

DISSERTATION

FAILURE ANALYSIS AND DURABILITY ENHANCEMENT OF POLYMERIC HEART
VALVE LEAFLETS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2024

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ABSTRACT

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Rheumatic and calcified aortic heart valve disease is prevalent globally among all aged people, and the number is rapidly increasing. Clinically accepted, minimally invasive xenograft-based transcatheter aortic heart valve replacement (TAVR) shows limited durability (<10 years). Hyaluronic acid (HA) enhanced linear low density polyethylene (LLDPE) shows excellent *in vitro and in vivo* anti-calcific, anti-thrombotic, and hydrodynamic performance, making it an excellent candidate for heart valve leaflets. The main problem, however, is durability. Cyclic loading causes premature failure during accelerated wear testing. This dissertation investigates leaflet premature failure mechanisms and provides two plausible solutions to improve heart valve durability without sacrificing performance.

To investigate failure mechanisms, representative areas of retrieved failed leaflets were examined under scanning electron microscopy (SEM) and small angle x-ray scattering (SAXS). The investigation found abrasive wear, wear polishing, fine scratching, and imprints of the metal stent on the leaflet surface, indicating surface wearing from the soft plastic rubbing against hard metal. A strong permanganate oxidizer was used to etch away low-energy amorphous domains to unveil stable spherulitic structures of approximately 3 μm , and the bridging and tie molecule domains of pristine LLDPE. The oxidizer partially etches away areas of polymeric material accumulation of failed leaflets only to reveal thinned-out and fractured spherulites beneath them, identifying the built-up material accumulated due to areas of stress concentration that are the precursors to crazing and tearing. SAXS revealed local lamellar disruption further confirming the SEM results. Notably, this is the first study, to our knowledge, to directly image the stable craze cross-tie microstructure that formed due to chain disentanglement from high amplitude cyclic

stress. The SEM images validate previous theoretical and computational molecular dynamics models of cross-tie structure architecture. Therefore, leaflet premature failures are the compound effect of cyclic fatigue-initiated crazing and surface wear.

Heart valve leaflet durability can be improved by controlling crazing and surface wearing. Both the crazing and surface wearing can be controlled by crosslinking of randomly folded amorphous chains. Because they are direct impacts of chain disentanglement under high amplitude cyclic stress. Crosslinked covalent bonds of polymers limit chain movements. LLDPE thin sheets were crosslinked at 50, 70, 100, and 150 kGy doses using 200 KeV (low energy) and 4 MeV (high energy) electron beams at room temperature in the air. Their effects were characterized by measuring gel content percentage, tensile testing, differential scanning calorimetry (DSC), nanoindentation, and nano scratch tests. Crosslinked LLDPE heart valve leaflets were tested in *in vitro* flow loops and wear testers to determine valve performance and durability, respectively. Low energy electron beam (LEEB) crosslinking formed 28% xylene insoluble gel whereas high energy electron beam (HEEB) resulted in 58 % gel at 100 kGy doses. LEEB did not affect mechanical properties, but HEEB significantly increased stiffness and yield strength. A slight reduction of melting temperature was found for LLDPE crosslinked by both of the energy sources. Nanomechanical tests showed crosslinking improved hardness and coefficient of friction, an indication of improving surface wear resistance, which should lead to durability improvement.

Heart valve durability was improved by strengthening the leaflet with fiber reinforcement. A thin plastic sheet was assembled into a cylindrical form by welding two ends. The welded areas never failed in long-term testing. The weld at the commissure post was found to be mechanically stronger than the rest of the leaflet, which protected this region. Braided fibers were embedded in the leaflet regions of the commissure post perpendicular to the valve circumference, mimicking the weld but at a much higher strength. Leaflet durability dramatically increased from a few million to 73 million cycles. The entire cardiac cycle of the heart valve with embedded fibers of varying angles, lengths, and numbers was simulated using finite element analysis (FEA) to study the effects on leaflet maximum principal stress and leaflet opening dynamics. Horizontal fibers wrap the

leaflet 360° to relax the leaflet completely during the peak diastolic phase. However, the leaflet has a higher coaptation gap and delayed opening. The heart valve with embedded horizontal fibers was physically manufactured and tested in an *in vitro* flow loop and wear tester, which showed improved durability, but compromised hemodynamics. Finally, strategically crosslinked leaflets simulated in FEA demonstrated leaflets open more easily than fully crosslinked leaflets.

Finally, the three-dimensional (3D) shaped LLDPE leaflet bio enhancement manufacturing process was developed. Leaflets were 3D shaped in a vacuum thermoformer followed by HA enhancement. Whole blood clotting resistance, platelet adhesion, activation, and cytotoxicity studies determined that $10^{-4} \mu\text{mol}/\text{mm}^2$ HA surface density was required to achieve the best blood compatibility. Generally, water contact angle, toluidine blue O (TBO) elution assays, and attenuated total reflectance Fourier-transform infrared spectroscopy were used to determine overall HA presence on the leaflet. This study reports TBO staining and elution as the most effective and accurate measurement tool for determining HA population density. Crosslinked LLDPE were HA-treated, and TBO staining predicted adequate and consistent HA surface density for blood compatibility.

ACKNOWLEDGEMENTS

I would like to show my heartiest gratitude to my advisor and my mentor, Professor Susan P. James. I probably could not ask for anything better than to have her as my advisor. She gave me the freedom yet walked with me on the path of research and innovation. I will always miss our one: one meetings not only because I see you in person but also because our conversations have led to all the achievements I made in graduate school.

I am forever grateful to Professor Lakshmi Prasad Dasi for allowing me to study in his lab for a month, expanding my breadth of knowledge beyond materials to cardiovascular principles and computational modeling.

To my committee, thank you for your advice, guidelines, and recommendations. Thank you for evaluating my progress and providing invaluable advice.

I would like to thank my co-advisor, Dr. Travis Bailey, for hiring me as a graduate student and facilitating work in his lab. You made CSU feel at home from day one of graduate school. Dr. Kirk McGilvray, thank you for teaching me computational modeling and allowing me to use your workstations at the beginning of my graduate school. I would also like to thank Dr. Kaka Ma for collaborating with us and allowing us to study our material using the nanoindentation system.

To all the professors and educators of CSU, Dr. Ketul Popat, Dr. David Prawel, Dr. Brian Newell, Dr. Rebecca Miller, Dr. Alyssa Winter May, Dr. Liszt Coutinho-Madruga, Dr. Hieu Bui, Dr. Joseph DiVerdi, Dr. Alisa Shaw, thank you all for sharing knowledge with me which paved my interdisciplinary research work.

I especially thank Dr. Suresh D. Pillai and Dr. Matt Pharr from Texas A&M University for your collaboration and research.

To my lab mates Justin Gangwish, Ethan Harrell, Julianne Kindsfater, Jo Kindsfater, Nelson Issacson, and Cara Leone, thank you for all the invaluable contributions and support during my graduate school. Thank you, Katie Vinterella and Sakhawat Hossain, for all your work to progress my research. My friend, my colleague, Miyurudarshi S A K V Piyathilake, your mere presence

inspires me. Janelle Hobbs, thank you for collaborating, conducting, and educating me on nanoindentation. Dr. Alee Klug and Will Boston Moris kindly shared their beamtime at APS with me to run SAXS on fatigued leaflets. A special thanks to Sam Wolyn for developing the MATLAB code used in chapter two, which facilitated handling SAXS text files.

To my undergraduate advisor, thank you, Dr. Hasan Shahariar, for hiring me as an undergraduate research assistant, and my undergraduate lab mate, Rashedul Islam, Mr. Rojal Hasan, for teaching me research 101.

Finally, my mother, my educator, my love Sabina Begum, and my father, my friend Abul Khair, thank you for everything. There are no words or gestures that could possibly encompass the benevolent sacrifices you made so that I can pursue education. Thank you, my sisters, Khadiza Begum Shetu, Jannath Ara, and Sadia Akhter for being there every time.

To Rashedul Islam, my husband, my best friend, and the father of my son, I don't know where I would be if you were not there. Thank you for pulling me up at the lowest of my times. Thank you for dropping me off at school and picking me up from school every day during my graduate school. Finally, thank you for showing me the mountains.

DEDICATION

*To the academic community, children, women, and men of Palestine, the martyrs of Bangladesh
for their benevolent sacrifice, and my little son Abdullah Afnan*

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Chapter 1

Introduction

1.1 Research Motivation

There were 21.5 million cases of calcified aortic valve diseases (CAVD) with moderate to severe aortic stenosis (AS) [9] and aortic valve disease caused by rheumatic heart diseases (RHD) globally in 2019 [9–11]. There is a need for durable and effective aortic valve replacements for all age patients because these two diseases affect two separate age groups [9]. Currently prescribed medical treatments either need highly invasive open-heart surgery or have poor durability due to thrombosis and calcification. Aortic restenosis occurs in both surgical and transcatheter tissue valves, and lifelong blood thinners are required for mechanical valves. A more hemocompatible and durable substitute for tissue TAVR leaflets will establish a long-term successful medical therapy for all patients, including young, low-risk patients (Society of Thoracic Surgeons score <4), without future interventions.

1.2 Current Scenario of Cardiovascular Disease

Valvular heart disease can be classified broadly into three main categories: RHD, CAVD, and Degenerative mitral valve diseases (DMVD). RHD frequently affects children and young adults in developing countries because of socioeconomic culture and overcrowding [12–14]. It causes moderate or severe tricuspid and bicuspid valve regurgitation [12], which damages three of the four human heart valves. Streptococcus bacterial infection causes Rheumatic Fever; later, this fever can damage the valves permanently [15]. In 2019, there were an estimated 40.5 million RHD-related active cases worldwide, making it primary heart disease. It is worth mentioning that developing regions like Oceania, South Asia, and central Sub-Saharan Africa had a majority share of the patients.

On the other hand, CAVD is the second primary valvular heart disease after RHD, which claimed

126,827 lives with an additional 9.4 million active cases globally in 2019 [9]. CAVD results in severe or moderate aortic stenosis i.e., thickening of leaflets reduces leaflet flexibility, resulting in central leakage and high-pressure gradient [16]. Leaflet calcification risk factors include but are not limited to aging, hypercholesterolemia, hypertension, smoking, and high BMI [9, 12, 17]. Therefore, CAVD cases are higher in developed countries than RHD cases [12, 13]. Probably because 16.783% of the population are 65 years old and above in developed countries, compared to 6.15% in South Asia [18]. In addition to CAVD, there were 24.2 million active cases of non-rheumatic mitral regurgitation globally in 2019, which is 157% higher than CAVD. However, there was 73% less death due to compromised mitral valve. Because of the mitral valve position, i.e., when Arteriors are damaged, it does not impact the mitral valve.

1.3 Native Heart Valve Architecture

The human heart uses four passive functioning valves: aortic, pulmonary, mitral, and tricuspid valves to fuel circulating nutrients of blood to the body [19]. All four healthy valve leaflets are composed of three layers named fibrosa, spongiosa, and ventricularis; they differ by thickness. Thickness changes with respect to their required transvalvular pressure gradient (ventricle to aorta pressure difference during diastolic). For example, the mitral transvalvular pressure gradient is the highest (120 mm Hg); which is why its leaflets are the thickest [20, 21]. Fibrosa consists of a complex network of collagen type-I fibers running primarily in the circumferential direction, but few fibers align in radial direction as shown in Figure 1.1 (A-B) [20, 22]. Collagen fibers are extremely strong, yet they have a corrugated architecture in leaflets. Therefore, these fibers act as the primary load bearer during the opening. In contrast to the fibrosa layer, spongiosa is a cushioning and lubricating layer between fibrosa and ventricularis made of glycosaminoglycans (GAGs) and proteoglycans (PGs). This layer is highly hydrated and transfers load from fibrosa to ventricularis. Similar to fibrosa layer, ventricularis layer has a mesh of collagen and elastic fiber running circumferentially. This thinnest layer prevents excessive radial strains during the valve

opening. The architecture of these three layers provides a strong yet flexible and robust structure that results in non-linear anisotropic mechanical properties [23].

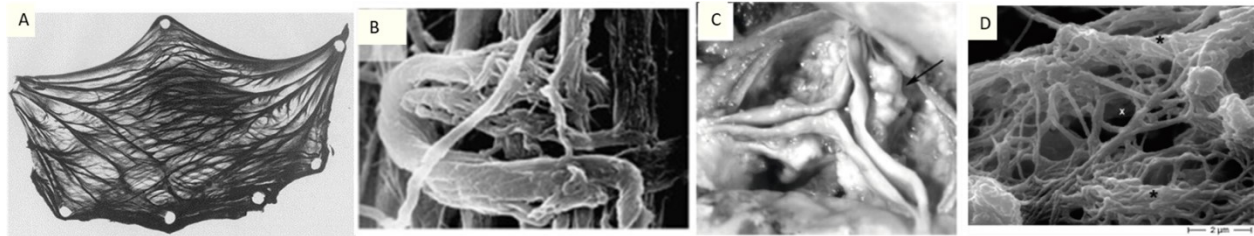


Figure 1.1: Optical and Scanning Electron microscopy images of collagen fiber arrangement in healthy (A-B) and diseased valve (C-D), respectively [1] Copyright 2014, John Wiley and Sons.

1.4 Aortic Valve Diseases and Treatments

CAVD is the most common cardiovascular disease among adults. Because mitral and aortic valves pump blood into the extremities of the body, they undergo a higher transvalvular pressure gradient (120 and 100 mm Hg, respectively) than right-side pulmonic valves (<30 mm Hg) [20]. Moreover, section 1.2 shows a significantly higher number of cases associated with these two valves. On the other hand, CAVD related deaths are higher than mitral valve since the aortic valve is damaged as the aortic root gets damaged. Therefore, the rest of the dissertation deals with aortic valve diseases and their treatments. CAVD causes mild to severe aortic stenosis, which thickens and stiffens leaflets, resulting in improper opening and closing. Sever AS can reduce effective orifice area (EOA) to less than 1 cm^2 , increase mean pressure gradient (ventricle to aorta pressure difference) to higher than 50 mm Hg, and increase blood peak velocity to more than 4 ms^{-1} [24]. This disease starts with the formation or deposition of calcium phosphate on collagen type I fiber (Figure 1.2 C-D), which limits fibers ability to slide past each other and increase bending stiffness [25]. Aortic valve regurgitation is another common disease that accounts for over 50% of total aortic valve replacement. It causes a backward blood flow from the aorta into the left ventricle, causing a heart-burning-like sensation. This disease is progressive but generally becomes symptomatic in advantaged stages.

Currently, there is no medical therapy to permanently prevent the progression of this disease [25–27]. Consequently, valve replacement is the only prescribed treatment for severe AS. The replacement procedure can be surgical or trans-catheter. Surgical procedure: open the sternum, bypass blood flow on external blood, remove the diseased leaflets, and suture a bioprosthetic or mechanical valve. This procedure takes 2-4 hours of exhaustive surgery and 4-8 weeks more to recover.

Since 2002, with the first-ever trans-catheter procedure and full FDA approval in the following years, doctors have practiced this minimally invasive process for valve replacement. Favorable endovascular access routes such as transfemoral, transapical, transjugular, trans atrial routes facilitated Transcatheter Aortic Valve Replacement (TAVR). Gold standard TAVR treatments have been skyrocketing since 2011 with the first FDA approval and have performed 276,316 cases of replacement [28]. Recently, this treatment procedure has exceeded surgical aortic valve replacement treatments. This number is expected to boom again with the FDA approval for intermediate-risk patients in 2016 and low-risk patients in 2019.

1.4.1 Transcatheter Aortic Valve Replacement (TAVR)

The native aortic valve is a passive functional system that opens and closes due to the pressure difference between the ventricle and the aortic side. During systole, leaflets stay open for around 330 ms to flow blood through the aorta. A few mm mercury pressure difference aortic ventricle side can continue the blood flow [29]. During opening (systolic), the corrugated nature of the fiber mesh network discussed in the previous section comes into action. Native aortic valve primarily has only leaflets or cusps, which are connected to the annulus. Surgical valves have a leaflet and can have a non-collapsible stent. Doctors suture the stented valve or only leaflets of the surgical valve to the annulus after removing the diseased native valve. On the other hand, TAVR must have a collapsible stent, one combined or three individual leaflets which are attached to the stent by suture or adhesion between polymer and stent.

TAVR stent holds, supports, and bears the cardiac stress over the leaflet (Figure 1.3) and can

collapse or crimp into a smaller diameter. This elite feature allows doctors to deliver TAVR by a catheter. Therefore, stent design and material consideration play a critical role in TAVR durability [1]. Tissue or bioprosthetic leaflets are thermo-chemically treated to fix the tissue and reduce thickness [1], cut into size, and sutured to the stent with surgical sutures. Most upgraded valves come with a paravalvular skirt to prevent leakage and migration. The skirt is generally a woven or knit textile with high porosity, promoting fiber in-growth and having a rough surface that provides frictional force to hold the valve in the annulus.

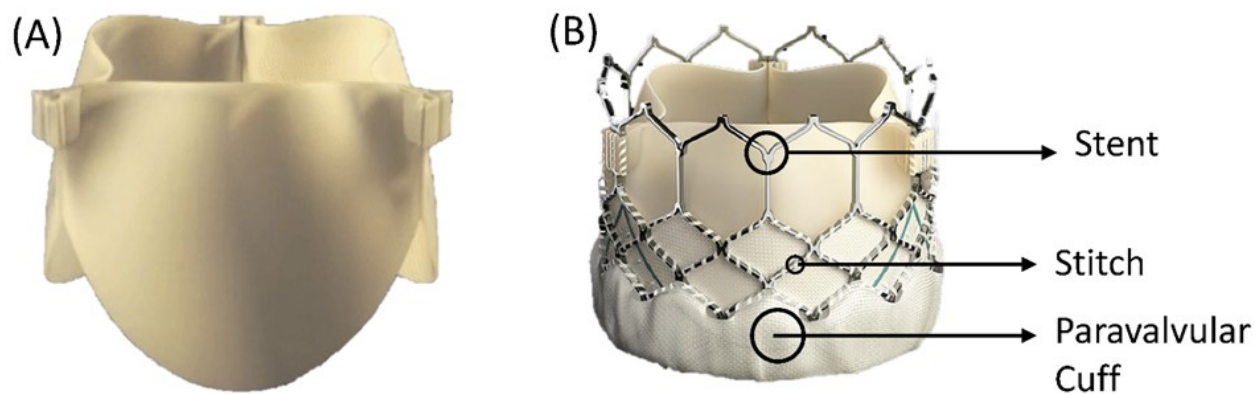


Figure 1.2: FDA-approved SAPIEN 3 valve parts (A) tissue leaflet (B) TAVR showing different parts [1]
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TAVR approaches the diseased valve during implantation after transfemoral insertion through a catheter. Beforehand, the valve is crimped to match the patient-specific blood vessels' size, followed by expansion at the annulus. The expansion can be self-regulated or balloon expansion, technically called self-expandable and balloon-expandable valves, respectively. This contraction and expansion regulate restriction in material consideration for both stent and leaflet. The stent design and leaflet flexibility must allow elastic crimping and expansion. Stainless steel, cobalt-chromium alloy, and nitinol have been widely used as stents [30]. On the other hand, the bovine pericardial sac and porcine aortic valve have been used for leaflet material in the bioprosthetic field. Surgical bioprosthetic leaflets were able to translate into TAVR leaflets after modification, whereas mechanical valve, being rigid, failed to do so.

During the TAVR procedure, the diseased valve remains on the annulus, which can be a savior that

facilitates initial anchoring and placement yet a curse that causes paravalvular regurgitation as de-calcification occurs over time [31]. Therefore, it is critical to maintain seamless contact between the stent and the aortic annulus to prevent de-calcification and regurgitation. Few parameters determine the success of the treatment and overall patient health condition, such as effective orifice area (EOA), pressure gradient (ΔP), peak velocity, and regurgitation factor, including paravalvular leakage, turbulence, vorticity, pinwheeling index. A successful TAVR would increase EOA $> 1.6 \text{ cm}^2$, reduce mean (ΔP) $< 10 \text{ mm Hg}$, and peak velocity $< 2 \text{ m/s}$ [24].

1.5 Commercial TAVR and Their Limitations

Current Food and Drug Administration (FDA) approved commercial TAVRs are from the Edwards Sapien Family (Figure 1.2) and the Medtronic Corevalve family. Other CE marked valves are Melody and Tendyne valves. The Lotus edge valve was removed from the market. All these valves are bioprosthetic or tissue valves with xenograft leaflets. This leaflet has excellent biocompatibility and hemodynamic properties. However, they suffer from calcification and tearing. On the other hand, only recently, the Foldax Tria surgical valve leaflet made of LifePolymer received FDA approval for a clinical trial, but the TAVR is still under research and development. This silicone polyurethane polymer patented valve has little published literature. The following two adapted sections will discuss more details of bioprosthetic valve leaflets and their shortcomings.

1.5.1 Bioprosthetic Leaflets

This sub-section is adapted from [1], previously published by Advanced Health Care Materials, John Wiley and Sons.

Caribier et al. first performed percutaneous transvalvular aortic valvuloplasty (PTAV) in 1986 [32]. A balloon catheter implantation was performed at the aortic position of elderly patients with aortic stenosis because surgical heart valve replacement was deemed impractical [32]. Unfortunately, 75% of PTAV performed patients had restenosis. Therefore, he theorized the need for a stented

valve and modeled the device [33]. Around the same time, Anderson et al. reported a balloon-expandable transcatheter aortic heart valve implantation in a pig [34]. The valve stent was made of medical grade stainless steel monofilament loops that were welded together to form a closed cylinder. The porcine aortic heart valve was mounted over the stent as a leaflet [34, 35]. The leaflet was attached to the stent using Prolene (medical grade polypropylene monofilament) suture. Over the last 60 years, bioprosthetic heart valve leaflets have made of tissues either collected from patients (autografts), human cadavers (homografts), or non-human animals (xenografts) [36]. Among them, xenografts are the most common due to their availability and wide ranges in size and thickness. However, these valve prostheses showed early failure caused by antigenic components inducing an immune response and the degeneration of collagen fibers. Moreover, cellular ingrowth in the cusp has been shown to cause severe inflammatory response [37]. Researchers treated tissue with glutaraldehyde which acts as a crosslinker of collagen molecules to improve durability and dissolve antigenic components to prevent the unwanted immune response [36–38]. However, researchers eventually realized the glutaraldehyde crosslinked leaflet also suffers from calcification, especially among younger patients. This is probably because more vital immune cells in young patients secrete calcium stimulating cytokine [38], which is potentially responsible for leaflet calcification. Interestingly, researchers observed that glutaraldehyde fixed bioprosthetic heart valve replacement caused an increase in anti-galactose- α 1,3-galactose (Gal) antibody since the heart valve leaflet had antigen Gal. It was later discovered that tissues having anti-Gal antibody would calcify less leading to the conclusion that anti-Gal is a prime suspect for calcification. This evidence prompt researchers to genetically modify tissue sourcing animals to grow more human compatible tissues [38].

Bovine and Calf Pericardial Sac

Pericardium, the fibroelastic envelope of the heart, is constructed of two layers: epicardium and parietal pericardium. There is pericardial fluid in between these layers, which prevents friction between them during the cardiac cycle. Epicardium is made of mesothelial cells nearest to the heart. Parietal pericardium is composed of type I collagen fiber and glycosaminoglycans [39, 40].

These three components are responsible for generating the viscoelastic nature of the pericardial sac, which helps to dissipate energy. Moreover, researchers found that the sac undergoes heterogeneous loads in the cardiac cycle. This physiological event enforced regionally varying collagen fiber architecture (Figure 1.1). This structural irregularity of collagen fibers resulted in anisotropic properties confirmed by bi-axial mechanical testing. Interestingly, left anterior specimens having comparably more regular fiber architecture show more isotropic mechanical properties. This anatomical variability of the collagen fibers could be mapped using small angle x-ray scattering technique [39,41]. This technique could directly control tissue variability that dictates anisotropic mechanical properties. Therefore, material constants could be derived for modeling and analysis. Different crosslinking techniques have significantly improved animal tissue. Lagrangian stress-strain response at a stress level below 200 kPa, but high stress-strain responses of crosslinked and native ones are comparable to each other. This is probably because the chemical treatment signifies an inter collagen fiber bond thus improving its mechanical properties to a great extent in a similar manner to fiber-reinforced composite but cannot change the fiber intrinsic properties which could affect high stress-strain response. While ultimate tensile strength and stress-strain behavior are vital for predicting failure properties, flexural rigidity is also critical in predicting hydrodynamic and fatigue properties [42]. Heterogeneity in the intrinsic mechanical properties of different layers and applied load during the cardiac cycle generates non-linear moment bending curvature behavior in epicardial and visceral surface directions. After crosslinking, the tissue becomes stiffer and behaves more non-linear elastic [42]. Moreover, post crosslinking, stiffness in collagen fiber direction and cross-fiber direction increases 211% and 68%, respectively, at .02 mm⁻¹ curvature [43]. Native fiber direction stiffness is lower than cross-fiber direction because of weak inter-fiber (parallel and near parallel) bonding. During crosslinking, parallel fibers get crosslinked, which significantly improves inter-fiber bonding strength, and therefore, stiffness increases more in the fiber direction [43].

Porcine Pericardium

Researchers found direct mesostructure-function relationships for collagen and elastin fiber in porcine aortic leaflets. Collagen and elastin fiber alignment dominate the mechanical properties of the fibers. Several researchers reported circumferential collagen fiber of the fibrosa layer is the main load bearer during diastole [22]. Whereas collagen and elastin fiber of ventricularis layer exert compressive pressure to keep fibrosa in highly rippled conformation. Spongiosa drives shearing and localized movement of two outer layers [40]. The aortic heart valve shows distinct mechanical anisotropic behavior in uniaxial and equibiaxial testing, i.e., stiffer in the circumferential direction (strain can be negative) than the radial direction. Moreover, native leaflets have distinct low modulus and high modulus regions providing load transfer from elastin to collagen regions [22]; whereas crosslinked leaflets have more regular and predictable stress-strain behavior in the equibiaxial testing [39]. In contrast to the pericardial sac, the flexural modulus of the porcine aortic heart valve leaflet is more linear in behavior for both belly and commissural regions. Before crosslinking, changes in stiffness with flexure are independent of flexural direction (i.e. against native curvature and with native curvature). However, it becomes approximately 40 times more difficult to flex against native curvature direction after treatment. The stiffness linearly decreases in the commissural region and increases in the lower belly region. This is probably due to the tissue buckling mechanism in the commissural region. Interestingly, crosslinked porcine tissue valve losses stiffness under cyclic fatigue significantly in a direction that is against the native curvature of the sac. The loss could be related to collagen fiber damage.

1.5.2 Limited Durability of Current TAVR

This sub-section is adapted from [1], previously published by Advanced Health Care Materials, John Wiley and Sons.

Ideally, the implanted prosthesis should be fully functional for a more extended period than the patient's expected lifespan. Unfortunately, these implanted valves have shown impaired functionality because of valve structural deterioration (SVD), infection, and thrombus formation

in many cases, depending on the patient's underlying health condition, age, sex, as well as device characteristics such as leaflet structure and stent design. Calcification and tearing are the main reasons for SVD, and they can be defined in two progressing stages. In the first stage, doctors diagnose leaflet morphological changes through imaging, and in the second stage, this morphological alteration results in a gradual increase of the transvalvular pressure gradient [44]. The bovine pericardial tissue-based valve tends to show calcific deterioration, whereas the porcine aortic valve tends to show tearing, probably because the latter has lower mechanical strength. Leaflet calcification has proved to be dependent on the tissue crosslinking method for the bioprosthetic valves [16].

Stent design has a significant impact on long-term leaflet durability because the stent partly matches the non-circular and calcified patient-specific root anatomy, leading to unequal stress on the leaflet [45]. For instance, photo-fixed bovine pericardial valve has reportedly failed due to poor stent design [46]. Leaflet mechanical properties (i.e. stiffness, yield strength, and viscoelastic behavior) also play critical roles in long-term success. Numerous clinical trials have been conducted since 2005 on TAVR such as Placement of AoRTic TraNscathetER (PARTNER) 1, PARTNER 2, Italian Registry, FRANCE-2 Registry; 657, 1011, 353, 4201 high risk patients with severe aortic stenosis and STS were treated in the respective trials [47]. In the five-year studies only 53 (PARTNER1), 373 (PARTNER 2), 108 (Italian Registry), 459 (FRANCE-2 Registry) patients were available for echocardiographic evaluation. Among them Italian Registry reports 4.2% SVD and FRANCE-2, 2.5% SVD [48]. Some early studies on TAVR treatment reported 3.5% of SVD among 84 patients available for evaluation after five years. One should not extrapolate any durability data from these because they carry little statistical information value because the 5-year end population is statistically small. Two primary clinical trial Placement of PARTNER Valves Trial II and Surgical Replacement and Transcatheter Aortic Valve Implantation (SURTAVI) are expected to end in 2024 and 2026 would provide more long-term studies might inform transcatheter bioprosthetic valve durability.

1.5.3 Paravalvular Leakage

Paravalvular Leakage or Paravalvular Regurgitation (PR) remains a consistent issue causing stroke and silent death post-treatment [24]. Although all the hemodynamics improved after TAVR, PR remained almost similar before and after. The most recent (2022) iteration of the Sapien valve, the Sapien 3, ultra-used a texturized Polyethylene Terephthalate textile that has high porosity to achieve a high frictional coefficient. This textile is sutured in the lower annulus part of the valve. This skirt is 40% taller than the previous Sapien 3 valve, which increased the contact of the annulus and reduced PR in vivo. Among 101 patients treated with this valve, 10.8% had mild PR compared to 36.5% with Sapien 3 30-day post TAVR evaluation. The credit was given to the tall texturized outer skirt [49, 50]. Therefore, a properly designed annular skirt can reduce PR and protect the annulus by reducing the radial force of valve expansion from crimping. New technologies are emerging with innovation in skirt material selection and design. For example, the Triskele valve has a sealing cuff and skirt, Accurate Neo2 has hydrogel, and Navigate has polyester microfiber fabric [51]. Polyester microfiber yarns have micropores, and the fibers are in the micron range diameter. Therefore, this promotes tissue ingrowth to reduce the gap between the valve and the annulus. Hydrogel conforms to the shape of the annulus and prevents PR but could cause valve migration due to less frictional co-efficient.

1.6 TAVR *in vitro* Testing

1.6.1 Hydrodynamic Testing

Hydrodynamic *in vitro* testing of TAVR is crucial to determine early-stage valve performances. Left heart simulator, particle image velocimetry (PIV) is used to image and trace fluid dynamics of a mixture of water and glycerin instead of blood across a TAVR [8, 52, 53]. This simulator causes a pulsatile flow mimicking physiological pressure, heart rate, and cardiac output. This tracer estimates the maximum velocity of blood to calculate the mean pressure gradient and EOA. For this, a pump ejects fluid through a stationary valve and a capacitance control fluid waveform. A systematic limitation can arise from using room temperature fluid instead of body

temperature since these subtle temperature differences can change fluid dynamics [8, 54, 55]. Moreover, standard parameters for a TAVR *in vitro* performance are provided in ISO 5840-3. On the other hand, *in vivo* hemodynamics is performed using Doppler echocardiography to calculate peak velocity, which calculates pressure gradient.

1.6.2 Fatigue or Wear Testing

Section 1.5.2 discussed *in vivo* long-term durability issues and shortcomings of the current bioprosthetic tissue valve. *In vivo* tests are conducted by computed tomography and echocardiography to image and evaluate the morphology of the leaflets. *In vitro* durability tests can provide insight into valve lifespan in their development stage. An accelerated wear tester (AWT) is generally used to run TAVR in physiological conditions except at an accelerated rate. The rate is crucial and unique for each leaflet polymer or tissue since both have viscoelastic properties. Generally, thin film leaflets such as polyethylene are tested at 15 Hz frequency, avoiding self heating and capturing the viscoelastic behavior. ISO 5840 provides standards on the performance requirement [56]. According to this standard, 200 million fatigue cycles are the standard performance in an AWT for a TAVR.

1.7 Research Scope

State-of-the-art polymers can be an effective alternative to tissue leaflets. Polymeric transcatheter heart valves have received much attention in recent years due to their promising biomechanical properties because polymers can be engineered to tune biomechanical responses. The classic advantages of synthetic biomaterial over xenografts are low cost, mass production, and consistent properties [57, 58]. On the other hand, the disadvantages originate from the absence of biomolecule and biomimetic properties in synthetic biomaterials. Therefore, a classic issue for heart valve implants is developing a biomaterial with a perfect blending of biomimetic and synthetic properties. Polyurethane is the oldest implanted leaflet in which biomechanical responses were improved over time with crosslinkers [58, 59], nanoparticle fabrication [60, 61],

and heparinization [62]. Nanoparticle incorporated polyurethane based Triskele valve [22, 60, 61, 63, 64], Siloxane Poly(urethane-urea) based Tria™ valve from Foldax Inc. [65], crosslinked SIBS based Polynova valve [54, 57, 58, 66, 67], silicone based Strati Access Technologies valve have been under research and development for the last decade. These are mostly polyurethane-based heart valves that could be thermoplastic, thermosets or nanoparticle incorporated. They had shown promising *in vitro* performances, but little is known about their *in vivo* performances [66]. Polyurethane based valve main drawbacks include but are not limited to hydrolysis under biological conditions and the presence of toxic crosslinkers. On the other hand, LifePolymer, one of the most recent iterations of the polyurethane to receive FDA approval for human clinical trial, has crossed 600M cycles of accelerated wear testing [57]. Besides, Polyester textiles (Dacron) [45, 53, 68, 69], and expanded polytetrafluoroethylene (ePTFE) [70, 71] have shown excellent hemodynamics because they can have virtually unlimited flexibility. However, biofouling [72] and regurgitation through the pores reduced their application in heart valve development [57]. Coating of textiles with hydrogels to mitigate regurgitation was observed with the sacrifice of its iconic flexibility [73]. A more detailed critical review of different heart valve leaflet materials was presented by Bui et al., [1]. Another relatively young research field is the interpenetrating polymer network (IPN) of guest biomolecule and host polymer instead of surface coatings. IPN has been shown to incorporate biomolecules in polymers without altering their mechanical properties [74]. One such iteration invented by the James research group and collaboration with Dr. Prasad Dasi's Georgia Tech lab is the current Rejuvenate™ valve which demonstrated excellent biocompatibility, biostability (*in vitro* and *in vivo*), and suitable mechanical properties [74–77]. This dissertation focuses on improving the performance of the polymeric Rejuvenate™ valve. Therefore, the following section discusses the details of the valve.

1.7.1 The Rejuvenate™ Valve

Polyethylene (PE) is used in more than 700 FDA-approved medical implants due to its availability, low cost, facile manufacturing process, versatility, adaptability, and biostability.

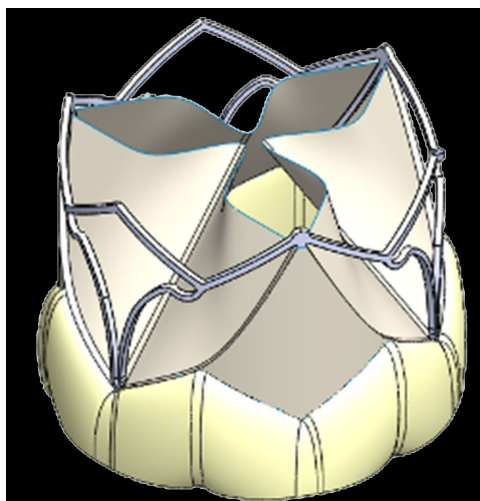


Figure 1.3: Rejuvenate™ valve assembled over stent reprinted with permission from YoungHeartValve, LLC.

High-density polyethylene, ultra-high molecular weight polyethylene, medium, and low-density polyethylene are some common categories of PE in medical implants. Tissue ingrowth ability and vascularization gave much of its success in the medical implant field. PE is considered a gold standard surgically implantable biomaterial [78]. In contrast to these implants, heart valve leaflets must be bioinert or near bioinert and prevent vascularization, biofouling, and protein adhesion cascade, which can lead to tissue ingrowth to maintain leaflet flexibility and functionality. Moreover, leaflets must be flexible yet have high tear strength to survive millions of cardiac cycles. Linear Low-Density Polyethylene (LLDPE) blown films have a moderate modulus of elasticity, great flexibility due to linear structure, and high tear strength. Dowlex 2045G (commercial resin of LLDPE) blown film has the maximum intrinsic tear strength among other resins [79] because it has an octane comonomer which is the largest side chain length. This material has been studied to obtain biocompatibility and non-biofouling nature by incorporating biomolecules [76]. In previous research, LLDPE amorphous region is swollen in tempered xylene to penetrate HA molecules, which are later crosslinked. This process gives a robust interpenetrative polymeric network (IPN) that significantly reduces protein adhesion cascade *in vitro*. Interestingly, *in vivo* chronic calcification and thrombus studies showed no calcification and thrombus formation under the naked eye, histology, Scanning electron Microscopy (SEM), Electron Dispersive Spectroscopy

(EDS), and Fourier Transform Infrared Spectroscopy (FTIR) (unpublished data). This novel biomaterial was achieved because HA is a naturally occurring polysaccharide consisting of repeating disaccharides of N-acetylglucosamine and glucuronic acid, making it hydrophilic. The flexible biomaterial was assembled over a balloon-expandable cobalt-chromium stent and sutured together using surgical threads to devise a TAVR.

1.7.2 Present Research Limitations and Proposed Research

Despite showing excellent biocompatibility and durability in the 6-month animal trial HA-LLDPE leaflets have shown tears beginning at 4 million cycles tested on a bare stent, around 50 million cycles on a nylon thread-wrapped stent, and 150 million cycles tested on a stent with a silicon shock absorber in the AWT. Although numerous investigations have been conducted on PE fatigue life prediction and modeling, few researchers have addressed the effect of PE part geometry, contact stress, and morphology on fatigue life, and fewer studies have focused on LLDPE. Therefore, there is a gap in understanding the LLDPE fatigue failure mechanism: polymer morphological changes with progressive fatiguing. Understanding this behavior could generate excellent fundamental scientific knowledge and provide solutions such as crosslinking. Leaflet tapering, i.e., changing leaflet thickness, could also reduce peak stresses, developing a gradient of flexural modulus [61]. Another way to improve the leaflet durability is strategically reinforcing the leaflet with high-performance fibers. The fiber polymer interphase in the composite will act as a load bearer and reduce LLDPE matrix stress concentration. Therefore, the main goal of this dissertation is to understand the failure mechanism of the polymeric leaflets and develop methods for improving the durability of polymeric TAVR.

The research has three specific aims:

1. **Specific aim 1:** Etching of Failed Polymeric Heart Valve Leaflets Reveals Cross-Tie Craze Microstructure
2. **Specific aim 2:** Cross-linked Low Density Linear Polyethylene: A Durable Biomaterial for Polymeric Heart Valve Application

3. **Specific aim 3:** Parametric Finite Element Modeling of Reinforced and Crosslinked Polymeric Leaflets for Improved Durability
4. **Specific aim 4:** Hyaluronic Acid Enhancement of Heart Valve Leaflet and Characterizations

Chapter 2

Etching of Failed Polymeric Heart Valve Leaflets Reveals Cross-Tie Craze Microstructure

2.1 Summary

Rheumatic and calcified aortic heart valve disease presents a global health concern, impacting millions of individuals across various age groups. The gold standard medical treatments recommend replacing the sick heart valves with xenograft-based bioprosthetic valves that are chemically fixed using glutaraldehyde, commonly sourced from bovine or porcine. Clinical investigations over more than two decades have revealed fixed tissues are prone to premature calcification and tearing, thereby limiting their durability. Moreover, challenges related to supply chains, inconsistent mechanical characteristics, and high costs have been constant issues for tissue valves. An innovative alternative approach involves enhancing polyethylene-based materials with hyaluronic acid (HA), a glycosaminoglycan naturally occurring in native heart valve leaflets. By incorporating small amounts of HA into a low-density linear polyethylene (LLDPE) thin film at the molecular level, a robust and hydrophilic biomaterial of in vivo anti-calcific and anti-thrombotic properties is achieved. The LLDPE thin film has high tear strength and excellent flexibility, making it an appealing choice for developing heart valves. Nonetheless, during durability testing according to ISO 5840-2005 standards, these valves exhibited premature failure. To uncover the fundamental mechanisms behind these failures, this study conducted a failure analysis of semicrystalline blown LLDPE thin film-based heart valves. The valves consistently tear and wear around highly stressed commissure posts. Ten of these worn posts were retrieved from failed valves and chemically etched. Low-voltage scanning electron microscopy (SEM) imaging was taken before and after chemical etching. The semicrystalline LLDPE polymer, with a crystallinity of 36% as determined by DSC, underwent chemical etching using a standard 2% w/v permanganate etching solution,

followed by multistep washing. The SEM analysis of pristine LLDPE unveiled distinctive spherulitic structures consisting of well-organized lamellae with diameters of approximately 3 μm and lamellae dimensions below 100 nm. The etching process effectively eliminated low-energy amorphous regions, revealing the spherulites. A similar study was carried out on the worn and torn LLDPE valves. The SEM images of the worn surfaces displayed signs of surface wear and aligned polymeric built-up oriented perpendicular to the principal stress direction. Following etching, some of the built-up remained partially intact, while others exposed the crystals beneath them. Some of these crystals within the fibrils lost their spherulitic superstructure, exhibiting thinned-out and fractured lamellae. The worn surface shows abrasive wear, wear polishing, tears, fine scratching, and imprints from the metal stent. Crystal disruptions were notably concentrated within 200-300 μm areas near the tear and wear, while the rest of the samples retained spherulitic morphology. Remarkably, one of the worn samples unveiled the Kramer craze microstructure "cross-tie," composed of aligned fibrils and interlinked cross-tie fibrils. The spacing between cross-tie lamella ranges between 100-200 nm, and the thickness remains 40-80 nm. To the best of the author's knowledge, the cross-tie structure has been only theorized with indirect evidence collected from lab-grown crazes. Direct images of cross-tie structure indicate cyclic fatigue initiated crazing and surface wears are responsible for premature failures. Stress disentangles polymer strands, causing lamellar fibrillation, but some do not disentangle. Instead, they pile up to bridge between fibrils. These findings are further confirmed with Small-angle X-ray Scattering (SAXS).

2.2 Introduction

Polyethylene thin films are excellent for heart valve leaflets because of their flexural and mechanical properties. Among the four valves, aortic valve engineering has advanced primarily due to their high clinical and market need and the presence of the cylindrical aortic annulus, which facilitates anchoring. Previously, TAVRs showed standard hemodynamics due to their unique flexural and mechanical properties but failed between 1-150 million cardiac cycles *in vitro*. The following reviews the established relationships between microstructure and failure mechanisms of

polyethylene.

Semicrystalline polymer morphology can be broadly divided into crystalline and amorphous domains. Depending on the molecular weight, molecular structure, and manufacturing techniques [80] the morphology of crystals can be lamellar [81], spherulitic [82], and shish kabab [80]. Theoretically, there is a boundary of electron density between amorphous and crystalline phases due to their distinct thickness throughout the structure. However, the regions are not entirely distinct. Instead, some amorphous chains enter the crystals as tie and bridging molecules for inter-region communication, which can also cause imperfections in crystal [83]. The growth, type of crystals, and fundamental energy balance behind their development are still controversial. On the other hand, amorphous domains consist of randomly folded, wiggling, low-energy polymer chains. The amorphous chain free ends protrude from one crystal while tie and bridging molecules connect two crystals. The tie molecules maintain communication between the domains and act as the stress transmitter during elastic deformation [2].

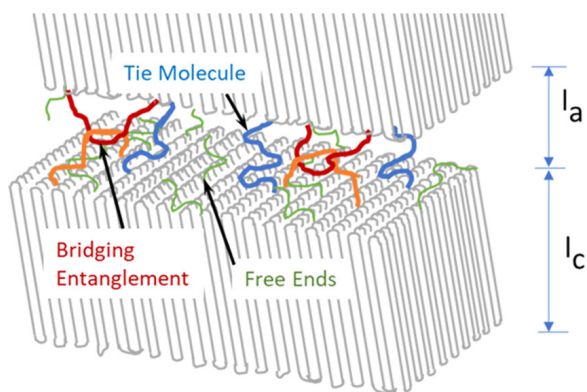


Figure 2.1: Schematic of semicrystalline polymer morphology showing arranged crystal lamella and amorphous region. Crystals have an intrinsic tilt depending on the polymer processing parameter. Reprinted (adapted) with permission from [2]. Copyright 2020 American Chemical Society

2.2.1 Failure Mechanics of Polyethylene

Andrews and Walker [84] conducted one of the earliest fatigue damage studies on low-density polyethylene (LDPE), including mathematical modeling and validation with experimental data.

They found LDPE compression molded sheets fatigue fails in three consecutive stages where a transition of micro-brittle crack to micro-ductile crack occurs. The fatigue life was estimated to depend on cyclic stress and spherulite size. High-density polyethylene (HDPE) crystallinity is almost double that of LLDPE, and HDPE failure mechanics are significantly different from LLDPE due to the higher concentration of tie molecules. However, HDPE is the closest in structure to LLDPE, which has been studied the most regarding fatigue and failure mechanisms. Therefore, the next section will discuss previous fatigue studies of HDPE and mathematical models that can predict its fatigue life. Amjadi et al. [80] examined the effects of temperatures (RT, 53 °C, 82 °C), manufacturing techniques (injection, compression, and blow molding), stress ratios, and thickness on HDPE polymer fatigue life. They claimed the equivalent fatigue life of blown and compressed sheets and the highest fatigue life of injection molded parts at the same temperature, stress ratio, stress amplitude, frequency, and thickness. Compressed films have higher isotropic crystal domain (zero Hermans orientation from small-angle X-ray scattering [79]), ordered crystal arrangement, longer period, and higher tear strength than blown film. Blow molding produces moderate anisotropic (< 0.2 Hermans orientation [79]) films with moderate crystal orientation towards the machine direction (MD). On the other hand, injection-molded sheets are anisotropic (> 0.2 Hermans orientation) and have folded shish-kebab morphologies [85]. The process uses rapid cooling after molding rejects more molecules from crystal regions that occupy the amorphous regions as tie or bridging molecules [79]. Therefore, the density of the tie molecules is the highest in injection molded parts, with a possibly higher number of crystal defects than blow molded films. Compressed sheets might have the highest tie molecule density. Consequently, crystals might reconstruct under stress, keeping tie molecules intact, and giving them high tear strength and fatigue strength. On the other hand, blow-molded sheets might have moderate tie density. The injection-molded parts might have the right amount of tie molecule density and lamellar thickness, resulting in better fatigue life than blown or compressed sheets. Therefore, the fatigue life of semicrystalline polymers depends on internal factors: density of tie molecules, bridging molecules, long period size, crystal arrangement, and lamellar dimensions, along with external

factors: applied stress conditions, stress ratio, the geometry of the part, stress amplitude, presence of contact stress, abrasion and nature of stress (symmetric, asymmetric). It is worth mentioning that under simple cyclic stress and strain in the absence of another contacting part, thick compression molded thermoplastic sheets have lower fatigue life than thin sheets [84].

Andrews and Walker developed a fatigue life prediction model for low-density polyethylene, which accounts for only internal cracks and applied stress for fatigue life [84]. The Walker equation for metals accounts for stress amplitude, stress ratio, and a material parameter found to fit thermoplastics fatigue data poorly [80]. A universal equation for the thermoplastic polymer is required to account for material properties, stress amplitude and ratio, frequency, and testing temperature. One such model was originally proposed for glass fiber reinforced thermoplastics [86] fits a few general thermoplastics such as HDPE, ultra-high molecular weight (UHMWPE), polypropylene glycol (PPG), etc [80]. The generalized equation

$$N_f = \left[1 + \frac{f^\beta (S_U - S_{max})}{S_U^{1-\xi} S_m a x^\xi (1 - R)^{\xi \alpha}} \right]^{\frac{1}{\beta}} \quad (2.1)$$

Here, S_U is ultimate tensile strength, s_{max} is maximum applied stress, f is frequency of testing is unit for frequency-independent materials, α , β , ξ are material properties. β is found to be constant (0.2) for various types of thermoplastics, and α depends on temperature, stress ratio, and thickness [80]. Equation (2.1) might be used to calculate the fatigue life of polymeric leaflets if the material constants were known. Stress ratio and stress amplitude can change over time depending on the viscoelastic behavior that causes complicated time-dependent stress and needs further mathematical modeling to account for stresses over each fatigue step [87].

Damage accumulation modeling can also predict fatigue failure. According to this model, visco-elastic and thermoplastic materials lose stiffness in each fatigue cycle [88]. This concept estimates modulus loss or damage created in each step, accumulates them, and predicts remaining fatigue life. However, these models are limited to certain materials, manufacturing techniques, thicknesses, and loading conditions.

Researchers extensively found that damage in crystal domains occurs in a plane or region

perpendicular to Principal stress [82, 89]. For instance, Raphael et al. [89] detected propagation of intra-spherulitic damage in the equatorial plane of injection molded nylon 66 part, as the nucleus accumulates stress on its core [82]. They also noticed the injection molded part is not pure radially spherulitic; instead, it has an orientation like shish-kebab morphology [85]. There have been limited studies on LLDPE fatigue behavior, [90] let alone LLDPE thin-film fatigue behavior. In the above sections, the fatigue behavior of HDPE, a close polymer to LLDPE, is discussed. The following section discusses morphological changes due to non-cyclic stress and strain on LLDPE films.

Butler and Donald [82] found increasing tie molecule density and disentanglement resistance increases toughness. They deformed 0.5-to-1-micron thick films in a tensile tester to 20% strain and found spherulite separation along the stress axis and intra-spherulitic damage as the film distorts. Tear strength could be an initial parameter for comparing fatigue life, because the more crystals are oriented in the machine direction (MD), the less tear strength in this direction and vice versa [79, 91]. Tearing in MD demands less energy to break van der Waals bond between polymer chains, resulting in high elongation to failure and strain hardening. The opposite is true for the transverse direction (TD). [91]. Tensile stretching of thin polyethylene films followed by their characterization leads to the discovery of structural phenomena happening at the molecular level [92]. Small and wide-angle X-ray scattering (SAXS/ WAXS) is one of the most popular techniques to analyze such polymer morphology. Crystalline and amorphous regions of semicrystalline polymers have varying thicknesses and electron density; therefore, x-rays scatter differently from each phase. It gives concrete information about the sizes and alignment of crystal lamellae [83, 93, 94]. Du et al. [92] found lamellae break and regrow in the stretching direction, whereas visible crazes generate and propagate perpendicular to the stretching direction because of favorable crystal realignment. Yoshizawa et al. [94] applied low amplitude cyclic loading i.e., stretch and shrink to LLDPE film, and found an intrinsic tilt of crystal arrangement (Figure 2.1) from the standard manufacturing directions. They found this angle of alignment reverted to zero with tensile stretching. The relaxation time of the tie molecules measured using solid-state proton

Nuclear Magnetic Resonance (NMR) is found to reduce drastically with stretching. These two phenomena imply stretching in the elastic region disentangles the tie molecules to straighten them and transmit stress from one crystal to another crystal as an act of disentanglement [2, 94].

Broadly, scanning electron microscopy, optical microscopy (polarized light, phase contrast), atomic force microscopy, and white light interferometry with phase-contrast are good techniques to illuminate polymers' morphology, especially semicrystalline polymers [95–99]. Polarized light and phase contrast optical microscopy can illuminate crystals i.e., lamellar, spherulites, and shish-kebab structure [82, 89, 98]. Polarized light microscopy (PLM) increases the contrast of the crystal phase. On the other hand, phase-contrast uses the birefringence (i.e., refractive indexes of both phases are different) nature of the polymer and increases the contrast of one phase to image crystal domains [100]. One limitation of PLM is it requires a thin polished surface to detect crystals.

