Spring 2021

The Effects of NaCl on the Triple Helix Structure of Collagen and the Reinforcement of Tung oil-based Polymers with Collagen Peptides

Christopher Dzorkpata

Recommended Citation

Dzorkpata, Christopher, "The Effects of NaCl on the Triple Helix Structure of Collagen and the Reinforcement of Tung oil-based Polymers with Collagen Peptides" (2021). *Electronic Theses and Dissertations*. 2261.
https://digitalcommons.georgiasouthern.edu/etd/2261

THE EFFECTS OF NaCl ON THE TRIPLE HELIX STRUCTURE OF COLLAGEN, AND THE REINFORCEMENT OF TUNG OIL-BASED POLYMERS WITH COLLAGEN PEPTIDES

by

CHRISTOPHER DZORKPATA

(Under the Direction of Rafael L. Quirino)

ABSTRACT

In this research, a collagen-reinforced bio-composite from tung oil was prepared using free radical initiation and thermal polymerization, leading to polyolefinic tung oil-based matrix. Two peptides (long and short) were synthesized and their secondary features were analyzed using circular dichroism spectroscopy. After this analysis, the long peptide exhibited more triple helix characteristics as compared to the short peptide. The full protein will ultimately have a better interaction with the tung oil, was used in the synthesis of the collagen-reinforced bio-composite. The presence of NaCl in the triple helix and its overall effect on the thermal and mechanical properties of the bio-composite was also investigated. Cure time was determined through differential scanning calorimetry (DSC). The thermo-mechanical properties were assessed by means of thermogravimetric analysis (TGA) and dynamic mechanical analysis (DMA), showing impressive improvement in storage modulus upon addition of the triple helix long peptides.

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

THE EFFECTS OF NaCl ON THE TRIPLE HELIX STRUCTURE OF COLLAGEN AND THE REINFORCEMENT OF TUNG OIL-BASED POLYMERS WITH COLLAGEN PEPTIDES

by

CHRISTOPHER DZORKPATA

B.S., Kwame Nkrumah University of Science and Technology, Ghana, 2004

M. Ed., University of Education, Ghana, 2015.

A Thesis Submitted to the Graduate Faculty of Georgia Southern University in

Partial Fulfilment of the Requirements for the Degree

MASTER OF SCIENCE

THE EFFECTS OF NaCl ON THE TRIPLE HELIX STRUCTURE OF COLLAGEN, AND THE REINFORCEMENT OF TUNG OIL-BASED POLYMERS WITH COLLAGEN PEPTIDES

by

CHRISTOPHER DZORKPATA

Major Professor: Rafael L. Quirino

Committee: Amanda White

Beverly Penland

Electronic Version Approved:

May 2021

DEDICATION

To **Deladem** for inspiring me even when he was not yet born. To **Selase** for taking care of my wife in my absence.

ACKNOWLEDGMENTS

To my committee members, Dr. Beverly Penland and Dr. Amanda White, I say a big thank you for your expertise, valuable time and insightful comments. To Dr. Amanda White, thank for allowing me to use your lab to run part of my experiments and for continuous encouragement. I would like to thank the Applied Physical Science program for allowing the use of their facilities, graduate tuition waiver and graduate assistant funding. I also thank Dr. McGibony for her support and guidance as the Applied Physical Science graduate program director

I am immeasurably indebted to my supervisor, Dr. Rafael Quirino, for the birth of this work, and his guidance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

BACKGROUND

1.1 *Polymers from Vegetable Oils*

With the rapid development of living standards and the consequent rapid consumption of resources, people have faced the challenges of survival from the aspects of renewable energy, environment, and population [1, 2]. In order to promote sustainable development, the effective use of renewable energy and resources has attracted more attention in recent years [3, 4]. Vegetable oils have become an area of keen interest for both academic and industrial research due to their ubiquitous availability, inherent biodegradability, and low price [5, 6]. Tung oil, which is derived from the seeds of tung trees, is an example of vegetable oil. Due to the advantages of fast curing, good corrosion, resistance and high temperature resistance, the products derived from tung oil have been widely used in various aspects such as military products, electronic appliances, construction decoration, waterproof coatings, and biodiesel [7]. In the case of biodiesel, which is a monoalkyl ester of long chain fatty acid derived from renewable feedstocks like vegetable oil, it is produced by transesterification. In this process, the oil or fat is reacted with a monohydric alcohol in the presence of a catalyst to give the corresponding monoalkyl esters as illustrated in figure 3 [8]. Tung oil is also widely used in polymeric resins [9] and plasticizers [10] in traditional coatings, and therefore constitutes an important bio-based building block. The main component of tung oil (Figure 1) is alpha-eleostearic acid (Figure 2**),** which contains three conjugated double bonds [11, 12].

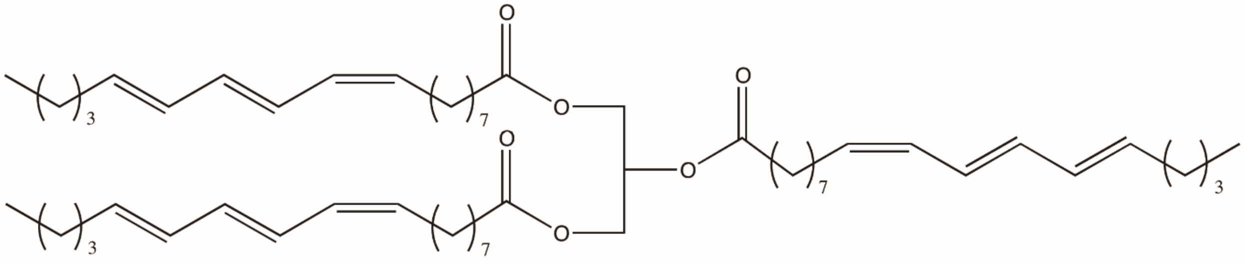

Figure 1: Chemical structure of tung oil. Adapted from reference [1]

Figure 2: Chemical structure of α-eleostearic acid. Adapted from reference [1]

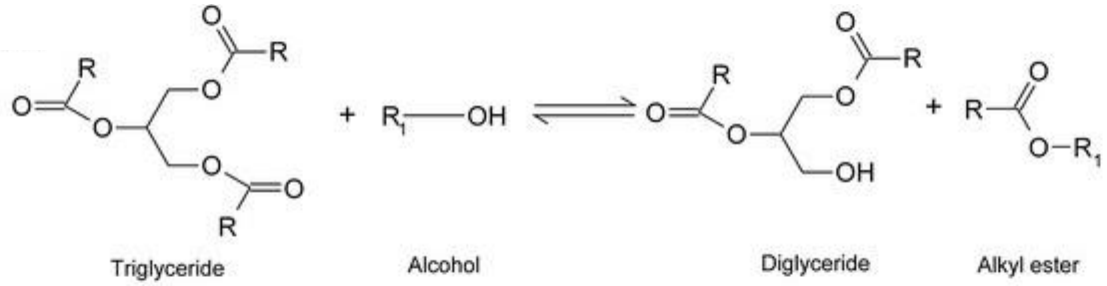

Figure 3. Reaction scheme for transesterification of vegetable oils (triglycerides). Adapted from reference [13]

For improving the properties of tung oil-derived polymers, a self-crosslinking monomer with epoxy and anhydride groups has been prepared from tung oil. This monomer has very good storage stability and can be cured with epoxy resins [14]. A biobased epoxy monomer with conjugated carbon-carbon double bonds was also synthesized from tung oil [15]. This monomer can react with both dienophiles and anhydrides via Diels-Alder and epoxy/anhydride ring-opening reaction to obtain thermosetting polymers with high performance but with lower thermal and mechanical properties than the petroleum-based epoxy resins as seen in figure 4.

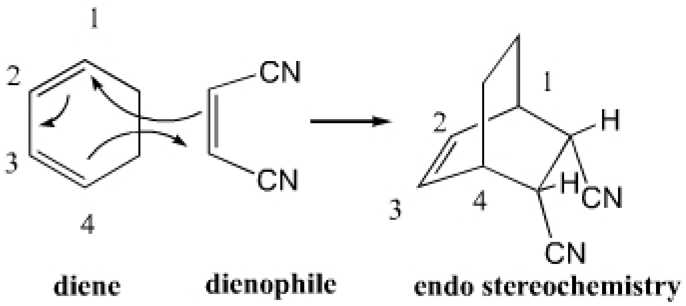

Figure 4: An example of a Diels Alder rection mechanism. Adapted from reference [16]

Vegetable oils like linseed and soybean oils have been used as major components in various resin formulations. Semi-conducting materials have been prepared from a poly (urethane amide) of linseed oil blended with poly (1-napthylamine) [17]. Linseed oil monoglyceride maleates have been co-polymerized with styrene to form a matrix for wood flour composites [18]. Clay composites have also been prepared using a matrix where conjugated linseed oil was co-polymerized with divinylbenzene (DVB) and acrylic acid (AA), and a similar resin containing linseed oil, DVB, styrene, and AA has also been studied [19]. Linseed oil has also been used as the starting material in the preparation of an AA-esterified monomer for later co-polymerization with styrene [19]. Also, polyester amides have been made from linseed oil [17]. Along the same lines, polyurethanes [11], an epoxy resin for glass fiber composites [20], and multicomponent thermosetting resins from soybean oil have been reported [19]. The high degree of unsaturation in these oils (~6 C-C double bonds per triglyceride for linseed oil and 4.5 for soybean oil) makes them very attractive as comonomers in free radical resins, as they serve as ideal reactive sites for free radical polymerization [21]. Despite the high number of carbon-carbon double bonds in these oils, it is known that a significantly higher reactivity toward free radical polymerization process is attained upon conjugation of the double bonds [22]. An example of a free radical polymerization is illustrated in Figure 5.

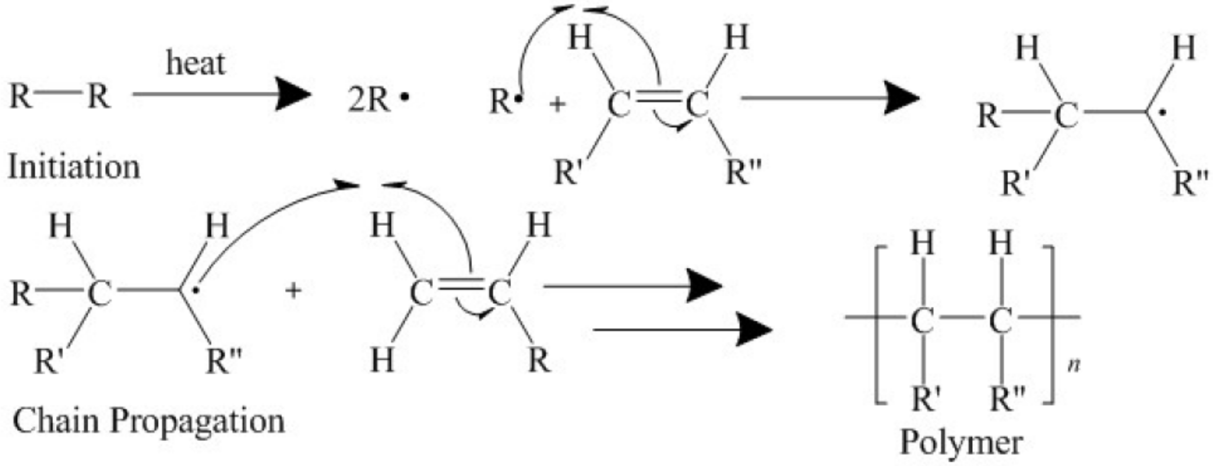

Figure 5: General free radical polymerization mechanism. Adapted from reference [23]

Composites are materials made from two or more different materials that, when combined, are stronger than those individual materials by themselves. The use of natural fibers to reinforce polymeric materials has been explored by several groups in the last decade. There have been reports on the reinforcement of polypropylene with sisal fiber [38], rice hulls, kenaf fiber [39], palm and coir fibers [40], wheat straw [41], sunflower hulls [42], and abaca strands [43]. High density polyethylene has also been reinforced with banana fiber [44], sugar cane bagasse, and wood flour [45]. Polyester and epoxy resins have been reinforced with jute fiber [46], and there have been reports on the use of hemp fiber [47] and poultry feathers as reinforcements for composites [48], among several other combinations of resin and natural fibers.

