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A Comparison of Tung Oil Resin Reinforcements Using Fibrous Proteins and Peptides for Biological and Materials Applications

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A COMPARISON OF TUNG OIL RESIN REINFORCEMENTS USING FIBROUS PROTEINS AND PEPTIDES FOR BIOLOGICAL AND MATERIALS APPLICATIONS

by

PARKER WILLIAMS

(Under the Direction of Amanda S. White)

ABSTRACT

In this study a tung oil based thermoset was reinforced with collagen and fibroin, and the resulting composites were analyzed for their physical properties. Tung tree seed oil is a great candidate for biobased polymer production because its triglycerides are primarily made of alpha eleostearic acid, a fatty acid with three conjugated carbon-carbon double bonds. These double bonds allow for mechanically strong crosslinking in the polymer. It has been observed that polymerized tung oil forms a gel. This issue has been addressed using divinylbenzene (DVB) and n-butyl methacrylate (BMA) as co-monomers. Similar bio-based polymers have been studied and tend to have overall weaker mechanical properties than their crude oil counterparts. Collagen and fibroin are two fibrous proteins that have strong mechanical properties that can reinforce the polymer. Collagen is a protein that forms a strong triple helix. However, after purification, collagen's triple helix structure has been observed to not be fully maintained due to the incorporation of sodium ions into the collagen structure. In this study, the effect of NaCl on collagen's reinforcing properties for a tung oil polymeric resin is thoroughly evaluated. A tung oil/DVB/BMA resin has been reinforced with collagen containing varying levels of NaCl. As an alternative to collagen, fibroin has been proposed as a potential reinforcement for tung oil/DVB/BMA resins. The thermo-mechanical properties of the resulting composites have been systematically assessed via thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), and dynamic mechanical analysis (DMA). DMA showed at 37 °C collagen reinforcement had increased the storage modulus of the tung oil thermoset from 97.45 MPa to a range of 99.40 MPa - 186.62 MPa. At 37 °C, fibroin reinforcement increased the storage modulus of the tung oil thermoset from 97.45 MPa to a range of 122.23 MPa - 140.98 MPa. TGA showed at T80 there was little change in thermal stability. The highest change being from collagen reinforcement

increasing the temperature from 382.30 °C to 424.50 °C. Neither the complete curing of the resin nor the thermal stability were significantly affected by the reinforcement. Collagen and fibroin samples effectively reinforced the resin, by increasing the storage modulus.

INDEX WORDS: Tung oil, Collagen, Fibroin, Free radical polymerization, Peptides, Bio-based materials, Sodium chloride

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by

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DEDICATION

This thesis was impossible for me alone to do. This thesis exists only because of God's uncountable blessings, and showing me that, despite my many intense fears, **"I can do all things through Christ which strengtheneth me." Philippians 4:13**. There is no limit to the things God can bless me to do. I thank my Lord for blessing me with a sister in Christ who prayed for me and helped me look at Him when I was the lowest.

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CHAPTER 1

INTRODUCTION

Importance of research

"A comparison of tung oil resin reinforcements using fibrous proteins and peptides for biological and materials applications." That title is filled with words that would end the conversation with anyone who is not a scientist. This now opens the need for someone to translate these words into common speech before anyone can understand that the work is important. In today's world, there are several different products that are made from crude oil-sourced organic molecules. Crude oil can be a versatile fuel for vehicles/cooking, a lubricant for moving parts, and even a basic building block for over 3,000 types of products. These products include asphalt, inks, lubricants, plastics, waxes, paints, etc.[1] There is one issue that arises with such a societal dependence on crude oil based products, and that is the fact that crude oil is a non-renewable resource. This research involves making a strong material from a polymer made from a vegetable oil. The vegetable oil being used is tung oil, and it is sourced from tung tree seeds (**Figure 1**).

Another motivator for this research is taking care of elderly people. All matter in the universe tends towards chaos. In chemistry that means that molecules over time will break, similar to how every object has "wear and tear". This phenomenon is known as entropy, and it affects biomolecules as well.[2] When an organism's biomolecules break down, it can cause many issues for that organism. Entropy will always catch up to elderly people and leave them with many bio-structural issues. This is currently remedied with non-biobased materials such as polymer dental fillings, or metallic bone implants. Because of the non-biobased nature of these materials, these materials are known to have complications. Silicone breast implants are known to have calcification build up over time. Metals are commonly used as organic catalysts, and it has been observed that metal implants cause catalytic biochemical complications. It has been observed that

malignant tumors have been found at the joint cites of implants made of PMMA cement, polyethylene, stainless steel, and alloys made from cobalt, chromium, nickel, and molybdenum.[3]

Figure 1. Tung tree leaf, and seeds.[4]

In past studies, tung oil-based polymers have been made, but have been observed to be mechanically weaker than crude oil-based polymer materials such as high density poly ethylene.[5] This project seeks to reinforce the tung oil polymer with collagen and fibroin. These two proteins are not only known for their high structural strength, but they also have been observed to aid in wound healing.[6]

Polymer basics

The polymer under investigation is prepared by bulk polymerization (**Figure 2**). This is a type of polymerization that uses no solvent. The lack of a solvent brings the disadvantage of increasing the polydispersity index (PDI) of the polymer. This results in polymers that have a wider range of chain lengths, which is not good for reproducibility. However, the lack of a solvent avoids the extra step of removing the solvent once the polymerization is finished and produces less waste. Because monetary cost is one of the leading reasons that biobased polymers are not being used over crude oil-based polymers, using bulk polymerization to save costs is a significant advantage for the chemical industry.

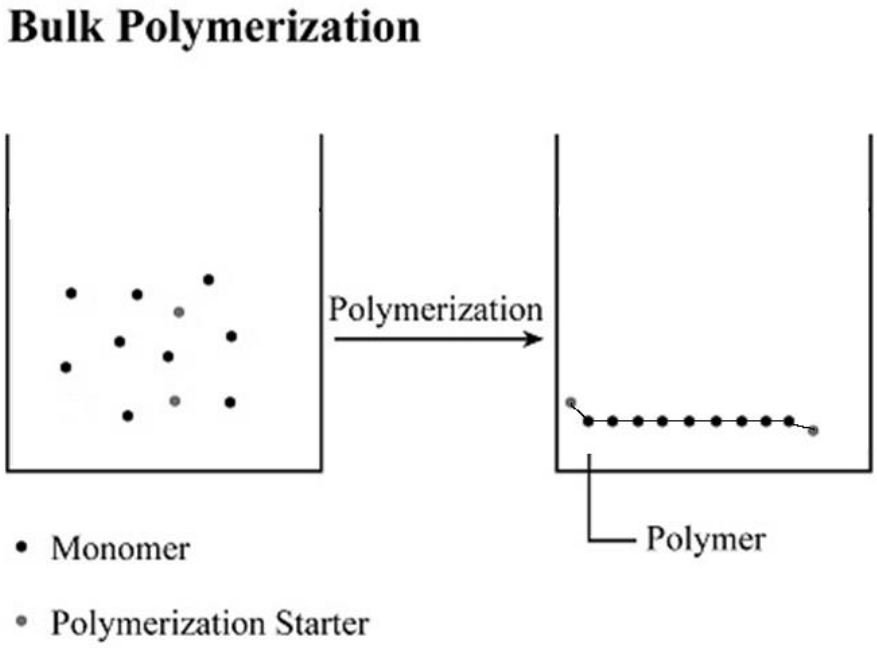

Figure 2. Bulk polymerization schematic.[7]

Polymers are very large molecules that consist of many small molecules, known as monomers, chained together. There are two general classifications for polymers, namely thermoplastics and thermosets (**Figure 3**).

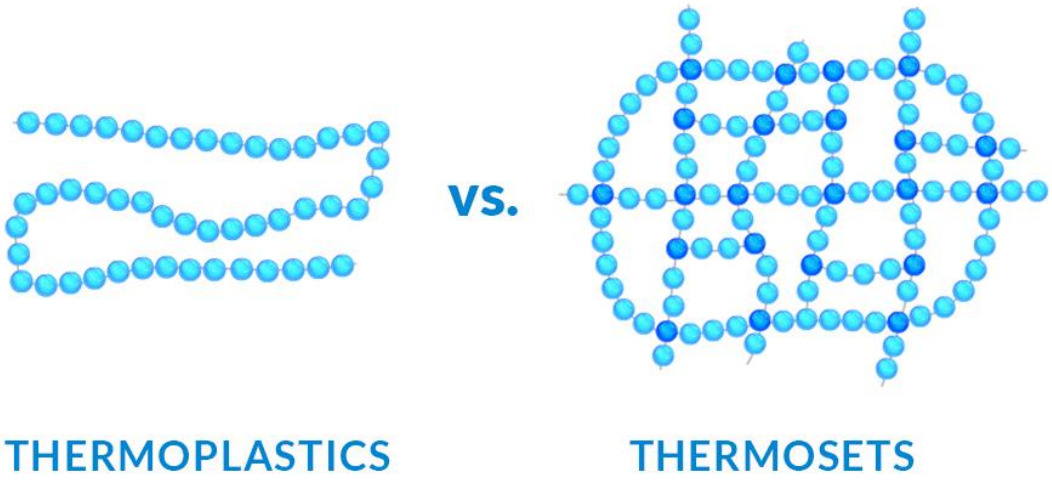

Figure 3. Schematic of structures of thermoplastics and thermosets. The darker blue circles represent crosslinkers.[8]

Thermoplastics are polymers made from monomers that can only create two covalent polymer linkages. These polymers are long linear chains of molecules. These polymers tend to be less mechanically strong but are able to be dissolved, reshaped, melted, and recycled. The monomers that make up thermosets include crosslinkers. These crosslinkers are molecules that can create more than two covalent polymer linkages. This crosslinking effect results in a polymer that creates chains in multiple directions. Crosslinked polymers as a result have many linkages that occur between other branching chains. This results in more of a matrix of chains, as opposed to the thermoplastic linear chain. The "-set" in the term thermoset refers to how these polymers are "set" in whatever shape in which they are polymerized. These polymers in general cannot be dissolved, reshaped, melted, or recycled.[9] Commonly used thermosets include silicone, epoxy, and polyurethane. The advantage to thermosets is that they are a much more mechanically strong polymer when compared to thermoplastics.[10] Because the goal of this project is to make a mechanically strong material, this project is using thermosets instead of thermoplastics.

Reinforcement

One way to further increase a material's mechanical strength is to use reinforcements. To make a reinforcement, one must physically add another material to the material being reinforced. Reinforcement is a powerful phenomenon where the two materials combine in a synergistic way. This results in the strength of the combination of materials being greater than the sum of its parts. One example of this is reinforced sand (**Figure 4**). The sand block in **Figure 4** has layers of fiberglass window screen as reinforcement. The resulting reinforced sand was able to hold the partial weight of a minivan vehicle. Neither sand nor fiberglass window screens could support the weight of a vehicle, but when combined, there is a great increase in the amount of load the sand can uphold.[11]

Figure 4. Sand being reinforced with layers of fiberglass window screen.[11]

Tung Oil Copolymer

Tung oil was chosen because its most abundant fatty acid is alpha-eleostearic acid (80% - 95%), a fatty acid with three conjugated carbon-carbon double bonds (**Figure 5**). These three carbon-carbon double bonds are very useful for free radical polymerization. In past studies, a polymer made from 100% tung oil resulted in a polymer that was a gel rather than a stiff material. Because a sturdy material was desired, the cross linker divinylbenzene (DVB) was added as a co-

monomer (**Figure 6**). The resulting co-polymer was very brittle, requiring the reactive a plasticizer *n*-butyl methacrylate (BMA **Figure 7**).[12]

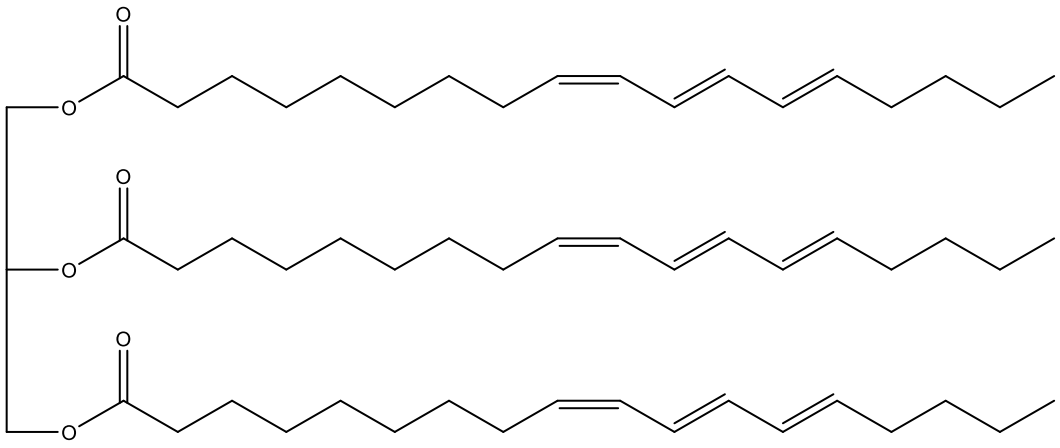

Figure 5. Tung oil triglyceride structure.[13] All chemical structures represented were generated in ChemDraw Professional

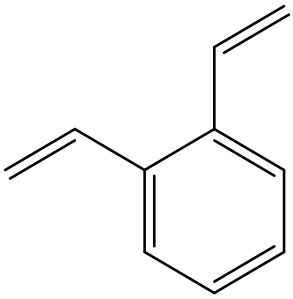

Figure 6. Divinylbenzene (DVB) chemical structure.[14]

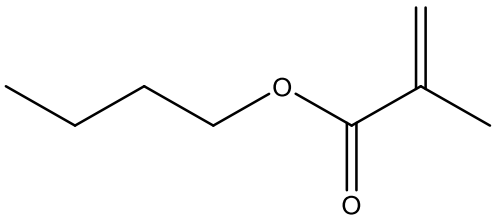

Figure 7. *n*-butyl methacrylate (BMA) chemical structure.[14]

One thing to note about tung oil is that it is a mixture of triglycerides with different fatty acids. Tung oil also has small amounts of oleic acid, palmitic acid, and stearic acid.[15] In past studies a polymer was made from just triglycerides with only alpha-eleostearic acid and compared with a polymer made from the tung oil mixture. The polymer made from the tung oil mixture had greater physical properties than the polymer made from the triglycerides with only alpha-eleost1earic acid. This is beneficial for the chemical industry as it removes the need for tung oil fatty acid purification. Protein reinforcement was then added to the polymer resin, and it was observed that the reinforcement was not compatible with the resin. The resin is a very nonpolar mixture, and the protein being used is slightly polar. This led to the addition of another monomer, the surfactant asolectin, to the copolymer (**Figure 8**).[12]

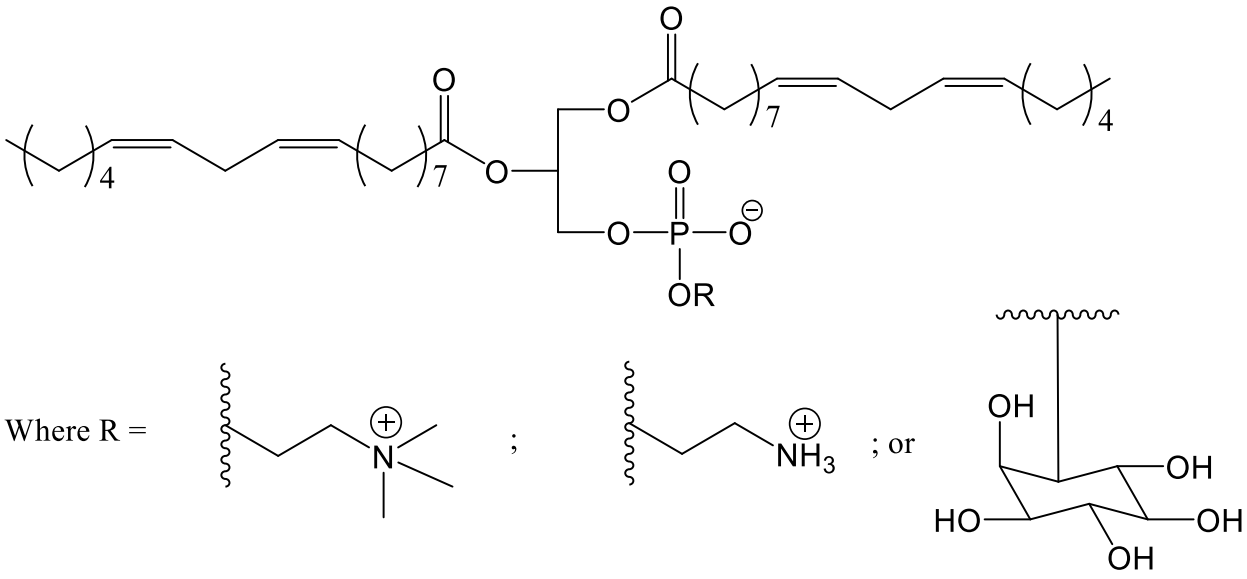

Figure 8. Asolectin general representation chemical structure. The asolectin used was sourced from soybeans.[16]

Asolectin is a mixture of phospholipids that is biobased, as it is sourced from soybeans. It has been successfully used as a surfactant in tung oil polymers.[16] The polar phosphate group of asolectin is compatible with the polar protein, and the non-polar fatty acids are compatible with the non-polar nature of the resin's components. The addition of the asolectin was observed to increase the compatibility of the resin and the protein reinforcement. This allowed more protein to be dissolved into the monomer mixture.[5] With the addition of all the comonomers, this copolymer is only half biobased. DVB and BMA are not biobased, but in future studies could be replaced with biobased crosslinkers and plasticizers. One potential crosslinker is vanillin, which is sourced from lignin,[17] and ozonized soybean oil is a potential biobased plasticizer.[18]

Free radical polymerization

The polymerizing reaction that was used to create the tung oil thermoset was free radical polymerization. This is a type of polymerization in which radicals are formed by separating paired electrons in a covalent bond. Free radical electrons are extremely reactive species and will react

with pi bonds to form both a covalent linkage and another free radical. This newly generated free radical then can react with another pi bond, repeating the process until monomers are completely consumed.[19] In this project the initial free radical species was generated using heat and a thermal free radical initiator. The initiator used was di-tert-butyl peroxide (DTBP) (**Figure 9**).