2.2.2 TAVR Premature Failure Mechanics

Polymeric TAVR was devised from LLDPE leaflet, and Co-Cr alloy stent was used to determine valve durability and associated failure region. A thin LLDPE film was welded at two ends to form a cylinder to assemble over a cobalt-chromium stent (Figure 2.2). The stent is a 23 mm diameter balloon-expandable cobalt-chromium alloy. The durability tests were performed at a 15 Hz cardiac cycle, using room temperature water-glycerin mixture as fluid. Valve (a) failed between 1-4 million cycles; failure is determined by visible tear and greater than 10% change in hydrodynamics value. The welded areas never failed and always tore at the cusp opposite the weld commissure post. The weld at the commissure post is mechanically more robust than the rest of the leaflet, protecting this region. Therefore, this research investigates failure mechanisms and provides methods to increase durability by strategically strengthening the leaflets in the region of the commissure posts. The failure results from complex multidimensional impacts: cyclic fatigue initiated crazing and impact wearing on the surface damage propagated by cyclic fatigue. Cyclic fatigue initiates craze as the local stress exceeds the yield stress. Therefore, this study aims to reduce the principal stress on the leaflets. The total cardiac cycle time is 0.8 seconds, and systolic closing happens in only 0.1

seconds. Therefore, the rise time is well below half the total cardiac cycle, causing impact loading at the leaflet stent contact region. Impact wearing effects can be minimized by preventing leaflet movements against the stent, especially around the leaflet region of the commissure post, and adding lubricating material to the leaflet, such as Hyaluronic acid (Chapter 5). Nylon and silicone wrapping initially handled the limited durability issue, but they are unlikely to be used *in vivo* because of increased complexity. The nylon wrap can be unstable and unwrapped during crimping and *in vivo* conditions. Therefore, there remains a need for a strong, robust, and durable leaflet that can survive *in vivo* conditions.

This research aims to reveal the morphological disruption of damaged LLDPE leaflets using SAXS and SEM and compare them with pristine LLDPE thin film. As discussed above, several phenomena that dictate valve life may happen at the molecular level. This research focuses on understanding the crystal morphology changes with impact fatigue damage and wear. Although numerous research studies were conducted on morphological evolution during fatiguing on other thermoplastic polymers, a gap exists in understanding how LLDPE behaves at the atomic scale under high amplitude (> yield strength) cyclic loading. TAVR undergoes complicated cyclic loading during the cardiac cycles. Also, the leaflet constantly deflects, translating over a metal stent, which causes impact wear or impact fatigue failure. This research aims to quantify wear damage caused in the crystal domain using SAXS and SEM. The specific aim is "Etching of Failed Polymeric Heart Valve Leaflets Reveals Cross-Tie Craze Microstructure."

2.3 Methodology

2.3.1 Leaflet Assembly

A thin film of 2045G resin (octane-1 comonomer, Ziegler Natta catalyzed) blown linear low-density polyethylene (LLDPE) with a thickness of 80 μm was utilized to create leaflets. These leaflets, measuring 23 mm in diameter and 30 mm in height, were sealed at the ends using either a heated Allen key or a plastic sealer. The cylindrical leaflet is inserted over the stent from the aortic side. Carefully, the snug fit leaflet is pulled down to the belly area. The cusps are inserted

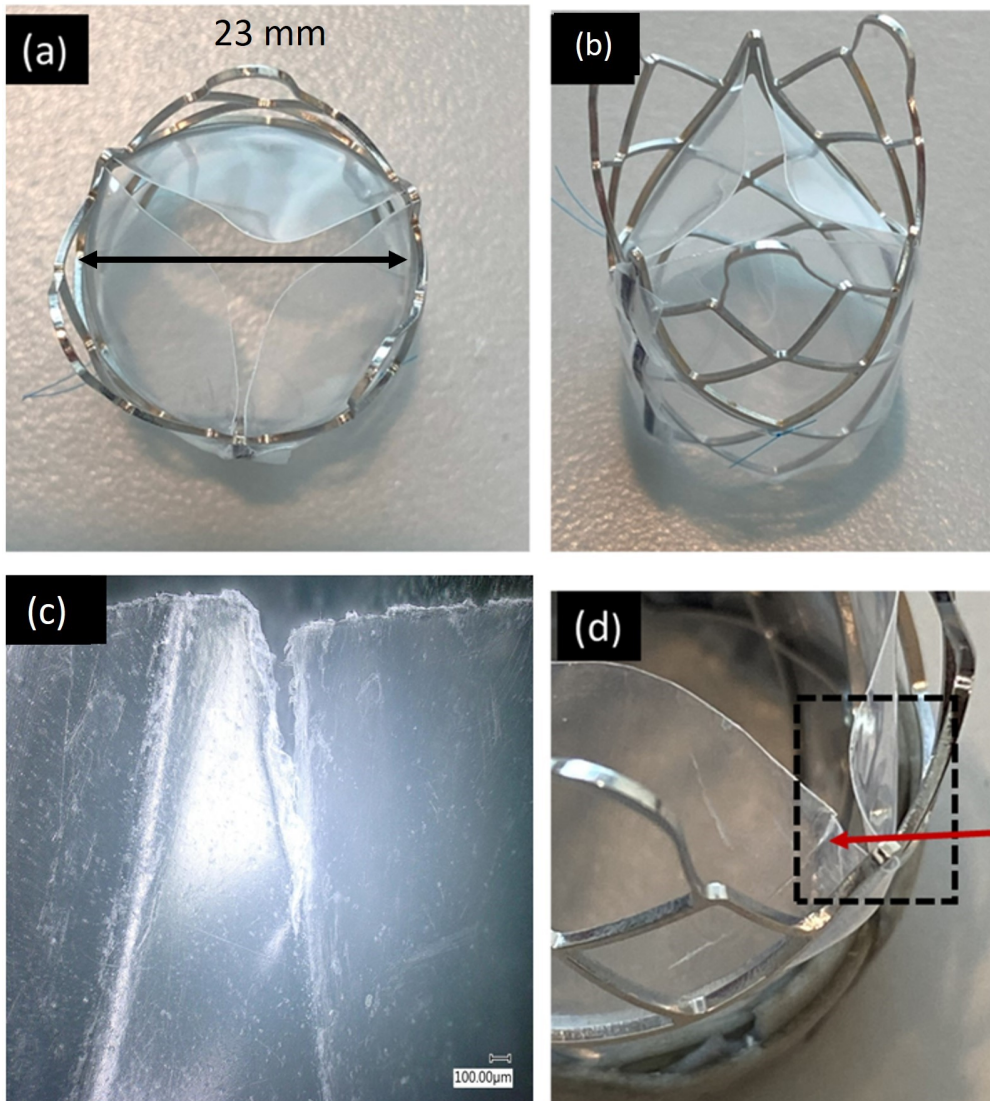
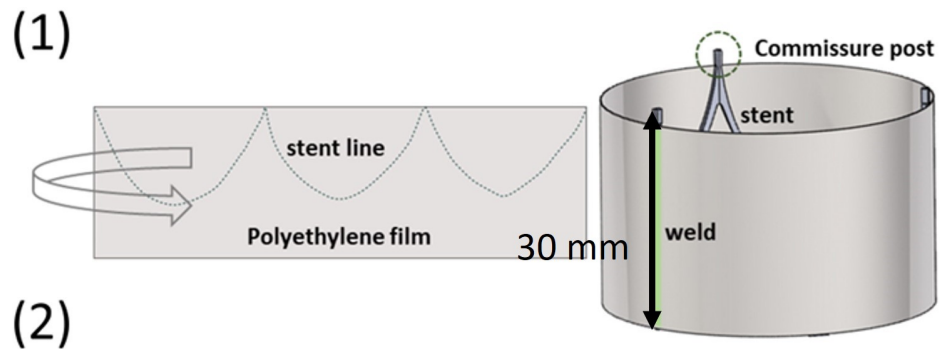


Figure 2.2: 1. Schematic of TAVR Assembly Showing Regions of Interest: commissure post and stent line
 2. Assembled TAVR over stent (a) top view (b) isotropic view (c-d) tear in leaflet region over the commissure post

back in the gap between the stent line and the crown. Fine adjustments are made to encourage proper coaptation. Three non-load bearing sutures are made around the inter-leaflet triangle to attach the leaflet to the stent securely. The assembly process requires positioning the leaflet snugly over the stent (see Figure 2.2), ensuring an even fit to enable uniform movement of all three cusps. Loose fitting of the valve assembly resulted in stenotic cusps, asymmetric coaptation, and lazy cusps showing delayed coaptation, thus hindering proper opening and closing akin to stenosis. The non-load bearing stitches cannot prevent leaflet translation during pressure gradients of the cardiac cycle. Leaflets move a few mm to the ventricular side (downward), rubbing against the stent. Valves were tested in two cohorts. One of them had ten valves, and the average durability for these components was 11 ± 10 million ISO standard cycles. These valves were studied in SEM. The second cohort had four valves, and their average durability was 2.5 ± 1.7 million ISO standard cycles. These cohort was studied in SAXS.

2.3.2 Accelerated Wear Testing

Durability testing, conducted *in vitro*, provides crucial insights into the lifespan of valves during their developmental phase. The ViVitro AWT System with DCT™, an accelerated wear tester (AWT), was employed to perform TAVR durability tests under physiological conditions, maintaining a mean pressure gradient of 10 mmHg. The heart rate was accelerated to 20 Hz, as opposed to the resting heart rate of 1 Hz, following the guidelines outlined in ISO 5840-3:2013 [56]. This accelerated heartbeat simulates years of cycles within a reasonable experimental timeframe. The choice of heart rate is contingent upon the viscoelastic properties of the leaflet material. Therefore, viscoelastic polyethylene leaflets were tested at a frequency of 20 Hz to prevent self-heating and to capture their viscoelastic behavior accurately.

2.3.3 Leaflet Imaging

The commissure post and the two adjacent cusps to the weld exhibited no failures, likely due to the thicker and stiffer nature of the weld, offering protection to these areas against metal stent rubbing. In contrast, all tested leaflets experienced consistent tearing, primarily around a

Table 2.1: Retrieved valve failure mode and their ISO cycles before failure. @Failed to meet ISO standard pressure gradient, * leaflet region over the stent commissure post

Failure mode	ISO cycles (millions)
Two tears on the posts including weld	1.04
Three tears on the posts including weld	3.88
Failed@	4.70
The weld tore	39.60
One of the posts* tore	11.04
One of the posts tore	7.24
The weld tore	0.63
The weld tore	12.62
The weld tore	3.75
One of the posts tore	12.09
Weld, and the one of the post tore	7.76
Weld and one of the post	4.73

cusps opposite the weld commissure post (Figure 2.2 D). Subsequently, ten failed leaflets were retrieved from the AWT for further investigation into the failure mechanisms using SEM. The average durability of testing for these leaflets was 11 ± 10 million ISO standard cycles. Leaflets utilized were composed of polyethylene 2045G resin blown LLDPE, with detailed materials characterization discussed in the subsequent chapter. Rolls for thin LLDPE film were blow molded in Film Tech LLC., without any antioxidant (PO-RF0157954701). Upon retrieval, the leaflet regions interacting with the stent commissure posts underwent visual inspection and were imaged using an optical microscope to assess wear and tear. The examination revealed signs of abrasive wear, wear polishing, tears measuring a few millimeters in length, fine scratching, and imprints from the metal stent. Based on the extent of wear observed, the leaflet regions over the stent commissure posts were categorized into two groups: a) torn posts and b) worn-out posts without visible tears.

Subsequently, this study investigated prematurely failed torn or worn leaflets to examine their morphological evolution and compared them with pristine leaflets. Before examination, the leaflets were meticulously cleaned with acetone to remove any contaminants from the water of the AWT and potential metal debris from the stent. Total six representative posts were then carefully excised

using scissors and securely affixed onto aluminum stubs using electrically conductive carbon tape. Among them, three were torn posts, and three were worn-out posts. The samples underwent a 10 nm gold coating process before being imaged using a JEOL-6500 FE SEM.

2.3.4 Etching of Semicrystalline Polymer

Following SEM observations, the crystalline lamellae of the six retrieved posts and another six virgin LLDPE were exposed using a standard oxidizing etching agent as described by Bischel et al. [101]. Briefly, samples were etched for 20 minutes in a solution containing 2% (w/v) potassium permanganate in a mixture of 1.5 parts concentrated sulfuric acid to 1 part concentrated phosphoric acid (see Appendix A.1). Immediately after etching, samples were rinsed in water to eliminate residual permanganate. This was followed by a sequential washing in a) 22% w/v sulfuric acid, b) 30% w/w hydrogen peroxide in water, and c) pure water. Subsequently, the samples were immersed in acetone for at least 5 minutes within an ultrasound bath at a temperature below 5 °C. The limitation of the study is that one side of the samples was gold-coated prior to etching, which was not etched by the etchant. Therefore, the opposite side was gold-coated at 10 nm and was observed under SEM.

2.3.5 Small Angle X-Ray Scattering

The current investigation involves sampling and analyzing representative sites from failed leaflets to measure their morphology evolution with impact wear. Carefully collected samples from torn and worn leaflets near the commissure post were rinsed with acetone and dried in a vacuum oven for 30 minutes. Pristine LLDPE was also cut using a standard biopsy punch of 5 mm size. On the other hand, samples from failed leaflets were cut using a 3 mm size sharp biopsy punch. Samples were collected from the region of interest: stent line, and commissure posts of the leaflets which areas have been frequently found to initiate wear [89]. These samples were mounted individually in the 5 mm diameter holes of the solid sample holder using Kapton tape. A list of samples with their durability and their locations can be found in Table 2.1 first three rows. They were tested in the ID-12B beam line of the Advanced Photon Source. ID-12-B beamline has

PILATUS 2M detector, a direct-detection system that prevents any geometry distortion [102]. The detector pixel size was $172\ \mu\text{m} \times 172\ \mu\text{m}$, and the sample to detector distance was 2m. The beam size was adjusted to $150\ \mu\text{m}$ in the x-direction and $100\ \mu\text{m}$ in the y-direction with a wavelength of $\lambda = 0.9322\ \text{\AA}$. The data set spans with a q range of 0.004 to $2.3\ \text{\AA}^{-1}$. The limitation of the method is that failed samples were smaller than the sample holder. Although multiple Kapton tapes were used to ensure samples are concentric to holder holes, there is a possibility of sample displacement during shipping and handling.

Data Collection and Analysis

The X-ray exposure time was 0.1 seconds for each measurement. The detector to sample distance was calibrated using standard sample silver behenate. Data reduction into 2D pattern and averaging over all available azimuthal angles into 1D data of scattering vector vs. Intensity (arbitrary unit) was done automatically in MATLAB interphase without requiring user interaction. Then, the background was subtracted from 2D pattern to report, but the background was not subtracted from 1D data. Additional scans were performed on Kapton tape to use as background data.

2.4 Results and Discussion

2.4.1 Spherulitic Morphology of Semicrystalline LLDPE

The SEM analysis of pristine Linear Low-Density Polyethylene (LLDPE) revealed the presence of spheroidal bumps, approximately $3\ \mu\text{m}$ in diameter, covered with surface features resembling clay (Figure 2.3 A). The SEM image also displayed straight micro scratches attributed to the blow molding machine roller. Upon chemical etching, distinct spherulitic structures became apparent (Figure 2.3 B), denoted by black arrows, comprising well-organized lamellae with diameters measuring approximately $3\ \mu\text{m}$ and lamellae dimensions ranging from 40 to 80 nm. Notably, the diameter of the spherulites closely corresponds to that of the spheroidal bumps. This etching process effectively eliminated low-energy amorphous regions, initially concealing

the spherulites with a dark, clay-like appearance.

The etching process for LLDPE was conducted at duration of 2, 5, 10, 20, and 30 minutes to determine the optimal etching time. Etching for 20 minutes proved to be the most effective, fully revealing the spherulites while eliminating amorphous regions. However, in some areas of the sample, over-etching occurred, resulting in the formation of random holes in the surface microstructure (Figure 2.3D, highlighted by the red arrow). Etching for less than 20 minutes only partially uncovered the spherulites, leaving details of the crystal domain obscured by the amorphous domain. This phenomenon was observed consistently, even after 30 minutes of etching (Figure 2.3C). Since etching efficiency does not improve beyond 20 minutes, this duration was chosen as both efficient and the minimum required etching time

SEM images of etched virgin LLDPE revealed details of the semicrystalline polymer crystal domain, amorphous domain, and tie molecule domain. The clay-like region marked by the white arrow in Figure 2.3 C represents loose, low-energy, randomly folded amorphous domains, which are the first responders to external environmental factors such as chemical etching, stress, strain, oxidation, and cross-linking. The dark spots marked by the red arrow in Figure 2.3 B may indicate tie and bridging molecule domain of the semicrystalline polymer. These molecules reside between spherulites, connecting them together, and are resistant to etching. This observation was supported by the inconsistently etched region indicated by the red arrow in Figure 2.3 D, where parts of the LLDPE 3D morphology, including arms of two spherulites connected through dark bridging and tie molecule domains, are visible. The dark, clay-like amorphous polymeric domains are connected to the bridging and tie molecule domain. The tie and bridging molecules themselves form strong connections with the crystal domains to communicate with the amorphous domain [103, 104].

2.4.2 Failed TAVR Leaflet Morphology

The SEM images of the worn surfaces displayed signs of surface wear (Figure 2.4) and crack precursor perpendicular to the principal stress direction (Figure 2.5).

Figure 2.4 illustrates concrete evidence of impact wear on the TAVR leaflet during Accelerated

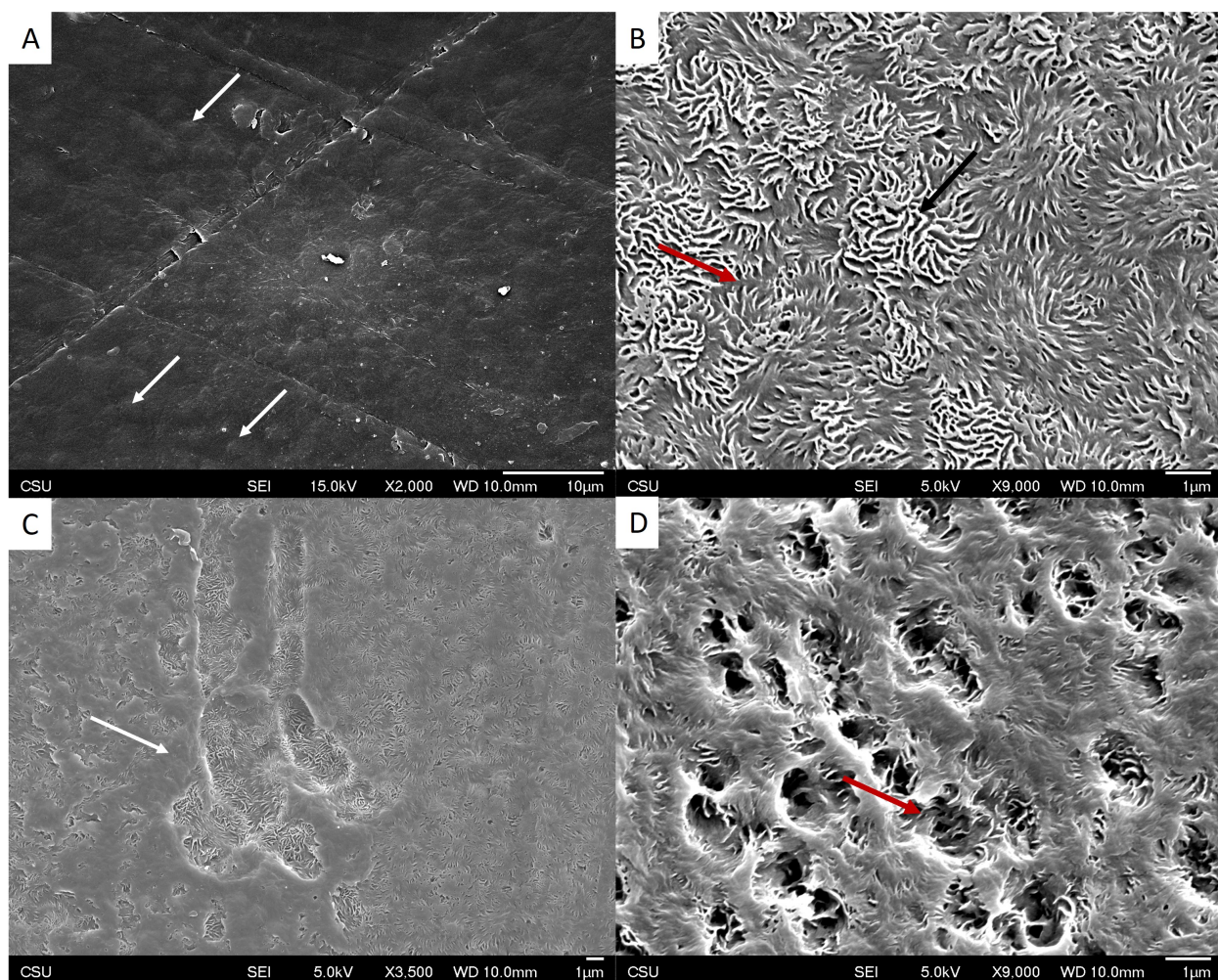


Figure 2.3: A) SEM images of virgin LLDPE before etching showing spheroidal bumps covered in dark clay-like amorphous domains marked in white arrow B) LLDPE after etching showing exposed spherulitic crystal microstructure C) partially etched LLDPE confirming amorphous and crystal domains D) over etched LLDPE revealing 3D morphology

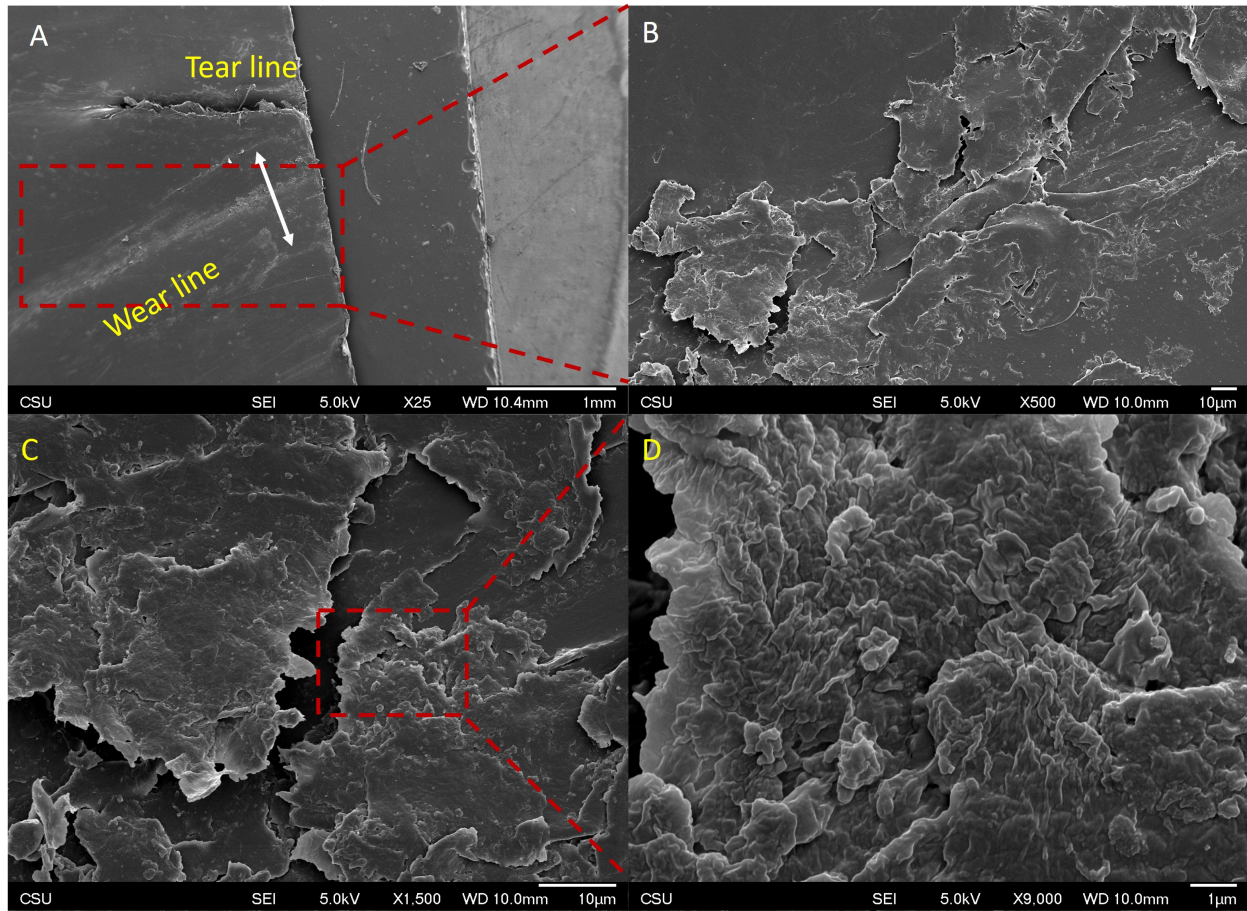


Figure 2.4: SEM images of LLDPE heart valve leaflets worn in an Accelerated wear tester for heart valve durability tested at different magnifications: ventricular side (rubbing against stent). (a) Showing a tear and wear caused by LLDPE wearing against metal stent. White arrows indicate the direction of applied principal stresses which cause tears at commissure posts (b) surface wear accumulation or debris at x500, (c) x1500 and (d) x9000 showing fibrillation on accumulated damage debris

Wear Testing (AWT), as discussed in Section 2.3.2. The ventricular side of the TAVR leaflet consistently experiences physical dynamic contact with a metal stent, resulting in impact wear. The abrasion between the leaflet and stent leads to a tear on one side and wear on the other side which has flakes, cracks, micro scratches, and debris. The white arrows in Figure 2.4 (A) represent calculated stress vectors derived from finite element analysis (Chapter 4). Magnified images of the wear reveal hyper-fibrillation of the worn surface, a consequence of disentanglement in the amorphous region, subsequently dragging the spherulitic crystal regions into nano-fibrillar oriented crystallites.

The leaflet side facing the aortic chamber does not exhibit surface wear and more likely fails due to cyclic loads during cardiac operations, as it never makes contact with a metal surface. The pressure gradient between the ventricular and aortic sides maintains the cardiac cycle. FEA results from the aforementioned study calculated a maximum principal stress on the leaflet of 18 MPa along the black arrow in Figure 2.5A. This stress surpasses the yield stress of polyethylene, which is 11 MPa. Upon examination of a low magnification image, leaflet region over the stent commissure post can be distinguished into two sections: (a) the tear line, exhibiting a physical tear and wear on the ventricular side, and (b) the wear line, which did not tear physically during testing but exhibits wear on the ventricular side. The wear line shows built-up of macroscopic polymeric material as a response to stress were highly oriented perpendicular to stress vectors. Interestingly, similar highly oriented built-up were observed at the bottom of the tear line, indicating the precursor of crack. Cardiac cycles of AWT apply diastolic stress during closing and then release during systolic opening. The application and withdrawal of stress create crack precursors where polymeric materials are built over time. The magnified edge of the tear line displays significant fibrillation, suggesting molecular movement, rearrangement, spherulitic breakdown, and movement. These SEM images provide insight into the polymer built-up, but they do not reveal the spherulitic microstructure beneath them. To address this, the samples underwent chemical etching to remove the low-energy amorphous domains, revealing the underlying spherulitic microstructure. After chemical etching, some built-up crack precursors remained partially intact, while others

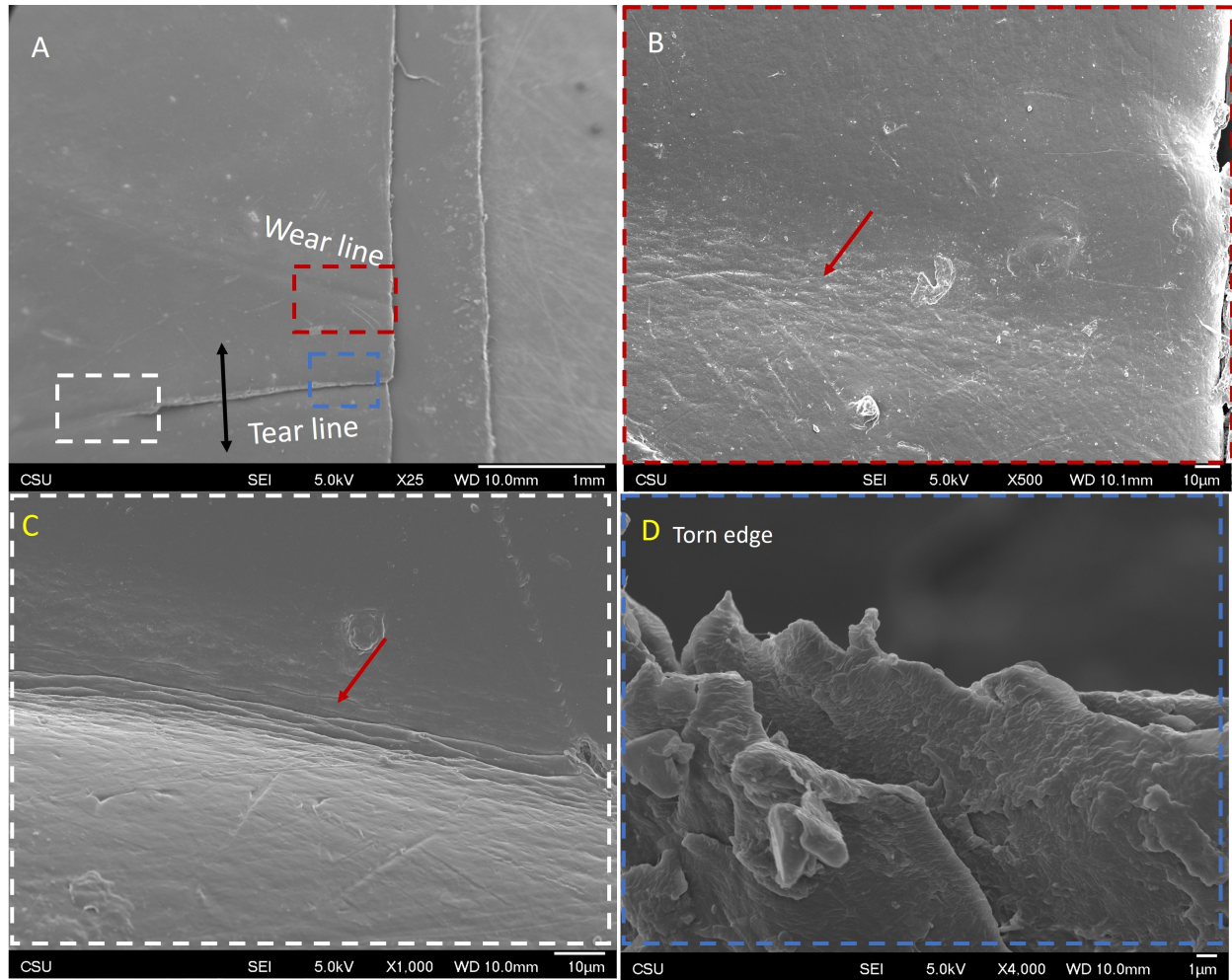


Figure 2.5: SEM images of LLDPE heart valve leaflet aortic side worn in AWT, at different magnifications: (A) Shows a tear and wear due to LLDPE wearing against metal stent. Black arrows indicate the direction of applied principal stresses that close the valve, which causes tears in leaflets at commissure posts. (B) Aligned polymer built-up perpendicular to principal stress created at the worn line. (C) Crack precursor of aligned polymer built-up (D) Severe fibrillation accumulated near the torn edge

exposed the crystal regions within them, as illustrated in Figure 2.6. The crystals within the built-up are thinned out and fractured. The spherulites are significantly thinner than virgin LLDPE spherulites, which are imaged in x15000 magnification and marked with red arrows. Crystal disruptions were concentrated within 200-300 micrometers near the tear and wear regions, while the remaining samples retained their crystal morphology.

Spherulites within the covering amorphous chains experience disruption at their center, with broken and thin lamellae compared to virgin LLDPE spherulites. Following etching, some built-up polymers on the ventricular side of the leaflet remained partially intact, while others exposed the crystals within them, as seen in Figure 2.7. These crystals within the crack precursor built-up lost their spherulitic superstructure, displaying thinned and fractured lamellae. Crystal disruptions were concentrated within 200-300 μm near the tear and wear regions, while the remaining samples retained their crystal morphology. The spherulites within the covering crack precursor exhibited disruption at their centers, with broken and thin lamellae compared to virgin LLDPE spherulites. Interestingly, the built-up polymers from the wear of the leaflet facing the aortic side did not etch away efficiently like the ones from the ventricular side.

Cross-tie craze microstructure

Remarkably, one of the worn samples revealed the Kramer craze microstructure known as "cross-tie," characterized by aligned and interlinked lamellae as depicted in Figure 2.7. This microstructure comprises a forest of primary fibrils with connected small cross-tie fibrils. Images capturing the cross-tie microstructure are displayed in Figure 2.7A, showcasing localized stable crazes at various stages of their life before eventual rupture. Upon chemical etching, amorphous domains within the craze microstructure are removed, leaving polymer fibrils intact. The roots of craze fibrils penetrate the less oriented amorphous domains from both ends. Parameters describing the craze cross-tie microstructure are outlined in Figure 2.7D schematic. These parameters include the spacing between the main fibrils (D_0), ranging from 70-200 nm, the diameter (D) of each fibril between 50-120 nm, the length of cross-tie fibrils (l_f), ranging from 60-260 nm, cross-tie spacing (R) between 230-1700 nm, and cross-tie fibril diameter (D_f) between 20-75 nm. Larger craze

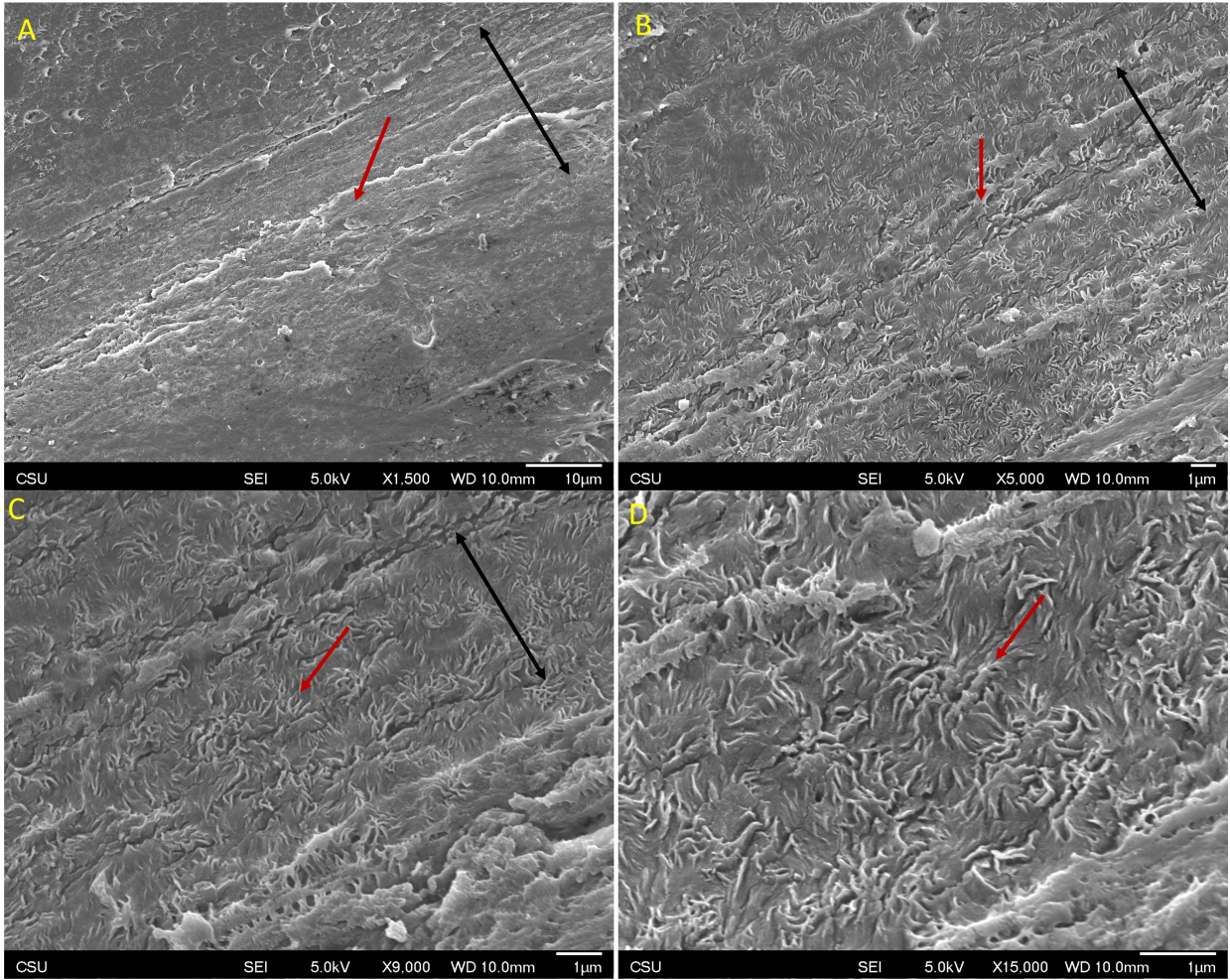


Figure 2.6: SEM images of chemically etched LLDPE heart valve leaflet ventricular side (rubbing against stent) worn in an accelerated wear tester for heart valve durability imaged at different magnifications: black arrows indicate principal applied stress direction. Worn section showing remnants of built-up polymers at (A) x1500 and thinned out spherulites at (B) x5000, (C) x9000, and (D) x15000 magnification.

length is associated with larger craze parameters, as summarized in Table 2.2.

Figure 2.7 B illustrates the early stage of the craze microstructure, which is notably thinner compared to the matured fat craze depicted in Figure 2.7C. Parameters such as diameter and cross-tie spacing are higher for the larger craze, with some main fibrils appearing broken, potentially leading to polymer tears. The cyclic pressure gradient exceeds the yield stress of LLDPE, creating favorable conditions for craze formation [3]. These low-impact crazes formed from single loading beyond yield strength are generally unstable and occur on a nanometer scale, necessitating immediate study within hours of formation in Transmission Electron Microscopy (TEM) [3]. Leaflets underwent Accelerated Wear Testing (AWT) for at least a week. SEM analysis was conducted at least two months after AWT, demonstrating the stability of the crazes even during the chemical etching process. Therefore, each cardiac cycle contributes to the formation of the cross-tie craze microstructure, and millions of cycles continue to stabilize, reinforce, strengthen, and propagate them until a crack opens. Note that measurements were made in ImageJ software at x9000 and x15000 magnifications to ensure accuracy. Magnified images of the cross-tie microstructure (Figure 2.7B and C) further confirm the slight angular orientation of the main fibril from the tensile axis as it widens. To the best of the author's knowledge, the cross-tie microstructure has only been theorized with indirect evidence collected from lab-grown low-strain crazes and simulated in molecular dynamics(Figure 2.7 D right).

Kramer extensively investigated crazing mechanics in amorphous polymers, as documented in [105, 105–108] . Their research laid the foundation for understanding crazing in semicrystalline polymers, as later explored in [108]. The craze microstructure is characterized by the formation of a forest of highly oriented polymer fibrils in response to tensile stress. These fibrils form a network of interconnected structures primarily responsible for load-bearing. As the fibrils grow and extend by pulling fresh material from behind, they eventually reach a critical size and rupture, leading to the formation of true cracks in the polymer (Figure 2.8). The process of craze formation, stabilization, and propagation before the occurrence of true cracks absorbs energy, thus enhancing polymer fracture toughness. The more stable the crazes that form, delaying or preventing actual

Table 2.2: Craze fibril microstructure parameters measured from Figure 2.7 B and C

Craze length (μm)	D (nm)	D ₀ (nm)	R (nm)	l _f (nm)	D _f (nm)
2.8 $\pm$ 0.35	73.35 $\pm$ 18	123.53 $\pm$ 22.1	380.1 $\pm$ 104.91	85.78 $\pm$ 20.74	38.9 $\pm$ 10.18
9.5 $\pm$ 0.24	86.87 $\pm$ 15.63	143.6 $\pm$ 46.7	989.6 $\pm$ 445.29	135.74 $\pm$ 51.87	44.77 $\pm$ 10.45

cracks, the tougher the polymer. Fibrillation observed near tears or wear regions are typically on the micron scale, as depicted in Table 2.2. These fibrillations propagate sufficiently to initiate crack formation, ultimately leading to catastrophic tears.

Kramer recorded nanometer-sized cross-tie crazes in TEM immediately after growing them in Polystyrene, as low-strain crazes are highly unstable. Their formation is hypothesized to nucleate from void formation due to stress, which progresses as primary fibrils while pulling fresh polymer chains. The primary fibrils are further reinforced with horizontal interconnecting cross-tie fibrils, controlling the load transfer to delay crack opening, ultimately increasing fracture toughness. Kramer established that cross-tie microstructure formations are crucial for enhancing polymer fracture toughness. Stress causes the polymer craze tip to soften or melt as a non-Newtonian fluid. The fluid air meniscus advances with stress, but their instabilities cause finger-like voids. Polymer chains accumulated between the voids continue to grow as they pull fresh material from the active zone.

2.4.3 Failed TAVR lamellae disruption

SAXS data obtained in previous studies [84] on LLDPE thin films indicated that the blowing process provides an anisotropic morphology with crystals oriented strongly in one direction. The crystal alignments change under strains or deformation [92]. This study investigated leaflets made of LLDPE-blown thin films using SAXS to trace evidence of morphological changes caused by impact fatigue. 2D pattern (after subtracting the background) and averaged 1D scattering profile (as collected) of failed leaflets were compared with pristine blown films. Figure 2.9 shows averaged 1D scattering profiles and 2D images (inset) of pristine blown films (a) and Kapton tape (b). The averaged scattering profile of 25 measurements (position 1,2,...,25) shows well-defined sharp

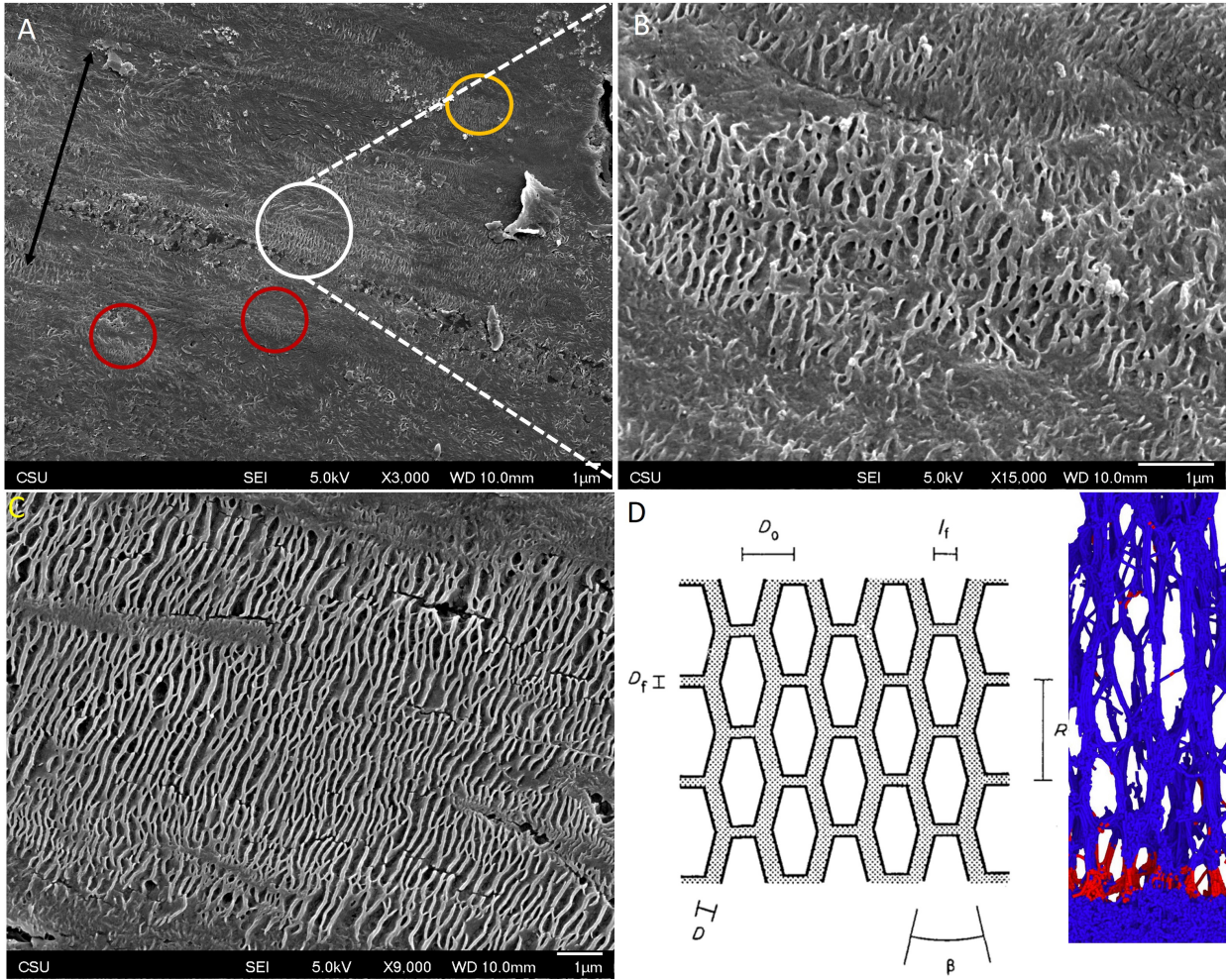


Figure 2.7: SEM image of craze Cross-tie microstructure at different stages of growth marked in white, red and, yellow circles (A), Matured (B), and developed (C) cross-tie crazes. Schematic [3] and simulated cross tie structure, Reprinted (adapted) with permission from [4]. Copyright 2017 American Chemical Society(D) A) 3000x, B)15000x, C)9000x

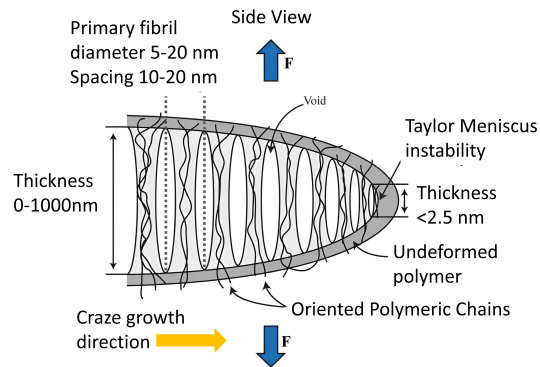


Figure 2.8: Schematics of Craze microstructure [5]

Bragg's peak, indicating organized lamellar packing and d-spacing in the films. The d spacing quantifies the distance between the center of one crystal to the center of another crystal. Since the data is not calibrated for absolute intensity, the conversion of peak scattering vector q into d spacing will be incorrect. Therefore, Lorentz Correction can be applied for calculating d-spacing [92]. On the other hand, the empty Kapton tape background shows the halo feature of the averaged scattering profile. These profiles are further confirmed with 2D images (inset Figure 2.9), which generated the 1d peaks after averaging azimuthal angles.

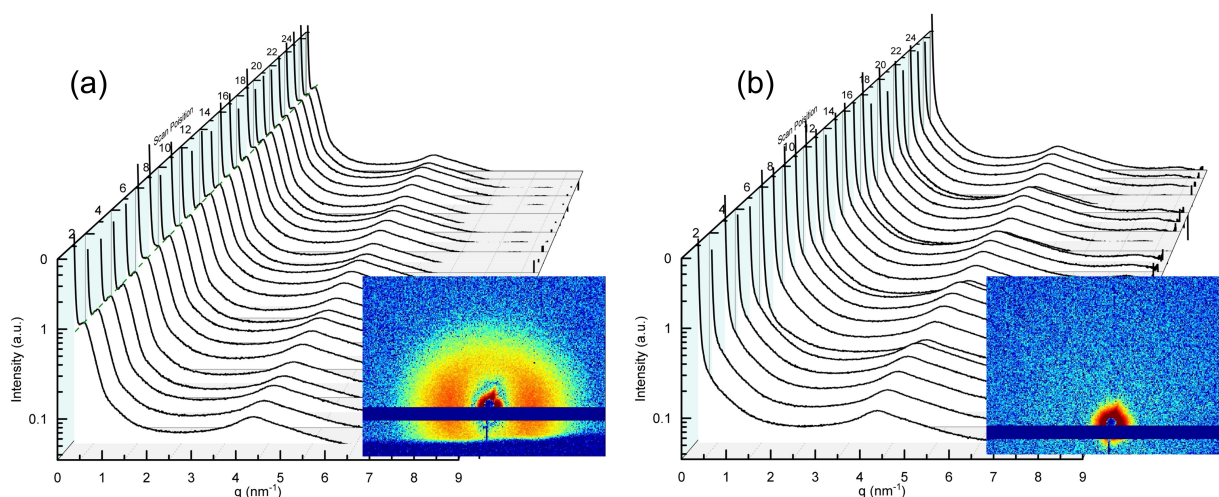


Figure 2.9: Averaged SAXS profiles and selected 2D SAXS images (inset) of (a) pristine LLDPE samples and (b) Kapton tape as the background.

Unlike 1D data, the background was subtracted from 2D patterns manually using MATLAB packages provided by APS, which facilitates the observation. Scattering intensity was higher in one direction than in the other direction. The high intensity direction is the MD for virgin films. The scattering direction indicates lamellar stacks oriented in one direction, which caused the SAXS pattern to scatter more in that direction. This prominent anisotropic nature of blown LLDPE is consistent with results obtained in previous studies [91, 109]

Figure 2.10 shows a selected averaged 1D scattering profile and 2D SAXS image (inset), which ran for 3.88 and 4.7 million cycles in AWT and were collected from the cusp and stent line area, respectively. As can be seen, in the 1D averaged profiles, the measurements from scan positions 1,

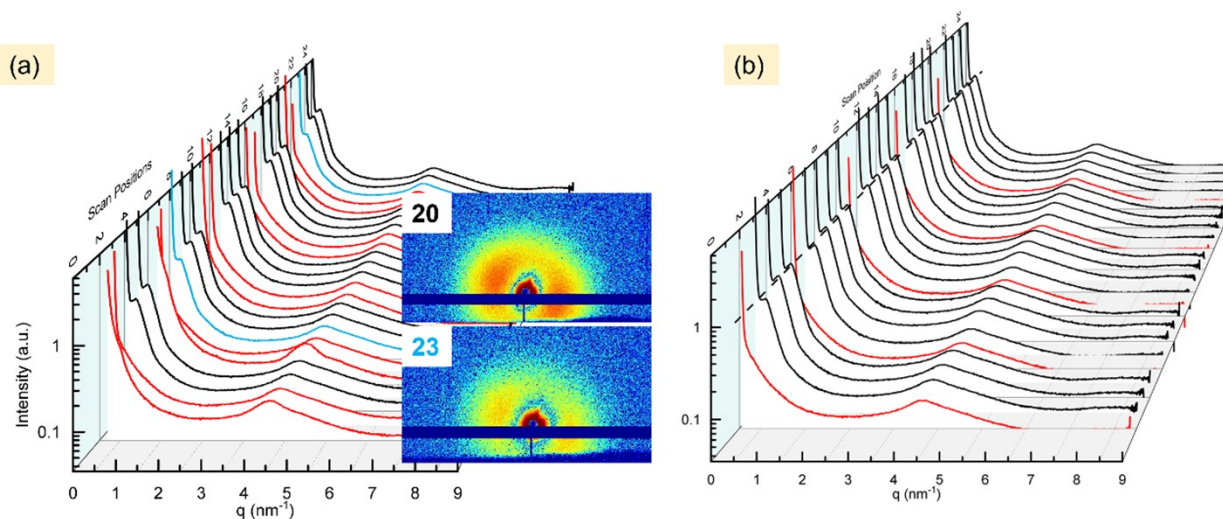


Figure 2.10: (a) Averaged SAXS profiles and selected 2D SAXS images (inset) of the commissure post, tested for 4.8 million cycles. The averaged profile shows a significantly higher number of featureless curves and broader peaks (b) Averaged SAXS profiles of leaflet cusp area tested for 3.88 million cycles

6, 11, 16, 21 (red lines) show featureless halo scattering curves. These positions always show featureless curves, as the beam might miss the sample at these positions. Theoretically, 3 mm size sample and 5 mm hole of the sample holder shall be concentric; however, it is nearly impossible to align the sample with the hole using a tweezer. The sample center moves, but the X-ray probe beam center remains constant. As a result, the X-ray beam was either partially or entirely missing the sample, generating featureless curves. Selected 2D SAXS images from these scans are like those obtained for Kapton tape only. During sample preparation, extreme care was taken to ensure the sample and the holes of the holder were concentric, and shear stress generated from cutting causes minimum morphology disruption along the edge. Once the samples are placed between Kapton, they are less likely to move because of Kapton tape glue. However, shipping and handling can off-center the samples.