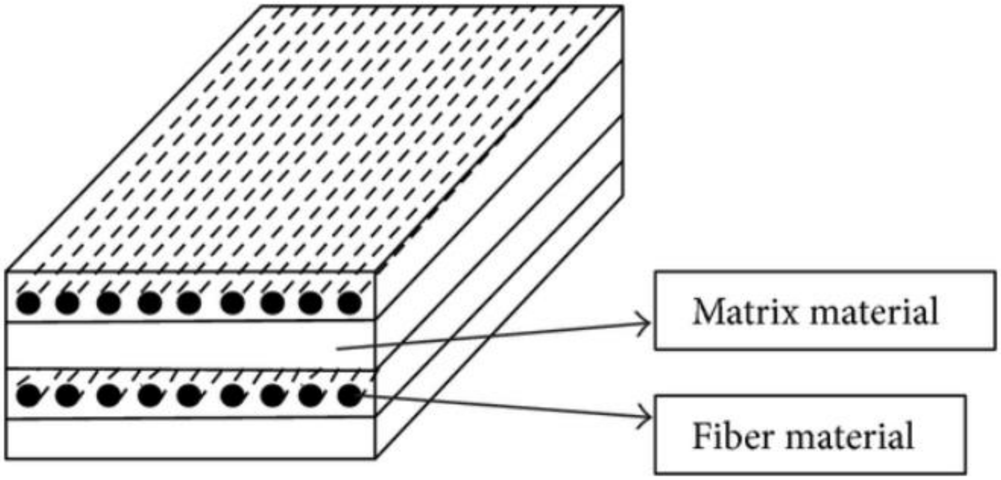

Figure 6: schematic of composite material reinforced with fiber. Adapted from [64]

In most of the examples, however, there is an inherent incompatibility between the hydrophobic resin and a hydrophilic reinforcement, which prevents maximization of the reinforcing effect imparted by the reinforcing materials used. Asolectin, a mixture of phospholipids naturally formed and obtained from soybeans, has been successfully used as a bio-based reactive surfactant in the emulsion polymerization of tung oil [24], and as a bio-based compatibilizer in tung oil-based/cellulose composites [24]. Asolectin contains both saturated and unsaturated phospholipids [25], which allow it to react with hydrophobic olefins, such as tung oil, and interact with hydrophilic groups, such as those present in collagen.

1.2 *Collagen*

Collagen is the main component of the extracellular matrix (ECM) and comprises 25-35% of the whole-body protein content in humans. The collagens are a large family of proteins, comprising 28 different types, which have widespread functions such as mediating cell adhesion, cell migration, tissue scaffolding and repairing. The most prevalent class of collagen – fibrous collagen – includes types I, II, and III collagens and is the main component of tendon, skin, cartilage and bone [26]. Although the architectures and roles of these collagens vary widely, they all comprise triple helix bundles [27]. The collagen triple helix comprises polyproline-II type helices held together by periodic interchain hydrogen bonding between the glycine amine in one chain and the carbonyl of the amino acid of an adjacent chain. Each strand of the helix consists of regularly repeated Gly-X-Y tripeptide repeats, with every third residue comprising glycine. Proline (Pro) and (4R)-hydroxyproline (Hyp) occupy the X and Y positions of the triplet repeats at the highest statistical frequency within native collagen [28], as illustrated in Figure 6.

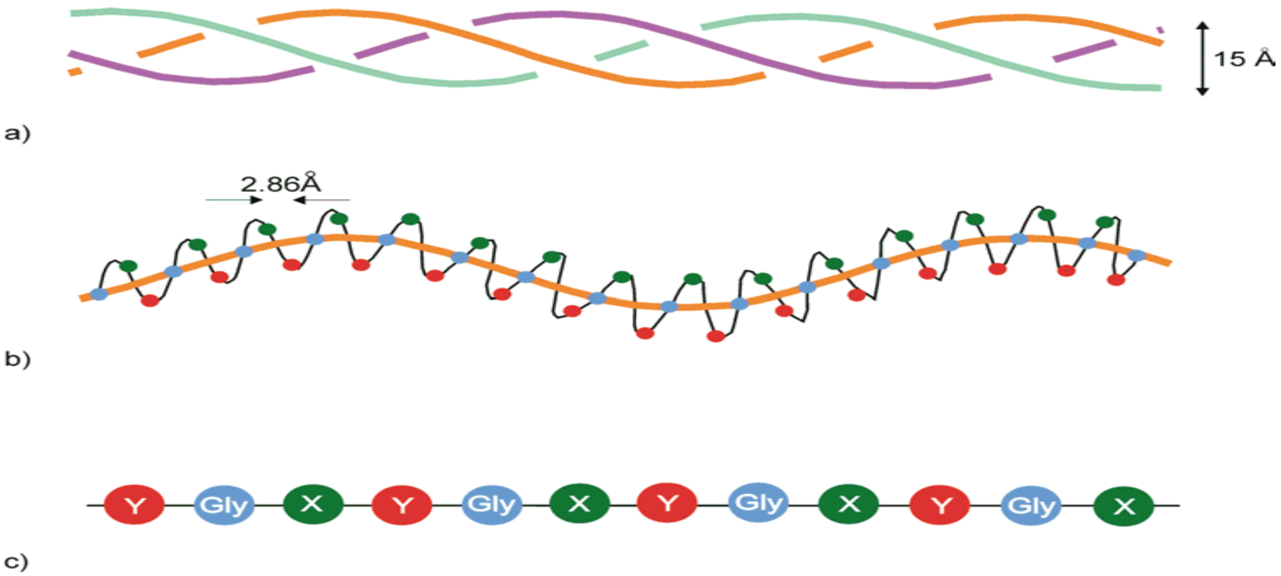

Figure 7. a) The collagen triple helix. b) Each α-chain forming a left-handed helix with every third residue located in the center of the triple helix. c) Collagen repeating sequence; where X and Y are often proline and hydroxyproline, respectively. Adapted from reference [17]

This helix is characterized by the right-handed intertwining of three polypeptide α-chains of pitch 9.5Å. Each α-chain forms a left-handed helix with every third residue located in the center of the triple helix. Here there is only a space for glycine, the smallest amino acid and there is hydrogen-bonding among the three chains. Therefore, the amino acid sequence of the α-chain is characterized by the Gly-X-Y repeat with X and Y often being proline and hydroxyproline residues [17], with chemical structures illustrated in Figure 8. The use of collagen in the synthesis of bio-based polymers has been scarcely reported, mainly as a reinforcement for castor oil-containing polyurethanes [29]. Collagen has a wide range of applications in the food, cosmetic, biomedical, pharmaceutical, and leather industries [30]. In biomedical applications, collagen helps rebuild damaged skin or helps with wound healing [31]. It is believed that collagen's polar segments are stronger than its hydrophobic segments, and the presence of the hydroxyl group of HyPro provides hydrogen bonding which stabilizes the triple helix. This makes the addition of a compatibilizer necessary in order to maximize its reinforcing effect in a polymer resin.

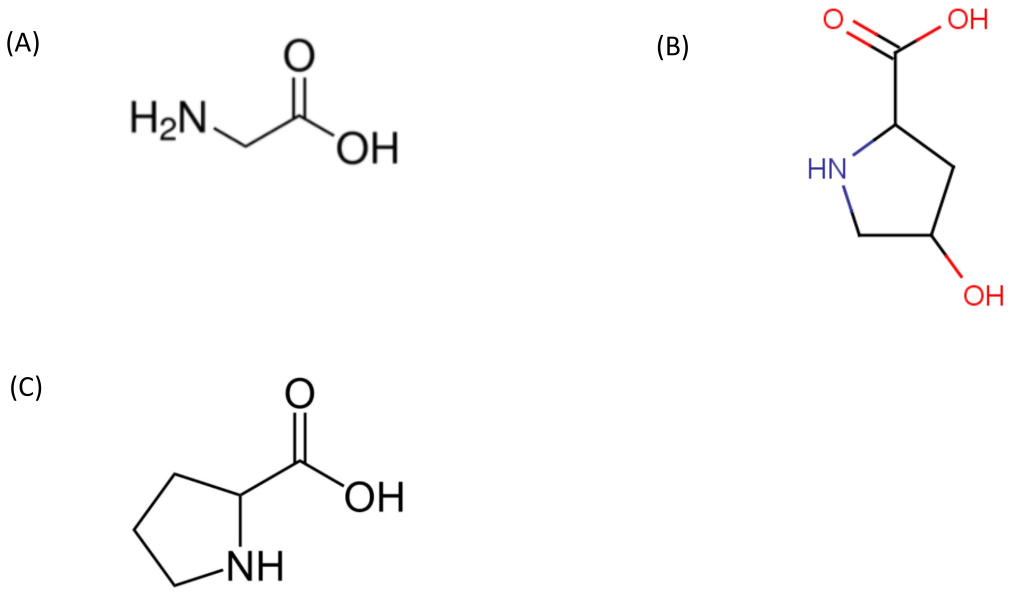

Figure 8: Frequently used essential amino acids in peptide synthesis; (A) glycine (B) hydroxyproline (C) proline. Adapted from chemspider.com [32]

1.3 *Previous work*

Based on previous work with tung oil-based composites with collagen [27], an initial resin composition containing 50 wt% of tung oil, 35 wt% of *n*-butyl methacrylate (BMA), and 15 wt% of divinylbenzene (DVB) was cured with 0.5-5 wt% of a commercial mixture of collagen I + III at 140^0C for 24 hours. In those preliminary trials, poor mixing of polymer matrix and collagen resulted in phase separation and a porous layer was observed above a tung oil-based polymer layer. Due to its long fatty acid chains and relative lower reactivity in comparison to DVB and BMA, tung oil cannot be used in concentrations higher than 50 wt%. In order to improve the miscibility of collagen and the resin, 10 wt% of tung oil was replaced with 10 wt% asolectin. In order to compensate for the lower reactivity of asolectin, an additional 5 wt% of DVB was added to the resin composition in detriment of BMA, resulting in a resin composed of 40 wt% of tung oil, 30 wt% of BMA, 20 wt% of DVB, and 10 wt% of asolectin. The overall bio-based content in the resin corresponds to 50 wt% (40 wt% of tung oil + 10 wt% of asolectin). Since the 10-75 wt% of collagen yielded poor results in the previous work, the resin was used for the preparation of composites containing 0 – 10 wt% collagen. Despite the absence of the porous layer upon cure, the resulting composites exhibited imperfections such as bubbles and cracks, indicating a need for adjustment of the cure schedule employed. Overall, the thermal and mechanical properties of tung oil-based resin improved upon reinforcement with collagen, exhibiting an impressive maximum improvement of ~100% for sample reinforced with 5.0 wt% of a commercial mixture of collagen I + III. These improved properties however do not meet the required standard for commercialization. It has recently been found that the presence of residual NaCl in collagen can adversely affect its structure thereby inhibiting further improvements in its properties.

Unlike linseed oil, corn oil, and soybean oil, which have non-conjugated double bonds, tung oil has all its double bonds conjugated which makes it more desirable in the synthesis of biobased composites. This is because the conjugated double bonds are very reactive. This means that less energy and time is required to bring the reaction to completion, which is not the case in other vegetable oils. The triple helix nature of collagen makes it mechanically resistant to tensile forces due to the hydrogen bonding that holds the helices together.

1.4 *Planned Work/Hypothesis*

From previous research, peptides have been used as model systems to study large protein structures, and these peptides have ultimately been used in the synthesis of bio-composites. In this study, the effects of NaCl on collagen structure will be investigated. Long and short peptides will be synthesized and studied to fully understand their mode of interaction and how they influence the properties of composites. In this context, the aim of this work is focused on the development of collagen-reinforced tung oil polymers, assessing the effect of the incorporation of long and short peptides on the thermomechanical properties of the resulting composites, and evaluating the effects of allyl glycine (seen in Figure 8) in the collagen sequence on these properties.

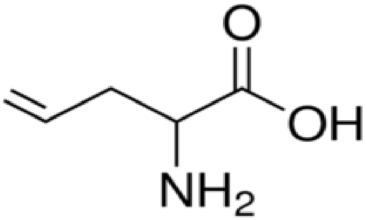

Figure 9. Chemical structure of allyl glycine. Adapted from reference [33]

In this research, the presence of NaCl in the triple helix and its overall effect on the thermal and mechanical properties of the bio-composite will be investigated. A collagen-reinforced bio-composite from tung oil will be prepared using free radical initiation and thermal polymerization, leading to polyolefinic tung oil-based matrix. Two peptides (long and short), that mimic natural collagen extracted from beef tendons, will be synthesized and their secondary structure will be analyzed using circular dichroism spectroscopy. After this analysis, the two peptides will be used in the reinforcement of a tung oil-based composite, and their properties will be compared. Cure time will be determined through dielectric analysis (DEA). The thermo-mechanical properties will be assessed by means of thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), and dynamic mechanical analysis (DMA). The peptide that yields the best composite will be resynthesized, but with the addition of allyl glycine. The thermomechanical properties of the composite formed from this new sequence will be analyzed and compared.

In this research a collagen-reinforced bio-composite from tung oil is to be prepared using free radical initiation and thermal polymerization, leading to a polyolefinic tung oil-based matrix reinforced with collagen.

From Figure 2, tung oil constitutes an ideal candidate for the preparation of biobased materials. This is mainly due to carbon-carbon double bonds it possesses as it can readily react with vinyl co-monomers, such as DVB, styrene, and dicyclopentadiene, via cationic, thermal, or free radical polymerizations, without the need for structural modifications. It has been shown that these reactions result in highly crosslinked polymer networks [18, 34, 35].

