanalytical Core Laboratory, Wake Forest University Center for Structural Biology).

The amino acid assay was performed using the standard protocol (Schroeder, Kay et al. 1955; Lombardi and Kaplan 1990). The extracted silk fibroin protein was hydrolyzed in 6N HCl for 24 hours. Then the hydrolyzed sample was derivatized with phenylisothiocyanate (PITC) to produce phenylthiocarbamyl (PTC) amino acid derivatives. The PTC-amino acids were separated and quantified using high performance liquid chromatography (HPLC).

C. Identification of the Molecular Weight of Silk Fibroin using Gel Electrophoresis

The molecular weight of extracted silk fibroin was determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). A 10% polyacrylamide gel was prepared according to the standard protocol. Upper gel (6.05 g Tris Base in 100 ml DDH₂O, pH to 6.8, 0.4 g SDS buffer), or lower gel buffer (36.4 g Tris in 100 ml DDH₂O, pH to 8.8, 0.8 g SDS) were mixed with polyacrylamide respectively, followed by chemical crosslinking with N,N,N,N’-tetramethylenediamine (TEMED) and 10% ammonium persulfate (APS). The lower gel was first poured into the middle of glass chamber to set for 2 hours until the gel was fully chemically crosslinked. Water was added gently on top of the lower gel to prevent the gel from drying. The upper gel was then gently poured
on top of the lower gel and sit to solidify through chemically crosslinking for 30 minutes. A comb was inserted immediately in the upper gel to create chamber cavities to load samples for electrophoretic separation. To prepare the loading sample, stored silk fibroin powder was dissolved in DDH$_2$O. Two treatments were applied to the concentrated silk fibroin solution. In the first treatment group, 100µl 8M urea was added to 100µl concentrated fibroin solution. For the second treatment groups, besides adding 8M urea, 20µl β-mercaptoethanol (BME) solution (5µl BME, 5 µl 10% SDS and 10µl Tris buffer at pH 8.0) was added to the urea treated concentrated fibroin solution. For each treatment a duplication of the sample was prepared. The treated samples were added to 1X Laemmli buffer supplemented with SDS (diluted 5X sample stock buffer: 10% w/v SDS, 10mM , 20 % v/v Glycerol, 0.2 M Tris-HCl, pH 6.8, 0.05% w/v Bromophenol blue) and boiled at 100°C for 5 minutes. The samples along with standard molecular weight marker solutions were then loaded onto the 10% polyacrylamide gel. The gel was run at 25mA and 200volts. Electrophoresis was stopped when the protein sample migrated to the bottom of the gel. The gel was transferred to the fixing solution (50%v/v methanol, 10% v/v acetic acid in DDH$_2$O) for 30 minutes and stained with Coomassie blue (0.1% w/v Coomassie blue R350, 20% v/v methanol, and 10% v/v acetic acid in DDH$_2$O) for 45 minutes. After destaining (30% v/v methanol, 5% v/v acetic acid in DDH$_2$O) for 3-5 hours, blue bands appeared on the polyacrylamide gel. Photographs were taken and the molecular weights of the protein were analyzed by comparing to the position of the bands of molecular markers.
D. Protein Structure Characterization of Silk and Extracted Fibroin

Chemical functional groups in silk fibroin were identified using Fourier transform spectroscopy. Silk fibers were tested before and after degumming. Extracted silk fibroin was tested as a powder form. All samples were tested on the Perkin-Elmer FT-IR spectrophotometer to obtain spectra graphs showing characteristic wavenumbers on x-axis.

II. Synthesis of PGS Prepolymer

PGS prepolymer can be easily synthesized from glycerol and sebacic acid which have been approved by FDA for medical applications (Wang, Ameer et al. 2002). The synthesis procedure followed the reported standard method with modification (Wang, Ameer et al. 2002; Pomerantseva, Krebs et al. 2009). Briefly, sebacic acid was heated at 180°C on an oil bath with nitrogen flow across the reaction flask for 10-20 minutes until all melted (Figure 5). An equimolar amount of warmed glycerol (60°C) was added. Then the pressure was decreased by attaching to a vacuum (General Medical, Richmond, VA) and the temperature was kept at 150°C for 4 hours to obtain PGS prepolymer in the form of a viscous amber color solution.

![Figure 5 Synthesis of PGS prepolymer](image)
Because the PGS prepolymer was in a viscous aqueous form, it usually needs to be thermally cured at 120°C for 48 hours to form the solidified PGS elastic polymer (Yi and LaVan 2008). In this study, PGS prepolymer was used in making the polymer and protein solution blends for electrospinning.

III. Production and Characterization of the Electrospun Mats

To produce the nanofiber construct, solutions made from type I collagen, silk fibroin, and PGS at different weight ratios of 9: 0: 1, 8: 1: 1, 4.5: 4.5: 1, 1: 1: 8, and 0: 9: 1 were prepared. The composites were acquired from extracted silk fibroin, commercially available type I collagen (Elastin Products Corporation, Missouri), and PGS prepolymers. Different proportions of type I collagen (Elastin Products Corporation, Missouri), silk fibroin and poly (glycerol-sebacate) (PGS) were dissolved in 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (HFP). Blended solutions were obtained for electrospinning with a 10% solute to solvent concentration (weight/volume). The solution was stirred for approximately 24 hours at room temperature. For electrospinning solution with an increased fibroin concentration, a longer stirring time was required to obtain a homogenous solution (up to 7 days) for the electrospinning.

A. Fabrication of Electrospun Mats using Electrospinning

Electrospinning was used to produce nanoscale fiber composites with collagen, fibroin and PGS polymer solutions at different weight ratios. Prepared polymer solution was loaded into a 3ml plastic syringe (BD, Franklin Lakes) with a stainless steel blunt needle (18 Gauge; 2.5cm length). The syringe tip was
attached to the negatively charged electrode. The collector was made by an aluminum metal plate and covered with aluminum foil to form a 12cm by 12cm square surface. The collector was attached to the grounded electrode and placed with the center facing the syringe tip at a distance of 20cm away from the syringe tip. The syringe was fixed on the Baxter infusion pump (Model AS50) to eject the polymer solution at a preset constant flow rate of 3ml/hour. When pumping started, a 35 kilovolts high voltage source (Gamma High Voltage Research, Ormond Beach, FL) was applied through the electrodes. The electrostatic force overcame the surface tension of the polymer solution at the syringe tip to form fibers that traveled toward the grounded collector. Nanofibers were spun and collected onto the whole field of aluminum foil. Thin sheets of nanofiber constructed mats (electrospun mats), in square shape approximately 12cm in length and width were fabricated.

The electrospun mats were then placed in a 120°C oven for 48 hours to thermally crosslink PGS (Yi and LaVan 2008). Glutaraldehyde was used to chemically crosslink collagenous tissues as reported (Sung, Chen et al. 2000). Glutaraldehyde solution (15%) was added in a glass dish in order to provide a vaporized atmosphere in a sealed desiccator to crosslink the electrospun mats at 22°C room temperature for 24 hours. Glutaraldehyde reacts with amino groups such as lysyl or hydroxylysyl residues, to form intermolecular and intramolecular crosslinking structures. Glycine solution (0.2M) was used to react with the remaining unreacted aldehyde group after crosslinking. Finally, electrospun mats were rinsed with DDH₂O for one hour. The thickness of the electrospun mats was
measured with a Vernier digital caliper. For storage, electrospun mats were air
dried and stored in a sealed plastic bag at 4°C.

B. Morphology Observation of Electrospun Mat using Scanning Electron Microscopy

The morphology and diameter of electrospun fibers were evaluated using
scanning electron microscopy (SEM) (JEOL JSM-6330F). Dried electrospun mats were cut into approximately 1cm by 1cm square. The sample was then
placed on the double-coated conductive carbon tab taped to a PELCO SEM pin stub. The sample was then sputter coated under vacuum with gold and palladium alloy (PELCO SC-4 machine). SEM images were taken at 5.0kV at a series of
magnification from 100X to 12000X for morphology observations. The images
with magnifications of 1500x and 2300X were used for fiber diameter measurements. Using NIH Image J software, sixteen measurements of the fiber diameters were taken from randomly selected area from two distinct SEM images. All measurements of fiber diameters were presented as the mean ±
standard error of the mean.

C. Characterization of Chemical Functional Groups in Electrospun Mat using FTIR

Chemical functional groups were detected with a Perkin-Elmer FT-IR
spectrophotometer to identify the polymer and protein structures. For individual composites, silk fibroin was tested as a powder form, type I collagen was tested using the sample directly from the manufacturer (Elastin Products Corporation,
Missouri), and samples of PGS after 48 hours of thermal crosslinking were punched using a 4mm biopsy punch (Miltex Inc, York, PA). For electrospun mats, three replicates of samples were made in the same manner punched from a 1-3mm thick electrospun mat using a 4mm biopsy punch. All samples were tested on the Perkin-Elmer FT-IR spectrophotometer to obtain transmittance spectra. A representative graph was presented for each sample to illustrate the major chemical functional groups in the composites. Structural changes of electrospun mats were compared and analyzed using the FTIR spectra.

D. Thermal Transition Analysis

The transition in physical state transition due to temperature change is important to implantable grafts. It is necessary to obtain a comprehensive thermal transition profile of the electrospun mats with temperature ranging from below storage temperature to above autoclave temperature. Phase changes of the materials are associated with exothermic (release heat) and endothermic (absorb heat) reactions. These thermodynamic changes can be detected by differential scanning calorimetry (DSC). Samples were prepared using 3mm biopsy punch and placed inside of standard Tzero aluminum pan/lid pairs (TA instruments, New Castle, DE). An empty pan of the same materials was used to be the reference. In each group, three samples were prepared and tested. The aluminum pan/lid pairs were weighed before and after loading the samples to calculate the sample weight. Thermal stabilization with no endo/exothermic events was typically achieved before the sample reached -60°C (Simone,
Dziubla et al. 2009). To obtain the complete profile of thermal transitions of the various composite ratios of electrospun mats, the DSC was run with the following steps:

- Equilibrate to -70°C Ramp 20° C/min to 300°C (heat)
- Mark end of cycle 0
- Ramp 20°C/min to -70°C (cool)
- Mark end of cycle 1
- Ramp 20°C/min to 20°C (heat)
- Mark end of cycle 2

The obtained thermal transitions depicted by heat flow over temperature were individually plotted and analyzed. By analyzing the thermal transition curves measured by DSC, changes in protein conformations and assemblies of the peptide chains could be studied. The glass transition seen as a step endotherm illustrated the state transition of the material from crystalline to amorphous phase. The melting point seen at the peak of a large endotherm depicted the material transition from solid to fluidic state of the material.