Flat featureless curves (red lines) measured from scan positions 2,3,7,12,17,22 and broad peaks 8, 23 (blue lines) indicate lamellae disruption within the spherulites. Each SAXS scan spot size was $150 \times 100 \mu\text{m}$, measuring an averaged polymer morphology within this 0.015 mm^2 scanned area. The featureless scans indicate the void created during crazing. The broad peaks may indicate

aligned polymer fibrils seen in crazing. The lamellae disruption within the spherulites was not directly observed in SEM images. Rather, their effect was seen in thinned spherulites Figure 2.6. The leaflet region near the commissure posts and cusp areas was viewed under X-ray to determine their morphological evolution. The sample collected from the cusp area shows a noticeably lower number of featureless curves. Figure 2.10 b shows positions 1, 6, 11, and 16 had featureless curves, indicating a significantly lower crystal disruption. Computational simulations have established that cusp areas undergo minimal principal stress during leaflet closing. Therefore, it is expected that the morphology would remain unaffected during AWT.

2.5 Conclusion

An extensive volume of knowledge has documented the morphological evolution of various types of polyethylene under uni or bi-axial tests. A fundamental gap exists in understanding the complex behavior of LLDPE semicrystalline polymer domains under the compound effect of cyclic loading and wearing. This study examines retrieved polymeric heart valve leaflets from an *in vitro* wear tester under an electron microscope and x-ray to determine the behavior of crystalline and amorphous domains under cyclic impact loading exerted during the cardiac cycle. A strong oxidizing chemical etchant successfully etch away the low-energy amorphous domain, revealing spherulitic structures in pristine LLDPE blow film. The failed LLDPE leaflets show polymeric material built-up on crack precursors within, which lays thinned-out spherulites. Debris, scratches, and worn pieces are found in the worn-out leaflet region of the commissure post. This indicates surface wearing from soft leaflets rubbing against hard metallic stent as the leaflet moves towards coaptation during testing. Polymer chains of one of the leaflets worn commissure show stable cross-tie microstructures found to be at different stages of their life. Most notably, this is the first study that, to the best of the author's knowledge, to image stable craze cross-tie architecture in nm scale directly. Furthermore, SAXS confirmed the findings of SEM imaging by measuring local lamellae disruption of the torn commissure post. Direct measurements of crazing in LLDPE heart valve failed leaflet instigates to study techniques that can reduce and optimize polymer chain

disentanglement to stabilize crazes without sacrificing overall toughness and unique stiffness. However, some limitations of the study are worth noting. Cross-tie microstructure was found only in one leaflet among ten others tested. The side of the leaflet observed under SEM was not etched away since it was gold-coated. Therefore, imaging before and after the exact spot was not possible. Finally, the leaflets were not bio-enhanced with HA during durability tests. The lubricating effect of HA might impact leaflet failure mechanics. Future work should, therefore, include follow-up work to test HA-treated leaflet durability. Spherulitic damages of failed LLDPE from tensile testing can also be explored using the etching method to correlate established spectroscopic analysis.

Chapter 3

Cross-linked Low Density Linear Polyethylene: A Durable Biomaterial for Polymeric Heart Valve Application

3.1 Summary

Heart valve leaflet durability can be improved by controlling crazing and surface wearing. Crosslinking randomly folded amorphous chains can control the crazing and surface wearing because they directly impact chain disentanglement under high amplitude cyclic stress. Crosslinked covalent bonds can limit chain movements to manage chain disentanglement. LLDPE thin sheets were crosslinked at 50, 70, 100, and 150 kGy doses using 200 KeV (low energy) and 4 MeV (low energy) electron beams at room temperature in the air. Their effects were characterized by measuring gel content percentage, tensile testing, differential scanning calorimetry (DSC), nanoindentation, and nano scratch tests. Crosslinked LLDPE heart valve leaflets were tested in *in vitro* flow loops and wear testers to determine valve performance and durability, respectively. Low energy electron beam (LEEB) crosslinking formed 28% xylene insoluble gel, whereas high energy electron beam (HEEB) resulted in 58% gel at 100 kGy doses. LEEB did not affect mechanical properties, but HEEB significantly increased stiffness and yield strength. A slight reduction in melting temperature was found for LLDPE crosslinked by both energy sources. Nanomechanical tests showed crosslinking improved hardness and coefficient of friction, an indication of improving surface wear resistance, which should lead to durability improvement.

3.2 Introduction

Polymer chains of the amorphous region are the most mobile, followed by tie molecules, bridging molecules, and crystal domains. Yoshizawa et al. used solid state Nuclear Magnetic

Resonance (NMR) to determine the higher molecular mobility of amorphous regions relative to crystal regions [94]. The failure analysis study discussed in Chapter 2 found that impact wearing and cyclic fatigue initiated crazing thinned out, broke, and restructured spherulitic superstructure. Crazing is predominantly the effect of chain disentanglement, which increases with chain mobility. Attempts to reduce chain mobility of the amorphous region can effectively reduce disentanglement and cyclic fatigue initiated crazing. One way to reduce amorphous region movement is to reduce their quantity by increasing crystallinity. However, increasing crystallinity will increase Young's and Flexural modulus ((3.1)), which might affect TAVR hemodynamics.

$$\text{Flexural Modulus} = \frac{(Ebt^3)}{(12)} \quad (3.1)$$

The Biomolecule enhancing HA treatment process (Chapter 5) largely depends on amorphous region and their swelling. Therefore, significantly increasing crystallinity can limit available amorphous chains to swell and interpenetrated biomolecules.

Cross-linking is a mature, state-of-the-art technology that has revolutionized traditional polymers for the last 65 years. Cross-linked polymer overarching applications include orthopedic implants [110], fuel cell membranes [111], insulation cables [112], and heat shrinkable polymers [113]. Polymer cross-linking implies creating covalent bonds between randomly wiggling polymer chains to limit their movements by reducing free volume and creating a 3D network akin to thermoset polymer. There are three main methods to cross-link polyethylenes: peroxide cross-linking, saline cross-linking, and radiation cross-linking [114, 115]. Chemical cross-linkers: peroxide and saline received much criticism due to their toxicity, environmental hazards, high cost, low speed, and variation in cross-linking density [116]. Generally, cross-linkers are mixed with polymer granules and activated during the high-temperature extrusion or molding process. Therefore, chemical cross-linking is the least favorable technique for blown films to synthesize biomaterial and eventually device cardiac implants. Radiation cross-linking or electron beam (E-beam) cross-linking is the most promising technique since it is extremely precise, efficient, fast, environment

friendly, and does not leave toxic hazardous waste. Moreover, E-beam cross-linked polyethylene is safer for FDA approval as e-beam cross-linked Ultra High Molecular Weight Polyethylene (UHMWPE) is already FDA approved and has revolutionized thousands of patients' lives.

E-beam crosslinked polyethylene has gained a considerable reputation since the advancement of e-beam equipment. Thanks to the prominence of primary carbon in polyethylene backbone, which ensures chain crosslinking instead of chain scission during the electron bombardment [117]. Muratoglu et al. received much success with UHMWPE for total hip arthroplasty. UHMWPE was irradiated with an electron beam and subsequently melt-annealed, which increased hip implant wear resistance from 2 years to 20 years of equivalent walking in a hip simulator. However, the irradiation technique also received its share of criticism because it generates free radicals, which hide in less mobile frozen crystal domains. The free radicals later are found to migrate into amorphous domains to cause oxidation in long-term *in vivo* performance [117, 118]. Therefore, irradiated polyethylene crystals require annealing or remelting after cross-linking to arrest the free radicals. Annealing may erase thermo-mechanical history depending on the temperature and time used. Remelting at a temperature above melting point may degrade mechanical properties but altogether remove the free radicals [110, 119]. In contrast, annealing at a temperature below melting point may significantly improve mechanical properties but retain some free radicals, which may later cause oxidation. Moreover, such changes in properties are not desired for medical implants. Interestingly, numerous methods were disclosed nationally and internationally to minimize mechanical performance alteration, such as annealing slightly below melting temperature, step-wise annealing, and using anti-oxidants [120–122]. Studies conducted on only E-beam crosslinked (without annealing) low-density polyethylene (LDPE) showed that crystallinity only slightly changed, tensile modulus increased from 17 MPa to 20 MPa, but elongation at break decreased from 700% to 450% [123]. Crystallinity slightly changed since irradiation happened in the solid state, which did not require restructuring the crystals from the molten state [124]. Melt-annealing after cross-linking, however, had lowered crystallinity, yield strength, ultimate tensile strength, elongation at break, and modulus [110], [119]. When

crystals start to regrow from completely molten cross-linked polymers, they are hindered by cross-linked chain joints, resulting in defective and small crystals. On the other hand, annealing at a temperature below the melting point of UHMWPE after crosslinking improved mechanical properties significantly [125]. Similar to UHMWPE, LLDPE thin film has improved its mechanical properties by annealing at lower temperatures [126]. However, concerns remain about annealing at lower temperatures after cross-linking since free radicals might still exist. Although one could imagine it might be easier to anneal (below melting temperature) free radicals out of LDPE or LLDPE compared to UHMWPE as molecules in LLDPE or LDPE are more mobile than the much larger molecules of UHMWPE.

Cross-linking without annealing has significantly improved the elastic and decreased the viscous properties of UHMWPE, LDPE, and LLDPE blown film [112, 118, 123, 124, 127, 127]. Polyethylene showed increased storage modulus, creep modulus, and decreased creep strain as they were irradiated with incremental doses of electron beam starting from 20-200 kGy. LLDPE blown film of 15 μm thickness showed higher storage modulus even at elevated temperature, shifting of $\tan\delta$ peak toward higher temperature, and decreased creep strain [124]. Moreover, all types of polyethylene developed a cross-linked network that was insoluble in xylene above polyethylene melting temperature. The formation of insoluble jointed amorphous domains swells less in xylene, even with added thermal energy, which can affect the bio-enhancing HA treatment process. The less viscous and more elastic properties provide insight into the cross-linked network to prove that cross-linking reduces amorphous chains' movements. Chapter 2 found evidence of chain movement directly affecting TAVR durability. Therefore, reduced chain movement should increase LLDPE TAVR leaflet durability.

3.3 Method

An amorphous domain with lower molecular mobility to minimize crazing and improve surface wear resistance may improve TAVR durability. This study crosslinked the LLDPE using electron beam irradiation. A pilot study was conducted in the National Center for Ebeam Research at Texas

A&M University. LLDPE thin blown film $6 \times 6''$ of $80 \mu\text{m}$ thickness was showered with 50, 70, and 100 kGy doses in a laboratory scale LEEB of 200 KeV energy at around 10 mA. Samples were irradiated at air and room temperature and stored in zip-lock bags. Later, they were characterized using gel content measurement, DSC, nanoindentation, and tensile testing.

A more comprehensive study was conducted at E-BEAM Services, Inc., Lebanon, OH. E-BEAM Services, Inc. is an FDA-registered facility that meets FDA standards for medical devices (21 CFR Part 820). LLDPE films were irradiated at 50, 75, 100, and 150 kGy doses using a 4MeV electron source in conditioned air $20\text{--}22^\circ\text{C}$ to prevent heat generation. Samples were irradiated in multiple passes, each pass radiating 25 kGy dose. Afterward, they were packed in zip-lock bags and remained sealed for characterization. The effect of irradiation was evaluated using the above-mentioned battery of characterizations.

Gel Content Measurement

Cross-linked polyethylene develops a relatively immobile insoluble network in xylene, which can be quantified by measuring gel content. The gel content amount is directly proportional to the degree of cross-linking. Pristine uncrosslinked polyethylene dissolves completely in hot xylene. Polyethylene thin films were enclosed in a 120×120 stainless steel mesh cloth and hung in a round bottom flask protruding from the reflux column like a tea bag. At first, pristine polyethylene was refluxed in xylene at 110°C according to ASTM D2765-16 standard (see Appendix A.2). The insoluble portion was dried in a vacuum oven at 150°C until a constant weight was found. The experiment was repeated for two samples. The gel content is the percent ratio of the final weight to the initial weight of the polymer.

ATR-FTIR

Attenuated total reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) was used to measure polyethylene oxidation groups. A Nicolet iS50 was used with a diamond ATR crystal and KBR beam splitter. Three spectra per sample were collected with 4 cm^{-1} resolution, and 64 scans per spectra at different sample locations. Spectra were corrected for baseline and background.

The ATR FTIR method determined the carbonyl (C=O) peak from 1650 to 1850 cm^{-1} since free radical-induced oxidation generates the carbonyl group in polyethylene. The collected spectra were corrected for baseline in the Omnic software.

Thermal Analysis

The thermal analysis was conducted on the virgin LLDPE thin films and samples with different irradiation doses to determine the effect of crosslinking on the crystal domain and morphology. Differential scanning calorimetry measurements were performed on TA Modulated DSC 2500 over a temperature range from 40 °C to 180 °C at heating and cooling rates of 10 °C /min under N_2 purge (see Appendix A.3. This first heating generates the first heating curve. Immediately after the first heating, the sample melted at 180 °C for five minutes and cooled to 40 °C at 10 °C/min. The samples were then heated back to 180 °C at the same rate to generate a second heating curve. Five mm diameter size samples were collected from pristine LLDPE thin and crosslinked films matching the aluminum pan inner diameter. Six to 8, 5 mm punches from each group weighing approximately 10 mg total were carefully stacked into the pan. Previously single-layer sample testing caused thermal leaks because they were extremely lightweight. Therefore, more than one layer was required to generate weight and create proper thermal contact between the pan and the sample. This method ensures consistent heat flow measurement. The crystallinity percentage was calculated by dividing the sample heat of fusion over LLDPE crystal heat of fusion for LLDPE. The exotherm peak was integrated into TA instrument' Trios software to determine the sample heat of fusion.

3.3.1 Mechanical Testings

Tensile testing

The effect of crosslinking on static mechanical properties was evaluated in Instron 4442 and Instron 68TM-10 according to ASTM-D880-18 for pilot study and comprehensive studies, respectively. The samples (n=8) were punched out using ASTM standard dogbone punch of 13 mm gauge length and 3.3 mm width and were tested at a 500 mm/min strain rate. Young's

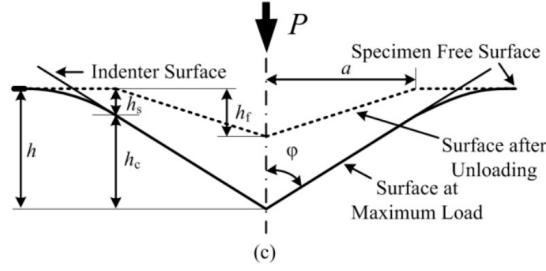


Figure 3.1: Schematic of nano-indentation where a is the contact radius [6]

modulus, elongation at break, yield strength, and toughness were measured and compared with pristine polyethylene samples. Modulus was determined from the best fit slope of the elastic region of the stress strain curves. Yield strength was probed from the yielding point of the stress strain graph, and toughness was the integration of the area under the stress strain curves.

Nanoindentation

Nanoindentation was performed on the crosslinked and virgin LLDPEs in a Hysitron TI Premier using a conical probe of 90° cone angle, probe radius $10.63 \mu\text{m}$ young's modulus of 1140 GPa , and Poisson's ratio of 0.07 . Samples were carefully taped over fused silica to make them as flat as possible. The conical probe indents more accurately on soft LLDPE than the Berkovich probe, which allows more accurate calculations of the projected area. In this experiment, a normal force of $500 \mu\text{N}$ at $50 \mu\text{N/s}$ loading rate was applied. Ten 10×10 arrays of indents were performed to create 100 indents per region to achieve statistical confidence. The load was held for 180 seconds to allow sufficient creep recovery (Figure 3.2), as below 180 seconds, the load vs displacement graph showed creep in the contact depth vs time graph. To avoid the effect of strain field from previous indents, nanoindentations were spaced $10 \mu\text{m}$ apart. Reduced modulus and indentation hardness values were measured using the Oliver and Pharr method to compare with virgin samples. Later, the reduced modulus was converted into an indentation modulus.

$$A(h_c) = C_0 h_c^2 + C_1 h_c^1 + C_2 h_c^{1/2} + C_3 h_c^{1/4} + C_4 h_c^{1/8} + C_5 h_c^{1/16} \quad (3.2)$$

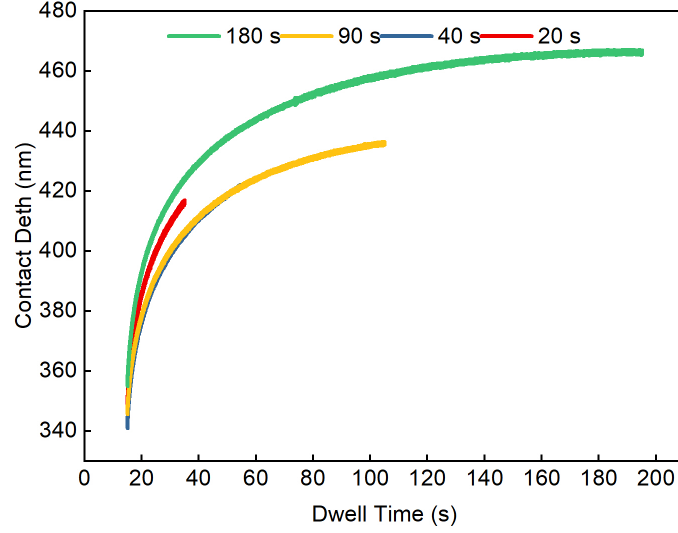


Figure 3.2: Indentation contact depth vs dwell time showing creep recovery at 20, 40, 90, and 180 seconds of holding/ dwell time

$$h_c = h_{max} - \epsilon \frac{P_{max}}{S} \quad (3.3)$$

$$\frac{1}{E_r} = \frac{1 - \nu_s^2}{E_s} + \frac{1 - \nu_i^2}{E_i} \quad (3.4)$$

$$E_r = \frac{S\sqrt{\pi}}{2\sqrt{A}} \quad (3.5)$$

$$H = \frac{P_{max}}{A} \quad (3.6)$$

Here, A is the resulted projected contact area which is a function of contact depth, C_0 is tip geometry co-efficient, C_{1-5} are variable coefficients obtained during indenter calibration with fumed silica. h_c is the indenter contact depth, S is the stiffness at the maximum indentation depth, ϵ is a correction factor = 0.75, P_{max} is the maximum load, E_r , is the reduced modulus, E_s , E_i are the Young's modulus of sample and indenter, ν_s and ν_i are the Poisson's ratio of sample and indenter, H is the hardness. Surface nano wear properties were assessed by nano scratch test using the same conical probe with these loading parameters: maximum normal load 500 μN at 50 $\mu\text{N/s}$, 15 seconds hold time, and unloading at 50 $\mu\text{N/s}$. Ten scratches were performed at each location to achieve statistical repeatability.

Accelerated aging

A Total of 10 samples, two from each group: virgin LLDPE of thickness 80 μm , films crosslinked at 50, 75, 100, and 150 kGy doses, were studied for accelerated aging. After irradiation samples were scanned in ATR-FTIR to find spectra for virgin LLDPEs and crosslinked LLDPEs. ASTM standard F1980-16 was followed to conduct accelerated aging that simulated 2 years of real-time aging. Samples were conditioned in 25 °C, and stored in the same bag for 28 days. Then, a 2/2 inch rectangle from each crosslinked group was placed in an air circulated oven at 70 °C preheated oven for 14 days. After aging for 14 days ATR-FTIR spectra were taken on aged crosslinked samples.

3.3.2 Hydrodynamic Testing

LLDPE irradiated at 100 kGy was used to assemble into TAVR as discussed in Section 2.3.1. The valve was tested for hydrodynamic performance in an *in-vitro* left heart simulator (LHS) under adult physiologic pressure (120/80 mmHg), heart rate (70 bpm), and cardiac output (5 L/min) using a 60/40 water/glycerin mixture to simulate blood properties [1, 8]. Eighty consecutive cardiac cycles of flow waveform data were recorded at a sampling rate of 100 Hz. The irradiated valve (n=1) was compared to a control valve made of pristine LLDPE. After averaging all cardiac cycles, effective orifice area (EOA) and mean transvalvular pressure gradient (PG) were calculated and compared to a control valve without any irradiation. In this study, the valve (n=1) was tested for durability in an AWT according to ISO 5840.

3.3.3 Statistical Analysis

Data sets were tested for equal variance using Levene's test and Anderson-Darling test to determine if the data was drawn from a normally distributed population. Origin Pro 2021 software package was used to determine statistical significance tested in one-way ANOVA with post hoc Tukey's test ($p < 0.05$).

3.4 Results and Discussion

3.4.1 Low Energy Electron Beam Irradiation of LLDPE

Previous research [124] on LLDPE thin films (15 μm) confirmed crosslinking at an 80 kGy irradiation dose using a 150 keV electron source. Gel content increased from 10% to 40% when the absorbed dose increased from 40 kGy to 80 kGy. This present study irradiated LLDPE at two energy levels, 200 keV and 4 MeV, and compared their properties with uncrosslinked LLDPE. The penetration depth of electrons depends on their energy and the irradiating material density. The higher energy electrons are, the deeper they can penetrate. For instance, a standard material's 120 μm depth absorbs 100 kGy, 240 μm depth absorbs 50 kGy, and 360 μm absorbs 0 radiation in the LEEB. This penetration depth is also influenced by material density and electron shielding properties.

LLDPE underwent crosslinking at doses of 50, 70, and 100 kGy in a low energy electron beam (LEEB), and its effects were assessed through gel content measurement, tensile tests, DSC, and nanoindentation. Gel content increased significantly after 50 kGy, reaching 26% at 100 kGy, forming an insoluble 3D network of LLDPE polymers. Only the sample irradiated at 100 kGy was further characterized due to its substantial gel content and possible effect on material properties. DSC analysis showed no changes in bulk crystallinity during the first and second heating cycles. However, a slight decrease in melting temperature from 124 $^{\circ}\text{C}$ to 121 $^{\circ}\text{C}$ was observed (Figure 3.3), along with a slight increase in heat enthalpy peak height for the first heating and a decrease for the second (Figure 3.4). Notably, the typical shoulder in the second heating cycle of uncrosslinked LLDPE representing small crystals disappeared.

At room temperature, LLDPE is well above its glass transition temperature. During LEEB (200 keV) irradiation at room temperature, mobile polymer chains generate excited free radicals in both amorphous and crystalline regions. Free radicals of the amorphous region cross-link immediately, but the free radicals of the crystalline region may remain relatively intact as their movements are restricted. They can later diffuse into amorphous regions to cause oxidation, or a thermal input can increase their movement to encourage crosslinking. The thermal energy generated during

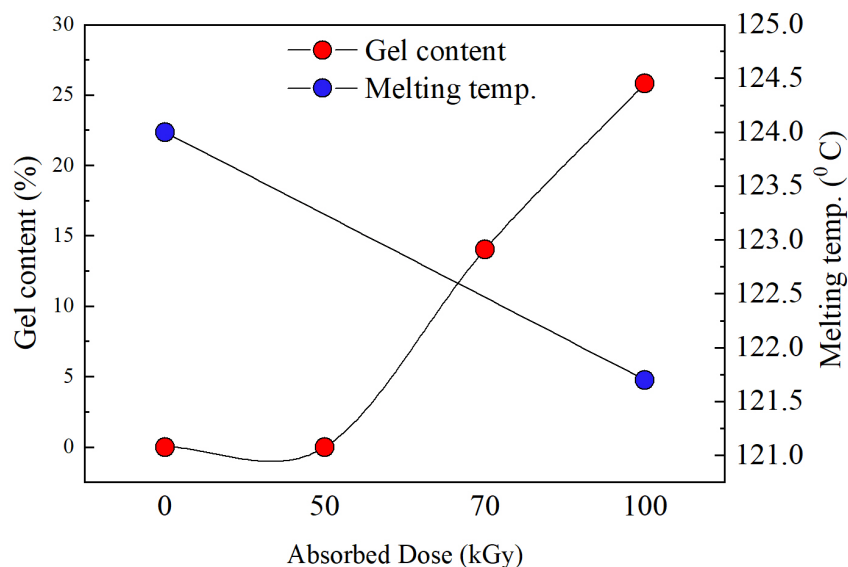


Figure 3.3: Increasing gel content % and decreasing melting temperature of crosslinked LLDPE with respect to absorbed radiation dose

irradiation or the heat provided at the vicinity of DSC caused the free radicals to react and crosslink, leading to a slight increase in DSC's first heating peak but a slight shift towards the left, reducing melting temperature. Because the newly crosslinked free radicals fold into branches of the main polymer chain instead of increasing the main chain length (discussed more in Section 3.4.2). Immediately after melting, as controlled cooling starts, the chains fold into spherulitic structures. Uncrosslinked LLDPE forms more perfect crystals, whereas cross-linked LLDPE forms relatively imperfect small crystals as the crosslinking limits macromolecular movements, which hinders crystal growth.

Figure 3.5 shows the effect of crosslinking on the mechanical properties: Young's Modulus, Yield Strength, tensile strength, and elongation at break of cross-linked and non-cross-linked virgin LLDPE. Statistically, there were no significant changes in any mechanical properties, but the average elongation at break was lowered for crosslinked LLDPE. Crosslinking restricts molecular disentanglement and chain extension by creating knots. The chains can stretch less than virgin films, thus reducing elongation before breaking. Figure 3.6 shows indentation hardness slightly improved for the irradiation side, facing the electron source, compared to the back side away from

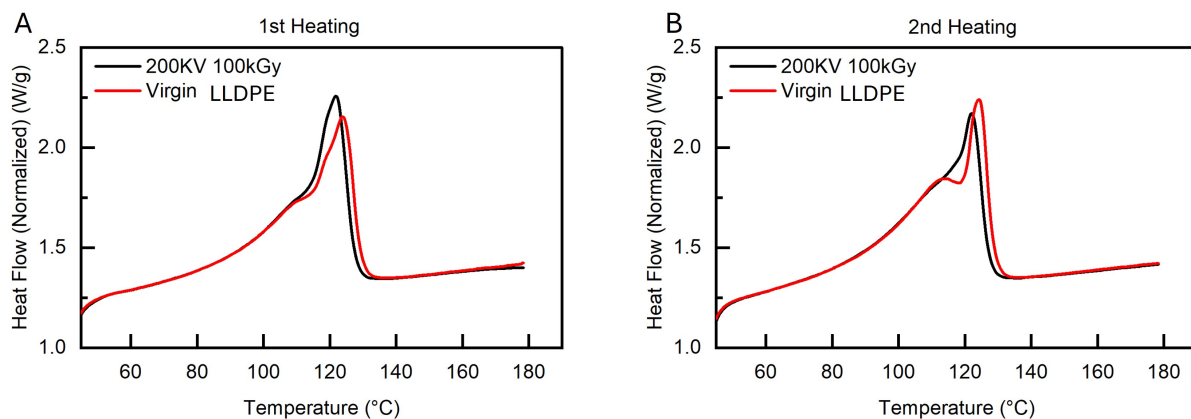


Figure 3.4: DSC (A)first and (B)second heating curves of crosslinked and uncrosslinked LLDPE

the electron source and virgin LLDPE. Similar to Young's modulus of bulk LLDPE, the indentation modulus calculated from the reduced modulus remained unchanged for all of them. Virgin LLDPE indentation modulus decreased from 115 and 51 MPa when contact depth increased 229 ± 15 nm to 939 ± 33 nm, respectively.

Indentation hardness is a measure of materials resistance to permanent surface deformation or damage, and modulus is a measure of materials resistance to elastic deformation [128, 129]. Hardness and modulus are affected when bonding energy, number of bonds, and crystallinity changes between molecules. The thermal test showed no changes in bulk crystallinity, and gel content measured the formation of a 26% insoluble 3D network in the amorphous domains. Unchanged crystallinity explains modulus (Young's modulus and indentation modulus) preservation. Crosslinking of amorphous domains limits their spontaneous wiggling even though they are well above the glass transition temperature. The knots created in amorphous chains hold the matrix to resist plastic deformation during indentation. As a result, the hardness increased for the side, facing electron source [129–134]. All these research irradiated polymer using an electron source of few MeV and found improvements in hardness at 100 kGy. LEEB is responsible for only slight improvement on the radiation side and unchanged hardness on the back side. Indentation modulus and Young's modulus (tensile test) are equivalent for isotropic material since indentation does not account for orientation [129]. In this study, Young's modulus of bulk polymer was

tested using a constant strain method, whereas indentation is a constant load method. During nanoindentation, the indenter probe depresses the sample surface under a maximum load and constant loading rate, causing plastic and elastic deformation. Load sensors monitor the contact depth (h_c), which determines the resultant contact area utilizing the Oliver and Pharr method of calibration ((3.2)). Reduced modulus is calculated from the elastic recovery unloading profile. During loading, the indents depress the surface to 939 ± 33 nm, which exerts compressive stress on the material surface as the material is fixed over fused silica. The elastic recovery during unloading determines material modulus and assumes that the probe is always in contact with the material during unloading. However, the probe contact with material varies with surface roughness and viscoelasticity, which in turn changes the contact area. Furthermore, the fused silica, a hard material, was used for calibration. Multiple indents were made on the fused silica to calculate the projected area as a function of contact depth ((3.2)). Since fused silica is a hard material, its indentation area will be significantly differ from the soft plastic material [135]. Indentation modulus and bulk modulus (measured in the tensile test) are theoretically equivalent but experimentally depend on material surface properties and viscoelasticity [129]. Because viscoelastic creep behavior affects the unloading profile. Amorphous chains of the semicrystalline polymer can rearrange on the surface to lower the surface energy, making it difficult for the indenter to depress the surface. Polymer chains are probably arranged homogeneously with less microstructure variation in the bulk sample than on the surface. Therefore, surface nanomechanical properties and calibration material can be blamed for underestimating the indentation modulus compared to Young's modulus. This present research maintained a constant loading rate, maximum load, and hold time for all tested samples.

Figure 3.7 shows the nano scratch behavior in terms of lateral force and friction as a function of lateral displacement of crosslinked LLDPE to compare with virgin LLDPE. At the end of the scratch test removed material from the sample surface piled up on the probe tip. As a result, the lateral force was inconsistent for the first and last $3 \mu\text{m}$ lateral displacement [136] but stabilized within -2 to $+2 \mu\text{m}$ of distance which was used to determine wear behavior. A total of ten

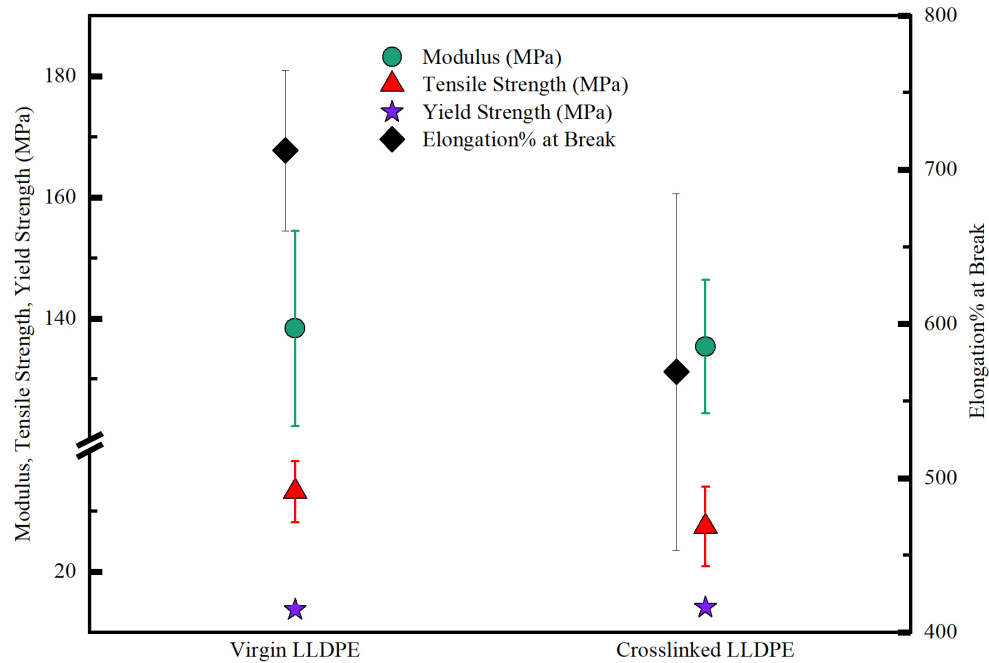


Figure 3.5: Mechanical properties: Young's Modulus, Yield Strength, tensile strength, and percent elongation at break of uncrosslinked and crosslinked LLDPE at 100 kGy

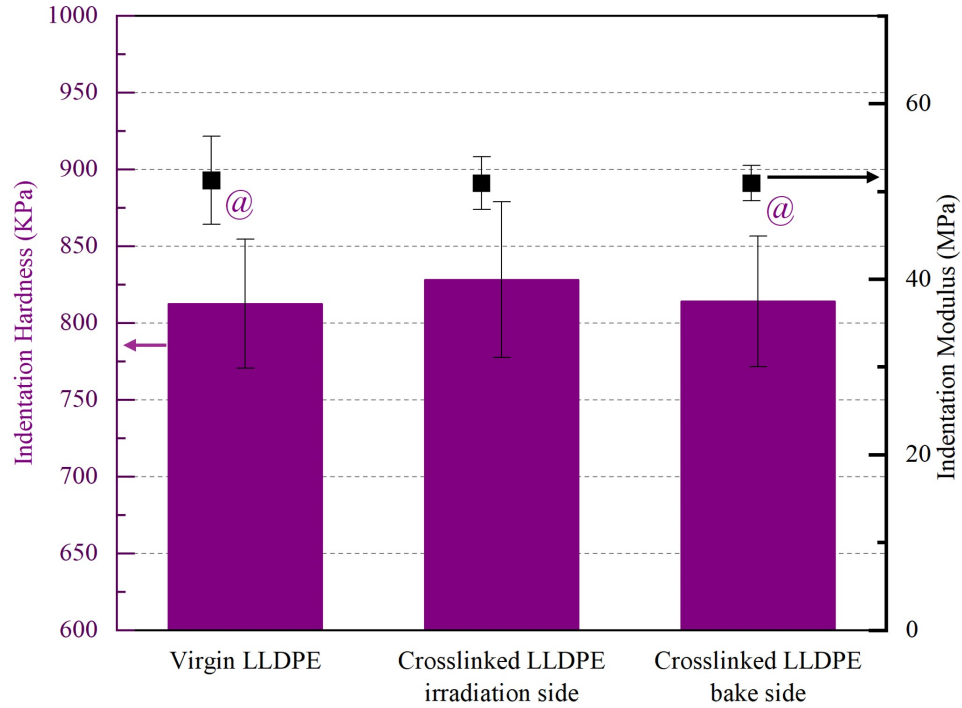


Figure 3.6: Variation of indentation hardness and modulus of virgin LLDPE crosslinked LLDPE irradiation side and back side

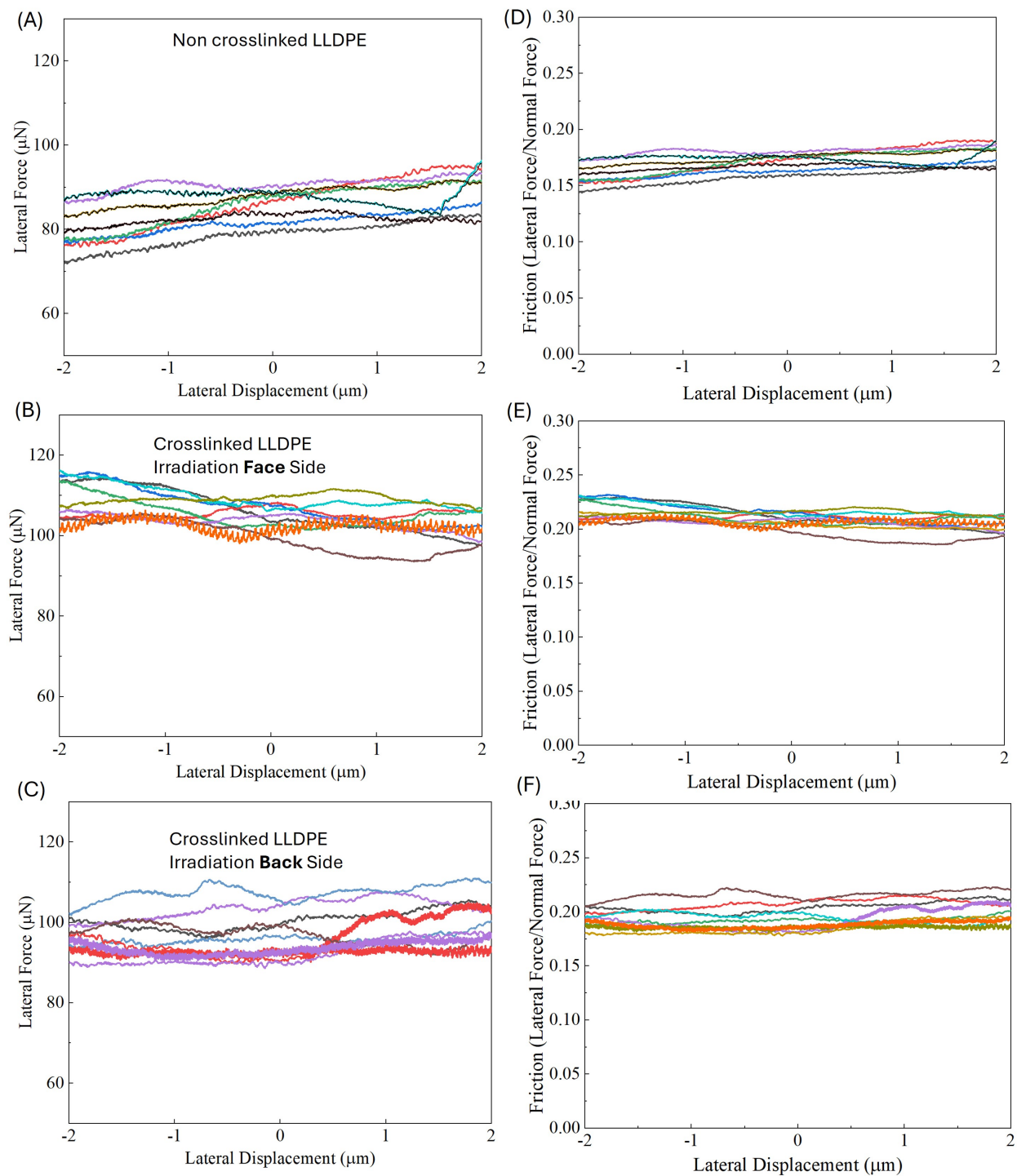


Figure 3.7: Variation of lateral force and friction with lateral displacement of virgin LLDPE, crosslinked LLDPE irradiation side and back side of ten repeated runs to achieve statistical confidence

representative spots were wear-tested to achieve statistical confidence. A higher lateral force indicates the probe requires more force to move the probe across the surface. The value of lateral force was found to be 109 ± 4 , 96 ± 5 , $86 \pm 3 \mu\text{N}$ at "0" lateral position for crosslinked irradiation face, back and virgin LLDPE respectively. An increment of $23 \mu\text{N}$ was found from virgin LLDPE to crosslinked LLDPE. Clearly, the back side, which was not facing the irradiation, has less insoluble gel or 3D net formation; hence, its resistance to wear improved less than the other side, which was directly hit by a shower of energized electrons.

From Figure 3.7 left it is seen that the lateral force experienced by the probe is the highest for crosslinked LLDPE irradiation face side. The crosslink bonds hold the amorphous matrix together by forming 3D network and hence resisting to scratch. Figure 3.7 right shows the variation of coefficient of friction or friction (ratio of lateral force over normal force) with lateral displacement. This parameter is an indicator of material resistance to scratch from indentation. The higher the friction value, the harder and more wear-resistant this material is [137]. Bautista et al. [138] found a linear negative correlation between the nano silica composite weight loss rate in the Taber abrasion test and the critical force required to start the nano scratch test. Nanoparticle presence affected elastic-plastic deformation of the nanocomposite, which reduced weight loss during the abrasion test and required more force to even start the nano scratch test. In general, it has been observed that the higher the coefficient of friction, the higher the material is wear-resistant. Unsurprisingly, friction is the highest for crosslinked LLDPE irradiation face side and significantly different from the rests. The value was found to be 0.21 ± 0.0062 , 0.18 ± 0.056 , and 0.17 ± 0.006 at the "0" lateral position for crosslinked irradiation face, back, and virgin LLDPE, respectively. The average was calculated from ten scratches at the "0" lateral position. The coefficient of friction for the crosslinked irradiation face side was significantly different from the uncrosslinked virgin LLDPE. It can be seen that there are improvements in scratch resistance from crosslinking for both sides of LLDPE. Even though the hardness (discussed earlier) did not improve for both sides of crosslinked LLDPE, the wear resistance improved for both sides. This finding is consistent with the findings

of Agarwal [139] where scratch resistance improved from adding Multi Walled Carbon Nanotube to UHMWPE, but hardness did not improve [110].

3.4.2 High Energy Electron Beam Irradiation of LLDPE

Figure 3.8 shows the effect of high energy electron beam (4MeV) irradiation (HEEB) on gel content % and melting temperature with respect to the absorbed dose. Virgin LLDPE completely dissolves in xylene at 110 °C. In contrast, only a small portion of irradiated samples were insoluble to xylene with an increased absorbed dose. Upon drying the gel at 150 °C, a thin film was retrieved as irradiation creates an insoluble crosslink network akin to thermoset polymer. Gel content skyrocketed to 42 % at 50 kGy and increased to 57% at 100 kGy dose. Beyond that, it decreased slightly as new gel formation saturated. Similar trends were reported in the previous studies but varied with electron energy. Low-Density Polyethylene (LDPE) formed 40% gel at 150 KeV [124], 60% gel at 1 MeV [140], 50% gel at 1.2 MeV [136], 65 % gel at 2 MeV [141], 80% gel at 10 MeV [142] at 100 kGy absorbed doses. Clearly, there is a linear relationship between gel content and the energy of the source electron. This present research reports that HEEB formed 57% of insoluble gel in LLDPE at the same 100 kGy absorbed dose, which matches with the published study explained earlier. The free radical reaction rate is higher for high energy electrons (4 MeV) than low energy electrons (200 KV), resulting in a higher number of radicalized polymer chains that react to form crosslinked LLDPE.

Figure 3.8 further shows changes in melting temperature with absorbed dose as measured in DSC. The temperature lowered by 3 degrees for 50 kGy from virgin LLDPE, followed by a gradual decrease of 1 degree till 150 kGy. These findings are similar to LEEB Section 3.4.1. A reduction in melting temperature is evidence of imperfect crystal formation.

Figure 3.9 shows DSC thermographs of crosslinked LLDPE with their absorbed dose. No changes in bulk crystallinity were measured from heat enthalpy for the first or second heating. Irradiation did not affect the endothermic cooling profile. Interestingly, the heating thermograph of irradiated LLDPEs changed for the first and second heating compared to virgin LLDPE. The melting

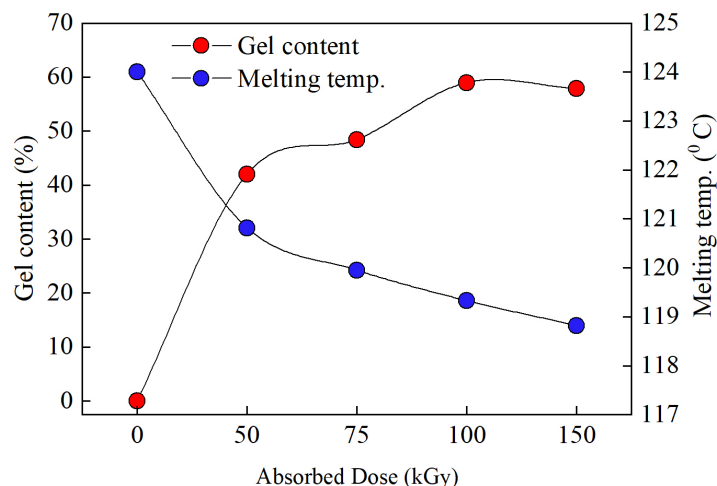


Figure 3.8: Gel content % increases and melting temperature decreases with the absorbed dose.

temperature of the first scan (Figure 3.9A) moved towards the left, but the peak height was slightly elevated. Similar behavior was observed for LLEB (Figure 3.4). A left shift or decrease in melting temperature is related to a decrease in crystallite size and crystal perfection. It indicates that crosslinked crystals were easier to melt than uncrosslinked crystals. On the other hand, peak height increases when crystallite size or thickness increases. This contradictory behavior of irradiated LLDPE is not seen for other branched or highly crystalline polyethylene. Khonakdar [140] et al. argued that due to immobility, crystalline regions do not crosslink during irradiation, though they may form free radicals. The thermal input of DSC increases their mobility, which favors crosslinking. The increase in melting peak height refers to increased crystallite size, as they crosslink and fold into the crystal domain. As mentioned earlier, this study found a slight increase in melting peak height, i.e., higher heat flow, but the bulk crystallinity remained fairly unchanged. On the other hand, the melting temperature measured as the location of peak maximum shifted toward the left, reducing the melting temperature slightly. The newly formed crosslinks might fold into branches of the main crystal domain and might not contribute to increasing the polymer main chain length. Because polymer chain length increment increases melting temperature [143], which would result in a more right shift of peak location. Rather, they branch out from the main chain, forming imperfect crystals. Since LDPE is highly branched, so formation of new branches during

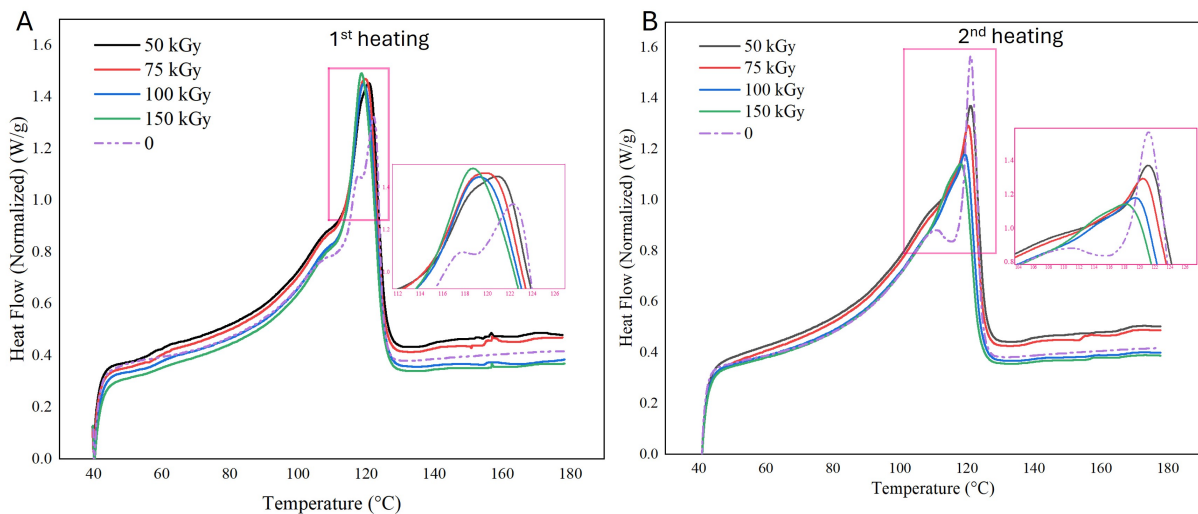


Figure 3.9: DSC thermograph 1st (A) and second heating (B) of crosslinked LLDPE as a function of absorbed dose. Insets show respective melting profiles, showing changes in normalized heat flow with absorbed doses indicating crystal size change.

and/or after irradiation does not affect the thermal profile [140].

The second heating graph matches with that of typical branched LDPE [140] where the second melting temperature gradually reduces and peak intensity (normalized heat flow) decreases. Similar effects were found in LEEB irradiation (Figure 3.4). Interestingly, the melting peak broadens with increased absorbed doses. Virgin LLDPE shows a minor melting peak at around 112 °C, referring to some small crystallites formed during cooling. Irradiated LLDPEs lose this minor melting peak and show a single peak. After first heating, the crystals completely melt, forming an isotropic amorphous phase. All the free radicals react with each other, forming crosslinks. The molten polymers start to crystallize as cooling starts. Virgin LLDPE recrystallization happens without any resistance. However, crosslinks resist macromolecular movements, impeding reorganization and chain folding. As a result, the crystals are smaller in size and less in content as seen in lower and broader melting peak heights.

Figure 3.10 shows the variation of mechanical properties with absorbed dose. Unlike low energy electron beam irradiation, Young's modulus increased with absorbed dose up to 100 kGy, then slightly decreased. The elongation at break decreased with an increased absorbed dose. Similarly, toughness and UTS slightly increased upto 100 kGy and decreased at 150 kGy compared to virgin

LLDPE. Yield strength was significantly affected by irradiation with doses as low as 50 kGy. Yield strength for all doses was significantly higher than uncrosslinked LLDPE ($p < 0.05$). The strength gradually increases with increased doses up to 100 kGy then decreases at 150 kGy. The change in yield strength is similar to that of elastic modulus as they both increase up to 100 kGy and decrease after that. These findings agree with previous studies [142]. The crosslinking of polymer chains reduces polymer free volume to create 3D networks of covalent bonds in the amorphous domain, and enhanced inter-crystal bonds are responsible for modulus increase. A slight reduction in modulus and yield strength at 150 kGy may indicate oxidation due to oxidative degradation causing polymer chain scission. The 3D network is again responsible for reduced elongation before the material breaks, as the covalent bonds between polymer chains act as knots, restricting the stretching of polymer chains. The change of elongation at break can be co-related to gel content. Gel content increased sharply at 50 kGy, then gradually increased. The average elongation at break decreased to 50 kGy and held steady for the rest of the dose. As the polymer chains got crosslinked, their disentanglement and ability to stretch were restricted; therefore, they strain less before breaking. Ultimate tensile strength and toughness followed the trend of modulus. They only slightly increased before decreasing at 150 kGy. It is important to note that there were significant differences between the modulus of LLDPE irradiated at 100 and 75 kGy compared to virgin LLDPE in one-way ANOVA tests at $p < 0.05$.