CHAPTER 2

MATERIALS AND METHODS

2.1 Collagen Purification

A standardized collagen purification protocol was adopted during the extraction of collagen from beef tendons. About 772 g of frozen beef tendons were cleaned and cut into small cubic pieces of about 1.5cm. These pieces were then incubated in cold water bath in four pitchers for 3 hours at approximately 4^0C and washed several times in deionized water. After that, the tendons were crushed using a blender and allowed to dry in the fume hood overnight before being placed in 5 L of 0.05 M Na_2HPO_4 buffer solution (using phosphoric acid, pH was kept between 8.7-9.1,), stirred and placed in the refrigerator for 24 hours. The pitchers were decanted, and the supernatant (labeled as first wash) was saved. Another fresh 0.05 M Na_2HPO4 buffer solution was prepared and added to the pellets, stirred and placed in the refrigerator for another 24 hours. This was decanted and the procedure was repeated for the last time; in total there were three washes [36].

The tissue was then added to 4 L of 0.5 M solution of acetic acid containing 5 mM EDTA. Since the required pepsin was 0.05 g per 100 g of beef tendons, the total pepsin used was 0.386 g. Exactly half of this amount was directly added to the solution, stirred and kept in the refrigerator. After approximately 7 hours, the remaining half of the pepsin was added, and the mixture stirred again and kept in the refrigerator overnight. This mixture was then decanted, and supernatant collected, and this process was repeated twice. After the final decantation, the collected supernatant which is rich in collagen, was poured into three centrifuge bottles in equal amounts and centrifuged at 4422 RPM for 15 minutes at 15 ^{0}C. After that, the supernatants were collected in a large pitcher and salted out by adding 4 M NaCl to the solution, mixing well, and then allowing it to sit in a refrigerator for 24 hours without stirring. The precipitate that formed at the top of the pitcher was separated from the solution with a 25 mL pipet and dissolved in 300 mL of 1 M acetic acid, followed by centrifugation of 5708 RPM for 15 minutes at 15 ^{0}C to be able to get the precipitate. A dialysis tubing which was allowed to equilibrate overnight in deionized water was washed with 300 mL of 0.02 M Na_2HPO_4 buffer solution. The precipitate was then dialyzed against 0.02 M Na_2HPO_4 buffer solution with a pH of 7.4 using the prewashed dialysis tubing and kept at 4 ^{0}C for 48 hours. It is important to note that some of the samples were dialyzed longer than 96 hours. This is to help investigate the samples for different salt concentrations and different properties based on varied salt concentrations. The final sample was then transferred into five falcon tubes and lyophilized for 48 hours. It was then weighed to be between 2.303 g and 2.512g.

About 10 mg of the lyophilized collagen was taken and analyzed through TGA to determine the amount of NaCl residue, which was found to be 68 wt%. The rest of the collagen was dialyzed for another 48 hours, dialyzed, and analyzed through TGA. The NaCl content reduced from 68 wt% to 20.5 wt%. This process was repeated and the NaCl content reduced to 11 wt%. It is important to note that after each process, about 200 mg of each was reserved to be used in the composite synthesis.

2.2 Circular Dichroism (CD) Spectroscopy Analysis

This technique is used to determine the secondary structures (helices, β – sheets, or random coils) of proteins and peptides. In this research, it was used to confirm the triple helix structure of collagen which has a maximum at approximately 222 nm and a minimum at approximately 195 nm in the CD spectrum [27]. About 2.500 mg of the collagen sample was initially dissolved in 0.5 mL of 1M acetic acid since it is insoluble in aqueous solutions. It was then diluted to final concentration of 0.1M acetic acid in 0.02 M Na_2HPO_4. The sample was then transferred into a cuvette. The cuvette with the sample and another cuvette containing Na_2HPO_4 were placed in the instrument and analyzed.

2.3 Composite Synthesis Via Free Radical Polymerization

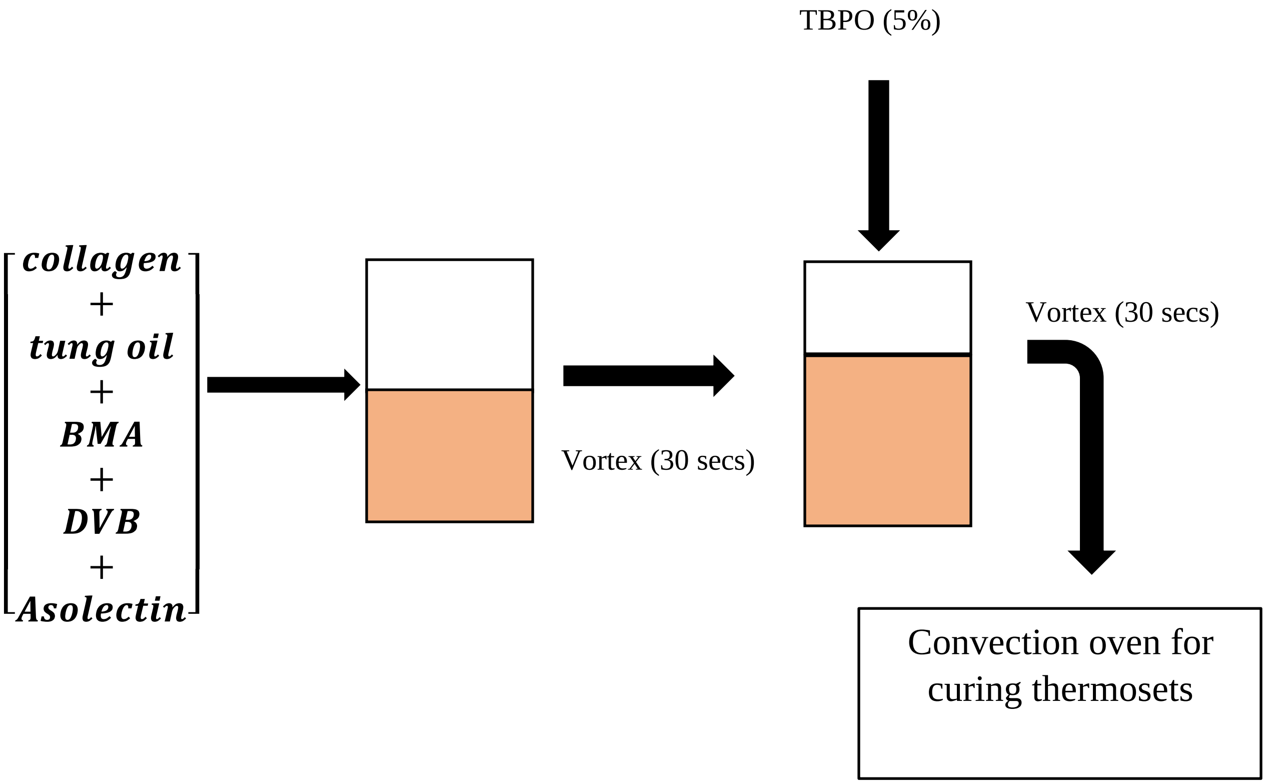

Figure 10. Schematic of the resin preparation cured in a convection oven under air.

2.3.1 *Materials*

Tung oil, *n*-butyl methacrylate (BMA), di-tert-butyl peroxide (TBPO), and asolectin were purchased from Sigma-Aldrich (St. Louis, MO). Bovine tendons were donated by Hunter Cattle Co. (Brooklet, GA). Sodium chloride (NaCl) was purchased from Fisher (Hampton, NJ). Divinylbenzene (DVB) was acquired from TCI America (Portland, OR). Differential scanning calorimetry (DSC) studies were conducted on a Q250 DSC instrument from TA Instruments, New Castle, DE, 5-10 mg of sample were heated from -20^{0}C to 200^{0}C at a rate of 10^{0}C/min. The mechanical properties of the composites were analyzed on a Q800 DMA (TA Instruments, New Castle, DE) using a three-point bending fixture with a 5 mm gap. Rectangular samples (5.0 x 9 .0

x 2.5 mm – length x width x thickness) were cut and subjected to a multi frequency strain experiment with an amplitude of 14 µm. The temperature was increased from -60^{0}C to 150^{0}C at a heating rate of 3^{0}C/min. The thermal properties were studied on a Q50 thermogravimetric analyzer (TA Instruments, New Castle, DE) using samples of approximately 9 mg. The experiments were performed under air, from room temperature to 600^{0}C.

2.3.2 *Resin Preparation*

To appreciate the effect of NaCl, I manually added NaCl in different quantities during the synthesis to determine whether it will affect the polymerization process. In the synthesis, 40 wt% of tung oil, 30 wt% of BMA (Figure 10A), 20 wt% of DVB (figure 10B), and 10 wt% asolectin was added to three different 20 mL scintillation vials resulting in 10.0 g each of crude resin. After capping the vials, the mixtures were vortexed until all the components were completely mixed. Subsequently, 0 wt%, 10 wt% and 15 wt% of NaCl were respectively added to the vials and agitated again until a homogenous mixture was obtained. Lastly, 0.5 g of TBPO was added to each vial and the contents were agitated. The vials were then placed in a convection oven and heated at 100 – 140^0 C over a period of up to 24 hours. The composites were then taken and characterized through DMA, TGA, and DSC.

Both BMA (Figure 11A) and DVB (Figure 11B) make ideal candidates for free radical polymerization due to their reactivity. The vinyl olefins in these monomers give rise to ideal reactive sites. In the absence of reactive co-monomers, the internal fatty acid olefins exhibit an exceedingly low polymerization rate leading to low conversion and poor properties [30]. Figure 10 shows a general schematic of the free radical polymerization initiated by heat and the free radical initiator, DTBP. For simplicity, BMA and DVB were not included in the reaction scheme but are incorporated in the polymer network in the same mechanism as shown in Figure 10. In the presence of a radical, the carbon-carbon bonds will break and share one of their electrons with the radical, forming a new covalent bond. As a result, a radical is formed on the adjacent carbon from the other electron generated from the π system and the polymerization continues until termination.

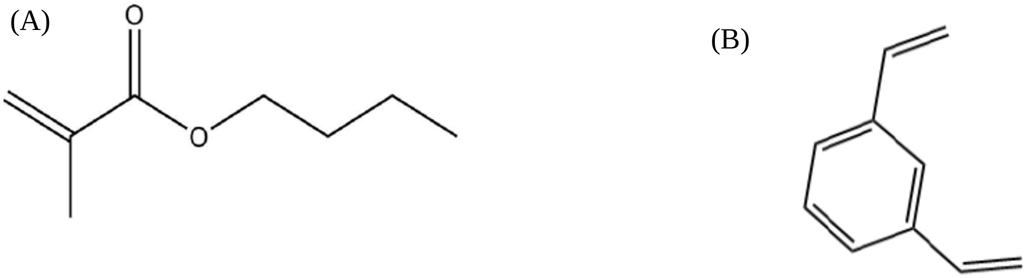

Figure 11: Chemical structure of (A) n-butyl methacrylate; (B) divinylbenzene (DVB). Adapted from reference [37]

(The reserved collagen samples were also incorporated in a different synthesis). After adding 40 wt% of tung oil, 30 wt% of BMA, 20 wt% of DVB, and 10 wt% asolectin to two different 20mL scintillation vials, 5 wt% and 10 wt% of the 68 wt% NaCl in collagen sample were respectively added to each vial and vortexed. This was to investigate thermomechanical properties of the composite for different amounts of the same collagen. This procedure was repeated for the 20.5 wt% of NaCl in collagen sample. The vials were then placed in a convection oven and heated at 100 – 140^0 C over a period of up to 24 hours. The composites were then taken and characterized through DMA, TGA, and DSC.

