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Development of cylindrical bacterial cellulose membranes for pulmonary heart valve prostheses

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DEVELOPMENT OF CYLINDRICAL BACTERIAL CELLULOSE MEMBRANES FOR PULMONARY HEART VALVE PROSTHESES

by

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CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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This is to certify that the Master's thesis of

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To my loving parents Padmaja and Sarathy and my awesome brother Arjun.

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Last but not the least, I would like to thank my family and friends for so much love and compassion throughout my educational career.

ABSTRACT

Novel biomaterials provide a spectrum of possibilities. They can be engineered in different forms to understand how they would perform as different bioprosthetic conduits. Bacterial cellulose membranes may be suitable candidates as prosthetic valve leaflets in valve replacement surgeries due to their functional properties (hemodynamics, resistant to thrombosis). Biomaterials used for most bioprosthetic heart valves are cut, trimmed and sutured. A major challenge for the bi-leaflet configuration is that the cutting and suturing of biopolymers fabricated as sheets into a cylindrical form increases failure risk due to greater number of suture points and irregular coaptation. The objective was to culture the bacterial cellulose membrane as a continuous cylindrical construct and evaluate its mechanical properties. Various design features of the fabrication process such as culturing media and the hollow carrier-mandrel characteristics were evaluated. A comparative study of how bacterial cellulose grows on different hollow carrier membranes was conducted and thin smooth surface silicone tubes fabricated in the lab were found to be most suitable. A bioreactor for culturing cylindrical bacterial cellulose tubes on the outer surface of the hollow carrier was designed and fabricated. The mechanical properties of the fabricated tubes, specifically, their tensile strength, flexure, suture retention and tear resistance were characterized. Mechanical characterization studies showed the cylindrical bacterial cellulose tubes to be anisotropic, with preferential properties in the longitudinal (axial) direction of the tube. Preliminary results show that cylindrical bacterial cellulose tubes can be a promising candidate for use in prosthetic valve conduits.

PUBLIC ABSTRACT

Biosynthetic materials have been extensively used as conduits for bioprosthetic valves. A major challenge is that biopolymers synthesized as sheets are cut, trimmed and sutured as a cylinder causing increased failure risk due to greater number of suture points and irregular coaptation of the leaflets. The objective was to fabricate this novel biomaterial as a continuous cylindrical construct and evaluate its mechanical properties.

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PREFACE

The BioMOST lab had been working on regenerative cellulose and bacterial cellulose for quite some time. As I joined the lab, Chaid Schwarz a PhD candidate then, had been working on the use of bacterial cellulose sheets for transcatheter valves. There was a genuine interest from few surgeons who questioned the possibility / viability of using a cylindrically cultured bacterial cellulose tube for preparing bi-leaflet pulmonary heart valves. Soon, I shadowed Chaid with his work and learnt the culturing techniques. From there on the objectives for my thesis were established and I moved forward with the project under the guidance of Dr. Raghavan and Chaid.

CHAPTER 1: INTRODUCTION AND MOTIVATION

Bioprostheses for valve replacement

There has been a steady increase in the number and quality of valvular surgeries being done in the recent years with an estimate 623,000 valve procedures done from 1993 to 2007[1,2]. With the societal trend in diabetes, hyperlipidemia, hypertension and coronary disease, it is expected that the North American demographic will worsen and therefore providing a persistent drive to improve the available valve technology[2]. At least 60,000 artificial heart valves are implanted in the US every year out of which it is estimated that about 60% are mechanical and 40% are bioprosthetic/tissue valves with a general shift towards more usage of bioprosthetic valves over past several years[3]. A bioprosthesis in general is an implanted device or artificial body part that has a non-synthetic origin. As the name suggests bioprosthetic heart valves come with inherent advantages such as low rate of thrombogenesis and the presence of less rigid occluders leading to hemodynamic properties similar to that of native valves with their limited durability being the biggest disadvantage, in contrast mechanical heart valves come with the risk of systemic thrombosis and thrombotic occlusion due to their non-physiologic surfaces and flow abnormalities created by rigid occluders but typically have high structural reliability/durability[3]. Figure 2 is the

Figure 2 : St. Jude Regent [23]

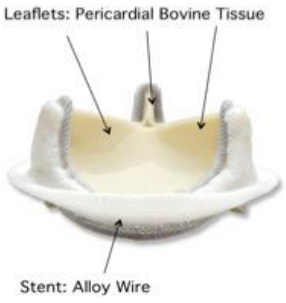

Figure 1: Carpentier-Edwards Perimount [4,5]

Carpentier – Edwards perimount heart valve, which was one of the first biomechanical

engineered aortic heart valves created in 1981[4,5]. Figure 1 is a depiction of St. Jude's regent mechanical heart valve.

Pulmonary and tricuspid valve replacements are common among children, approximately 8 out of 1000 babies are born with congenital heart diseases, 20% of whom have deformities that involve the right ventricular outflow tract (RVOT) and pulmonary valve[6]. Pediatric patients born with these complications such as the tetralogy of fallot generally undergo surgical procedures within the first days or months of life, which include the implantation of some sort of prosthetic conduit or artificial heart valve[6]. The challenge with these procedures is that the conduit cannot grow with the patient, therefore when implanted in small children is a major limitation. As they are subjected to progressive degeneration, multiple surgeries are generally required over the lifetime of the patient. Hence considering the inherent disadvantages of open heart surgeries and the high probability of repeat procedures, percutaneous implantation of an artificial heart valve / transcatheter valve replacement procedure is highly desirable. On similar lines, the drive for this project is to obtain a material that can show a good level of mechanical strength, perform like a native tissue hemodynamically, easy mountability onto a stent and can be implanted percutaneously. The Melody heart valve from Medtronic is one of the first commercially

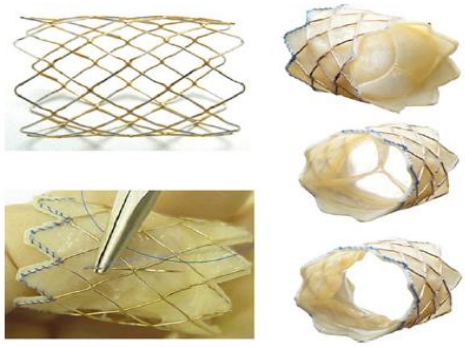

Figure 3 : Depiction of the Melody TPV at various stages of its production[6].

Figure 4 : Melody valve's transcatheter delivery system. From top to bottom, the system with the sheath on, the sheath withdrawn and valve exposed, inner balloon expanded followed by the outer balloon expanded[6]

available bioprosthetic heart valves for the pulmonary position that is implanted

percutaneously[6]. The Melody transcatheter pulmonary heart valve combines the use of a valved segment of the Bovine jugular vein sewed onto a balloon expandable Platinum-iridium stent.

BC – Bacterial cellulose

Cellulose has been isolated from plants successfully for many years, but in the last decade or so bacterial cellulose has been attracting more research due to their superior purity and supra molecular structure [7]. Bacterial cellulose fibers are typically 0.1 micrometer thick, which is about one-hundredth the size of wood fibers[7]. In the present project, bacterial cellulose is synthesized by culturing the bacteria Gluconacetobacter Xylinus (Acetobacter Xylinum) or Komagataeibacter Xylinus. Starting from the water- soluble, non-toxic monosaccharide D-glucose the non-pathogenic bacteria builds up pure cellulose extracellularly within some days[8]. In the presence of carbohydrates, the bacteria synthesizes cellulose microfibrils in the form of ribbons. The matrix of the interwoven ribbons constitutes the bacterial cellulose membrane or pellicle[8]. Therefore, the cellulose is produced in the form of pellicles at the air/water interface of the culture medium in a static culture. It is said that the pellicle protects the cells from the lethal effect of ultraviolet light, enhances colonization, retains moisture to prevent drying, and holds the bacteria in the aerobic environment[8]. Bacterial cellulose differs from plant cellulose with respect to its ultra-fine architecture, high hydrophilicity, and moldability during formation[8]. These desirable properties of bacterial cellulose such as its high mechanical strength in wet state, enormous water retention values, low roughness of inner surface, and strong tear resistance when dried makes it an attractive material for biomedical applications. Present research

shows the use of bacterial cellulose sheets as a barrier in the regeneration of periodontal tissue, repair of abdominal defects and as a dura mater substitute[9].

Figure 5 : Wet bacterial cellulose pellicle after purification. Photo courtesy of Chaid Schwarz

Bovine and autologous pericardia are predominantly used for cardiac valve, patch and conduit repair. But due to many of their limitations such as patch thickening, calcification and retraction, the search for novel patch / bioprosthetic conduit materials is an active area of investigation[10]. CorMatrix® extracellular matrix is a material of interest. This novel material is synthesized from decellularized porcine small intestinal sub-mucosa. It consists of an absorbable biocompatible framework and therefore acts as an ideal tissue scaffold[10]. The CorMatrix material is commercially being used for pericardial repair, cardiac tissue repair and vascular repair.

