Silk Hydrogels Incorporated with Melanin

Anne Lutz

Wright State University

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SILK HYDROGELS INCORPORATED WITH MELANIN

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biomedical Engineering

by

ANNE LUTZ
B.S.B.E, Wright State University, 2019

2021
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Anne Lutz ENTITLED Silk Hydrogels Incorporated with Melanin BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science in Biomedical Engineering

______________________________
Tarun Goswami, PhD.
Thesis Director

______________________________
Subhashini Ganapathy, PhD.
Chair, Biomedical, Industrial, and Human Factors Engineering

Committee on Final Examination:

______________________________
Tarun Goswami, PhD.

______________________________
Ulas Sunar, PhD.

______________________________
Jaime E. Ramirez-Vick, PhD.

______________________________
Lawrence Drummy, PhD.

______________________________
Barry Milligan, PhD.
Interim Dean of the Graduate School
Melanin is a naturally produced pigment found within the human body. Melanin is known for its ability to protect against Ultra Violet light, but also its ability to allow for mechanical protection. Making melanin a good addition to biomedical devices such as hydrogels. Silk hydrogels are weak in their load bearing capabilities but are known for their biocompatibility, biodegradability, and porosity. This makes the silk hydrogel a good material to incorporate melanin into in order to improve the mechanical properties.

For the silk solution to form a solid, there must be a presence of both a catalyst and an oxidizer to cross-link. Once the solution is crosslinked it forms a gel, also referred to as a hydrogel due to its water retaining abilities. In this work, eumelanin was produced from bacteria and mixed into a silk solution to form hydrogels that were studied and tested. Results showed that the hydrogels incorporated with melanin were stronger than the control, and that the melanin mixed with the silk solution without the presence of a catalyst was able to cause gelation.
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1. Introduction

Skin injuries are a severe health risk to the human body. The skin provides thermal insulation, helps with body fluid retention, and protects from exogenous pathogens. After a wound occurs, within minutes platelet aggregation begins. The platelets form fibrin clots. Growth factor is locally released, and inflammatory cells move to the wound site. The inflammatory response clears foreign bodies, bacteria, and damaged cells from the wound site. Near the end of the inflammatory phase, both fibroblasts and epithelial cells induced by growth factor proliferate and migrate into the wound. The proliferation phase initiates new blood vessel, and tissue formation. The overall wound healing process is dependent upon the health and age of the patient, or the presence of foreign bodies or infection. Typically, complete wound healing takes between several weeks to months. Acute wounds go through this procedure and are completely finished within 8-12 weeks. Chronic wounds stall during the inflammation process. During the inflammation process of chronic wounds; exudate, pain, infection, and necrosis of cells causing the full wound healing process to take several months or years to heal.

Chronic wounds require frequent hospital visits, and patient physical restriction and limitations. This can greatly depreciate the patient’s quality of life. There are approximately 20 million people worldwide suffering from chronic wounds. The cost of chronic wounds is expensive for both the healthcare provider and the patient. Wound dressings on chronic wounds help the healing process and prevent infections. By
protecting the wound site, the dressings allow the wound to heal much quicker. There are many different types of dressings that can be used. An ideal wound dressing needs to provide a moist environment and protect against foreign bodies, microbes, or damaging forces. The wound dressings need to have the mechanical strength for both application and removal. Hydrogels provide excellent healing conditions and allow for minimal damage when removed, making them desirable as an infrastructure for further development.

Hydrogels are a bundle of hydrophilic polymer chains. They are used in the biomedical field specifically in tissue engineering as carriers for bioactive molecules, and as a scaffold to produce a backbone to help with regeneration of tissue. Hydrogels are able to absorb large quantities of water or biological fluids, allowing them to be used for drug delivery, biosensors, matrices and carriers for cells within the body. Hydrogels are advantageous due to their biocompatibility, biodegradability, and porous structures. However, due to their low mechanical strength, they have very limited practical applications. Hydrogels can be made from both synthetic and natural materials such as hydrophilic polymers. Natural hydrophilic polymers include both polysaccharides and polypeptide.

Hydrogels can be utilized for both acute and chronic wounds. They are promising in wound care based on their ability to keep the wound moist, are adhesion free, pain reduction through cooling, and the ability for intervention during the healing process. With a growing popularity and an aging society, continuous expansion in hydrogel
materials is necessary. Current problems within hydrogel manufacturing and use are; high cost, safety concerns regarding drug delivery, and lack of mechanical stability. In order to meet clinical necessity, a new hydrogel without the drawbacks would be most impactful and further the progression of hydrogels.