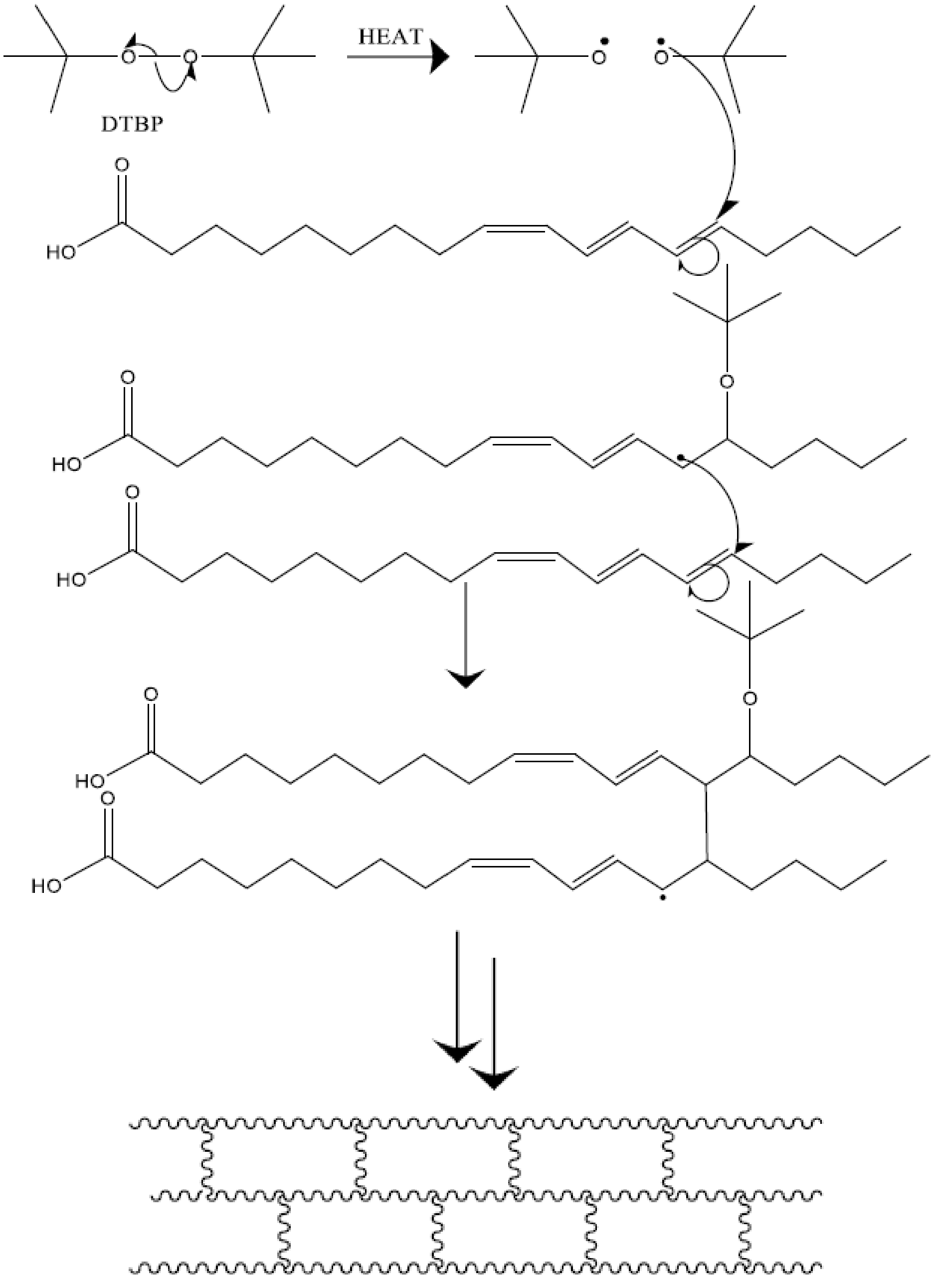

Figure 12: General schematic of free radical polymerization with *di-tert* butyl peroxide (DTBP) as the free radical initiator. For simplicity, cross-linking agents BMA and DVB are not included in the diagram.

2.4 Composite Characterization

In previous literature, various amounts of bio-based monomers have been used in combination with the petroleum-based monomers like DVB or BMA, to create co-polymers with viable and competitive properties. The vegetable oil weight percentage compositions in these co-polymers range from 40% to 70%. Normally, vegetable oils are not as reactive as their petroleum counterparts. Hence, the creation of hybrid polymers is composed of bio-renewable starting materials and petroleum-based products. When establishing the resin composition, it is important to account for the differences in reactivity, which will affect the resulting polymer's morphology, and subsequently, the mechanical properties. In this research, it was found that the best combination of monomers was 40 wt% of tung oil, 30 wt% of BMA, and 20 wt% of DVB.

2.4.1 *Cure Verification Via Differential Scanning Calorimetry*

Differential Scanning Calorimetry (DSC) is a commonly used thermal analysis technique that measures the difference in heat flow between polymer sample and empty reference pan with respect to temperature. The sample pan and reference are heated, cooled or held isothermally, and the respective heat flow differences are recorded. DSC analysis allows users to obtain a thorough understanding of the cure kinetics and process. Depending on the chemical processes taking place during the analysis, the sample can either absorb or release heat/energy to maintain its heating rate equilibrium with the reference pan. This energy release or absorption trend plots nicely on the DSC graph as an exothermic or endothermic peak. An absence of an exothermic peak can be an indication of a fully cured polymer. If the polymer was not fully cured, the heat from the DSC analysis would regenerate the polymerization, resulting in exothermic peaks on the DSC curves. Exothermic peaks can also be indicators of the polymer crystallizing, while enothermic curve shifts are more likely to be indicators of a glass transition temperature (Tg), or the polymer melting. In the experiment, a Q20 DSC instrument (TA instruments, New Castle, DE) was used under a nitrogen atmosphere, to heat each sample of approximately 10 mg from – 20 ^{0}C to 200 ^{0}C at a rate of 10 ^{0}C per minute.

2.4.2 *Composite Thermo-Stability Via Thermogravimetric Analysis (TGA)*

The thermal stability of the composites was assessed using a Q50 TGA instrument (TA instruments, New Castle, DE). A piece of each composite was heated from room temperature to 600^0C at a rate of 20^0C per minute. During this time, the weight of the sample was being measured under an air atmosphere as a function of time.

2.4.3 *Composite Mechanical Properties*

Vegetable oil-based co-polymers are commonly used in research due to their tunable mechanical properties by adjusting the resin composition, as well as their inexpensive design. The most conventional mechanical property evaluation is completed on a dynamic mechanical analysis (DMA) instrument. DMA measures the response of the sample to a sinusoidal or oscillating stress. This stress can be representative of changes in force, temperature, or frequency and sample's respective deformation response. The amount of sample deformation is related to its stiffness or elastic behavior, and is measured by the storage modulus, E' or G'. Under sinusoidal force conditions, the modulus can be divided into two components, an in-phase component and an out of phase component. The in-phase component is known as the storage modulus and the out of phase component, the loss modulus. The tan δ curve is the ratio of the loss modulus to the storage modulus, and is often referred to as damping. It is a measurement of the energy dissipated, and an indication of how well the sample can absorb energy. In this experiment, a Q800 DMA instrument (TA instruments, New Castle, DE) was used under tension mode.

To measure the stiffness and damping of the composites, the Q800 DMA instrument (TA instruments, New Castle, DE) was used under tension mode. A multi-frequency strain method was employed, using the three-point bending clamp with an amplitude of 14.0 μm and a frequency of 1 Hz. The composite samples were cut into specimens of 10.00 mm x 5.00 mm x 2.60 mm (length, width, thickness), as shown in Figure 13, to examine their dynamic mechanical behavior between – 60^0C and 150^0C. Storage modulus (MPa) and tan delta were measured as a function of temperature under a heating rate of 3 ^{0}C/min.

Figure 13. Image of composites and DMA samples

Two composites prepared by Kody Burrows (a former student) were also analyzed. One composite contained one strand of alpha 1 peptide while the other contained a triple helix mixture with two alpha 1 and one alpha 2 peptide strands. These composites were analyzed through DMA, TGA, and DSC.

2.5 Peptide Synthesis

In future research, peptides will be synthesized using a Biotage Initiator+ Alstra to mimic collagen peptides. The peptides to be synthesized are listed (see Figure 14). Another synthesis should be performed where the length of these peptides will be doubled. In this method, a solid-phase peptide synthesis technique, a valid technique currently used to produce peptides [8], will be employed. The activators used will be hydroxybenzotriazole (HOBT), hexafluorophosphate benzotriazole tetramethyl uronium (HBTU), and Diisopropylethylamine (DIEA), all in dimethylformamide (DMF). Twenty percent piperidine in DMF will be used for all deprotection steps. After washing and drying, the peptides must be cleaved from the rein by treatment with a mixture of TFA/TIPS/H2O (95 %/2.5 %/2.5 %). The cleaved peptides will be lyophilized to obtain a solid product. After lyophilization, the peptides must be separated using a Shimadzu SCL-A10 reversed phase HPLC and a gradient solvent of acetonitrile with increasing deionized water. MALDI-TOF is an appropriate and valid testing method for peptides so the fractions should then be tested using a Bruker microflex MALDI-TOF Mass Analysis on a 96 well-plate high polished stainless-steel plate. The collagen will then be self-assembled by combining 40mg of α1 chain mimic dissolved in 1 mL of 0.1 M acetic acid in 20 mL of Na_2HPO_4 buffer and 20 mg of α2 chain mimic dissolved in 1 mL of .1 M acetic acid in 10 mL of Na_2HPO_4 buffer at pH 7.4 at 4 ^{0}C for at least 48 hours.[6] Circular dichroism (CD) is a common technique for protein analysis [18]. CD uses circularly polarized light to detect chiral centers. CD will be used at pH 7.4 in 1mmol/L concentrations from 260 nm to 190 nm to confirm that the peptides are in a triple helix conformation. The thermal properties of the peptides will investigated through thermogravimetric analysis (TGA). The peptide that has a better thermal property will be resynthesized with the addition of allyl glycine.

a) *α1 short*: Gly-Pro-Lys-Gly-Glu-Hyp-Gly-Pro-Ala- Gly-Arg-Hyp-Gly-Pro-Gln-Gly-Lys-Hyp

α2 short: Gly-Pro-Glu-Gly-Arg-Hyp-Gly-Pro-Ala-Gly-Asp-Hyp-Gly-Pro-Gln-Gly-Lys-Hyp

b) *α1 long*: $(\text{Gly-Pro-Lys-Gly-Glu-Hyp-Gly-Pro-Ala- Gly-Arg-Hyp-Gly-Pro-Gln-Gly-Lys-Hyp})_2$

α2 long: $(\text{Gly-Pro-Glu-Gly-Arg-Hyp-Gly-Pro-Ala-Gly-Asp-Hyp-Gly-Pro-Gln-Gly-Lys-Hyp})_2$

c) *α1 long (AllylGly)*: $(\text{Gly-Pro-Lys-Gly-Glu-Hyp-Gly-Pro-}\textbf{AlGly}\text{- Gly-Arg-Hyp-Gly-Pro-Gln-Gly-Lys-Hyp})_2$

α2 long (AllylGly): $(\text{Gly-Pro-Glu-Gly-Arg-Hyp-Gly-Pro-}\textbf{AlGly}\text{-Gly-Asp-Hyp-Gly-Pro-Gln-Gly-Lys-Hyp})_2$

Figure 14: Type I collagen sequence, where (b) is a repeated sequence of (a). In (c), Ala is replaced with the allyl glycine.

After completion of the peptide synthesis, each peptide will be washed three times in a 10mL isopropanol and dichloromethane solution. After washing and drying, the peptides must be cleaved from the resin by treatment with a mixture of Trifluoroacetic acid (TFA)/Triisopropylsilan (TIPS)/H_2O (95% / 2.5% / 2.5%).

CHAPTER 3

RESULTS AND DISCUSSION

The following section will discuss the dialysis of collagen, characterization of collagen with different NaCl concentrations, and the analysis of reinforced/unreinforced composites. Composites were examined using DSC, TGA, and DMA, and the results were compared.

3.1 NaCl Content Analysis

3.1.1 *Circular Dichroism (CD) Spectroscopy of Collagen*

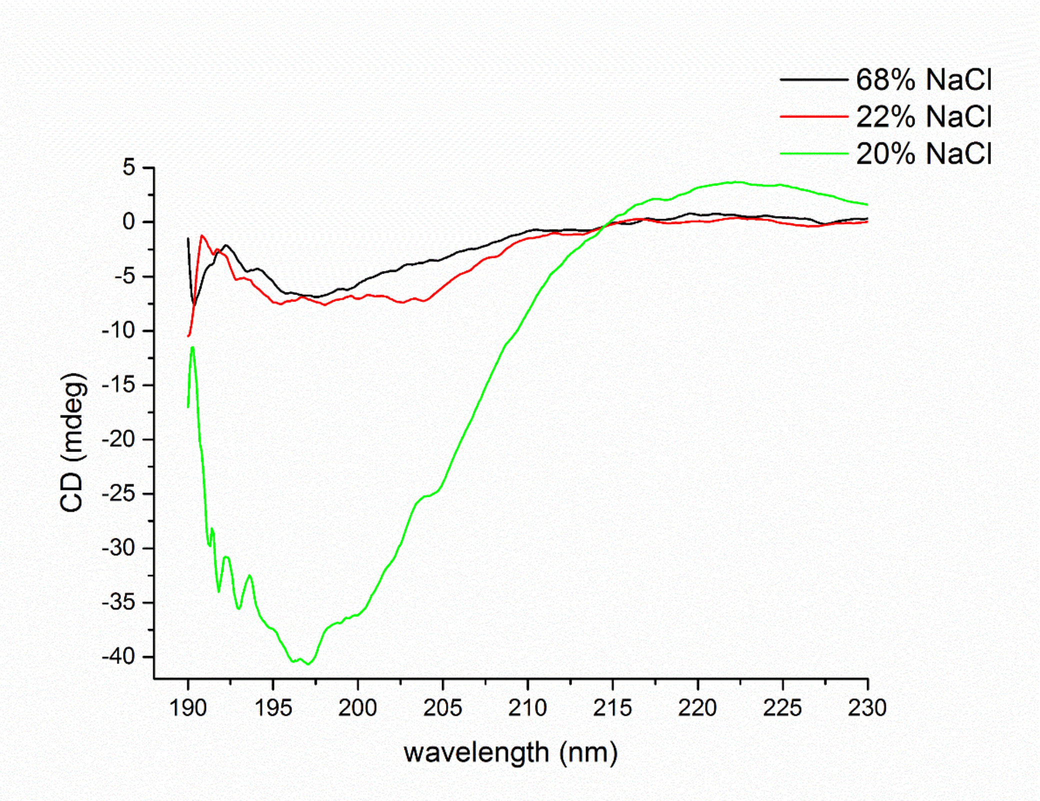

Figure 15: Circular dichroism spectroscopy of collagen with different amounts of NaCl

Circular dichroism spectroscopy was used to get an insight into how sodium ions interact with the hydrogen bonding that holds the three helices together to form a tripe helix. From Figure 15, the sample with the lowest amount of sodium ions showed as triple helix structure as it had an upward peak of approximately 220 nm and a downward peak of approximately 196 nm. The other two samples represent a denature collagen structure.