Bacterial cellulose membranes can potentially be used for similar functions. Unlike the CorMatrix material, bacterial cellulose is not absorbable but provides the advantage of moldability during formation and tunable compaction. Tubular-shaped bacterial cellulose has been suggested for use as a blood vessel replacement and for nerve regeneration[9]. In this

Figure 6 : An aortic valve prototype using flat sheet Bacterial cellulose cut and sutured on to a stent [21]

project we use the commercially available Kombucha tea as the starter culture for obtaining the required bacteria and bacterial cellulose. It has been observed that bacterial cellulose/microbial cellulose with high purity and high degree of crystallinity could be successfully produced from the fermentation of Kombucha and therefore is comparable with that of commercial microcrystalline cellulose[11].

Shape – continuous cylindrical membrane

Most commercially available tissue/bioprosthetic valves are mounted on and supported by a metal or plastic stent, typically with three posts and surrounded by a sewing cuff at the base [3]. To produce bi-leaflet valves, flat sheets of the bioprosthetic membrane are sutured axially along a line to obtain a cylinder which is then sutured on to the stent. Bioprosthetic valve cusps are also generally composed of pieces of tissue that are artificially trimmed and attached to a stent[3,12]. Regurgitation and bioprosthetic valve failure are frequently caused due to noncalcific cuspal perforations and tears. The tears in the

bioprosthetic valves such as pericardial valves are frequently associated with a commissural suture ("alignment stitch") and also occur along the attachment points of cusps or in the zone where the cusps are attached to the axially arranged struts[3,12]. This method of cutting and suturing also makes it highly impossible to get the complete coaptation of the leaflets i.e., owing to the lack of symmetry after the material is sutured into a cylinder as two sheets

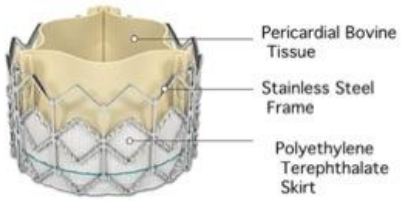

Figure 7 : Edwards Sapien bioprosthetic valve with prericaridial Bovine tissue trimmed, treated and stitched on to a balloon expandable stainless steel stent [4,5]

overlapping each other, it is bound to have a certain defect in the way the leaflets close and open. This irregular coaptation of the leaflets can lead to the formation of congested zones and vortex-type of blood flow after opening or closing of the valves [12]. The ability to have a flexible and durable membrane as a continuous cylinder to begin with would first avoid the need of suturing the material into a cylinder which saves production time and more importantly limits the points of incisions made on to the membrane.

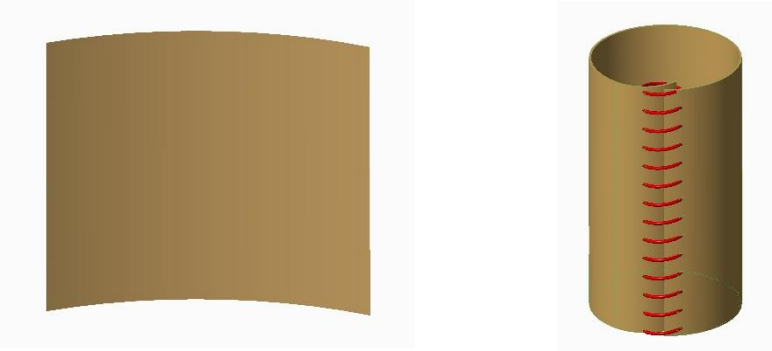

Figure 8 : Schematic depiction of a flat sheet of membrane being sutured horizontally to form a cylinder for a bi-leaflet configuration

Certain suturing techniques can be used to fix the continuous cylindrical construct on to the stent with limited points of suturing on to the stent such that the free edge of the cylinder would collapse on itself to coapt as a bi-leaflet valve. The underlying premise of this project is that by avoiding the suture, the symmetry of the cylinder allows us to have a valve that will completely coapt minimizing the regurgitation of blood. If every point of suture is seen as a potential point of failure, by reducing the number of sutures we hope to increase the longevity of the valve.

Aims and objectives

- Develop a protocol to fabricate a continuous cylindrical bacterial cellulose membrane
- Assess the effect of the chosen fabrication parameters on mechanical characteristics of the bacterial cellulose tube, specifically:
    - Role of hollow carrier : Polymer (ePTFE) vs. Silicon vs. Porous plastic
    - Role of culture media : Tea vs. modified Hestrin-Schramm
    - Role of oxygen concentration : 21% (air) vs. 100% (pure oxygen)

CHAPTER 2: METHODOLOGY

Culturing bacterial cellulose

Two different media types were used to culture the bacteria Gluconacetobacter Xylinus. The recipe for each of the media types were put together from previous work[7–9,11,13].

Table 1: Composition of the first media used - Tea media

Ingredient	Quantity
Distilled water	1L
Stash Green Tea (steep – 15min)	3g/L
Turbinado Raw Sugar	80g/L
Starter Culture/Kombucha Tea	142mL/L

Table 2: Composition of second media used – modified Hestrin-Schramm media

Ingredient	Quantity
Distilled water	1L
Stash Green Tea (steep – 15min)	3g/L
Corn Steep Liquor	40g/L
Mannitol	20g/L
Na_2HPO_4	2.7g/L
Citric Acid	1.15g/L
Starter Culture/Kombucha Tea	142mL/L

Water is heated to about 80°C and the green tea steeped for 15 minutes, following which the required components are added. After the solution is completely cooled the inoculant/starter culture is added. This solution is then kept in an incubator at 30°C and checked for cellulose growth every 5 days. Commercially available Kombucha tea can be

used as the starter culture to grow, culture and eventually isolate the cellulose producing bacteria[11,13]. Once an existing culture shows good cellulose growth at the air-water interface, the cellulose pellicle's surface can be scrapped to obtain the more active bacteria.

This culture can now be mixed and used as an inoculant/starter culture.

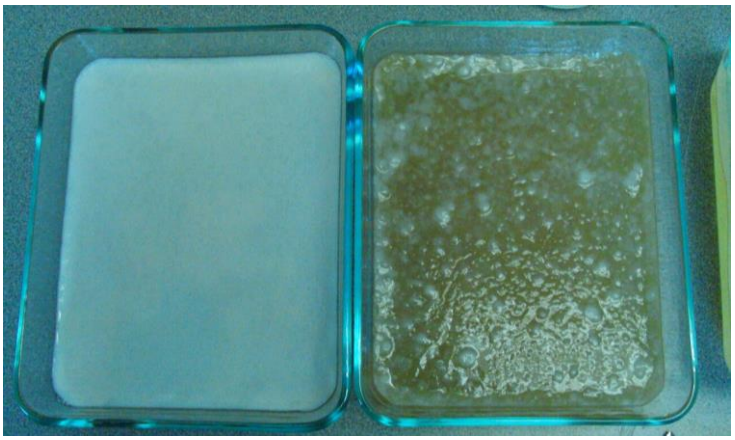

Figure 9 : To the left is a culture with confluent layer of bacterial cellulose grown at the air-media interface. And to the right is a culture in the initial period of its incubation, where we can see the start of cellulose growth at the surface. Photo courtesy of Chaid Schwarz

Culturing bacterial cellulose as a cylinder

The basic principle followed is that a hollow bacterial cellulose tube can be obtained by culturing a cellulose producing bacteria on the outer surface of a hollow cylindrical carrier composed of a gas permeable material with desired porosity and high oxygen permeability[8,9,14–16]. In that one side of the gas permeable material is contacting a gas containing oxygen whereas the other one is contacting the liquid culture medium so that the bacterial cellulose is formed on the latter side and will subsequently be isolated [8]. The growth of the cellulose does depend on the type of hollow carrier/gas permeable membrane and its thickness, therefore preliminary test cultures were done using four different types of membranes – an ePTFE membrane 1.5mm thick with an OD of 19mm, a silicon membrane

extruded using a 3D printed mold to be 1mm thick and have an OD of 20mm, a smooth silicon membrane extruded by coating on a rotating metal rod to be 0.3-0.4mm thick and have an OD of 20mm and a porous plastic tube obtained from GenPore® with an OD of 20mm. These were selected keeping in mind the cellulose tube production would be affected by the porosity of the membrane, its ability to maintain a cylindrical shape with continuous movement of air through it and its oxygen permeability. The outer diameter of each of the hollow carriers/ membranes were 18-20mm to meet the requirement of a bioprostheses that could be implanted at the pulmonary

The hollow carrier/membrane is connected to an air outlet and a continuous air supply is established. This setup is immersed in a tall glass vase filled with the culture media such that the hollow carrier/membrane is in a vertical position. The hollow carrier is now connected to an outlet tube which is directed outside the vase (U-tube path). This set up is kept in an incubator for 15 days at a temperature of 32°C and a constant air pressure level is maintained.