For collagen, temperature induced denaturation disrupt the hydrogen bonding between the polypeptide chains, and disassembles the helical structures to random coil. For highly crystalline fibroin, the temperature induced phase transformation is important to understand the protein conformation. These changes can be reflected by endotherms in the thermal transition curve.
E. Porosity Measurements

The porous matrix structure in the electrospun mat provides a large surface area for cell to adhere and proliferate, and for nutrients and oxygen to transport. Therefore, measuring the porosity of the electrospun mats gives important information for understanding this cellular integration and remodeling process.

The stored electrospun mats were hydrated with DDH$_2$O and kept flat on parafilm. The mats were cut into 1cm by 5cm rectangular shape samples (n=3) with a thickness between 0.1 – 0.3mm measured using a Veiner digital caliper. Each sample was placed in a 15ml conical tube for lyophilization. Kimwipes (KIMTECH, Roswell, GA) secured with tape were used to cover the top of the tubes. Samples were lyophilized for 1 hour. The weights of the dried samples were measured immediately. Then, the samples were immersed completely in 5ml DDH$_2$O in the tube with the top sealed and placed on the mechanical shaker for 30min at 22°C. Three replicates of samples in each group were blotted dry with filter paper and the weights were measured again. The density of type I collagen (1.40g/cm$^3$), silk fibroin (1.31g/cm$^3$), and PGS (1.13g/cm$^3$) were used (Minoura, Tsukada et al. 1990; De Cupere, Van Wetter et al. 2003; Pomerantseva, Krebs et al. 2009). Knowing the weight ratios of the composites, the densities were calculated using the equation shown below. The volume of the electrospun mat samples were obtained by dividing the weight by the density of the mat.

To calculate the porosity of the samples, the following equation was applied:
porosity: \[ \varepsilon = \frac{V_{liq}}{V_{liq} + V_{MAT}} \]

(Soliman, Sant et al. 2011)

\( V_{liq} \) is the volume of the intruded water and \( V_{MAT} \) is the volume of the electrospun mat. The calculated porosities were averaged and expressed as mean ± standard error of the mean.

F. Mechanical Tensile Testing using Inston Mechanical Tester

Heart valve grafts need to withstand the physiological pressure. Therefore, the mechanical tensile properties of the electrospun mats were evaluated. An Instron 5500R mechanical tester (Instron Corporation, Norwood, MA) with a 500N load cell and BlueHill software was used to perform uniaxial tensile tests of the electrospun mats. The Instron mechanical tester was calibrated and the testing rate was set to be 10mm/min. To prepare the testing specimens, electrospun mats were gently flattened on parafilm. The standard dumbbell stamp (ASTM D638-IV cutting die, Pioneer-Dietecs Corporation, Norwood, MA) was used to obtain 6 testing specimens cut out from electrospun mats of various weight ratios. Specimens were air dried and stored at 4°C cold room in a sealed bag before use. Three batches of specimens were tested for each weight ratio of composites. To perform the uniaxial tensile testing, each specimen was rehydrated individually on the mechanical shaker for 10 minutes by completely soaking in 100ml DDH₂O. The thickness and width was measured immediately after 10 minutes of soaking using a Vernier digital caliper as the sample
maintained in the hydrated state. The specimen was then loaded onto the Instron mechanical tester with two dumbell edges secured in the upper and lower grips (Figure 6). The complete stress-strain curves were recorded until the specimen broke. Three measurements from each type of electrospun mat were used to calculate mean ± standard error of the mean (SEM) of elastic modulus, stress, and strain.
Figure 6 Photo illustrating the set-up of a uniaxial tensile test for electrospun mats using Instron mechanical tester
G. Modified Suture Retention Testing using BOSE Electroforce Mechanical Tester

Suture retention test is a standardized procedure commonly performed in the manufacture of heart valve prosthesis (Trowbridge, Lawford et al. 1989). To better facilitate the needs of surgical procedure for the heart valve replacement, this mechanical test was performed with modification using a BOSE Electroforce mechanical tester. In detail, specimens were prepared in 2cm in length and 0.5cm in width. The monofilament prolene suture (3-0 monofilament; Ethicon, Somerville, NJ) was placed 3mm from the 0.5cm edge to form a single loop. Another loop was created to fix the suture to the upper holder BOSE-Electroforce mechanical tester (Figure 7). Due to the delicate nature of the sutured specimen and the probability of structural failure at pre-loading, a total of 5 replicates of each sample were prepared. The loading rate was set to 10mm/min. The maximum tensile load was recorded as the suture ruptured from the specimen.

Figure 7 Photograph illustrating the set-up of modified suture retention mechanical test for the electrospun mat using Electroforce BOSE mechanical tester
IV. Functionality Assessment of Electrospun Mats

A. Material Degradation

To assess degradation in vitro, electrospun mats made from 4.5: 4.5: 1 weight ratio of collagen: fibroin: PGS composites (PFC mats) were incubated in phosphate buffered saline (PBS) solution at 37°C supplemented with 0.1% sodium azide to prevent bacteria growth. The weights of the PFC mats were measured at the end of every 7-day period for a total duration of 9 weeks. In detail, four samples of the electrospun mats were cut into long narrow pieces and used to measure the weight loss during degradation. The weights of empty conical tubes were weighed. The electrospun mats were dried and weighed after putting in 15ml conical tubes individually. Each sample was soaked separately in 10mL of PBS with 0.1% sodium azide at 37°C. The samples were removed from the incubator every 7 days at the same time point. The pH was measured for each sample. Then the 0.1% sodium azide solution was poured out and each sample was rinsed 2 times with 15ml DDH₂O to remove the residual PBS and sodium azide. Each sample was freeze-dried using a lyophilizer for 24 hours. Samples from three different batches were repeated for the degradation experiments. The percentage of weight loss was determined and presented from one of the representative groups. The data were calculated as the ratio of mass change after degradation to the original mass of the scaffold (Liu, Teng et al. 2010; Okhawilai 2010).

\[
\text{Weight loss (\%) } = \frac{W_i}{W_i} \times 100\%
\]
$W_i$ represented the initial weight of the electrospun fiber mats. $W_i$ was the weight loss of the same fiber mats after exposure in degradation solution. The values were expressed as the mean ± standard error of the mean (n = 4).

One additional sample was prepared for taking scanning electron microscopy of fiber morphology during degradation. The samples were retrieved at week 3, 5, 6, 7, 8, and 9. After freeze-drying, the samples were sputter coated and observed under scanning electron microscope.

**B. Cell Culture Study**

To prepare the substrate for cell seeding, PFC mats were first soaked in sterilized 0.02 M glycine for 1 hour, following 1 hour PBS soaking. The mats were then sterilized with 70% ethanol for 1 hour and soaked in PBS with ultra-voilet (UV) exposure for 1 hour for each side of the mat. The thickness of the PFC mat used for cell culture was between 0.1mm – 0.3cmm as measured by a Veiner digital caliper. Fibronectin was used as the pre-coating on the material to mimick \textit{in vivo} process of initial protein adsorption onto the material. Both PFC mats in the culture dishes and the plain culture dishes were coated with fibronectin at a concentration of 100µg/ml. After incubating for 30 min, the PFC mats and culture dishes were rinsed with excess PBS once. Equal amount of culture media were added to culture plate with PFC mats and plain culture dishes and placed at 37°C incubator with 5% CO$_2$ before cell seeding. The cell compatibility of PFC mats was first assessed by seeding three cell lines of endothelial cells (HUVECs: CRL-
1730), fibroblast (HEPM: CRL-1486), and epithelial cell (GMMe: CRL-2674). All cell lines were purchased from ATCC (American Type Culture Collection). To further study the proliferation and endotheliation, human endothelial cells derived from umbilical veins (HUVECs) were used. The cells were cultured at 37°C and 5% CO₂ incubator with a complete change of culture medium twice a week (Lonza Endothelial Cell Basal Medium with EGM-2 Single Quots supplement, Walkersville, MD). For subculturing, the plate was washed once with PBS and cells were trypsinized using 6ml of 0.25% trypsin and 0.02% EDTA for 1 minute. Culture media (6ml) was added to stop the reaction when cells detached and started to float. The media with cells was collected into a 15ml conical tube and centrifuged at 1000g for 4 min. The supernatant was aspirated and the cell pellet at the bottom was resuspend. For this experiment, cells were seeded at a high initial density (50,000 cells/well in 48-well plate) in order to examine cell interaction within a short culturing time. Cells were counted and gently seeded at a cell density of 50,000cells/well onto PFC mats in plain polysterene culture dishes that were all pre-coated with fibronectin as described earlier (diameter: 1cm; area: 0.75cm²). Media (0.5ml) was added after 24 hours of culturing. The cell morphology was observed microscopically on a daily base. Cells were cultured for 3 and 7 days respectively before staining for F-actin and nuclei using rhodamine-phalloidin and sytox green (Invitrogen, Eugene, OR). On day 3 and day 7, the cells were fixed with formalin for 15 min, and then washed with PBS with 0.2% Triton X-100 for 10 min to permealize the cell membrane at 22°C. The samples were then incubated with 1% bovine serum albumin (BSA) for 10 min.
Rhodamine-phalloidin and sytox green dyes were made into 1:40 and 1:1000 dilutions in 1% BSA and TBS (Tris buffered saline). Each staining dye was added to incubate with cells for 1 hour, followed by two washes with PBS. Then the stained samples were prepared in mounting media on the glass slides for confocal microscopy.

To compare the cell morphology and proliferation rate, four confocal images that represented the entire mats or culture dishes were chosen (20X, 0.46cm by 0.46cm area on the sample). The cells were easily identified by stained nuclei and F-actin. The cell numbers for each picture were labelled and counted using Image J software. The measurements were presented as mean ± standard error of the mean (SEM). Cell proliferation on day 3 and day 7 were also analyzed by comparing the percentage changes in total cell numbers. Statistical analysis was performed using ANOVA following post hoc Fisher’s test.