ATR-FTIR analysis was performed on the surface of irradiated and virgin LLDPE before and after accelerated aging. Irradiated samples were studied in ATR-FTIR to assess oxidative degradation around 965 (trans-vinylene) and 1718 cm^{-1} (carbonyl) wavenumbers. Figure 3.11 shows ATR-FTIR spectra of e-beam irradiated (before accelerated aging), and accelerated aged after irradiation from 600-2000 cm^{-1} wavenumber. Before aging, the samples had no peak between 800-1200 or 1600-2000 cm^{-1} wavenumbers for virgin, 50, 75, and 100 kGy. A small bump is found for LLDPE irradiated at 150 kGy at around 1718 cm^{-1} . The bump is so insignificant that upon correcting the baseline, it disappears to match the uncrosslinked LLDPE. Accelerated aged LLDPE of 100 kGy and 150 kGy doses might again have small bumps at 1718 cm^{-1} wavenumber. The double bond

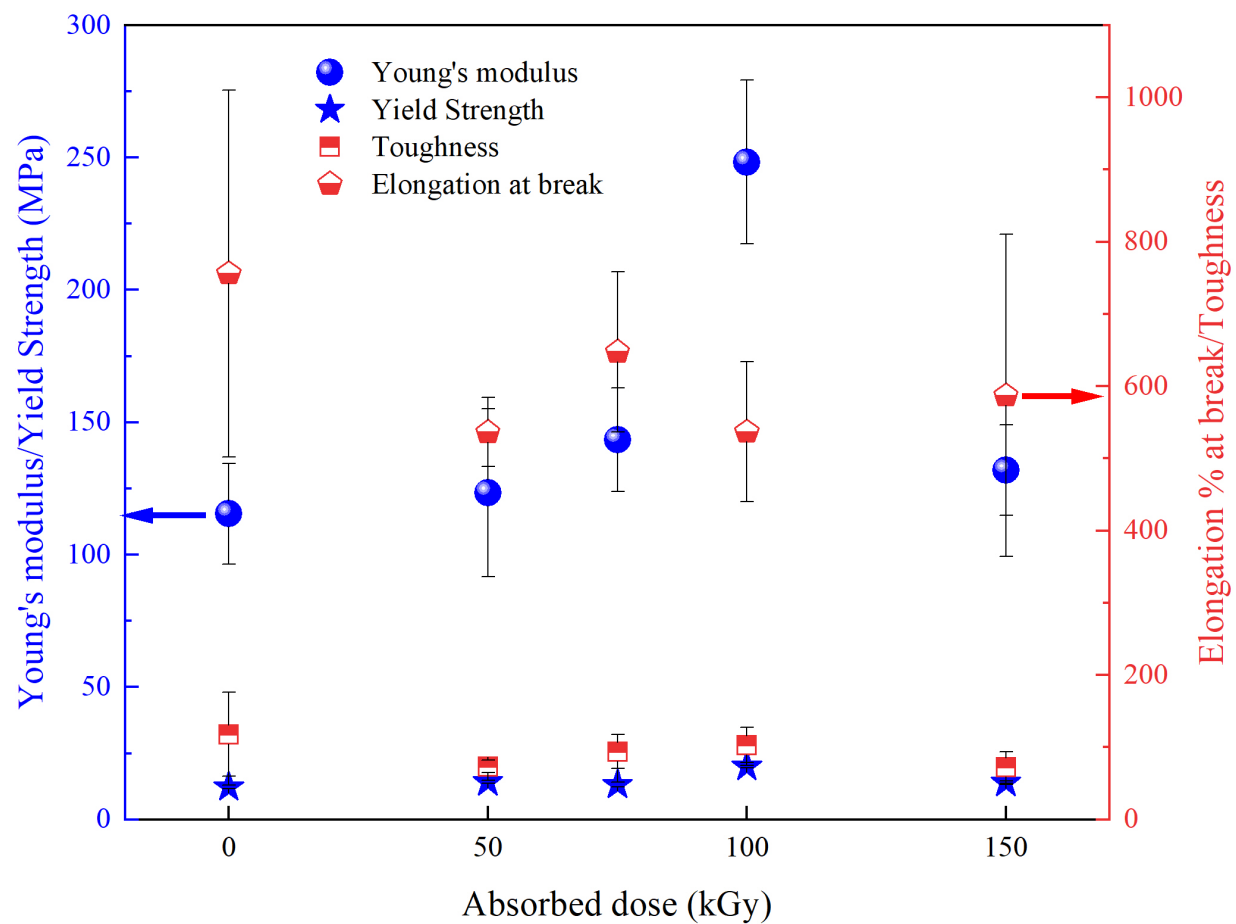


Figure 3.10: Variation of mechanical properties: Young's Modulus, Yield strength, Elongation% at break, Toughness with absorbed dose

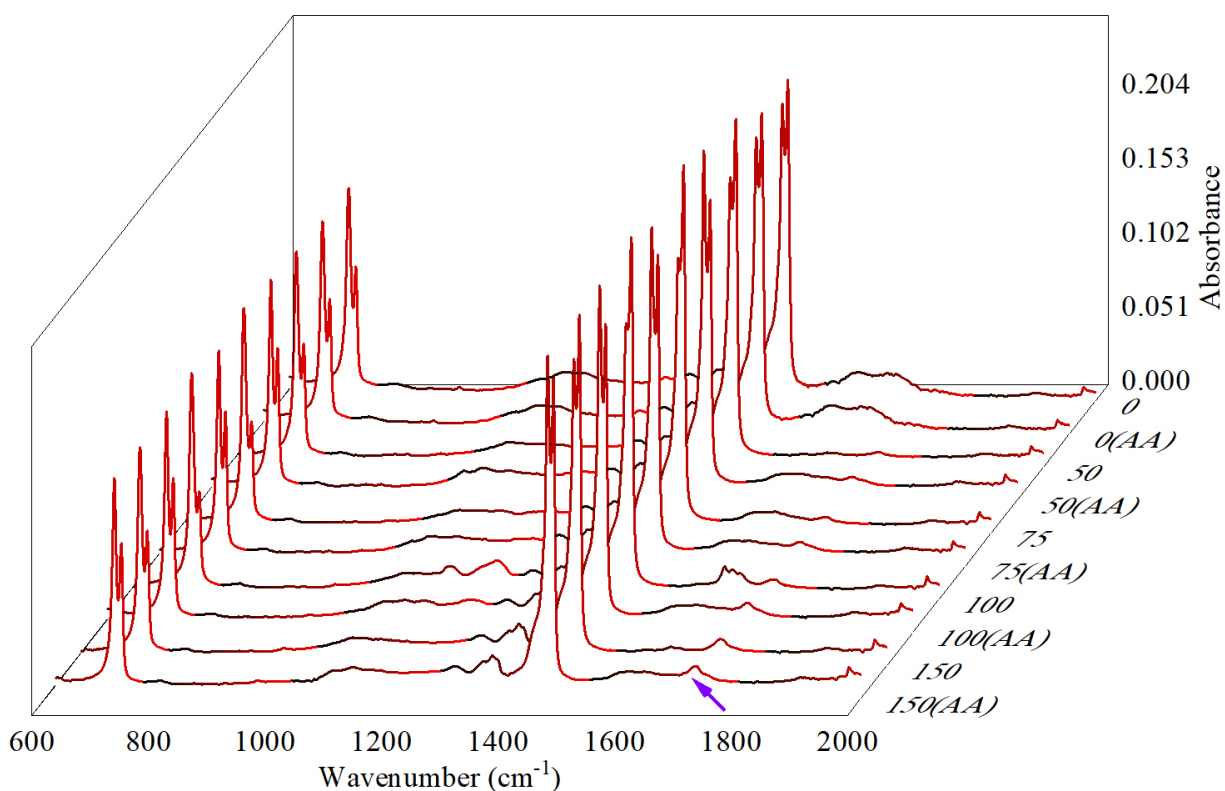


Figure 3.11: ATR-FTIR spectra of irradiated, accelerated aged after irradiation as a function of absorbed dose 0-150 kGy at wavenumber 600-2000 cm^{-1}

of trans vinylene peak formed during crosslinking of polyethylene chains is missing both before and after accelerated aging of irradiated LLDPE. This double bond is formed when hydrogen is released to form carbon-free radical. The ketone carbonyl peak at 1718 cm^{-1} is an indication of surface oxidation. Their presence on high-dosed and aged LLDPE indicates some oxidation happened, at least on the surface of thin ($80 \mu\text{m}$) LLDPE films. It is unknown if there were enough free electrons on the surface to diffuse further into bulk LLDPE. The oxidative product ketone group aligns with the mechanical properties of irradiated LLDPE. As discussed in the previous section, the modulus decreased at 150 kGy from oxidative degradation (non accelerated aged), and evidence of oxidation is found in the ATR-FTIR spectra for this dose.

A TAVR was assembled (Section 2.3.1) from an LLDPE flat sheet irradiated at 100 kGy, as the maximum effect of crosslinking was found in mechanical and chemical tests. Chain scission or oxidation might have happened beyond this dose, which can affect mechanical properties in the

long term. One valve was tested in LHS and compared with the unirradiated valve. The crosslinked valve had a pressure gradient of 18.1 mm Hg and EOA of 1.2 cm². As of writing this dissertation, the valve is still running in the AWT, crossing 6 million ISO cycles. The pressure gradient is slightly higher than the control valve, and the EOA is slightly lower. This is because the modulus is double that of virgin LLDPE. According to (3.1), flexural modulus is directly proportional to Young's modulus, and the higher bending stiffness makes it more difficult for the leaflets to open and close compared to uncrosslinked leaflets. Future research can crosslink the valve strategically so that the stiff yet the highest crosslinking happens only in the leaflet area of the commissure post, and interleaflet triangle, where failures have been shown to initiate (Chapter 2). These areas are in contact with the stent and do not undergo coaptation. Changes in the modulus of these areas do not affect valve hydrodynamics. Moreover, since valve thickness is not increased, TAVR crimping into smaller diameter will not be altered either. Therefore, highly crosslinked abrasion-resistant LLDPE should prevent crazing and wear in areas that do not undergo coaptation to improve valve durability without altering valve hydrodynamics.

3.5 Conclusion

Electron beam irradiation has revolutionized the UHMWPE orthopedic implant field by controlling polymer chain disentanglement to provide wear resistance and skyrocketed durability. LLDPE thin films are used as leaflet material in polymeric transcatheter heart valves but face premature failure due to cyclic fatigue-initiated crazing, which is related to chain disentanglement and surface wearing. This study irradiates LLDPE energized electrons to cause crosslinking, which can control crazing and surface wear by limiting polymer chain movements. The mechanical property improvement, insoluble gel formation, and thermal profile melting temperature shifts confirm the crosslinking of LLDPE instead of chain scission. Crosslinking also improves nanomechanical behavior surface hardness and scratch resistance. The crosslink bonds hold the amorphous matrix together by forming 3D network and hence resisting to scratch. These findings agree with established knowledge of the behavior of crosslinked polyethylene and extend beyond

that to apply the material as a leaflet in the heart valve. Leaflet lifespan is expected to improve with crosslinking but alters valve performances in response to increased stiffness. This study, therefore, indicates the necessity of crosslinking to control crazing and surface wear. However, some limitations are worth noting. Irradiation was performed in air in the presence of oxygen, which can cause oxidation in the long run. Crosslinking improves material stiffness as new bonds are created in the interpolymer chain. The stiffening of the leaflet cusp affects valve performance and must be avoided. Therefore, future research should avoid high dosage crosslinking on the cusp by masking or protecting the cusps using electron shielding material so that the leaflet cusps form enough crosslinked bonds to control crazing and surface wear but not too much that can change material stiffness.

3.6 Future Research

LLDPE blown films will be irradiated using an electron beam accelerator in an inert environment (N_2) to prevent in situ oxidation and related damage and perform cross-linking. Crosslinking can be performed in the first step of TAVR manufacturing assembly line. During crosslinking, the cusps regions must be properly covered to avoid exposure to high irradiation. The flat sheet then could be melt pressed at 125 °C or beyond to completely arrest the free radicals. Then, the flat sheet could be vacuum-formed into a 3D shape. Care must be taken when selecting the masking area to identify and map plastic how the plastic sheet folds into a 3D shape. The map should be able to predict which areas of the sheet will form the 3D cusps and, therefore, only cover those areas. Then 3D-shaped leaflets can be HA-treated for bioenhancement using standard protocol.

Chapter 4

Parametric Finite Element Modeling of Reinforced and Crosslinked Polymeric Leaflets for Improved Durability

4.1 Summary

Heart valve durability can be improved by strengthening the leaflet with fiber reinforcement. A thin plastic sheet is assembled into a cylindrical form by welding two ends, which never fails. The weld at the commissure post is mechanically stronger than the rest of the leaflet, which protects this region. Braided fibers are embedded on the leaflet regions of the commissure post perpendicular to the valve circumference, mimicking the weld but at a much higher strength. Leaflet durability skyrockets from a few million ISO 5840-2005 cycles to 73 million. The entire cardiac cycle of the heart valve with embedded fibers of varying angles, lengths, and numbers is simulated in Finite Element Analysis (FEA) to study their effects on leaflet maximum principal stress and leaflet opening dynamics. Horizontal fibers wrap the leaflet 360° to relax the leaflet completely during peak diastolic. However, the leaflet has a higher coaptation gap and delayed opening. The heart valve with embedded horizontal fibers is physically manufactured and tested in an *in vitro* flow loop and wear tester, which shows improved durability but compromised hemodynamics. Finally, a strategically crosslinked leaflet is simulated in FEA, where leaflet regions of the commissure post and stent line are assigned with stiff crosslinked LLDPE material properties. The three cusps are assigned with uncrosslinked LLDPE material properties. Results show that strategically crosslinked leaflets open more easily than fully crosslinked leaflets.

4.2 Introduction

Ideally, TAVR shall endure the patient's expected life and maintain as-implanted hemodynamics. However, FDA-approved commercially available TAVRs lose "as-implanted" hemodynamics over an extended period because of calcification, tearing, and thrombus formation, depending on the patient's underlying health condition, age, and sex [144]. Since TAVR is a comparatively new medical treatment, the data on long-term clinical durability (>10 years) is limited. French-2 registry conducted 5 years of post-implantation studies on 509 patients, and 2.5% of patients had deteriorated leaflets [48]. Therefore, with the relatively recent approval for low-risk patients, TAVR durability raised major concerns. An alternative approach to bioprosthetics is leaflets made from state-of-the-art polymers with tunability to achieve hemocompatibility and long-term durability to address this issue. Despite compelling research in the growing polymeric heart valve field [8, 145–147], only The Tria™ polymeric surgical aortic and mitral valve achieved approval for clinical trials from FDA. The Tria™ TAVR is in the pilot chronic animal model testing phase ([148]). The absence of biomolecules at the molecular level and the limited fatigue strength of investigating polymers might be attributed to the lack of commercially successful polymeric TAVR.

Our research group has previously developed a framework for hyaluronan (HA) enhanced biomaterials for TAVR that showed excellent *in vitro* hemodynamics, *in vivo* anti-thrombotic, and anti-calcific properties in pilot juvenile sheep studies. *In vitro*, hemodynamics results that meet ISO standards originate from accurate mechanical flexibility of LLDPE thin film and optimized 3D shape. Superior hemocompatibility originated from an interpenetrating host polymer LLDPE and guest polymer HA network. Despite excellent *in vivo* and *in vitro* performances and high tear strength, the TAVR prototype leaflet has limited durability, as shown in Chapter 2. Leaflets were welded into cylinders from an 80-micron flat thin film (Figure 2.2 Chapter 2). Interestingly, all the AWT leaflets tore near the commissure post region opposite the weld-associated commissure post. Therefore, this research will investigate two leaflet reinforcing techniques to improve durability.

4.2.1 TAVR Premature Failure Mechanics

Numerous computational modeling and frameworks on the diastolic aortic phase have established that cuspal, commissure, and basal attachment of leaflets are the primary load bearers during the diastolic phase [146, 149]. These computational analyses were further validated experimentally under physiological conditions. Polymeric TAVR was devised from LLDPE leaflet and Co-Cr alloy stent to determine valve durability and associated failure region. A thin LLDPE film was welded at two ends to form a cylinder to assemble over a cobalt-chromium stent (Figure 2.2). The stent is a 23 mm diameter balloon-expandable cobalt-chromium alloy. The durability tests were performed at a 15 Hz cardiac cycle, using room temperature water-glycerin mixture as fluid. Valve (a) failed between 1-4 million cycles; failure is determined by visible tear and greater than 10% change in hydrodynamics value. The welded areas never failed and always tore at the leaflet region near the commissure post opposite the weld-associated commissure post. The weld at the commissure post is mechanically stronger than the rest of the leaflet, protecting this region. Therefore, this research provides methods to increase durability by strategically strengthening and crosslinking the leaflets in the region of the commissure posts. As discussed in Chapter 2, SEM images of the torn surface revealed crazing, surface wearing, and damage accumulation where fibrils propagate towards stress direction. The failure is the result of complex multidimensional impacts: cyclic fatigue initiated crazing and impact wear on the surface damage. Cyclic fatigue initiates craze as the local stress exceeds the yield stress. Therefore, this study aims to reduce the principal stress below yield stress. The cardiac cycle is 0.8 seconds, and diastolic closing happens in only 0.1 seconds. The rise time is well below half the cardiac cycle, indicating impact loading. Impact wearing effects could be minimized by preventing leaflet movements against the stent, especially around the leaflet region of the commissure post.

4.2.2 Motivation of Research

As discussed in Section 1.3 the native heart valve has an elite architecture made from collagen and elastic fiber [150]. The strong collagen fibers are the main load bearer, and their unique

orientation contributes to its known durability. One would argue that bioinspired native leaflets like fiber-reinforced polymer composite can significantly improve valve durability. Liu et al. [151] conducted a computational study on stress distribution of fiber-reinforced polymer composite leaflets and found an 86% reduction of maximum principal stress compared to virgin polymer. The composite fiber orientation reportedly follows native leaflet collagen fiber orientation and architecture. This study provides excellent proof of the concept that collagen fiber in native leaflets might play a key role in reducing maximum principal stress [152, 153]. Although this optimized reinforced valve survived a fatigue life of 10.35 years *in vitro*, the valve prematurely failed within 6 months of *in vivo* studies. Because the base polymer calcified and torn, exposing the fiber [152]. Other techniques, such as leaflet tapering, leaflet geometry optimization, and stent geometry optimization, have been studied thoroughly over the decade [61]. This chapter used computational simulations to upgrade strategic locations of the TAVR leaflet properties (Figure 4.1 and Figure 4.2).

4.3 Methods

4.3.1 Valve Prototyping

TAVR leaflets were fabricated by reinforcing the commissure posts with polyester fiber. One polyester fiber of 0.2 mm diameter (braided surgical thread) was hand-laid upon a thin sheet of LLDPE (80 μm) along the commissure post region of the leaflet perpendicular (90°) to circumference. In another leaflet, three polyester fibers of 0.1 mm diameter were hand-laid on the most aortic side of the leaflet. Extreme care was taken to ensure fibers were aligned to the proper angle and straight, avoiding fiber wrinkles. Extreme care was taken to ensure fiber spacing was constant and fibers did not overlap. A thin sheet of the same LLDPE was laid over the fiber, covering them completely. Carefully, the laid structure was melt-pressed in a bench top press at 500 lbs pressure at 125°C for 1 minute. The flat laminate was further melt-pressed (i.e., welded) at the two ends to create cylindrical leaflets assembled over the stent (Figure 4.1).

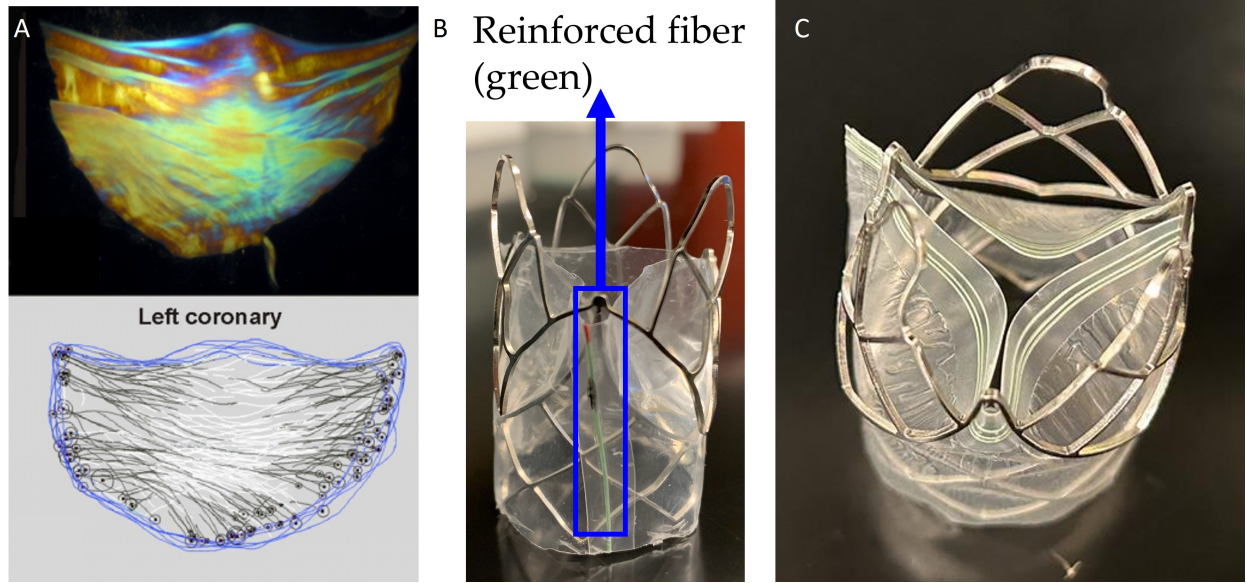


Figure 4.1: (A) Polarized light image and generalized tracing of collagen fiber bundles of aortic valve left coronary cusp; color denotes fiber diameter black: thick, white: thin [7] (B-C) Bioinspired fiber reinforced TAVR. (B) Single fiber running along valve height (C) Three fibers running along valve circumference

4.3.2 Constitutive Model

SolidWorks was used to create a CAD model assembly of cylindrical leaflets and stents to simulate the TAVR in ABAQUS. The internal diameter and height of the stent (cobalt chromium) were 26 mm and 18.5mm, respectively (Figure 4.2a). One of the fiber-reinforced valves was created by thickening a 3 mm width of the leaflet at the commissure post region with a peak thickness of 0.28 mm, which was tapered down to 0.08 mm to match the rest of the valve thickness. The thickest region housed a fiber (0.2 mm diameter, Modulus 1GPa) **perpendicular** (90^0) to the valve circumference (Figure 4.2c). In the second set of studies, three fibers (0.1 mm diameter each, Modulus 1 GPa) were tied to the commissure post region of the leaflet at an angle of 0, 15, and 30^0 to leaflet circumference (Figure 4.2c, d, and e) at 0.1 mm spacing to optimize the orientation of fiber.

In the third set of studies, fiber length was optimized: 12 mm long, covering only around the commissure post region of the leaflet, and 86 mm long, covering the entire leaflet (360^0) at the topmost region of the leaflet (Figure 4.3). Eight additional fibers were added through the height of the valve to find their effect on valve opening dynamics.

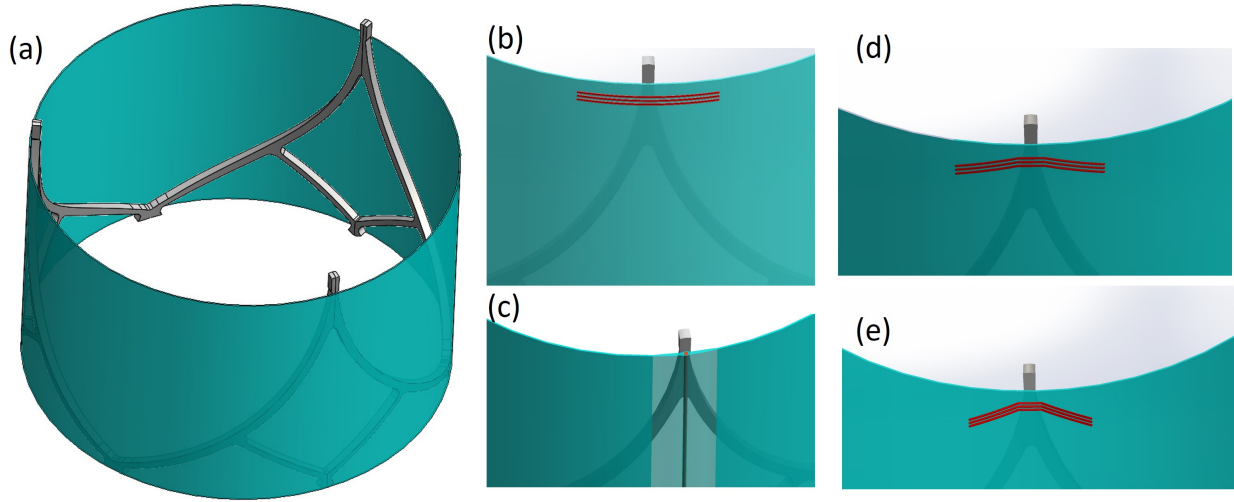


Figure 4.2: CAD model for optimizing fiber orientation. (fiber length 6mm, dia 0.1 mm): (a) TAVR valve assembled over the stent and (b-e) highlighted one commissure post showing fiber reinforcement at (b) 0° (c) 90° (d) 15° and (e) 30° fiber orientation

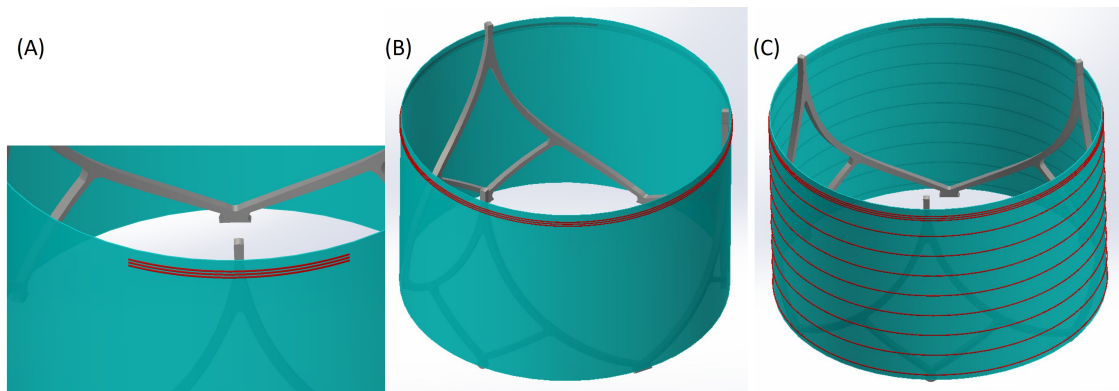


Figure 4.3: CAD model for optimizing fiber length. (fiber orientation 0° , dia 0.1 mm): (A) 12mm (B) 80 mm (C) 8 additional fibers through the height

The effect of crosslinking on valve performance was also simulated in this study to compare with results from the *in vitro* model. The material property of virgin LLDPE in the simulation of the control leaflet was changed to that of stiffer crosslinked LLDPE measured in tensile tester (Section 3.4.2). Stiff crosslinked leaflet showed a higher mean pressure gradient than uncrosslinked leafletSection 3.4.2. Failure analysis showed that the leaflet region around the commissure post is the most vulnerable to stress and tear, where the leaflet experiences impact stress over the stent. Therefore, the leaflet was simulated by applying crosslinking properties only around the region of the commissure post and stent line area where the leaflet and stent meet.

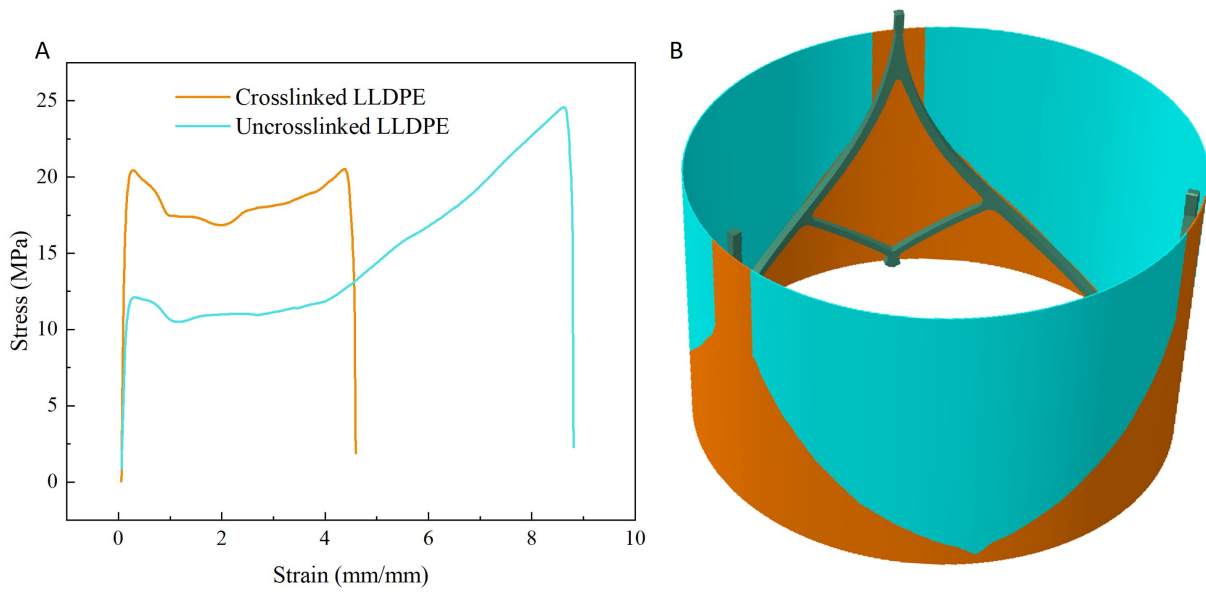


Figure 4.4: (A) Stress-strain graph of virgin 2045 G LLDPE and crosslinked LLDPE (100 kGy doses, 4MeV electron source) (B) Strategically crosslinked LLDPE where orange and blue color denotes crosslinked LLDPE and uncrosslinked LLDPE, respectively

4.3.3 Finite Element Model

The current investigation conducts parametric studies on polymeric transcatheter aortic valve leaflets to improve their durability. This study uses computational modeling to determine the maximum principal stress of leaflets with strategic reinforcement and crosslinking [149]. The model was simulated in the ABAQUS 2021 commercial package. The leaflet was discretized in Hypermesh 2021 using eight-node linear brick elements (C3D8 element type in ABAQUS), and mesh convergence was achieved on the leaflet. The stent was discretized using 4-node linear tetrahedron elements (C3D4 element type in ABAQUS). Cobalt-chromium alloy was modeled using the linear elastic material model: elastic modulus 210 GPa and Poisson's ratio of 0.33. LLDPE leaflet was modeled with 2nd order Ogden hyperelastic material properties (Figure 4.4). Fibers (0.1 mm and 0.2 mm diameter) were discretized using a 6-node linear triangular prism element (C3D6 element type in ABAQUS).

All parts were assembled in SolidWorks. ABAQUS applied a general contact (explicit) between all the parts with a coefficient of friction of 0.48. Fiber surfaces and their projected area on the

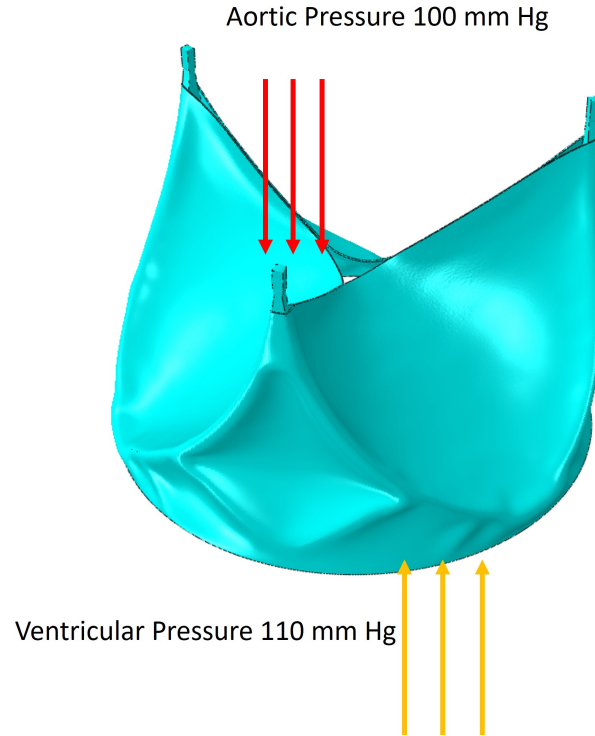


Figure 4.5: Aortic and ventricular pressure applied to the leaflet

leaflet were excluded from the general contact as they were tied. The tie constraint between fiber and leaflet assumed no slippage in the fiber leaflet composite. The leaflet closing and opening were then simulated by conducting two-step dynamic simulation (Explicit modulus in ABAQUS) where geometric nonlinearities (NLgeom=ON in ABAQUS) were activated. In order to reduce the computational time and cost of simulation, a mass scaling of 1×10^{-8} was applied to the whole model at the beginning of both steps. Leaflet closing was simulated in the first step by applying 100 mm Hg pressure for 0.55 part of the normalized cardiac cycles on the aortic side, which was propagated in the second step to maintain the pressure gradient. Leaflet opening from the closed state was simulated by applying 110 mm Hg pressure on the ventricular side for 0.44 part of the normalized cardiac cycle (Figure 4.5). Appropriate boundary conditions were applied to the leaflet bottom edge and the entire stent to restrict their movement. A mesh convergence study was conducted to evaluate the accuracy of the simulation with balanced computational time. This study was performed with four mesh sizes (element sizes 0.3 and 0.2, one element through the

thickness, and element sizes 0.3 and 0.09, two elements through the thickness) for the leaflet. More information on the leaflet element size is provided in Table 4.1.

Table 4.1: Details of leaflet mesh size

	Number of elements (Element size, through-thickness element)
Coarse	77492 (0.3, 1)
Medium	93794 (0.3, 2)
Fine	97589 (0.2, 1)
Finest	414574 (0.09, 2)

4.3.4 Hydrodynamic Testing

Fiber reinforced valves of fiber aligned 0 and 90⁰ were tested for hydrodynamic performance in an *in-vitro* left heart simulator (LHS) under adult physiologic pressure (120/80 mmHg), heart rate (70 bpm), and cardiac output (5 L/min) using a 60/40 water/glycerin mixture to simulate blood properties. Eighty consecutive cardiac cycles of flow waveform data were recorded at a sampling rate of 100 Hz. The fiber-reinforced valves (n=2) were compared to an unreinforced (control) valve. After averaging all cardiac cycles, effective orifice area (EOA) and mean transvalvular pressure gradient (PG) were calculated and compared with a control valve without fiber reinforcement. In this study, particle image velocimetry (PIV) was also performed to measure flow velocity magnitude for 90⁰ fiber reinforced leaflet. Time-resolved recordings were acquired at a temporal resolution of 1000 Hz. Later, the valves 0 and 90⁰ fiber orientation (n=3) were tested for durability in an AWT according to ISO 5840.

4.4 Results

4.4.1 The Unreinforced valve

The mesh convergence study was conducted on the control valve devised from the leaflet and stent without any reinforcement. The leaflet was discretized using decreasing element size. The maximum principal stress value and location were considered for this study. As can be seen in

Figure 4.6, the maximum principal stress increases with smaller element sizes and an increasing number of elements through the thickness. However, the value remained reasonably constant in the finer and the finest group. Element size 0.09 with two elements through the thickness “the finest” was used for further meshing to capture the stress distribution through the thickness of LLDPE within a reasonable amount of time.

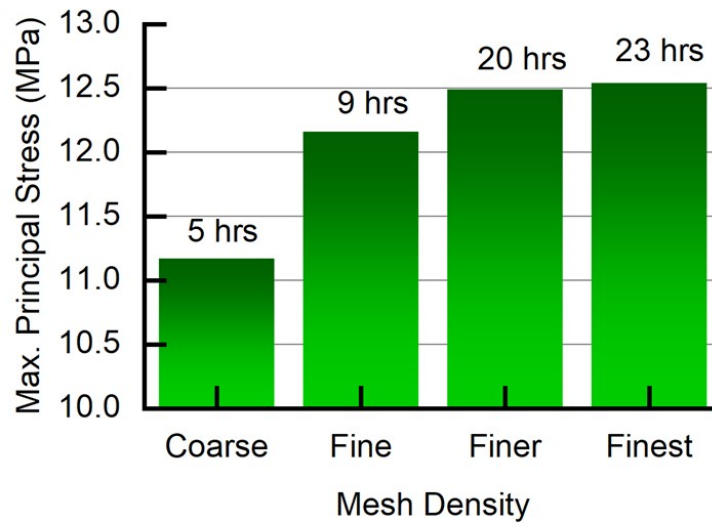


Figure 4.6: Mesh convergence study showing maximum principal stress stabilizes for finer mesh

The research aims to conduct parametric finite element modeling of reinforced polymeric leaflets for improved durability. Data obtained in the previous studies [146, 149, 154] using tissue leaflet on a TAVR indicated that valve design optimization could reduce maximum principal stress or stress distribution on the leaflet. The peak principal stress ranges from 2-3 MPa for the SAPIEN 3 valve depending on the stent design and material model used [149].

The failure analysis conducted in Chapter 2 study found the three commissure post regions of the leaflet are the most vulnerable areas, making them the Region of Interests (ROIs). Figure 4.7 shows the maximum Principal stress and stress vectors at the ROI. The maximum principal stress around this region is 16-18 MPa, and the stress vectors are parallel to the circumference. Stress is the highest at the topmost elements of the ROI, which gradually decreases along the stent line.

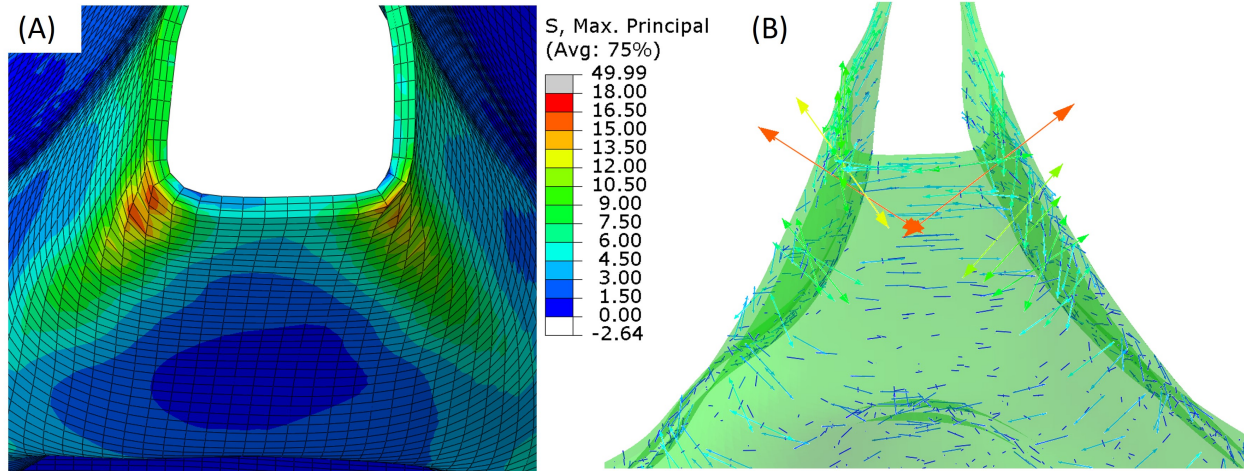


Figure 4.7: Max. Principal stress (A) and stress vector (B) of the control valve without reinforcement showing the Region of Interest leaflet region around the commissure post

The stress profile (magnitude and direction) aligns with previous studies on the prosthetic valve and native aortic valve [7, 155]. Native aortic valve commissure posts are elite architecture of multiple layers that can easily slide past one another. The layers house collagen fiber in a complex orientation and crossing arrangement, branching fiber bundles, and sub-fiber where fiber bundle diameter ranges from 0.5-3 mm [7].

4.4.2 Orientation Optimization

Figure 4.8 shows the maximum principal stress reduced from an average of 18 MPa to 15 MPa ($n=3$) for fiber parallel to valve height (90° with valve circumference). In addition to reducing the maximum principal stresses, the fiber-reinforced groups showed compression on the inner surfaces of the highest bend region compared to tension in the control group. The stress distribution pattern is consistent with the previous studies [146] for bioprosthetic tissue leaflets. Preliminary studies discussed in Section 4.2.1 found strong weld protects ROI. Perpendicularly fiber-reinforced valves showed drastic improvements in durability as the highly stressed commissure posts were strengthened and protected. However, FEA simulation results did not show significant peak stress reduction. This indicates that the strong composite at the post was more of a surface protection from abrasion between the leaflet and stent than stress reduction in the leaflet.

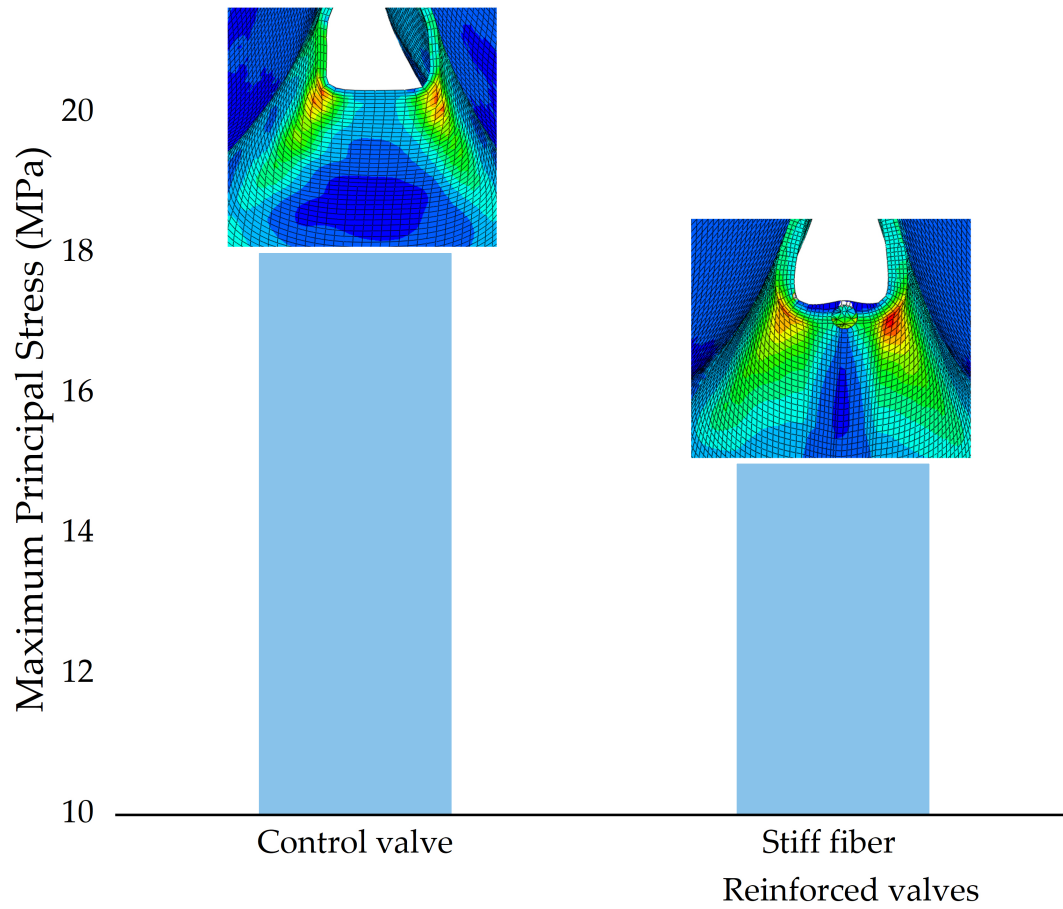


Figure 4.8: Max. Principal stress and ROI of control valve and fiber placed at 90^0

Tensors of the deformed valve show high stresses are parallel to the circumference, not to valve height (Figure 4.7). Therefore, three fibers were placed parallel to the valve circumference (0^0) to transfer the maximum load from the matrix to the fiber. Fiber reinforcement immediately reduces leaflet peak stress at the ROI (Figure 4.9). Three fibers embedded on the leaflet at the ROI at zero-degree alignment decreased the peak stress from 18 MPa to 5 MPa, a solid 72% stress reduction of leaflet stress. The principal stress of embedded fiber was 175 MPa. On the other hand, native leaflet like fiber orientations increase stress on leaflets but lower stress on the fiber. These findings are in alignment with previous studies [155]. Coulter et al., designed valves with simple horizontal fiber and bio-inspired design comprising fiber at 30^0 angles that run the entire cusp. Fibers were 3D printed on the whole cusps, which could increase cusp surface roughness and activate platelets easily *in vivo* environments. Horizontal and bio-inspired fibers reduced maximum principal stress by 26% and 21%, respectively. Since they housed fibers along the entire cusp, stiff leaflet opening dynamics were slightly compromised. So, research conducted in this chapter holds advantages as fibers are placed only in the ROIs instead of the entire cusp and can reduce 72 % stress. However, the biggest concern is that the fiber aligned parallel to the valve circumference sheds the carried stress at the tip of the fiber on the leaflet as there is a vast geometry and property mismatch. The leaflets of aligned fibers have higher stress concentrations on the leaflet along the fiber and at the tip of the fiber. The stress concentration was above 34 and 26 MPa for 15^0 and 30^0 aligned fibers, respectively.

Figure 4.10 shows closed-up ROI sides and front views to emphasize the principal stress reduction. Leaflets and fibers both undergo maximum strain in this region. Therefore, unreinforced leaflets show maximum stress in this region. Similarly, embedded fibers experience maximum stress and strain in this region as they carry away the stress of the leaflets, but the leaflet regions stay relaxed. The close-up of the ROIs reveals some compressive stress imparted from the fiber on the soft plastic as it strains. Notably, fibers need to be embedded at the topmost of the commissure post region of the leaflet to achieve maximum reinforcement effect, as stress is the highest on the topmost part (aortic side) of the leaflet. Again, the concern about embedding 6 mm fibers at the ROIs is the stress

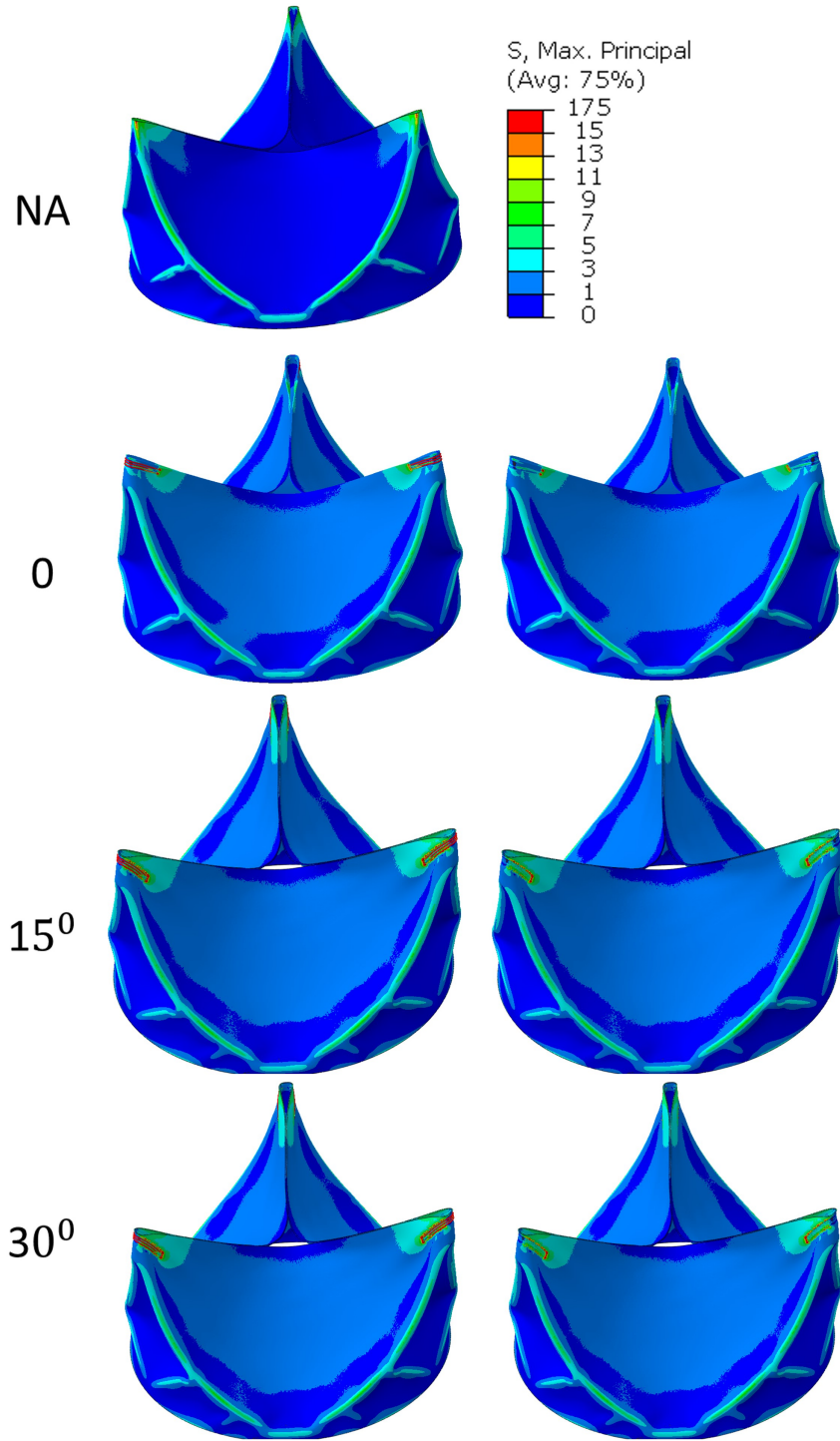


Figure 4.9: Effect of fiber orientation on leaflet maximum principal stress during peak diastolic closing. The left column simulations show stress on fiber, and the right column shows stress on ROI beneath the fibers. Stress near the commissure post region of the leaflet is the lowest for fiber running at 0° angle

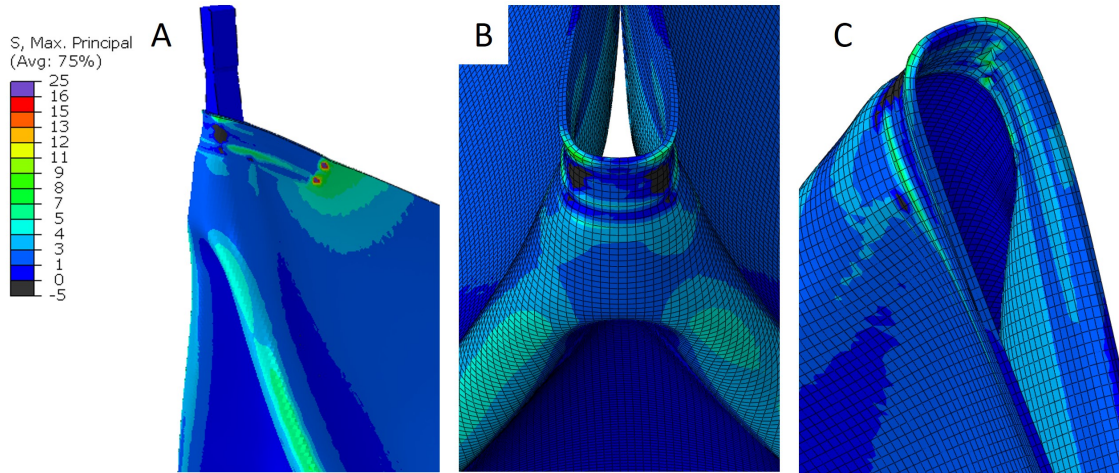


Figure 4.10: Closed-up views of leaflet commissure post region sides and front view of parallel fibers

concentration at the fiber tip on the leaflet, which could cause a new failure location. Because the stress concentration is higher than the maximum principal stress of the control valve the length optimization studies shall address this issue.