Preparation of silicon tube using 3D printed mold

The 3D printed mold consists of 4 pieces – the first piece is a solid cylinder of diameter 20mm (+/-0.5mm), the second piece which is a hollow cylinder of ID 22mm(+/-0.5mm) acts as the shell and the other two pieces are top and bottom pieces designed to act like caps. The solid cylinder and the shell are sprayed with mold release 15 minutes prior to pouring the Silicone. An appropriate amount of silicon is then measured out, mixed well with the curing agent and degassed in a vacuum chamber to remove air bubbles. The Silicon is then poured into the shell with bottom cap on following which the solid cylinder is pushed in to position. The mold is closed with the top cap and left to cure for a day, following which the silicon tube can be removed.

Preparation of silicon tube by coating on metal rod

This process uses a custom lathe on which a metal rod of required OD is rotated. A smooth metal rod having an OD of 19.75mm was purchased. Small pieces of heat shrink wrap of similar diameter are heated on to both ends of the rod using a hot air gun. These heat shrink wraps with a thickness of about 0.3-0.5mm create an offset in the diameter of the tube and hence correspond to the thickness of the silicon tube coated on the rod. Once the heat shrinks are in place, the rod is set to rotate at a slow/suitable rpm. The mixed and degassed silicon is now uniformly poured on to the rotating metal rod with a wide glass slab being gently pushed against the offset/pieces of heat shrink wrap to cut out the excess silicone. When the surface of the silicon looks uniform enough the glass slab is pulled back and the silicone is left to cure on the rotating rod overnight. The silicon tube can be gently peeled/pulled off the metal rod the next day.

Bioreactor design

After the preliminary cultures there was a need to optimize the set up. A new "bioreactor" was designed to culture the cellulose tubes. It consists of a top and bottom piece 3D printed. The top piece have the inlet and outlet connectors on one side and have respective connectors on the other side that will be connected to the hollow carriers. The bottom piece acts as a U-joint and connects to both the hollow carriers, therefore completing the path of the air. The bio reactor is held solid in place with the support of two all thread rods going through the 3D printed pieces.

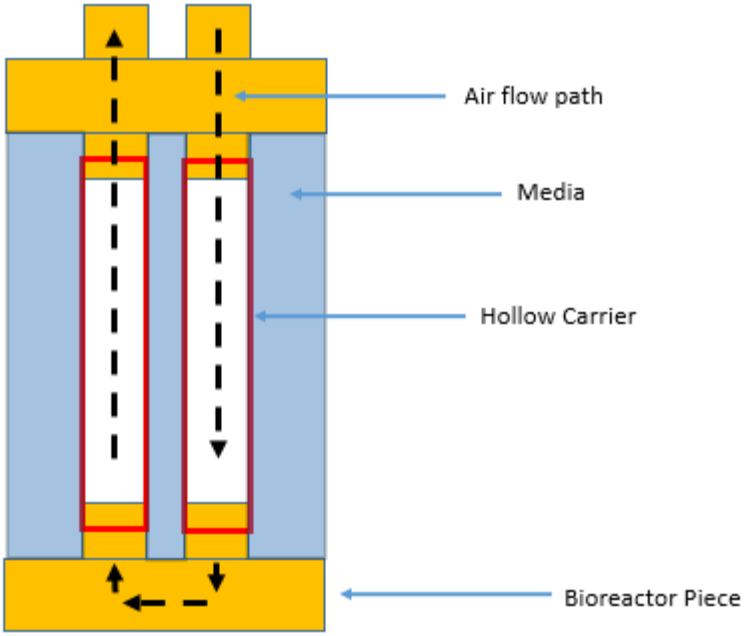

Figure 10 : A schematic showing basic idea based on which the bioreactor was designed.

The PLA plastic used to print these pieces are basically porous/permeable to water and hence there was a need to seal / water proof them. The pieces were sealed using silicon glue and gorilla glue.

After few trials the bioreactor design was further modified to tackle the challenge of air / O_2 leakage through the 3D printed joints. In our new design the 3D printed pieces were only used as a frame to hold the set-up in place. The joints connecting to the hollow carrier were now replaced with commercially available pipe fittings / connectors. And therefore the air pathway did not consist of the PLA plastic.

Purification of cultured bacterial cellulose

The cellulose pellicles obtained from the cultures were put in a solution of 1% by weight NaOH at 75°C for 2 hours. The solution is then discarded and the pellicles are rinsed with distilled water for the next couple of days. This step helps in disrupting the cells in the cellulose pellicle and reducing the impurities present[7,8,16].

Compaction

The cellulose pellicle in its "never dried" form has a high swollen fiber network and show water retention values of up to 1000% [8]. At this wet state the thick cellulose pellicle fibers are still loosely bound and show weak mechanical properties. A process of compaction that is performed by drying out water from the pellicles[7,8], is used to make it thinner by compacting the fibers into its ultrafine network structure. After rehydrating the dried pellicle for more than 2 hours we can see decreased water retention values, comparable with those of plant cellulose[8]. The rehydrated cellulose pellicle now is thinner and shows better mechanical properties.

In order to perform compaction, the cellulose pellicles after the purification process are dried for 10 hours in a dehydrating unit following which they are rehydrated for 6 – 10 hours. The process is then repeated 3 more times to a total of 4 compaction cycles for every sample.

Scanning electron microscope (SEM) Imaging

Bacterial cellulose tube structure and fiber orientations were analyzed using scanning electron microscopy. The samples were cut into appropriate size and dried out completely. They were then mounted on SEM Specimen Pin Stubs with the help of Carbon tape and sputtered for 1 min using Palladium-gold in a sputter coater.

CHAPTER 3: MECHANICAL TESTING

Heart valves throughout the cardiac cycle undergo constant cyclic loading, and need to accommodate to extraordinary and repetitive changes in shape and dimension. This is usually accomplished because the heart valves have a layered, complex architecture and highly specialized, functionally adapting cells and extracellular matrix (ECM) [3]. As bioprosthetic materials lack these inherent features, durability has been the biggest challenge for bioprosthetic valves. It is important to understand how the material will perform when they undergo in-plane tension, bending and shearing within the tissue due to the cyclic loading a typical valve leaflet would experience. The mechanical tests performed help us asses the materials potential to be used a valve membrane that can be collapsed and delivered using a catheter.

Uniaxial extension

The act of extending or the condition of being extended can be defined as extension. As a material is put in this condition of being extended, their response vary and an idea of how the material is going to stretch under tension can be obtained. The material stretches until a point called failure tension, after which it begins to tear leading to the failure of the material. As the material is being extended the highest load the material can withstand before failure is the failure tension of that material. Therefore this test allows us to gauge the strength / failure strength of the material under uniaxial extension. Correspondingly, the maximum stretch the material can undergo before failure is also obtained and known as failure strain.

Materials can differ in their elastic stretch properties, while some materials have linear regions and consistent failure tension other materials like biological tissues are more

complex with non-linear elastic loading and larger variability in their failure tension. These properties calculated as stress is force normalized by the cross sectional area [17,18]. But while considering bacterial cellulose membranes wherein the thickness can be decreased by not removing any material, it is recognized that the cellulose production is random and there will be natural variability in resulting data depending on the sample. Because of these considerations failure tension data is reported based on tensile load per sample width.

The uniaxial extension test basically consists of two clamps separated by a gauge distance. The rectangular sample is placed in between with either end clamped. A controlled unidirectional extension is applied to one end while the other end remains fixed, therefore stretching the material.

The samples were tested using a vertically oriented axial tester (AMETEK Inc, PA). The samples were cut using a custom cutting block to a width of 5mm. One end of the sample is taken and carefully clamped on top first. The other end is then carefully aligned to be vertical and clamped at the bottom. The gauge length / length between the two clamps was maintained at 20mm for the test. This was considered the zero load position. Care was taken to constantly hydrate the sample, therefore maintain the sample in its wet state

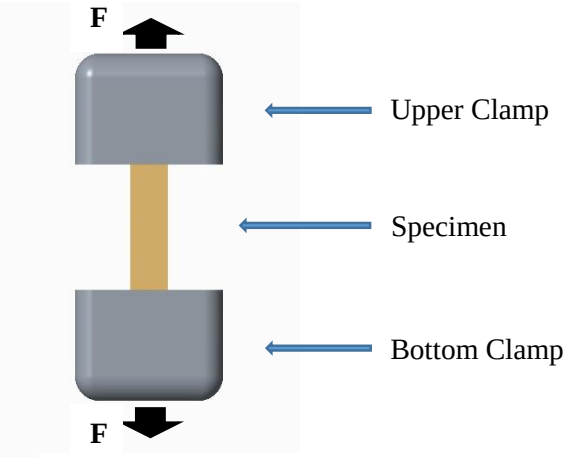

Figure 11 : Schematic depiction of uniaxial extension

throughout the test. First, preconditioning was initiated for 10 cycles at a rate of 15-

30mm/min at 2.5-5% strain. The preconditioned samples then underwent uniaxial testing at 10 mm/min until tissue failure.