C. Platelets Study

a. Preparation of Platelet Rich Plasma

Adhesion and morphology of the platelet on various substrates were observed using confocal and electron microscopy. The microscopic images were used to quantify the amount of adherent platelets. Resting platelet had a discoid shape, while the shape of activated platelet was spiky and nonuniform. Briefly, human whole blood (a kind gift from the Laboratory of Dr. Roy Hantgan at Biochemistry Department, Wake Forest University) was drawn into a 2.7 ml BD Vancutainer® Coagulation Tube (BD, Franklin Lakes, NJ). To isolate platelet-rich
plasma, the whole blood was centrifuged at 800 rpm for 15 min at 25°C with Harrier 18/80 centrifuge (Sanyo Gallenkamp, Loughborough, UK). Platelet rich plasma (PRP) was collected from the supernatant (about 1/2 of the total volume) and counted using a cytomter.

b. Platelet Adhesion Test on PFC Mat

PFC mats, type I collagen mats, rat tail type I collagen (BD Biosciences, Bedford, MA) hydrogels were placed in the culture dish. After the concentration of platelets in PRP was determined, PRP was diluted (1:2) in 37°C PBS solution to seed on the substrates at $2.16 \times 10^8$ platelets/ml. The same amount of 100µl diluted PRP was carefully applied in the center of the substrates to cover the whole surface of PFC mats, type I collagen mats, collagen gels, and plain polysterene culture dish. To select an optimal incubation time for comparing platelet adhesion and activation on these substrates, a series of incubation time points (15min, 30min, or 60min) were performed. F-actin, which is the contractile protein expressed in platelets, was stained with rhodamine-phalloidin to observe platelets adhered on the substrates using confocal microscopy. Samples for microscopic observation were prepared as follows. After two gentle washes with phosphate buffer solution (PBS, pH=7.4), samples were fixed with 2.5% glutaraldehyde. The whole field of the material was first observed under low magnification (10X) and photomicrographs (20X) were used for platelet counting (n=3). The quantity of platelets could be easily visualized using the confocal microscopic images and quantified by counting the total number of platelet
adhesion using Image J software. Three distinct fields were used from representative areas (6% of the total area in images) in the photomicrograph that was divided by a 4 by 4 grid (magnification: 20X). The platelets were labelled and counted using a cell counter in Image J software and the average amount of platelets were presented as mean ± standard error of the mean (SEM).

Platelets on different substrates were also observed using scanning electron microscopy with high magnification. Platelets activation was identified by shape changes which were classified as discoidal shape, early dendritic pseudopodial shape, spread dendritic shape, spreading phase with pseudopodial and hyaloplasm spreading, and fully spreading stage with hyaloplasm (Wan and Xu 2009). Scanning electron microscopic images were taken to observe the morphology of platelets in order to identify the platelet activation stages. After fixing the samples of platelet seeded PFC mats with 2.5% glutaradehyde for 1 hour, the samples were washed with PBS. To prepare samples for scanning electron microscopy, the samples were dehydrated by processing with a graded ethanol (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%) following critical point drying with hexamethyldisilazane (Cellular Imaging Shared Resource, Comprehensive Cancer Center of Wake Forest University).

c. Platelet Adhesion in Association with Cells

To understand the influence of endothelial cell on platelet adhesion, a second treatment group was used by first culturing HUVECs on different substrates for 24 hours followed by platelets seeding. Platelets and cells were fixed with 2.5%
glutaraldehyde following rhodamine-phalloidin staining for F-actin and sytox green for nuclei. Images were acquired using confocal microscopy. For the treatment groups co-incubated with HUVECs, the fluorescence intensity of platelets F-actin staining was higher than HUVECs. Therefore, the amount of platelets could be distinguished from endothelial cells by their size, shape and fluorescence intensity. The gains of rhodamine, FTIC (fluorescein isothiocyanate), DIC (differential interference contrast), and overlay channels were adjusted to obtain the optimal intensity for counting the platelets. The same counting procedure was used as following: Three distinct fields were used from representative areas (6% of the total area in images) in the photomicrograph which was divided by a 4 by 4 grid (magnification: 20X). The platelets were labelled and counted using a cell counter in Image J software. The average number of counted platelets were presented as mean ± standard error of the mean (SEM). In order to understand the role of co-incubated endothelial cells in affecting platelet adhesion, two types of fields were selected for counting. One was platelets associated with HUVECs in the counted area (n=3), and the other was platelets associated with no HUVECs in the counted area (n=3).
V. Statistical Analysis

Data were presented as mean ± standard error of the mean (SEM) unless otherwise noted. Statistical significance was determined using one-way analysis of variance (ANOVA) following post hoc or nonparametric tests for multiple groups when appropriate. A value of $p < 0.05$ was considered statistically significant. StatView 5.0 (SAS Institute, Cary, NC) was used to perform the statistical analyses.

For comparing elastic modulus, stress, and strain of different electrospun mats, one way ANOVA following post hoc Fisher’s protected least significant difference (LSD) test was used. Kruskal-Wallis one way analysis of variance on ranks following post hoc Tukey test was performed to compare the differences in fiber diameters during degradation in PBS solution. Cell proliferation on PFC mats and culture dishes at day 3 and day 7 were compared using one-way ANOVA following Fisher’s test. The effect of different substrates on platelet associated with and without cells in the counted area were also tested using a one way analysis of variance (ANOVA) following post hoc Fisher’s LSD test. Moreover, the percentage differences and changes in folds of total adhered cells or platelets numbers were calculated from the mean.
RESULTS

I. Characterization of Silk Fibroin

After boiling and washing, the yield of degummed silk fibroin was 88% of the total weight of initially used raw silk. Degummed silk was dissolved in 5.0M CaCl$_2$ solution and an amber color silk fibroin solution was obtained. Dialysis removed the salt from fibroin solution which was then freeze dried to obtain white colored silk fibroin powder for further characterization.

A. Amino Acid Composition

The amino acid composition of the extracted fibroin agreed with protein sequence database (Swiss-Prot accession number for fibroin: PO5790 and for sericin: PO7856) and published literature data of silk fibroin (Schroeder, Kay et al. 1955). Mole fraction of major amino acids glycine, alanine, and serine comprised 82% of the total amino acid composites in the extracted silk fibroin (Table III). This can be compared to the reported 88% total mole fraction of glycine, alanine, and serine composition. Therefore, a purity of 93% was estimated for extracted silk fibroin used in this study. Glycine, alanine and serine accounted for 43%, 25% and 13% in fibroin extraction respectively. These were close to the reported values (Table III). However, the values were very different from sericin in silk that has 11% glycine, 70% alanine, and 33% serine (Swiss-Prot accession number for sericin: PO7856).
Table III Amino acid composition of silk fibroin (*Bombyx mori*)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mole Fraction</th>
<th>Measured</th>
<th>Reported</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>Alanine</td>
<td>25</td>
<td>28</td>
<td>34</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.4</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>1.5</td>
<td>N/A</td>
<td>2.3</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>1.5</td>
<td>N/A</td>
<td>1.7</td>
</tr>
<tr>
<td>Glycine</td>
<td>43</td>
<td>44</td>
<td>43</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.93</td>
<td>N/A</td>
<td>0.90</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.91</td>
<td>N/A</td>
<td>0.81</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.45</td>
<td>N/A</td>
<td>0.56</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.94</td>
<td>N/A</td>
<td>1.3</td>
</tr>
<tr>
<td>Serine</td>
<td>13</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.6</td>
<td>1.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6.0</td>
<td>N/A</td>
<td>11</td>
</tr>
<tr>
<td>Valine</td>
<td>3.0</td>
<td>N/A</td>
<td>3.1</td>
</tr>
</tbody>
</table>

B. Molecular Weight of Extracted Silk Fibroin

To further identify extracted silk fibroin protein, gel electrophoresis was used to determine the molecular weight. There are two major proteins in raw silk, silk fibroin and sericin. Fibroin is composed of heavy and light polypeptide chains with molecular weights of 390kDa and 25kDa. The molecular weight of sericin is 119kDa (Chen, Priewalder et al. 2010). Consistent bands indicating these two molecular weights were observed for each sample of fibroin at around 390kDa and 25kDa. The intensities of both 8M urea and beta-mercaptoethanol (BME) solution treated samples (Lane 2 and 4) were slightly decreased compared to only 8M urea treated samples on Lane 1 and 3 but the positions of the bands remained the same (Figure 8). The band indicating the molecular weight of sericin protein at 119kDa was absent. The results of the gel electrophoresis suggested that fibroin was the only protein presented in the extract (Figure 8). It also suggested the sericin which potentially causes an in vivo inflammatory response was removed by the degumming process. This result agreed with the molecular weight of silk (Bombyx mori) reported on protein sequence database (Swiss-Prot accession number for fibroin: PO5790 and for sericin: PO7856). Therefore, this experiment identified the molecular weights of extracted silk fibroin and suggested that it was successfully purified by degumming to remove the other silk protein sericin.
Figure 8 Photograph showing the region of silk fibroin molecular weight on a 10% polyacrylamide gel after electrophoresis. The samples from left to right column were labelled as 1, 2, 3, 4 and 5. Lane 1 and 3: 8M urea treated solution. Lane 2 and 4: 8M urea and BME treated solution. Lane 5: molecular weight marker. The molecular weight of extracted silk fibroin heavy and light chain were approximately at 390kDa and 25kDa.
C. Protein Structure Characterization