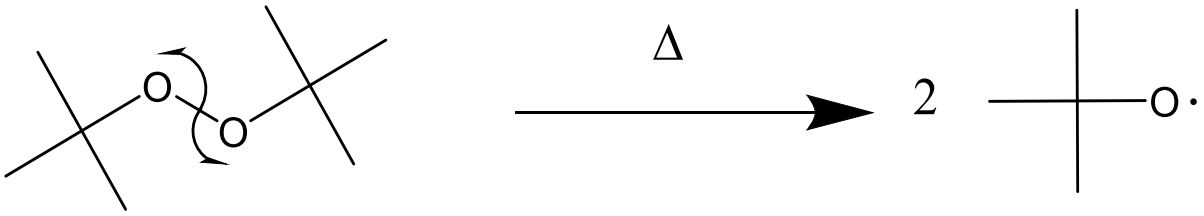

Figure 9. Di-tert-butyl peroxide thermal radical initiation. Heat generates two tert-butyl peroxide radicals.

To begin polymerization the mixture of the resin (tung oil, DVB, BMA, asolectin, and DTBP) is heated in a convection oven. This heat initiates the radical formation of the tert-butyl peroxide radical as shown in **Figure 9**. This radical will then react with the monomers to begin polymerization (**Figures 10, 11, 12**)

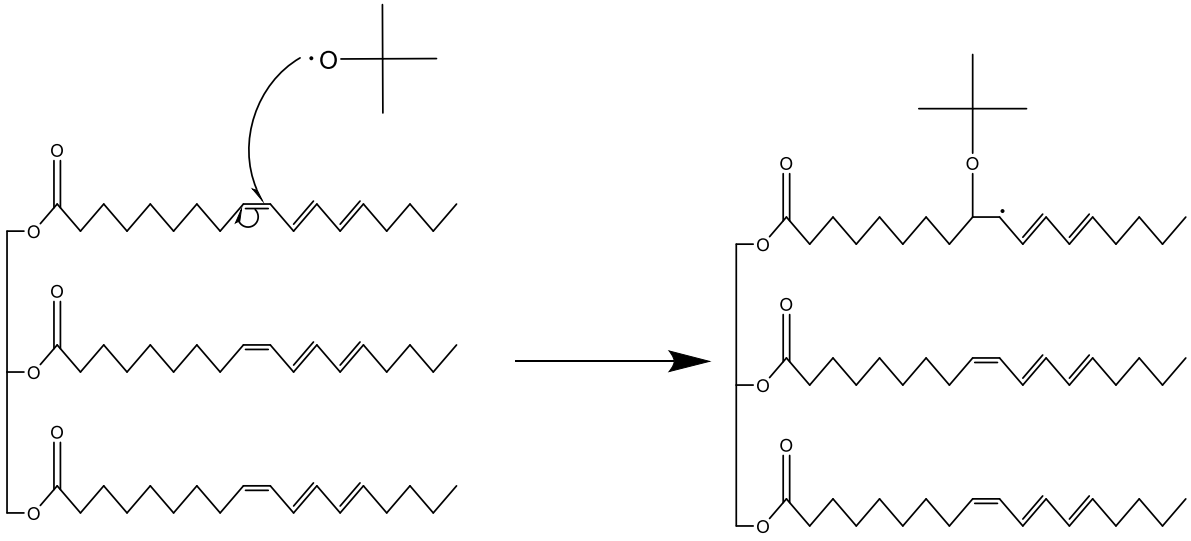

Figure 10. Formation of the tung oil triglyceride radical via radical attack.

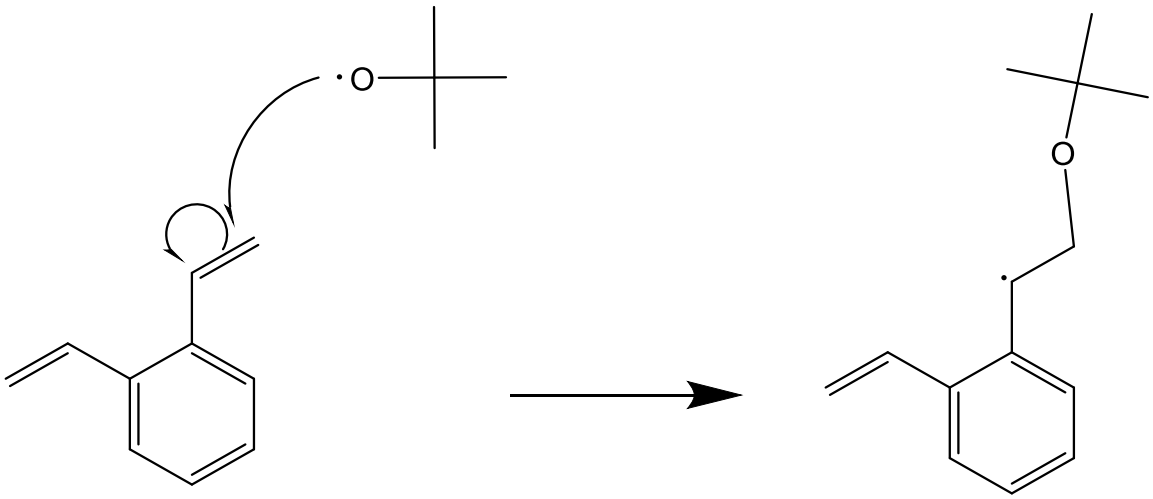

Figure 11. Formation of the divinylbenzene radical via radical attack.

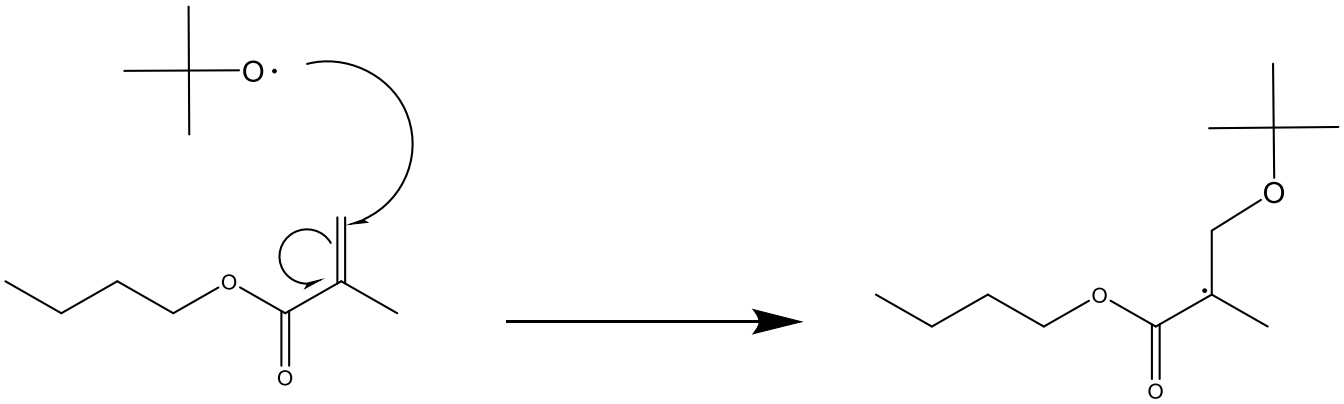

Figure 12. Formation of the butyl methacrylate radical via radical attack.

Because these molecules are randomly dispersed in the reaction vial, the resulting copolymer is a random distribution of these monomers. There are many double bonds across these monomers. In total there are 32 different possible polymer linkages that can occur. This results in a highly crosslinked thermoset (**Figure 13**).[5]

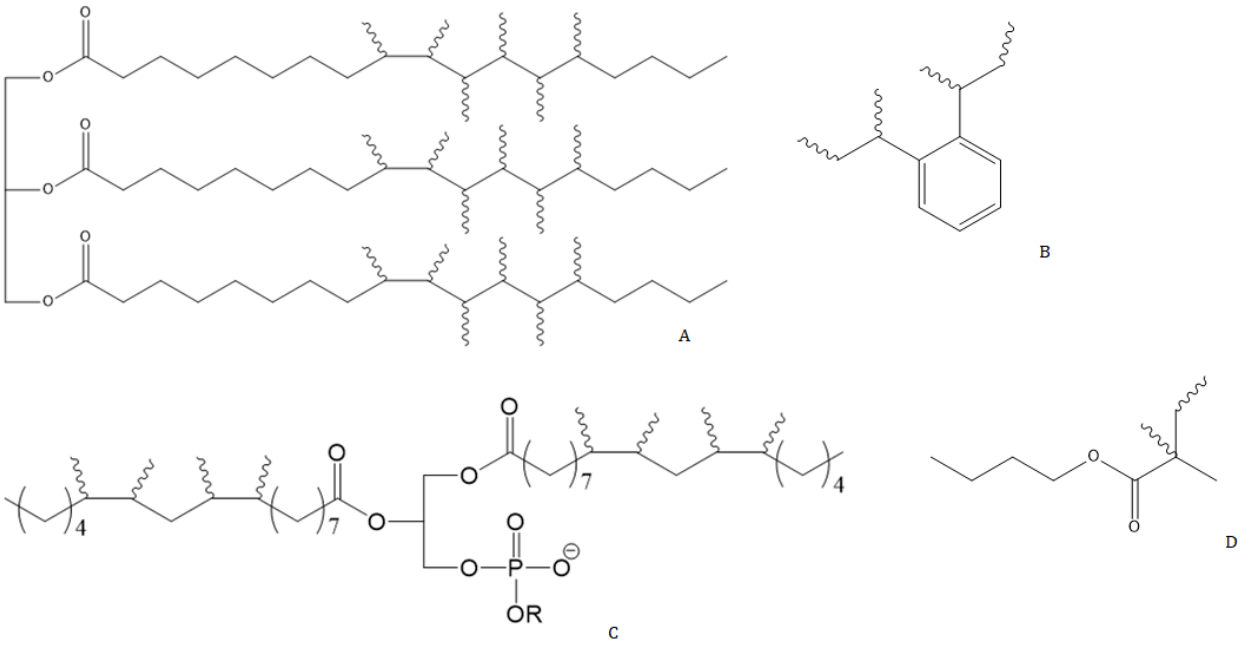

Figure 13. A schematic of 32 different possible polymer bonds in comonomers. The possible polymer bonds are represented as wavy lines. A: Tung oil triglyceride, B: Divinylbenzene, C: Asolectin, D: Butyl Methacrylate.

Other Successful Tung Oil Polymers

There are many other tung oil based polymers that have been made successfully. Researchers at the Institute of Chemical Industry of Forestry Products in Beijing China synthesized monomer using tung oil (TO) triglycerides, pentaerythritol (PER), and maleic anhydride (MA) to create a monomer known as TOPERMA. They then copolymerized TOPERMA with styrene using thermally initiated free radical polymerization to create a rigid thermoset matrix with high tensile and flexural strength.[20] Tung oil polymers can also be copolymerized by cationic polymerization with DVB and styrene. This polymerization for this polymer was initiated using boron trifluoride ether as a photoinitiator. Photoinitiators are initiators that are activated by UV light.[21]

While crude oil based polymers tend to not be biodegradable, it has been observed that tung oil polymer films do have some biodegradability. In a study where a tung oil polymer film was buried in soil in the garden of Wuhan Polytechnic University for three months, it was observed that biological mechanisms uniformly degraded the polymer. Evidence suggests that the soil microbes

degraded the carbonyl groups, and it is thought that the carbonyl groups are weak spots in the polymer.[15]

Tung oil polymers have also been used to create self-healing polymers through the process known as microencapsulation. The method involves polymerizing tung oil with catalysts being suspended in the polymer matrix. Also suspended in the polymer matrix are capsules of liquid monomer. When stress causes microcracks within the polymer it exposes the liquid monomer to the catalysts, which catalyze a polymerization reaction that seals the microcrack. One limitation to this technique is that if a healed area experiences another microcrack, there is no more liquid monomer to polymerize. This can still significantly increase the longevity of tung oil polymers.[22]

Collagen as a resin reinforcement

Collagen is a fibrous protein that in nature is used as a structural biomaterial. These properties make it desirable to use as a reinforcement to the tung oil resin. Collagen is a protein that is made up of three polypeptide helices that combine into a triple helix via hydrogen bonding. Collagen polypeptides are primarily made up of three amino acids: glycine, proline, and hydroxyproline (**Figure 14**). The proline amino acids are responsible for causing a twist that forces the polypeptide into a helical shape. The hydroxyproline has a hydroxyl group that allows the hydrogen bonding needed for the triple helix to occur. In the triple helix, there must be amino acids that have small enough R groups that can fit in the center of the triple helix. Glycine has the smallest R group of hydrogen to fit this purpose. Every three amino acids there must be a glycine. Every three amino acids there must be a proline or a hydroxyproline, then the next three amino acids must have a proline or a hydroxyproline, whichever was not in the first three amino acids.[23]

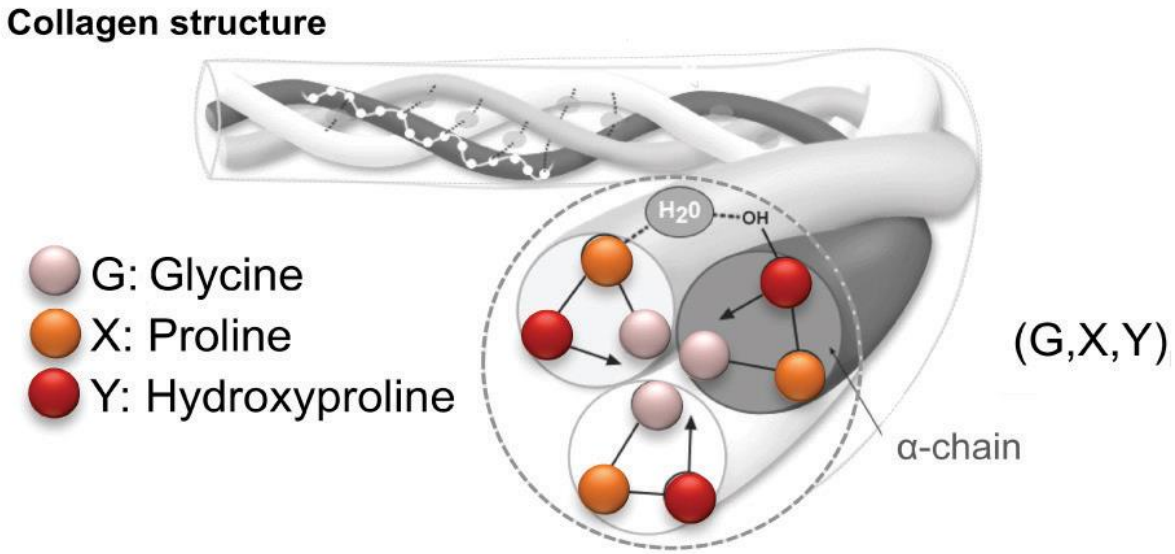

Figure 14. Collagen triple helix diagram.[24]

This project originally reinforced the tung oil resin by using type 1 collagen purchased from Sigma Aldrich. It became a question if collagen purified in house would have a greater reinforcing effect. Like any polymer, collagen has been observed to have stronger mechanical properties with longer chain lengths.[5, 25] Type 1 collagen was purified in house from beef tendons provided by the Hunter Cattle Company. The reinforcing effects were compared between the purified collagen and the Sigma Aldrich collagen, and the purified collagen was observed to have a greater reinforcing effect.[5] The project moving forward then used only collagen purified in house from beef tendons. The collagen purification procedure involved salting out the collagen using NaCl. This step introduced a significant amount of NaCl contamination. An error in the in house Georgia Southern University deionized water system also introduced salts into the water carboys, verified by ICP-MS.[26] This increased ion contamination of the collagen samples. It was observed by Christopher Dzorkpata using circular dichroism spectroscopy that the collagen with higher amounts of % weight NaCl lost its triple helix peak (**Figure 15**).