3.1.2 *Thermogravimetric Analysis of Collagen*

After dialyzing the collagen sample for 48 hours, the sample was lyophilized or freeze-dried for 48 hours. To determine the amount of sodium ions in the collagen after dialysis, TGA was run on the sample to determine the amount of residue**.** Figure 15 shows how the collagen sample degrades over time as a function of temperature. C-C bonds should break around 400^0C so the fact that part of the sample remains at 650^0C means a metal is more likely to be present. Based on the sodium in which the sample was placed during the dialysis, and collagen's affinity for sodium, it is more than likely that this mystery metal is sodium. The end of the curve representing the 48 hours shows sodium ion content in the collagen after a 48-hour dialysis which was found to be approximately 55 wt%. After an additional 48-hour dialysis in a fresh solution, the concentration reduced further to 31 wt%. Comparing the two curves, it was observed that from room temperature to about 100^0C, the 96-hour sample degraded relatively faster as suggested by the slope. This is due to the difference in sodium ion concentration of the two samples. They both, however, began to assume the sample profile between 250^0C and 650^0C as a greater percentage of the collagen had degraded.

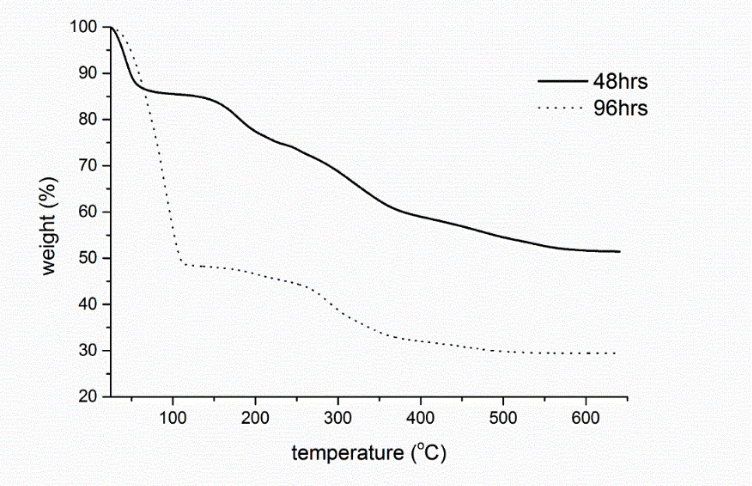

Figure 16. TGA of collagen dialyzed at different times, from room temperature to 650^0C at a heating rate of 10^0C /min

To get more collagen samples for elaborate analysis, a new set of beef tendons was secured and purified. After dialyzing for 48 hours, the sodium ion content was determined via TGA. The end of the curves in Figure 16 shows the residue (sodium ion) after each dialysis. For the first 48 hours the salt content observed was approximately 70 wt%. After the sample was dialyzed again for 48 hours, the amount of residue was determined to be approximately 20 wt%. Though the amount of residue was expected to reduce upon the third dialysis, it was found to be 20.8 wt%. This is because the dialysis buffer is a sodium phosphate buffer so the dialysis may still allow for binding of some sodium ions. Thus, it is difficult to completely remove sodium ions from the collagen.

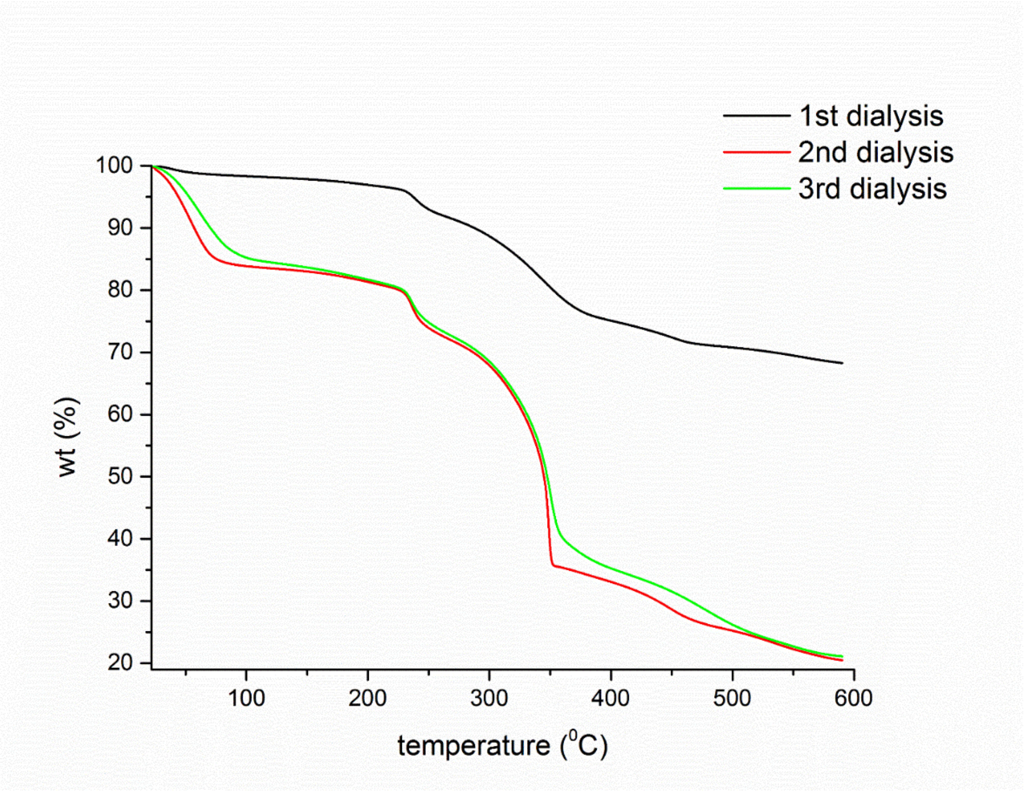

Figure 17. Overlay of TGA data for different stages of dialysis

3.1.3 *Cure Verification by Differential Scanning Calorimetry*

The use of DSC allows insight into the cure kinetics and thermal events of materials as a function of temperature. The sample under analysis can either absorb or release heat/energy. This release or absorption results in either an exothermic (up peak), or an endothermic (down peak) peak. The absence of exothermic peaks indicates a fully cured polymer sample. An uncured sample typically exhibits an exothermic peak that indicates further polymerization reactions (crosslinking). Exothermic peaks could also be an indication of the polymer undergoing crystallization. With endothermic peaks, the polymer could be melting, or this is an indication of glass transition temperature (T_g) of the polymer. From Figure 17, it is shown that the composites were fully cured as the curves remained fairly flat. Thus, the presence of NaCl did not affect the reaction.

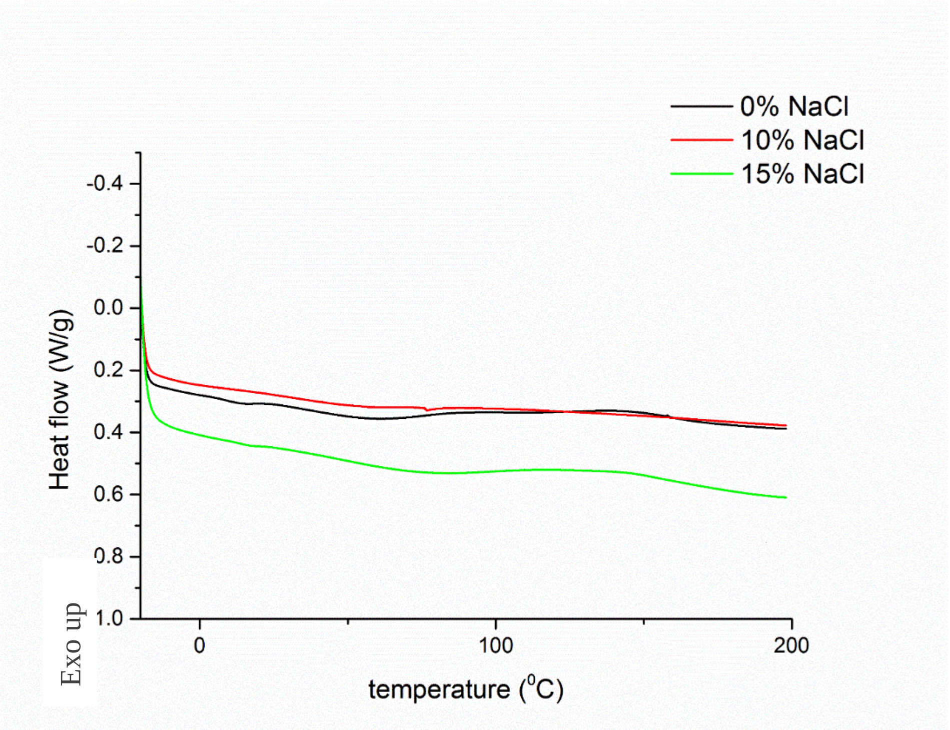

Figure 18. DSC of composite with different amount of NaCl

3.1.4 *Cure Verification by Thermogravimetric Analysis*

The thermal properties of the composites with different percentages of NaCl were evaluated by TGA. From Figure 18, the 0% and 15% showed the expected results as the end of the curves confirmed the amount of NaCl added. They also began to degrade about the same temperature (250^{0}C approximately), which is expected because NaCl does not affect the thermal stability of the composite. The 10% sample however showed a rather surprising profile as the residue does not match. This is because not all the salt dissolved in the resin before it was placed in the oven. Therefore, some parts of the composite tend to have more salt than other parts. Additionally, it began to degrade faster than the other two samples. The sample will be rerun to hopefully get a better result.

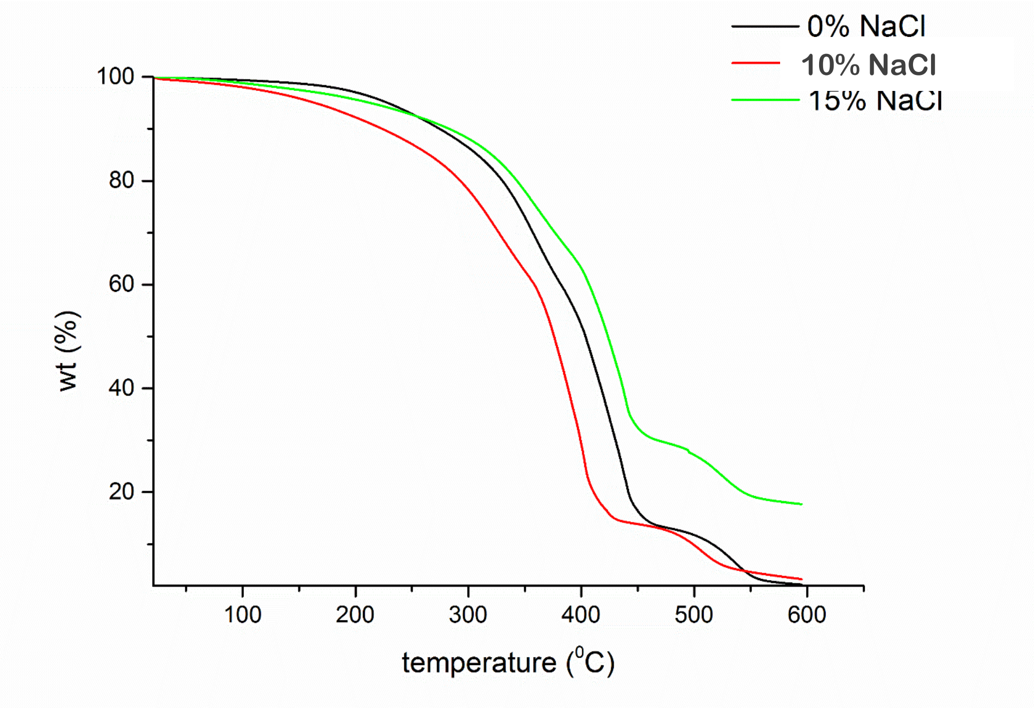

Figure 19. Overlay of TGA of composite with different amount of NaCl

3.1.5 *Mechanical Properties of Composites*

Dynamic mechanical analysis (DMA) was used to examine the mechanical properties of the composites at room temperature. As thermosetting polymers would, these composites exhibited the three distinct viscoelastic regions: the glassy state, glass transition state, and the rubbery plateau states. At very low temperatures, all resins are in their stiff, glassy states. From Figure 19, the storage moduli of the three composites peaked at approximately – 50^0C. Thereafter, the samples entered their glass transition stage as there was an otherwise sharp decrease in their storage moduli till 0^0C. As expected, the storage modulus decreases as the temperature increases. At room temperature, the storage moduli of the samples were evaluated and will be discussed subsequently in the summary table. A tan delta curve, which is a ratio of the loss modulus to the storage modulus, was used to evaluate the glass transition temperature of the composites at room temperature.