Flexure / Bending

Valve leaflets are under constant movement and stress, a major mode of deformation is due to dynamic flexure. The leaflets tend to show elevated stresses especially near the commissures and along the stent-attachments. The leaflets are in a dynamic condition of being bent or curved and hence the flexure / bending test will give us an idea of the materials flexibility which contributes to the durability of the valve. A material offering less resistance to bending would suffer from smaller regions of localized stress and hence be favorable. Saying that, it is important to realize a potential material at this region needs to be exceedingly durable.

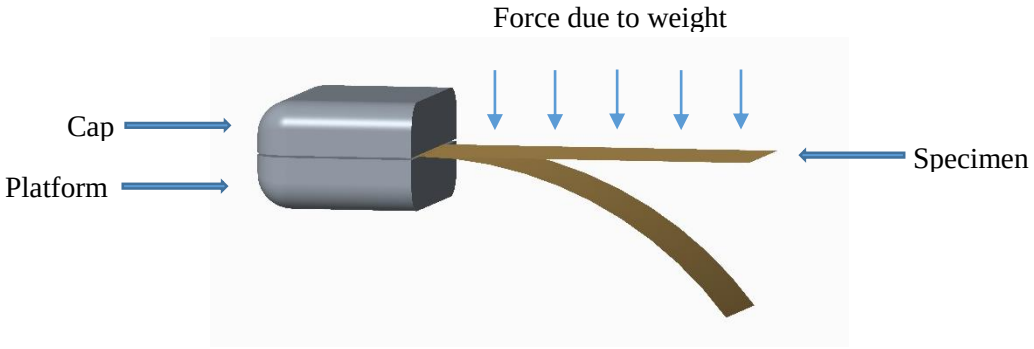

Figure 12 : Schematic depiction of bending test

In this flexure test, the flexibility of the material is measured using the weight of the material. The samples were cut using a custom cutting block to a width of 5mm and length of 48-52mm. The weight of the sample was first measured using a precision balance. Following which flexure was evaluated with a sample placed flat on an elevated platform against a calibrated sheet with an X and Y axis. The samples were placed with 40mm over hanging and a constant plastic capping block. The capping block is the same size as the

platform and is placed on top of the sample to resist arching at the start of the cantilever. As the sample is left hanging it experiences continuous deformation with time due to constant gravitational load. The vertical displacement (distance from the x-axis) was measured using a digital Vernier caliper. This measurement was used to give us a preliminary understanding of the flexibility of the material.

Suture retention

A bioprosthetic material used for a transcatheter valve is sutured to the inside of a collapsible stent. As every suture is created, a point defect is introduced to the membrane and this point experiences a local load. The maximum force required to locally tear through the material gives the suture retention strength. In work done elsewhere, the suture strength was calculated as the peak load before pullout/(suture diameter * thickness) [19]. Another group evaluated suture strength of small diameter cellulose tubes by placing a 6-0 prolene suture 6mm from the edge and obtained results that indicated that 2-3.5mm thick cellulose tubes attained a suture strength of approximately 4-5 N.

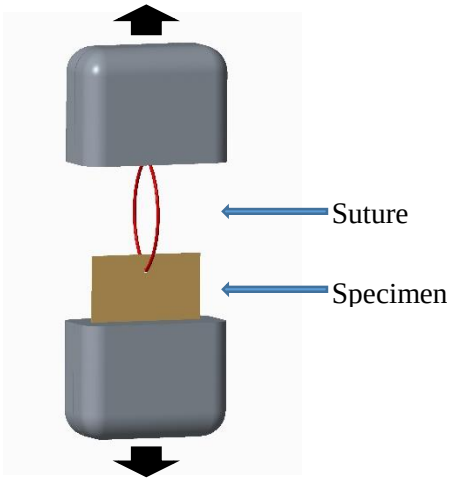

Figure 13 : Schematic depiction of suture retention test

For the test, samples were cut to a width of 10mm and length 30mm. The bite size picked was 4mm, therefore an Ethicon 2-0 prolene suture was placed 4mm from the top edge of the sample. Just as in the uniaxial extension test the gauge length is set to 20mm. The bottom edge of the sample is clamped first and the top suture ends were carefully then fixed in the upper clamp. The top clamp was then extended at a rate of 10mm/min until the suture tore through the sample. The maximum load value is reported from the generated plot and indicated the highest force the material can withstand at any point in time.

Tear propagation

To understand how the material resists to tear after an initial tear has started, the trouser tear tests were performed. In our study we used a method based on the Spanish standard UNE 53-516-83 method A [20].

The samples are cut 30mm long and 10mm wide. A slit is made 20mm from one end therefore creating two legs. The two legs are clamped carefully to opposite ends of the vertical tensile tester and extended at a rate of 250mm/min. Care was taken to make sure the trouser legs were aligned before the start of the tests. In our data analysis, the average value

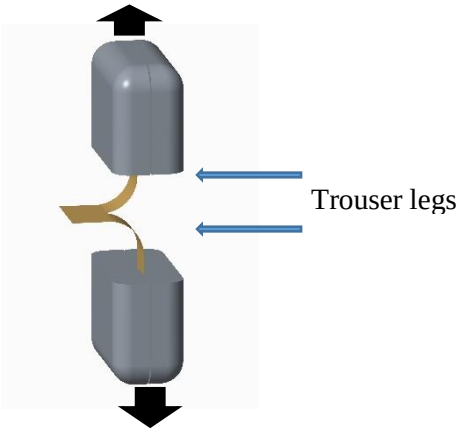

Figure 14 : Depiction of the tear propagation test

of the cyclic loading reported was calculated and the maximum force experienced was also reported.

In summary, this report documents the methodology to obtain large diameter confluent bacterial cellulose tubes.The preliminary mechanical tests on the bacterial cellulose tubes show how its mechanical features can be influenced by its configuration/shape of growth. The mechanical test results set up the path for future work, which would include the durability tester to test how the material / configuration would perform as a bioprosthetic valve. The test parameters and techniques used for the tests were similar to that done by Schwarz 2016[21].

CHAPTER 4: OBSERVATIONS & RESULTS

Culturing of Bacterial Cellulose

Bacterial cellulose was cultured successfully in both the media types. As the cultures were observed through the 15 day period, it was observed that the bacteria produced cellulose at a faster rate in the mHS media. At the end of the 15 day incubation period the cellulose pellicles at the air-media interface were observed to be thicker in the mHS cultures when compared to the tea media cultures.

Kombucha tea was used successfully as a starter culture to produce bacterial cellulose. Cultures started using Kombucha tea can be passaged regularly to obtain active cultures of Gluconacetobacter Xylinus.

Culturing of Bacterial Cellulose as a Cylinder: Choice of Hollow Carrier

The hollow carrier used plays a large part in the production of a continuous, confluent and thick bacterial cellulose tube. All the preliminary cultures were done using tea media. The results of our preliminary tests with different types of hollow carrier membranes were as follows:

- The ePTFE tube was observed to be very porous. When enough air pressure was given for the tube to maintain its cylindrical structure, bubbling through the media was excessive and therefore unfavorable. To rectify this challenge, a short piece of plastic tubing was inserted into the ePTFE tube. This way the cylindrical structure of the ePTFE tube was maintained at a low enough pressure such that bubbling is not seen through the media.

- After the 15 day period a thick pellicle was seen at the media-air interface as expected (control). As the tube was removed from the culture, an extremely thin layer of cellulose growth was seen aroud the tube.
- This thin film of cellulose growth was extremely delicate and it was not possible to pull it off without damaging the cellulose membrane.

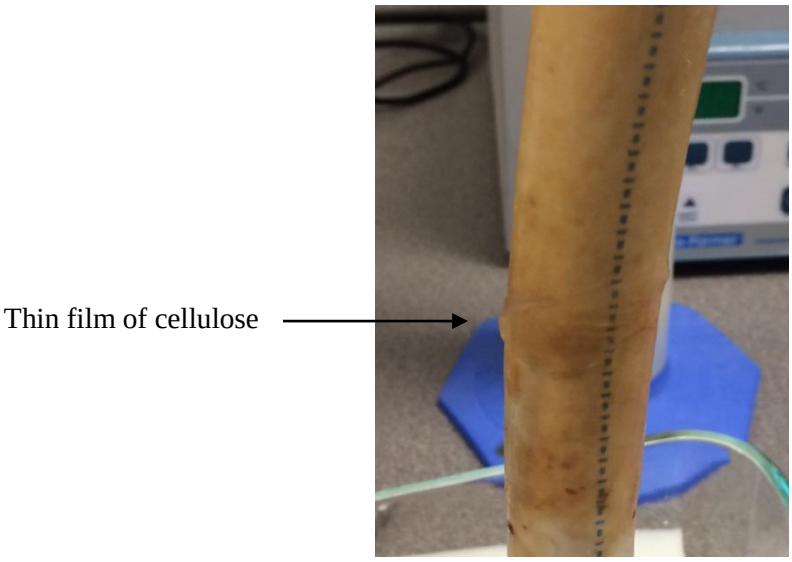

Figure 15 : Thin film cellulose growth on ePTFE tube

- The GenPore porous plastic tube as required was permeable to air but impermeable to water. At the end of the 15 day incubation period, there was no cellulose growth seen on the surface of the porous plastic tube.