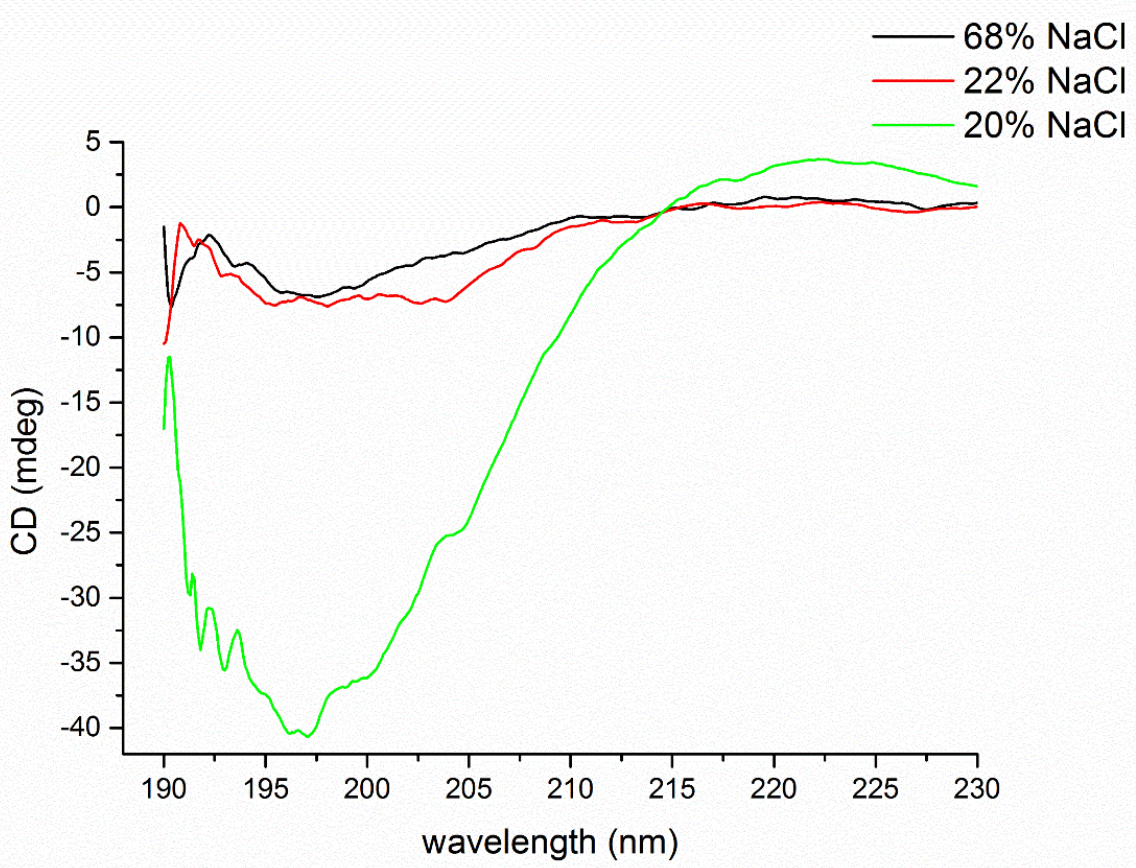

Figure 15. Circular dichroism (CD) spectroscopy of collagen with varying amounts of NaCl. 2.500mg of collagen was dissolved in 0.5M acetic acid, then diluted to 0.1 M acetic acid with 0.02 M pH 7.4 Na_2HPO_4. CD scanned from 190nm to 230nm.[27]

It was observed that collagens with 20% NaCl showed a characteristic triple helix peak at 222 mdeg.[27] This indicates that high concentrations of NaCl affect the triple helix formation in collagen.[28] In this study collagen was further desalted using different methods: dialysis and ultra filtration. These studies compare how the reinforcing effects vary with high NaCl collagen and desalted collagen.

Other Collagen Materials

Collagen has been observed to be blended, mixed, or dissolved in the same solvent, with other biomolecules such as Chitosan, Elastin, Keratin, and Silk Fibroin. These biopolymer blends have unique biochemical properties.[29] The collagen/chitosan blend has been 3D printed into

scaffolds[30] and had therapeutic benefits to rats with completely transected spines.[29] Collagen blended with elastin has successfully been electrospun into a skin substitute for treating wounded skin.[31] Keratin and collagen bio composites have been used in the agriculture industry. These composites have been used in finishing leather, and to stimulate rape seed germination by controlling nitrogen release.[32] Blending silk fibroin with collagen can aid biomedical applications. The addition of collagen to silk fibroin materials has been observed to result in increased biocompatibility. Fibroin-collagen can be electrospun into scaffolds that have been observed to increase the viability and the adhesion of mammary epithelial cells.[33]

Fibroin as a resin reinforcement

Fibroin is another protein that is known for its very high tensile strength in nature.[29] Fibroin is an antiparallel beta sheet protein. Most beta sheets are not able to form long fibers like fibroin. Fibroin can do this because of its amino acid composition. Fibroin is mostly made up of glycine and alanine. Glycine and alanine both have small R groups, which allow the fibroin to tightly pack into a fiber. Serine is also present in small quantities and contributes hydrogen bonding interactions to strengthen the protein. The beta sheet structure is formed by the arrangement of backbone atoms due to their hydrogen bonding. (**Figure 16**).

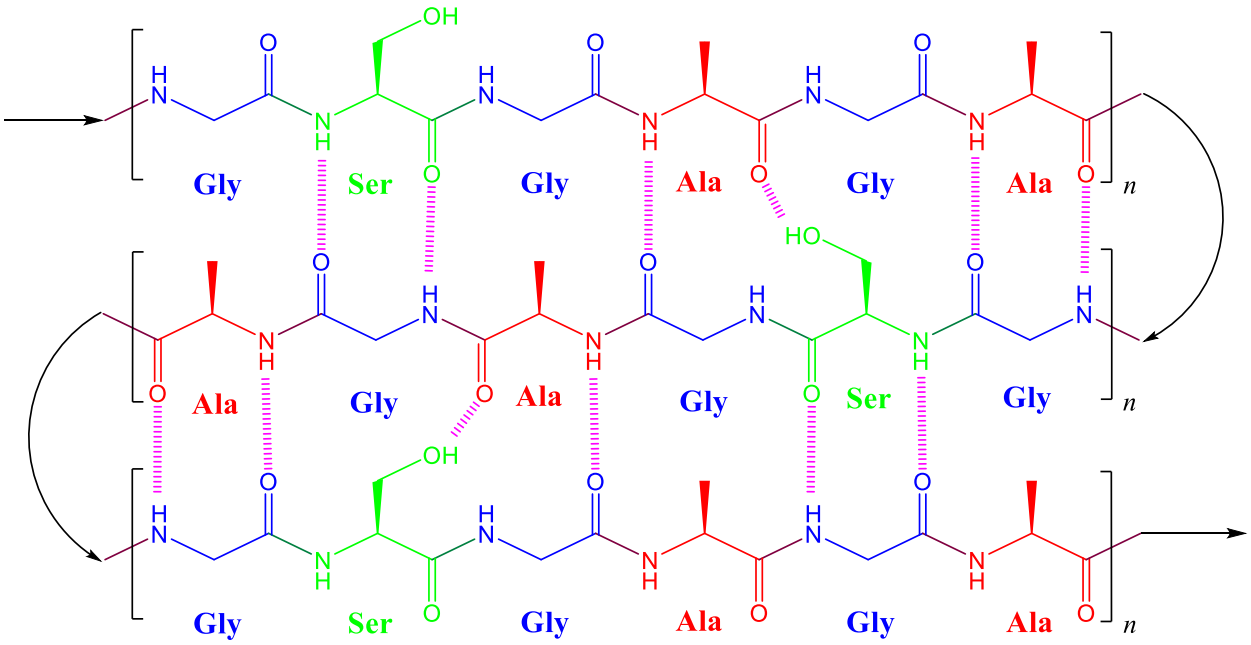

Figure 16. Fibroin antiparallel beta sheet chemical structure.[34]

Because fibroin's beta sheet is not formed by covalent linkages, it allows the other strands to slide horizontally, and still be able to form hydrogen bonding among backbone atoms. This extent of hydrogen bonding is why fibroin has a high tensile strength, and the lack of covalent linkages allows fibroin to be stretchy and flexible.[34] The fibroin being used in this study was not purified in house. It was instead purchased from Advanced Biomatrix and was originally sourced from silkworm cocoons. Similar to collagen, it may be beneficial to purify the silk cocoons in house, but before that is pursued, vendor purchased fibroin was examined in this study.

Other Fibroin Materials

For centuries the silk from the Bombyx mori silk worm has been used both as a suture material and for clothing.[35] Silk fibroin is capable of forming strong networks that can absorb water known as hydrogels. Silk fibroin can be used as a medium to grow cell lines. Scaffolds of silk are useful in wound healing and tissue engineering including bone, cartilage, tendons, and ligaments.[36] Silk fibroin materials have applications in water purification. Silk fibroin was observed to be able

to remove iron ions from contaminated water at 98% efficiency.[37] The protein also has applications in dental surgery recovery. Nano-hydroxyapatite crystal deposits on the surface of silk fibroin produces a scaffold that has been observed to promote new bone growth in areas where teeth are extracted.[38]

Peptide mimics as a resin reinforcement

Peptides are also a desirable reinforcement. With collagen and fibroin, they are both crudely sourced from organisms. Proteins have many different uncontrollable variables, such as exact amino acid composition, and polypeptide length. With peptides these variables can be directly controlled to further analyze how they change reinforcing affect. The goal of the peptides is to mimic collagen and fibroin for comparisons to the full proteins. Type 1 collagen is made up of two identical α1 polypeptide chains, and a α2 polypeptide chain. Because type 1 collagen is being mimicked, the proposed collagen peptide is made up of two α1 chain mimics with sequence: (Gly-Pro-Lys-Gly-Glu-Hyp-Gly-Pro-Ala-Gly-Arg-Hyp-Gly-Pro-Gln-Gly-Lys-Hyp), and one α2 chain mimic with sequence: (Gly-Pro-Glu-Gly-Arg-Hyp-Gly-Pro-Ala-Gly-Asp-Hyp-Gly-Pro-Gln-Gly-Lys-Hyp). Similar collagen peptide mimics have been observed to form stable triple helix structures.[39] To analyze how the chain length affects the reinforcement, the same collagen sequences are repeated to make a peptide that is twice as long. One further addition that may be interesting to study is the addition of the amino acid allyl glycine (**Figure 17**).[27]

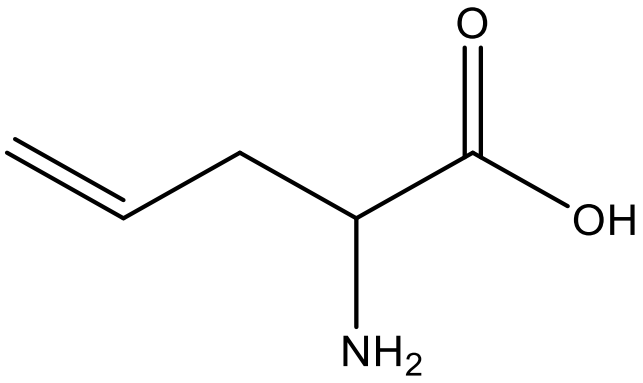

Figure 17. Allyl Glycine chemical structure.[40]

Allyl glycine is an amino acid that has a pi bond. If a peptide with this amino acid is used as a reinforcement, the radicals formed during polymerization would also attack the pi bond in allyl glycine. This would result in the peptide reinforcement being covalently bonded to the resin, which may increase the physical properties further.[27]

A peptide that mimics fibroin would also be interesting to study. The fibroin peptide sequence proposed is $(Ala\text{-}Gly\text{-}Ser\text{-}Gly\text{-}Ala\text{-}Gly)_5$. It would be interesting to see if this peptide has similar properties to the fibroin proteins.

Peptides were not analyzed during this study but will be a path that this project pursues in the future.

CHAPTER 2

MATERIALS AND METHODS

Chemical/Material Vendors

Beef flexor tendons (59991039) were donated by the Hunter Cattle Company (19.25 kg) The Beef flexor tendons (5991039) used were donated by the Hunter Cattle Company. The beef tendons were packaged on February 15, 2019; Lot: 011902153710715. The beef tendons were kept frozen until use. The 5% silk fibroin solution was purchased from Advanced BioMatrix Catalog No. 5154 Lot 7951. The silk fibroin was stored at -70 °C until use and was manufactured in August 2019. The glacial acetic acid was purchased from Fisher Scientific Lot 121507.

The ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) was purchased from Fisher Scientific, JustPURE Lot 107343. The pepsin was purchased from ACROS Organics. Lot: B0134189. The o-phosphoric acid, 85% was purchased from Fisher Scientific. Lot: 116094. The sodium chloride was purchased from Fisher Science Education Lot: 6GIRI16127007. The sodium phosphate dibasic anhydrous was purchased from VWR AMRESCO Life Science Lot: 3107C221. The sodium phosphate monobasic was purchased from Fisher Scientific Lot: 061137. The divinyl benzene (DVB) 80% mixture of isomers with 1000ppm 4-tert-butylcatechol was purchased from Alfa Aesar Lot: Y21D032. The asolectin from soybean was purchased from Sigma Aldrich Lot: BCBM5625V. The butyl methacrylate (BMA) was purchased from ACROS Organics Lot: A0342684. The di, tert-butyl peroxide was purchased from Sigma Aldrich Lot: MKCD9470. The tung oil was purchased from Sigma Aldrich Lot: MKCD2169. The dialysis membrane standard regenerated cellulose (RC) tubing was purchased from Spectrumlabs Fisher Scientific; Molecular Weight Cut Off: 3.5 kDa Lot: 9201590. The stirred ultrafiltration cell model 8200 was purchased from Amicon. The ultrafiltration discs (1 kDa) were purchased from Amicon Bioseparations Lot: C5CA86278. The E-Pure deionized water system was purchased from Barnstead. The Millipore

UltraPure (Type 1) deionized water system was purchased from Millipore. The FreeZone 4.5 plus lyophilizer was purchased from Labconco. The mechanical convection oven DKN 600 was purchased from Yamato. The differential scanning calorimeter (DSC) 250 discovery series was purchased from TA Instruments. The dynamic mechanical analysis (DMA) Q800 was purchased from TA Instruments. The Thermogravimetric Analysis (TGA) Q50 instrument was purchased from TA Instruments.