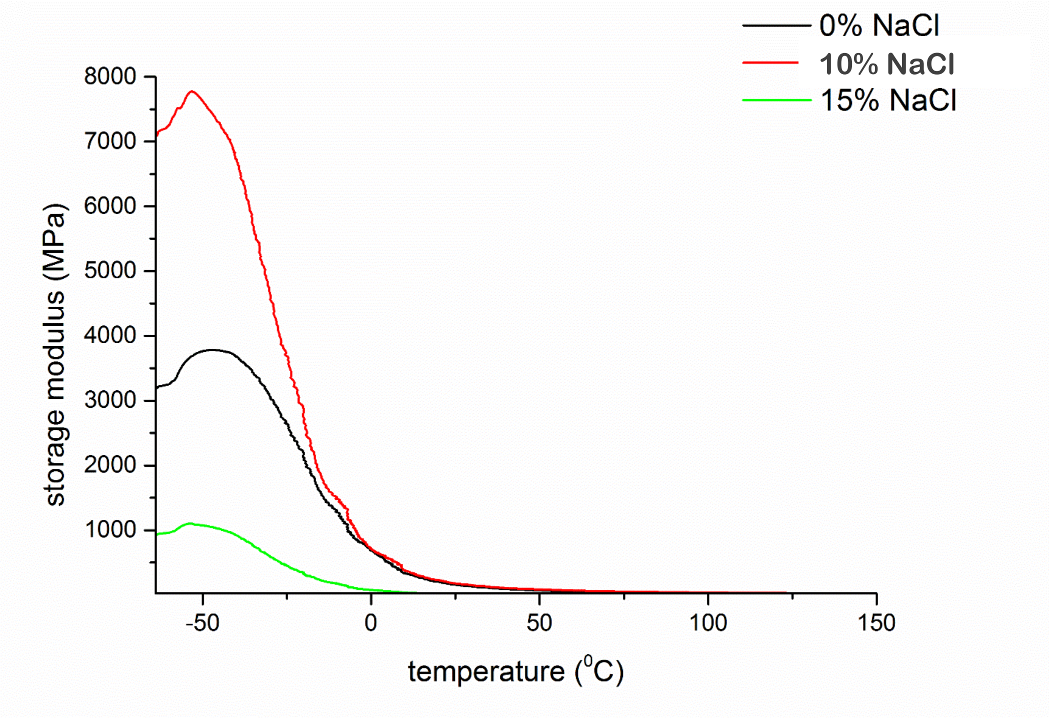

Figure 20. Overlay of DMA of composite with different amount of NaCl

3.2 Composite with Different Amounts of NaCl in Collagen

In this research, the purification of collagen was done twice. The composite synthesized with the collagen from the first purification broke on two occasions during the dynamic mechanical analysis, and there was not enough collagen to resynthesize the composite. This made it necessary for another purification of collagen.

3.2.1 *Cure Verification of Composites*

Differential scanning calorimetry was used to determine whether the polymerization reaction was complete. From Figure 20, there was no indication of incomplete polymerization or evaporation as the curves are fairly flat, thus, no prominent peaks. The one broad barely noticeable peak is from some water leaving the polymer and was expected.

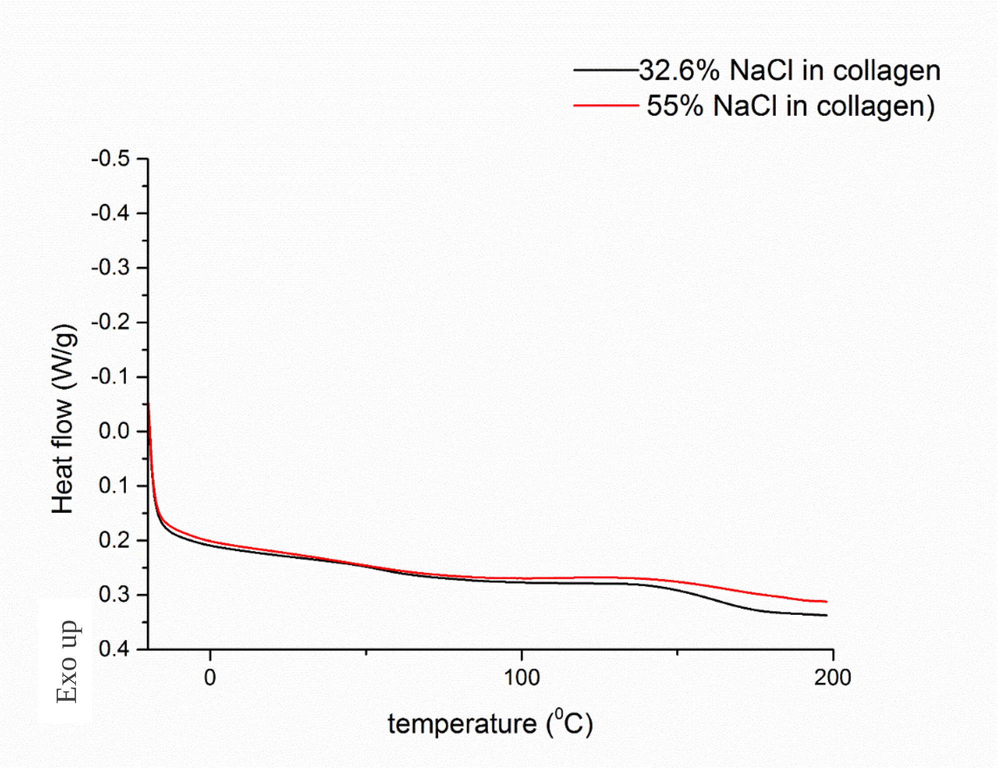

Figure 21. Overlay of DSC of composite with different amount of NaCl in collagen

3.2.2 *Thermal Analysis of Composites*

Thermogravimetric analysis technique was used to study the thermal stability of composites reinforced with collagen. The composites were reinforced with 10 wt% of collagen containing different amounts of NaCl because of dialysis previously performed. This means that the actual NaCl content in the 32.6 wt% and 55 wt% samples are 3.26 wt% and 5.5% respectively. Figure 21 illustrates the comparison of the composites with the same amount of collagen but different NaCl concentrations. The sample with the least amount of NaCl seemed to be less thermally stable as it began to degrade at a much lower temperature (approximately 160^0C). This is unexpected because the amount of collagen or NaCl in the sample should not affect its thermal stability so it is necessary for the synthesis to be repeated. The end of the curves however accurately represents the amount of NaCl residue in the samples.

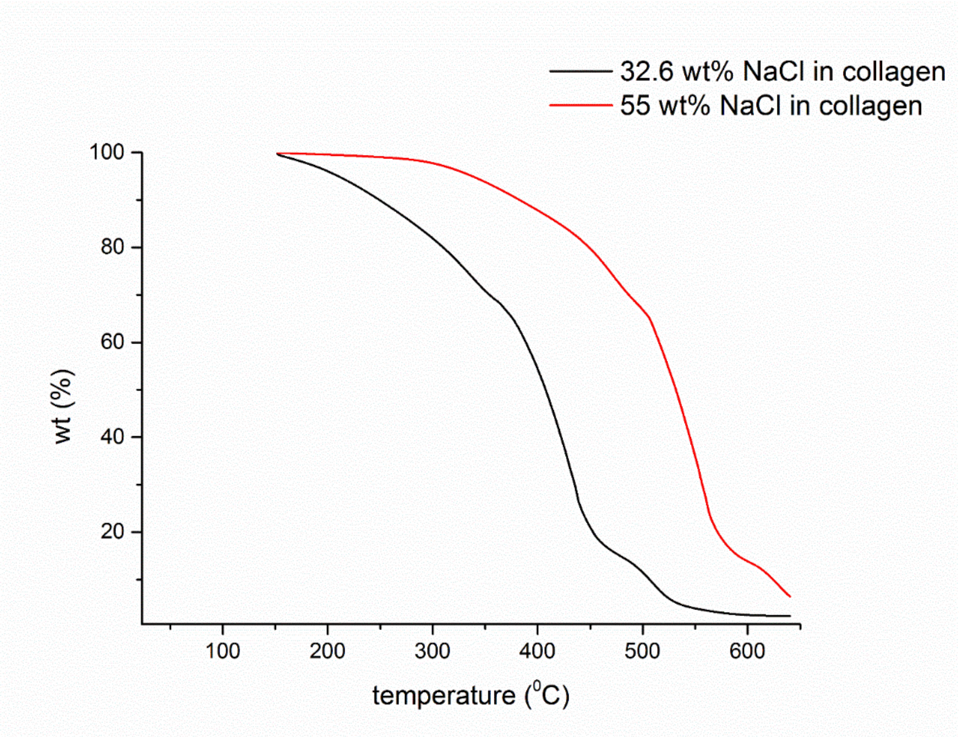

Figure 22. Overlay of TGA of composite with different amounts of NaCl

3.2.3 *Cure Verification of Composites from Second Purification*

The collagen from the second purification was incorporated in the synthesis of the composites and analyzed. Figure 22 shows two samples containing the same amount of NaCl but different amounts of collagen being analyzed via DSC. Around 15^{0}C, both samples display endothermic peaks representing sample degradation or the glass transition temperature. Further analysis with DMA and TGA can further explain the phase changes and are discussed later in the chapter.

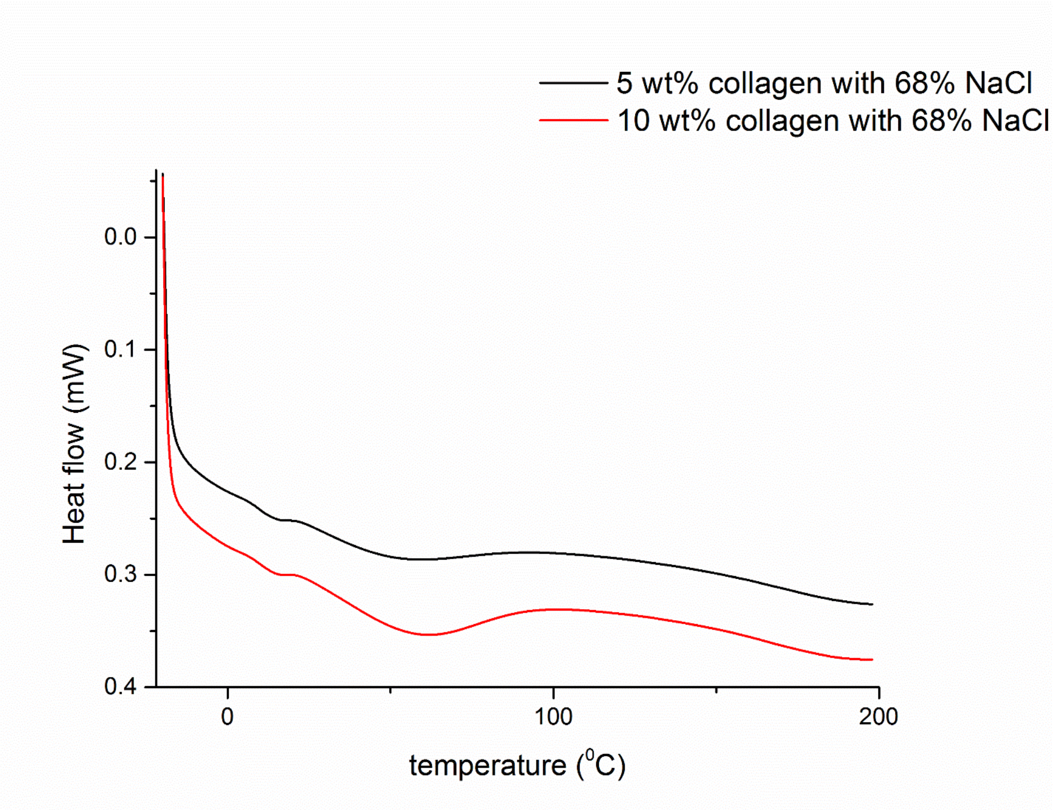

Figure 23. Overlay of DSC of composite with same amount of NaCl in different amounts of collagen

3.2.4 *Thermal Analysis of Composites*

Figure 23 illustrates the comparison of collagen composites with the same of amount of NaCl but different amounts of collagen. As expected, both samples begin to degrade at the same temperature, at approximately 200^{0}C. As indicated earlier, the curves do not reduce to zero due to the presence of NaCl, and also corresponds, approximately, to the NaCl residue in the collagen.