Protein structures are of great importance for silk fibroin. The formation of anti-parallel β-sheet is the primary structure contributing to protein assembly, insolubility in water, and strength. The protein structures of raw silk, washed degummed silk and extracted silk fibroin are presented at different designated wavenumbers (cm$^{-1}$) in the FTIR spectra (Figure 9). By comparing to literature values, the results suggest the amide groups of raw silk and extracted silk fibroin were in the β sheet conformation (Um, Kweon et al. 2001; Cilurzo, Gennari et al. 2011). More specifically, the β sheet structure was illustrated by wavenumbers of amide I, II, III corresponding to the carbonyl stretchs 1619-1622 cm$^{-1}$, 1509-1516 cm$^{-1}$, and 1225-1233 cm$^{-1}$ respectively (Table IV). The wavenumber ranging from 3275-3282 cm$^{-1}$ was indicative of the β-N-H stretching vibration in amide A. A broad wave was observed for amide A in the extracted fibroin. The β sheets structure was shown with an absorption peak at 1700 cm$^{-1}$ which was present in both raw silk and washed silk. The peak at 1260 cm$^{-1}$, indicating amide III β sheets became weaker after washing and extraction. These two peaks were assigned to be the C=O stretch in amide I and the C-N stretch and C-N-H bend in amide III β sheets structure respectively (Hu, Kaplan et al. 2006; Hayashi and Mukamel 2007). The absorption at 1225-1233 cm$^{-1}$ was indicative of the amide III β sheets. Two strong peaks were shown at the regions of 1619-1622 cm$^{-1}$ for C=O stretch of amide I and 1509-1516 cm$^{-1}$ for the C-N stretch and C-N-H bend for amide II (Horan, Antle et al. 2005). In summary, FTIR spectra illustrated the structural features of raw silk, degummed and extracted silk fibroin. They also
suggested the primary β-sheet conformation in silk protein structure. Secondary
structure of β sheets were found in all raw silk, degummed and extracted silk
fibroin which suggested the extraction procedure did not change the structure of
silk protein significantly. The β sheets in the extracted silk protein could potentially
contribute to mechanical strength and stability of the graft materials.
Figure 9 Diagram presenting the spectrum which illustrates the chemical functional groups in silk using Fourier transform infrared (FTIR) spectroscopy.
Table IV Wavenumber ranges for the major functional groups

<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
<th>Description</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3275-3282</td>
<td>N-H stretch(^1)</td>
<td>amide A</td>
</tr>
<tr>
<td>1700</td>
<td>C=O stretch(^2)</td>
<td>amide I β sheets</td>
</tr>
<tr>
<td></td>
<td>(shoulder)</td>
<td></td>
</tr>
<tr>
<td>1619-1622</td>
<td>C=O stretch(^3)</td>
<td>amide I β sheets</td>
</tr>
<tr>
<td></td>
<td>C-N stretch(^4)</td>
<td></td>
</tr>
<tr>
<td>1509-1516</td>
<td>C-N-H bend(^5)</td>
<td>amide II β sheets</td>
</tr>
<tr>
<td>1260</td>
<td>C-N stretch(^6)</td>
<td>amide III β sheets</td>
</tr>
<tr>
<td></td>
<td>C-N-H bend(^6)</td>
<td>(shoulder)</td>
</tr>
<tr>
<td>1225-1233</td>
<td>C-N stretch(^7)</td>
<td>amide III β sheets</td>
</tr>
<tr>
<td></td>
<td>C-N-H bend(^7)</td>
<td></td>
</tr>
</tbody>
</table>
II. Production and Characterization of Electrospun Mats

To mimic the structure and create a strong valvular ECM, electrospinning was used. Electrospinning followed the standard method that was described earlier to form randomly aligned fibers constructed mats. The electrospun mats were 12cm by 12cm in width and length and 0.1-0.3mm in thickness, measured by a Veiner digital caliper. After crosslinking, the color of electrospun mats changed from white to light yellow. After 1 hour soaking in 0.2M glycine solution and 1 hour rinse in DDH$_2$O, the electrospun mats were air dried. Samples were taken from the dried electrospun mat for fiber morphology observation and fiber diameter measurements using images taken by scanning electron microscopy.

A. Fiber Morphology Characterization using Scanning Electron Microscopy

The fiber morphology including size and construct porosity has a significant role in microenvironment cues and nutrient transport for in vivo applications. The interconnected fiber network structures of electrospun mats at various collagen, fibroin and PGS weight ratios were compared after crosslinking using scanning electron microscopy (SEM). Figure 10 shows the SEM images of fibers prepared with different collagen: fibroin: PGS weight ratios of 9:0:1, 8:1:1, 4.5:4.5:1, 1:8:1, and 0:9:1. The fiber diameters were measured for 16 randomly selected fibers from two representative SEM pictures of different areas. The fiber diameters ranged from 600 - 3000 nm, and the measurements are shown as mean ± standard error of the mean in Table V. Highly porous and interconnected matrix structures were observed in all electrospun mats, while the fiber diameters and
morphologies were influenced by different weight ratios of the composites. Thinner and rounder fibers were observed for the electrospun mats (Figure 10 D and E) with higher fibroin content (90% and 80%) as compared to the thicker and flatter fiber structures for electrospun mats (Figure 10 A, B, and C) with high proportions of collagen (90%, 80%, and 45%). The porous structure was also present in greater homogeneity and quantity but smaller in size when there was a higher fibroin composition (Figure 10 D and E). In summary, the results suggest that even when the concentrations of electrospinning solution are the same, different composites at various ratios in the blends show changes in fiber morphologies and matrix structure of the constructs which could be an effect of dynamical evaporation of solvents out from the electrospun fibers.
Figure 10 Scanning electron microscopic images illustrating the morphologies of the electrospun fibers. The diameters of the random arrays of electrospun crosslinked fibers after crosslinking, and treatment with glycine and water are shown. (A) Collagen: PGS (9:1); (B) collagen: fibroin: PGS (8:1:1); (C) collagen: fibroin: PGS (4.5:4.5:1); (D) collagen: fibroin: PGS (1:8:1); (E) fibroin: PGS (9:1). With higher silk fibroin protein content, smaller fiber diameters were observed (Magnification 2300X).
Table V Fiber Diameter Measurements

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Fiber Diameters (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen : PGS (9: 1)</td>
<td>2067 ± 168</td>
</tr>
<tr>
<td>Collagen: Fibroin: PGS (8: 1: 1)</td>
<td>4577 ± 697</td>
</tr>
<tr>
<td>Collagen: Fibroin: PGS (4.5: 4.5: 1)</td>
<td>2952 ± 240</td>
</tr>
<tr>
<td>Collagen: Fibroin: PGS (1: 8: 1)</td>
<td>784 ± 77</td>
</tr>
<tr>
<td>Fibroin: PGS (9: 1)</td>
<td>694 ± 43</td>
</tr>
</tbody>
</table>

Measurements are presented as mean ± SEM (n=2).
B. Characterization of Chemical Functional Groups in Electrospun Mats using FTIR

To further identify the effect of various blends to the protein and polymer structural changes, electrospun mats were analyzed using FTIR. Pure proteins and synthetic polymer were first measured individually to show the characteristic spectra of the composites. Amide A band was observed in extracted silk fibroin as a sharp peak at 3299 cm\(^{-1}\) indicating the \(-N-H\) stretch (Figure 11). The type I collagen had a characteristic broad peak at the absorption of 3275 cm\(^{-1}\) which was indicative for the \(-O-H\) stretch and N-H stretch in this region (Figure 11-1). PGS polymer (after the 48 hour heat treatment at 120\(^{\circ}\)C) had a distinct FTIR spectrum which showed a broad OH stretch at 3458 cm\(^{-1}\). This characteristic was reported to indicate the hydrogen bonded hydroxyl groups in PGS (Wang, Ameer et al. 2002). Sharper transmittance peaks at 2928 cm\(^{-1}\) and 2855 cm\(^{-1}\) (Figure 11-2) were shown to represent the sp3 C-H stretch (asymmetry and symmetry). Moreover, the intense ester C=O stretch at the absorption of 1734 cm\(^{-1}\) (Figure 11-3) was another unique feature in PGS which was not presented in either collagen or fibroin proteins. In the collagen, fibroin, and PGS blended electrospun mats, there was a decrease in the broadness of the peak in the region from 3280 – 3306 cm\(^{-1}\) as the content of collagen decreased and the content of fibroin increased. A weak transmittance peak suggested the presence of PGS in the electrospun mats, showing the absorption peak at 1734 cm\(^{-1}\) as well as 2927-2931 cm\(^{-1}\).
Amide III was at the absorption range of 1228 – 1237 cm\(^{-1}\) (Figure 11-6). Amide I, II were present at the absorption range of 1622-1630 cm\(^{-1}\) (Figure 11-4) and 1546-1515 cm\(^{-1}\) (Figure 11-5) respectively. All these characteristic protein structures were present in the designated wavenumber regions which suggests homogenous blends of each composite (collagen, fibroin, and PGS). Regions of amides slightly shifted from higher wavenumbers to lower wavenumbers as the collagen content decreased and fibroin content increased. These shifts corresponded well to the random coil structure that was predominant in collagen and the β sheet structure that was characteristic in fibroin as the composite ratio changes (Hu, Kaplan et al. 2006; Zoccola, Aluigi et al. 2008).
Figure 11 Diagram illustrating the spectrum characterized chemical functional groups of composites in electrospun mats using Fourier transform infrared (FTIR) spectroscopy. The spectrum of different electrospun mats revealed the presence of incorporated collagen, fibroin, or PGS composites blended in the fabricated construct.
C. Thermal Transition Analysis of Electrospun Mats

Evaluating the phase transitions of biomaterials due to temperature changes is important for in vivo applications. Graft materials need to maintain the mechanical integrity when implanted into the body (37°C) from room temperature (22°C) or storage (4°C). Therefore, conformational and phase change of proteins and polymer was examined using DSC to perform thermal analysis (Figure 12). Electrospun mats were tested along with the pure composites for their thermal transitions with increasing temperature at a constant rate as described in the methods. Fibroin has been reported to transform from crystalline to amorphous phase at a glass transition temperature at around 178°C (Zoccola, Aluigi et al. 2008). An endotherm with a transition temperature around 180°C in the DSC curve was observed in this study (Figure 12). This suggests that silk fibroin is thermodynamically stable at body temperature (37°C). On the other hand, collagen denatures at a lower temperature which can be observed as a broad endotherm near 100°C. This denature temperature suggested the disruption of hydrogen bonds between the polypeptide chains and the conformational changes from an organized triple helical structure to a random coil structure.