1.1. Motivation

The motivation behind this project was to produce a new hydrogel composite to improve mechanical strength, provide antimicrobial protection, allow for UV protection, and be cost effective. From previous research, melanin has been known to improve the mechanical strength of materials when added. Melanin has also been known to provide antimicrobial properties along with Ultra Violet protection. Silk hydrogels are fairly cost effective however the mechanical strength of silk prevents it from being a viable hydrogel. Silk is a cost-effective material and is known for its biocompatibility properties. In addition to the silk hydrogel, the melanin provides strength to the hydrogel which otherwise is too weak for practical application.

Melanin is naturally produced within the human body, it is also biocompatible, making the combined hydrogel an exemplary choice for practice. Melanin has also been investigated as a drug delivery system due to its biodegradation and its ability to bind to drugs. However, in a drug delivery system, researchers have found that melanin binds to different drugs without understanding why and the binding preferences of melanin. It is speculated that because melanin protects the body, its binding properties are exemplary to prevent damage from foreign bodies.
2. Literature Review

2.1. Silk as a Biomaterial

Silk is natural fibrous protein polymers, spun by both silkworms and spiders. Among silk variants, there is an increased interest in the silkworm silk of B. Mori (Bombyx Mori). B. Mori is a type of silkworm that is entirely domesticated. It is a primary producer of silk and is only able to reproduce with human intervention due to its availability in large quantities and its material properties. Silk fibroin can be extracted from the cocoons of the B. Mori silkworm and combined synergistically with other biomaterials to form biopolymer composites. Silk proteins can be processed in aqueous environments into various forms including films, sponges, electrospun mats, and hydrogels. The FDA has approved silk medical devices for sutures and as a support structure after reconstructive surgery. With increasing needs for implantable and degradable devices, silkworm silk has attracted interest for both implantable and degradable medical devices, along with a broader range of utility in different device applications.

The silkworms form cocoons which are dissolved forming silk fibroin solution. Once the silk fibroin is in an aqueous state, the concentration can be adjusted. This silk solution was used to make silk hydrogels. Silk hydrogels were formed from the silk solution crosslinking which causes the aqueous silk solution to gel. Gelation occurs from intermolecular and intramolecular cross-linking between protein chains such as hydrogen bonding, hydrophobic, and electrostatic interactions. The silk solution to form the hydrogel is very sensitive. It is reactive to heating, shearing, water evaporation, or solvent
exposure. Although it is sensitive in its aqueous state, this also allows for the choice of reaction based on the experiment being done. Once the slightest changes occur it starts the chain of events to form β-sheets which supply the gel with both stability and strength.

Figure 1: Silk Fibroin Protein

Figure 1 shows the chemical formula of the silk fibroin from the B. Mori silkworm. The silk fibroin from the B. Mori is composed of three amino acids; glycine, alanine, and serine. They are present in the molar ration of 3:2:1, respectively. Percent composition is Glycine (45%), Alanine (29%) and Serine (12%), with the remaining 13% composition of tyrosine, valine, aspartic acid, glutamic acid, etc. This can be seen in in the table below which also compares the percent composition of fibroin to sericin. The strength of the silk fibroin comes from the hydrogen bonds tightly packed in the center. The lateral dimensions are 20x60 angstroms on average.
Table 1: Silk protein composition of amino acids present in B. Mori silk