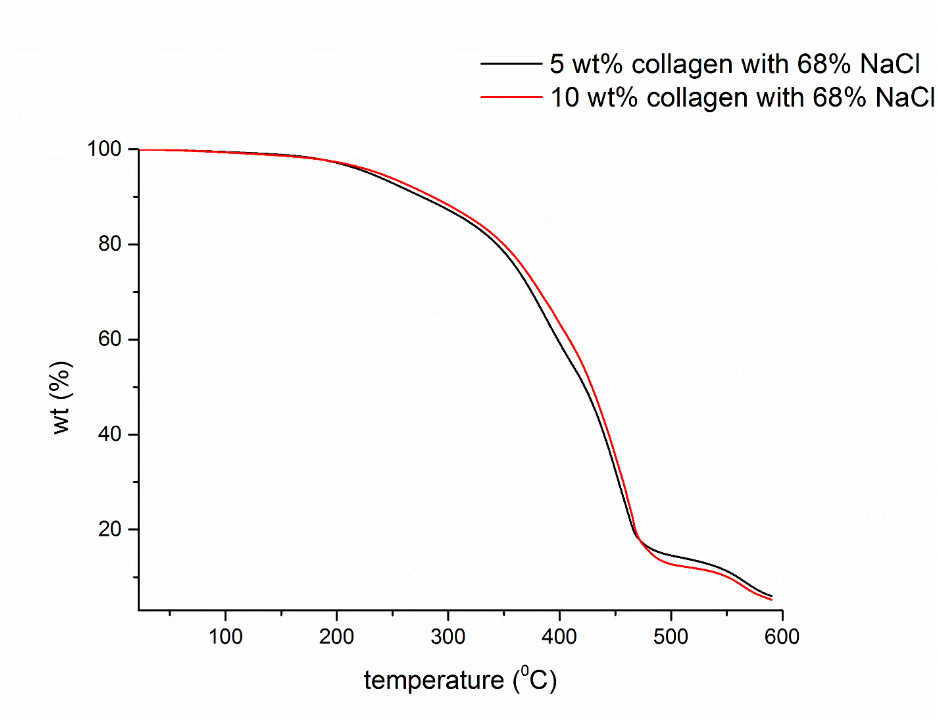

Figure 24. Overlay of TGA of composite with same amount of NaCl in different amounts of collagen

3.2.5 *Composites Mechanical Properties*

Under sinusoidal force conditions, the modulus can be divided into two components, an in-phase component, and an out-of-phase component. The in-phase component is known as the storage modulus and the out-of-phase component, the loss modulus. The tan δ is the ratio of the loss modulus to the storage modulus and is often referred to a damping. It is a measurement of the energy dissipated, and an indication of how well the sample can absorb energy. The peak of the tan δ curve gives the glass transition temperature (Tg) of the polymer. Its evaluation will be discussed in subsequent sections. Even though the sample displayed similar characteristics in the

TGA, their mechanical strengths differ. Figure 24 shows the DMA of the samples with 68 wt% NaCl and different amount of collagen. Their respective storage modulus and T_gs at room temperature were measured and will be discussed in the summary table.

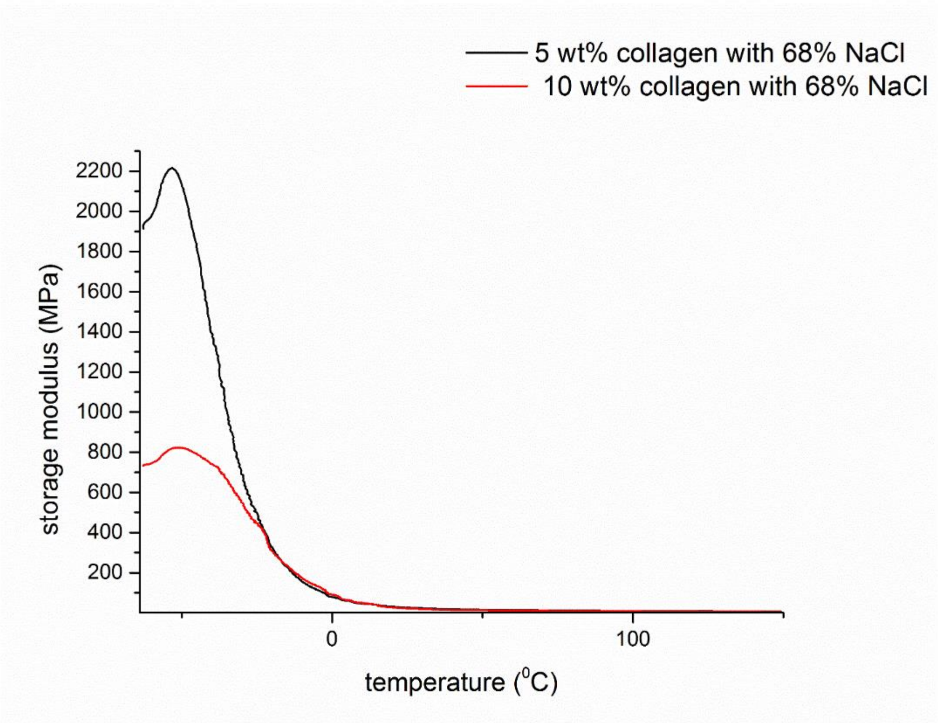

Figure 25. Overlay of DMA of composite with same amount of NaCl in different amounts of collagen

3.3 Characterization of Composites with Peptides

To better understand the interaction between collagen and the composites, peptides mimicking the collagen sequence, were to be synthesized. The synthesis could not be completed as I ran out of DIEA chemical. However, a former student, Kody Burrows, had already synthesized two composites, one containing 0.5 wt% alpha 1 peptide and the other containing a mixture of three helices (two alpha 1 and one alpha 2). I therefore had the opportunity to analyze the composites.

3.3.1 *Cure Verification of Composites*

DSC measures the heat flow in a temperature range. This detects endothermic and exothermic reactions that are occurring along the temperature range for that specific sample. It also analyzed the thermal transitions in the sample. Figure 25 shows the comparison between the single stranded peptide and the mixture of three helices. The composite with mixture of the helices showed a loss of water around 160^{0}C. This peak corresponds to the curing reaction. The presence of the flat peak could be moisture evaporating from the sample or the mixture of the helices is trapping some of the free radicals and not allowing full polymerization. Further polymerization would be required to diminish the peak around 160^{0}C.

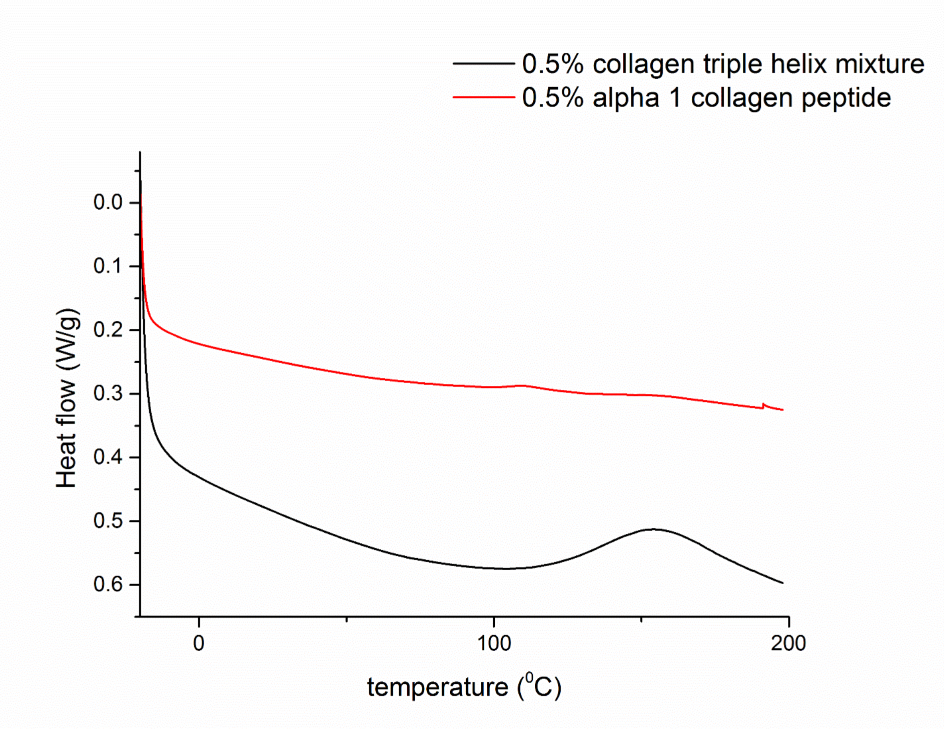

Figure 26. Overlay of DSC of composite with alpha 1 peptide and mixture of three helices

3.3.2 *Thermal Stability of Composites*

From the TGA curve in Figure 26, both composites were thermally stable until around 200^{0}C. The composites exhibit characteristics of a three-stage thermal degradation mechanism above 200^{0}C. The first stage of degradation is from around 200^{0}C to 300^{0}C and represents the evaporation of soluble, unreacted materials, as well as their subsequent decomposition. These materials are the easiest to evaporate/degrade since they are not included in the cross-link network. The second stage is from around 300^{0}C to 490^{0}C, and is the region with the fastest degradation, expressed by the steepest slope. This degradation stage is representative of the cross-link network degrading. In the last stage from 490^{0}C to 600^{0}C, the degradation slope starts to plateau, as the polymer residue reaches its maximum weight loss, often referred to as char. The last stage degradation corresponds to the steady, oxidative behavior of the char as it evaporates/degrades. The mixture of the three helices did not have any effect on the thermal stability of the composite, as it shows similar properties as the single strand peptide.

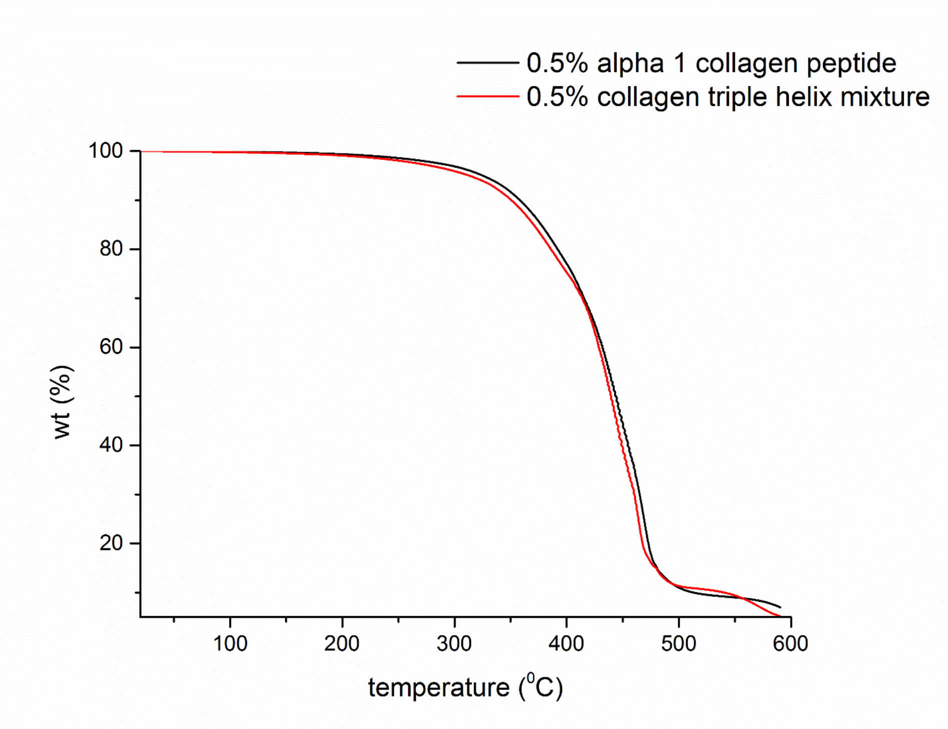

Figure 27. Overlay of TGA of composite with alpha 1 peptide and mixture of three helices

3.3.3 *Resin Mechanical Properties*

The strength of the composites was investigated via DMA. The storage modulus in the DMA indicates how much force it takes the composite to be displaced the same amount. If the entire DMA graph is at a higher MPa value at every temperature value, then the composite has been reinforced. From Figure 27, the graph indicated that the mixture of three helices had no reinforcement abilities in the composite. This is partly due to the incomplete curing of the sample as discussed earlier in subjection 3.3.1. The value of the storage moduli of the composites was recorded at room temperature and will be discussed in the summary Table 1.