The electrospun mat made with a high collagen content such as collagen: fibroin: PGS (9:0:1) had a strong endotherm for the collagen denaturation. As the fibroin content increased in the electrospun mat composition, this endotherm became smaller. This phase transition change in terms of DSC endotherm curves suggested fibroin introduced a relatively thermodynamically stable
component into the overall electrospun mats. Both denature temperatures and glass transition temperatures could be clearly identified for collagen: fibroin: PGS (4.5: 4.5: 1 and 8:1:1) (Figure 12). The endotherms of thermal transition also suggested the existence of both amorphous and crystalline regions in the composites of electrospun mats. This versatile combination of fibroin with collagen and PGS creates a material that has high phase transition temperatures. This high phase transition temperature property of the material provided the electrospun mats with thermodynamical stability to maintain their structural and mechanical integrity as heart valve tissue substitutes.
Figure 12 Diagrams illustrating thermal transition temperatures of electrospun mats using differential calorimetry
D. Porosity of the Electrospun Mats

Porosity measurements of electrospun mats are shown in Figure 13. The results are presented as means ± standard error of the mean (SEM) with a sample size of 3 replicates for each group. High porosity with an interconnected porous network in electrospun mats could potentially improve nutrient transport and surface area of cell attachment, as compared to materials with low porosity. The porosity of the electrospun mats ranged from 67% to 80%. Statistically significant differences were found in collagen-fibroin-PGS (in 0:9:1 and 4.5:4.5:1 weight ratios) compared to all other groups (p<0.05). Fibroin: PGS (9:1) had the lowest porosity value of 67% and collagen: fibroin: PGS (4.5:4.5:1) had a porosity of 73%. The mat made using collagen: fibroin: PGS (1:8:1) was too fragile to obtain a sufficient piece of intact sample for the porosity test. The porosities for collagen: fibroin: PGS (8:1:1, 9:0:1, and 1:0:0) were 79%, 80% and 78%, respectively. From this experiment, the porosities of electrospun mats made of collagen: fibroin: PGS (8:1:1, 9:0:1, and 1:0:0) were not significantly different from each other. All the electrospun mats had a porosity greater than 60%, which could potentially allow efficient gas and nutrient transport as well as sufficient surface area for cell to attach.
Figure 13 Measurements of porosity for electrospun mats. Each bar represents mean ± standard error of the mean of the porosity measurements for electrospun mat with various composite weight ratios (n=3). Bars designated with the same letter are not significantly different, whereas bars with different letters are significantly different (p<0.05).
E. Mechanical Tensile Testing

Mechanical properties of the electrospun mats were characterized and compared with the reported values of collagen-based aortic valves. Table VI presents the uniaxial tensile mechanical testing results (mean ± SEM, n=5) of electrospun mats. The elastic modulus of the electrospun mats ranged from 2.25 Mpa (Collagen: PGS = 9: 1) to 4.97 Mpa (Fibroin: PGS = 9: 1). The electrospun mats with 4.5: 4.5: 1 and 8: 1: 1 collagen to fibroin to PGS weight ratios had elastic moduli of 4.11 Mpa and 2.76 Mpa respectively. The elastic modulus of electrospun mats with 0:9:1 collagen to fibroin to PGS weight ratio was significantly different from all other groups (p<0.05). Whereas elastic modulus of electrospun mats with 4.5: 4.5: 1 collagen to fibroin to PGS weight ratio was significantly different from all other groups (p<0.05), except electrospun mats with collagen composites alone. Electrospun mats with collagen alone generated in this study had a similar elastic modulus of 3.67 Mpa compared to collagen-based porcine valvular grafts: 3.68 Mpa fresh valves and 3.95 glutaraldehyde fixed valves (Vesely and Noseworthy 1992). Modified electrospun mats with an increased fibroin content (>45% by weight) showed a comparable elastic modulus to the collagen-based heart valve grafts.

Tensile stress is defined as the force per surface area. Values of stress were found between 0.69 Mpa to 1.45 Mpa for electrospun mats. Highest and lowest tensile stresses were found to be 1.45 Mpa and 0.69 Mpa respectively in electrospun mats with collagen: fibroin: PGS at 4.5: 4.5: 1 weight ratio and pure
collagen electrospun mats. Electrospun mats with collagen: fibroin: PGS 4.5:4.5:1 had tensile stress significantly different from all other electrospun mats except mats of 9: 0: 1 (collagen: fibroin: PGS composites). These values were 100-fold higher than the physiological blood pressure in normal or hypertension stage (Table II). Therefore, the electrospun mats could be considered to be sufficient in withstanding the physiological blood pressure as they performing the function of a heart valve grafts.

Usually after glutaraldehyde crosslinking, the elastic properties of collagen-based grafts become compromised as the grafts become less elastic compared to the fresh aortic valve tissues (Vesely and Noseworthy 1992; Newton, Mahajan et al. 2009). This effect was predominantly found in pure collagen electrospun mats with a strain of 23%. In the present study after crosslinking with 15% glutaraldehyde vapor, the percentages of elongation over the original length (tensile strain) of the electrospun mats ranged from 33% to 62%. These values of electrospun mats tensile strain were similar to the reported strains of fresh aortic valves: between 20% to 62% (Vesely and Noseworthy 1992). Based on the results of uniaxial tensile test, the stresses, strains and elastic moduli of electrospun mats were found to be similar to the reported values of fresh aortic valves. This similarity in mechanical properties suggests that the electrospun mats could potentially be used as an aortic heart valve graft while blood is being pumped from the heart.
Table VI Uniaxial Tensile Testing

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Elastic Modulus (Mpa)</th>
<th>Stress (Mpa)</th>
<th>Strain (mm/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>3.67±0.12^a^</td>
<td>0.69±0.06^a,d^</td>
<td>0.23±0.03^a^</td>
</tr>
<tr>
<td>Collagen: PGS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(9:1)</td>
<td>2.25±0.16^b^</td>
<td>1.30±0.09^b,c^</td>
<td>0.62±0.02^b^</td>
</tr>
<tr>
<td>Collagen: Fibroin: PGS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(8:1:1)</td>
<td>2.76±0.20^b^</td>
<td>1.10±0.09^b^</td>
<td>0.44±0.03^b,c^</td>
</tr>
<tr>
<td>Collagen: Fibroin: PGS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4.5:4.5:1)</td>
<td>4.11±0.13^a,c^</td>
<td>1.45±0.05^c^</td>
<td>0.41±0.01^a,b,c</td>
</tr>
<tr>
<td>Collagen: Fibroin: PGS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1:8:1)</td>
<td>N/A: too brittle to be determined</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroin: PGS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(9:1)</td>
<td>4.97±0.27^d^</td>
<td>0.82±0.09^d^</td>
<td>0.33±0.12^a,c^</td>
</tr>
</tbody>
</table>

Table illustrating the tensile stress, strain and modulus of electrospun mats

Data are presented as mean ± standard error of the mean (n=3). Numbers designated with the same letter are not significantly different, whereas numbers with different letters are significantly different (p<0.05).
**F. Modified Suture Retention Strength Testing**

The suture retention test assessed the maximum pull-out force that could be held by electrospun mats and procine heart valve leaflets at single punctured suture sites (Table VII). Collagen: Fibroin: PGS (4.5: 4.5: 1) showed the highest suture pull-out strength with a maximum load of 0.32 N as compared to the maximum load of 0.64 N for fresh porcine heart valve. Sutured samples made using collagen: fibroin: PGS (1: 8: 1) were too fragile to test, as they ruptured while loading the suture and electrospun mat on to the mechanical tester. Fibroin: PGS (9: 1) and collagen: fibroin: PGS (8: 1: 1) ruptured when loading the suture specimen onto the mechanical tester or broke at an average low load of 0.05N.
<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Maximum load at the break (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen: PGS</td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>0.12</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.15</td>
</tr>
<tr>
<td>Collagen: Fibroin: PGS</td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>0.07</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.03</td>
</tr>
<tr>
<td>Collagen: Fibroin: PGS</td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>0.35</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.28</td>
</tr>
<tr>
<td>Collagen: Fibroin: PGS</td>
<td>N/A: specimen too fragile to obtain</td>
</tr>
<tr>
<td>Sample 1</td>
<td></td>
</tr>
<tr>
<td>Sample 2</td>
<td></td>
</tr>
<tr>
<td>Fibroin: PGS (9:1)</td>
<td>N/A: Tear at the suture site at preloading</td>
</tr>
<tr>
<td>Fresh Porcine</td>
<td>Sample 1</td>
</tr>
<tr>
<td>Heart Valve</td>
<td>Sample 2</td>
</tr>
</tbody>
</table>
III. Functionality Assessment

Based on results of electrospun mats characterizations, collagen-fibroin-PGS electrospun mats with a 4.5: 4.5: 1 weight ratio (PFC mats) were found to have the optimal mechanical properties that were similar to a fresh aortic valve. The chemical and physical properties of the PFC mats were clearly identified and showed sufficient properties for porosity and thermodynamic stability. Therefore, the PFC mats were selected to perform the functionality tests for evaluating degradation and biocompatibility by interacting with endothelial cell and blood platelets.

A. Degradation of PFC Mats

Degradation was tested by measuring the weight loss of PFC mat soaked in PBS solution at 37°C. This test was designed to facilitate the understanding of graft material degradation in vivo. To become a functional heart valve graft, neither the composition nor the structure should be lost significantly in order to maintain the mechanical integrity and structural geometry. There was a negligible weight loss with a 0.01% increment in total weight loss per week (Figure 14). With an unaided eye, PFC mats stayed intact during the degradation time period with no visible physical changes observed. Microscopically, unchanged fiber morphologies of the PFC mats were observed and illustrated in Figure 15. From the scanning electron microscopy images, a consistent morphology of fibers was observed (Figure 15). No visible surface erosion of the fibers was observed with increasing exposure time in PBS solution at 37°C. The structure of the randomly
aligned fiber meshwork was also maintained during the study. Measurements were acquired using Image J software (NIH) and further confirmed an unchanged mean of fiber diameters (Figure 16). The results were presented as mean ± standard error of the mean (SEM). No significant difference was found in fiber diameters of PFC mats at different time points during soaking and incubation.
Figure 14 Graph illustrating percentage weight loss of PFC mat over time. Over a 9-week period, percentage weight loss versus time shows a linear increment with 1% decrease in weight per week. Each data point is presented as mean ± SEM (n=4).
Figure 15 Scanning electron microscopic images (A, B, C, D, E, and F) illustrating the morphological features of PFC fibers at week 3, 5, 6, 7, 8, 9 (Magnification 1500X).
Figure 16 Graph showing the fiber diameter measurements of PFC mat at 3, 5, 6, 7, 8, and 9 weeks of degradation in PBS. Data were presented as mean ± standard error of the mean (n=2). No significant difference in fiber diameters among groups at different time points.
B. Cell Adhesion and Proliferation Study

a. Cell Compatibility Test

Biocompatibility is defined as the ability of biomaterials to perform appropriate host response (Ratner 1996). A heart valve graft needs to be biocompatible in order to be functional. The biocompatibility of PFC mats was tested by observing cell adhesion and spreading on the material. An initial experiment was completed to determine the interactions of cells with the PFC mats as a mean to assess material biocompatibility.