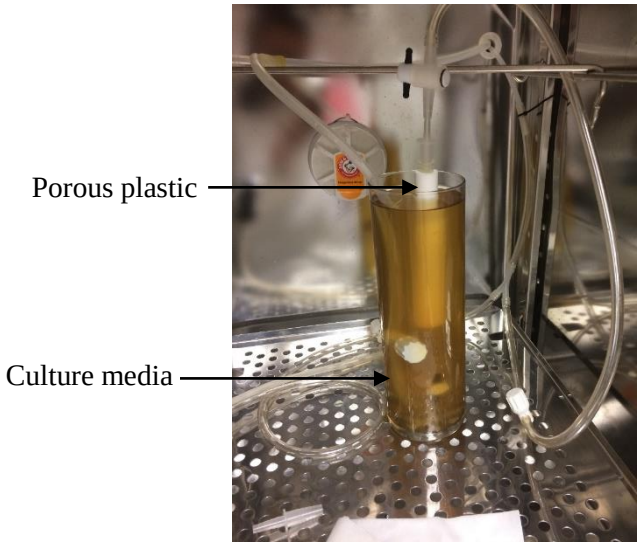

Figure 16 : GenPore porous plastic tube in tea media

- The silicon tube fabricated in the lab using a 3D printed mold showed striations on the surface caused due to the PLA plastic.
    - The Si tube was non-porous did not let any air pass through at plain sight.
    - At the end of the 15 day period, there was growth of cellulose seen all around the tube. The bacterial cellulose membrane grown around the tube was of reasonable thickness but still remained as a delicate membrane.
    - It was interesting to observe that the bacterial cellulose had grown as a pattern (striations) which was similar to how the surface of the Si tube looked.

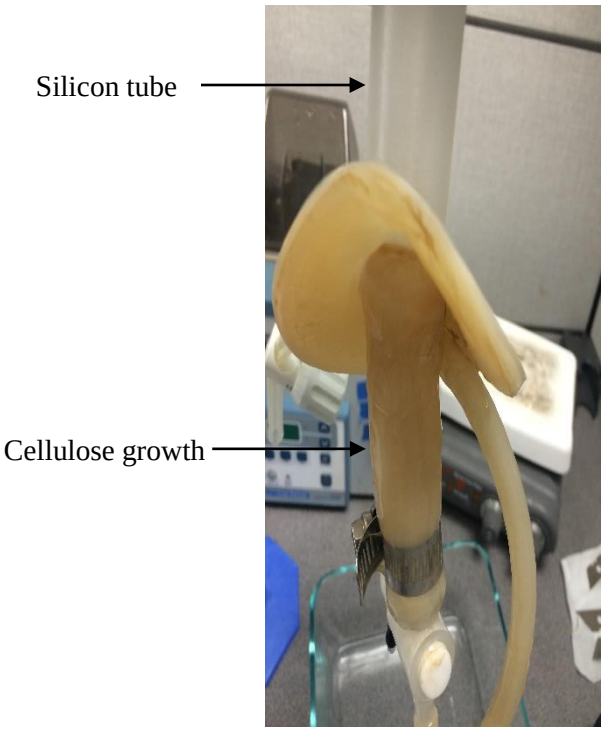

Figure 18: Bacterial cellulose growth on Si tube

Figure 17: Striations seen on the cellulose tube

- The Silicon tube fabricated in the lab by coating on a metal rod had a smooth surface. Just as the previous Si tube it was visibly a non-porous material.
    - There was good bacterial cellulose growth all around the Silicon tube.
    - The cellulose growth was continuous, confluent and thick.
    - It clearly maintained a cylindrical structure and was easily removed off the Silicon membrane.
    - The thickness of the cellulose membrane did evidently alter along the length.

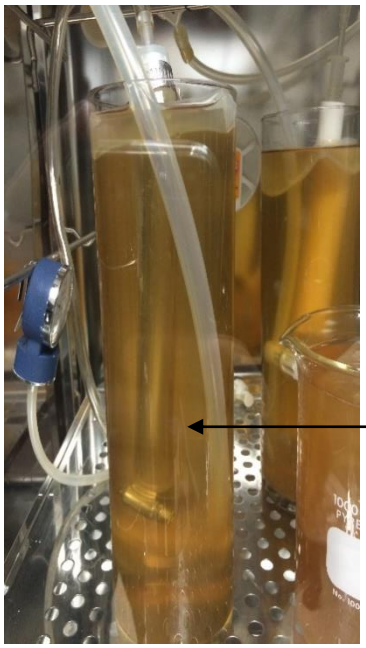

Figure 19 : Smooth Si tube being cultured in tea media

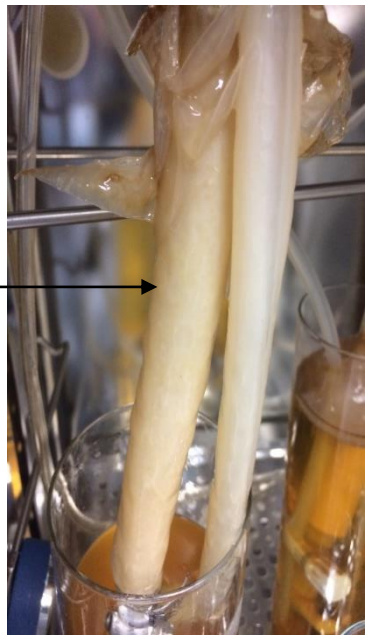

Figure 20 : Confluent Bacterial cellulose tube

- Therefore these preliminary tests helped in coming to the conclusion that the most suitable hollow carrier membrane to be used further would be the thin walled Silicon membranes fabricated in the lab using coating technique.

Table 3 : Table summarizes preliminary tests with different hollow carriers

Type	Diameter (mm)	Porosity	Surface	Thickness of BC growth	BC tube removable
ePTFE	19.75	High	Smooth	<0.3mm	✗
GenPore	20	High	Smooth	0mm	✗
Silicon (molded)	20	Non-porous	Rough / striated	~1mm	✗
Silicon (coated)	20	Non-porous	Smooth	~2mm	✓

Bioreactor

The bioreactor allowed us to use the hollow carrier as the inlet and outlet tubes, therefore enabling us to culture two tubes simultaneously. It offered stability to the culture and enhanced the speed at which a new culture can be started.

It came with few challenges the biggest one being the porous nature of the print material – PLA plastic. When trying to culture using controlled pressure it did not do well as the air leakage over longer periods of incubation was obvious. The leakage did evidently affect the cultured tubes. We tried coating the bioreactor pieces with commercially available waterproof Silicon glue, Gorilla glue and Rubber Evaporation was the next biggest challenge which hindered the growth of the cellulose tube along its length.

The modified version of the bioreactor that used pipe fittings instead of a completely 3D printed model was successful. It maintained the pressure in the system and totally curbed evaporation, resulting in healthy cultures overall.

Compaction

The tubes were compacted to about 20% their original thickness after the first cycle and further compacted to about 12% their original thickness by the end of their fourth cycle. The wet thickness measurements showed that the thickness did vary along its length and to an extent circumferentially. The compaction cycle reduces these thickness variations.

They were few challenges faced during the compaction/drying process. The tube needs to be held as a cylinder during the drying process or the tube collapses on itself as a flat cylinder and causes a crease along the tube length. Thick walled lab silicon tubes of slightly smaller diameter were inserted into the bacterial cellulose tubes before the start of the compaction process. Or the bacterial cellulose tubes were put a balloon / latex material and inflated. Both these methods were done to maintain the celulose tube as a cylinder during the compaction process.