Collagen Purification

Collagen was purified from beef flexor tendons. The beef tendons were left outside the freezer to thaw. Once thawed, 4.72 kg of beef tendons were weighed out and cut into small, approximately 1.5cm x 1.5cm x 1.5cm pieces. The beef tendon pieces were then placed in large containers, and ultrapure deionized water was added to suspend the beef tendon pieces. The suspension was covered, and stored at 4 °C for three days, until crushing/blending. The liquid was then decanted from the beef tendon suspension and washed with fresh deionized water. The tendon pieces were then removed from the deionized water and crushed in a blender. Once crushed, the liquid was decanted, and the crushed tendons were placed in a fume hood to dry overnight. The tendons were then flipped and left to dry for a few more hours. The dry mass of tendons was then weighed (4.303 kg of dry crushed tendons). The tendons were then suspended in 0.05 M Na_2HPO_4 buffer adjusted to ~pH 9 using 85% o-phosphoric acid. The beef tendons were covered and stored at 4 °C for at least 24 hours. This was repeated two times (three Na_2HPO_4 buffer washes total), decanting as much liquid as possible, and replacing it with buffer each time. The beef tendon tissue was then added to a solution of 0.5 M CH_3COOH in 5 mM Ethylenediamine tetra acetic acid (EDTA). Pepsin enzyme was then added (0.025g of pepsin per 100g of tissue), and the solution was mixed. This suspension was then covered and stored at 4 °C for 12 hours. After initial pepsin degradation, the same amount of pepsin was added and mixed into the tissue suspension. The tissue suspension was covered again and stored at 4 °C for 12 more hours. After 24 hours total mixing time, the liquid was decanted and saved as the first pepsin wash. This procedure was repeated twice

to obtain a second and a third pepsin wash. Unfortunately, the second pepsin wash was done with EDTA that was not fully dissolved because acetic acid was accidentally added before the EDTA was fully dissolved. The third buffer wash had a volume of 650mL. Each buffer wash was covered and stored at 4 °C. The third buffer wash was then centrifuged at 3000 g for 15 minutes at 15 °C. The supernatant was separated from the pellet, and NaCl was added to make the solution 4 M NaCl to salt out the collagen. The solution was stirred for one hour, and then covered and stored at 4 °C for 24 hours. After 24 hours, a white precipitate formed on the top of the solution. The precipitate was collected and dissolved in 1M CH_3COOH. The mixture was then centrifuged at 5000g at 15 °C for 15 min. The precipitate was then collected and dialyzed against 0.02 M Na_2HPO_4 buffer adjusted to pH 7.4. Standard Regenerated Cellulose (RC) Tubing with a MWCO of 3.5kDa was used. The collagen was dialyzed for 48 hours, and after the first 24 hours, the buffer was replaced with fresh buffer (two buffers total). After the total 48 hours, the collagen and solvent were then removed from the tubing and lyophilized for 48 hours.[41]

Lyophilization of Proteins

Both collagen and fibroin were lyophilized using the Labconco FreeZone Plus 4.5 Liter Cascade Benchtop Freeze Dry System. The samples were put on a vacuum at approximately 0.2 torr - 0.5 torr, and cooled to -80 °C. Liquid samples were frozen with liquid nitrogen before lyophilization.

Tung Oil Resin/Composite composition and curing

Each resin/composite was a 5g sample. The resin is composed of 40% weight tung oil, 30% weight of n-butyl methacrylate (BMA), 20% weight of divinylbenzene (DVB), 10% weight of asolectin from soybeans. In each resin sample there was an additional percentage of 5% di-tert-butyl-peroxide (TBPO) initiator.[12] In the composites, fibrous protein reinforcement was added as an additional 1%, 2%, or 5% weights, depending on the sample. These “additional percentages” make the total percent above 100% but were represented this way for ease of reporting.[5] The actual percent weights are listed in **Table 1**.

Table 1. Table of percent weights of composite components.

	LITERATURE % WEIGHTS (%)			
Tung Oil	40			
BMA	30			
DVB	20			
Asolectin	10			
Literature TBPO	5			
Protein	0	1	2	5
Total %	105	106	107	110
	ACTUAL % WEIGHTS (%)			
Protein (1%, 2%, 5%)	0	0.9433962264	1.869158879	4.545454545
TBPO	4.761904762	4.71698	4.67290	4.545454545
Tung Oil	38.0952381	37.73585	37.38318	36.36364
BMA	28.57142857	28.30189	28.03738	27.27273
DVB	19.04761905	18.86792	18.69159	18.18182
Asolectin	9.523809524	9.43396	9.34579	9.09091
Total %	100	100	100	100
	REAL WEIGHT COMPOSITION OF “5g” COMPOSITES (g)			
Protein (1%,2%,5%) (g)	0.05	0.1	0.25	
TBPO (g)	0.25			
Tung Oil (g)	2			

BMA (g)	1.5			
DVB (g)	1			
Asolectin (g)	0.5			
Total actual mass (g)	5.3	5.35	5.5	

Proteins were lyophilized before adding to the composite mixture to remove any volatiles gained from freezer storage. For the high NaCl collagen composites, there was 44.62% NaCl weight in the collagen. For each of the desalted collagen composites, Ultrafiltration collagen samples 2 and 4 (**Table 2**) were both used in equal amounts to make up the reinforcement, due to collagen scarcity. All the resin components except for the TBPO were then mixed in a 20mL scintillation vial to make samples approximately 5g. The composites with 5% desalted collagen, 2% desalted collagen and 5% fibroin were too massive to properly be added to a composite. These proteins were compressed in the scintillation vial using just the handle of a plastic syringe plunger (**Figure 18**).

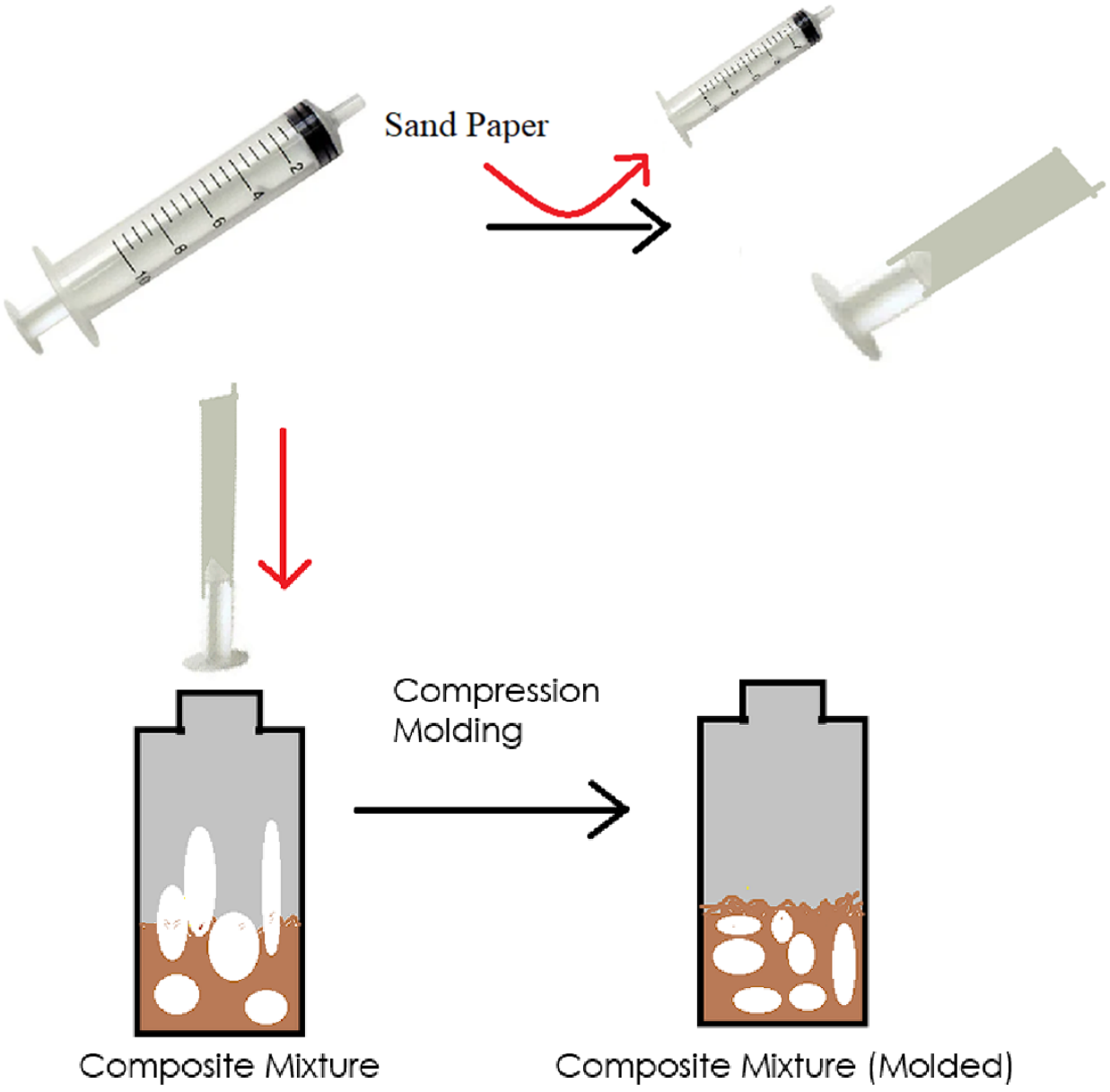

Figure 18. Production of compression molding tool for compression molding of proteins. The plunger of a plastic syringe was obtained and sanded to be able to fit into the scintillation vial. The proteins in the 5% and 2% desalted collagen and 5% fibroin composites were too massive and absorbent to be properly cured. The compression molding tool was used to compress the proteins to make proper composites.

The resin mixtures were then vortexed for 20 min for homogenization. The samples were then capped and left to rest at room temperature to allow the removal of bubbles. The TBPO was then

added, and the mixture was gently swirled to agitate without creating bubbles. The samples were then capped and placed in a convection oven to cure. The samples were heated for 25.7 hours in a stepwise process. Over 20 min the samples were heated to 100 °C, then remained equilibrated at 100 °C for 2 hours. Then they were heated to 110 °C over 20 min and remained equilibrated at 110 °C for 6 hours. The samples were then heated to 120 °C over 20 min and remained equilibrated at 120 °C for 6 hours. The samples were then heated to 130 °C over 20 min and remained equilibrated at 130 °C for 6 hours. The samples were then heated to 140°C over 20 min and remained equilibrated at 140°C for 4 hours.[5]

Thermogravimetric Analysis (TGA)

TGA was used to quantify the percent weight of NaCl in collagen. The TGA Q50 produced by TA Instruments was used for the analysis. Five to ten milligrams of collagen was placed on a platinum pan and heated from room temperature (~24 °C) to 850 °C at a heating rate of 20 °C per minute. During the heating, the sample weight was measured over the time of the run. The reference pan was in a nitrogen environment that had a gas inlet flow rate of 20 mL/min. The sample and its pan were in an air environment achieved by a gas inlet with a flow rate of 20 mL/min. After the heating, the residual mass was assumed to be NaCl. Initially samples were taken from the top of the collagen sample. After seeing different results, the collagen vial was then homogenized with a glass stir rod to get a more homogeneous TGA sample. Samples that had a higher amount of NaCl content were not dense enough to stay on the pan. Those samples were flattened in the folds of weigh paper to decrease their volume.

All polymer/protein composite samples' thermal properties were also analyzed using the same methods and parameters. The composite samples contained 1-2 pieces of composite, with no flattening required.

Collagen Ultrafiltration

To further purify the collagen, the collagen was dissolved in 0.1M acetic acid made from deionized water from the University of North Carolina at Chapel Hill. This deionized water

dissolved the collagen better than the in house Millipore. The collagen was then filtered using the Amicon Ultrafiltration Stirred Cell model 8200 unit. The dissolved collagen was washed 3 times with 0.1M acetic acid (150 mL per wash). The collagen was washed against an Ultracel® 3 kDa nominal molecular weight limit Ultrafiltration Disc. The unit was stirred and under pressure using nitrogen gas. The glassware used to collect the elutant was rinsed ten times with in house deionized water to eliminate ion contamination of the collagen. The collagen was then lyophilized and stored in a freezer.

Differential Scanning Calorimetry (DSC)

DSC was used to analyze the complete polymerization of the polymers. Small pieces (2 mg-10 mg) of the composite were placed in an aluminum hermetic pan, and the pans were sealed shut. The samples were heated from -20 °C to 200 °C, then cooled back down to -20 °C for a second heating to 200 °C at a heating/cooling rate of 10 °C per min.

Dynamic Mechanical Analysis (DMA)

DMA was used to analyze the mechanical properties of the composites. The samples were sawed and sanded with P120 grit sandpaper to make a smooth rectangular prism shape. The DMA Q800 by TA Instruments was used in "DMA Multifrequency – Strain" mode. The samples were placed on a three-point bending set up and heated from -60 °C to 150 °C at a heating rate of 3°C per min. During heating the samples were placed under load with an amplitude of 14.0000 μm.

CHAPTER 3

RESULTS AND DISCUSSIONS

% NaCl Content in Collagen

During the purification of collagen from beef tendons, 5M of NaCl must be added to precipitate the collagen. Since NaCl has been observed to disrupt collagen's triple helical structure, it became a goal of the project to desalt the collagen. Due to this, the original purification dialysis step (48 hours, changing the buffer after 24 hours) was included to desalt the collagen. The percent NaCl content in the collagen was analyzed by completely oxidizing all of the organic components of a sample through the TGA (heating from room temperature to 850°C at a rate of 20°C/min). Only inorganic components, and the TGA measured the mass before and after that complete oxidation to get the percent NaCl content. The exact chemical makeup of the post-TGA residue was not identified fully. It was assumed that a large majority of the inorganics were introduced in the aforementioned NaCl from the purification process. The data in **Table 2** assumes that all the residue is NaCl. It was observed that the dialysis in the purification step did not sufficiently remove the NaCl from the collagen. The collagen was then dialyzed for longer times, with a varying number of buffer changes to attempt to remove the NaCl.

Table 2. Summary table of desalting collagen using dialysis and ultra filtration. After dialysis with 0.02M sodium phosphate buffers, the collagen was desalted using ultra filtration with a MWCO of 1kDa. The percent weight of NaCl was obtained by TGA.

Dialysis Time (Hours)	# of Dialysis Buffers total	% weight NaCl after Dialysis (%)	% weight NaCl after Ultra Filtration (%)
48	2	49.48 (42.79)[a] (44.62)[b]	N/A[e]
96	4	36.46	N/A[e]
144	6	54.18	0.247
66.5[c]	1	57.08 (61.05)[a]	N/A[e]
114.5[c]	3	48.16	N/A[e]
162.5[c]	5	54.75	No Residue Detected
96[d]	1	20-60	0.3815

[a]This sample was not homogenized.
[b]This was a repeat run from a second purification done by Yarami Lopez.
[c]These samples were dialyzed for longer, but with fewer buffers used.
[d]This sample was a mixture of collagen samples purified by Christopher Dzorkpata.
[e]All collagen in these samples was dialyzed again and became a subsequent table entry.

Collagen Homogeneity

The collagen once lyophilized is in one large piece, and the homogeneity of this collagen was in question. The collagen sample dialyzed for 48 hours with 2 buffers and the collagen sample dialyzed for 66.5 hours with 1 buffer were used to test the homogeneity. After homogenization, another small collagen sample was taken. Both samples were used in TGA. The collagen that had been dialyzed for 48 hours with 2 buffers had a higher %NaCl content when homogenized. The collagen that had been dialyzed for 66.5 hours with one buffer had a lower %NaCl content when homogenized. This supports the idea that the single piece of collagen was less homogeneous than the homogenized sample. Due to this result the rest of the collagen in the study (see **Table 2**) were homogenized before use.