For the first experiment, three different types of cell lines, HUVEC (endothelial), HEPM (fibroblast), and GMMe (epithelial) were cultured on PFC mats. All cell lines tested adhered to PFC mats and some cells appeared to stretch between fibers. The general, the morphology of each cell type was maintained (Figure 17). It is apparent from Figure 17 that cells interacted with the fibers to moderately affect their morphology that compared to that reported by ATCC for each cell line grown on culture dishes.
Figure 17 Confocal image illustrating the adhesion and morphology of three different cell lines on the PFC mats. PFC mats for cell seeding were prepared using the same fabrication, crosslinking and sterilization procedures. Cells were plated at 30,000 cells/well onto the PFC mats having 0.1-0.3mm in thickness in a 48-well plate. The images were acquired after 3 days of culturing. Association and interaction of all cells with fibers were observed. Cells were stained for F-actin protein (red) using rhodamine-phalloidin and for nuclei (green) using sytox green (Magnification 40x).
b. Cell Proliferation and Endothelial Monolayer Formation

Formation of a monolayer of endothelial cells on blood contacting biomaterials is physiologically significant because it is a natural protectant to prevent thrombosis. In another experiment, the proliferation of HUVECs on PFC mats was tested and compared with cells cultured on a polystyrene tissue culture dish (Figure 18). The amount of cells on PFC mats or culture dishes was quantified by counting cell numbers with four different confocal images (n=4) at two different time points (Figure 19). From day 3 to day 7, the cell number on PFC mats and culture dishes increased significantly (p<0.001). Total cell numbers on PFC mats and culture dishes increased 2.5-fold and 1.8-fold respectively from day 3 to day 7. Although there was more cell growth on PFC mats compared to culture dishes on day 7, the cell numbers were not statistically different.

Cells cultured on PFC mats appeared morphologically more distended. At 3 day of culture, HUVECs on culture dishes appeared evenly spread without significantly cell-cell interactions, whereas on PFC mats, cells appeared as foci of cells (Figure 18). This was especially apparent at day 7, where a more organized uniform cell sheet was observed for cells on PFC mat compared to cells on culture plate.
Figure 18 Confocal microscopic image illustrating HUVEC morphology and proliferation on the PFC mat (A and B) or culture dish (C and D) after 3 day (A and C) or 7 day (B and D) of culture. Cells were seeded at 50,000 per dish on a 48-well plate. Representative photomicrographs depict increased cell number on cells grown on both substrates. Cells were stained for F-actin protein (red) using rhodamine-phalloidin and for nuclei (green) using sytox green (Magnification 20x; Scale bar: 50µm).
Figure 19 Cell numbers for HUVEC after 3 or 7 days of growth on culture dishes and PFC mats. Bars represent the mean ± standard error of the mean (SEM) for each observation from representative photomicrographs (n=4). An increase number of HUVECs was observed from day 3 to day 7. For cells on both substrates, cell numbers increased significantly from day 3 and day 7 (*p < 0.001).
Upon examining the cells at a higher magnification, areas demonstrating tight cell-cell interactions were observed for cells cultured on PFC mats (Figure 20). As reported, F-actin indirectly binds to the endothelial tight junction protein such as VE-cadherin along the cell-cell junction. The intense staining pattern of F-actin at the cell borders suggests the formation of tight junctions. To better observe the interaction and association of cells with PFC mats, confocal images in Figure 21 revealed the relationship of the PFC mats with cells. A uniform endothelial monolayer was observed on the surface of the PFC mat. It is apparent that the cells interact significantly with PFC mat. This suggests that PFC mat effectively functions to organize the cells into an intact monolayer. To further investigate the formation of endothelial cell monolayer on the surface of the PFC mat, a confocal image constructed by a 32-slice stack of optical sections (z-series) was obtained. The three-dimensional image was reconstructed slice by slice from the top of the mat seeded with cells into the mat until reach the middle layer without cell presence. Side view and frontal view images illustrate the formation of a monolayer of HUVECs on surface of PFC mat (Figure 22). The formation of this endothelial monolayer suggests that PFC mats are capable of promoting endothelial cell grow into a functional protectant layer. This cell monolayer has physiological significance for potentially preventing thrombosis and improving the mechanical strength of the graft materials.
Figure 20 High magnification confocal image of HUVEC cultured on PFC mat for 7 days. A magnified region from Figure 18B clearly illustrated the cuboidal shape of endothelial cells. Formation of an intact cell monolayer and intercellular tight junction was suggested (white arrows). Cells were stained for F-actin protein (red) using rhodamine-phalloidin and for nuclei (green) using sytox green (Magnification 40x; Scale bar: 50µm).
Figure 21 Confocal images of HUVECs on PFC mats on day 3 (A, B, C, D) or day 7 (E, F, G, H). Confocal image channels of A, B, E, and F are identical to C, G, D, and H, except they overlay an additional transmission diascopic (DIC) channel to show PFC mats underneath. On day 7, increased amount of HUVECs covered the PFC mat surface was observed compared to day 3. HUVECs on the PFC mats showed cuboidal morphology. Cells were stained for F-actin protein (red) using rhodamine-phalloidin and for nuclei (green) using sytox green (A, C, E and G: Magnification 10x; B, D, F, and H: Magnification 40x; Scale bar: 50µm).
Figure 22 Projected confocal image (z-series) of a PFC mat with HUVECs seeded on top. On the top shows three dimensional cross-sectional view stacked from coronal stacked slices of the mat revealing the formation of endothelial monolayer on the PFC mat surface. The projected top view of the mat surface shows clear adjacent cell borders. Cells were visualized by staining F-actin (red) using rhodamine-phalloidin and nuclei (green) using sytox green (Magnification 20x; Scale bar: 50µm).
C. Platelet Adhesion Study on PFC Mats

a. Quantifying Adhered Platelets on Various Substrates

Several studies were completed to assess the thrombogenic nature of the PFC mats without and with HUVECs by coincubation with isolated blood platelets. For these studies, equal amounts of PRP (100µl, 2.16x10^8 platelets /ml) were incubated for 15min on various substrates. Platelet adhesion was determined microscopically by evaluating the number and size of the platelets or platelet aggregates formed on the substrates. Confocal images were acquired to count adherent platelets using Image J. In initial studies, platelets on different substrates incubated for 15, 30, and 60 minutes respectively were evaluated. Fifteen minutes was found to be an optimal incubation time for platelet adhesion studies. At 15 minutes, no spontaneous aggregates were observed, whereas at longer time periods some spontaneous platelet aggregates were observed (data not shown). Confocal images of platelets stained with rhodamine-phalloidin demonstrated a low level of adhesion of platelet on polystyrene culture dish (Figure 23C). Many of the platelets were present as single platelet or as small clumps of 2-3 platelets. In sharp contrast, platelets adhered to the thrombogenic collagen gel control revealed increased platelets numbers as well as increased sizes of aggregates (Figure 23D). Fewer platelets and platelet clumps were observed on PFC mats (Figure 23A) as compared to the collagen controls (Figure 23B).
Figure 23 Confocal images illustrating platelet adhesion on different substrates. PRP were plated with $1.08 \times 10^7$ platelets/dish in 48-well plate. The images were acquired after 15min of incubation on various substrate surfaces at 37°C. A: PFC mat, B: collagen mat, C: culture dish, D: collagen gel. There are more adhered platelets and formation of microthrombi on the collagen gel and collagen mat as compared to the culture dish and the PFC mat. Platelets (white arrows) were visualized by rhodamine-phalloidin staining (red) for F-actin protein (Magnification 40x; Scale bar: 50µm).
The numbers of adhered platelets on various substrates were counted using cell counter in Image J software. Data were presented as means ± SEM (Figure 24). One way ANOVA showed there was significant differences among the groups (p<0.001). Platelet numbers on the PFC mat, collagen mat, and collagen gel were, respectively, 2.4, 3.6 and 19 folds higher than on the culture dish. Differences in platelet number on the culture dish and collagen mat, culture dish and collagen gel were found to be significant (p<0.05). No statistically significant difference was found between number of adhered platelets on culture dishes and PFC mats. There was a 1.5 and 8 fold reduction in adhered platelet numbers on PFC mat comparing to the collagen mat and collagen gel respectively.