4.4.3 Length Optimization

Fiber length plays a critical role in managing stress concentration at the end of the fiber tip on the leaflet. Increasing the fiber length was found to reduce stress shedding on the leaflet. Fibers embedded on the topmost of the commissure post region of the leaflet relaxed it by carrying away principal stress. Figure 4.12 shows the principal stress on the leaflet and the fiber. While the 12 mm long fiber stress concentration magnitude and location are lower than short fibers (6 and 3 mm), it was still higher than the unreinforced leaflet. The physically manufactured leaflet may not tear along the ROI, but a new tear region can be generated at this stress concentration. Therefore, fiber length was increased to wrap the leaflet 360° at the topmost region. Finally, fibers carried away all the stress from the leaflet ROI without having any stress concentration. Similar to 6 mm long fibers, the principal stress was 5-6 MPa around the commissure post region of the leaflet. Eight more fibers at equal spacing were embedded on the leaflet through the valve height to strengthen the leaflet along the stent line. Unlike the former iteration, the leaflet got more stressed on the cusp areas. All the simulations mentioned here maintained a constant fiber stress of 175-180 MPa as a

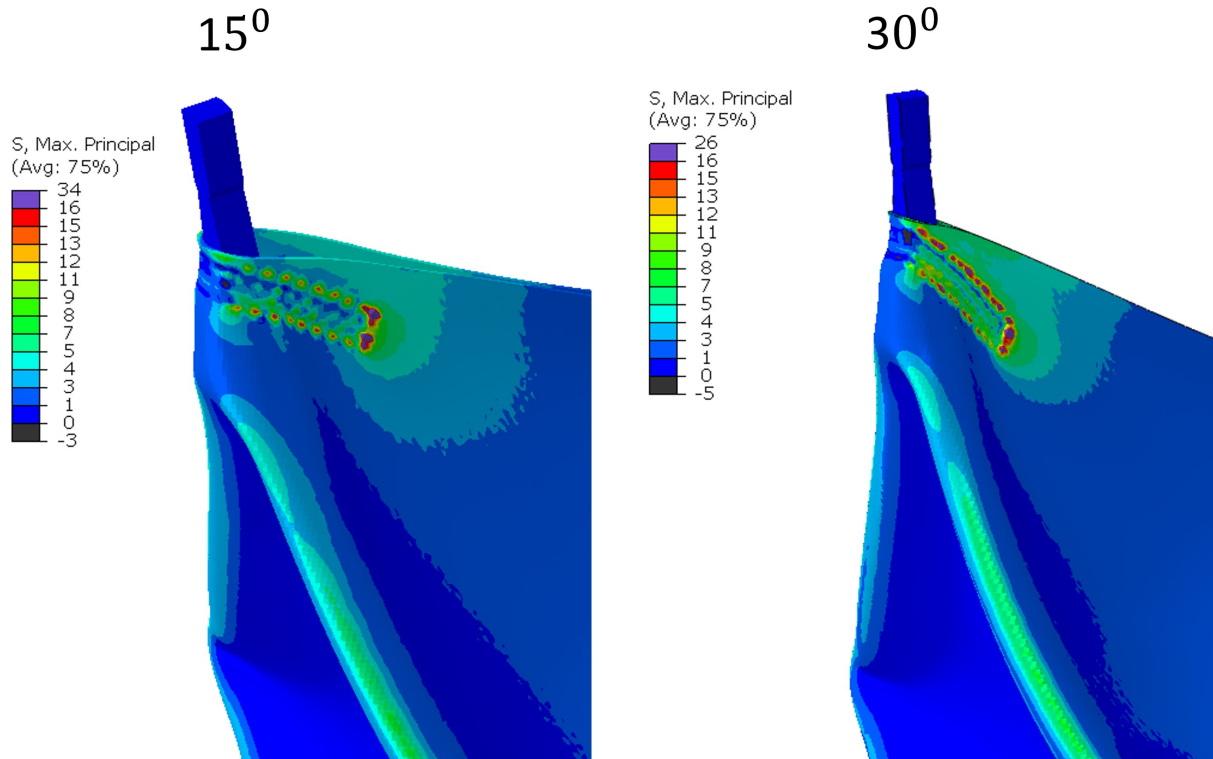


Figure 4.11: Closed up view of leaflet commissure post regions of aligned fibers

constant (1GPa) fiber modulus was maintained.

The unreinforced LLDPE thin film leaflets showed excellent performance *in vitro* flow loops in previously published data due to their unique flexural modulus or bending stiffness. Any alteration of stiffness of the cusps can result in poor hemodynamic performance. Therefore, leaflet opening was also simulated in this study. Figure 4.13 shows GOA over the normalized cardiac cycle. Leaflets reinforced with 12 mm long fiber and unreinforced leaflets followed the same opening dynamics. The leaflet reinforced with 86 mm long fiber that covers the topmost of the cusp shows delayed opening. Embedding an additional eight fibers might help the valve open more during early systolic. Both of these valves had higher GOA for the early systolic state. However, a limitation of the study is noteworthy. The simulation ignored suture points, which attach leaflets to the stent at the commissure posts to ensure secure attachment between them. This secure attachment prevents leaflets from completely opening, unlike the peak systolic shown in the simulation. Rather, the commissure post region of the leaflet remains bent during the entire cycle (Figure 4.13 inset).

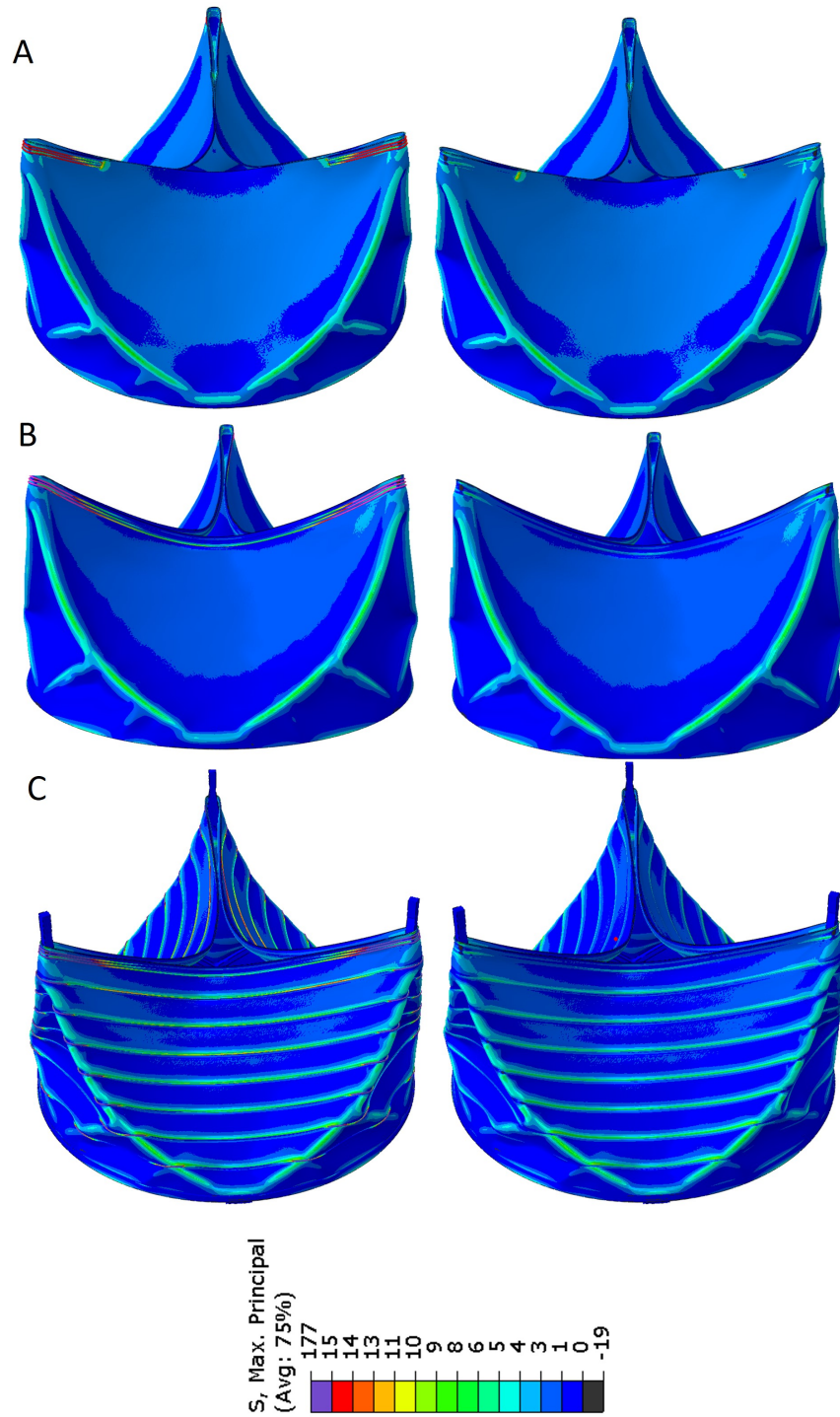


Figure 4.12: The principal stress profile of horizontal fiber-reinforced valves, the left column showing the fibers and their stress, and the right column hides the fibers to expose the relaxed commissure post region of the leaflet. A) fiber length 12 mm, B) fiber length 86 mm, C) 8 fibers through the height with the length of 86 mm

Therefore, a displacement/rotation boundary condition was applied over the 3.5 mm^2 area of the ROI during leaflet opening so that the ROIs stay bent. Figure 4.14 shows changes in leaflet opening dynamics from a simulated closed diastolic phase, which agrees with previous simulations of linear elastic leaflet [155]. However, previous studies on *in vitro* flow loops showed leaflet cusps are not completely open during peak systolic because the leaflet is assembled inside the stent, which prevents unrestricted opening. In contrast, in this simulation, the leaflet is assembled over the stent [8]. Furthermore, the solver of ABAQUS did not apply computational fluid dynamics principles. Therefore, the opening dynamics may behave differently than *in vitro* model.

In this simulation, leaflet GOA gradually increases to reach the peak systolic state and immediately drops, indicating the late systolic state, as seen in Figure 4.15 . Un-reinforced leaflet coaptation gap is 4.9 mm^2 during peak diastolic because cylindrical cusps do not have any 3D curvature to assist coaptation. Furthermore, the cusps are cylindrical and not optimized for shape; there was no extra material at the peak of the coaptation area to eliminate the gap. The area of this gap increases to 18.6 mm^2 with increasing fiber length from 12 to 86 mm. The value increased slightly when eight more fibers were added through the valve height. The stiffening of the cusp required more energy to close the valve completely, which can increase the mean pressure gradient. Since the fiber-reinforced leaflets do not completely coapt during peak diastolic, the GOA is slightly higher than the unreinforced leaflet during early systolic. At the peak systolic, the GOA magnitude rank is unreinforced, reinforced through the valve height, and reinforced at the topmost of the valve height. Opening dynamics of the leaflet reinforced with an additional eight fibers through the height catch up the GOA of unreinforced leaflets at mid-systolic state but have lower GOA during the peak systolic state. After peak systolic, all the leaflet GOA drops, indicating a late systolic phase. However, the fiber-reinforced at the topmost of the valve shows delayed opening and does not completely open during peak systolic. The leaflet reinforced with 12 mm fiber opened immediately after the ventricular pressure was applied. The fiber reinforcement acts similar to spring which facilitates leaflet opening and can significantly improve valve hemodynamics. The stress shedding at the end of the fiber can be further optimized using less stiff fibers.

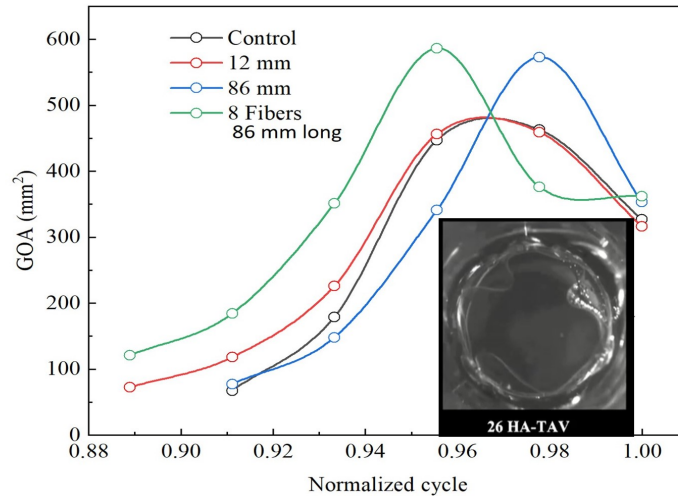


Figure 4.13: Measured Geometric Orifice Area of the unreinforced leaflet (control), 12 mm long fibers, 86 mm long fibers, and 86 mm long eight fibers over the normalized cardiac cycle, inset is leaflet peak systolic opening dynamics tested *in vitro* flow loop, Reprinted from Publication [8] Copyright (2019), with permission from Elsevier

Fiber reinforcement technique effectively reduces leaflet principal stress and should significantly upgrade valve durability. However, it also increases leaflet stiffness, increasing the mean pressure gradient to 44.7 mm Hg compared to 9 mm Hg of unreinforced leaflet. Stiffening the leaflet near the commissure post with strong welds improved durability without affecting hemodynamics. One way to achieve stronger ROIs is to embed strong fibers on the commissure post at 90° so that fiber ends can not dump high stress on the soft plastic polymer. This technique does not relax the leaflet but improves durability (mentioned below). Another way to improve durability is to stiffen the leaflet on the ROIs and stent line region but leave the three cusps untouched. One way to physically achieve this is to crosslink the LLDPE using e-beam irradiation selectively. E beam irradiation crosslinking method was extensively studied in Chapter 3. Crosslinking improved surface hardness and wear resistance determined by nanoindentation and could potentially prevent cyclic fatigue-initiated crazing. However, the method stiffened the leaflet, increasing the mean pressure gradient.

In this FEA study, a crosslinked leaflet was simulated to estimate principal stress using the uniaxial tensile test property (measured physically in a universal tensile tester) of crosslinked LLDPE. The principal stress was found to be 2 MPa higher than that of uncrosslinked LLDPE during peak

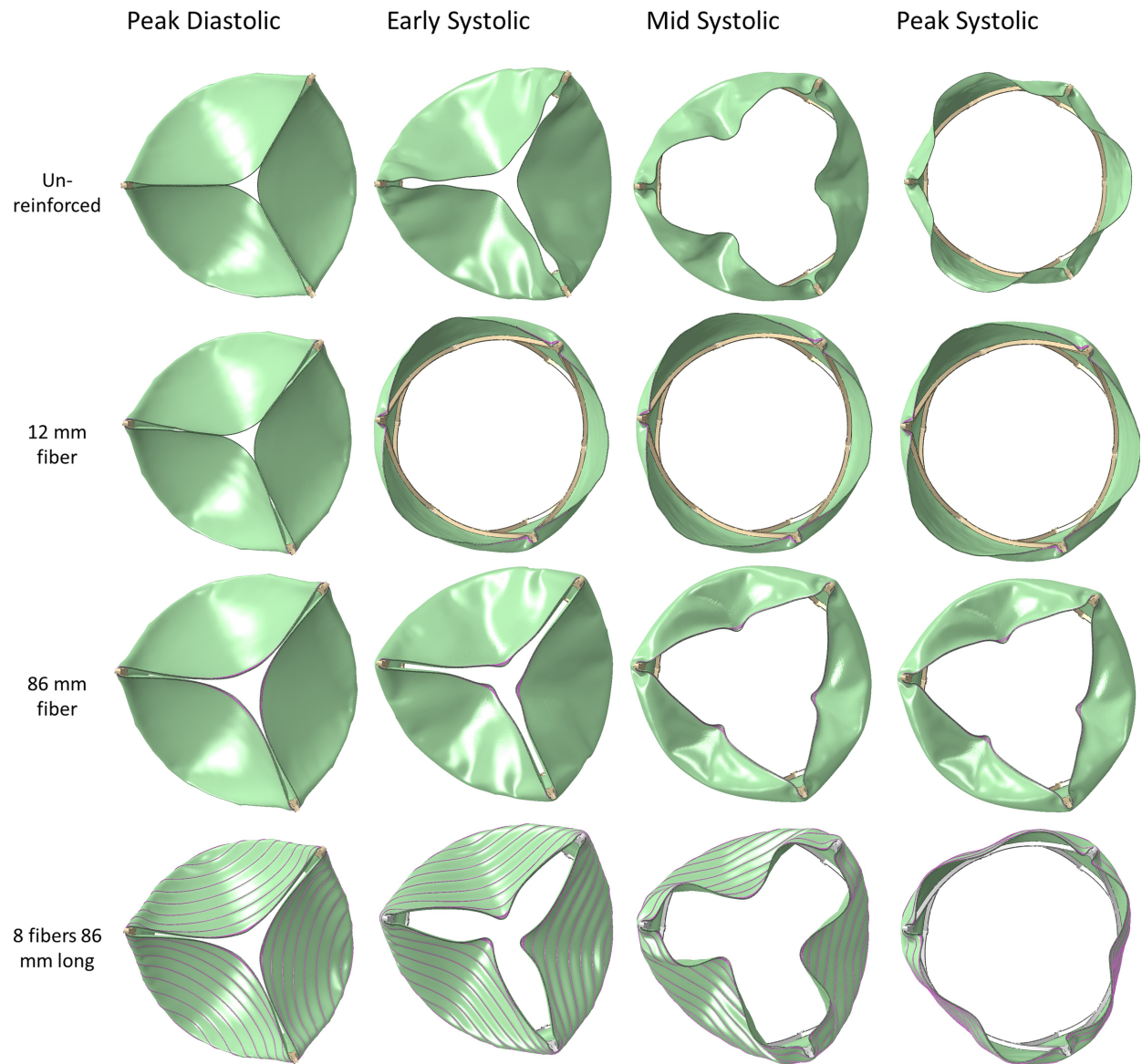


Figure 4.14: Leaflet systolic and diastolic dynamics change with fiber length and number increment after applying translation restricting the leaflet on the commissure post region

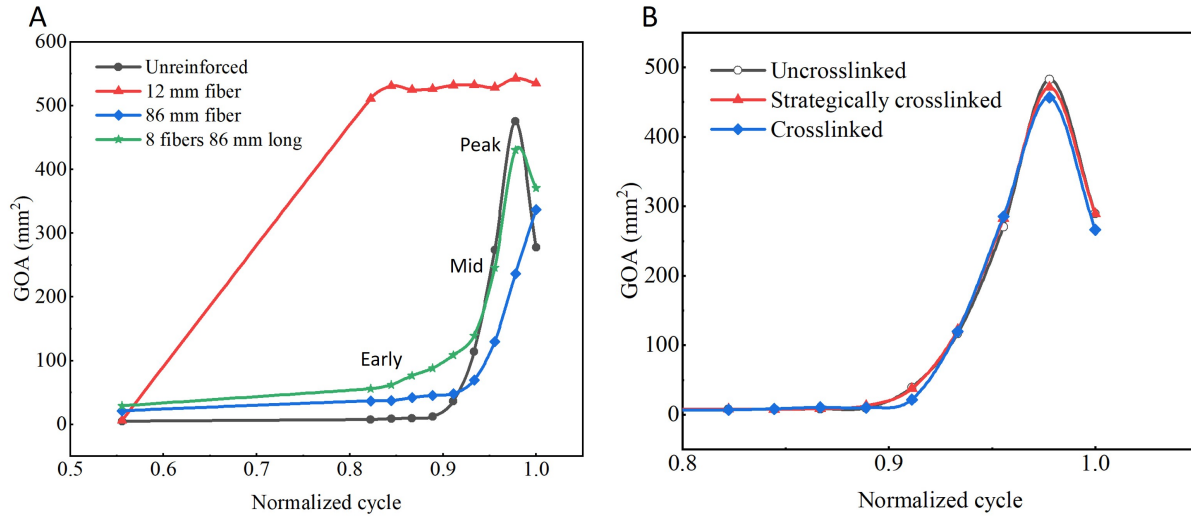


Figure 4.15: Measured Geometric Orifice Area of (A) unreinforced leaflet (control), 12 mm long fibers, 86 mm long fibers, and 86 mm long eight fibers (B) uncrosslinked, strategically crosslinked, and crosslinked leaflet over the normalized cardiac cycle

diastolic closing. A strategically crosslinked leaflet was further simulated where only the elements of ROIs and the leaflet region of the stent line were assigned with crosslinked LLDPE properties. Elements of the rest of the leaflet (three cusps) were assigned with uncrosslinked LLDPE (uniaxial tensile testing properties). The principal stress of partially and fully crosslinked leaflets was equivalent. Because leaflets experience maximum principal stress at the ROI, these elements were assigned with stiff crosslinked LLDPE properties. Selective crosslinking can be achieved experimentally by masking the cusp with electron-shielding thick material so that the cusp is never irradiated with high energy electrons and remains pristine like uncrosslinked LLDPE. Crosslinked leaflet opening dynamics were simulated to compare with uncrosslinked leaflets. Figure 4.18B shows that the GOA of all three leaflets is similar during the entire cycle except during the early and peak systolic state. During peak systolic state, the completely crosslinked leaflet GOA (456 mm^2) was less than the other two partially and uncrosslinked leaflets (482 mm^2). Figure 4.15 B further shows that crosslinked leaflets GOA is the least between three of them during early systolic. The difference in GOA during peak systolic should explain the moderately high mean pressure gradient found *in vitro* model (mentioned below). However, it is noteworthy to mention that the simulation

was run for one time. Therefore, at least two more iterations may find statistically confident GOA and comparison.

4.4.4 Hemodynamics and Durability

The perpendicular fiber-reinforced valves were tested in LHS and compared with the unreinforced valve. The fiber-reinforced valves had a pressure gradient of 13.2 ± 2 mmHg and EOA of 1.5 ± 0.5 cm². Reinforced valve peak velocity was 2.2 m/s (Figure 4.16) compared to 2.2 m/s for the control group [8]. The fiber-reinforced valve hydrodynamics were comparable to the previously published control group (pressure gradient 9.5 mm Hg and EOA 1.91 cm²) because the strong reinforcement was tucked behind the stent. The results showed a slightly higher standard deviation, indicating inconsistent valve assembly during testing. Interestingly, the fluid velocity did not change significantly, indicating the overall performance comparable to unreinforced leaflet [8]. This perpendicular fiber-reinforced valve showed drastic improvements in durability as the highly stressed commissure posts were strengthened and protected. However, FEA simulation results did not show significant peak stress reduction due to the parallel fiber reinforcement, even with the thickened commissure region. The horizontal fiber reinforced valve had a mean pressure gradient of 44.7 mm Hg and EOA of 0.484 cm². The valve is severely stenotic as it requires more pressure to open. In AWT, the perpendicularly reinforced valve survived 73 million cycles compared to 4 million cycles for the control group. On the other hand, the 86 mm horizontal fiber reinforced valve survived only 9.5 million cycles in the AWT. The leaflet tore at the cusps near the coaptation region where LLDPE meets fiber.

4.5 Discussion

Over a decade of polymeric TAVR studies with numerous techniques is yet to develop a hemocompatible valve that can survive over 20 years *in vivo*. Polymer and leaflet material includes Poly (styrene-b-isobutylene-b-styrene) (SIBS), crosslinked SIBS, Dacron, PEGDA coated textiles, silicone, expanded Polytetrafluoroethylene (ePTFE), fiber reinforced xSIBS [1]. Despite

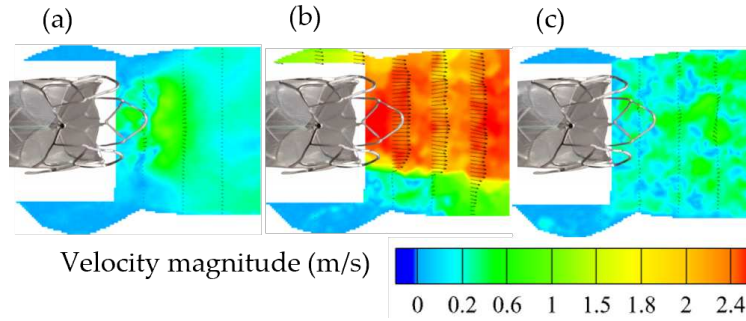


Figure 4.16: Velocity magnitude of fiber (perpendicular) reinforced valve: mid to late acceleration (a), peak (b), and mid to late deceleration (c)

in vivo performances, these valves fail to meet full FDA durability regulations due to thrombosis or oxidation. Still now, fiber reinforcement remains one of the most promising techniques for improving thin polymer durability. Coulter et al. [155], simulated and 3D printed fiber reinforced valve with excellent hemodynamics. Fibers were 3D printed on the cusp, which might increase cusp surface roughness. Increased surface roughness has a high probability of activating platelets (Chapter 5), resulting in thrombosis. Furthermore, their bioinspired fiber-reinforced leaflet survived for 40 million cycles in AWT [155].

This study strategically designed a valve to improve durability without altering hemodynamics or surface roughness. A computational model was established by assembling a simple cylindrical leaflet over a cobalt chromium stent design. The failure analysis study revealed a strong weld that connects two ends of the flat plastic sheet used to make a cylindrical leaflet protects adjacent areas of the leaflet. Therefore, three strong weld-like fiber reinforcements were made around the commissure post of the leaflet by placing fiber perpendicular to the valve circumference. The reinforcement drastically improved durability without altering hemodynamics but did not reduce maximum principal stress in simulations. *In vitro* flow loop hemodynamics did not alter because stiff fibers are tucked behind the stent. Stiff fibers act like protective clothing for the leaflets to prevent the leaflet from rubbing the stent on the commissure post region and protecting it from premature failure.

Fiber orientation was then studied to reduce the maximum principal stress. Simulations calculated

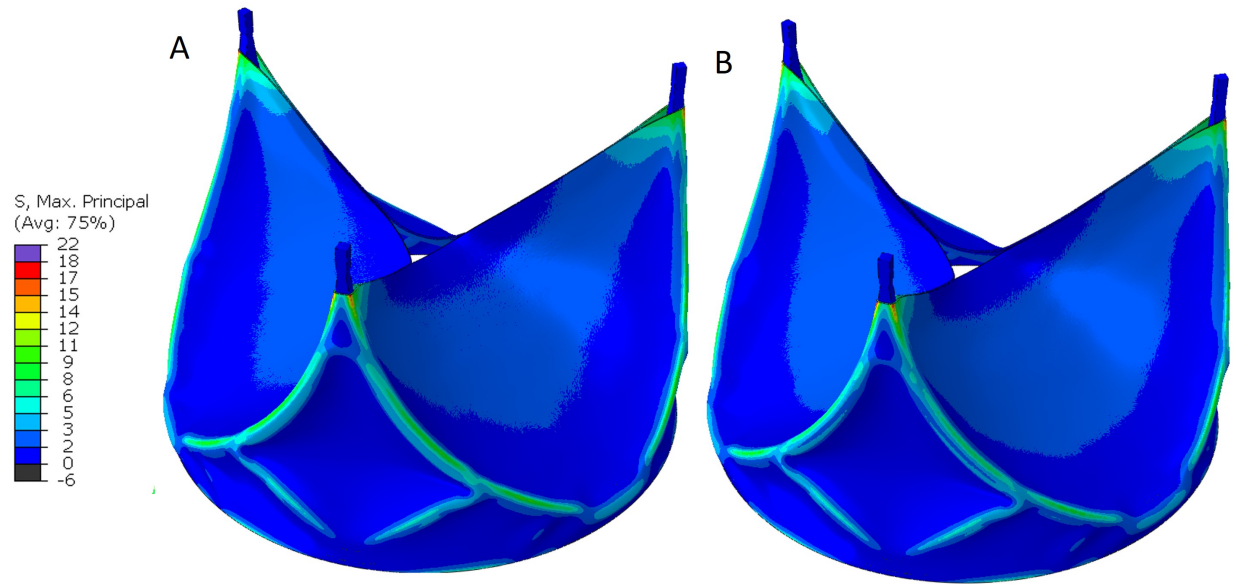


Figure 4.17: Principal stress profile of (A) crosslinked leaflet (B) strategically crosslinked leaflet

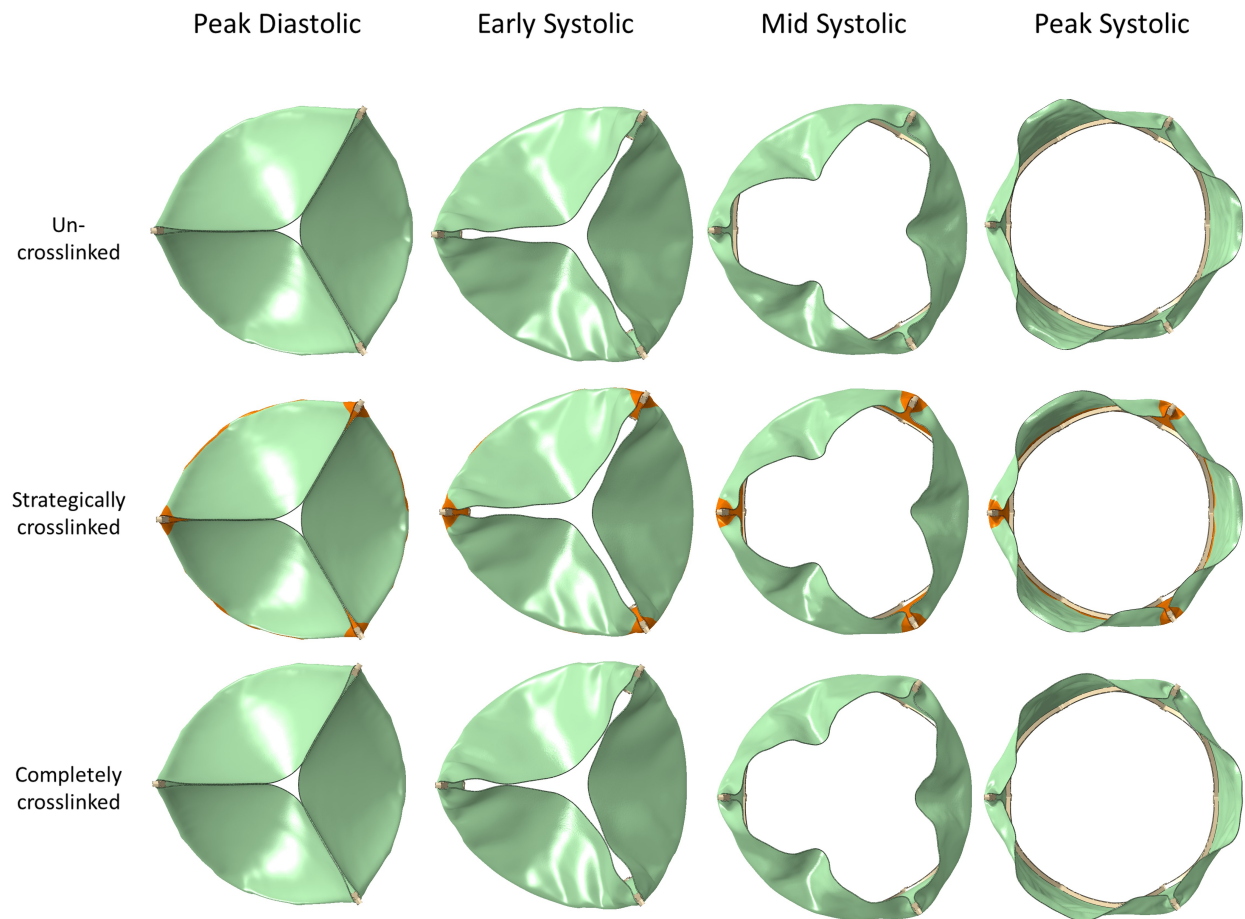


Figure 4.18: Leaflet systolic and diastolic dynamics change of uncrosslinked leaflet, strategically crosslinked leaflet, and completely crosslinked leaflet

stress vectors are parallel to valve circumference, as seen in the native heart valves (Figure 4.1A). Native heart valve has bundles of horizontal/parallel to valve circumference collagen fiber near the commissure post, which later branches out to a smaller diameter and orients at 15-30° angle relative to the circumference. The commissure post of the native valve is specially designed to facilitate flexible systolic and diastolic dynamics and survive one's lifetime. Inspired by this natural valve, a bundle of three fibers comprising three polyester fibers, each 0.1 mm diameter, was placed on the commissure post parallel to the valve circumference. Fiber length was kept constant at 6 mm to avoid leaflet cusp stiffening. In another two sets of simulations, fibers were placed at 15 and 30° angles and compared with parallel fibers. Maximum principal stress was reduced by 72% for horizontal fiber around the commissure post region of the leaflet compared to the control leaflet without any reinforcement during peak diastolic closing. The 6 mm long fiber bundle is the simplest solution to reduce valve principal stress. However, there is a huge stress concentration on the leaflets at the fiber tip on the leaflet. One can argue that the stress concentration can be ignored as it is away from ROI using Saint Venant's principle. The geometric and property mismatch between leaflet and fiber can also cause stress singularity and thus be ignored. Attempts were made to reduce this stress singularity by adding fillets between fiber and leaflet, wrapping the fiber with LLDPE material, and making the fiber tip hemispherical [156] in modeling. However, none of the attempts were successful in reducing the stress concentration. Therefore, fiber lengths were doubled and found to improve the stress concentration but not completely eradicated. In later simulations, fiber length was increased to wrap the entire leaflet covering the commissure post; only this iteration completely relaxed the leaflet. Later, eight more fibers were placed at equal spacing to address the stress along the stent line through the height.

Native heart valve architecture comprises thick collagen fiber bundles near the commissure post region. The thick bundle diameter reduces into thin fibrils and feathers away as they move deep into the leaflet (Figure 4.1 black lines denote thick bundle, and white lines denote feathered fibrils). So, the fibers closer to the commissure post are thick, and fibers closer to the center of the cusp, which undergoes coaptation, are thin. This architecture was traced by Doebling et

al., [7] from five overlapped cusps. This elite architecture of fibers must not be a coincidence but intentionally designed to minimize the geometric mismatch between matrix and collagen to avoid stress concentration without increasing leaflet stiffness. The fiber-reinforced leaflet architecture studied in this research roughly matches this native leaflet elite architecture in terms of housing fibers. Still, native leaflets consist of much more complicated architecture where fibers are embedded between layers that can slide past each other.

Long fibers that run 360° around the leaflet are found to be the most optimal in terms of stress reduction. However, an 18 mm² coaptation gap area was calculated during leaflet peak diastolic closing. *In vitro* flow loop found a central leak or back regurgitation during peak diastolic closing. Leaflets with an additional eight fibers through the valve height, which has an even higher coaptation gap, can be estimated to have a large central backflow in the flow loop. Interestingly, the RejuvenateTM valve has suppressed this issue by optimizing the leaflet shape into 3D curvature and housing additional material in the leaflet to prevent any coaptation gap. The major limitation of the simulation is that it fails to predict extreme high-pressure gradients and low EOAs found in the flow loop. The simulation predicts that long fibers will help open dynamics. Meanwhile, in the flow loop, the fibers stiffened and increased the mean pressure gradient. A more accurate representation of a real scenario would calculate low GOA during peak systolic and much-delayed opening.

The simulation makes two assumptions: 1. fiber is a linear elastic material, and 2. there are no secure attachment points between the stent and the leaflet. The following section discusses the accuracy of these two assumptions

LLDPE is a soft plastic with an average modulus of 250 MPa, whereas polyester braided fibers are stiff with a 4 GPa modulus, around eight times stiffer than LLDPE. Monofilaments of polyester are twisted to manufacture braided polyester thread that provides the structural stability and bending stiffness required for suturing. In contrast, the FEA simulation conducted here assumed the fibers to be a linear elastic material. The transverse properties of fiber were ignored, which can alter the leaflet opening dynamics. During diastolic closing, they experience maximum stress. Fiber tends

to return to its original shape as a linear elastic material in a zero-stress condition. Modeling linear elastic spring-like material showed that untwisted monofilament fiber can help leaflets to open effortlessly from zero-stress conditions. Furthermore, the simulation completely ignores a layer of LLDPE that covers fiber during physical composite laminate manufacturing. The necessary extra layer of LLDPE can also be responsible for the high mean pressure gradient measured *in vitro* model.

The leaflet is attached securely to the stent using three non-load-bearing stitches to avoid leaflet detachment from the stent. The cylindrical leaflet is assembled over the stent; the cusps are brought together to coapt. The stitches and the initial bending of the leaflet around the commissure post guide the leaflet to coapt. Unlike the simulation result, this initial bend of the leaflet region around the commissure post is maintained throughout the cycle and never fully straightens up. Leaflet bending around the commissure post prevents EOA from reaching the total cross-sectional area of the 23 mm diameter cylinder, which is 4.1 cm^2 . Instead, the EOA was found to be 2 cm^2 , measured in the flow loop. Furthermore, the leaflet is simulated in a 26 mm stent and experimentally tested in a 23 mm stent, automatically reducing EOA.

One of the limitations mentioned here was overcome in FEA by applying displacement/rotation boundary conditions on the 3.5 mm^2 area of ROI during leaflet opening. The leaflet reinforced with 12 mm long fibers opens immediately after ventricular pressure crosses aortic pressure and remains open for the rest of the cycle. Therefore, this leaflet could improve valve hemodynamics. However, stress concentration on the leaflet at the end of fiber on the leaflet could create a new failure region. Moreover, the melt press technique used to manufacture physically can leave stress concentration at the junction of leaflet and fiber, which could cause failure. The leaflet, reinforced with eight fibers 86 mm in length, has comparable GOA to the unreinforced valve. Probably because fibers are placed homogeneously through the height compared to placing fibers only in the topmost part of the leaflet. However, the stiff fibers can make the leaflet severely stenotic, as only a bundle of fibers at the topmost part of the ROI (86 mm fiber) made them stenotic. One of the fundamental differences between FEA and *in vitro* models is the absence of fluid dynamics

principles in the former. The leaflet nonlinear opening dynamics could probably be better estimated in ANSYS, which can use fundamental fluid dynamics principles. Clearly, FEA simulation could predict trends in results to compare with the *in vitro* model.

Strategically Crosslinked Leaflet

LLDPE-based leaflets experience premature failure due to fatigue-initiated crazing and surface wearing from cyclic impact loading. Their yield strength is well below the cyclic impact stress they experience during durability testing. The leaflet has all the reasons for failing prematurely in the AWT unless properties are changed. Perpendicular fiber reinforcement tucked in the back of the stent has upgraded leaflet durability without altering hemodynamics as measured in PIV and flow loop. Horizontal fiber has slightly improved durability, but some significant alterations of hemodynamics were imminent. In this context, electron beam irradiated strategically crosslinked LLDPE holds the promise to upgrade durability by (1) reducing polymer chain disentanglement and increasing yield strength to prevent fatigue-initiated crazing, (2) improving the surface coefficient of friction to prevent surface impact wearing. The pressure gradient measured *in vitro* hemodynamics model was slightly higher for crosslinked leaflets than uncrosslinked LLDPE because 1. crosslinked LLDPE is stiff, 2. 23 mm stent was used, which reduced EOA, and 3. According to (3.1), stiffness is directly proportional to bending stiffness, and bending stiffness is positively related to the mean pressure gradient. *In vitro*, a flat, thin, crosslinked sheet was assembled into a leaflet, resulting in at least a 5 mm Hg mean pressure gradient compared to the uncrosslinked leaflet. This dissertation has identified the leaflet commissure post region as the most vulnerable region during peak diastolic that needs property upgrades. The rest of the cusp is generally relaxed under diastolic closing and systolic opening. The unique flexibility of the cups should be left untouched by only crosslinking the ROIs and leaflet region of the stent line that experience surface wearing and crazing. Simulation results of strategically crosslinked leaflets showed no stress concentration at the junction of crosslinked and uncrosslinked regions, which was found in horizontal fiber reinforcement due to their geometric and mechanical mismatch. The opening dynamics of the systolic phase predict that selectively crosslinked leaflets may have

comparable hemodynamics to the uncrosslinked leaflets because they have GOA similar to that of uncrosslinked leaflets during early and peak systolic. Like perpendicular fiber, the strategically crosslinked leaflet stiff LLDPE regions are tucked behind the stent, so the GOA is unaffected from strategic crosslinking.

4.6 Conclusion

This study conducted parametric computational FEA modeling on fiber reinforced and electron beam irradiation crosslinked leaflets to estimate maximum principal stress during a complete cardiac cycle. The study further compares how easily and how much they open during systolic opening and compare with measured EOA and pressure gradient of *in vitro* models. This stress based analysis finds that horizontally embedded fibers at the leaflet near the commissure post region can significantly reduce maximum principal stress, which could improve leaflet durability. However, stress concentration on the leaflet at the fiber tip might create a new failure zone. Therefore, fiber length was increased to wrap the leaflet 360°, and eight more additional fibers were added throughout the valve height. The simulation predicts a low stress concentration where fiber wraps the leaflet 360° during peak diastolic closing. However, it predicts significantly low GOA. Experimentally, fiber running at the topmost of the commissure post region of the leaflet was manufactured by embedding polyester braided fibers to test in an *in vitro* flow loop. As predicted by FEA, the EOA was significantly low, which increased the mean pressure gradient, making the valve moderately stenotic. Generally, a plastic sheet is assembled into a cylindrical form by welding two ends. The welded areas never fail in long-term testing. The weld at the commissure post was mechanically stronger than the rest of the leaflet, which protected this region. Braided fibers were embedded in the leaflet regions of the commissure post perpendicular to the valve circumference, mimicking the weld but at a much higher strength. Leaflet durability dramatically increased from a few million to 73 million cycles with minimal to no change in mean pressure gradient or EOA. The perpendicular leaflet was further simulated in FEA but found no effect on maximum principal stress during diastolic closing. Finally, this study uses the mechanical properties of

electron beam crosslinked LLDPE leaflet to predict its effect on the cardiac cycle, which is stiffer than uncrosslinked LLDPE but has a higher friction coefficient to upgrade durability. The FEA estimates a slight reduction in GOA for crosslinked compared to uncrosslinked. The trend agrees with *in vitro* model hemodynamics. The simulation further estimates a selectively crosslinked leaflet might have hemodynamics of uncrosslinked LLDPE.

One limitation of the study is the solver ABAQUS is less accurate in predicting fluid dynamics that take place in the heart chambers. Future studies, therefore, should simulate leaflet openings using software packages like ANSYS. The selectively crosslinked LLDPE and perpendicular fiber reinforcements are the most promising for drastically improving durability. Therefore, combining these two techniques might be explored to get their compound effect.

Chapter 5

Hyaluronic Acid Enhancement of Heart Valve Leaflet and Characterizations

5.1 Summary

This chapter discusses the development of three-dimensional (3D) shaped LLDPE leaflet bio enhancement manufacturing process. Leaflets were 3D shaped in a vacuum thermoformer followed by HA enhancement. Whole blood clotting resistance, platelet adhesion, activation, and cytotoxicity studies determined that $10^{-4} \mu\text{mol}/\text{mm}^2$ HA surface density was required to achieve the best blood compatibility. Generally, water contact angle, toluidine blue O (TBO) elution assays, and ATR-FTIR were used to determine overall HA presence on the leaflet. This study reports TBO staining and elution as the most effective and accurate measurement tool for determining HA population density. Fiber-reinforced LLDPE, and crosslinked LLDPE were HA-treated, and TBO staining predicted adequate and consistent HA surface density for blood compatibility.

5.2 Introduction

Chapter 2, Chapter 3, and Chapter 4 investigated failure mechanics and provided two methods to improve leaflet durability. This chapter focuses on HA-treated bio enhancement of crosslinked leaflets, fiber reinforced leaflets, and 3D shaped leaflets. The study investigates the minimum amount of HA surface density required to achieve biocompatibility and proposes the most accurate and reliable method to characterize HA treated bio-enhanced LLDPE. Previous studies on improving TAVR hemodynamics found that optimizing curvature-shaped leaflets significantly improved hemodynamics than cylindrical leaflets [145, 157, 158]. The Rejuvenate™ valve is optimized for 3D curvature to achieve a mean pressure gradient of 5 mm Hg. Challenges remain in shaping the TAVR leaflet, which has a wall thickness in the micron range but other dimensions

in the mm range. Also, cross-linking proposed in the Chapter 3 might significantly alter the polymer chain swelling, which could affect the HA treatment process. Critical questions remain on understanding the effect of shaping on the bio-enhancing process and finding the appropriate stage, i.e., bio-enhancing before or after shaping. One can argue that the bio-enhancing process's chemical and thermal input (discussed in the methods section) may erase the shape if shaped before treatment. On the other hand, thermomechanical input from the shaping process may burn and degrade the HA molecules if shaped after the treatment.

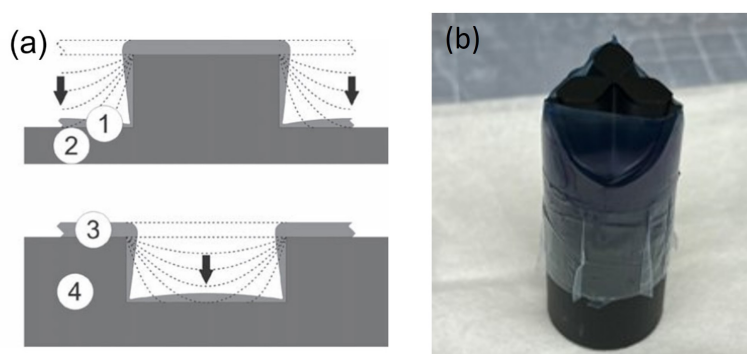


Figure 5.1: (a) Schematic of vacuum forming process using a positive mold (top) and negative mold (bottom) (b) HA treated leaflet for closed state showing leaflet curvature and shape retention after chemical treatment.

5.2.1 Shaping Methods

Injection molding, blow molding, and thermoforming are three popular forms of forming LLDPE thermoplastic polymer for bulk production [159]. Injection molding and blow extrusion molding melt polymerized granular resin, which originates from the thermomechanical history of the molded part. Injection molding is known for forming a defect-less, chain-folded shish-kabab structure [160] that provides unique mechanical and wear resistance properties [161]. On the other hand, blow molding generates stable spherulitic superstructure in the molded part, providing unique toughness (Chapter 2). Blow molding introduces compressed air into a molten tube, blowing the tube out against a mold cavity [162]. However, both have relatively high tooling costs and lead times for producing a new design. A miniature medical device, such as a TAVR

leaflet, has a wall thickness in the 80-micron range but other dimensions in the mm range. A miniature version of injection molding is microinjection molding used to form microelectronic chips for a long time [161, 163]. Micro injection molding is a potential process for shaping TAVR leaflets because it is not only a miniature version of injection molding but also works at extremely high pressure with other sophisticated techniques, such as mold heating, to support uniform crystal growth. The screw and barrel systems help prevent polymer degradation due to high pressure. However, this state-of-the-art technique requires a high cost for making the mold and may not work for leaflets where one dimension (leaflet thickness) is several orders of magnitudes less than the dimension of the other part [164]. Therefore, thermoforming is an efficient and inexpensive alternative with allowable tolerance of manufacturing.

Thermoforming processes preheat polymeric sheets or thin films to a soft yet solid state. In this process, heating is predominantly controlled using a halogen lamp, which generates uniform heat distribution over the heating zone [159, 165]. Semicrystalline polymers are heated to a temperature above the glass transition temperature of the amorphous region and below the melting temperature of the crystalline domains to prevent complete melting yet retain the solid-state that eases stretching. Stretching can be performed in two ways: (1) pre-stretch and then forming over the mold [166], and (2) concurrent 3D stretching and forming. Pre-stretching pumps a certain amount of air at specified pressure into softened films to create a bubble formed over the mold [159, 166]. This two-step process minimizes the thinning of the film to maintain consistent wall thickness. The other method is more conventional: vacuum pulls (“vacuum forming”), applying pressure or forms with plug assistance over the negative or positive mold in a single step Figure 5.1. This research aims to evaluate the thermoforming process for shaping a leaflet for an optimized semi-open-shaped heart valve and characterize the morphology of the thermo-shaped leaflet. Moreover, leaflet hemocompatibility was tested *in vitro* to determine the minimum amount of HA molecules required to achieve minimal blood clotting, cytotoxicity, and platelet adhesion. The study evaluates the three traditional HA-treated bioenhanced characterization techniques

to recommend the most reliable one. The specific aim of this research is **Hyaluronic Acid Enhancement of Heart Valve Leaflet and Characterizations**.

5.3 Methods

5.3.1 Manufacturing Leaflet for Closed State

This study aims to manufacture and characterize bio-enhanced leaflets for a heart valve in the semi-open state using LLDPE from Dowlex 2045G blown film. Thermoforming or heat shaping techniques, which are frequently used for low cost plastic shaping, were used to shape the leaflets in the semi-open. [159, 165]. Ziegler Natta catalyzed Dowlex 2045G blown LLDPE thin film of thickness 120 microns was purchased. Shaping was performed in Formech 450 DT thermoformed, which “shapes” or “forms” the heated and softened film by vacuum pulling. In this process, a negative mold was 3D printed using a dental-grade resin that provides a smooth surface finish (Appendix A.11). The one-step shaping process involves carefully placing LLDPE film (cleaned in xylene and acetone overnight) over the negative mold and under the heater which heats the film by radiation. The heater was programmed to reach its maximum given power (70%) within 20 seconds and hold the heat. Generally, the films soften within 5-10 seconds of further heating at maximum power. The heating reaches a temperature slightly below the crystalline domain’s melting temperature and above the glass transition temperature of the amorphous domain. Immediately after softening (visually perfectly straightened film), the heater was removed, the mold was raised upwards, and the vacuum pulled down the film. Vacuum forming causes 3D stretching and thinning to form over the mold. This resulted in variations of thickness on the shaped leaflet. The thickness of the leaflet varied from 30-50 μm near the commissure post region, and on the cusps, it varied from 50-60 μm , measured in pressure-sensitive thickness gauges of low tolerance level. The vacuum forming continues for 5-10 seconds more, and then the shaped leaflet gets quenched at room temperature. Finally, the leaflet is trimmed and removed from the mold extremely carefully to remove extra areas and used for bio-enhancing.

5.3.2 Leaflet Bio-enhancing

The bio-enhancing process developed earlier [167] can be broadly divided into three parts: (1) complexation and silylation of hyaluronan (SHACTA), (2) Treating LLDPE with silylated hyaluronan, and (3) Hydrolysis to revert back to hyaluronan (HA). The complexation process introduces the ammonium salt of a long hydrophobic chain to hyaluronan since it cannot be directly silylated. Silylation is critical to converting hydrophilic HA into hydrophobic HA so that it can be dissolved in xylene and enter the amorphous region of a xylene swollen hydrophobic polymer.

Materials

2045G resin (octane-1 comonomer, Ziegler Natta catalyzed) blown LLDPE thin film (thickness 80 μm), was sourced from Film Tech LLC (2011), medical grade sodium hyaluronate (Na-HA), molecular weight 750 kDa was purchased from Lifecore Biomedical and stored at 2-8 ° C. Cetyltrimethylammonium ammonium bromide (CTAB), and Acetic acid were purchased from Fisher. Xylene, sodium chloride, Toluidine Blue O, Acetone, Dimethyl sulfoxide ($\geq 99.9\%$) ReagentPlus (DMSO), Hexamethyldisilane ($\geq 99.9\%$ ReagentPlus), and Tolyene-2,4-diisocyanate were obtained from Sigma.

Complexation and silylation of hyaluronan

The CTA-HA complex is formed in this step, followed by inserting a trimethylsilyl group(Appendix A.6). This trimethylsilyl imparts hydrophobicity to the hydrophilic Na-HA. In the complexation step, 1% aqueous CTAB solution is slowly added to a 0.3% Na-HA aqueous solution under high-speed stirring, which precipitates as HA-CTA. The CTAB is dissolved in water under constant stirring at 40 °C to form the CTAB aqueous solution. White HA-CTA precipitates are then centrifuged, washed to remove residual CTAB, and dried in vacuum oven at 50 ° C to evaporate water and cryo grounded into powder form. The powder has a higher surface area which facilitates the reaction. In the silylation step(Appendix A.6), HA-CTA powder is dissolved in DMSO solvent under dry N_2 , and constant stirring at 50 °C for 24 hours which swells and dissolves HA-CTA. Then, HMDS reactant containing silyl group is added and the temperature is raised to 75 ° C

for 48 hours under dry N₂. The product of the reaction, silylated HA-CTA (SHACTA), and the solvent DMSO result in a two-phase solution: a thin top layer contains SHACTA and HMDS, and the bottom layer contains thick DMSO. At the end of the step, the top layer is washed five times with xylene in a rotary evaporator and dried at 50 °C in a vacuum oven.

Developing HA Incorporated Thin Film

In this process, LLDPE thin films, and crosslinked LLDPE were volumetrically swollen in 1% w/v SHACTA-xylene solution at 50 °C to introduce SHACTA into the amorphous regions for 1.5 hours. Then the samples were drained from the solution at a slow and constant rate of 2.5 cm/hr under vacuum using an automatic elevator. Later, HA incorporated films were vapor crosslinked over 25% v/v 2,4 diisocyanate (TDI)-xylene solution at 60 °C for 1 hour. Afterward, they are dried under vacuum overnight. Previous research found that vapor cross-linking provides a uniform and consistent layer of SHACTA. Later on, SHACTA is reverted to HA through hydrolysis described elsewhere. Later on, only the uncrosslinked and the films were shaped into a 1 mm³ cube using the thermoforming method described above to determine the shaping ability of HA incorporated thin film while maintaining the integrity and molecular structure of the HA network.