Thermogravimetric Analysis (TGA) of Dialyzed Collagen

After a second dialysis, the % weight NaCl was observed to decrease. After a third dialysis, the % weight NaCl increased. Using fewer buffers in dialysis resulted in collagens with higher % weight NaCl, even when dialyzed for longer amounts of time. Due to the % NaCl content increasing after the third dialysis, dialysis was deemed insufficient to fully desalt the collagen

samples. The high NaCl collagen composites used collagen that were dialyzed with only the dialysis in the purification procedure (48 hours with two buffers). This collagen was purified by Yarami Lopez and had 44.62 %NaCl content (**Table 2**).

Ultrafiltration of collagen

Ultra filtration is a more active form of desalting. While dialysis uses passive diffusion, ultra filtration uses pressure to filter the collagen through a membrane of 1kDa MWCO, allowing small ions to be washed through while retaining the collagen. The collagen was washed three times (450mL total) with a low salt 0.1M acetic acid solution. Due to water carboy hard water contamination issues, the deionized water used to make the solution came from the University of North Carolina at Chapel Hill.

Thermogravimetric Analysis (TGA) of Ultra filtered Collagen

The collagen samples that had been dialyzed three times were desalted in an ultrafiltration apparatus. The collagen %NaCl content decreased from approximately 50% NaCl to virtually no residue being detected by the TGA. In attempts to gain more desalted collagen, collagen samples purified by Christopher Dzorkpata were pooled together and washed in the ultrafiltration apparatus. These collagen samples had an %NaCl content ranging from 20%-60%. The collagen post ultrafiltration also had a virtually zero %NaCl content. Ultra filtration of all collagen samples effectively removed almost all of the salt contamination across multiple samples (**Table 2**).

Resin/Composite Curing

The tung oil resin components, excluding the initiator, and protein reinforcements were added and vortexed until all the components had completely dissolved or for a maximum of 30 min. This maximum was put in place to prevent the vortex from denaturing the proteins. Due to this time limit, the protein reinforcement did not fully dissolve in the resin mixture which is observable in **Figure 19**. After the components were mixed, the sample vials were filled with resin mixture froth. Curing the resin/composites with the froth would result in a weaker material due to the many air pockets. To avoid this, the samples were left to sit at room temperature for at least 30

min. After the 30 minute period, a majority of the froth dissipated. At this point the TBPO initiator was added, which removed the rest of the froth. The vial was then swirled by hand to agitate the sample to start polymerization, but gently enough to not produce more froth. After gently swirling, the samples were then immediately placed in the mechanical convection oven to be heated in a stepwise process to 140°C over 25.7 hours.

The proteins in the 5% and 2% desalted collagen and the 5% fibroin mixtures were very massive. These composite mixtures were the only ones whose proteins absorbed the oil instead of being suspended in the oil (as shown in **Figure 19**). If the composites were cured in this state, large amounts of protein would not be effectively incorporated as reinforcement. These proteins were compression molded prior to the addition of TBPO initiator as seen in **Figure 19**.

Figure 19. Composite mixtures before curing. The 1% and 2% fibroin, and 1% desalted collagen are shown on the top left, top middle, and top right, respectively. The 5% and 2% desalted collagen, and the 5% fibroin are shown: bottom left, bottom middle, and bottom right, respectively. These three composites required compression molding. All samples were photographed before curing.

The composites post cure show the limits of incorporating proteins into the composites. With the collagen composites the 5% desalted composite had many pockets of undissolved collagen. This supports the idea of the resin being oversaturated with collagen. This was not observed in the 2% or 1% for both the high NaCl and desalted collagen composites. The 5% fibroin

composite had some undissolved protein pockets, but much less than the 5% collagen. This supports the idea that fibroin may dissolve better in the resin mixture.

After curing, the resin and all the composites had a uniform flaky white layer (remnants can be seen on the 10 % sigma Aldrich collagen composite in **Figure 20**). During the curing process the resin mixture is heated above DVB's boiling point, and some DVB evaporates. It is thought that DVB polymerizes with itself as it is separated from the resin mixture, then deposits onto the tung oil resin/composite as poly DVB.

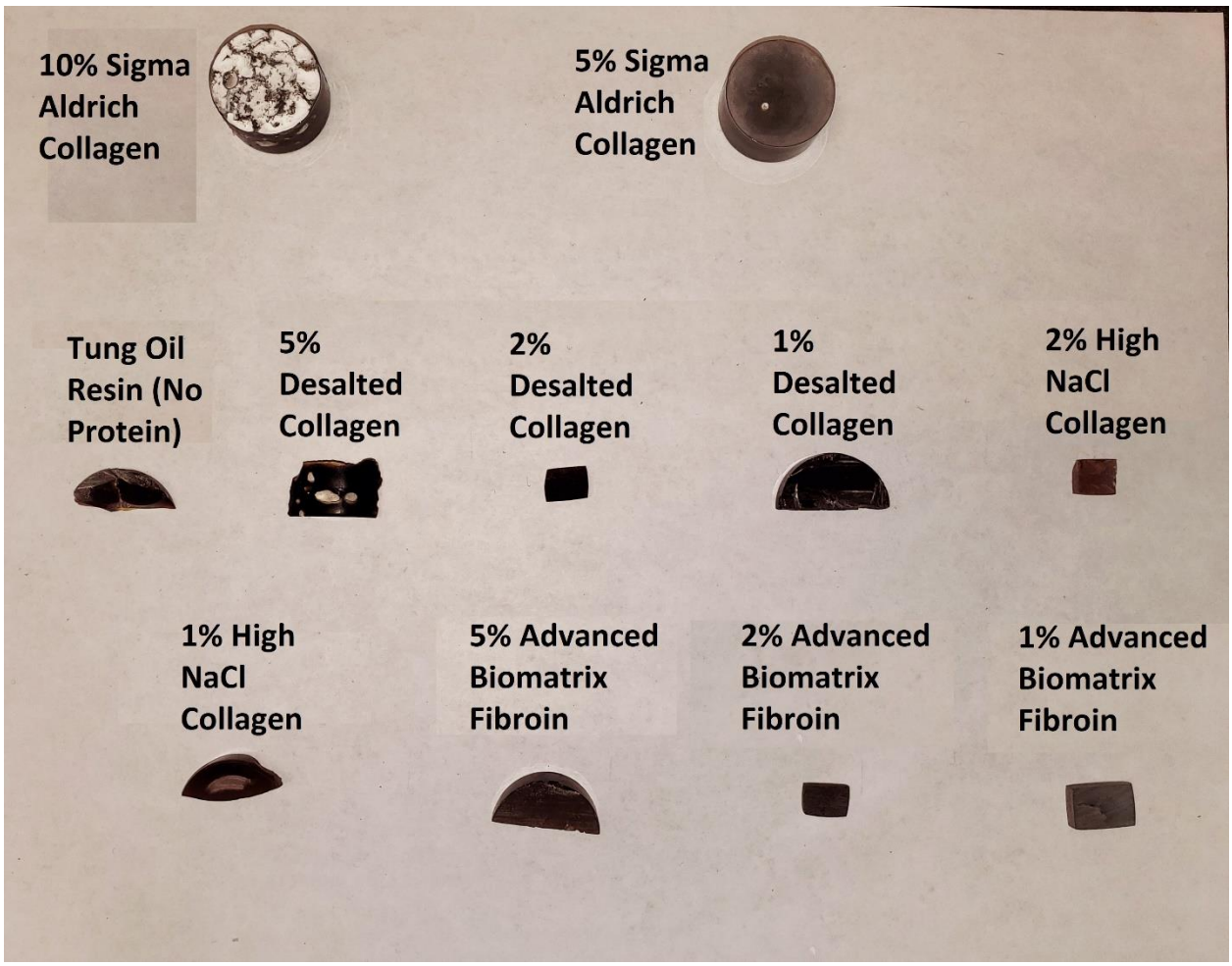

Figure 20. Composite samples post cure, with varying levels of processing for DMA. Note Sigma Aldrich composites were not analyzed in this study. The composites all cured with layers of white poly divinylbenzene deposited on the surface, as seen in the 10% Sigma Aldrich collagen sample. The 5% desalted collagen composite shows pockets of undissolved collagen. The 1% and 2% high salt collagen composites were made by Yarami Lopez.

DSC analysis of the resin/composites

DSC analysis was performed to analyze if the curing method resulted in a complete polymerization. DSC analysis heats the samples and measures energy changes in the form of heat flow. Small 2mg – 10mg samples of the resin or composites were taken and sealed in aluminum hermetic pans. The samples were then heated from -20°C to 200°C at a heating rate of 10°C/min. This heating range was selected because it provides a range above and below the curing temperature. If there are any unreacted monomer components, the heating will cause them to react, which will be detected as an energy change peak by the DSC. Energy changes can also be interpreted as the chemical bonds breaking, making DSC a measure of the polymer's thermal stability as well. Once the samples were heated to 200°C, the samples were cooled to -20°C for a second heating to 200°C. The second heating was done to gain more information on a potential heat flow peak. If the second heating results in a similar peak as the first heating, then the peak is likely not an unreacted monomer reacting in the DSC because the free radical polymerization reaction is an irreversible reaction. It may instead be the polymer chains softening due to increased molecular motion from the heat. This is generally shown in **Figure 21** as the heat flow gradually decreased as the heat was increased, and the second heating shows that is a reversible heat flow decrease.

The DSC of the resin shown in **Figure 21** has smooth heating curves. This indicated that there were no distinct chemical reactions occurring during the heating process. This supports the idea that the resin was completely polymerized. All composites were analyzed using DSC (see appendix "DSC ANALYSIS OF PROTEIN COMPOSITES") and had smooth curves. This indicates that the protein composites did not affect the ability of the curing technique to fully cure the polymer. For the resin and the protein composites, the smooth DSC curves provide evidence that these polymers are thermally stable between -20°C and 200°C.

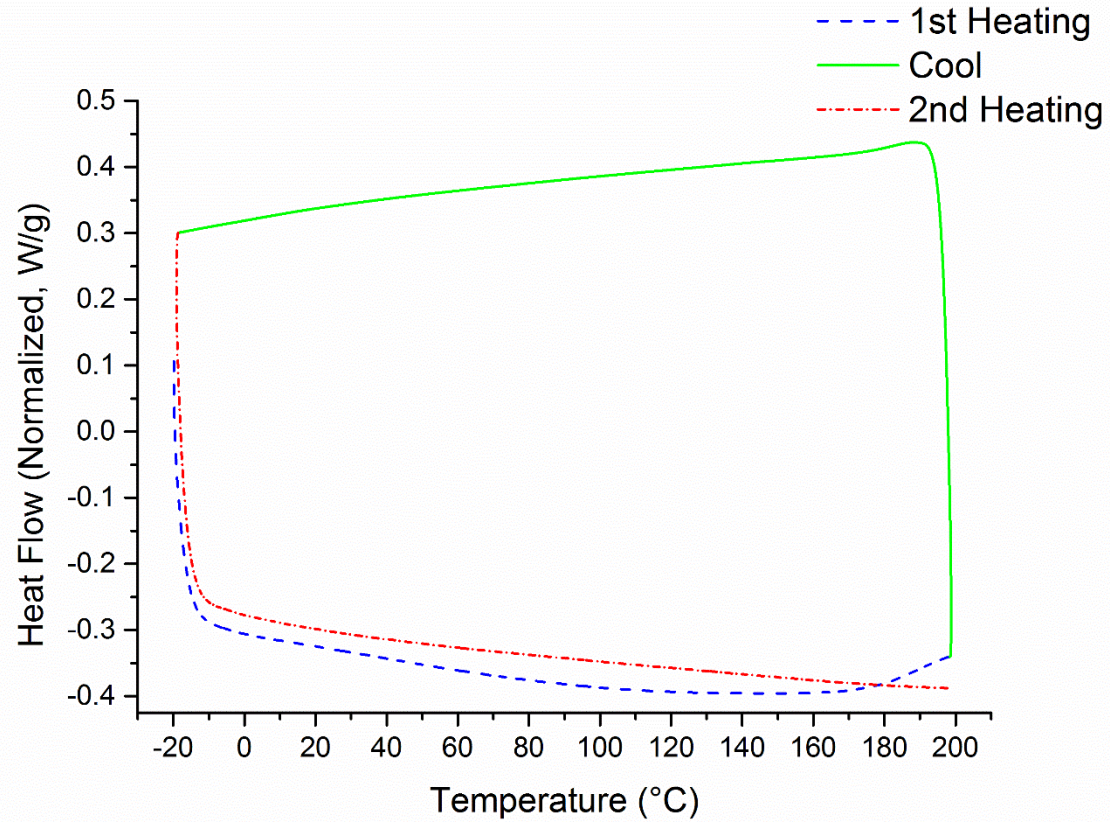

Figure 21. DSC analysis of the tung oil resin with no protein reinforcement. Samples were sealed in aluminum pans and heated from -20°C to 200°C. The samples were then cooled to -20°C, to be heated to 200°C a second time. Heating and cooling were done at a rate of 10°C/min. Samples were run in triplicate.

Dynamic Mechanical Analysis (DMA) of the Resin/Composites

DMA was conducted with the resin and all protein composites. DMA measures the mechanical strength of the material. The DMA used in this study was a three point bending set up, which applies load to the material several times as it oscillates. The DMA measures the storage modulus, which is a measurement of how much a material can store energy through elastic deformation. The DMA done in this study involved heating the sample during the application of load. The sample was heated from -60°C to 150°C with a heating rate of 3°C/min. This heating is done to view how the elasticity of the material changes over a thermal profile. Because temperature directly affects the elasticity of materials, this information gives insight on how the material will mechanically perform at different temperatures. In general polymers have higher storage moduli

when they are colder, as the polymer chains are more rigid. As the sample temperature increases, the storage modulus decays exponentially as the polymer chains become more fluid like. The decay eventually levels out in what is known as a "rubbery plateau," which is where the polymer chains are at their most flexible state. The temperature at the inflection point of the decay is the glass transition temperature (Tg) of the material. The Tg is described as the transition of a material from a glassy state to a rubbery state. When a material's temperature is lower than its Tg, then the material is more rigid and brittle like glass. When the temperature is higher than the material's Tg, the material becomes more flexible like rubber. The inflection point of the storage modulus exponential decay is difficult to view graphically. Instead, the peak of the tan delta curve can better show the Tg. The tan delta curve is a ratio of the loss modulus (a measure of the energy lost due to the material's internal friction when load is applied) to the storage modulus.

The results of the DMA analysis are given in **Figures 22, 23, and 24**, as well as **Table 3.** In **Table 3**, two temperatures were chosen to make numerical comparisons of the storage modulus between the samples analyzed. The lower temperature, physiological temperature of 37°C, was chosen because a possible application for the protein composites are in human implants. The higher temperature, Tg + 50 °C, was chosen to compare the samples while they are all at rubbery states. The addition of protein reinforcement reguardless of NaCl content increased the storage modulus across both temperatures. This supports the idea that these proteins are true reinforcements. The job of a reinforcement is to distribute forces from the resin, to the reinforcement. The reinforcing effect may come from the force being distributed into the collagen and fibroin fibers, which are known for high tensile strength.

The highest storage modulus observed for both temperatures was the 1% high NaCl collagen. The high NaCl composites, while having the highest storage modulus, were also the most brittle, even to the point where there were fewer usable DMA samples for the 2% high NaCl collagen composite. With the 1% sample being less brittle and having a higher storage modulus, there may be a solubility cap with the high salt collagen that has been reached. This may be

beneficial for the chemical industry, as only little reinforcement is needed for maximum effect, and said reinforcement does not need to be fully desalted.

The desalted collagen composites had differing trends based on the temperature. At 37 °C as the amount of desalted collagen increases, the storage modulus decreases. At Tg + 50, the storage modulus increases from 1% to 2% desalted collagen but decreases from 2% to 5%. The 2% desalted collagen needs more trials, as the trends do match between the temperatures if the 2% sample is treated as an outlier. That could mean the desalted collagen trends match the trend for the high NaCl collagen composites. That trend being adding 1% collagen increases the storage modulus but adding above 1% collagen decreases the storage modulus. If the current trend persists after additional trials, then it may be due to the 5% desalted collagen composite's drastic decrease in Tg value when compared to the 2% desalted collagen composite. Because the Tg is a property of the resin, it may be that once enough desalted collagen is added it chemically affects the resin's polymerization, or the resin undergoes conformational changes that affect the storage modulus.