To further investigate the platelet adhesion on structurally similar PFC mats and collagen mats, the optical sections (z-series) were acquired by confocal microscopy to show adhered platelets on these two substrates (Figure 25). More platelets in larger aggregates were observed on the collagen mat surface than the PFC mat surface.

b. Morphological Identification of Platelets Activation

The morphologies of platelets were further observed using scanning electron microscopy to evaluate the degree of activation (Figure 26). The results demonstrate extensive activation and fused degranulated platelets when platelets were plated on collagen gels for 15 minutes (Figure 26 A and D). Fewer aggregates were observed on the electrospun collagen mat compared to the
collagen gel. It was apparent that platelets were activated based on the spreading morphology (Figure 26B and E). The majority of platelets associated with the PFC mat were spherical with defined border (Figure 26C and F) and less activated compared to the collagen mat and the collagen gel.
Figure 24 Number of adhered platelets on different substrates (culture dish, PFC mat, collagen mat, and collagen gel) after 15min incubation at 37°C. Numbers of adhered platelets counted from representative photomicrographs (n=3). Data were expressed as means ± SEM. Bars having the same letters are not significantly different, whereas bars with different letters are significantly different (p<0.05).
Figure 25 Confocal optical section images (z-series) illustrating association of platelets on PFC mat and collagen mat after 15min incubation at 37°C. A: z-series stack of adhered platelets on PFC mat (20µm with 21 slices into the material from the top until no platelet present in the mat); B: z-series stack of adhered platelets on collagen mat (58µm with 59 slices into the material from the top until no platelets were present in the mat); On the top is the cross-sectional side view of the coronal slices of the mat, while bottom is the top view of the mat. More platelets and larger aggregates were observed on the collagen mat surface than on the PFC mat surface. Platelets were visualized by rhodamine-phalloidin staining (red) for F-actin protein (Magnification 40x; Scale bar: 50µm).
Figure 26 Scanning electron micrographs of collagen gel (A and D), collagen mat (B and E) and PFC mat (C and F) after 15 minutes of incubation with PRP. The images demonstrate the presence of a single layer of platelets on the PFC mat and minor platelet activation in comparison to activated platelets on the collagen mat and collagen gel (Magnification: 1500X for A, B, and C, scale bar: 10µm; 5000X for D, E, and F, scale bar: 1µm).
D. Coincubation of Platelets with Cells

To further mimic the *in vivo* conditions under which the grafts will be implanted, morphological features of platelets co-incubated with same amount of HUVECs (50,000 cells/well in 48-well plate) were examined (Figure 27). The interactions of cells, platelets, and PFC mats were quantified and analyzed by counting the number of adhered platelets (Figure 28). There were more adhered platelets on collagen mat and formation of microthrombi on collagen gel as compared to single or clumps of 2-3 platelets on culture dishes and PFC mats. Both the size and number of rhodamine-phalloidin stained platelets on collagen gels were larger than all three other groups.

For platelets co-incubated for 15min with HUVECs that were pre-seeded and cultured for 24 hours (Figure 28), one way ANOVA following Fisher’s LSD test indicated that there was a significant difference among the groups (p<0.05). A 60% reduction in total adhered platelets on the PFC mat was observed as compared to the culture dish (p <0.05). The number of platelets decreased 2.9 fold on PFC mat comparing to the collagen mat (p<0.05), and 2.2 fold comparing to the collagen gel (p<0.05). There was no significant difference between dish and collagen mat; dish and collagen gel; and collagen mat and collagen gel.

For platelets associated with no cells in the counted area (Figure 28), there was a significant difference among different substrates (p<0.001). The amount of platelets associated with no cells on PFC mat was 2 fold higher than the amount of platelets on culture dish (p<0.05). There was a 27% reduction of platelet
number on the PFC mat compared to the collagen mat. No significant difference was found between PFC mats and collagen mats. There were very large increases in the number of adhered platelets on collagen gels compared to PFC mats, and the difference of numbers was significant (p<0.001). On the collagen mat and collagen gel, the total numbers of platelets found to be 2.6 and 5.1 fold higher compared to the negative control polystyrene culture dish (p < 0.05). Three-dimensional cross-sectional and frontal views of the confocal images revealed the association of platelets with cells on PFC mats and collagen mats. An increased quantity and size of platelets was observed on the collagen mat surface compared to the PFC mat surface (Figure 29).
Figure 27 Confocal images illustrating the association and interaction of platelets with HUVECs on different substrate. PRP were plated at $1.08 \times 10^7$ platelets / dish in 48-well plate. The images were acquired after 15min of incubation on various substrate surfaces at 37°C. A: PFC mat, B: collagen mat, C: culture dish, D: collagen gel. Quantity and size of the adhered platelets (white arrowheads) co-incubated with cells (yellow arrowheads) was observed. Formation of microthrombi on the collagen gel and increased size of platelet aggregates was observed on the collagen gel and collagen mat as compared to the culture dish and PFC mat. F-actin (red) of cells and platelets were stained using rhodamine-phalloidin and nuclei (green) of cells were stained using sytox green (Magnification 40x; Scale bar: 50µm).
Figure 28 Number of associated platelets with cells on different substrates (culture dish, PFC mat, collagen mat, and collagen gel) after 15min incubation at 37°C. Numbers of adhered platelets were counted from representative photomicrographs (n=3). Data are expressed as mean ± standard error of the mean, p<0.05 was considered significant. Bars having the same letter are not significantly different, whereas bars with different letters are significantly different (p<0.05).
Figure 29 Confocal optical section images (z-series) illustrating association of platelets with HUVECs on a PFC mat and collagen mat after 15min incubation at 37°C. On the top is the cross-sectional side view of the coronal slices of the mat, while bottom is the top view of the mat. A: z-series stack of adhered platelets and cells on the PFC mat (66µm with 67 slices into the material from the top until no cells or platelets were present in the mat); B: z-series stacked for adhered platelets and cells on the collagen mat (42µm with 43 slices into the material from the top until no cells or platelets were present in the mat). More platelets were observed on the collagen mat surface than the PFC mat surface. F-actin (red) of cells and platelets were stained using rhodamine-phalloidin and nuclei (green) of cells were stained using sytox green (Magnification 20x; Scale bar: 50µm).
DISCUSSION

Creating heart valve grafts is one of the main targets in cardiovascular tissue engineering (Yacoub and Takkenberg 2005). The goal is to reproduce the composition, structure, as well as repopulate the grafts into a healthy, durable analogous valvular tissue (Mol 2004; Yacoub and Takkenberg 2005; Mirensky and Breuer 2008). Decellularization of xeno or allografts, cell seeding and graft material preconditioning are common strategies in creating a tissue engineered heart valves (Dohmen, Lembcke et al. 2011; Ruzmetov, Shah et al. 2012). These methods reduced foreign body reaction through the removal of donor cells followed by seeding with autologous cells from patients (Hopkins 2005; Cebotari 2011; Ruzmetov, Shah et al. 2012). However, potential challenges remain in how to control graft mechanical properties, material degradation, and biological response in order to obtain a functional, durable and mature heart valve.

Proliferation of endothelial cells forming the monolayer and interstitial cells depositing ECM proteins usually have been considered as the first landmark for valvular grafts to become functional (Butcher, Mahler et al. 2011). Cell proliferation, cell-cell/ECM interactions, and cell function such as the production of non-thrombogenic glycocalyx are essential. However, cell growth and production of the ECM composites cannot occur simultaneously upon implantation (Butcher, Mahler et al. 2011). Tissue remodeling requires the valvular implants to adapt to native physiological condition, as well as to provide suitable chemical and mechanical microenvironment cues for cell proliferation to
occur. Therefore, it is vital to not only reproduce analogous native compositions, but also to modify the grafts in order to offer mechanical strength under physiological conditions.

PFC mats produced in this study had superior strength and flexibility. The β sheet structure of fibroin resulted in a stiff but brittle material. However, blending silk fibroin with collagen and PGS created a tough material with elastic properties. Elastic modulus, tensile stress, and strain of the PFC mats were found to be similar to fresh heart valve tissue. This suggested that the PFC mats have the potential to meet bending and tensile stress requirements during cardiac cycles while valvular leaflets opening and closing. These mechanical properties suggested PFC mats were able to providing instantaneous structural and functional support when implanted in vivo.

In the suture retention tests, PFC mats showed sufficient pull-out strength as compared to electrospun mats of other composite ratios and the fresh porcine heart valve. This resistance and stability at the suture site implies PFC mats could be used as surgical implants. Although there was weaken effect caused by the suture, it could be improved in vivo as the tissue grows and remolds (Edwards, Draper et al. 2005).

Creating valvular grafts that could retain mechanical integrity by resisting cell-mediated mechanical buckling and microstructure failure is another major challenge (Cebotari 2011; Dijkman, Driessen-Mol et al. 2012). Cell mediated
structural shrinkage due to buckling could lead to functional failure of the grafts in forms of valvular regurgitation (Cebotari 2011; Dijkman, Driessen-Mol et al. 2012). An approach was introduced previously to first having cells seeded on synthetic grafts in order to deposit ECM composites. Prior to the use of these ECM “conditioned” grafts, cells would be removed to prevent further contraction (Dijkman, Driessen-Mol et al. 2012). In this work, a different strategy was applied to fundamentally improve the strength of the graft material in order to prevent cell-mediated buckling. Silk fibroin protein was extracted and incorporated into the composites to improve mechanical strength. This was based on the unique anti-parallel β sheet structures of silk fibroin which are attributed to the crystalline domains and make the composite stiffer (Jiang, Wang et al. 2007). Electrospun mats were chemically crosslinked with glutaraldehyde vapor to further provide superior resistant to cell mediated material shrinkage (Billiar and Sacks 2000). The present study incorporated silk fibroin with helical collagen and the elastic polymer PGS to create an interesting combination of both strength and flexibility (Billiar and Sacks 2000; Beun, Beaudoux et al. 2011). The brittleness of silk fibroin was modulated by incorporating collagen and elastic PGS. An interconnected porous meshwork was fabricated using electrospinning.

In this study, PFC mats showed minimal weight loss and structural integrity that was superior compared to the reported structurally similar, fully crosslinked collagen or polylactic acid electrospun constructs (Kim, Yu et al. 2003; Zong, Ran et al. 2003; Horan, Antle et al. 2005; Liu, Teng et al. 2010). Even after degradation, the bulk properties did not change significantly and the
structure remained intact. No statistically significant difference was observed in fiber diameters over the 9-week degradation test. These characteristics suggest that PFC mats would be stable at 37°C when surgically implanted in patients. *In vivo* degradation tests indicate that silk fibroin degrades slower *in vivo* than most of other collagen based scaffolds such as poly caprolactone-collagen scaffold which completely degraded in one month (Tedder 2009; Zhou, Cao et al. 2010). Zhou et al. illustrated that electrospun pure silk fibroin lasted 8 weeks post implantation, without tissue fibrosis observed. Traditional fabricated synthetic grafts released degradation byproducts during destruction *in vivo* and elicit immune response by macrophages (Hopkins 2005; Tedder 2009). The host response to the PFC mats was not examined in the current study but each individual material of the composite has been shown in published studies to have a low or minor immunogenic response *in vivo* (Wang, Ameer et al. 2002; Zhou, Cao et al. 2010; Rockwood, Preda et al. 2011).