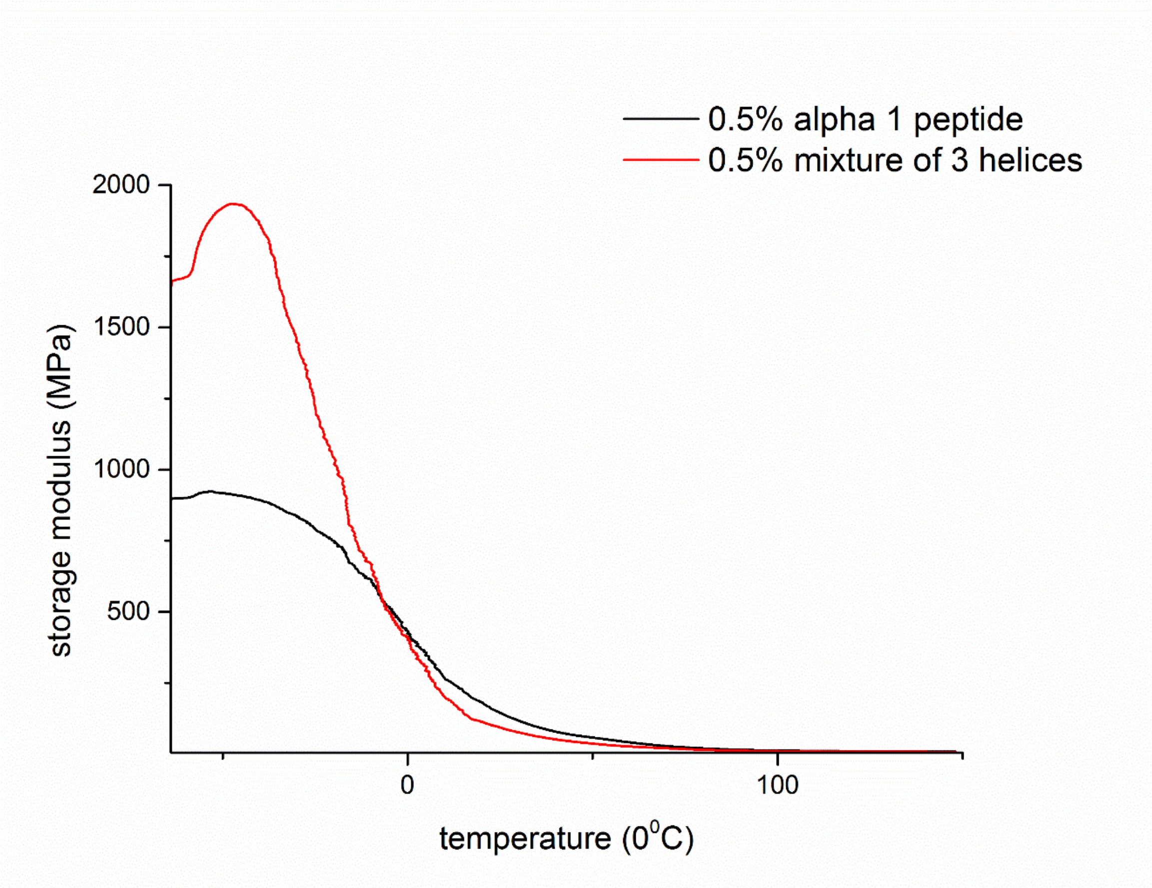

Figure 28. Overlay of DMA of composite with alpha 1 peptide and mixture of three helices

3.4 Summary of DMA Results

The table below, Table 1, shows a summary of the mechanical strengths of all composites synthesized in this research. In entries 1, 2 and 3, it is observed that the glass transition temperature and storage modulus, at room temperature, reduce with increased NaCl load. Obviously, the mechanical strength of the composite is negatively affected by the presence of NaCl. For entries 4 and 5, even in the presence of collagen, the 20 wt% of NaCl in collagen shows a better mechanical property compared with 68 wt% NaCl, though both have the same amount of collagen. This goes to reinforce the data from entries 1, 2, and 3, that NaCl is playing a major role in affecting strength of the composite. With increased amount in collagen for entries 7 and 8, the strength of the composite in entry 8 saw significant increase in the storage modulus. Entry 7 was, however, inconsistent with the trend and the composite would have to be resynthesized and analyzed. Because the composite with the mixture of the three helices did not fully form, its storage modulus was relatively low as seen in entry 10. The alpha 1 peptide however did have a slight positive impact on the strength of the composite.

Entry	**Collagen wt%**	**NaCl load wt%**	**Glass Transition Tg (^{0}C)**	**Storage modulus E' at 25 ^{0}C (MPa)**	**E' at Tg + 50 ^{0}C (MPa)**
1	-	-	-1.67	158.3	72.55
2	-	10	-7.37	84.85	53.34
3	-	15	-16.47	14.50	10.82
4	5	20	-4.49	47.61	
5	5	68	-50.22	26.05	78.89
6	10	20	-40.45	98.45	154.78
7	10	68	-22.94	20.20	17.15
8	10	70	-40.2	165.50	324.45
9	0.5% alpha 1 peptide	-	31.63	144.7	19.04
10	0.5% triple helix mixture	-	0.70	92.59	35.89

Table 1. DMA results for unreinforced resin and composites prepared with NaCl, collagen extracted from beef tendons, and peptides from Kody Burrows.

CHAPTER 4

CONCLUSIONS

In conclusion, this thesis discusses the effects of NaCl on the tripe helix structure of collagen and its effects on collagen-reinforced tung oil-based composites. The resulting enhancement in compatibility between the resin and reinforcement should consequently lead to better thermo-mechanical properties in the bio-based composites. Comparison between tung oil composites, composites with only NaCl, and composites with NaCl and collagen were completed to examine the effects of thermo-mechanical properties based on the composition of the composite.

From the data gathered, NaCl lowered the mechanical properties of the composites formed and future research will look into the interaction between NaCl and the resin to be able establish the chemistry behind how NaCl is lowering the mechanical strength of the polymer. Collagen was originally extracted for the purpose of incorporation into bio-based composites. Comparisons of unstructured collagen protein were analyzed via CD, TGA, and DSC. After going through a series of dialysis to remove NaCl, the corresponding CD data for each dialysis was compared. Removing NaCl affected the triple helix structure of collagen. Overall, the collagen had a positive impact on the strength of the composite. Future research will investigate how to completely remove NaCl from the collagen, which will in turn, be used for future experiment to contribute to the global goal of engineering bio-based polymers that are robust, biorenewable and eco-friendly.

Future Directions

Long and short peptides mimicking the collagen sequence will be synthesized and incorporated in the tung oil polymer. Given which peptide gives a better interaction, the peptide will be tuned using allyl glycine, which is an amino acid with double bonds that can form covalent linkages with tung oil resin.

REFERENCES

1. Liu, C.-M. and S.-Y. Wu, *From biomass waste to biofuels and biomaterial building blocks.* Renewable Energy, 2016. **96**: p. 1056-1062.
2. Ji, N., et al., *Catalytic conversion of cellulose into ethylene glycol over supported carbide catalysts.* Catalysis Today, 2009. **147**(2): p. 77-85.
3. Wang, Z., M.S. Ganewatta, and C. Tang, *Sustainable polymers from biomass: Bridging chemistry with materials and processing.* Progress in Polymer Science, 2020. **101**: p. 101197.
4. Zhao, X., et al., *Low-temperature microbial and direct conversion of lignocellulosic biomass to electricity: Advances and challenges.* Renewable and Sustainable Energy Reviews, 2017. **71**: p. 268-282.
5. Ronda, J.C., et al., *A renewable approach to thermosetting resins.* Reactive and Functional Polymers, 2013. **73**(2): p. 381-395.
6. Ourique, P.A., et al., *Synthesis, properties, and applications of hybrid polyurethane–urea obtained from air-oxidized soybean oil.* Progress in Organic Coatings, 2017. **108**: p. 15-24.
7. Chen, Y.-H., et al., *Biodiesel production from tung (Vernicia montana) oil and its blending properties in different fatty acid compositions.* Bioresource Technology, 2010. **101**(24): p. 9521-9526.
8. Keera, S.T., S.M. El Sabagh, and A.R. Taman, *Transesterification of vegetable oil to biodiesel fuel using alkaline catalyst.* Fuel, 2011. **90**(1): p. 42-47.
9. Weishaar, A., et al., *Evaluation of self-healing epoxy coatings for steel reinforcement.* Construction and Building Materials, 2018. **191**: p. 125-135.
10. Ribeiro da Silva, V., et al., *Polyurethane foams based on modified tung oil and reinforced with rice husk ash II: Mechanical characterization.* Polymer Testing, 2013. **32**(4): p. 665-672.
11. Liu, C., et al., *Phase-separation dominating mechanical properties of a novel tung-oil-based thermosetting polymer.* Industrial Crops and Products, 2013. **43**: p. 677-683.
12. Seniha Güner, F., Y. Yağcı, and A. Tuncer Erciyes, *Polymers from triglyceride oils.* Progress in Polymer Science, 2006. **31**(7): p. 633-670.

13. Andreo-Martínez, P., et al., *Advances on kinetics and thermodynamics of non-catalytic supercritical methanol transesterification of some vegetable oils to biodiesel.* Energy Conversion and Management, 2018. **173**: p. 187-196.
14. Huang, K., et al., *A self-crosslinking thermosetting monomer with both epoxy and anhydride groups derived from tung oil fatty acids: Synthesis and properties.* European Polymer Journal, 2015. **70**: p. 45-54.
15. Baldock, C., et al., *The Supramolecular Organization of Collagen VI Microfibrils.* Journal of Molecular Biology, 2003. **330**(2): p. 297-307.
16. Wu, J.-L., et al., *Synthetic studies toward lindenane-type dimers via Diels-Alder reaction.* Tetrahedron, 2018. **74**(47): p. 6749-6760.
17. Riaz, U., C. Nwaoha, and S.M. Ashraf, *Recent advances in corrosion protective composite coatings based on conducting polymers and natural resource derived polymers.* Progress in Organic Coatings, 2014. **77**(4): p. 743-756.
18. Meiorin, C., M.I. Aranguren, and M.A. Mosiewicki, *Polymeric networks based on tung oil: Reaction and modification with green oil monomers.* European Polymer Journal, 2015. **67**: p. 551-560.
19. Sharma, V., J.S. Banait, and P.P. Kundu, *Spectroscopic characterization of linseed oil based polymer nano-composites.* Polymer Testing, 2008. **27**(8): p. 916-923.
20. Selladurai, M., P.R. Sundararajan, and M. Sarojadevi, *Synthesis, thermal and mechanical properties of modified PMR/carbon fiber composites.* Chemical Engineering Journal, 2012. **203**: p. 333-347.
21. Bhuyan, S., et al., *Effect of crosslinking on the friction and wear behavior of soybean oil-based polymeric materials.* Wear, 2007. **263**(7): p. 965-973.
22. Andjelkovic, D.D., et al., *Novel thermosets prepared by cationic copolymerization of various vegetable oils—synthesis and their structure–property relationships.* Polymer, 2005. **46**(23): p. 9674-9685.
23. Lovell, P.A., et al., *Studies of rubber-toughened poly(methyl methacrylate): 1. Preparation and thermal properties of blends of poly(methyl methacrylate) with multiple-layer toughening particles.* Polymer, 1993. **34**(1): p. 61-69.

24. Johns, A., et al., *4 - Plant Oil-Based Polyhydroxyurethanes*, in *Bio-Based Plant Oil Polymers and Composites*, S.A. Madbouly, C. Zhang, and M.R. Kessler, Editors. 2016, William Andrew Publishing. p. 55-71.
25. Šestáková, I., Š. Skalová, and T. Navrátil, *Labile lead phytochelatin complex could enhance transport of lead ions across biological membrane.* Journal of Electroanalytical Chemistry, 2018. **821**: p. 92-96.
26. Shoulders, M.D. and R.T. Raines, *Interstrand Dipole-Dipole Interactions Can Stabilize the Collagen Triple Helix*.* Journal of Biological Chemistry, 2011. **286**(26): p. 22905-22912.
27. Ramachandran, G.N., M. Bansal, and R.S. Bhatnagar, *A hypothesis on the role of hydroxyproline in stabilizing collagen structure.* Biochimica et Biophysica Acta (BBA) - Protein Structure, 1973. **322**(1): p. 166-171.
28. von Arx, T., et al., *Evaluation of a prototype trilayer membrane (PTLM) for lateral ridge augmentation: an experimental study in the canine mandible.* International Journal of Oral and Maxillofacial Surgery, 2002. **31**(2): p. 190-199.
29. Oprea, S., et al., *Synthesis and characterization of poly(urethane-urea-acrylate)s.* European Polymer Journal, 1999. **35**(7): p. 1269-1277.
30. Huang, C.-Y., et al., *Isolation and characterization of fish scale collagen from tilapia (Oreochromis sp.) by a novel extrusion–hydro-extraction process.* Food Chemistry, 2016. **190**: p. 997-1006.
31. Hong, H., et al., *Amylase enhances production of low molecular weight collagen peptides from the skin of spent hen, bovine, porcine, and tilapia.* Food Chemistry, 2021. **352**: p. 129355.
32. Campbell, A.C., et al., *Structural analysis of prolines and hydroxyprolines binding to the l-glutamate-γ-semialdehyde dehydrogenase active site of bifunctional proline utilization A.* Archives of Biochemistry and Biophysics, 2021. **698**: p. 108727.
33. Abshire, V.M., et al., *Injection of L-allylglycine into the posterior hypothalamus in rats causes decreases in local gaba which correlate with increases in heart rate.* Neuropharmacology, 1988. **27**(11): p. 1171-1177.
34. Montero de Espinosa, L. and M.A.R. Meier, *Plant oils: The perfect renewable resource for polymer science?!* European Polymer Journal, 2011. **47**(5): p. 837-852.

35. Bhuyan, S., et al., *Effect of filler composition and crosslinker concentration on the tribological behavior of spent germ particle-based polymeric composites.* Tribology International, 2010. **43**(1): p. 171-177.
36. Caporale, A., et al., *Automatic procedures for the synthesis of difficult peptides using oxyma as activating reagent: A comparative study on the use of bases and on different deprotection and agitation conditions.* Peptides, 2018. **102**: p. 38-46.
37. Ma, Y., et al., *Synthesis and characterization of microencapsulated phase change materials with comb-like acrylic co-polymer shell as thermal energy storage materials.* Solar Energy, 2019. **179**: p. 410-423.