The fibroin composites showed the opposite trend from the collagen composites. The collagen composites overall were observed to increase in storage modulus as the amount of fibroin content increased. At Tg + 50 °C, the storage modulus of the fibroin composites increased as the amount of fibroin content increased. At 37 °C the storage modulus decreased from 1% to 2%, and then increased from 2% to 5%. This is similar to the desalted collagen at Tg + 50°C, where it may be possible that if more trials were run on the 2% fibroin sample, then the sample may fit into the trend the fibroin composites at Tg + 50 °C exhibits. If this is not the case after more trials, then the drastic decrease in Tg value of the 5% fibroin composite may be a clue that the resin is being chemically affected more significantly once enough fibroin is added. It may be that adding enough fibroin or collagen causes a drastic change in the resin's polymerization or causes a resin conformational change that affects the storage modulus. Despite this, the highest storage modulus of the fibroin composites at both temperatures is still the 5% sample, and the lowest at both

temperatures is the 1% sample. This supports the idea that it may be possible to dissolve more fibroin into the resin and increase the storage modulus further.

The Tg values vary significantly with added protein reinforcement across all samples. Because the Tg is generally a property of the resin itself, this indicates that the protein reinforcement is not only acting as a physical reinforcement, but chemically affecting the resin itself. The addition of the proteins and NaCl may be causing polymer conformational changes in the resin which affect the physical properties of the resin. While the Tg values are affected, the data does not display many trends. The Tg value of the resin was observed to increase and decrease depending on the type of reinforcement and the amount of reinforcement added. Adding 1% high NaCl collagen decreased the Tg, but when 2% high NaCl collagen was added the Tg increased higher than the original resin. When adding 1% fibroin the Tg increased, but upon adding more fibroin the Tg decreased for both the 2% and 5% samples. Because the samples were polymerized using bulk polymerization, the polydispersity index (PDI) is relatively high, leading to variable polymer chain lengths. The chain lengths of the proteins were also variables that were not being controlled both in the collagen purification and in the fibroin from Advanced Biomatrix. Chain lengths of both the resin and the proteins do affect the physical properties, including the Tg value. More trends may be unveiled if more repeat experiments were analyzed.

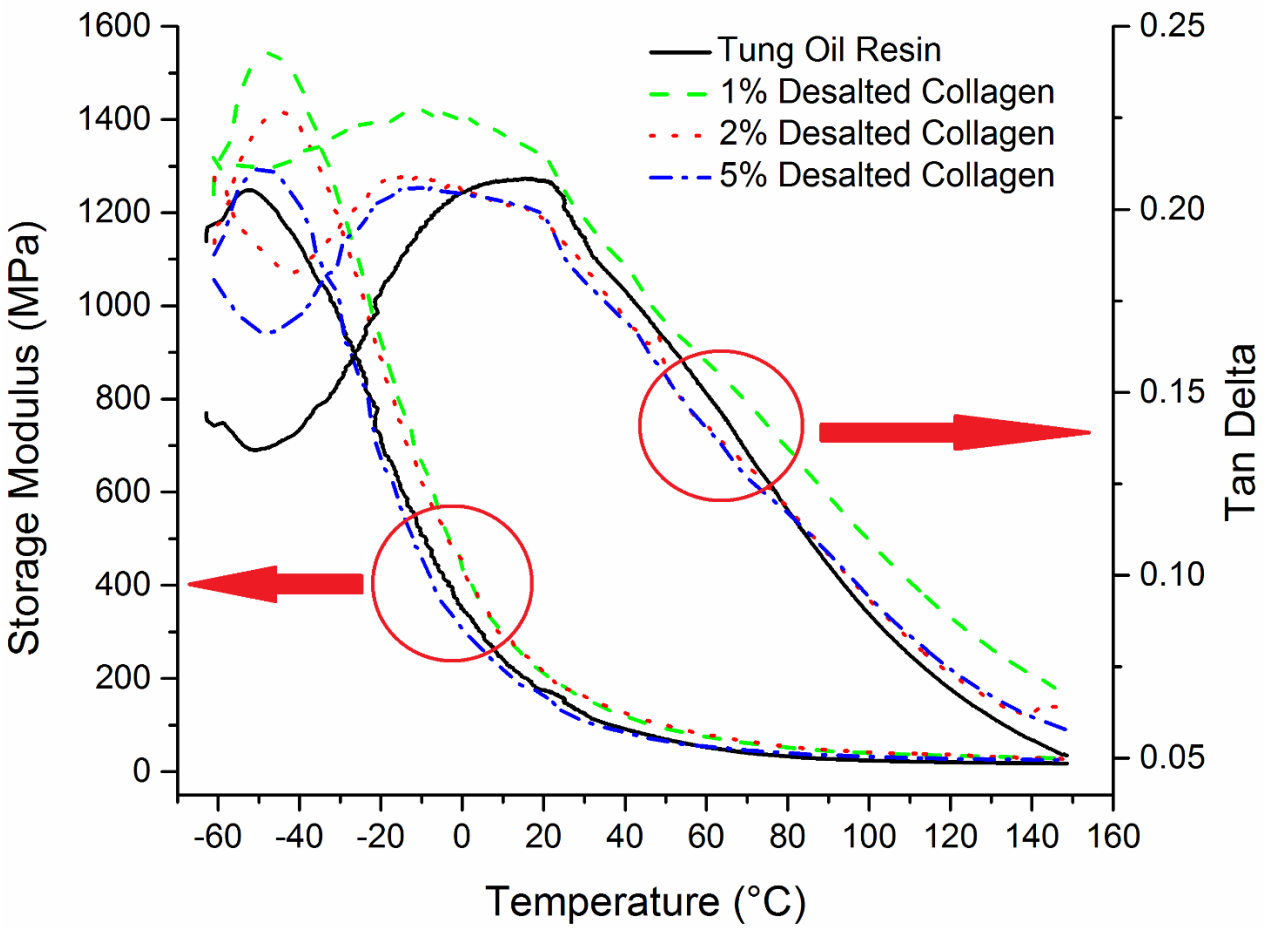

Figure 22. DMA comparison of desalted collagen composites and polymer resin. Samples were heated from -60°C to 150°C with a heating rate of 3°C/min. The strain load amplitude was 14.0000 μm. Storage modulus and tan delta curves are represented with the same color/line format.

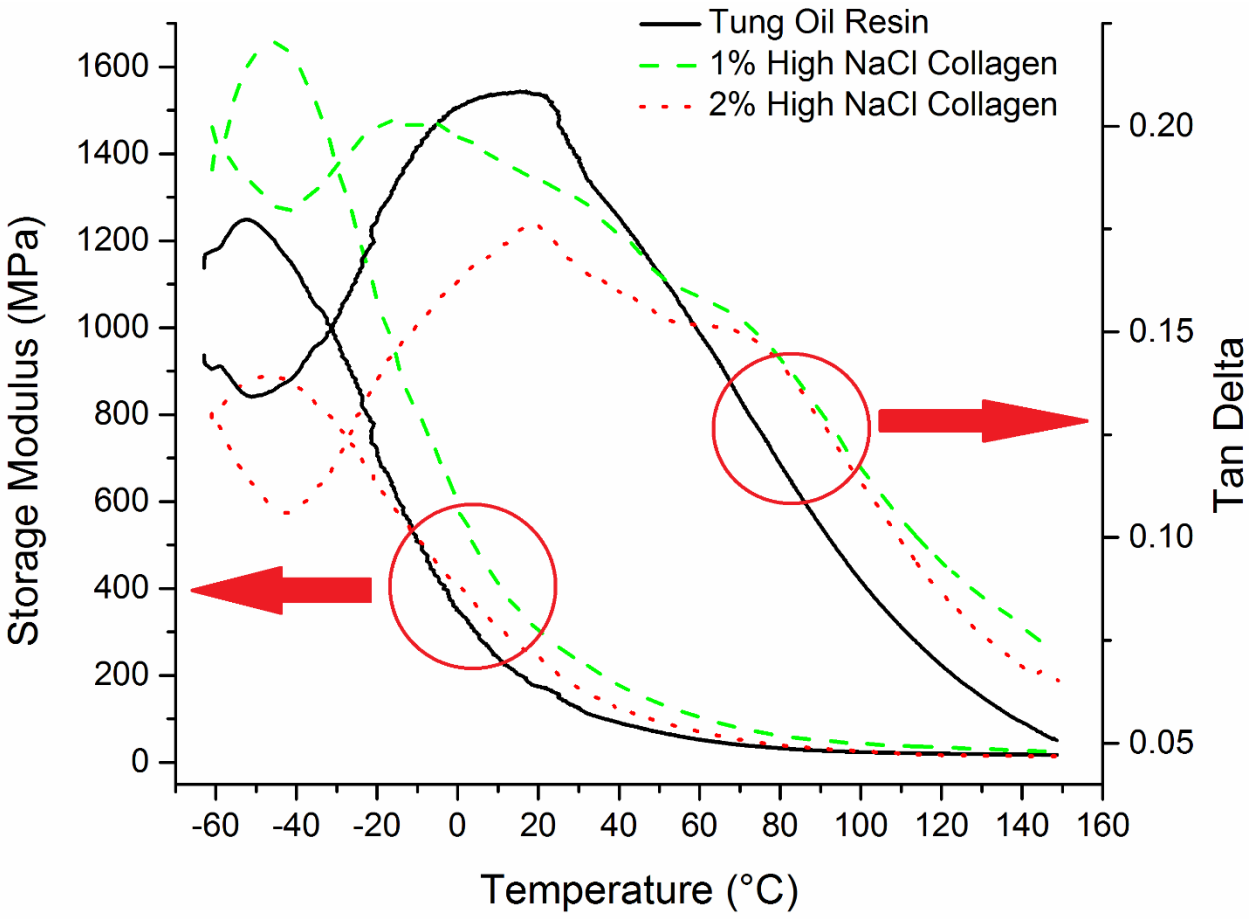

Figure 23. DMA comparison of high NaCl collagen composites and polymer resin. Samples were heated from -60°C to 150°C with a heating rate of 3°C/min. The strain load amplitude was 14.0000 µm. Storage modulus and tan delta curves are represented with the same color/line format.

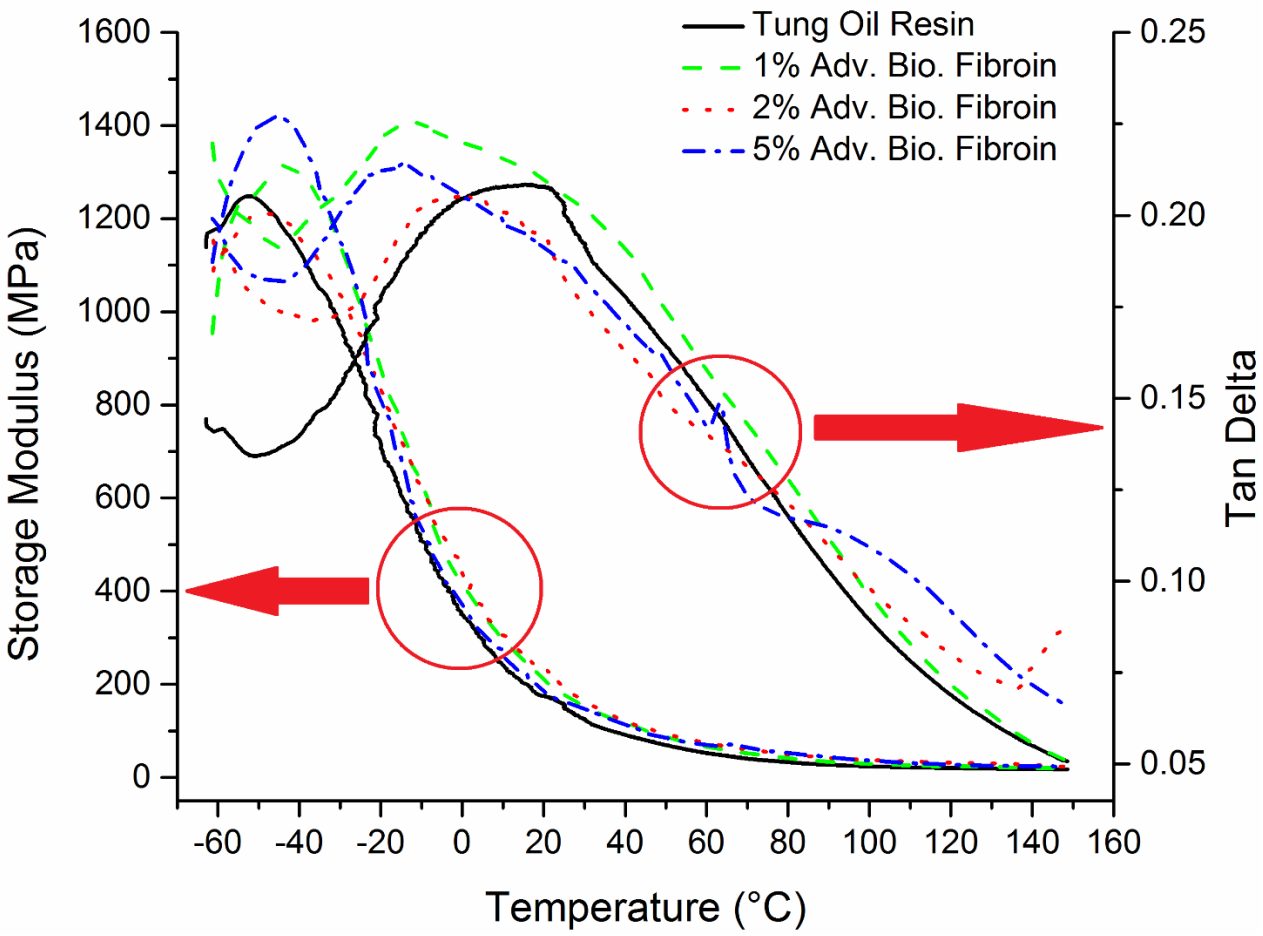

Figure 24. DMA comparison of Advanced Biomatrix fibroin composites and polymer resin. Samples were heated from -60°C to 150°C with a heating rate of 3°C/min. The strain load amplitude was 14.0000 µm. Storage modulus and tan delta curves are represented with the same color/line format.

Table 3. Summary DMA table of samples run in triplicate, with outliers removed as necessary.

Sample	**Storage Modulus at 37 °C**		**Storage Modulus at Tg + 50 °C**		**Glass Transition Temperature Tg**	
	Average (MPa)	**Standard Deviation**	**Average (MPa)**	**Standard Deviation**	**Average (°C)**	**Standard Deviation**
Tung Oil Resin (No Protein)	97.45	2.57	47.44	2.30	13.56	1.71
1% Desalted Collagen	161.52	45.43	105.41	15.30	5.17	3.61
2% Desalted Collagen	115.01	28.54	130.12	16.77	6.97	6.78
5% Desalted Collagen	99.40	13.15	88.30	7.25	-8.06	1.31

1% High NaCl Collagen[b]	186.62	10.30	160.77	68.17	-21.72	14.44
2% High NaCl Collagen[a,b,c]	167.48	41.67	73.90	28.16	17.98	1.56
1% Advanced Biomatrix Fibroin	122.23	4.50	61.29	4.91	17.17	6.39
2% Advanced Biomatrix Fibroin	103.36	39.81	71.22	24.58	3.03	1.30
5% Advanced Biomatrix Fibroin	140.98	25.65	121.19	10.47	-18.65	5.29

[a]This sample was very brittle, and only two samples could be analyzed.
[b]This sample was created by Yarami Lopez
[c]This sample was analyzed partially by Yarami Lopez

Thermogravimetric Analysis (TGA) of the Resin/Composites

TGA was conducted to analyze the thermal stability of the resin and composites. Small samples weighing 8mg to 12mg were heated from room temperature to 850°C with a heating rate of 20°C/min. This was done in an air environment to allow the oxidation of all organic components. The mass is measured during the heating to give a measure of how the samples degrade with increasing temperature. In general, TGA curves first show a drop in mass around 100°C as any volatiles are evaporating from the sample. This could be water or any other organic solvents. Around 400°C to 500°C is the range in which the first major breaking of carbon-carbon bonds occurs. There is a second degradation around 550°C to 650°C where the more highly crosslinked bonds start to break such as the areas in the polymer that contain DVB. Because of this it may be possible to observe a change in polymer crosslink density by analyzing this second degradation. To better numerically compare the data an arbitrary temperature was chosen to compare the % weights. The T80, the temperature at which 20% of the sample has degraded, was chosen because at that temperature, the sample curves were not overlapping for a better comparison.