Cell adhesion and proliferation on the PFC mats revealed the superior biocompatibility of PFC mats. Cell attachment and growth is considered to be the first step for achieving sufficient tissue remodeling and maturation *in vivo* (Butcher, Mahler et al. 2011). While specific binding kinetics of cells to the PFC mat was not studied in this investigation, previous findings suggested that individual composites in PFC mats provided binding sites and microenvironment cues for guiding cell adhesion and proliferation (Gu and Masters 2010; Shekaran and Garcia 2011; Zou, Cao et al. 2012). A highly organized monolayer of endothelial cells was formed on the PFC mats. Strong F-actin staining of
HUVECs suggested a high level of cell adhesion and potential for improved mechanical strength of the graft material (Edwards, Draper et al. 2005).

The special structural features of PFC mats could potentially contribute to the long term viability of valvular cells. These interconnected fiber networks only had a thickness of 100 - 300µm for efficiently supporting nutrient, oxygen transport and soluble cell signal transmission (Soliman, Sant et al. 2011). Electrospun fibers of PFC mats mimicked the highly porous matrix structure of valvular ECM which is essential in providing large surface area for cell attachment and growth. Further investigations could be performed to perfect the microscopic architecture and function of the PFC mats by incorporating more complex structural and compositional features. Glycosaminoglycan, growth factors or specific cell signaling markers can be incorporated to provide mechanical and chemical cues to cells through physical absorption or chemical modification based on previously reported methods with other biomaterials (Yamada, Kennedy et al. 1980; De Cock 2010; Deng 2011; Jordan, Williams et al. 2012). These enhanced mechanical and chemical cues through modification may further contribute to a higher level of mature valvular tissue formation by attracting and integrating larger amount and more viable native valvular or progenitor cells.

Previous research showed that there were elevated thrombogenic risks associated with decellularized collagen-based grafts (Schopka 2009). This work investigated the thrombogenicity through both PFC mats associated with platelets in plasma and PFC mats associated with co-incubated platelets in
plasma and endothelial cells. Compared to the structurally similar electrospun collagen mats, there were fewer platelet aggregates and a more scattered distribution of platelets on PFC mats. The decrease in platelet adhesion and aggregation on PFC mats implies the hemocompatibility which might be a result of the PGS as previously suggested (Motlagh, Yang et al. 2006). The morphology of platelets observed by scanning electron microscopy depicted a state of decreased activation on PFC mats compared to the conventionally used collagen-based substrates. Only a few discoidal shape or early dendritic pseudopodial shape platelets were seen on the PFC mats while platelets having a spread dendritic shape were primarily observed on the collagen control. These results suggested that the PFC mats were more hemocompatible than structurally similar traditional collagen materials.

Because the PFC mats are directly synthesized and fabricated from proteins and synthetic polymer, they offer an unlimited off-the-shelf alternative supply and minimal risk concerns with disease transmission as there are for processed bovine or procine grafts. The electrospinning fabrication techniques can be used to rapidly produce PFC mats compared to other heart valve graft processing procedures (Sacks, Schoen et al. 2009). As a feasible fabrication technique, electrospinning on molds could be used to recreate the native geometry of a heart valve tissue for the in vivo tests. Other uses might include fabrication onto stents for use as a new generation of transcatheter heart valves in minimal invasive cardiothoracic surgery (Dijkman, Driessen-Mol et al. 2012).
CONCLUSION

A major accomplishment of the current investigation was the formulation of a new composite of natural and synthetic material that can be used for heart valve replacement. In this study, composites of collagen, fibroin and PGS were successfully created and fabricated using electrospinning. The optimal electrospun material was a PFC mat with collagen: fibroin: PGS at 4.5:4.5:1 weight ratio. The compositional and structural similarities of PFC mats to native valvular tissues potentially offer cellular binding sites and microenvironment cues for cell adhesion and growth. The interconnected fiber structure and high porosity of PFC mats provides a large surface area and internal space for tissue maturation to occur. Mechanical testing demonstrated the PFC mats had comparable mechanical strength to fresh heart valve tissue, and therefore could withstanding the physiological pressures. The functionality test showed minimal weight loss and sustained fiber structural integrity over a 9-week of degradation. Organized endothelial monolayer formed on PFC mats. Reduced platelet adhesion and aggregation size suggested PFC mats were less thrombogenic compared to collagen mats and collagen gels. In summary, the novel PFC mats created in this study could be potentially used as durable, biocompatible, and nonthrombogenic grafts. The feasible fabrication process in producing the PFC mats using electrospinning could be further implemented to mimick the geometry of native heart valve in order to investigate the in vivo functionality and performance.
REFERENCES


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CURRICULUM VITAE

Rui Wang

HIGHER EDUCATION

University of Miami: May 2010
B.S. in Biomedical Engineering, Minor in Mathematics, Coral Gables, FL
Illinois Institute of Technology: July 2007 Transferred
Majoring in Biomedical Engineering Chicago, IL

RESEARCH EXPERIENCES

Wake Forest University, Winston-Salem, NC

Graduate student, 2010-Present
- Investigating tissue repairs and drug delivery using silk fibroin and collagen-based biomaterials

Diabetes Research Institute, Miller School of Medicine, Miami, FL

Senior project, May 2009-2010
- Investigated the biological effects of the controlled release of immuno-suppressive and anti-inflammatory glucocorticoid dexamethasone, on pancreatic β-cell line using a silicon-based device. The purpose of this study is to examine whether this macro-environment specific treatment of the dexamethasone provides a better immuno-suppressive and anti-inflammatory outcome after the islet transplantation, as compared to a routine dexamethasone administration.

Miami Veterans Affairs Healthcare System, Miami, FL

Research Assistant, April 2009-2010
- Volunteered in the stem cell tissue engineering laboratory for cartilage reconstruction research
• Assessed both office-based test (PFP-10) and physical test with elderly veterans (over 65-year of age). The purpose of this study is to compare progressively resistance training to periodized prescription training and find out which is more beneficial for the daily activities of elderly veterans.
• Developed a software program using MATLAB interface to easily record and organize data for these assessments.

**Bascom Palmer Eye Institute McKnight Research Building, Miami, FL**

*Volunteer, May 2008-March 2009*
• Observed the signal acquisition for Pattern Electroretinography (PERG) and optical coherence tomography (OCT). Observed the surgical procedure on DBA/2J mice for Visual Evoked Potential (VEP) measurements.
• Worked on distortion correction using MATLAB for OCT images.

**Tissue Engineering Lab, Illinois Institute of Technology, Chicago, IL**

*Research Assistant, March 2007-July 2007*
• Worked on *in vitro* reconstructions of adipose tissue using basement membrane-based hydrogel prepared from the dermal and adipose tissues, as well as Matrigel. Applied 3D on-top culture method for cell culturing, and Oil-Red O for cell staining. Used confocal microscopy to quantifying the 3D tissue formation. BCA protein assay, Western Blot, and ELISA, were also applied. The purpose is to study fat tissue reconstruction and angiogenesis in 3D hydrogel environment both *in vitro and in vivo.*

**Biology and Physiology Lab, Illinois Institute of Technology, Chicago, IL**

*Student Assistant, September 2006-July 2007*
• Prepared frozen tissue sections and performed immunohistochemistry staining of brain tissue in order to study the signaling change in olfactory bulb. Image analysis, Western blot, DNA Extraction, spectrophotometer
measurement, PCR, and TA-vector cloning of PCR product were also applied. The purpose of this study is to investigate the olfactory signal pathway in mice and fish model.

Physiology and Pathophysiology Lab, Peking University Health Science Center, Beijing, China

High School Student Researcher May 2004- January 2006

- Isolated and cultured preadipocytes and primary adipocytes from rat adipose tissues. Differentiated the preadipocytes to matured adipocytes. The intracellular lipid droplets in rat differentiating adipocytes at different stages were stained with Oil-Red O. The lipolysis (triglyceride hydrolysis) of rat primary adipocytes was determined after the cells were treated with pioglitazone, an anti-diabetic medicine. Molecular changes such phosphorylations of the signal proteins: perilipin and hormone-sensitive lipase in the lipolytic cascade were examined by using SDS-PAGE and Western blot. The purpose is to test how the effect of pioglitazone on preadipocytes differentiation and lipolysis of mature adipocytes.

PUBLICATIONS


ABSTRACTS

1. R. Wang, N. L. Polyachenko, and W. D. Wagner. “A novel nano-fiber constructed graft for heart valve replacement” as a poster presentation in
Twelfth Annual Graduate Student and Postdoctoral Fellow Research Day, Wake Forest University, Winston-Salem, NC March, 2012

2. R. Wang, S.H. Uriel, and E. Brey. “Isolation of fat basement membrane and preadipocytes for tissue reconstruction applicants” as a poster presentation (P2.190) in Biomedical Engineering Society Annual Fall Meeting in Los Angeles, CA September, 2007

HONORS

- Outstanding Service Award, College of Engineering, University of Miami
- Member of Alpha Eta Mu Beta National Biomedical Engineering Honor Society, 2010-Present
- Member ofEta Sigma Phi National Classics Honor Society, 2010-Present
- Member of Tau Beta Pi National Engineering Honor Society, 2007 – Present
- Provost’s Honor Roll, Dean’s List, University of Miami, 2007 – 2008
- 1st Prize, Youth Science Innovation Competition, Beijing, 2005

ACTIVITIES

- Member, Society for Biomaterials, 2010 - Present
- Member, Biomedical Engineering Society (BMES), 2006 - Present
- Biomedical Engineering and Sciences, Department Representative, Wake Forest University, 2010-2012
- President, Society for Biomaterials National Student Chapter at Wake Forest University, 2010-2012
- Project Coordinator, EMBS, IEEE at Wake Forest University, 2010-2011
- Volunteer, Winston-Salem Children Museum, 2010-2011
- Representative, Biomedical Engineering Department, University of Miami, 2009-2010
• Event coordinator and Spotlight editor, Biomedical Engineering Society, University of Miami, 2009-2010
• Organizer, Note Card Fund Raising Project for Miami Children’s Hospital, 2009-2010
• Member, Engineering Advisory Board (EAB), University of Miami, 2009-2010
• Volunteer, Service Learning Program, Illinois Institute of Technology 2006-2007
• Volunteer, Mercy Hospital, Chicago, Illinois 2006-2007