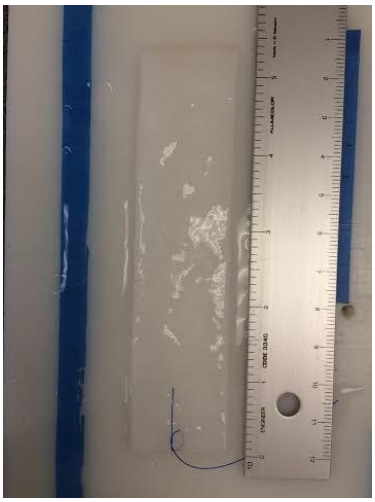

Figure 21 : Wet BC tube

Figure 22 : Dry BC tube

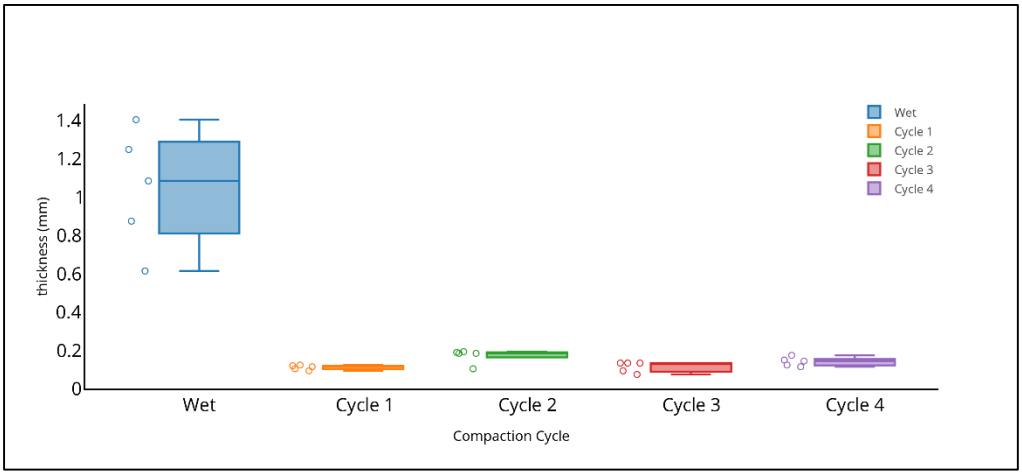

Figure 24 : Thickness measurement along the length of BC tube between compaction cycles – front face

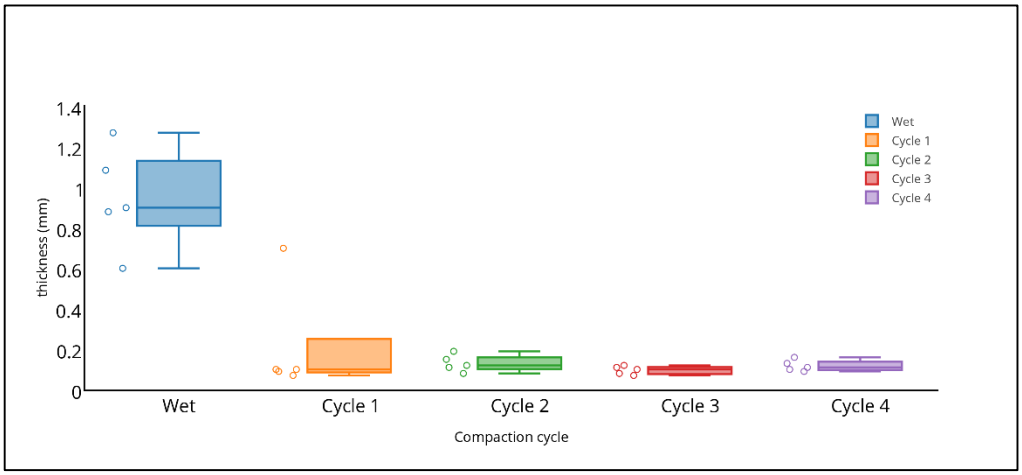

Figure 23 : Thickness measurement along the length of BC tube between compaction cycles – rear face

Fiber Orientation

One of the more distinctive features of the hollow bacterial cellulose tubes in contrast with bacterial cellulose cultured as flat sheets is their anisotropy. The cellulose fibers are said to be layered in parallel to the wall of the vessel or axially aligned [14].

Data from uniaxial extension tests show that the failure strength or the maximum force the material could withstand before complete fracture of the material was significantly higher when the sample was oriented axially during the extension.

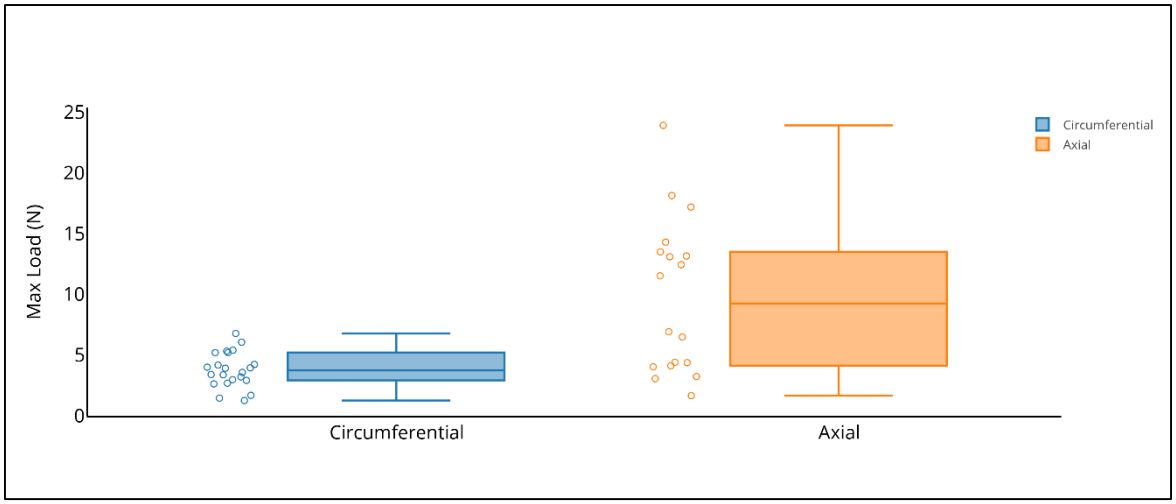

Figure 25 : Failure strength comparison between samples oriented in circumferential and axial directions

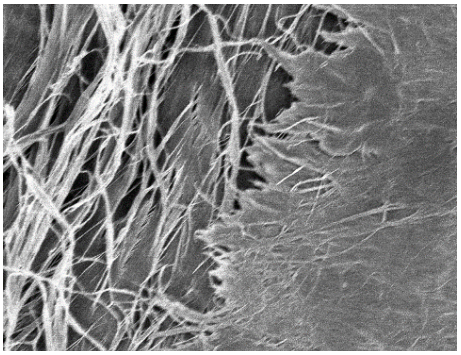

Figure 26 : SEM image of the BC tube material taken at the edge as a cross-section.

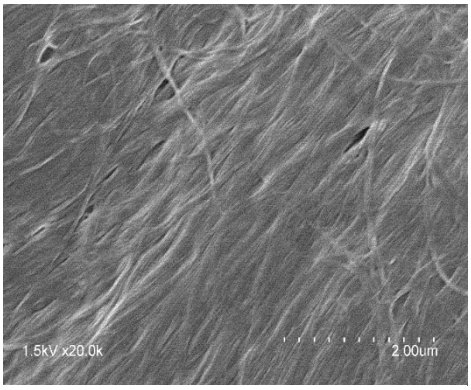

Figure 27 : SEM image of the BC tube material showing fiber orientation

SEM images were then taken to check fiber orientation. The images do show a preferential orientation of the cellulose fibers, therefore showing evidence of anisotropy.

Mechanical Testing: Assessing Parameters

The strength of the membrane is usually given by the fiber concentration. Wet measurements were compared to see if there were significant differences in the wet thicknesses of the cellulose tubes obtained between the two media. From figure it is evident that the bacterial cellulose tubes obtained from both the media had comparable wet thicknesses and no significant differences. But the cultures in mHS media with 100% oxygen used instead of air result in cellulose tubes that are significantly thicker (in wet state) and show regions that are close to 3.5mm thick.

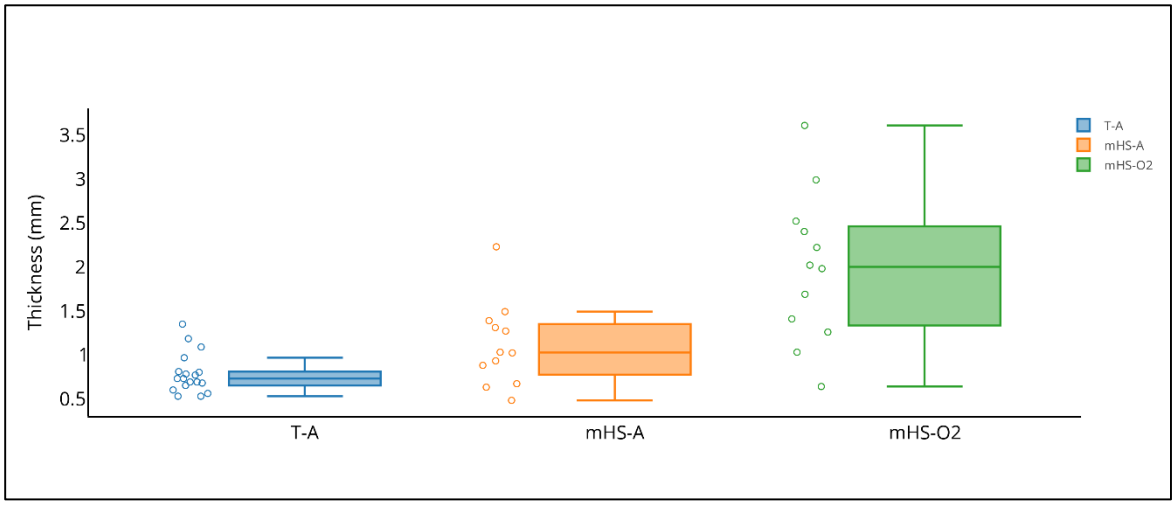

Figure 28 : Wet thickness of BC tube comparison between tubes in tea and mHS

- Uniaxial Extension : It was observed from the preliminary data analyzed that cellulose tubes grown in tea media and cellulose tubes grown in mHS media with air as the gas phase fail at a comparable range of max load for samples taken circumferentially and axially. It can also be observed that the mHS samples grown with 100% Oxygen does not follow the trend in showing axial samples having