Developing HA Incorporated Heart Valve Leaflet Biomaterial.

Thermoformed leaflets were also treated to incorporate HA biomolecules after shaping, as shaping post-treatment can degrade HA molecular weight. Thermo-shaped leaflets of 26 mm diameter were carefully cut to the valve height of 42-45 mm (Figure 5.1 (b)). The leaflet was assembled using Teflon^(R) rings with and without holes and magnets to hang vertically cusp down position in a glass jar for swelling (Appendix A.7). The PTFE rings facilitated withdrawal from the solution at an ultra-slow withdrawal speed at 50 °C under -15 inch vacuum condition. The PTFE ring assembly was also used in the vapor crosslinking step. In this process, LLDPE thin films and shaped leaflets were volumetrically swollen in 1% w/v SHACTA-xylene solution at 50 °C to introduce SHACTA into the amorphous regions for 2.5 hours. Then, the samples were drained from the solution at a slow and constant rate of 1.5 cm/hr under vacuum using an automatic elevator.

Draining under a vacuum is expected to evaporate xylene from the meniscus of leaflet air and xylene quickly, leaving a constant and even layer of grafted SHACTA. Immediately after, the films are dried under vacuum overnight. Later, HA incorporated films were vapor crosslinked over 10% v/v 2,4 diisocyanate (TDI)-xylene solution at 60 ° C for 1.5 hours. Afterward, they are dried under a vacuum overnight. Previous research found that vapor cross-linking provides a uniform and consistent layer of SHACTA. SHACTA is then reverted to HA through hydrolysis, as described elsewhere. In brief, LLDPEs are sonicated for 3 hours in a solution of 0.2M NaCl in 1:1, v/v DI water, and ethanol solution. Then they are sonicated in 0.2 M NaCl DI water solution for one hour, rested in 3:2 water ethanol solution for 2 hours, and finally cleaned by sonicating in water for 30 minutes. This same process was used to make HA sham treated leaflets where the whole process above mentioned was used except no HA was dissolved in xylene.

5.3.3 Surface Characterization

Contact angle

HA incorporated thin films and shaped leaflets, which were tested using a goniometer to determine the water contact angle. HA has an abundance of hydroxyl groups resulting in a strong affinity towards water creating H-bonds. As a result, HA treated biomaterials show distinct hydrophilic natures [77]. This study utilized the captive bubble water contact angle to ensure the films were evenly and continuously hydrated during testing. HA treated thin film and shaped leaflets were punched out using 8 mm biopsy punches. Cusps have distinct 3D curvature, which adds errors to the traditional contact angle measuring methods, which demand extremely flat samples. Therefore, 8 mm diameter small punches were collected from the entire leaflet and measured for contact angle individually. The punches were taped on a glass slide using 12 mm wide carbon tape, swelled in water for 1 hour, hung inverted in water, and an air bubble was introduced with a curved needle in the goniometer 260-F4 Ramé-Hart Instrument. The angle between the solid surface and liquid is measured for static contact angle. In addition to static contact angles dynamic contact angles were also measured to estimate HA surface coverage consistency. The air

bubble was increased in size to a metastable bubble for receding and reduced to size for advancing contact angle. A hysteresis graph was utilized to validate the stability of dynamic contact angle data. Samples with average static contact angle 30° were grouped as heavy HA, and $40-50^\circ$ were grouped as moderate HA. Because the lower the contact angle higher the amount of HA presence in samples. The samples were removed from carbon tape using pure ethanol and saved for FTIR and TBO staining.

Quantitative ATR-FTIR

Attenuated total reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) was used to investigate new chemical groups introduced to LLDPE during HA bio-enhancement. A Nicolet iS50 was used with diamond ATR crystal and KBR beam splitter. One spectrum per sample was taken with 4 cm^{-1} resolution and 64 scans per spectra at different locations of the sample. Spectra were baseline-corrected before analysis. The Omnic software calculated the area under the peak of hydroxyl peak from $3006-3694\text{ cm}^{-1}$ and the methylene peak from $3004-2690\text{ cm}^{-1}$. The ratio of the area hydroxyl peak to methylene peak (A_{OH} over A_{CH_2}) was used to quantify and compare HA presence in the treated samples.

TBO staining and elution study

Toluidine Blue O (TBO) is used to stain glycosaminoglycans in histology samples. This is a rapid testing kit to determine the presence of HA on LLDPE. Samples after spectroscopy were stained with 0.1% TBO solution of 8M urea for 15 minutes. Then, the loose TBO molecules were washed away with $\text{Di H}_2\text{O}$ followed by drying at 50°C (Appendix A.8). Later the attached TBO was eluted in 50% acetic acid water solution for an hour). Immediately after, $100\text{ }\mu\text{L}$ of the eluent was plated in triplicate for each sample and measured at 630 nm. The TBO concentration in the eluting solution was quantified based on known standards.

5.3.4 Effect of Thermoforming on LLDPE Morphology

Thermal analysis

Thermal analysis was conducted on the virgin LLDPE thin films, shaped leaflet for semi-open state, and HA sham-treated films to determine the effect of heat shaping and HA treatment on LLDPE morphology. Moreover, a Dowlex 2056 resin LLDPE blown film (Dow Corning), successfully bio-enhanced in previous research was compared with Dowlex 2045 G blown film (Film Tech LLC.)(Appendix A.12). Differential scanning calorimetry measurements were performed on TA Modulated DSC 2500 over a temperature range from 40 °C to 180 °C at heating and cooling rates of 10 °C /min under N₂ purge (Appendix A.3). This first heating generates the first heating curve. Then the sample melt was held at 180 °C for five minutes and then immediately started cooling down to 40 °C at 10 °C /min. The samples were then heated back to 180 °C at the same rate to generate the second heating curve. Samples were collected from pristine LLDPE thin films and shaped leaflets for the closed state to match the aluminum pan inner diameter. A total of four 5 mm size samples from each group weighting a total of approximately 6±.1 mg were carefully inserted one at a time into the pan using tweezers to ensure that samples were properly touching the pan. Single-layer samples caused thermal leaks since they were extremely light weight. Therefore, more than one layer was required to create a proper thermal contact between pan and sample. This method ensures consistent heat flow measurement. Later, the crystallinity percentage was calculated by dividing the sample heat of fusion by the theoretical heat of fusion for a perfect LLDPE crystal [77]. The exothermic peak was integrated with TA instrument' Trios software to determine the sample heat of fusion.

$$\text{Crystallinity \%} = \frac{\text{Sample heat of fusion}}{\text{Crystalline LLDPE heat of fusion (288 J/g)}} \times 100\% \quad (5.1)$$

Small angle X-ray Scattering

The effect of shaping and HA treatment on the crystallinity of the thin films was further evaluated by X-ray diffraction. X-rays are scattered by the periodicity of the crystalline regions

in a semicrystalline polymer such as LLDPE. Scattering is governed by $n\lambda \propto d \sin\theta$, meaning information about crystal thickness (d) which is in nm scale can be found by analyzing small angle X-ray diffraction patterns. Therefore, LLDPE films (1) 2045 G pristine LLDPE and (2) 2056 pristine LLDPE (3) 2045 G blown film shaped for closed state leaflet, and (4) 2045 G HA sham treated films were analyzed by small angle X-ray scattering (SAXS) to determine changes in the morphology with respect to the processing described above.

Samples were rinsed with acetone and dried in a vacuum oven for 30 minutes before analysis. Samples were cut using a standard biopsy punch of 5 mm size and mounted individually in the 5 mm diameter holes of the solid sample holder using Kapton tape. They were tested in the ID-12-B beam line of the Advanced Photon Source at Argonne National Laboratory. ID-12-B beamline has a PILATUS 2M detector a direct-detection system that prevents any geometry distortion [102]. The detector pixel size was $172 \mu\text{m} \times 172 \mu\text{m}$, and the sample to detector distance was 2 m. The beam size was adjusted to $150 \mu\text{m}$ in the x-direction and $100 \mu\text{m}$ in the y-direction with a wavelength of $\lambda = 0.9322 \text{ \AA}$. The data set spans scattering vector q (the ability of a material to scatter X-ray) range of 0.004 to $2.3 \text{ \AA}^{-1}$. The detector to sample distance was calibrated using a standard sample of silver behenate. Data reduction into a 2D pattern and averaging over all available azimuthal angles into 1D data of scattering vector vs Intensity (arbitrary unit) was done automatically in MATLAB interphase without requiring user interruption. Additional scans were performed on only Kapton tape (without any sample between Kapton tapes) to use as the background. Since absolute intensity calibration was not performed, the 1D data was correct using the Lorentz correction factor to determine the long period. Long period measures the distance between one lamella to another lamella which includes the amorphous regions. The Lorentz correction uses the peak q_{max} from q vs Iq^2 plot, to determine the long period using the equation bellow

$$L = \frac{2\pi}{q_{\text{max}}} \quad (5.2)$$

5.4 Biocompatibility characterization

Whole Blood Study

Six sample punches from uncrosslinked and unreinforced that were tested in contact angle and ATR-FTIR were used to study whole blood clotting resistance. Thrombosis has been one of the primary reasons for valve failure *in vivo*. Therefore, three groups of HA-treated leaflets were tested for anti-thrombosis using human whole blood to evaluate sample and blood interaction. Samples were collected using TCPS 24-well plates (Appendix A.9). Human blood drops were added to the sample for the treated group, and TCPS well for the negative control group, which is known to cause blood clotting. Blood was allowed to clot for 15 and 30 minutes. Then, DI water was added to each well at a blood to water ratio of 1:100 and agitated on a plate shaker to release non-clotted blood and free hemoglobin. Hemoglobin concentration was measured in a spectrophotometer (FLUOstar Omega) by measuring the absorbance at 540 nm. The study was repeated with three blood donors with n=(6) on each of the following sample types:

1. TCPS (negative control: empty well plate)
2. Untreated LLDPE (No HA, vacuum-shaped leaflets clean in xylene and acetone)
3. Heavy HA Leaflet (HA treated)
4. Moderate HA Leaflet (HA Treated, collected from cusps)
5. Positive control (TCPS with water)

Platelet Adhesion, Activation and Cytocompatibility

When blood comes in contact with a foreign biomaterial, the resulting protein adhesion cascade initiates the platelet adhesion cascade, which leads to thrombosis. The untreated shaped leaflet and two groups of HA treated shaped leaflets were studied for the platelet adhesion cascade and compared with the positive control group TCPS. All the groups were sterilized and hydrated beforehand and incubated in platelet-rich plasma (PRP) for two hours. The incubated PRP is then reacted with Caymann LDH cytotoxicity assay (Appendix A.10). This study tested HA treated

three groups of leaflets with lactate dehydrogenase (LDH) release assay. LDH enzyme release is a biomarker of cell and tissue damage. Then, phosphate buffer saline rinsed the samples and gently removed free platelets. The adhered platelets were fixed by submerging them in a 3% (v/v) glutaraldehyde, 0.1 M sucrose, and 0.1 M sodium cacodylate aqueous solution for 45 minutes to collect SEM images. The fixation process ends by placing them in a sodium cacodylate and sucrose secondary fixative solution for one hour and dehydrating them in ethanol /DI water. Finally, the samples was imaged in SEM x500, x1000, and x5000, with two representative spots per sample. Similar to whole blood clotting assay two HA treated groups were studied and compared. The attached platelet's surface coverage was estimated by thresholding in ImageJ (NIH). The study was repeated with three blood donors. Following are groups of samples with n=3 for the study,

- TCPS pucks made by cutting a well plate into 8mm punch.
- Heavy HA Leaflet
- Medium HA Leaflet
- Virgin LLDPE
- Empty cells that contain only PRP (spontaneous release)
- Empty cells that contain PRP, then 10% Triton X-100 solution (maximum release)

5.5 Statistical Analysis

Data sets were tested for equal variance using Levene's test and Anderson-Darling test to determine if the data was drawn from a normally distributed population. Origin Pro 2021 software package was used to determine statistical significance tested in one-way ANOVA with post hoc Tukey's test ($p < 0.05$).

5.6 Results and Discussion

5.6.1 Bio-enhancement of flat substrate

Data published on HA incorporated 2056 LLDPE blown film showed a remarkable reduction in static contact angle with as low as 30° mean static contact angle, indicating the presence of uniform and consistent HA on LLDPE [74,77]. This research aims to manufacture and characterize the HA molecule incorporation in the 2045G leaflet. 2045 G blown film was treated with HA and then shaped into a cube. The presence of HA was confirmed by qualitative ATR-FTIR, TBO staining, and contact angle goniometry, to evaluate the feasibility of shaping HA incorporated film. Furthermore, SAXS and DSC evaluated the shaping effect on morphology. Figure 5.2 shows the contact angle of HA treated flat films. As can be seen, HA treated LLDPE static contact angle reduces by at least 50% to that of pristine LLDPE, which is consistent with the result obtained in previous studies for 2056 LLDPE [74,77]. However, the static contact angle has a high variation indicating an inconsistent surface. Also, the difference between advancing and receding contact angles is considerably high, indicating the surface chemical heterogeneity. Later, HA presence was confirmed on TBO stained samples, as shown in Figure 5.3. Heavy HA areas of TBO stained samples indicate that the surface is highly populated with HA. The blue areas indicate the presence of HA because TBO attaches to the carboxyl group of HA. It can be seen that the crosslinked HA treated LLDPE also appears dark blue after TBO staining. Which might indicate high concentration of HA population. ATR-FTIR, TBO elution and contact angle measurements were not performed in this study for this particular LLDPE which could be conducted in future experiments. Pristine LLDPE having no carboxyl group, stays colorless. The presence of HA was further confirmed by FTIR (Figure 5.4 bottom black line) in bands 1250 cm^{-1} (C-O stretching), 1060 cm^{-1} (C-O-C stretching), TDI group in 1540 cm^{-1} , 1600 cm^{-1} (C=C aromatics), 1660 cm^{-1} (aromatic amide).

In another set of experiments, LLDPE flat thin film was treated with HA and then vacuum shaped over a 1 cm^3 cubic mold producing a five sided hollow cube. The goal here is to test whether HA is retained on HA-incorporated films during thermoforming. Figure 5.2 also shows that the water

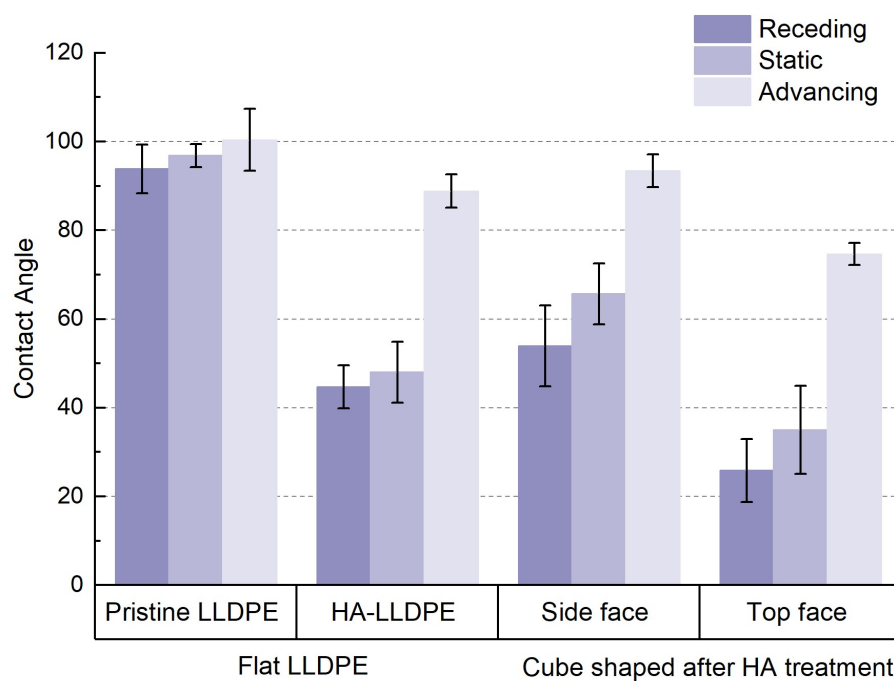


Figure 5.2: The dynamic and static water contact angle of flat LLDPE and Cube-shaped after HA treatment LLDPE showing hydrophobicity significantly reduces after HA treatment but increases after thermoforming. Flat LLDPE includes contact angle of pristine LLDPE and HA treated LLDPE. Cube-shaped HA-LLDPE includes contact angle of side faces and top face. Interestingly top face had a significantly lower water contact angle implying strong hydrophilicity than the side faces. All columns are significantly different at $p < 0.05$, $n=3$

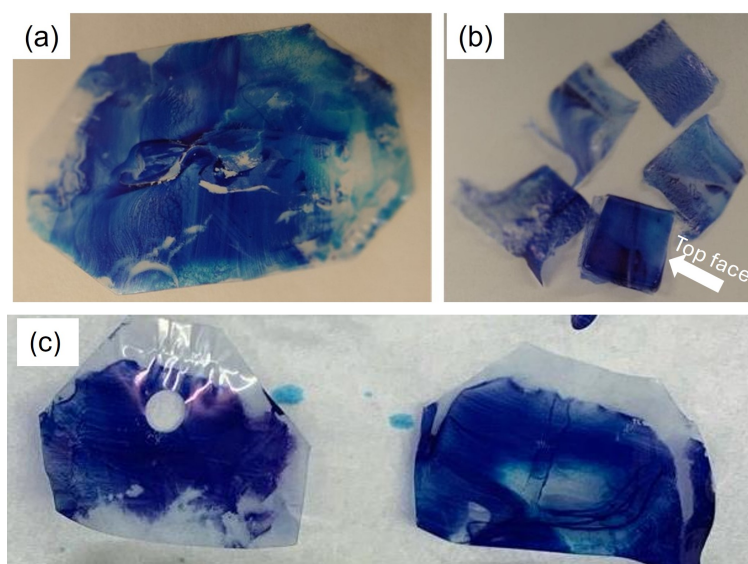


Figure 5.3: TBO stained (a)HA-LLDPE (b) Cube shaped after HA-treatment white arrow showing top face and rests are four sides of hollow cube (c) crosslinked HA LLDPE

static contact angle increased significantly for the side faces after the HA treated film was vacuum shaped, indicating the return of hydrophobicity. However, the top face contact angle was improved and was lower than the contact angle of HA treated films before forming (Figure 5.2). In addition, vacuum forming developed physical heterogeneity on the side faces visible to naked eyes. This physical disturbance on the side faces can be seen in Figure 5.3 (b). Interestingly the top face did not show heterogeneity and maintained heavy HA color from staining. The TBO staining results agree with contact angle results. Figure 5.4 shows FTIR spectra collected from the faces of the cube. The top face and the 3rd side face retained the OH molecule bending, but the other three faces lost TDI and HA bending. The data were collected from at least three spots of the faces, and other sites had shown a similar trend of reduced peak height for the HA and TDI groups. This suggests that few regions had thermally degraded HA to its lower molecular weight while other regions had retained its molecular weight. This observation agrees with previous studies [168, 169], where high molecular weight HA degrades to low molecular weight HA when heated above 90 °C. In this study, HA treated films were softened above 90 °C for at least 10 seconds and the heating may have cause HA to degrade in molecular weight.

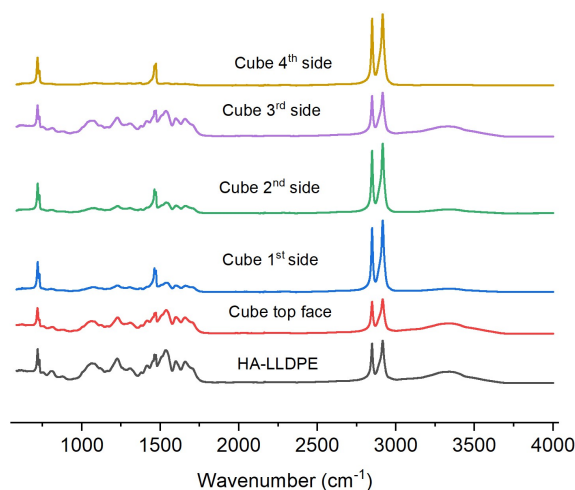


Figure 5.4: FTIR spectra of HA-LLDPE and faces of cube shaped HA-LLDPE

5.6.2 Effect of Shaping on LLDPE Morphology

The LLDPE films discussed above were further investigated to trace morphological changes due to heat shaping and HA treatment and were compared with previously successful 2056 blown film morphology. Figure 5.5 (a) shows the crystallinity percentage calculated using equation 5.1 from the thermal analysis. As can be seen, 2045 G, 2056 pristine LLDPE and 2045 G shaped leaflet crystallinity were not statistically different. However, 2045 G HA sham treated films were slightly more crystalline than the rest. In addition to total crystallinity, Figure 5.5 (b) shows normalized heat flow obtained from DSC for both 2045 G pristine film and shaped leaflet. The DSC partially holds the history of film processing techniques in the thermograph. The first heating curve of the pristine LLDPE blown film thermograph had double melting peaks: one principal peak and one “shoulder”, which agree with previous studies on blown film [79]. *In situ* SAXS conducted during LLDPE blowing [170] on the bubble showed that long lamellar are formed at the beginning of cooling and small lamellar are formed as cooling tends to end. Therefore, the DSC thermograph presented here agrees with this phenomenon where the shoulder peak representing weak crystals could have been formed at the end of bubble cooling. The melt peak representing long crystals could have been formed immediately after the bubble had formed and cooling started. Because at the beginning of blowing, there was higher thermal energy available to bubble, which is required to form long and strong crystals. However, the shoulder was replaced with a valley and a single melting peak in the second heating curve. This implies that the morphology developed during blowing was erased, and the constant cooling rate regrew new crystal domains and morphology. Interestingly thermomechanical inputs from vacuum forming did not delete the history of blow molding in crystal domains. As can be seen, both the small and long crystals were preserved. This is probably because the heating temperature stays slightly below the melting temperature of crystal domains during the relatively short thermoforming. Therefore, the crystal domain regrowth was not required, thus persevering blow molding morphology. However, 3D stretching of the softened film might have caused the slight displacement of the weak crystal domains since the shoulder peak moved slightly compared to the pristine LLDPE shoulder peak.

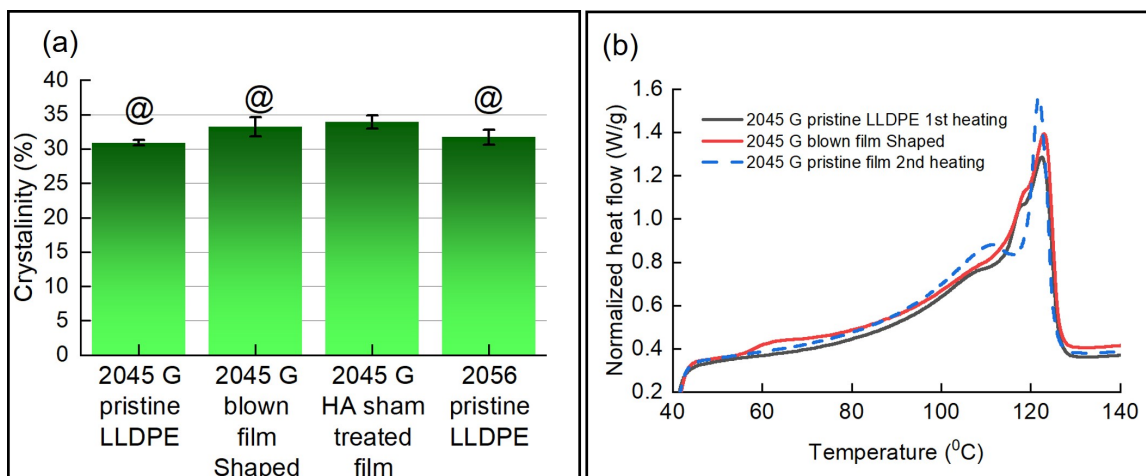


Figure 5.5: Crystallinity percentage of two pristine LLDPEs, vacuum formed LLDPE and HA sham treated LLDPE. Columns sharing the same symbol are not significantly different at $p < 0.05$, $n=4$, indicating that vacuum shaping does retain crystal morphology.

The above-discussed phenomenon was further validated with SAXS since X-rays can quantitatively measure long period (distance between one lamellar to another) and reveal more information about the crystal domain [79, 94, 109, 170]. Numerous research conducted on LLDPE blown film [81, 91, 170–172] found the signature Bragg's peak indicating organized lamellar packing in averaged 1D scattering profile. SAXS generated a 2D pattern of intensity which is then averaged for all available azimuthal angles and converted averaged scattering vector q . Figure 5.6 (a) shows q (/nm) vs. Intensity (I) in arbitrary unit for all four LLDPEs discussed in DSC curves. As can be seen, all three 2045 G films: pristine, shaped and HA sham treated films showed similar sharp Bragg's peak indicating well organized lamellar packing structure. Interestingly, the 2056 film shows a broader peak and the scattering vector q moved slightly on the right, indicating a smaller long period. This also implies that 2045 G film morphology might have remained fairly constant with processing which agrees with thermal analysis.

X-ray scattering vector q measures the difference between scatter beam and incident beam wave vector; as a result, this parameter is solely dependent on crystals ability to scatter the X-ray [83]. This study also calculated the long period by applying Lorentz correction to the 1D SAXS data (Figure 5.6 b). As predicted from thermal analysis, the long period of both virgin (13.67 nm) and shaped (13.34 nm) leaflets were almost equal. Since their crystallinity percentage is statistically

similar, lamellar thickness also remained relatively constant. Therefore, it could be assumed that heating below melting temperature for 30 seconds and 3d stretching of semicrystalline polymer LLDPE may not alter polymer morphology significantly.

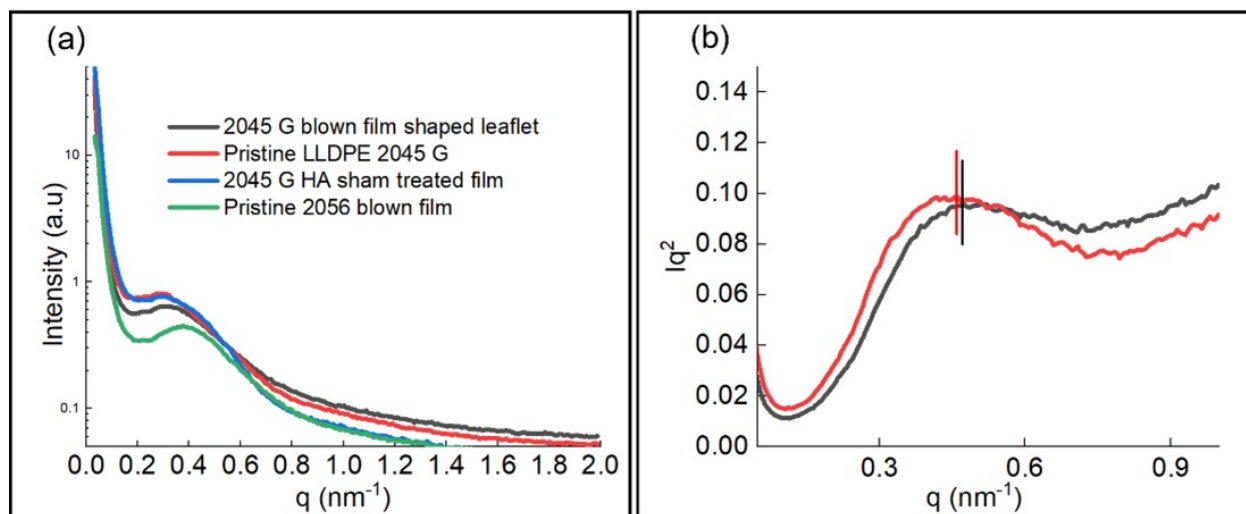


Figure 5.6: (a) Averaged scatter vector vs intensity of two pristine LLDPEs, vacuum formed LLDPE and HA sham treated LLDPE (b) Lorentz correct intensity vs scatter vector of pristine and vacuum formed LLDPE showing no significant difference in lamellar thickness

5.6.3 Bio-enhancement of shaped TAVR leaflets

Initial investigations emphasized the importance of first shaping the leaflet and then applying hyaluronic acid (HA) treatments, as opposed to treating with HA and then shaping in the assembly line for Transcatheter Aortic Valve Replacement (TAVR). The thermoforming technique utilized for shaping sometimes produces defective parts due to under or overheating. Further challenges were encountered during the removal of leaflets from reusable 3D-printed molds. Consequently, integrating HA treatment as a post-process of thermoforming emerges as a more economically viable approach. Established methods entail using silylated hyaluronic acid (SHACTA) to treat flat plastic sheets, resulting in a uniformly interpenetrated HA network. This facet of the research focuses on investigating the HA treatment process for shaped TAVR leaflets with specific curvature in the cusps.

Shaped leaflets are immersed in a SHACTA solution and then withdrawn at an ultra-low speed ranging from 15-25 mm/hr at 50 °C under -15 in mmHg vacuum. Figure 5.7 provides a detailed analysis of the surface characteristics of HA-treated leaflets, confirming the presence of HA both quantitatively and qualitatively. Quantitative analysis includes measurements of static and dynamic water contact angle (using the captive bubble method), available HA surface density (determined from eluting TBO molecules attached to HA), and the ratio of the area under OH/CH₂. Qualitatively, insets in the figure depict TBO stained leaflets, showing relatively uniform staining across the leaflet base, interleaflet triangles, and cusps. However, the quantitative results only somewhat support this observation. Leaflets treated at all withdrawal rates exhibit a consistent distribution of HA over the interleaflet triangles of no 3D curvature. The average static, advancing, and receding contact angles around these regions are $48 \pm 12^\circ$, $82 \pm 14^\circ$, and $40 \pm 12^\circ$, respectively. These values are approximately 10 degrees higher for the cusps, suggesting either a lower amount or inconsistently populated HA over the cusps. Available HA surface density results measured by TBO elution (Figure 5.7) demonstrate a statistically significant reduced HA surface density on the cusps compared to other areas of the leaflet, consistent with findings from contact angle measurements. The only exception is observed in the area ratio A_{OH} over A_{CH_2} from ATR-FTIR spectra. The ratio serves as an indicator of HA presence and does not exhibit statistical differences. This discrepancy may arise because only one scan was performed on each sample center, covering and testing a far smaller surface area than TBO staining, elution, and contact angle measurements. Adjustments to treatment parameters such as withdrawal rate, temperature of withdrawal, and swelling time before starting the withdrawal process were made to discern fundamental factors behind low HA presence on leaflet cusps. While temperature and swelling time did not significantly alter the results, withdrawal rate yielded intriguing effects on the HA treatment process. As illustrated in Figure 5.8, a leaflet treated at 25 mm/hr displayed horizontal strips of TBO stain perpendicular to the withdrawal direction (indicated by black arrows). These horizontal strips suggest that HA was integrated along these lines. Decreasing the withdrawal speed to 20 mm/hr and 15 mm/hr significantly reduced the spacing between these strips. This phenomenon

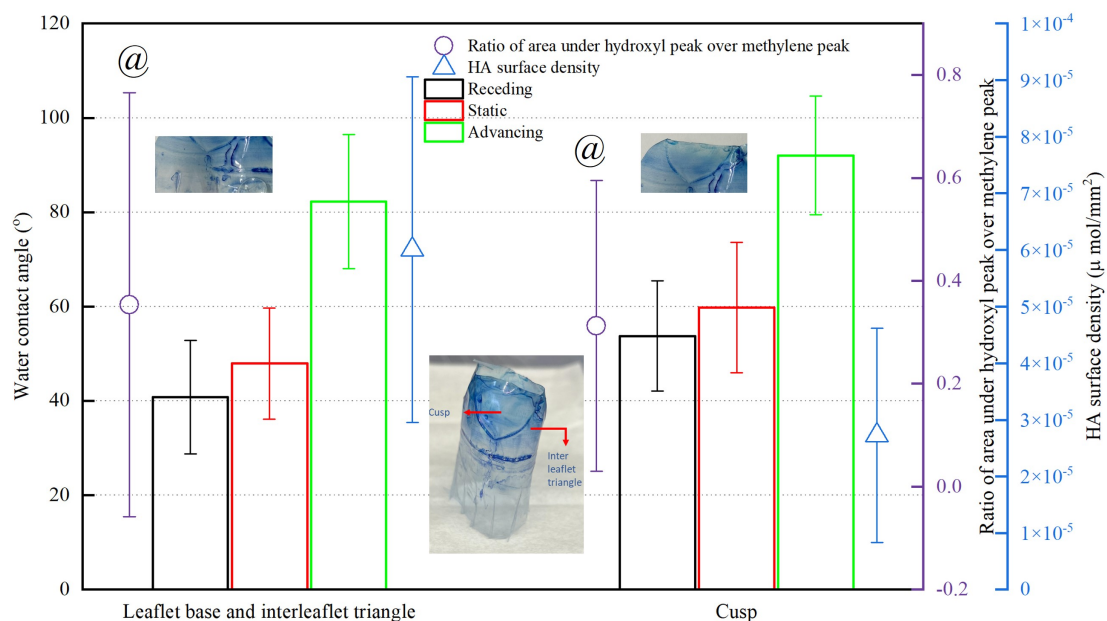


Figure 5.7: Surface characteristics of HA-enhanced custom-shaped TAVR and their components. The inset displays a TBO stained leaflet highlighting two distinct regions: 1. the cylindrical leaflet base and interleaflet triangles. These regions exhibit low contact angles and high HA surface density; Conversely, 2. the curved cusps demonstrate contrasting values, suggesting a lower presence of HA. The area ratio of OH/CH₂ for both regions shows no statistically significant difference, indicated by @, $p < .05$, $n = 15$

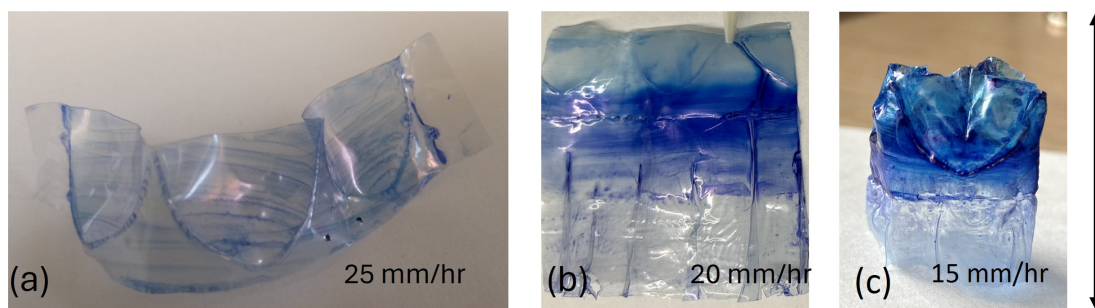


Figure 5.8: TBO stained HA treated leaflets: withdrawn at 25 (a), 20 (b), and 15 (c) mm/hr from SHACTA solution. Leaflets showing horizontal strips of stain perpendicular to the withdrawal direction, marked as a black arrow. The clear portion of these leaflets (b-c) are used for assembly which does not get HA treatment

resembles natural occurrences on sea beaches due to receding waves, natural striations, and dip coating at ultra-low withdrawal speeds. Uchiyama et al., [173–179] have published a series of articles on dip coating ceramics over silicon substrates to elucidate this phenomenon. They discovered that at an ultra-slow withdrawal speed of 12 mm/hr, stripes of the coated film are perpendicular to the withdrawal direction, and coating film thickness increases with decreasing withdrawal speed, aligning with HA treatment findings at an ultra-low withdrawal speed. This phenomenon is often explained using the "stick-slip" or "coffee ring" theory: ultra-slow speed allows solvents to evaporate before capillary action raises the coating solution to the edge of the meniscus. The edge of the coating solution is pinned to the previously dried substrate, preventing gravity from flowing back the solute, thus causing a linear pattern.

Solvent, evaporation-driven dip coating can also elucidate why the curved cusps receive significantly lower HA despite being withdrawn at an ultra-slow speed. A constant 90° angle is maintained between the substrate (here, the leaflet interleaflet triangle and bottom) and the solvent. However, as soon as the leaflet cusp touches the solvent, the angle changes, altering the meniscus and resulting in inconsistent HA integration. Future research could explore angular controlled dip coating, where either the substrate or the solution is tilted to maintain a constant angle between the cusp surface and the solution [180].

5.6.4 Correlation among contact angle, ATR- FTIR, and TBO eluate

The discussions about contact angle, ATR-FTIR, and TBO staining and elution prompted an inquiry into their interrelationships. Initial observations suggest a negative correlation between water contact angle and available HA surface density measured from TBO elution. In other words, a material exhibiting a lower water contact angle will likely appear dark blue from TBO staining, and its eluates will yield higher HA surface density. Conversely, a material containing a higher quantity of HA tends to exhibit a more substantial hydroxyl peak and a weaker methylene peak in ATR-FTIR spectra, resulting in a positively correlated ratio. To test this hypothesis, 28 samples of 8 mm diameter were collected from HA-treated leaflets at three withdrawal speeds: 15, 20, and 25 mm/hr, encompassing cusps, interleaflet triangles, and leaflet bottoms. These samples were evaluated for contact angle, scanned for ATR-FTIR, subjected to TBO staining, and then eluted to quantify TBO attached to HA during staining. The data was analyzed to determine the linear Pearson correlation coefficient.

Figure 5.9 illustrates the correlation among these surface characteristic parameters. Black and red circles represent available HA surface density and static water contact angle. The linear Pearson coefficient was calculated to be $r = -0.51$ for water contact angle and HA surface density. The dynamic hysteresis, i.e., the difference between advancing and receding contact angles, displayed a similar relationship. These surface characterization techniques utilize the entire surface area and provide a more accurate estimation of surface properties, as discussed in section 5.6.1. A low water contact angle indicates a hydrophilic surface, achievable through chemical and physical homogeneity, as well as the presence of functional groups capable of bonding with water molecules. With its abundant hydroxyl groups, HA can generate hydrophilicity on extremely hydrophobic surfaces like LLDPE. Since both techniques utilize the entire sample area, they offer enhanced precision in quantifying HA presence.

On the other hand, the ratio of A_{OH} over A_{CH_2} weakly correlates negatively ($r = -0.31$) with water contact angle and positively ($r = 0.27$) with HA surface density. These findings align with the hypothesis that increased HA surface density corresponds to heightened hydroxyl peak intensity

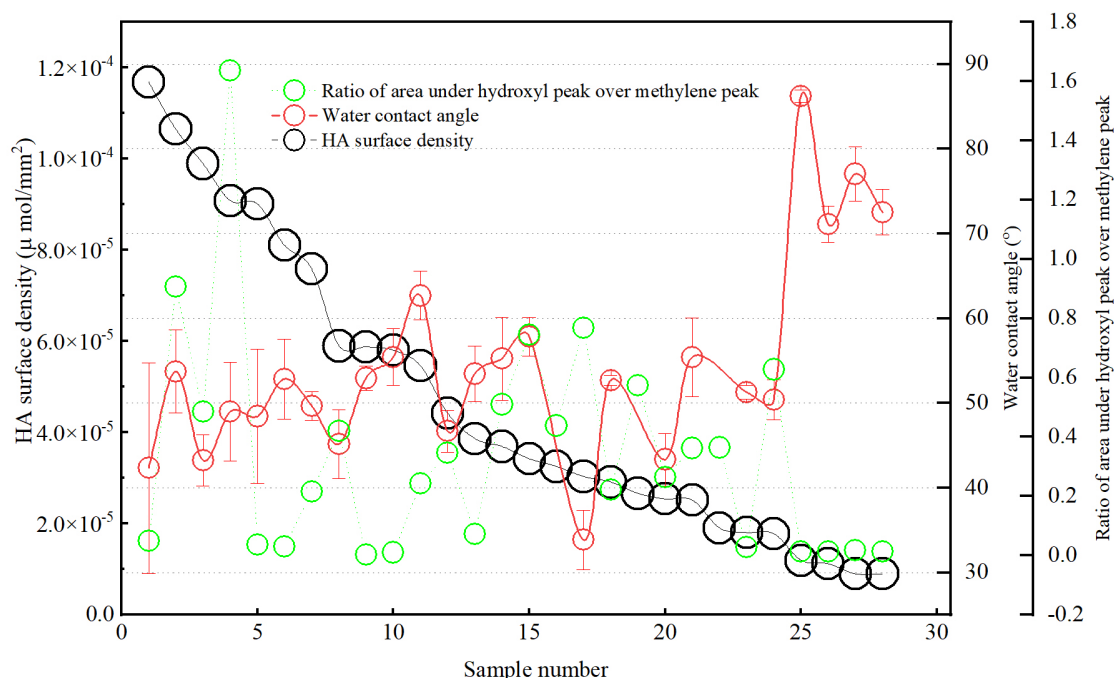


Figure 5.9: Correlation among surface characteristics parameters: HA surface density, water contact angle, and the ratio of A_{OH} over A_{CH_2} . Linear Pearson coefficient $r = -0.51$ for contact angle and HA surface density, representing a moderate negative relation. $r = 0.27$ for HA surface density and A_{OH} over A_{CH_2} ratio; $r = -0.31$ water contact angle and A_{OH} over A_{CH_2} ratio showing weak positive and weak negative relationships respectively

and reduced water contact angle. However, no linear quantitative relationship was established between them. Future research could produce a relationship between HA density and ATR-FTIR absorbance to quantify its presence more accurately.

5.6.5 Influence of HA Quantity on Resistance to Whole Blood Clotting

HA-integrated biomaterials are anticipated to exhibit biocompatibility and anti-calcific and anti-thrombotic properties, making them suitable for incorporation into TAVR leaflets. Previous research by Bui et al., [77] demonstrated these attributes through *in vitro* blood studies, including assessments of whole blood clotting resistance, cytotoxicity, and platelet adherence. However, the minimum amount of HA required to achieve biocompatibility remains unknown. This segment of the research conducted *in vitro* blood studies on TAVR leaflets to explore the relationship between the amount of HA presence in biomaterials and their responses to the biological environment.

Samples were collected from HA-treated leaflets and subjected to water contact angle testing and ATR-FTIR scanning. The samples were categorized into two groups:

1. Heavy HA leaflets: Average static contact angle of $30^0 \pm 15$, collected from leaflet interleaflet triangles and bottom areas.
2. Moderate HA leaflets: The average static contact angle of $50^0 \pm 16$ was collected from leaflet cusps.

5.10 illustrates the whole blood clotting resistance, measured by free hemoglobin absorbance, of the groups above in comparison with the negative control (TCPS), control (virgin LLDPE), and positive control (water) groups. The clotting resistance of both positive and negative controls differs significantly from the other groups. However, no statistical differences were observed between the three sample groups. Although virgin LLDPE (control) and moderate HA samples exhibit similar clotting resistance levels, their high standard deviations prevent statistical significance.

Clotting resistance for 30 minutes is not statistically significant between the positive control and virgin LLDPE. There is also no statistical significance between the negative control, moderate, and heavy HA samples. However, the clotting resistance of virgin LLDPE is significantly higher than that of the heavy and moderate HA samples. Notably, virgin LLDPE displays higher clotting resistance than HA-treated samples for the 30-minute clotting period.

The *in vitro* whole blood clotting resistance assay for HA-treated LLDPE has been criticized for its high error percentage. Visually, TCPS appears to exhibit the highest clotting for both time points. The positive control, representing maximum free hemoglobin release, is obtained when blood is pipetted into water to prevent clotting. Interestingly, the clotting resistance of all three LLDPEs (treated and untreated) displays higher absorbance or clotting resistance at 15 minutes compared to the positive control. Only at the 30-minute does the clotting resistance of virgin LLDPE decrease to match that of the positive control. The high clotting resistance of plain polyethylene is expected, as

it is considered a gold standard biomaterial for implants, as discussed in Section 1.7.1 established in previous works [77].

For HA-treated LLDPEs, clotting resistance does not significantly differ between heavy and moderate HA samples at both 15 and 30-minute time points, despite significant differences in water contact angles between the two groups. This suggests that the quantity of HA does not influence LLDPE clotting resistance. However, these results may be criticized due to the random and unrestricted movement of clotted hemoglobin over the samples in water. During testing, 200 μL of water and blood mixture was collected from the bottom of each well plate for experimental consistency. Since the clotted hemoglobin was randomly moving in water, some samples only pipetted the clot, leading to inconsistencies in the test. Furthermore, a linear Pearson coefficient of less than 0.4 was found when comparing each sample's clotting resistance with its respective contact angle and the ratio of the A_{OH} over A_{CH_2} . Thus, no concrete evidence was found to establish a relationship between the quantity of available HA on LLDPE and whole blood clotting resistance.

5.6.6 Influence of HA Quantity on Platelet Activation, Adherence and Cytotoxicity

In this study, sample interaction with human platelet cells was studied to determine platelet adherence and activation, as well as cytotoxicity, using the Caymann LDH assay. The study was replicated using samples from three different blood donors. This section presents the results from each donor separately to elucidate the relationship between the quantity of HA present on LLDPE and platelet activation and adherence and the differences in platelet behavior of the different donors.

The initial investigation comprised two groups of HA-treated samples, along with virgin LLDPE and TCPS controls. Heavy HA samples were collected from the HA-treated leaflet bottom area. Samples were collected from this same area and tested for water contact angle, TBO staining, and elution. Average HA surface density was $1.5 \times 10^{-4} \pm 2.73 \times 10^{-5} \mu\text{mol}/\text{mm}^2$, $n=6$, average static

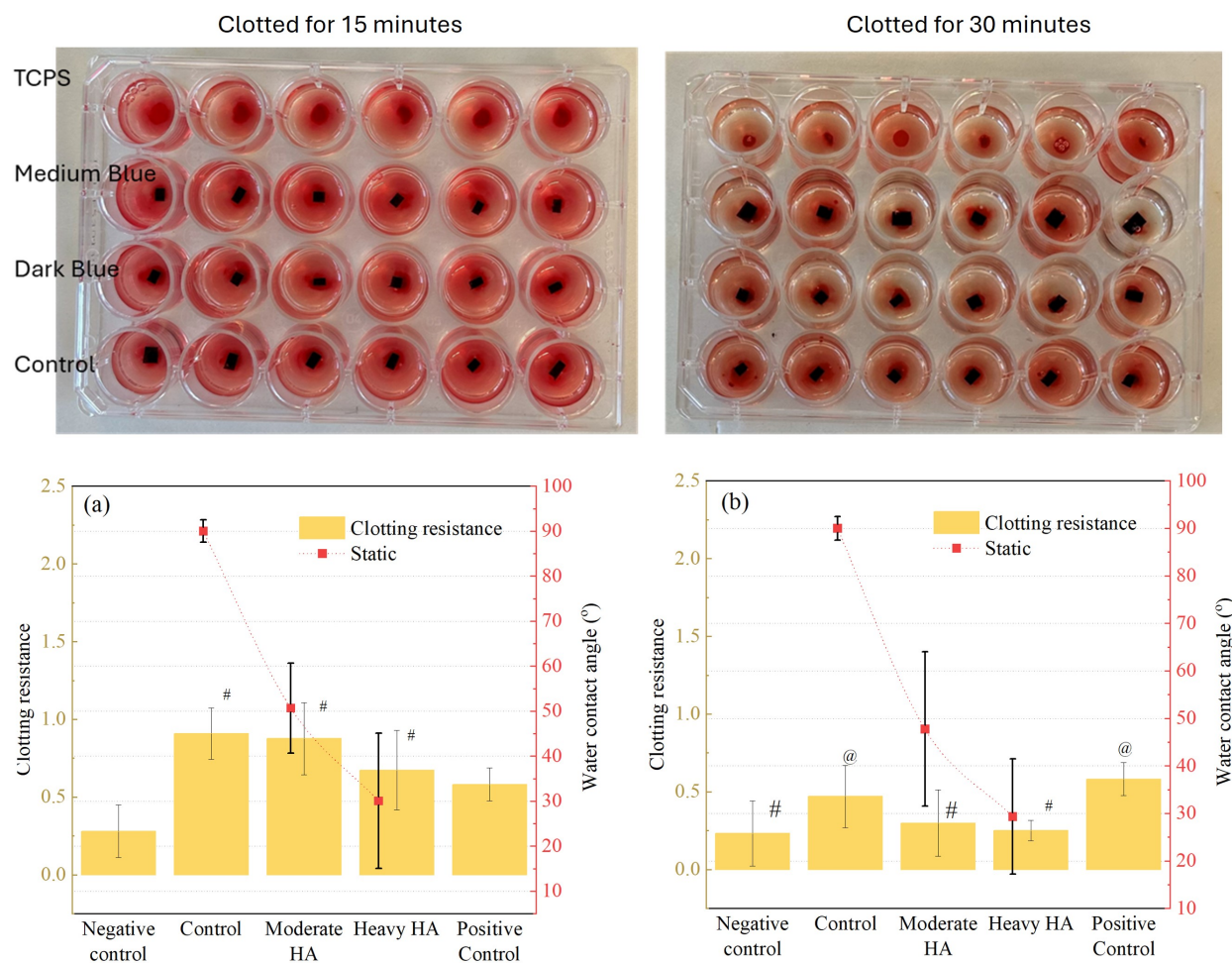


Figure 5.10: Effect of HA quantity on biomaterials on whole blood clotting resistance for 15 min (left) and 30 min (right). Top row: optical images of blood clotting; samples in each row are the same. Bottom row: (a) clotting resistance vs static water contact angle showing no statistical differences between samples of average static contact angle 30 (heavy HA) and 50 (moderate HA). Columns having asterisks are not statistically different, $n=6$, $p<.05$

water contact angle was $18.5 \pm 4.7^\circ$, dynamic hysteresis was $33.17 \pm 13^\circ$, $n=7$. Individual samples from the heavy HA group were not surface-characterized. Conversely, surfaces of samples from the moderate HA group were individually characterized, with an average static contact angle of $57.24 \pm 6.3^\circ$ and a dynamic hysteresis of $36.7 \pm 1.5^\circ$ ($n=3$). Additionally, two samples collected from the adjacent area of the moderate HA group were tested to determine HA surface density, yielding values of 8.1×10^{-5} and $3.4 \times 10^{-5} \mu\text{mol}/\text{mm}^2$. Although theoretically, elution of TBO-stained samples should revert to the original HA-treated samples, preliminary contact angles and whole blood study results revealed minor changes in surface properties. Consequently, TBO-stained and eluted samples were not used for blood studies.

Platelet adherence and activation studies for all three donors demonstrated that platelets were fully aggregated and spread, forming thrombi in all samples of TCPS and virgin LLDPE. In contrast, platelets in the moderate HA and heavy HA groups were only activated to a dendritic state (SEM images provided in Appendix A.13). For the first donor, the percentage of area coverage by platelets was quantified using ImageJ. Notably, the heavy HA group exhibited a significantly lower percentage coverage of platelets ($10.6 \pm 4.8\%$) compared to the moderate HA group ($16.1 \pm 3.4\%$). TCPS and virgin LLDPE displayed significantly higher percentage coverage, with values of $17.8 \pm 7.8\%$ and $20.38 \pm 5.5\%$ respectively. Comparisons between TCPS and virgin LLDPE with the other groups may yield inaccurate results. Because large clots of platelets aggregated in one spot rather than covering the entire surface, rendering their surface coverage less precise. These findings suggest that HA on LLDPE reduces platelet adherence, even with a moderate quantity of HA population in the sample. However, It is highly recommended to populate the leaflet with an available HA surface density of 10^{-4} HA on LLDPE is recommended to achieve optimal anti-thrombotic properties.

Regarding the second donor platelet adherence and activation to the samples, aggregated platelets formed a layer that covered platelets in virgin LLDPE, TCPS, the moderate HA group, and two of the four samples in the heavy HA group. It was evident that the donor's blood contained a higher amount of platelets, or the platelets were more likely to clot, forming a layer of cells that covered

the activated platelet of the sample surface. Additionally, a notable presence of white blood cells was observed in these groups. The presence of white blood cells on the material surface indicates an immune response.