The TGA analysis shows that the protein reinforcement of the polymer, both collagen and fibroin, does have a small effect on the thermal stability (see **Figure 25**, and **Table 4**). The tung oil resin is the first sample to begin its first major degradation at around 325°C. Then at 350°C the fibroin and desalted collagen composites begin to degrade. At 375°C the salted collagen begins to degrade. The desalted collagen and fibroin degrade the fastest, and despite the tung oil resin beginning its major degradation first, it appears to degrade at the slowest rate. The tung oil resin has the highest percent weight from 525°C to 650°C. The salted collagen composites have the highest percent weight from 400°C to 500°C. The proteins in this study are biodegradable, and the resin is highly crosslinked. This crosslinking makes the resin much more thermally stable in comparison to the proteins. This may be a reason that the desalted collagen and fibroin composites

degrade at lower temperatures when compared to the tung oil resin. One other possibility is that the proteins are chemically interacting with the resin in some way. The DMA data also supports this with the reinforcement affecting the Tg values, a property of the resin, not the reinforcement. It is possible that the addition of these proteins caused some resin conformational changes that affect the thermal stability. Conformational changes are generally dictated by the intermolecular forces present that affect the polymer. The fact that the high NaCl collagen degraded at higher temperatures may be due to the increased ionic intermolecular forces that induce different conformational changes on the resin. NaCl in general is much more thermally robust in comparison to proteins and thermosets, which may be another reason for the increased thermal stability. Even with these disparities, while looking at the T80 values in **Table 4**, the differences in the thermal stability across all samples is very miniscule. The largest difference across the samples is between the tung oil resin and the 1% high NaCl collagen composites. This difference is only 42.20°C, which suggests that the protein reinforcement does not greatly affect the thermal stability of the resin.

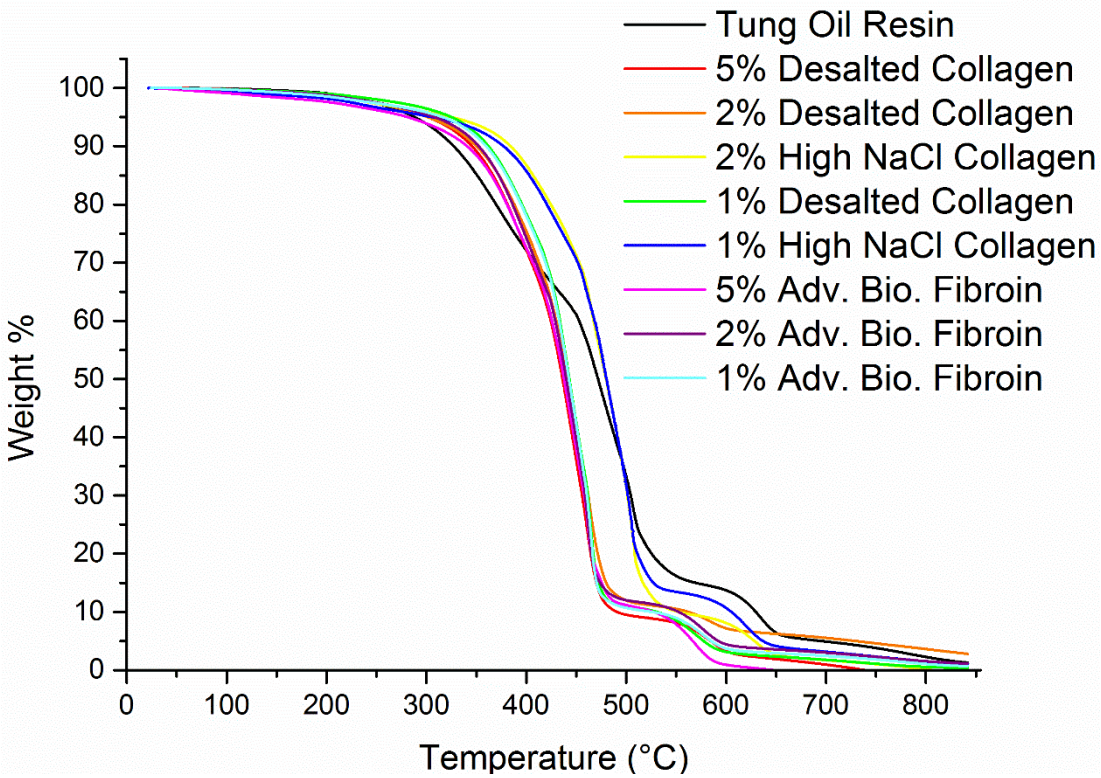

Figure 25. TGA analysis of all composites. Samples were heated in an air environment from room temperature to 850 °C with a heating rate of 20°C/min.

Table 4. Summary of TGA Data. Samples were run in triplicate.

Sample	**T80: Temperature at 20% Thermal Degradation**	
	Average (°C)	**Standard Deviation**
Tung Oil Resin (No Protein)	382.30	10.92
1% Desalted Collagen	391.78	3.82
2% Desalted Collagen	389.81	1.98
5% Desalted Collagen	384.96	5.65
1% High NaCl Collagen	424.50	3.30
2% High NaCl Collagen	421.74	10.15
1% Advanced Biomatrix Fibroin	386.51	10.20
2% Advanced Biomatrix Fibroin	387.64	1.21
5% Advanced Biomatrix Fibroin	382.05	4.02

CHAPTER 4

CONCLUSION

Most protein based material studies involve the protein as the primary material that is being reinforced. This study provides more insight into how proteins act as reinforcement, which is not well studied in the literature. In all the samples adding proteins increased the storage modulus of the material, which supports the idea that desalted collagen, high NaCl collagen, and fibroin are all true reinforcements. It is also certain that salt affects the reinforcement, as it was observed to increase both the thermal stability and the storage modulus. However, the samples were much more brittle. To mitigate this issue, it may be beneficial in the future to increase the percentage of BMA for its plasticizing effects in high NaCl collagen. The collagen that is purified from beef tendons has been shown to be virtually completely desalted after using ultra filtration. Ultra filtration was much more effective than dialysis was in desalting collagen. However, it may be that desalting collagen is not as beneficial as maintaining salt in the composites. In general, increasing the amount of both collagen past 1% decreases the storage modulus. Fibroin had the opposite effect, with increasing fibroin causing a decrease in the storage modulus. It may be possible to increase the amount of fibroin to increase the storage modulus further. At lower temperatures, the 1% desalted collagen composite was observed to have a higher storage modulus than the 5% fibroin composite, indicating that the desalted collagen was a greater reinforcement at lower temperatures. The opposite conclusion can be drawn when looking at the higher Tg + °C temperature. Fibroin may be a greater reinforcement for more plastic resins. The Tg values were affected by the reinforcement, indicating that the resin is being affected chemically. The Tg values varied, and more future trials may unveil trends. The DSC analysis showed that all of the samples were thermally stable from -20°C to 200°C and that the curing process is effective in achieving a complete polymerization, despite the type or amount of protein added. The TGA shows that the addition of these proteins has

a minimal effect on the thermal stability of the resin. The desalted collagen and fibroin composites had very similar thermal stabilities, while the high NaCl collagen composite had a higher stability.

In the future it would be interesting to use circular dichroism analysis to verify the collagen triple helix in a desalted collagen sample. More thermal analysis of trials of the samples is needed to unveil trends and further support the currently observed trends. The identity of the collagen contaminant is assumed to be NaCl because past collagen sample contamination was verified by doing ICP-MS of the saved desalting washes.[28] In the future ICP-MS could be done with the collagen desalting washes from the samples used in this work. This project also has peptide research in its future. The idea is to make peptides that are similar to the protein composites to make more finely tunable physical properties. One interesting aspect of the peptide composites is the addition of the allylglycine amino acid. This amino acid has a pi bond and would covalently bond the peptide to the resin under free radical polymerization. This may shift the physical properties of a composite due to the extra covalent linkage. It is known that proteins denature with heat, but currently there is not a known method for analyzing the tertiary/quaternary structure of the proteins after they are cured in the resin.

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APPENDIX

DSC ANALYSIS OF PROTEIN COMPOSITES

Figures 21, 26, 27, 28, 29, 30, 31, 32, and **33** all conclude the same result that all of the composites cured were completely polymerized, due to having no distinct heat flow peaks when heated well above the curing temperature.

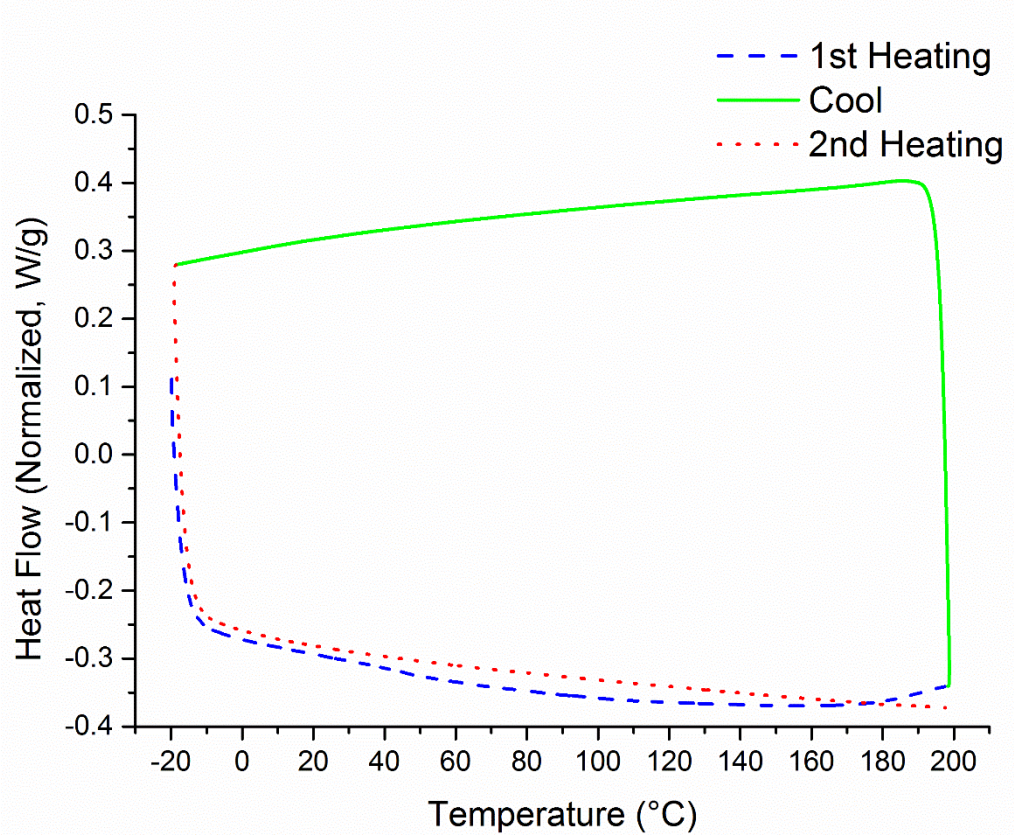

Figure 26. DSC analysis of the 1% desalted collagen composite. Samples were sealed in aluminum pans, and heated from -20°C to 200°C. The samples were then cooled to -20°C, to be heated to 200°C a second time. Heating and cooling were done at a rate of 10°C/min.

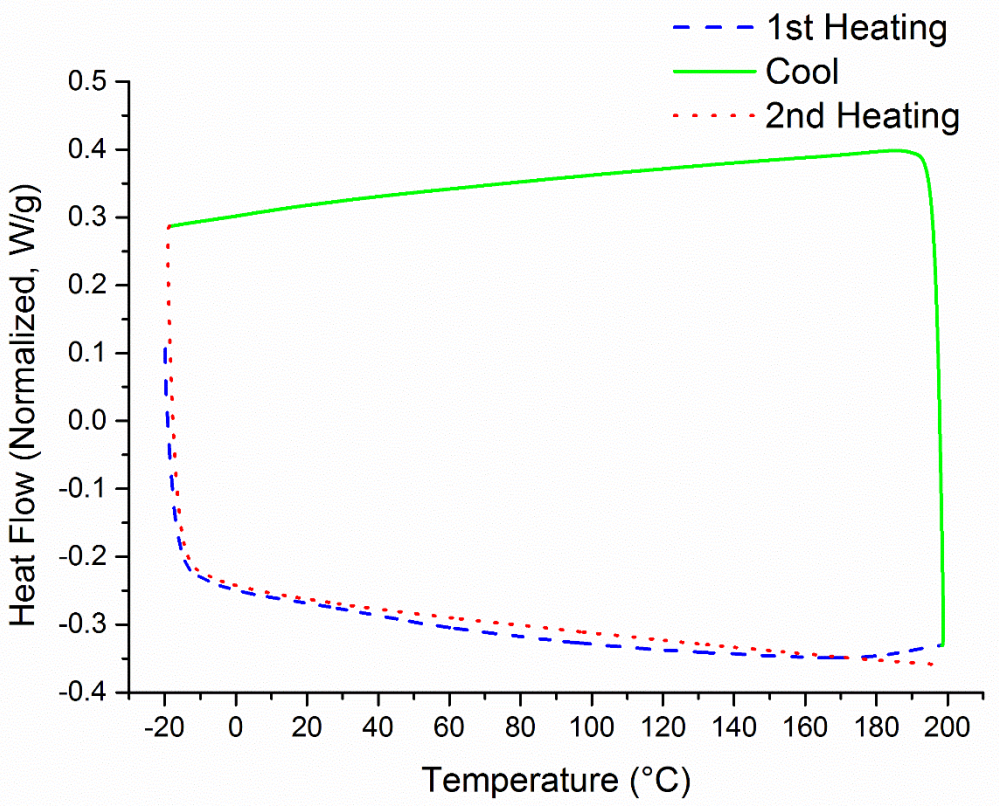

Figure 27. DSC analysis of the 2% desalted collagen composite. Samples were sealed in aluminum pans, and heated from -20°C to 200°C. The samples were then cooled to -20°C, to be heated to 200°C a second time. Heating and cooling were done at a rate of 10°C/min.