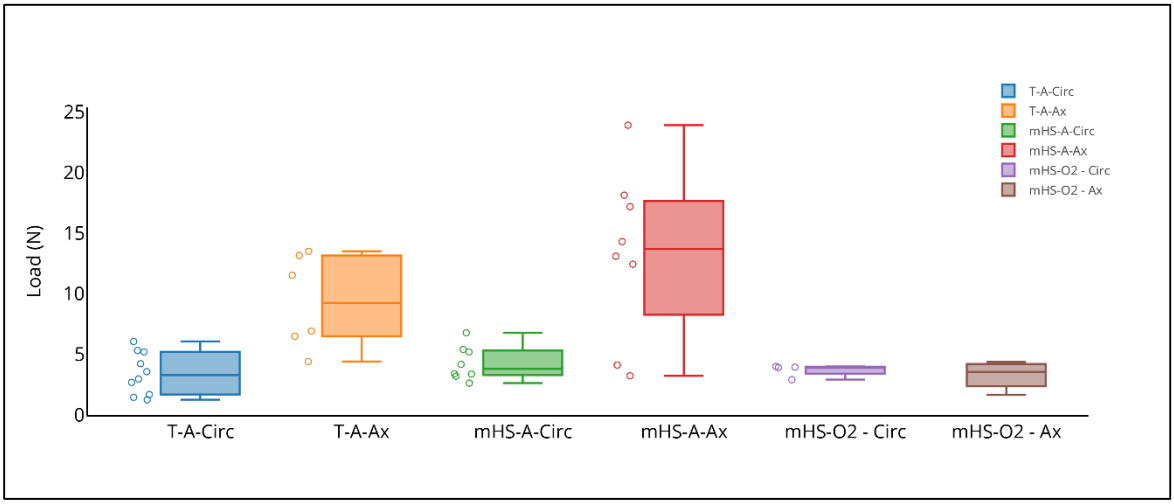

Figure 29 : Failure strength comparison between BC tubes grown in different media

higher failure strength. Can be noted that the axially oriented samples taken from tubes with mHS-Air combination show the highest failure strength.

- Flexure / bending test: Considering the cantilever length used for the test is 40mm, the x axis from figure 23 has a range between 0 and 40. A greater displacement from the x axis shows more flexibility. As you can see from the data

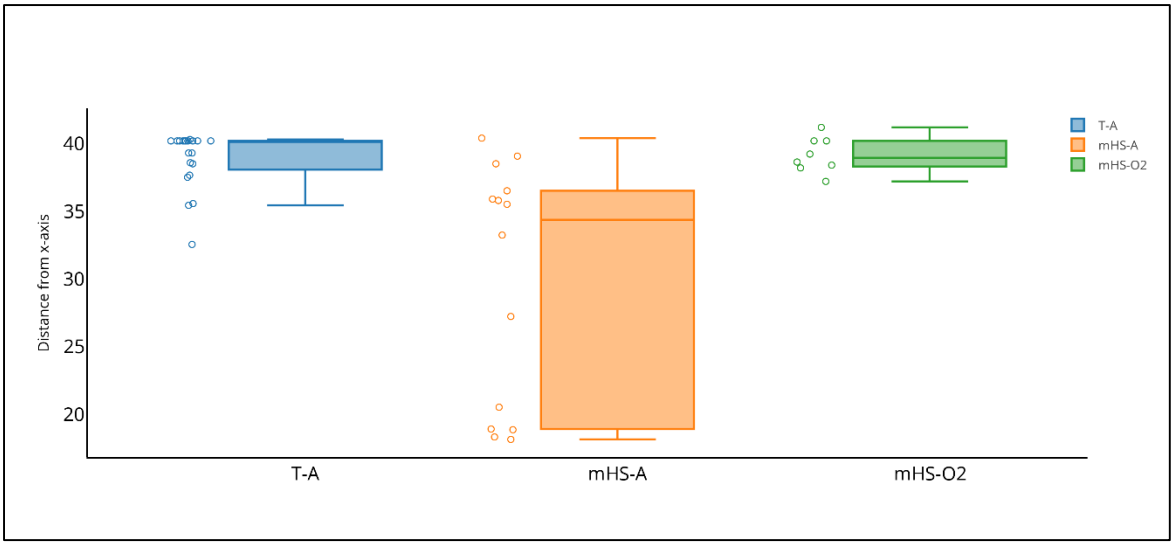

Figure 30 : Flexibility comparison based on displacement of the material from the x-axis for BC tubes grown in tea media and mHS

analyzed, the cellulose tubes grown in general show good flexibility. The flexibility between the material obtained from both the culture media with air and 100%O_2 as the gas phase do show the tubes grown in mHS media with air as the gas phase seem to have samples that show lesser flexibility, tubes grown in the tea media seem to be slightly more flexible.

- Tear Propagation: The average load / tension value is calculated as the sample experiences cyclic loading as the material tears. From the data analyzed, we can see the average tension at which the materials tear are fairly comparable for tubes grown in both the media types. Although tubes grown in tea media with air and mHS media with 100% Oxygen do show a higher average load value in

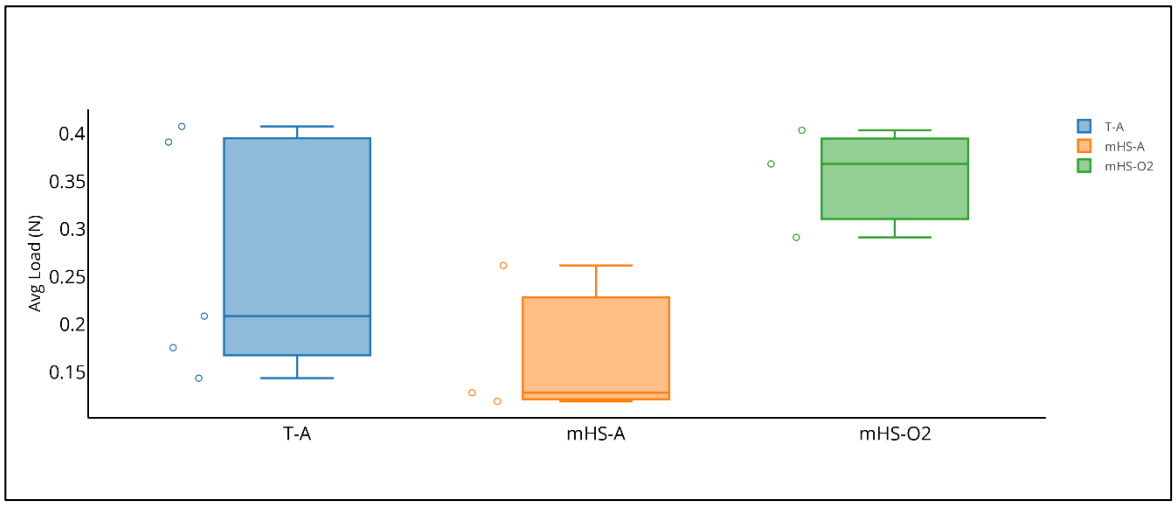

Figure 31 : Comparison of data obtained from the tear propagation test for BC tubes grown in tea media and mHS media

comparison with samples taken from tubes that are cultured in mHS media with air as the gas phase.

- Suture Retention: From the data analyzed it can be seen that the BC tubes cultured in mHS media with 100% O_2 as the gas phase seem to perform slightly better than the rest with a higher failure/retention strength. Following which, samples from tea media showing a higher failure/retention strength than samples from mHS media for the tubes grown with air as the gas phase.