The samples from the moderate HA group underwent water contact angle testing before contacting with the platelet, revealing a static water contact angle of $49.13 \pm 3.9^\circ$ and dynamic hysteresis of $37.5 \pm 2.6^\circ$. Conversely, four samples of the heavy HA group can be categorized into two subgroups:

1. Two samples had an average contact angle of $29.1 \pm 9.1^\circ$ ($n=3$ per sample) and dynamic hysteresis of 41.4° and 28.9° for the first and second samples. These samples were collected from the leaflet bottom area. Samples were collected from an adjacent area determined their average contact angle of $45.2 \pm 8.81^\circ$, dynamic hysteresis of $44.7 \pm 6.34^\circ$, and HA surface density of $9.36 \times 10^{-5} \pm 1.8 \times 10^{-5} \mu\text{mol}/\text{mm}^2$, $n=8$. These two samples were selected based on their low water contact angle. Available HA surface density was in the order of 10^{-5} , indicating a lower population of HA for samples collected from adjacent areas. Similar to the virgin LLDPE and moderate HA group, these samples displayed severe platelet adhesion and activation, forming a network of platelet layers covering most of the sample surface.
2. Two samples collected from another leaflet bottom area exhibited an average contact angle of 38.2 ± 16 , dynamic hysteresis of 40.62 ± 11.8 , HA surface density of $1.5 \times 10^{-5} \pm 2.73 \times 10^{-4} \mu\text{mol}/\text{mm}^2$, $n=9$. Individual contact angles were unavailable, but as they were collected from the leaflet bottom, they should be represented by the average values. Remarkably, these two samples were the only ones in the study that did not display layers of activated platelets. Instead, the platelets were observed to be in a dendritic or spreading dendritic activation stage, with an adhered platelet area coverage percentage of $9.75 \pm 2.5\%$ (Appendix A.13).

Figure 5.11 and Figure 5.12 illustrate the outcomes of third donor platelet activation and adherence to the HA-integrated biomaterial. SEM images confirm the observed trend of the previous two donors' platelet adhesion and activation to the HA. In the x500 image, fewer platelets are attached

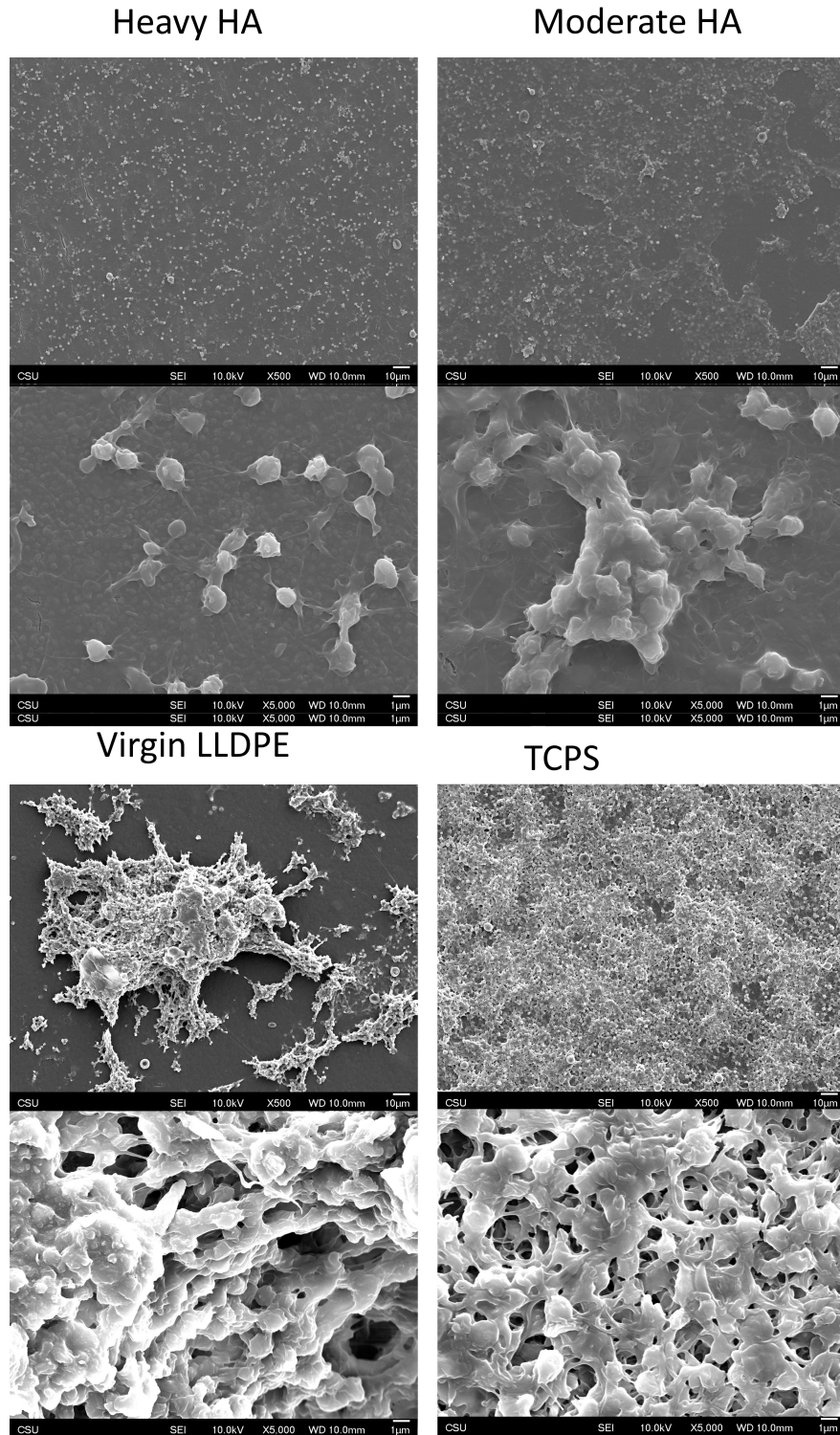


Figure 5.11: SEM images of fixed platelets on samples at x500 and x5000 magnification (third donor). Platelets of heavy HA samples (average static contact angle 30°) are in the early dendritic activation stage. Platelets of moderate HA samples (average static contact angle 40°) are fully spreading. Platelets of virgin LLDPE platelets are aggregated, creating a network of thrombosis. Platelets of TCPS are all aggregated and spread to cover the sample surface area, causing severe thrombosis entirely.

to the heavy HA group, with those present appearing in the dendritic activation stage in the x5000 magnification. Notably, the number of platelet SEM images analyzed was 4 (sample number 2), as one image displayed severe thrombosis and was thus deemed an outlier and excluded from the study.

Platelets from the moderate HA group exhibited notable activation, forming aggregated clots that covered platelets. Severe thrombosis was evident in virgin LLDPE and TCPS samples. Low-magnification SEM images of virgin LLDPE revealed large clots accompanied by multiple white blood cells, indicating potential future thrombosis and calcification. TCPS samples displayed maximum platelet adhesion, adherence, and activation.

Figure 5.12 presents the percentage coverage of groups alongside their respective static water contact angles. Notably, an average contact angle of 32° significantly reduced surface coverage and activation for the heavy HA group. However, heavy HA and moderate HA group static contact angle values did not exhibit significant differences nor the dynamic hysteresis (not depicted in the graph) in one way ANOVA test. Interestingly, HA surface density, measured in the adjacent location of these two group samples, showed statistical differences. The average HA surface density was $5 \times 10^{-5} \pm 1.9 \times 10^{-5} \mu\text{mol}/\text{mm}^2$, $n=9$ for the moderate HA group and $1.5 \times 10^{-4} \pm 2.73 \times 10^{-5} \mu\text{mol}/\text{mm}^2$, $n=9$ for the heavy HA group.

This study reports reduced platelet adherence and activation on HA-treated leaflets in agreement with previous studies. This study Section 5.6.4 discusses a moderate negative relationship between HA surface density and water contact angle measurements. HA surface density measured from TBO staining and elution better represents active HA molecules interpenetrated in LLDPE than spot scanning performed in ATR-FTIR. HA treated leaflets require to be heavily populated with HA ($10^{-4} \mu\text{mol}/\text{mm}^2$) and a static water contact angle of less than 40° in order to prevent thrombosis or future calcification. These findings confirm that consistent HA molecules prevent platelet adhesion and activation.

Figure 5.13 depicts the cytotoxicity in terms of absorbance of LDH release when human platelet cells are incubated in the groups mentioned above. The Negative control had maximum

LDH release because platelet cells were intentionally lysed. LDH release absorbance was not statistically different for virgin LLDPE and moderate HA treated leaflets. Conversely, the heavy HA group, with significantly higher HA content, showed the lowest absorbance for LDH release. Even lower than the positive control TCPS. TCPS is expected to have the least LDH release than any other group [181] as cells are grown in standard conditions for spontaneous LDH release. LDH release is an indicator of cell membrane damage and cell death. Interestingly, the number of dead cells and damaged cells is the least in the heavy HA group even lower than the standard conditioned TCPS measured from LDH release. TCPS is an alien material to cells, whereas HA is native material to platelet cells as HA is abundantly found in the bloodstream [182]. Therefore, cells are more viable in heavily populated HA treated samples and releases less LDH than TCPS. The findings reiterates findings of platelet adhesion and adherence that heavy HA samples containing the available HA surface density in the order of 10^{-4} do not have a cytotoxic effect on human platelet cells.

5.7 Conclusion

LLDPE biomaterial fabrication through the HA enhancement process is well documented in previously published literature. The thin plastic sheet biomaterial with HA integration was ready to be devised into the cardiovascular heart valve. However, the thin sheet biomaterial was not optimized for the heart valve leaflet, which is 3D shaped with specific curvature necessary for the best leaflet hemodynamics. This research tested the ability of the thermoforming technique to shape LLDPE thin-film pre-processed with HA biomolecule without degrading HA. It was found that the heater and 3D stretching degraded HA locally, resulting in increased hydrophobicity and physical heterogeneity. Therefore, 3D-shaped leaflets were bio enhanced with HA treatment and characterized for quantifying the available HA population. Leaflet cusp areas that have maximum 3D curvature housed significantly lower amounts of HA compared to relatively 2D parts of the leaflet. Because the curved features do not allow stable meniscus between sample and solution, which prevents the "stick-slip" mechanism from getting consistent HA presence

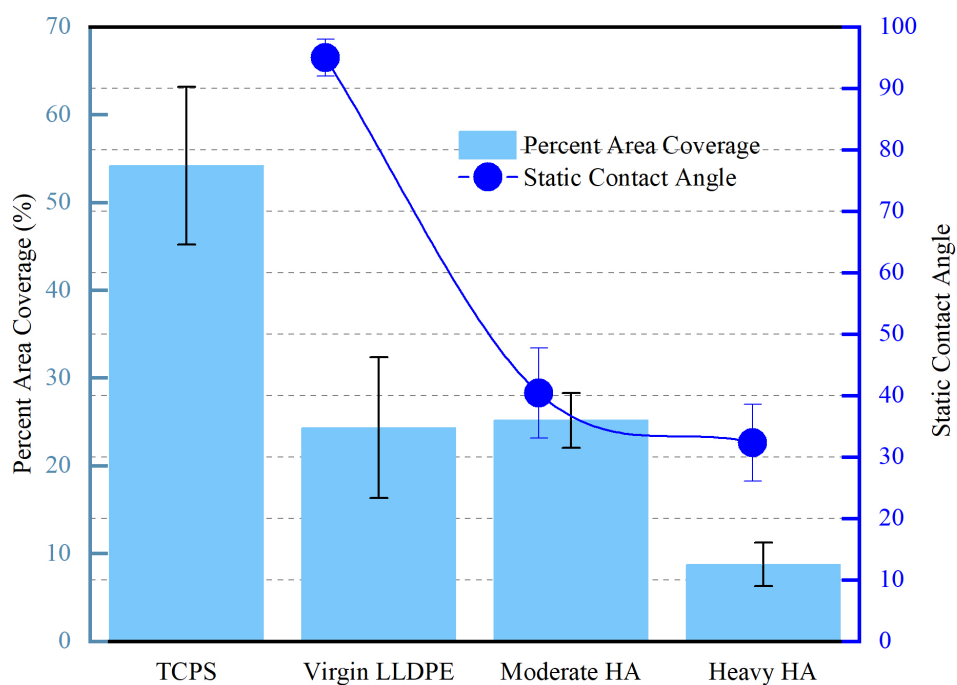


Figure 5.12: Percent area coverage (measured using ImagJ thresholding) of adhered platelets with group respective static water contact angles. Heavy HA samples of average contact angle 30 °have significantly lower platelets covering surface area. Number of samples =3, number of SEM images analyzed =6, $p < 0.05$

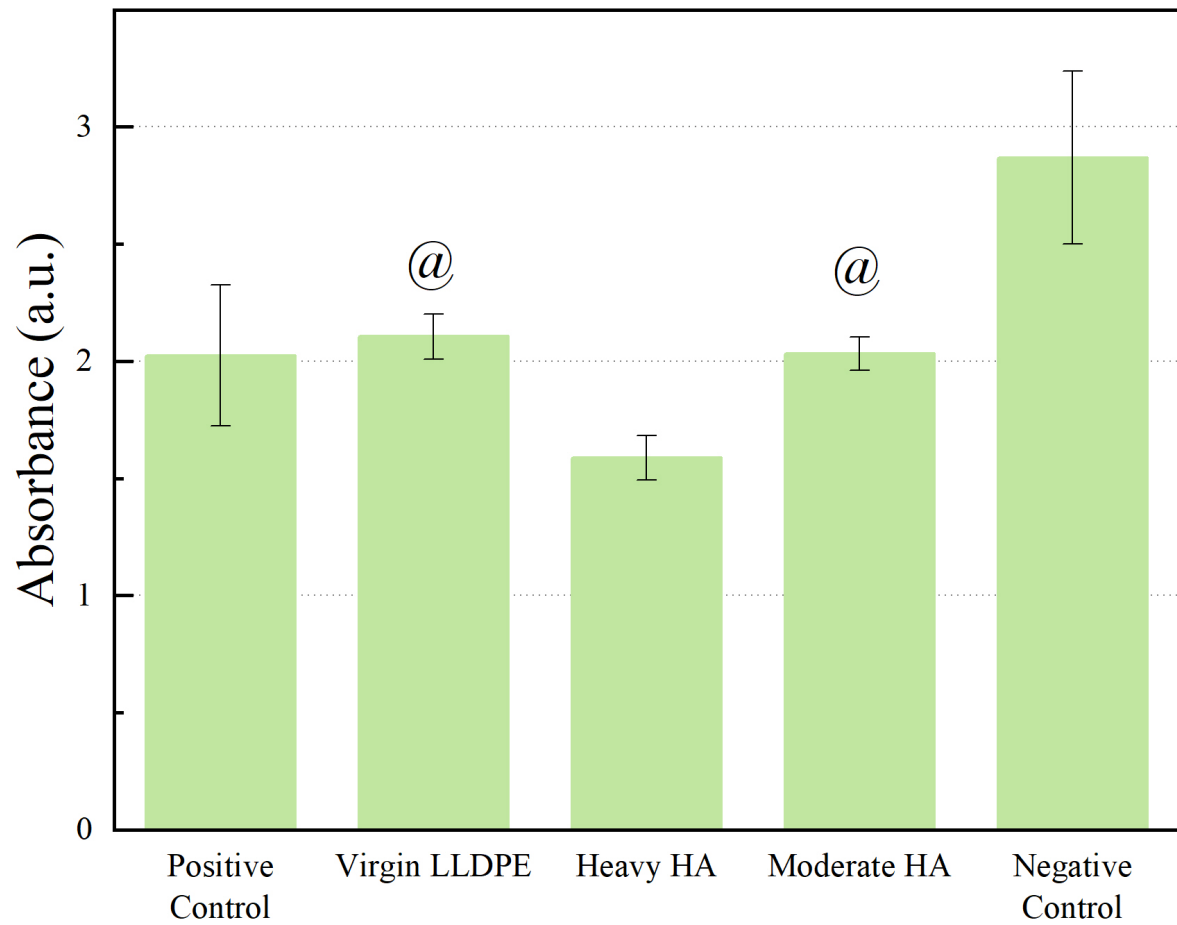


Figure 5.13: LDH assay absorbance measurements for platelet cells. Virgin LLDPE and moderate HA groups are not significantly different, rest of the groups are significantly different. $n=3$ $p<0.05$

throughout the leaflet. Interestingly, only two leaflets were housing HA molecules consistently and homogeneously throughout the leaflet but were not able to reproduce. Therefore future research work should invest in angular controlled dip coating, where either the substrate or the solution is tilted to maintain a constant angle between the cusp surface and the solution

HA treated LLDPE has proven to be biocompatible and anti calcific. However, the optimum amount of HA required to impart biocompatibility has yet to be studied. Therefore, this study compared the biocompatibility of two groups of HA-treated LLDPE populated with HA heavily and moderately. The classification of heavy and moderate was based on the available HA surface density measured in TBO and water contact angle. The whole blood clotting assay could not find any significant clotting resistance between the virgin LLDPE (without any HA) and moderately and heavily populated HA-treated LLDPE, even though a higher sample size and three repeats of the study were conducted. Heavy HA LLDPE had significantly less human platelet adhesion and activation, indicating anticalcific properties. Furthermore, the LDH assay revealed platelet cells were more viable in the heavy HA group than in the standard positive control TCPS. The heavy HA group had an available HA surface density of $10^{-4}\mu\text{mol}/\text{mm}^2$, significantly higher than the moderate HA group ($10^{-4}\mu\text{mol}/\text{mm}^2$). The static water contact angle was 33.17 ± 13 and $57.24\pm 6.3^\circ$ for heavy HA and moderate HA groups, respectively. A general notion is HA surface density is negatively correlated with contact angles, but this study found a moderate linear correlation between them.

Based on the biomaterial characterizations study reported in this chapter, a few conclusions are made:

1. TBO staining and elutes to determine available HA surface density is a better tool for determining the overall HA quantity on biomaterial than water contact angle.
2. ATR-FTIR is a good tool for qualitative and non-destructive methods to confirm HA presence.

3. HA treated LLDPEs must have an available surface density of HA in the order of $10^{-4} \mu\text{mol}/\text{mm}^2$ and a water contact angle of between 20-30° for the most negligible platelet adhesion and adherence.
4. Whole blood clotting resistance assay can be skipped for estimating biocompatibility and anti calcific properties of HA-treated LLDPE biomaterial.

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Appendix A

A.1 Etching of LLDPE using Potassium Permanganate

Objective

Reveal crystalline Lamellae of semicrystalline polymer for SEM imaging. Etching solution conc and time is for LLDPE 80 micron thick 2045G resin.

Materials & Equipment

Personal Protective Equipment

1. Nitrile Gloves
2. Goggles (may need over-the-glasses goggles if using eyeglass)
3. Lab coats

Note: before starting the experiment make sure fire extinguisher and spill kits are readily available. KMnO_4 is extremely oxidizing and can cause fire in contact with acetone or other flammable chemicals.

General Equipment and or glassware

4. 250ml glass beaker
5. Teflon-coated tweezers.
6. Large Crystalizing dish
7. Pipette suction bulb
8. 5 and 10ml macro pipette
9. Stir bar
10. Scintillation vial

11. 100ml glass jar with appropriate cap for storing acid stock solution

Chemicals

1. conc. Sulfuric acid (H_2SO_4), 95% w/w (chem stockroom)
2. 85% orthophosphoric acid (H_3PO_4) (chem stockroom)
3. Potassium Permanganate (KMnO_4 supplied by EHS)
4. 30% w/w. Hydrogen peroxide (H_2O_2) (chem stockroom)
5. Acetone
6. Ice and DI water

Safety Concerns

1. Remove all acetone, xylene, ethanol, methanol, and other flammable chemicals from the fume hood and store them in the designated cabinet.
2. Hydrogen peroxide is highly oxidizing and can intensify fire. Always maintain extra caution while handling.
3. Always add acid to water for dilution slowly dropwise
4. Only use Teflon-coated tweezers while etching
5. Never use water for acid spill, instead use strong bases and absorbent from the spill kit
6. Assess and remove any source of heat before starting the experiment
7. Submit eRFD on the same day of etching
8. Conduct the whole test with another person in the lab

Procedure Preparing all stock solutions They can be prepared the day before etching.

1. Place a 150ml beaker in a large crystallizing dish and fill the crystallizing dish with ice. Prepare 9M, 30 ml (88.2% w/v or 47.9% w/w) sulfuric acid by slowly (dropwise and look for any vapor formation) adding 15 ml (1 part) acid to 15 ml DI (1 part) water under constant stirring. Use macro pipette suction or bulb and 10ml pipette. Don't cross-contaminate the pipette. Store them in the acid cabinet 100 ml glass jar.
2. Place another 150ml beaker in a large crystallizing dish and fill the crystallizing dish with ice. Prepare 22% w/w, 70ml (40% w/v or 4.13M) H_2SO_4 dilute acid solution for washing by adding 20ml of conc sulfuric acid to 54 ml di water under constant stirring. Transfer 5 ml solution to one 20 ml scintillation vial for each sample. For example: 14 samples would need 14 vials and each vial will have 5 ml of the solution to use a total of 70 of the solution. Adjust stock solution volume as necessary. Store them in the acid cabinet.
3. Transfer 5 ml conc. Hydrogen peroxide to one 20 ml scintillation vial for each sample. Keep the peroxide bottle in ice and vials in large crystallizing dish filled with ice to always maintain peroxide temperature below 5 °C. For example: 14 samples would need 14 vials and each vial will have 5ml of the solution to use a total of 70ml of the solution. Adjust stock solution volume as necessary. Store them in the refrigerator under 0-5 °C.

Preparing etching solutions:

1. Place a 250 ml glass beaker in a large crystallizing dish and nicely fill the gap with ice. Place a stir bar inside the glass beaker.
2. Prepare 1.5:1 diluted H_2SO_4 : H_3PO_4 by slowly adding 30ml 9M H_2SO_4 (prepared in previous step 1) to 20ml of H_3PO_4 . Avoid Pipette cross-contamination. Care must be taken during adding to avoid any heat formation. Wait for the mixture to go to room temperature
3. Measure 1g (2% w/v) of KMnO_4 and extremely slowly (a pinch in every 2-3 mins) add to the sulfuric acid and phosphoric acid (step 2 in preparing etching solution) solution. It may take around 30 min to an hour to add all of the permanganates.

4. Maintain 0-5°C temp of the solution and look for color change. If the color changes to green immediately close the fume hood, call EHS, and use a fire extinguisher in case of fire. KMNO₄ can be measured in the balance outside of the hood and carried to the hood, but care must be taken to not leave anything in the balance.
5. Wait 30-45 mins for KMNO₄ to dissolve in the acid solution and the entire solution to go to below 5 °C. Use a thermometer to constantly monitor temperature

Etching Process

6. For 80micron LLDPE use a Teflon coated tweezer to hold and submerge in the oxidizing/etching solution for 20mins. Multiple tweezers can hold multiple samples at the same time, use negative tweezers to hold them so that samples are not lost in the solution. The location that holds the tweezer will not get etched.
7. After 20mins remove the sample, and rinse in water (needle and face side).

Washing

8. Immediately after that put them in scintillation vial with 10ml 22% w/w sulfuric acid, cap the vial and sonicate in ice bath for 5mins.
9. Remove the sample and put it in conc. hydrogen peroxide and follow step 8 for 20 minutes. This is the most critical step for washing
10. Repeat step 8 with water and acetone but 5 minutes for each.
11. Store in scintillation vial in one desiccator 3
12. Gold coat with 10 nm and take SEM images

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A.2 Gel Content Measurement

Objective: Measure gel content of irradiation crosslinked LLDPE

Materials

- 2x Stainless Steel Mesh Cloth (mesh size 120x120, from McMaster-carr , part number 85385T433)
- Stainless Steel String (chem stock room)
- 2x Stainless Steel Binder Clips
- Reflux Column
- 500 mL Round Bottom Flask
- LLDPE Film
- Oil Bath or Heating Mantle

Process Considerations

- Gel Content Measurement is heavily dependent on xylenes, which can be dangerous when inhaled. Take proper precautions when handling xylenes.
- The reflux apparatus utilizes running water to cool xylenes, ensure **NO** leaks are present and that no water will end up in the oil bath if one is used.
- Process can be repeated twice for two samples at a time if needed, however much more caution must be used when putting dissolving samples into the vacuum oven.

- Run the the reflux for few hours after setting up everything before turning on the heat, to observe any water leakage. Preferably, during the whole day samples sit in the xylene and the system runs and at around 8/9 pm turn on the heat. Next morning at 8/9 am after 12 hours samples are removed.

Preparation of Sample and Sample Pouch

- Cut a 40mm x 70mm portion of the stainless steel mesh cloth and fold in half to achieve a 40mm x 35mm piece.
- Cut a 30mm x 26mm portion of the LLDPE crosslinked film to analyze.
- It is more important to have the sample fit securely in the pouch, rather than match the exact measurements.
- Weigh the sample and record.
- Place the sample into the folded pouch so that the sample is touching the folded side.
- Put three staples on the left and right side of the folded stainless steel mesh cloth for a total of six staples used and that one side is still open.
- Weigh the sample pouch with the sample inside and record.
- This can be repeated twice as the procedure can measure the gel content of two samples at once.

Reflux Apparatus Preparation

- Thread a long portion of stainless steel string through the main chamber of the reflux column so that a loop containing two binder clips is hanging from the bottom of the column. Tape steel string on the outside of the column to ensure the string doesn't move.
- See figure 1 for a visual example.

- Assemble a reflux column so that water flows countercurrent to the direction of xylenes. Zip tie all the water connections tightly and use hose clamps after zip ties so no water leakage is found.
- Place an oil bath or heating mantle beneath the column.

Gel Content Measurement Procedure

1. Fill 500 mL round bottom flask with 400mL of xylenes, or until almost full (It is more important to use a volume that covers the entire sample when hanging)
2. Clip sample pouch, or pouches, to the binder clips.
3. Attach the 500 mL flask to the bottom of the reflux apparatus while inserting the sample pouches into the xylenes. (This process can be tricky, but is doable. The sample pouches will need to be folded in thirds, or in any way to push them into the round bottom flask)
4. Once samples are submerged in xylenes and the round bottom flask is securely attached to the reflux apparatus, turn on the oil bath or heating mantle to 110 °C.
5. Let the reflux apparatus run for 12 hours so the sample can dissolve.
6. Preheat a vacuum oven for 150 °C and -25 inHg.
7. Carefully remove the samples from the xylenes and place into a crystallization dish, put the dish into the vacuum oven.
8. Removing the samples can be challenging, utilize long tweezers and any other tools needed.
9. Ensure that the fume hood sash is lowered as much as possible to avoid xylene inhalation.
10. After 1.5 hours, shut off the heat but let the sample keep drying until all xylenes are removed.
11. Remove the pouch from the crystallization dish and record its weight. This weight will be the sample and the pouch together.

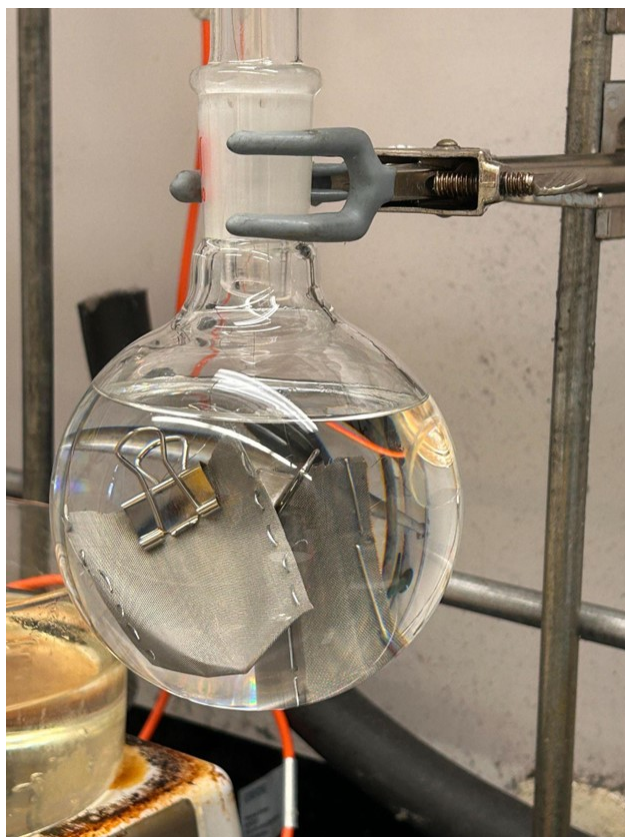


Figure A.1: Stainless steel mesh cloth in xylene

12. Subtracting this weight from the original pouch weight will yield the current weight of the sample.
13. Calculate the gel content percentage by dividing the post-procedure sample weight by the original sample weight.

$$\text{Gel content}\% = \frac{(\text{Sample and pouch wt before reflux} - \text{Dry Sample and pouch wt after reflux})}{(\text{only sample wt before reflux})}$$

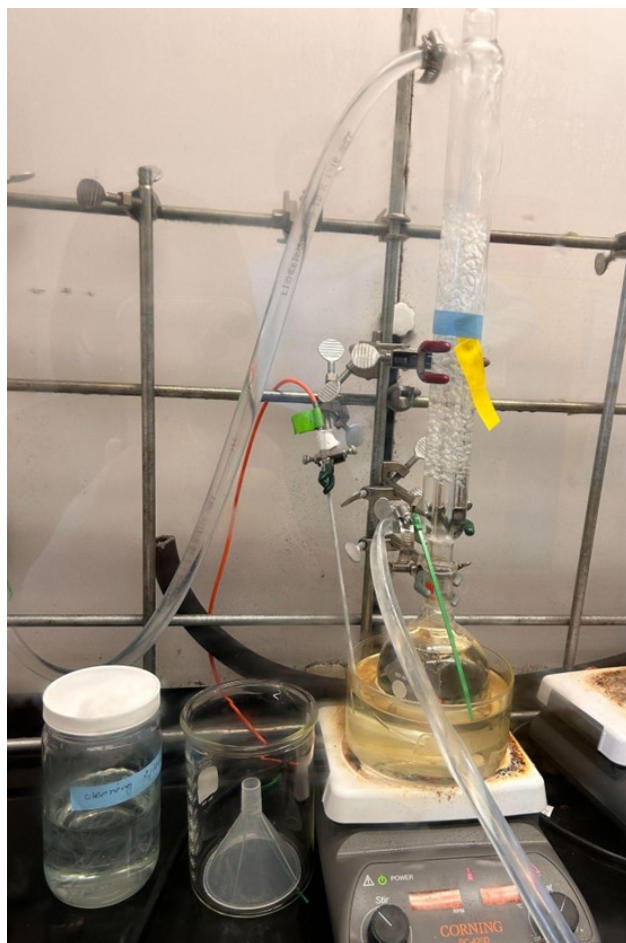


Figure A.2: Reflux Setup

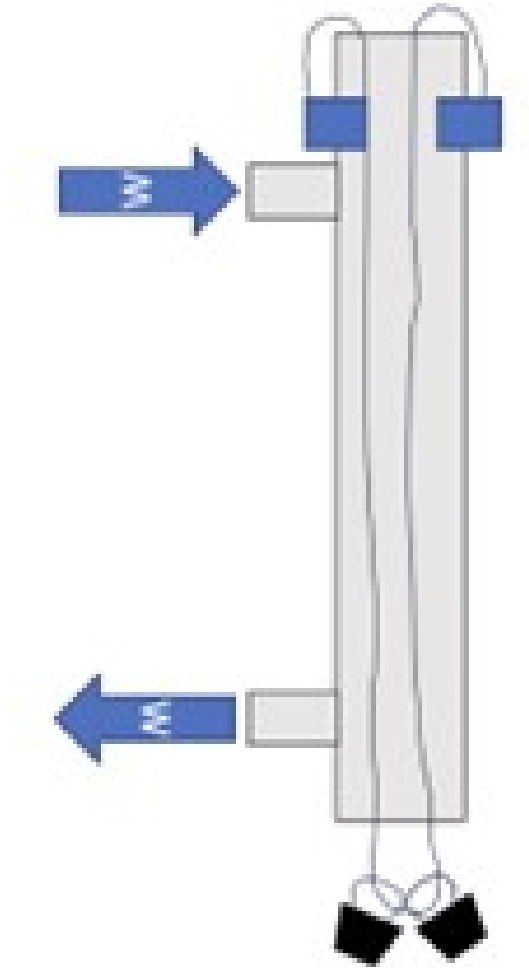


Figure A.3: Schemetic of water inlet and outlet

A.3 Differential Scanning Calorimetry (DSC)

Objective

The purpose of this process is to evaluate several material properties of LLDPE including the peak melting temperature and the crystalline/amorphous composition of the material.

Materials & Equipment

- Personal Protective Equipment (PPE)
- Black Nitrile Gloves
- 80 or 120 micron LLDPE
- Aluminum DSC Hermetic Sample Pan and Cover Set (5mm diameter, bottom flat)
- 5 mm disposable biopsy punch
- Cutting board
- Crucible sealing press and crucible box
- Tweezers
- Scale accurate to 3 decimal places

****NOTE:When operating the machinery, be sure to avoid disturbing the countertop or the cabinetry beneath the DSC in order to avoid inaccurate readings**

A.3.1 Procedure

Setting up the Software

1. Login to the computer and open the DSC software
2. Once the software has loaded, look to the right-hand side of the screen and find the 'RCS 90' button. Click on the button to change it from 'off' to 'on.'

3. Go to TA data > DSC > Group of choice (James) > New folder (enter experimenter's name)
> date of experiment
4. Making the Sample Pan:
5. Remove the crucible sealing press and crucible box from the cabinet and drawer beneath the DSC.
6. Open the crucible box to find two round, blue implements.
7. Take the smaller implement and insert this into the magnetic opening of the crucible sealing press. The larger implement will hold the pan and will go beneath the magnetic opening.
8. Take an aluminum pan and lid and record the mass using a scale.
9. To create a sample, choose your material and lay it flat on the cutting board.
10. Take the 5 mm biopsy punch and make approximately 6-8 punches of the material in order to obtain a sample of approximately 10 mg.
11. Each punch can be extracted one after another using tweezers or the 6-8 samples can be punched successively and all 6-8 samples can be removed at the same time from the punch).
12. Using the tweezers, delicately place the samples in the 5 mm aluminum pan making sure to push the samples as far down into the pan as possible in order to have the most contact with the base of the pan and the material.
13. Place the pan with the samples back on the scale with the lid and record the mass.
14. Subtract the pan and lid mass from the mass of the pan containing the material and the lid to get the mass of the material. (Aim for around 10 mg)
15. Take the larger round, blue implement and place the pan in the opening using the tweezers.
16. Place the implement beneath the magnetic opening of the crucible.

17. Place the aluminum lid on top of the pan, making sure that the pan and lid are aligned.
18. Take the handle of the crucible and move it towards yourself in order to lower the magnetic component of the press to hermetically seal the lid onto the pan.
19. Once lowered all the way, move the handle away from you to raise the press and remove the pan.
20. Take the pan over to the DSC machinery and place the pan in one of the numbered sample spaces (take note of the reference pans, do not place samples in these spaces).
21. Record the sample space selected in your notebook
22. Repeat steps 7-19 for each desired sample.
23. Once all samples have been made, place the crucible back into the cabinet and the crucible box back into the drawer beneath the DSC. This should be done before the DSC runs in order to avoid inaccurate readings from the machinery.
24. Once each sample has been placed in its chosen numbered space, go to the left-hand side of the screen to find the Running Queue.
25. Right click and click "Insert run" for the same number of samples you have prepared.
26. For each run, put the name of the sample, the mass of the lid and pan, and the sample in the spaces where it prompts you to do so.
27. To load the proper procedure, go to the yellow folder icon beneath the section entitled 'Procedure.'
28. Select windows TA > TA data > DSC > James group > Nipa > NK-4014 (make sure this is the procedure file and not the raw data file)
29. To save the data to the correct folder, go to 'File Name' and follow the same instructions in step 3.

30. Check the box that says 'dispose of pan following run'
31. Double check that the correct data for each sample has been filled into each space before selecting 'add to running queue.'
32. Be sure to begin the kiosk through iLab to document how long the machinery was used for.
33. Once added, press the green play button at the top left of the screen. The procedure will now run.

A.4 Tensile Testing using Instron 4442

Objective

The purpose of this process is to determine material properties of LLDPE including tensile strength, tensile yield strength, and elongation at break.

Materials & Equipment

Personal Protective Equipment (PPE)

- Black Nitrile Gloves
- 80 or 120 micron LLDPE
- Instron 4442 Mechanical Tester
- Mechanical press
- Dogbone sample punches (SAMD)
- Tweezers
- X-ACTO blade

**** NOTE: Be cautious when using the X-ACTO blade as it is extremely sharp Procedure Making Dogbone Samples:**

1. Place a sheet of LLDPE on the cutting board surface of the mechanical press.
2. Take the dogbone stencil and place it over the sheet of LLDPE. SAMD has 12mm gauge length custom dogbone punches
3. Pull the lever of the press (SAMD has this press to punch out samples) downwards to press the dogbone stencil into the LLDPE.
4. Take an X-ACTO blade and follow the pattern of the dogbone stencil to make individual LLDPE dogbone samples. Extreme care must be taken to NOT cut the sample other than

the dogbone pattern. Any notch or scratch on the sample can lead to premature failure in validating the results. Make sure to have a very sharp blade to cut sharp and precise samples. Also, change blades often as they lose sharpness

Loading the Software and the Sample:

1. Turn on the Instron 4442 mechanical tester by flipping the switch on the back of the machinery.
2. Be sure to verify that the clamps attached to the Instron are the **50N** load cells, not 500N and respected grips.
3. Click on the tensile testing software and select the desired procedure (JPG 500mm/s)
4. Before loading the sample and adjusting the parameters, be sure to hit IEEE48 to calibrate the machinery so it can detect the load force.
5. To load the sample into the machinery, take a set of tweezers and hold the sample in the middle in order to leave the top neck of the sample exposed.
6. Align the sample in the top clamp so that approximately 2mm of the neck is exposed and not in the clamp.
7. Once you have the desired placement, step on the right clamp peddle that is placed on the floor just beneath the machine to close the pneumatic clamps
8. To ensure there is a strong grip from the clamps, the pressure reading on the air compressor should read between 90 and 120 psi.
9. Align the bottom end of the sample in the same manner with the same 2 mm of neck exposed from the clamp.
10. Ensure that the sample is as straight as possible before clamping the bottom end of the sample

11. When ready to clamp the bottom end of the sample, press the right clamp peddle one more time to have both ends of the sample clamped.
12. If adjustments need to be made, step on the left peddle to removed the grips from the sample.
(check video tensile testing)
13. Once the sample has been loaded, insert your particular parameters including gauge length of the sample (the portion that is not in the clamps) and the thickness of the sample (either 120 or 80 microns).
14. Make sure that the load force is around 0 N (+/- .5 N). This force can be adjusted by using the up and down controls on the bottom left side of the machine. If length adjustments are made, be sure to account for this by changing the gauge length.
15. Following all adjustments to length, recalibrate the change in length to 0.0 mm by clicking the icon in the far top-right of the screen. A non-zero value will not give accurate data.
16. Once all parameters are set, you can test your sample.
17. When the sample tears, remove the sample from the clamps by pressing the left clamp peddle. Make sure the tear happens at least 1 cm above the grips.
18. If you have additional samples to test, click the arrow at the top of the parameters section to move to sample 2.
19. If you do not have additional samples, press 'finish' and save your data to the desired folder.
20. To shut down the machinery, close out of the software.
21. Turn the switch off on the back of the machine.
22. Be sure to clean up any residual material from the area.

A.5 HA-CTA Complexation

Objective

Hydrophobic modification of hyaluronan for reaction in anhydrous solvents

Materials and Equipment

- Sodium hyaluronan (NaHA)
- Cetyltrimethylammonium bromide (CTAB)
- Fresh Deionized water (DI H₂O)
- 1000 ml beaker or flask
- 500 ml beaker or flask
- Magnetic stir bars
- Stir plates
- Freezer mill/Cryogrinder
- Liquid nitrogen
- Hyaluronan-cetyl trimethylammonium complex (HA-CTA)
- Vacuum oven
- Vapor trap
- Vacuum pump
- Thermal gloves
- Safety glasses
- Buckner funnel

- Filter paper
- Erlenmeyer flasks (1L)

Procedure

1. Prepare a 0.30% w/v solution of sodium hyaluronan in DI H₂O. Minimize large clumps when adding NaHA.

a. Example: 1.5g NaHA in 500 ml DI H₂O

b. Stir the reaction at room temperature until the NaHA is completely dissolved. This can take several hours depending on the molecular weight of the NaHA. Stir for 15 hours to 3 days. Parafilm the beaker.

c. When fully dissolved, the solution is clear.

d. To get the NaHA into solution, turn the stir bar RPMs high enough to get a vortex on the top part of the stir bar for at least 5 mins. Then, turn the RPMs down to a low setting to form a little vortex.

e. Record the following in lab notebook:

Date, Time, Mass of NaHA (g) used, Volume of DI H₂O (mL) used, Dissolve Time (from start of mixing to when CTAB is added), Manufacturer, Lot Number, Part Number, and when the bottle of NaHA was opened.

2. Prepare a 1.00% w/v solution of CTAB in DI H₂O.

a. Example: 1.69 g CTAB in 169 ml DI H₂O

b. Stir the reaction at 40°C until the CTAB is completely dissolved. When dissolved, the solution will be clear. This takes 10-15 mins.

c. Record the following in lab notebook:

Date, Time, Mass CTAB (g) used, Volume DI H₂O (mL) used, Dissolve time, Manufacturer, Lot Number, Part Number, and when bottle of CTAB was opened.

3. Slowly add the CTAB solution to the NaHA solution while under magnetic stirring. Parafilm the beaker. The mixture will become increasingly opaque as the CTAB solution is added. When

the reaction is complete, a white precipitate forms and the supernatant is clear. Varying the addition rate affects the size of the precipitate (a slower addition rate produces a smaller precipitate). Stir for 15 hours – 36 hours.

a. Record the following in lab notebook:

Date, and Time of addition.

4. The precipitate is HA-CTA. Collect and wash the HA-CTA to remove excess CTAB by vacuum filtration using a Buchner funnel. Use a vapor trap on the oven.

a. Set up a Buckner funnel to two Erlenmeyer flasks. (pic)

b. Place filter paper on the funnel and wet it using DI H₂O.

c. Pour the HA-CTA/DI H₂O solution into the Buchner funnel slowly to prevent HA-CTA from getting under the filter paper.

d. Rinse the HA-CTA with 500 ml DI H₂O.

e. Use a spatula to scrape the HA-CTA into an Erlenmeyer flask with 300 ml DI H₂O.

f. Cover the Erlenmeyer flask with a serum stopper and shake it for 30 seconds.

g. Pour the contents of the Erlenmeyer flask in the Buchner funnel and vacuum off the water.

h. A second time, rinse the HA-CTA with 500 ml DI H₂O.

i. A second time, use a spatula to scrape the HA-CTA into an Erlenmeyer flask with 300 ml DI H₂O.

j. Cover the Erlenmeyer flask with a serum stopper and shake it for 30 seconds.

k. Pour the contents of the Erlenmeyer flask in the Buchner funnel and vacuum off the water.

l. A third time, rinse the HA-CTA with 500 ml DI H₂O.

m. A third time, use a spatula to scrape the HA-CTA into an Erlenmeyer flask with 300 ml DI H₂O.

n. Cover the Erlenmeyer flask with a serum stopper and shake it for 30 seconds.

o. Pour the contents of the Erlenmeyer flask in the Buchner funnel and vacuum off the water.

p. A fourth time, rinse the HA-CTA with 500 ml DI H₂O.

q. A fifth time, rinse the HA-CTA with 500 ml DI H₂O.

r. Move the HA-CTA to the center of the filter paper, and carefully place the filter paper inside a petri dish. Spread the HA-CTA out.

s. Place the petri dish and filter paper in a vacuum oven to dry at 50°C for 3 days. Occasionally wipe the water off the inside of the oven door. Be sure to watch vapor traps to make sure they don't fill and are functioning correctly. «note: combine with 5 below»

t. Record the following in lab notebook:

Date, Process Start Time, Process End Time, and Oven in Time.

5. Dry HA-CTA in a vacuum oven (-25 in Hg, 50°C) for 3 days or until no change in weight is observed. A yield of about 2.5 g HA-CTA is expected for a starting NaHA weight of 1.5 g.

6. Grind the dried HA-CTA to a powder using a freezer mill/cryogrinder.

a. Wear thermal gloves and safety glasses.

b. Slowly fill the cryogrinder with liquid nitrogen to the fill line. This typically requires about 5L of liquid nitrogen and will cool the cryogrinder down. Close the top cover and let the cryogrinder sit until vapor stops coming out of the rear vent.

c. Weigh the HA-CTA and record the weight.

d. Place the bottom cap on a cryogrinder tube, and place half of the HA-CTA into the tube with a magnet.

e. Place the top on the cryogrinder tube, with the slotted end towards the outside so that it can be removed using the "tool".

f. Insert the cryogrinder tube into the cryogrinder so that the cap slot is aligned with the end of the tube chamber.

g. Use a low impact frequency for a total of 15 mins. Increased cryogrinding time will make the HACTA in fine powder. 15 min cryogrinding time was found to increase particle surface area by decreasing particle size. For three minutes cryo grinding particle size was few mm which was reduced to average 50 μm by 6 mins cryogrinding and further to 25 μm by 10 minutes of cryogrinding. See SEM images attached. Therefore it is recommended to cryogrind for 15 minutes which can give as high as 80% yield of SHACTA

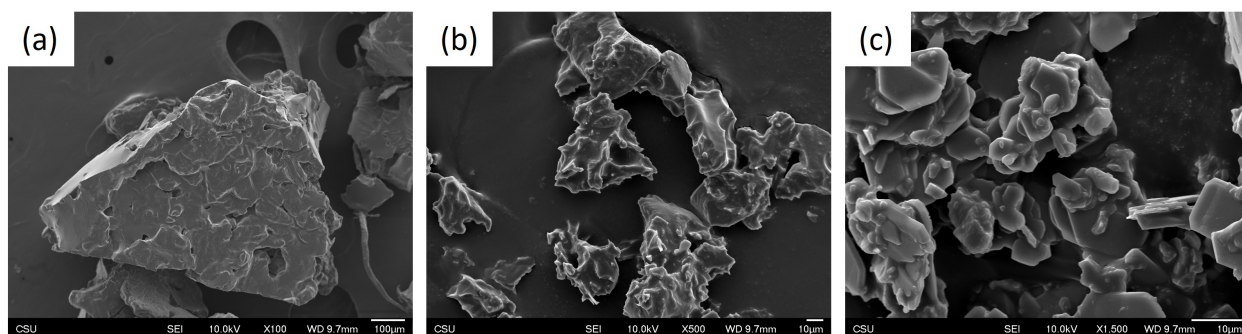


Figure A.4: SEM images of HACTA particles (a) cryoground for 3 minutes (b) cryoground of 6 minutes (c) cryoground for 10 minutes showing particle size reduction with increased grinding time. Also, 10 minutes grinding smoothen particle surfaces

- h. Collect the HA-CTA powder in a 20 ml vial.
- i. Repeat the previous steps to cryogrind the remaining half of the HA-CTA.
- j. Periodically check the liquid nitrogen level and add more if needed.
- k. Clean cryogrinder tubes with 2% Liquinox and DI H₂O. **Do not use solvents, including acetone.** Clean metal caps and magnet with 2% Liquinox, DI H₂O, and acetone.
- l. Record the following in lab notebook:
Date, Process Start Time, Grind Time, Process End Time, and Oven in Time
7. Dry the ground HA-CTA in a vacuum oven (-25 mm Hg, 50°C) for 24 hours or until no change in weight is observed.
8. When dry, HA-CTA should be stored in a desiccator.

A.6 HA-CTA Silylation

Objective

Hydrophobic modification of hyaluronan for reaction in anhydrous solvents

Materials & Equipment

- Hyaluronan-cetyl trimethylammonium complex (HA-CTA)
- Dimethyl sulfoxide $\geq 99.9\%$ ReagentPlus (DMSO) – Part #276855
- Hexamethyldisilazane $\geq 99.9\%$ ReagentPlus (HMDS 99.9% ReagentPlus) – Part # 44019
- Xylenes dried over sieves (250 ml)
- 500 ml Round Bottom Flask (RBF) – 2x
- Graduated cylinders (2x 100 mL)
- Crystalizing dish
- Separatory funnel -125 mL,
- Serum stoppers
- Copper wire
- Needle nose pliers
- Keck clips
- Condenser
- Dry Nitrogen (N₂) gas
- Magnetic stir bars
- Stir plates

- Vacuum oven
- Vapor trap
- Vacuum pump

Before beginning the procedure, ensure that the HA-CTA has been sitting in the oven at 50° C the night before to dry the reactant as much as possible. **Procedure**

Glassware preparation

Acid Bath Cleaning Method for Glassware:

Before beginning, remember to take proper safety precautions. Cover yourself with the appropriate PPE (lab coat, face shield, acid resistant gloves in addition to rubber latex gloves, closed toed shoes, etc.) in order to avoid contact with the acid that you will be using. Tuck lab coat into the acid resistant gloves for optimal coverage. If the acid resistant gloves are loose around the wrist, use rubber bands to create a tighter fit.

1. Begin by turning on the faucet and allow for water to flow constantly.
2. Bring the acid bath over to the sink and remove the lid. This can be accomplished by pushing in the red clip on the cap of the bucket while simultaneously rotating the cap counterclockwise until the cap detaches from the threads of the bucket.
3. Take each piece of glassware needed for silylation and ensure that it is submerged in the acid bath, all the while taking note of any splashing or bubbling that could potentially cause bodily harm. This is especially important in regard to RBF's as they tend to sputter if they are submerged too quickly, so it is always best to do this step slowly.
 - (a) Make note of which hand is being used to transfer the glassware from the counter next to the sink (dry hand) and which hand is submerging the glassware in the acid bath (wet hand).
 - (b) It is important to keep the wet hand over the bucket in order to minimize dripping or spilling.

4. Once all glassware has been placed in the acid bath, liberally rinse the acid-resistant gloves with running water. Make sure to take time on this step, washing between folds in the rubber and between fingers where acid could potentially remain.
5. Once rinsed thoroughly, dry with paper towels.
6. After rinsing the gloves, cap the bucket. Then move the securely closed acid bath with all glassware back to the holding area at the north end of the lab. While transferring the acid bath, do so gently. The glassware is fragile, and oftentimes placed in awkward positions in the bath itself to ensure the best submersion possible.
7. Remove rubber bands and acid resistant gloves and set atop the acid bath bucket.
8. Allow for the glassware to sit for at least 24 hours in acid bath.
9. Before removal, once again make sure that all safety precautions are being taken before working with the acid bath and that all PPE guidelines are being followed accordingly.
10. Once again, allow for both tap and DI H₂O to run constantly in the sink before bringing the acid bath over.
11. Begin by removing each piece of glassware while remembering to maintain a wet acid hand and a water/rinse hand. Once again, the acid hand should be held over the bucket to reduce the chance of drips/spills.
12. Each piece of glassware should be rinsed thoroughly first with tap water, and then finally with DI water.
13. Once all pieces of glassware have been rinsed, place them in the oven until dry.
14. The capping and transfer process of the acid bath does not change from the previous process. Always take care to move slowly and surely with the bath and be on the lookout for any missed drips on the floor.

Silylation of Glassware:

1. Once the glassware is dry, remove it from the oven and allow for it to cool to room temperature
2. When cool, reserve one 25 mL graduated cylinder for 5 mL of HMDS, the rest of the cooled glassware can be placed in the oven.
3. Add 5 ml HMDS to the 25 mL graduated cylinder
4. Place the 25 mL graduated cylinder containing the 5 mL of HMDS in the oven with the rest of the glassware.
5. Turn off the main (blue) vacuum pump and disconnect the outlet tubing.
6. Attach the solvent resistant welch vacuum pump on top of the vacuum pump with the red tubing
7. Pull vacuum to -20 in.Hg, close the vacuum pump valve and shut off the pump itself. At this point, both the atmosphere and vacuum valves should be closed.
8. Turn the heat knob to 150 °C and turn on the heat switch on the vacuum oven. At this point the glassware will be left in the oven for 1.5 hours
9. Wait 30 minutes and check the temperature of the oven by looking at the internal thermometer.
10. After 1.5 hours vent the chamber 5 times.
 - (a) Open the oven to atmosphere letting the pressure reach 0 in.Hg
 - (b) Close the oven to atmosphere and use the welch pump to pull vacuum to -20 in.Hg
11. Repeat these steps 3 times.
12. Remove the red tubing and reattach the main vacuum pump.