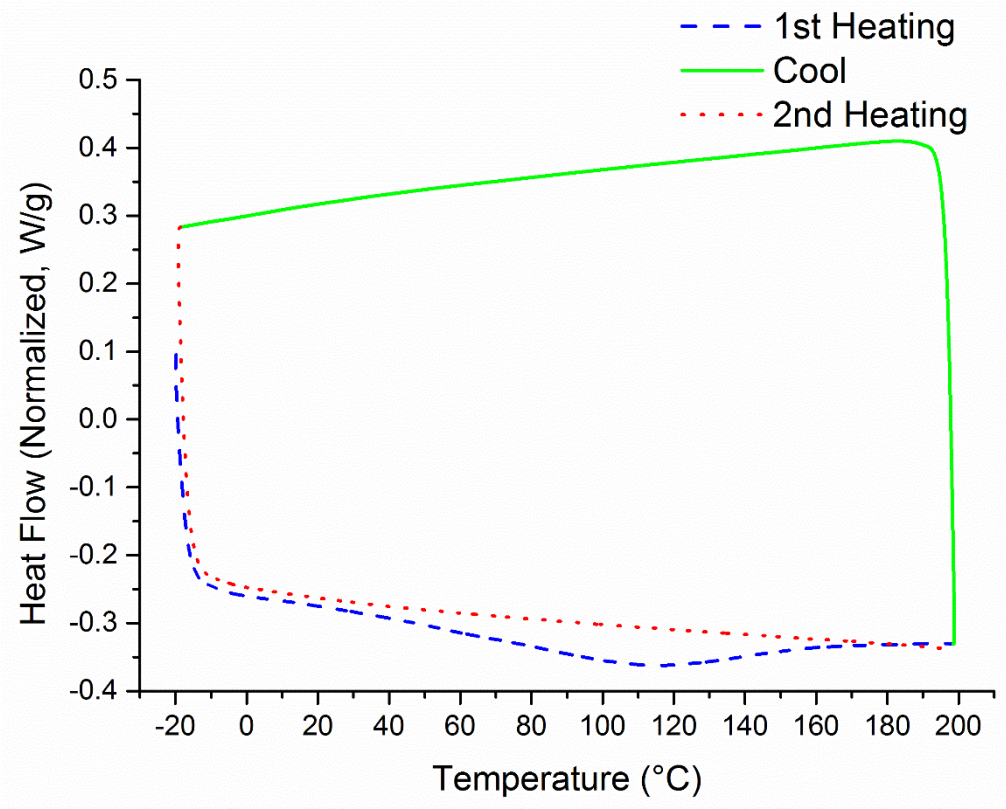

Figure 28. DSC analysis of the 5% desalted collagen composite. Samples were sealed in aluminum pans, and heated from -20°C to 200°C. The samples were then cooled to -20°C, to be heated to 200°C a second time. Heating and cooling were done at a rate of 10°C/min.

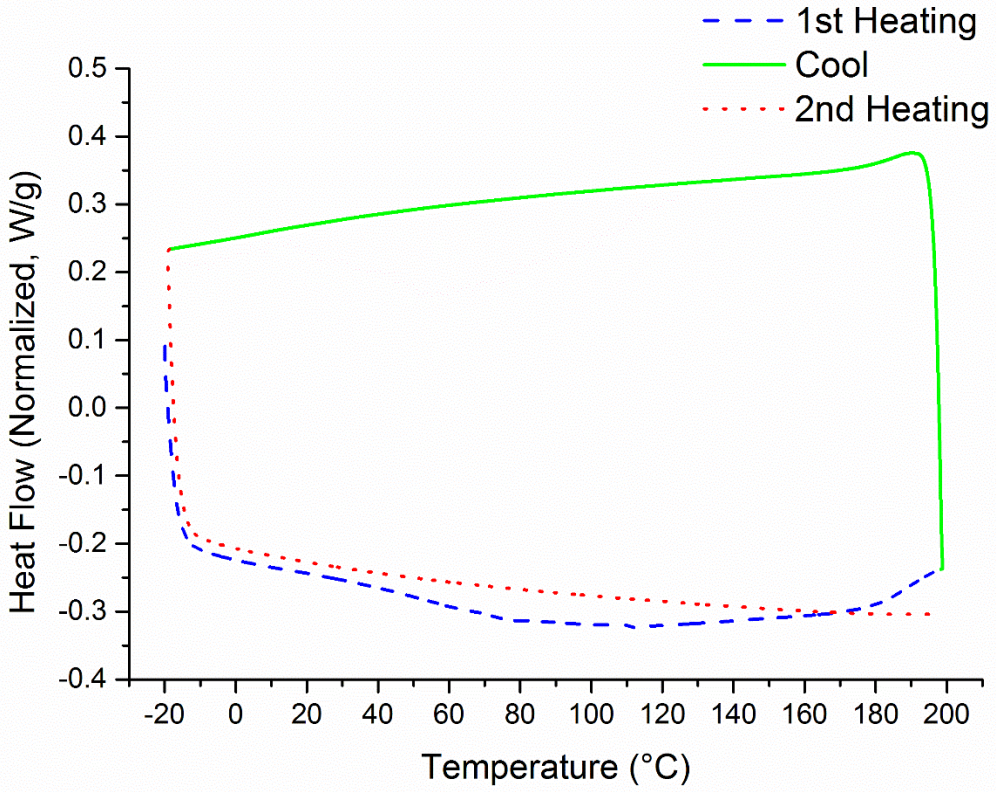

Figure 29. DSC analysis of 1% high NaCl collagen composite. Samples were sealed in aluminum pans, and heated from -20°C to 200°C. The samples were then cooled to -20°C, to be heated to 200°C a second time. Heating and cooling were done at a rate of 10°C/min.

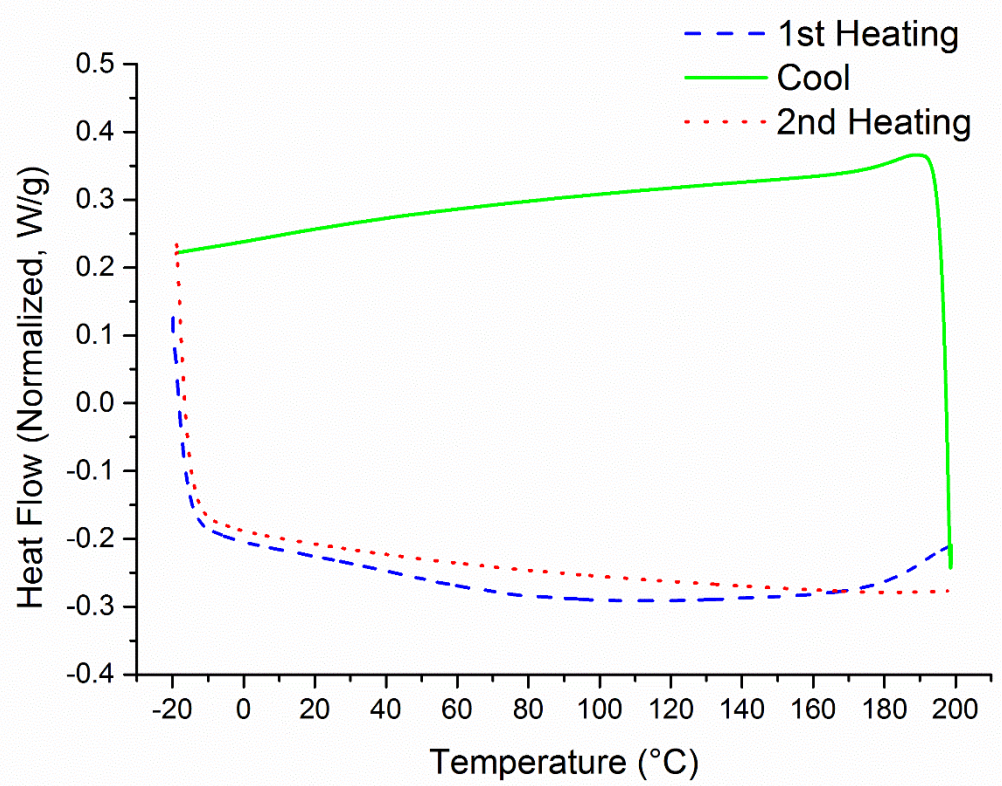

Figure 30. DSC analysis of the 2% high NaCl collagen composite. Samples were sealed in aluminum pans, and heated from -20°C to 200°C. The samples were then cooled to -20°C, to be heated to 200°C a second time. Heating and cooling were done at a rate of 10°C/min.

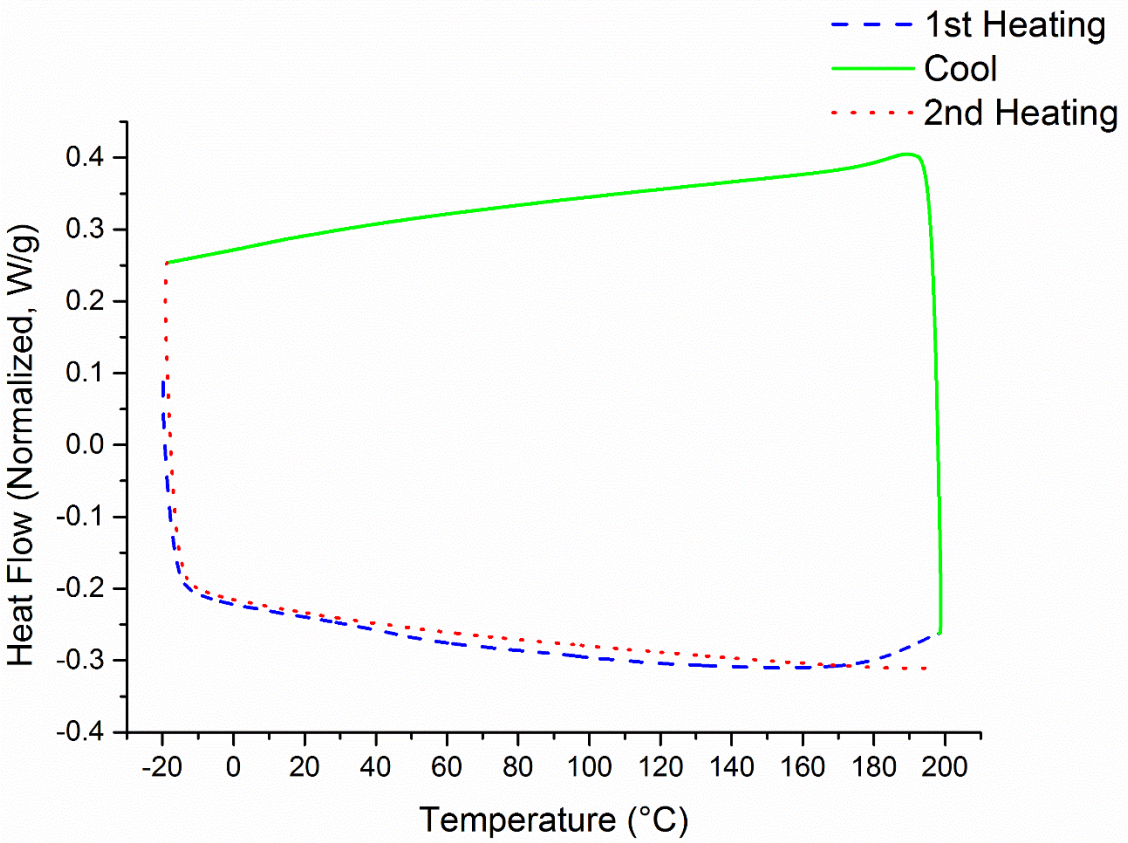

Figure 31. DSC analysis of the 1% Advanced Biomatrix fibroin composite. Samples were sealed in aluminum pans, and heated from -20°C to 200°C. The samples were then cooled to -20°C, to be heated to 200°C a second time. Heating and cooling were done at a rate of 10°C/min.

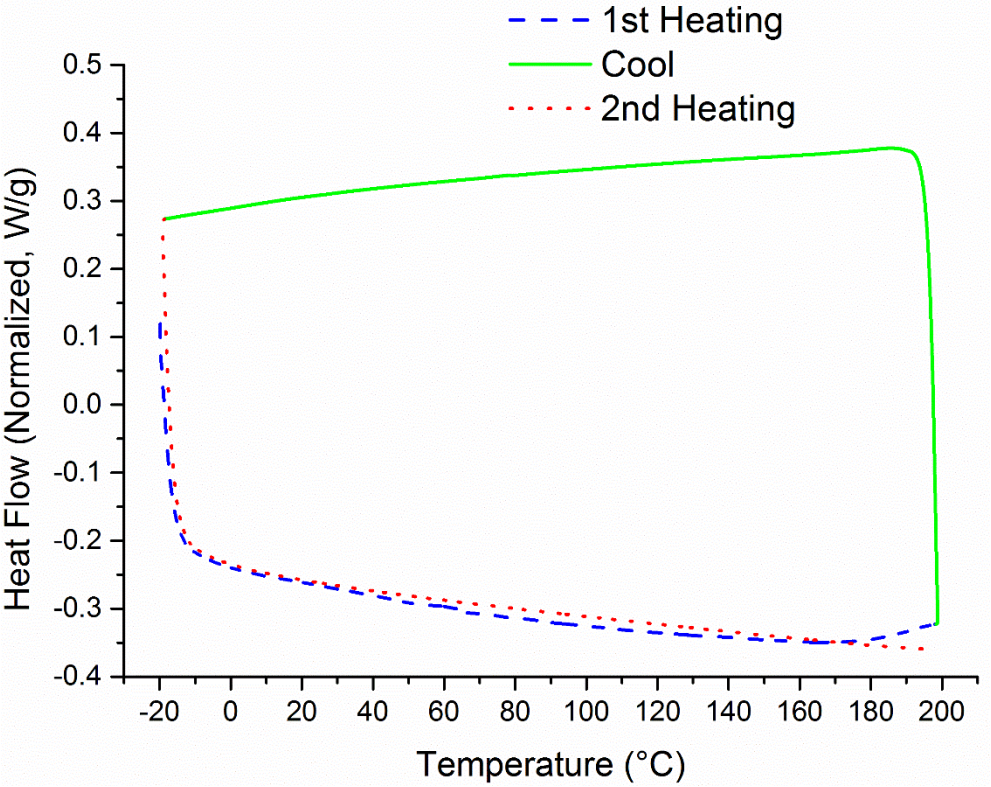

Figure 32. DSC analysis of the 2% Advanced Biomatrix fibroin composite. Samples were sealed in aluminum pans, and heated from -20°C to 200°C. The samples were then cooled down to -20°C, to be heated to 200°C a second time. Heating and cooling were done at a rate of 10°C/min.

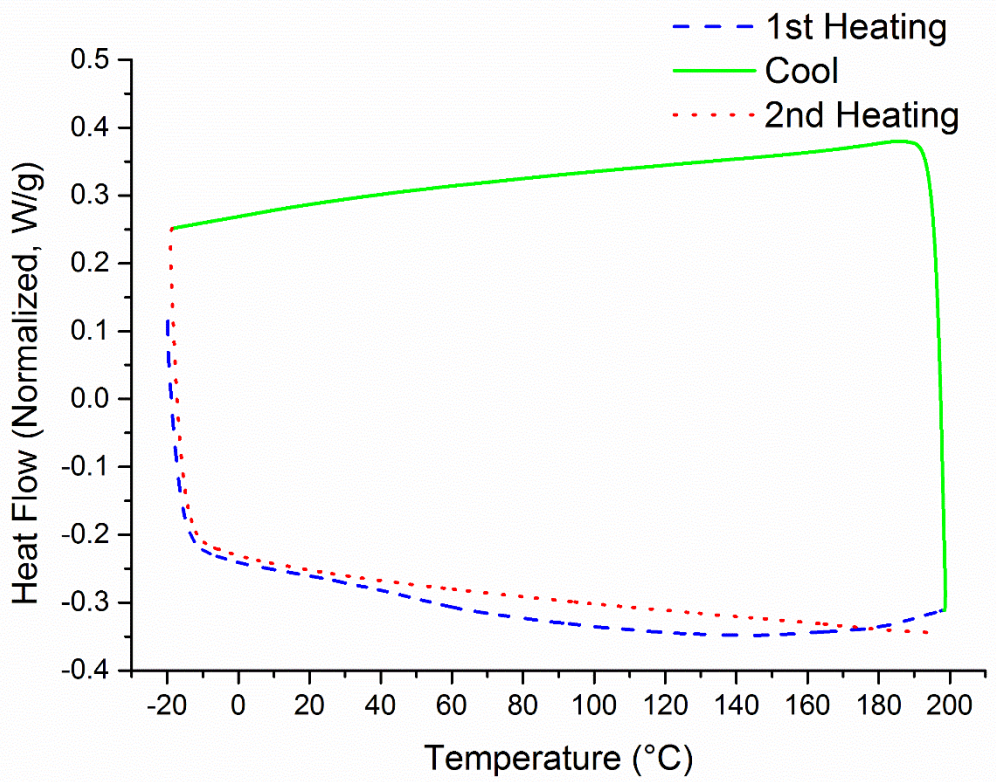

Figure 33. DSC analysis of the 5% Advanced Biomatrix fibroin composite. Samples were sealed in aluminum pans, and heated from -20°C to 200°C. The samples were then cooled down to -20°C, to be heated to 200°C a second time. Heating and cooling were done at a rate of 10°C/min.