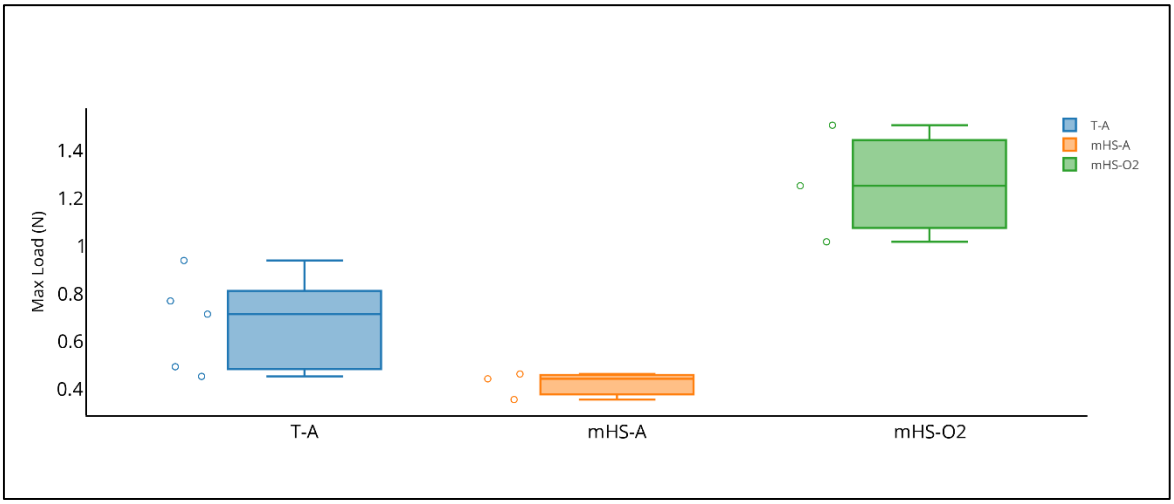

Figure 32 : Comparison of the failure tension values obtained from suture retention test between BC tubes grown in tea and mHS media

CHAPTER 5: DISCUSSION

The first heart valve was implanted in 1951, and it is undeniable how far we have come since then with the vast improvements in technology. Patients are being saved everyday with the use artificial heart valves and the need for newer technology is always high. With the introduction of bioprosthetic heart valves and percutaneous implantation, we see the drive to engineer devices with more native qualities, devices that are minimally invasive and devices that not only intervene but provide a good quality of life for the patient after implantation. Our interest being to develop a bi-leaflet bioprosthetic pulmonary heart valve, the biomaterial of interest would have to be very thin, flexible, durable and show some sort of biocompatibility.

A thin walled Silicon tube fabricated in the lab can be used to produce hollow continuous bacterial cellulose tubes. The moldability of the bacterial cellulose and the hollow carrier (which is Silicone in this case) opens up a variety of options for the future. It provides a much desired symmetry and can help reducing sutures. Conceptually, a desired design of the hollow carrier should be able to produce a corresponding bacterial cellulose membrane. During the preliminary test for choice of hollow carrier, the drive to begin with was to obtain a material that was porous and could push air through easily. But as the tests progressed it was observed that, more than just convection of air through the media, the bacteria responded to a membrane that was mainly non-porous but had a high Oxygen permeability and therefore gradually diffused Oxygen into the media. Also, with a material that is highly porous, it was seen that even a small portion that was not immersed into the culture media would end up being the path of least resistance for air flow and therefore most of the air could escape through the small portion on top exposed to the atmosphere affecting

the culture and slowly causing the media at the surface to evaporate. The success with the smooth silicon tube shows that the smoothness of the surface of the hollow carrier membrane also plays a role in cellulose tube synthesis.

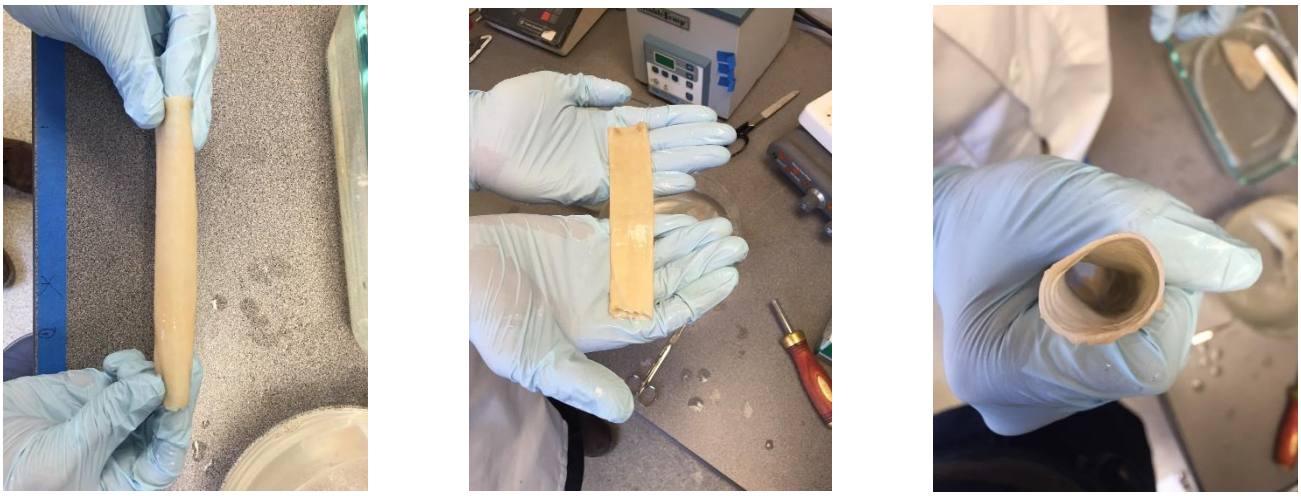

Figure 33 : Images of cultured bacterial cellulose tubes

The cellulose membrane as a continuous cylinder and its ability to be produced in various size and shapes gives surgeons the opportunity to be creative in their valve fabricating techniques. From reducing the number of sutures, to introducing tapered ends, designs can be optimized to attain better fastening of the membrane on to the commissure and with more progress could evolve into a stent less bioprosthetic valve.

The initial tests of the fiber orientation shows us that cylindrical bacterial cellulose tubes might show anisotropy. Therefore their mechanical properties can differ from flat sheet bacterial cellulose and be dependent on the fiber orientation. This can be further researched to characterize the burst pressure of the tubes and how cutting and suturing can be tuned to obtain maximum durability.

The mechanical tests helped us get an idea of how the material would perform when put into a valve configuration. And also helped us compare how the material performs when grown in two different media. The results show us that the material is a potential candidate

for heat valve prostheses and that most test results do not show a difference in the mechanical properties of the tubes obtained from the different media. So the factor that might actually affect the tubes material properties could be the tubes wet thickness when removed from the culture.

In a flat static culture, the thickness of the cellulose pellicle at the air-water interface is greater in the mHS media culture. This is not seen in the wet measurements of the BC tubes, and this could be due to the introduction of more limiting parameters in the cylindrical culture set up such as the membrane properties of the hollow carrier, the thickness of the hollow carrier, oxygen concentration and the pressure at which the air / oxygen is flowing through the carrier.

Limitations

In this study, the effects of the thickness of the carrier membrane were not studied. The wall thickness of the silicon membrane directly relates to its oxygen permeability and hence influences celluloses growth. A membrane with non-uniform thickness may relate to the cellulose growth on its surface and it is something that needs to be further studied.

The next big limitation is the sample size. Considering the culture time and the tubes ruled out due to contamination. The sample size was restricted to a couple of tubes for each type of media. Though this sample set can show trends and features, a much larger sample size is required to establish certain results.

The incubation period for all the cultures were kept constant at 15 days. The growth of cellulose does seem to plateau after 15 – 18 days according to literature [22]. But in a cylindrical culture where a lot of the growth is dependent on the slow diffusion of oxygen through a membrane, comparing with longer incubation periods is something that should be done in the near future.

Throughout the study 4 compaction cycles were done to obtain a thin membrane. But how / and if number of compaction cycles affect the mechanical properties of the bacterial cellulose tubes was not studied.

When considering any bioprosthetic conduit, biocompatibility of the material used is of great importance. Though different products of this material are currently being used and shown to have good biocompatibility, performing some in vitro tests (sensitization assays, hemocompatibility etc) would have helped establish some sort of biocompatibility of the material.

The orientation of the samples during the suture retention and tear propagation tests were not controlled. As the bacterial cellulose tube is shown to be anisotropic, the tear properties in both cases would be affected by the orientation of the samples.

Future Direction

The scope of current effort is limited to fabricating a cylindrical membrane. Much future work remains before this may be translated to the clinic. First, many of the limitations detailed above may be addressed. These include, study of additional fabrication parameters and additional experiments for a larger sample size in measurements. A better understanding of the relationship between various fabrication parameters and the resulting membrane features would help optimize fabrication. Subsequently, a bi-leaflet pulmonary valve could be prototyped by suturing this membrane onto a stent frame. Durability tests should be performed to assess the quality of the prototype and for comparing against a similar prototype fabricated with sheet-membranes to assess whether a cylindrical membrane is indeed superior in durability.

Additional studies such as biocompatibility tests followed by animal model studies and clinical trials would need to be finally performed.

In conclusion, a methodology for fabricating cylindrical conduit of bacterial cellulose membrane has been developed and evaluated. Three fabrication parameters were studied and reported upon. The mechanical properties of the cylindrical membrane have been characterized and shown to have preferential directions. This membrane has potential as a candidate biomaterial for developing catheter-deliverable pulmonary valves.

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