13. Turn on the main pump and leave the glassware under vacuum for the duration of the silylation reaction except when retrieving glassware for use.

Add DMSO

Note: DMSO is light sensitive. Be sure to minimize exposure to hood light and outside light. Below calculations and glassware lists are for one RBF or one batch (containing $2 \times 1.5\text{g}$ starting NaHA), which can be repeated in another RBF in another hot plate at the same time to get double production or around 10 g of SHACTA in one week as each RBF will result in around 5g of SHACTA

1. Use a pre-silylated 100 ml graduated cylinder and a 500 ml RBF.
2. Place a stir bar and the cryoground HA-CTA powder (2 vials of HACTA each containing around 1.5g of starting NaHA or around 2.5g of HACTA) into a 500 ml single neck RBF. Be sure a sample for FTIR was taken.
3. Cap the RBF and a graduated cylinder with rubber stoppers and copper wire. The copper wire should be tight and pinch into the rubber.
4. Turn on the dry nitrogen and adjust to a low flow rate.
5. Vent the RBF and graduated cylinder with dry N_2 . Depending on the nitrogen flow, venting five times for five seconds each time is recommended.
6. Add 50 ml of DMSO for every 1.5g of starting NaHA to the RBF via a cannula and dry N_2 , i.e. 100 ml of DMSO for $2 \times 2.5\text{ g}$ of HACTA or $2 \times 1.5\text{ g}$ of starting NaHA. Maintain positive pressure in the graduated cylinder and RBF. Mark the number of punctures in tally form on the bottle.
7. Swell the HA-CTA in the DMSO at room temperature. (about 4-12 hours).
8. Lower the RBF into a 50°C oil bath and continue to stir until the starting material is fully dissolved (4-24 hrs). DMSO/HA-CTA solution should gel preventing the stir bar from

turning once the HA-CTA is “dissolved” **Make sure the thermocouple light on the hot plate is turned on.**

9. Record the following in lab notebook: Date, Time, Manufacturer, Lot number, Part number, date the bottle of DMSO was opened, and time the heat was turned on.

Add HMDS

1. Silylate a 100 ml graduated cylinder.
2. Cap a graduated cylinder with a rubber stopper and copper wire. The copper wire should be tight and pinch into the rubber.
3. Vent the graduated cylinder with dry N₂ before adding HMDS. Depending on the nitrogen flow, venting five times for five seconds each time is recommended.
4. Add 25 ml of HMDS $\geq$ 25 mReagentPlus for every 1.5g of starting NaHA to the RBF via a cannula and dry N₂ while maintaining positive pressure in the graduated cylinder and RBF i.e. 50ml of HMDS for 1.5 $\times$ 2 g of starting NaHA or 2.5 $\times$ 2 g of HACTA . Increase the temperature of the oil bath to 75 °C for 24 hrs. Vigorous stirring is important to mix the HMDS and DMSO layers. Mark the number of punctures in tally form on the bottle. Solution should go from gel to liquid within 10-15 minutes **Make sure the thermocouple light on the hot plate is turned on.**
5. Turn the heat to 60 °C after 24 hrs and allow to stir for an additional 48 hrs. (72 hours total)
6. Periodically check stirring and hot plate temperature. Stirring is important for mixing the DMSO and HMDS to increase the degree of silylation.
7. Record the following in lab notebook: Date, Time, Manufacturer, Lot Number, Part Number, and when the bottle of HMDS was opened.

Separating and washing silyl HA-CTA

1. Cool the reaction to room temperature.
2. Silylate a separatory funnel and a crystallizing dish.
3. Pour the reaction mixture into a 250 ml separatory funnel (125 ml works as well), and let the two phases separate for at least 60 minutes.
4. The **upper** layer contains HMDS and silylated HA-CTA.
5. The bottom layer is DMSO.
6. Let the DMSO drain into an Erlenmeyer flask, and dispose of the DMSO into a hazardous waste bottle **only after you have washed (see below) SHACTA** to avoid disposing SHACTA into hazardous waste .
7. Let the upper layer drain into the second vapor silylated RBF. This RBF now contains the silyl HA-CTA.
8. Close the separatory funnel stopper and add 10 ml of **dry** xylenes. Cap the funnel and swirl the xylenes to rinse the funnel. Collect the xylenes into the RBF containing the silyl HA-CTA. The purpose of this rinse step is to increase the yield of silyl HA-CTA.
9. Wash the silyl HA-CTA using a rotavap. (This helps remove residual DMSO from the final Silyl HA product).
10. Fill the bowl of the rotavap with DI H₂O.
11. Heat the DI H₂O to 60-70 °C. If the water heats to 75 °C, cool it down to prevent degradation of the silyl HA-CTA.
12. Apply vacuum grease to the two stopcocks, to the top surface of the cold finger, and to the inside surface of the RBF condenser as needed (see how to grease a stopcock by Mike).
13. Place the cold finger inside the outer condensing column.

14. Place the rubber gasket flat against the rotavap arm as seen in Figure XX.
15. Screw the grey clamp partially on as seen in Figure XX.
16. Hold the edge of the coldfinger flat against the gasket and screw the grey piece until snug.
17. Attach the RBF condenser using a keck clip.
18. Fill the inner cold finger with ice.
19. Check vacuum tubing connection between the coldfinger and pump.
 - a. Follow pump protocols taped to front of fume hood along with below.
 - i. Make sure the exhaust port of the pump is not blocked by the back wall of the fume hood (or anything else).
 - ii. Run the pump for a few (2-3) minutes before connecting it to the rotovap until it has warmed up.
 - iii. Once finished rotovapping, let the pump run for three minutes disconnected from the system to make sure no vapors remain in the pump.
20. Lower the rotavap arm using the lever so that the RBF containing silyl HA-CTA is partially submerged in water but still able to rotate.
21. Turn the vacuum pump strength to low, and turn the pump on. Wait for the vacuum to pull through the system.
22. Set the rotation speed to 60RPM.
23. Slowly increase the strength of the vacuum until vapor is pulled into the cold finger. Be careful to avoid boiling the solution because this could decrease the yield of silyl HA-CTA by pulling it into the cold finger.
24. When the silyl HA-CTA is mostly dry, turn the rotation off, turn the vacuum pump strength down, and turn the vacuum pump off. At this point, the silyl HA-CTA should be thick like honey, or thicker.

25. Raise the rotavap arm using the lever.
26. Release the vacuum from the system using the upper stopcock, and let air back into the tubing by opening and closing the stopcock a few times.
27. Gently twist and pull the RBF containing silyl HA-CTA off of the rotavap.
28. Add 40 ml of **dry** xylenes to the RBF, cover with a serum stopper, and dissolve the silyl HA-CTA by swirling the flask.
29. When dissolved, uncap the RBF and attach it to the rotavap using a keck clip.
30. Wash the dry xylenes as in the previous steps.
31. Add dry xylenes 4 more times and wash as described, for a total of 5 washes with xylenes (in addition to the first wash in HMDS). The sample should be allowed to crystallize on washes 2 and 4 and the time recorded for the sample to dissolve back into xylenes recorded.
32. On the last wash, leave a few milliliters (5 ml) of xylenes in the flask.
33. Pour the silyl HA-CTA/xylenes into a silylated crystallizing dish.
34. Add 5 ml more xylenes to the RBF to dissolve any remaining silyl HA-CTA, and pour it into the same crystallizing dish.
35. Dry Silyl HA overnight at room temperature. Cover with aluminum foil that has holes in it. Puncture the aluminum **before** covering the silyl HA in order to avoid contamination.
36. Dry the silyl HA-CTA at 50°C using a vapor trap until no weight change is observed. Save a sample for NMR analysis. A yield of 2.5-3.0 g of silyl HA-CTA is expected when starting with 1.5 g NaHA. Dry Silyl HA overnight at room temperature. Cover with aluminum foil that has holes in it. Puncture the aluminum **before** covering the silyl HA in order to avoid contamination.

37. Record the following in lab notebook: Date, Time, Oven in Time, Oven out Time, and Final Weight.

38. Record all information in the Silyl HA Tracker in the BREL drive.

Note

Cannula transfers should be done with at least two people. Never try a cannula transfer alone.

A.7 SHACTA Treatment of LLDPE Shaped Leaflet with Vapor Cross-Linking

Objective

Shaped Linear low-density polyethylene (LLDPE) will be integrated with silylated hyaluronan-cetyltrimethylammonium complex (SHACTA) by swelling the LLDPE in a solution of SHACTA and xylenes followed by vapor cross-linking. The volumes and masses given in this procedure are specific for 40-45 mm long LLDPE shaped leaflets. ***Materials & Equipment***

-
- One 500 mL round-bottom flask (*Vapor Silylation*)
- 100 mL graduated cylinder (*Vapor Silylation*)
- 25 mL graduated cylinder (*Vapor Silylation*)
- 100 mL glass jars with caps (*Vapor Silylation*)
- Six small circular magnets (1x1/4x1/8 inch (Diameter x Inner Diameter x Thickness)) magnets.
- Teflon ring (Outer diameter 1 inch, inner diameter half inch, height 10mm, 3mm hole)
- Teflon disk 2 inch diameter , 15 mm long
- Hose clamp (18-32mm)
- 125 ml Erlenmeyer flask (*Vapor Silylation*)
- Two 150 ml beakers (*Vapor Silylation*) (*One for each film being treated*)
- Two Glass funnels (*Vapor Silylation*)
- One 600 ml beaker (*Vapor silylation*) (*Optional for transfer of SHACTA/xylene solution*)

- Large crystallizing dish
- Dowlex 2045G LLDPE films vacuum shaped to a length of 42-45mm
- Xylenes on activated sieves
- Solvent vacuum oven
- Teflon stoppers
- Teflon stir bars
- SHACTA
- Hot plate with thermal probe
- Heating block
- Infrared thermometer
- Needle-nosed tweezers
- Toluene 2,4-diisocyanate (TDI)
- P1000 Micropipette and tip
- NaCl
- Ethanol

Notes before starting

Untreated LLDPE Leaflets can be cleaned in bulk and stored

TDI is extremely toxic; handle with extreme care.

All xylenes used should be dried on activated molecular sieves (except for xylenes used in first step “Preparing LLDPE films”)

Procedure

Preparing LLDPE Films

1. See Thermoforming protocol for preparation of shaped LLDPE leaflets.
2. Soak the films in xylenes for 12 hours at room temperature. Xylenes do not have to be distilled.
3. If treatment is planned for the next day, prepare the following glassware (acid bath for 24 hours and dry in blue oven for 24 hours followed by silylation):
 - a. 1x 500 mL round-bottom flask
 - b. 1x 100 mL graduated cylinder
 - c. 1x 25 mL graduated cylinder
 - d. 4x100 mL glass jars
 - e. 2x 150 ml beakers
 - f. 2x Glass funnels
 - g. 1x 125 ml Erlenmeyer flask
4. After 12 hours pull leaflets from xylenes, rinse with acetone.
5. Line a large crystallizing dish with a large kimwipe.
6. Place clean leaflets into lined crystallizing dish. Ensure they do not touch the glass.
7. Dry films in solvent vacuum oven for at least 3 hours. Store in a labeled jar.

Silylation of Glassware

1. Once the glassware is dry, remove it from the oven and allow for it to cool to room temperature
2. When cool, reserve one 25 mL graduated cylinder for 5 mL of HMDS, the rest of the cooled glassware can be placed in the oven.
3. Add 5 ml HMDS to the 25 mL graduated cylinder
4. Place the 25 mL graduated cylinder containing the 5 mL of HMDS in the oven with the rest of the glassware.
5. Turn off the main (blue) vacuum pump and disconnect the outlet tubing.

6. Attach the solvent resistant welch vacuum pump on top of the vacuum pump with the red tubing

7. Pull vacuum to -20 in.Hg, close the vacuum pump valve and shut off the pump itself. At this point, both the atmosphere and vacuum valves should be closed.

8. Turn the heat knob to 150 °C and turn on the heat switch on the vacuum oven. At this point the glassware will be left in the oven for 1.5 hours

9. Wait 30 minutes and check the temperature of the oven by looking at the internal thermometer.

10. After 1.5 hours vent the chamber 5 times.

a. Open the oven to atmosphere letting the pressure reach 0 in.Hg

b. Close the oven to atmosphere and use the welch pump to pull vacuum to -20 in.Hg

11. Repeat these steps 3 times.

12. Remove the red tubing and reattach the main vacuum pump.

13. Turn on the main pump and leave the glassware under vacuum for the duration of the silylation reaction except when retrieving glassware for use.

Sample holding apparatus preparation

1. Place the teflon ring with hole top down, insert elevator rod through the hole.

2. Place one small magnet on top of the ring.

3. Place small teflon ring (5mm height) on top of the magnet.

4. Place shaped leaflet sample on top of the stack so that the stack is covered by LLDPE, but the hole of the rod-holding teflon is exposed.

5. Using a hose clamp, tighten the apparatus to ensure the sample is secure.

See figure 1 and 2 for visual clarification. 6. Repeat for two samples for the treatment.

Swelling in SHACTA and Xylenes Solution

1. Prepare a 1% (w/v) solution of SHACTA and xylenes, 100 mL for every sample.

Example 1 – 1 gram of SHACTA in 100 ml dry xylenes

Example 2 – 0.5 grams of SHACTA in 50 ml dry xylenes

- a. Measure out the SHACTA into the silylated RBF.
 - b. Collect a Teflon stir bar, Teflon stopper, RBF with SHACTA, silylated graduated cylinder, and dried xylenes.
 - c. Add the appropriate volume of xylenes for a 1% (w/v) SHACTA xylenes solution.
 - d. Stir at 50 °C until all the SHACTA is dissolved.
2. Warm heating block to 55 °C. Setting on block should be around 63 °C causing the average measured by the infrared thermometer to be 55 °C from top and bottom of tape on aluminum mantle.
 3. Using two magnet on the top of the glass jars caps, place the samples underneath the caps and secure the samples in place.
 4. Place glass jars in the block.
 5. Use an infrared thermometer to confirm the heating block has reached 50 °C.
 6. Measure out 100 mL of SHACTA solution into each jar.
 7. Cap jars and swell for two hours.
 8. Preheat vacuum oven to 50 °C

9. Film Elevator Prep

- a. Ensure batteries are fully charged
 - b. Determine rate of withdrawal and check “Rate of Elevation vs Dial setting” PDF. Generally to get the max HA on the sample withdrawal rate should be 20/32 inch per hour. Dial on between 1 and zero generally gives this rate. If HA over the whole sample is desired with lower amount, 1 inch/hr withdrawal rate should be used.
 - c. Reset screw to 11/32 inch (drop from seat at about 6/32”)
 - d. Turn on Elevator briefly to ensure motor is functioning and gears are locked in and turning
10. Disconnect the solvent vacuum from the pump and connect it to the blue vacuum pump
 11. Remove gasket from oven and place film elevator inside along with 2 inch dia Teflon discs inside of 150 ml beakers, HDPE blocks replace gasket

12. After swelling is complete (two hours) unscrew caps and remove samples from lids and insert aluminum rod through the rod-holding teflon spacers hole.

13. Hang films from elevator aluminum rod

14. Pour the SHACTA solution into the beakers

15. Place films into beakers with SHACTA solution with Teflon block underneath

16. Keep air flowing out of the oven while the house vacuum continually pumps it down to maintain a pressure at -15 in. Hg while films are lifted from solution

17. When elevation is complete hang films over beaker or place in clean crystalizing dish with kimwipe. Generally it takes 1.5 hours to complete elevation. The elevator might jam when trying for lower speed and not withdrawal at all. At those time remove the elevator and adjust the rotor outside of the oven. If the rotor is engaged with base gear it shall move.

Vapor Cross-Linking:

1. Prepare a 10% (v/v) toluene diisocyanate (TDI) solution in xylenes, 10 mL per sample.

Note: TDI is extremely volatile and toxic, use with great care.

- Collect a P1000 micropipette (set at the correct volume for TDI), its tip, TDI, a kimwipe, distilled xylenes, a silylated graduated cylinder, a 125 mL Erlenmeyer flask with a stir bar, and a Teflon stopper
 - Measure out the correct volume of TDI with the micropipette into the Erlenmeyer.
 - Measure out the correct volume of xylenes into the Erlenmeyer.
 - Place stopper in the Erlenmeyer and stir for 15 minutes.
2. Keep the pipette tip that contacted TDI in the fume hood.
3. While TDI is dissolving, replace the rod-supporting teflon spacer with another small teflon spacer in the sample's holding apparatus.
4. Warm the heating block up to 60 °C. (Set at 68 °C)

- Note the heating block heats unevenly and should have an average of 60 °C the bottom will be about 8-10°low and the top 8-10 °C high

5. Using a magnet on the top of the glass jars caps, place the samples underneath the caps and secure the samples in place.
6. Use the infrared thermometer to confirm that the heating block has reached 60 °C.
7. . Pour 10 mL of the TDI solution into each jar.
8. Twist the caps back on the glass jar. Make sure the samples do not touch the solution.
9. Cross link for one hour and thirty minutes in glass jars.
10. Remove samples from their respective holding apparatus and dry in the solvent vacuum oven on a kimwipe-lined crystallizing dish.

Hydrolysis

1. Prepare a 1:1 solution of 0.2M NaCl to ethanol, about 150 mL per sample.
 - a. Ex 2.34g NaCl in 200 ml DI water then add 200 ml ethanol
 - b. $0.2(\text{mols NaCl}) \times 58.44(\text{g/mol NaCl}) \times 0.2(\text{l DI water}) = 2.34\text{g NaCl}$
 - i. Note 0.2 M solution is for NaCl in DI water and the final solution is 0.25 M
2. Run sonicator for 30 minutes to de-gas.
3. Place each sample into a small glass jar cusps down. Fill each jar with roughly 30 mL of hydrolyzing solution and sonicate for 60 minutes. Keep the temperature between 30 °C and 40 °C.
4. Repeat for 3 total cycles, 60 minutes each. Switch the solution out each time.
5. Prepare a 0.2 M NaCl solution, about 30 mL per sample.
6. Use tweezers to pull the samples out of the NaCl ethanol solution and rinse with DI water.
7. Place samples in new small jars.
8. Fill the jars with roughly 30 mL of the 0.2M NaCl solution.
9. Sonicate for 60 minutes. Keep the temperature under 40°C.
10. Prepare a 3:2 solution of water:ethanol, 30 mL per sample.
11. Use tweezers to pull the samples out of the 0.2M NaCl solution and rinse with DI water.

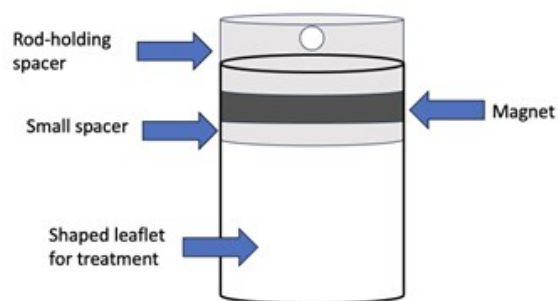


Figure A.5: *Sample holding apparatus without hose clamp for visualization purposes.*

12. Place samples in new small jars.
13. Fill the jars with water:ethanol solution, cover with watch glasses, and let sit for at least 2 hours.
14. Rinse samples with DI water and put them in new small jars.
15. Sonicate for 30 minutes in DI water.
16. Dry in the water oven on a kimwipe-lined crystallizing dish.

Figures

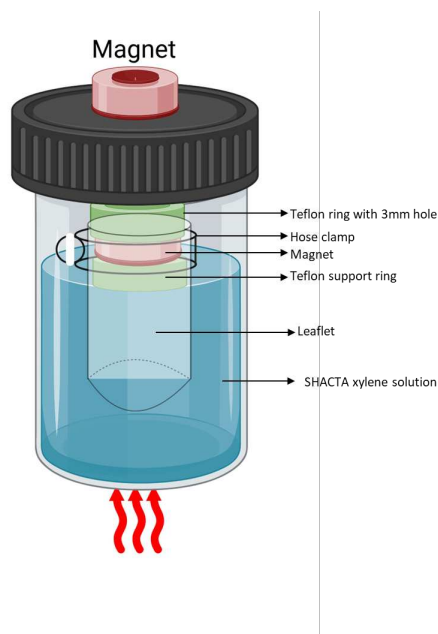


Figure A.6: *Sample holding apparatus during swelling*

A.8 Toluidine Blue O Staining Assay

Objective

TBO (toluidine blue O) assay is used to identify acidic tissue molecules. On HA-LLDPE treated samples, TBO binds to the carboxyl groups in HA staining them blue/purple, differentiating them from the cross-linker and the LLDPE film.

Materials & Equipment

- Scintillation vial & cap
- Small stir bar
- TBO
- Urea
- X-Acto knife
- Needle

Notes before starting

TBO stained items can be cleaned with ethanol or 2% Liquinox.

Use stir bars that already appear to be stained blue if possible. **Procedure Prepping HA-**

LLDPE Samples

- Hydrate samples in DI H₂O for at least 24 hours prior to staining
- Dry samples with a Kim Tech wipe prior to placing in TBO assay to avoid diluting the solution.

Making 10mL 0.1 % TBO Stain in 8M Urea

1. Measure 10mL DI H₂O and add to cleaned scintillation vial with stir bar.
2. Add 4.8g urea to scintillation vial.
3. Stir at room temperature for 30 minutes.
4. Add 0.01g TBO to aqueous urea solution allowing it to dissolve completely

Staining Samples

1. Place sample on needle by puncturing an edge*
2. Submerge sample in TBO solution for 20 minutes at room temperature.
3. Rinse excess TBO using DI H₂O, leaving behind bound TBO.
4. Dip stained sample in fresh DI H₂O and agitate until excess dye is leached out
5. Dry sample on Kim Tech wipe.
6. Place sample in vial and allow to dry in drying oven.
7. Take photos of samples from each treatment group .

A.8.1 Toluidine Blue Elution

Objective

TBO stain on HA-LLDPE samples can be completely eluted in 50% Acetic Acid leaving behind a blue solution. The solution's absorbance can be measured in a plate reader at 630nm in order to approximate HA surface density on a sample.

Materials & Equipment

- TBO stained HA-LLDPE films
- Stock Glacial Acetic Acid (17.4M , pH = 1.25)
- DI H₂O
- Scintillation vials
- Micropipettes and tips
- Beaker
- Stir bar
- Macropipettes
- Kim Tech wipes
- Vortex machine
- Well plate (96 flat bottom)

Notes before starting

- See TBO Assay Protocol for staining procedure
- Acetic acid waste should be placed in its own container
- Take photos of samples before and after elution

Procedure

Making 50% Acetic Acid (50mL solution)

Place 100mL beaker with magnetic stir bar on stir plate and add 25mL of DI H₂O using a macropipette

Slowly add 25mL stock glacial acetic acid to DI H₂O while stirring using a macropipette

Be sure to use two different pipettes in order to avoid cross contamination

Stir for 10 minutes before using

Making Standards and Serial Dilution Ladder

Keep 50% Acetic Acid in DI H₂O constant. Test varying TBO concentrations. See TBO Elution Calculations below to see example dilution and ladder calculation

Make 10mL of 1000 μ M TBO solution in 50% Acetic Acid*. **Note this can also be completed at 10x concentration so as to use a higher mass of TBO as most balances only measure to the 1/1000th of a gram**

1. Add 5mL stock glacial acetic acid to 5mL DI H₂O, mix well
2. Add 0.00305g TBO, vortex for 30 seconds to mix well (*0.0305 grams for 10x concentration*)
3. Dilute down to 2mL of 100 μ M TBO solution in 50% Acetic Acid
4. 200 μ L of 1000 μ M TBO solution and 1800 μ L of 50% Acetic Acid in DI H₂O
5. Perform serial dilutions to create standards
6. Recommended TBO Concentration Range: 0-25 μ M TBO
7. Measure absorbance of 100 μ L of each dilution in triplicate in plate reader at 630 nm
8. Plot Absorbance vs. TBO concentration to create ladder
9. *1000 μ M TBO solution made due to accuracy restrictions of measuring small amounts of TBO

Prepping Samples

- Cut samples into relatively equal rectangles*(or 8mm punch), and measure surface area.
- Be sure to **double the area** when doing calculations in order to account for both sides of the sample
- Attempt to cut representative sample (not a completely dark region, not completely light region, do not include grooved edge);
- If using 8mm punch, use entire sample
- Hydrate TBO stained samples for at least one hour prior to elution
- Dry samples with Kim Tech wipe before placing in elution solution to avoid dilution
- *rectangles allow for more accurate area calculations

Eluting TBO from Samples

1. Submerge sample in 10mL of 50% Acetic Acid solution for one hour
2. If using 8mm punch, use 3mL of 50% Acetic Acid solution for elution
3. Agitate solution occasionally (ideally at least 3-4 times over the hour of elution)
4. After one hour, remove samples from solution
5. Rinse samples well with DI H₂O
6. Dry samples with Kim Wipe
7. Take 3, 100 μ L samples from each solution and place in well plate
8. Measure absorbance at 630nm
9. Save excel file and calculate HA surface density with equation below.
10. Note 0.003 L is assuming 3 ml 50% Acetic Acid solution was used for elution

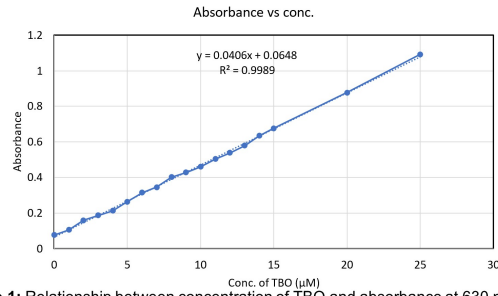


Figure 1: Relationship between concentration of TBO and absorbance at 630 nm.

Figure A.7: TBO elution ladder

Example Calc

$$TBO \text{ Surface Concentration}(\mu\text{mol}/\text{mm}^2) = \frac{TBO \text{ concentration}(\mu\text{mol}/L) \times 0.003(L)}{2 \times \pi \times (4(\text{mm}))^2}$$

A.9 Whole Blood Clotting Protocol

Objective

To determine if the material treatment has reduced acute whole blood clotting.

Materials & Equipment

- Petri dishes (for pipetting blood onto the samples, one for each treatment group)-Never used
- 24 well plates (for freeing hemoglobin)(VWR Tissue culture plate, cat 7342324)
- 96 well plates (for plate reading) (96 Grenier well plate F bottom)
- DI water
- DI water is used to release free hemoglobin from red blood cells that were not trapped in a blood clot, after clotting time has elapsed.
- Timer
- 20 µl and 1000 µl pipettes and pipette tips (popat lab)
- Vacuum tubes which have not been coated in anticoagulant. (BD Vacutainer REF 366703)
- Plate Reader (Dr. Popat's Lab)

Safety Considerations

Work carefully and avoid contact with blood at all times.

Remember to take the Blood-borne Pathogens (BBP) Precautions and Training offered by CSU. Wipe pipettes with 70% ethanol after finishing everything. Avoid bubbles when pipetting blood

Note: Blood must be drawn on site so that it can be immediately transferred onto samples/well plates!!! You must make an appointment to have the phlebotomist come to the lab. Have screening and consent form printed out and signed by the donor.

Preparation

Determine how much blood and how many samples are needed prior to the study. Use six samples for each time point and a total of 12 samples per group for two different time points (15 min and 30 min). Total samples needed per treatment group are 12. Each sample will have 10 μ L of whole blood pipetted onto the surface. A 1:100 Blood-to-DI water ratio will be used for data analysis.

- **Treatment groups are as follows**

1. TCPS (negative control: empty well plate cells)
2. Untreated LLDPE (No HA, vacuum-shaped leaflets clean in xylene and acetone)
3. Heavy HA (HA treated)
4. moderate HA samples (HA Treated, including cusps)
5. Positive control (TCPS cells with water)

- Determining Number of Samples and Total Volume of Blood for 4 treatment groups.

12 samples x 10 μ L of blood= 120 μ L of blood per treated group 120 μ L of blood x 4 groups= 480 μ L total blood for treatment 480+ 60 of blood for t_0 (10 for 6 wells) = 540 μ L total blood for the study

- One blood vacuum tube holds approximately 6 ml of blood. Total three vials of blood each vial containing 3ml was collected from donor. First vial was discarded
- Make sure to label the 24-well plate according to the time points and materials which will be used. Only one 96 well plate is needed for plate reading. At least 3 wells should be blanks.

Soak samples at least 2 hours prior to the study to swell the hyaluronan.

- For 8 mm samples, be sure to use carbon tape to seal them on center of the 24 well plate

- and have them hydrated for at least 2 hours prior to the test. Samples should be removed from DI water, blotted dry, and taped to well plate immediately prior to blood draw. The well plate should be covered to prevent HA from drying out.
- Pipette 1000 μL and 200 μL of DI water into each T_0 and blank well, respectively
- Cover all well plates while waiting for the phlebotomist

Procedure

1. Have phlebotomist draw blood into vacuum tubes which have not been coated with anticoagulant. The first will be thrown away as it contains the skin plug and locally activated platelets. The second will be used for the study.
2. Pipette 10 μL of blood into each T_0 well of the 24-well plate. These should have 1000 μL of DI water in them. Gently agitate on the Titer plate shaker for 30 seconds at 100 RPM. Let wells sit to allow the free hemoglobin that was not trapped in the blood clot to be released into the water for 5 minutes.
3. Transfer 10 μL of whole blood onto each of the T_{15} samples. Start the timer as the first drop is placed on the samples. Ensure the blood is in the center of the materials. Be sure to change pipette tip each time to avoid cross contamination. DO NOT reuse pipette tips.
4. Repeat step 3 for T_{30} samples.
5. As each time point arrives, transfer 1000 μL of DI water (in the same order in which blood was transferred) to the corresponding wells on the 24-well plate.
6. Gently agitate on the Titer plate shaker for 30 s at speed 1. Let sit for five minutes to allow the free hemoglobin that was not trapped in the blood clot to be released into the water.
7. After 5 minutes, pipette 200 μL of the blood +DI solution into the 96-well plate. Be sure to change pipette after pipetting each group tip to avoid cross contamination.

8. Analyze results using the plate reader at 540 nm wavelength. Open Whole Blood method on the plate reader in Popat's Lab.

A.10 Platelet Activation and Cayman LDH Cytotoxicity Assay

Objective To expose a material to human platelet cells for later evaluation of how those cells interact with the material surface.

Materials & Equipment for platelet activation study

- Plate Shaker
- 20, 200 and 1000 μ L pipettor and tips (sterile in biosafety cabinet)
- 48 and 96 well plates (96 well plates need to be sterile, take the whole pack into the cabinet after sterilizing the packet)
- Sterile Phosphate buffer saline (PBS) (The Biosafety cabinet has sterile PBS in 50 ml conical tubes, refile them with sterile PBS from outside of cabinet)
- Sterile tweezers for transferring samples
- EDTA coated Vacutainer tube (popat lab)
- Sterile water (same as PBS, Popat lab has them)
- 50 ml conical tube for PBS and water

Materials & Equipment for platelet fixation for SEM study

- Sodium cacodylate Trihydrate (Popat lab)
- Sucrose
- Glutaraldehyde 25% in DI water (371 refrigerator, -20 degree C)
- DI water
- Ethanol
- beaker (to make buffer/fixative solution)

Materials & Equipment for LDH cytotoxicity study

- Cayman LDH cytotoxicity Assay Kit (item no 601170)(one for each donor study)
- Sterile 15ml centrifuge tubes (popat lab)
- sterile 600 μ L centrifuge tube (popat lab)

Safety Considerations

Work carefully and avoid contact with blood at all times. Remember to take the Blood-borne Pathogens (BBP) Precautions and Training offered by CSU.

Process Considerations

1. If using aluminum or styrene pucks to submerge samples, be sure to sand the puck well enough to remove rough edges that can activate platelets. **No Pucks were used. Samples were directly taped to the well plate.**
2. Be gentle, work diligently and slowly to prevent activation of platelet.
3. Avoid bubbles when pipetting blood and plasma. It was not possible to redo the LDH study with one Caymann assay, and predicted that the assay was not saved properly. it is recommended to use new assay per donor

Reagent preparation for LDH Assay: prepare in sterile environment little before using

1. **Assay Buffer:** Dissolve the cell-Based Assay Buffer Tablet (item No. 10009322) in 100ml of sterile water using the same bottle the tablet comes in. This buffer should be stable for approximately one year at room temperature.
2. Add 150 μ L sterile water to LDH Diaphorase vial (10009318) vial. This solution can be thawed only once, therefore, remove 55 μ L of the solution and leave them in 600 μ L sterile centrifuge tube (-20 degree C). Later on thaw only one vial to use.

3. LDH Reaction Solution: To make 4ml of LDH Reaction Solution add 40 μ L of the following to 3.84 ml of Assay Buffer

- NAD⁺ (100X) (item No. 10009319)
- Lactic Acid (100x) (Item No. 10009320)
- INT (100x) (item No 10009328)
- LDH Diaphorase

Procedure

1. Sample Sterilization: Sterilize each sample container, tape, 48 well plate (packet) and take them to biosafety cabinet. Tape all samples to the well plate, remember to mark sample groups. Add 70% ethanol to samples for at least 30 mins. Rinse twice with sterile water, 2x with PBS and leave them in water overnight. One hour before test, aspirate water and add PBS.
2. Treatments groups are as following with n=3
 - TCPS pucks made by cutting a well plate into 8mm punch. Punches are cut from bottom of 48 well plate in chemistry B1 (Tom Frederick) . Sand rub the edges to avoid platelet activation
 - Heavy HA treatment group
 - moderate HA treatment group
 - LLDPE
 - Empty cells which will contain only PRP (spontaneous release)
 - Empty cells which will contain PRP then 10% Triton X-100 solution (maximum release)

3. Collect blood from the phlebotomist and store them in EDTA coated tubes to prevent coagulation. Each tube can hold approximately 2 ml of plasma.
4. Using a centrifuge, spin blood at 150 g for 15 minutes and let it rest for another 15 minutes. Place tubes of the same level of blood opposite to each other in the centrifuge.
5. There should be two visible layers of solution in the centrifuged blood tube: platelet rich plasma (PRP) on top, and blood cells (bottom). The buffy coat is located in the middle, but this is not always noticeable with the naked eye.
6. Aspirate PBS from the wells containing samples,
7. Pipet 400 μL of PRP into each well of the 48 well plate..
8.
 - (a) Draw 400 μL of PRP at each well plate and prevent liquid entering and sucking blood.
 - (b) Draw only 2/3 of plasma layer to prevent the risk of drawing blood and the buffy coat.
 - (c) If the blood is mixed accidentally, centrifuge the tube for 5 minutes.
 - (d) Avoid pipetting PRP onto the sample.
9. Sterilize the shaker and leave it inside the popat incubator and plug to outside outlet. Put well plate on the shaker storing in the cell culturing incubator (37 C, 5% C02), and shake at 100 RPM for 2 hours. While waiting for the two hour prepare the fixation and buffer solution for SEM fixation which can be done bench top and does not require sterile environment. Also prepare the LDH reaction solution in the meantime in sterile environment.
10. Add 40 μL of 10% Triton X-100 solution to three cells that does not have any sample or TCPS and has only PRP. Then put well plate on a shaker storing in the cell culturing incubator (37 C, 5% C02), and shake at 100 RPM for 15mins
11. Transfer 100 μL of PRP cell supernatant from each cells of 48 well plate to a new 96 well assay plate. Save the remaining 300 μL PRP cell supernatants from each cells of 48 well plate to a another 96 well plate in case one of them is corrupted.

12. Add 100 μ L of LDH reaction solution prepared in **Reagent preparation for LDH assay** to each well.
13. Incubate the plate in incubator for 30 minutes at 37C
14. Dilute each well with PBS into half of the concentration. i.e. remove 100 μ L from each well and add 100 μ L of PBS into each well.
15. Use a cover from 24 or 48 well plate while taking the sample to the plate reader and read the absorbance at 490 nm with a plate reader
16. Aspirate the rest of the PRP from the 48 well plates while working on LDH assay. Avoid aspirating direction the sample that could suck platelets.
17. Rinse the samples twice with PBS. Avoid pipetting directly at the sample to prevent adhered platelets from washing away.
18. Move onto SEM fixing (morphology analysis)

A.10.1 Platelet Fixation for SEM

Process Considerations

Make fixing solution and buffer solution simultaneously to save time.

10 ml of each solution in step 1 of the procedure is usually enough.

Fixative and Buffer Preparation^{1,2}

Fixative: 3% glutaraldehyde in 0.1 M sodium cacodylate and 0.1 M Sucrose

To make 10 ml buffer and 10 ml of fixative:

1. Obtain 19.4 ml of DI water
2. Add 0.68 g of sucrose and 0.43 g of sodium cacodylate to DI water, and swish until everything is fully dissolved.
3. Remove 10 ml the mixing solution. This is the buffer solution.

4. Add 0.3 ml of glutaraldehyde to the remaining solution. Swish gently. This is the fixative solution.

Procedure^{1, 2}

1. Prepare the following solutions: fixative solution, buffer solution, 35% ethanol, 50% ethanol, 70% ethanol, 100% ethanol in 20ml small vials. Pipete 0.5ml of fixative solution into each sample of 48 well plates and let it sit for 45 minutes.
2. Aspirate the fixative solution and Pipete 0.5ml of buffer solution and let sit for 10 minutes. If crunched on time, the samples can be incubated in the buffer solution overnight.
3. After incubating in buffer solution, aspirate buffer solution and each of the followings to the samples for the designated times:
 - (a) 10 min- 35% ethanol
 - (b) 10 min- 50% ethanol
 - (c) 10 min- 70% ethanol
 - (d) 10 min- 100% ethanol
4. Allow air dry of ethanol residue and store them in the desiccator prior to SEM imaging. Care must be taken during sample removing from the wells so that tweezers does not scrub away platelets

A.11 Thermoforming LLDPE

Objective

The purpose of this process is to shape flat LLDPE into formed leaflets using vacuum and heat

Materials & Equipment

Personal Protective Equipment (PPE)

- Nitrile Gloves

Other General equipment and or glassware

- Formech 450DT Thermoformer
- 3D printed mold of choice
- 6"x6" 120 micron LLDPE
- Ethanol (for cleaning mold)
- Scissors
- Kimtech Kimwipes

****Use caution when heating the Formech 450DT Thermoformer in order to avoid burns.**

Give the heater 10-15 minutes to warm up before starting shaping. The first few leaflets may need to be heated for double the recommended time if the heater is not warmed up enough. The first few leaflets may not be properly shaped as well and may need to be discarded if the heater is not properly warmed up.

Procedure

1. Turn on the Formech 450DT Thermoformer by first plugging in the machine to the nearest outlet and then turning on the red switch located on the left side of the machine
2. When the screen turns on, select the heating logo on the left side of the screen
3. Once heating is complete, select the play button

4. Press the “settings” logo with the gear icon to either manually enter in specific parameters to the machine or choose a pre-existing file
5. Select the icon with the hand logo to manually enter the heating parameters
6. The specific parameters should be entered as follows:
 - All heating zones labeled 1-4 should be set to 70%
 - Temperature should be set to 70% capacity
 - The heater icon should be set to 20
7. Once the parameters have been adjusted, you can now begin preparing the mold
8. Wearing nitrile gloves, use a Kimwipe and ethanol to clean the mold (mold should be cleaned every time in between samples)
9. Take the square base of the mold and attach it to the bottom of the valve mold using molding clay for increased stabilization
10. Take the assembled mold and place it on the metal crosshatched surface on the thermoformer, also known as the table
11. Push the table lever on the right-hand side of the machine as far away from you as possible in order to lower the table all the way down inside the machine
12. Place a 6”x6” clean LLDPE square over the rectangular hole where the metal crosshatched surface once was (see SHACTA Treatment protocol for cleaning LLDPE)
 - Ensure that the LLDPE is fully covering the rectangular hole in order to pull the maximum vacuum
13. Once the LLDPE is properly adjusted, lower the clamp frame and use the material clamps to stabilize the material in place
14. Use the back arrow to get to the screen with the heater icon and the vacuum icon (arrows pointing down)

15. Pull the heater towards you (This should start a timer which can be seen counting upwards on the heating logo on the left-hand side of the screen)

- You can watch the material by slightly pushing the heater away from you
- It should appear slightly loose as it is being heated, but then pulled taught as the heating time comes to completion which is signaled by a high-pitched beep

16. Once the machine beeps, push the heater all the way back or wait few seconds for LLDPE to become completely straight indicating enough softening. For 80 micron it generally takes 20 seconds and for 120 microns it can take 28 seconds.

17. Press the vacuum icon and then pull the table lever towards you to raise the table with the mold on it

****NOTE if the heater is not pushed back all the way, the table lever will not be able to be pulled and the table will not be raised in time**

18. Pull vacuum for 2-3 seconds by selecting the vacuum logo in the middle of the screen and then select the vacuum logo again to stop vacuum

- Steps 16-18 should be completed as quickly as possible in order to prevent malformation of the LLDPE

19. Let the mold rest for a few seconds before unclamping the frame clamp and removing your mold

20. Once the mold has been removed from the machine, remove the thermoformed LLDPE from the mold

- Use the excess LLDPE at the base of the mold as leverage for turning the thermoformed LLDPE inside out off of the 3D-printed mold
- Once the LLDPE is removed from the mold, it should be inside out
- Carefully flip the mold right-side-out using your fingers to gently manipulate the LLDPE

- Remove the excess LLDPE at the base of the mold using scissors

21. Place the shaped film in a plastic bag for safe keeping

22. Repeat steps 10-21 for the required number of leaflets being made

23. When finished using the Formech 450DT, lower the clamp frame and make sure to turn off the machine using the red switch on the left-hand side

24. Dispose of plastic waste appropriately

See below:

GA Tech 2019 & 2022 Dowlex
2045G Blend

Ken
92122

Sample	gauge (MIL)	Machine Direction				Transverse Direction			
		%E	T break (psi)	T yield (psi)	secant (psi)	%E	T break (psi)	T yield (psi)	secant (psi)
2019 2054G Blend	3.2	645	5615	1970	53200	655	5210	2010	58800
22-7098	3.2	655	6345	1700	42000	690	5865	1730	53000
% Deviation from 2019		1.6%	13.0%	-13.7%	-21.1%	5.3%	12.6%	-13.9%	-9.9%

2019 2054G Blend	4.7	695	5320	1785	43000	670	4760	1791	51300
22-7099	4.7	700	6350	1735	41700	720	6100	1790	47700
% Deviation from 2019		0.7%	19.4%	-2.8%	-3.0%	7.5%	28.2%	-0.1%	-7.0%

Figure A.8: Properties of Dowlex 2045G blown LLDPE films from Film Tech LLC. CSU rolls were purchased in 2011 and YoungHeartValves were purchased in 2019 by Hieu Bui

A.12 LLDPE Information

CSU LLDPE information

Film Tech LLC, 799 W Broad way

12”/.0032”,

Lot no: SA-19-2102A

Customer PO: RF 015795470

Set No:01

Roll No-04

Roll Position: D

Roll Wt: 23lbs

Packed by DH

Date 11-19-19

YHV LLDPE information

Film Tech LLC, 799 W Broad way

12”/.0032”,

Lot no: 22-7098

Customer PO-062222LD-01

Set No:04

Roll No-15

Roll Position: C

Roll Length 500ft

Roll Wt: 8lbs

Treat: No

Packed by CK

Date 07/28/2022, GT

Spec No: Sample Material

A.13 Supplementary Images

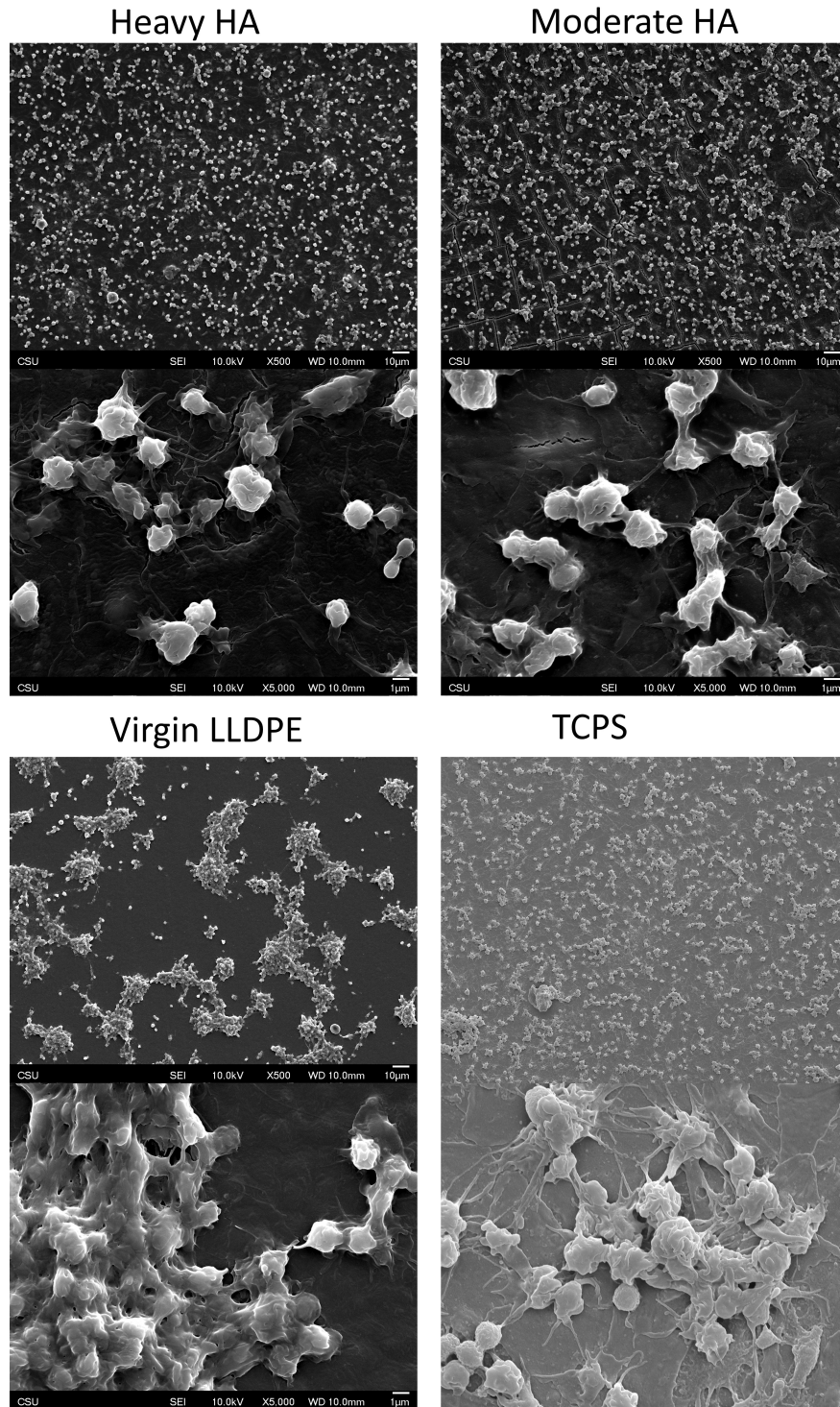


Figure A.9: SEM images of fixed platelets on samples at x500 and x5000 magnification (first donor). Platelets of heavy HA samples (average static contact angle 30°) are in the early dendritic activation stage. Platelets of moderate HA samples (average static contact angle 40°) are fully spreading. Platelets of virgin LLDPE platelets are aggregated, creating a network of thrombosis. Platelets of TCPS are all aggregated and spread to cover the sample surface area, causing severe thrombosis entirely

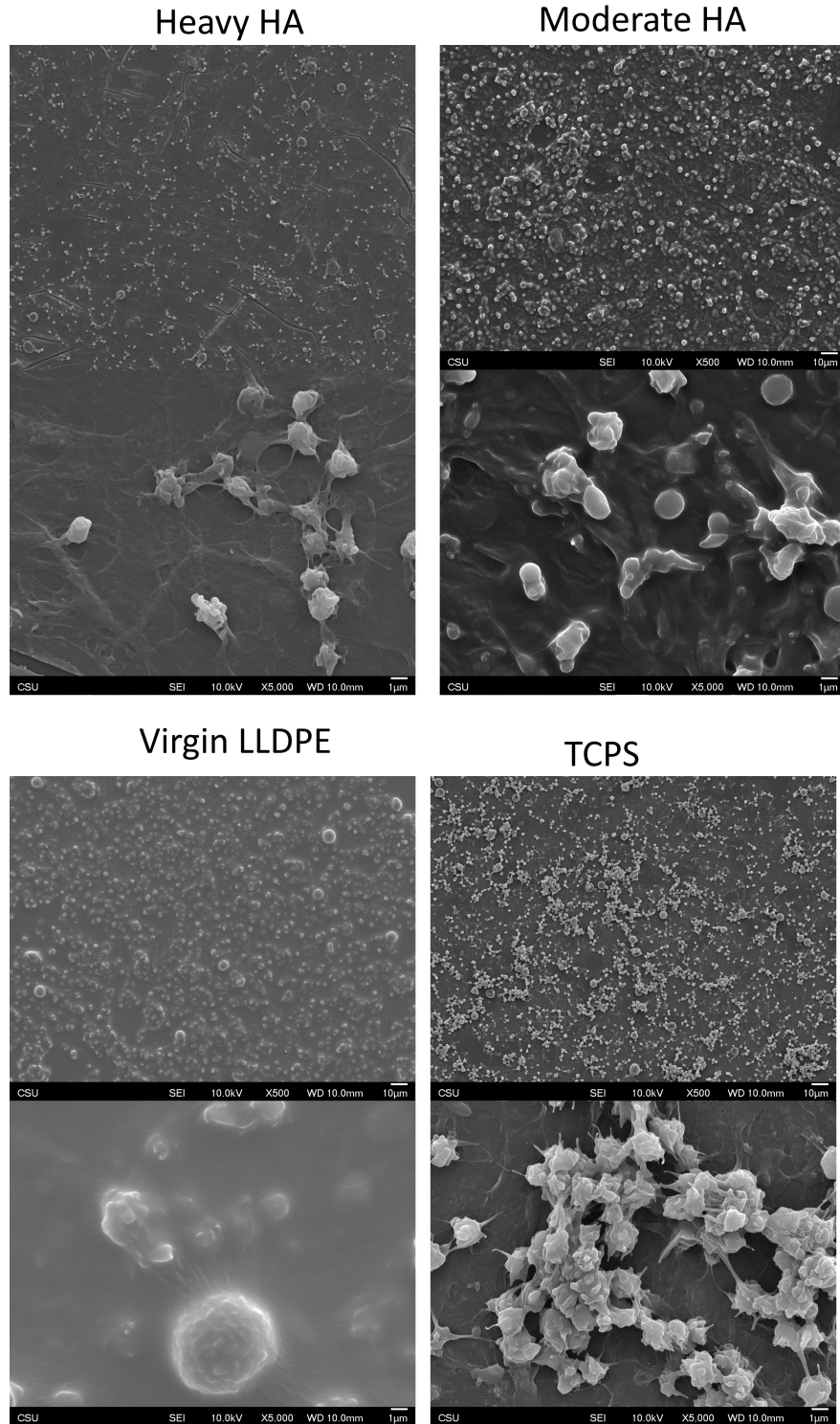


Figure A.10: SEM images of fixed platelets on samples at x500 and x5000 magnification (second donor). Platelets of heavy HA samples (average static contact angle 30°) are in the early dendritic activation stage. Platelets of moderate HA samples (average static contact angle 40°) are fully spreading. Platelets of virgin LLDPE platelets are aggregated, creating a network of thrombosis. Platelets of TCPS are all aggregated and spread to cover the sample surface area, causing severe thrombosis entirely