<table>
<thead>
<tr>
<th>symbol</th>
<th>Amino acid</th>
<th>Fibroin</th>
<th>Sericin</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>(glycine)</td>
<td>45</td>
<td>14</td>
</tr>
<tr>
<td>A</td>
<td>(alanine)</td>
<td>29</td>
<td>5</td>
</tr>
<tr>
<td>S</td>
<td>(serine)</td>
<td>12</td>
<td>33</td>
</tr>
<tr>
<td>Y</td>
<td>(tyrosine)</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>V</td>
<td>(valine)</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>D</td>
<td>(aspartic acid)</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>R</td>
<td>(arginine)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>E</td>
<td>(glutamic acid)</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>I</td>
<td>(isoleucine)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>L</td>
<td>(leucine)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>F</td>
<td>(phenylalanine)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>T</td>
<td>(threonine)</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>C</td>
<td>(cystine); half</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H</td>
<td>(histidine)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>K</td>
<td>(lysine)</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>M</td>
<td>(methionine)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P</td>
<td>(proline)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>W</td>
<td>(tryptophan)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Silk scaffolds are formed using water-based methods without the addition of organic solvents which is preferable when in-vivo applications are concerned. Although silk hydrogels are researched heavily for drug delivery systems and in-vivo applications, silk hydrogels can be too weak for in-vivo applications. The loadbearing capabilities of the silk hydrogel are too low which can be quantified by the low Youngs modulus. Researchers are now looking into ways in order to improve the mechanical strength of hydrogels.
Silk is regarded as a semi-crystalline biopolymer with highly organized β-sheet nanocrystals imbedded within the amorphous matrix. When a silk hydrogel is formed the β-sheet formations can be seen using FTIR in the 1500-1700 range as two large peaks. This can be seen for all silk naturally produced in insects.

![FTIR of single silk fibers](image)

**Figure 2:** FTIR of single silk fibers (a) B. mori, (b) A. pernyi, and (c) N. edulis.

2.2. Cross-linking in hydrogels

Crosslinking is necessary for hydrogels to form, they prevent the dissolution of the polymer chains within the aqueous environment. Hydrogels are advantageous due to their biodegradability. Labile bonds are frequently introduced into the gel. Labile bonds present themselves in crosslinking or the polymer backbone of the hydrogel. There are

7
many ways that crosslinking can occur. Some crosslinking methods are: radical polymerization, chemical reaction, addition reactions, condensation reactions, high energy irradiation, crystallization, physical crosslinking, and enzymatic crosslinking. Silk hydrogels are able to be enzymatically crosslinked by the presence of horseradish peroxidase (HRP) and hydrogen peroxide. This specific enzymatic reaction forms hydrogen bonds. Hydrogen bonding is the quickest of the bonds allowing the silk fibroin solution to get faster by use of enzymatic crosslinking.

When a large protein or polymer is introduced in silk fibroin solution, crosslinking is able to occur. Natural proteins with α-helices are characterized by both a hydrophilic and hydrophobic side of an amino acid chain. This coil is also able to form crosslinking within a silk hydrogel, making it able to cross-link without the presence of an enzyme.
Figure 3: Structural representation of hybrid hydrogel primary chains and the attachment of coil proteins.  

2.3. Wound Dressings

The skin provides a barrier to help protect against infection and environmental changes. Approximately 1-2% of people in the US will sustain a chronic wound during their lifetime. Autografts are the standard replacement for skin injuries, however, they
are not always able to be used if large areas of the body are affected.\textsuperscript{3} Using wound dressings is a good alternative to autografts. An ideal wound dressing provides a moist environment at the interface between the dressing and the wound, prevents gas exchange, and act as a barrier against micro-organisms.\textsuperscript{6} The skin damage after the incident causes the death of skin cells, leading to a loss of body fluids followed by dehydration, electrolyte imbalance, and the possibility for renal and circulatory failure.\textsuperscript{8} The cumulative effect of cell-sheets, scaffolds, cell-scaffolds, and hydrogels with healing helps promote factor triggers, accelerate, and enhances wound healing and re-epithelialization leading to reduction in scar formations.\textsuperscript{8} Skin is composed of three main layers (epidermis, dermis, and hypodermis) with its appendages (hair, sweat and sebaceous glands, sensory neurons, blood and lymph vessels, etc.)\textsuperscript{8}
Figure 4: Classification of Serious Burn Injuries. (A). First-degree burns induce epidermis injury with skin redness and pain. Second-degree burns affect the epidermis and part of the dermis layers with the formation of blisters. Third-degree burns destroy the deeper layer of skin, the hypodermis. Fourth-degree burns involve injuries to the muscles, tendons, and bones. (B) Grafted hydrogel containing cells, growth factors, and antibacterial agents in the burned skin.
One of the major concerns regarding wounds and dressings is scarring formed after the skin has healed. Silk fibroin is useful because of its ability to stimulate proliferation of keratinocytes and fibroblasts within a burn. Although silk fibroin has been shown to provide the proper environment for burns, currently the only tissue-engineered constructs for burns are able to provide protection. This means that thermoregulation, sensation, excretion, perspiration, and UV protection are not restored.

2.4. Ultra Violet light on Scars

UV avoidance is recommended while a wound is healing and for years after a wound has healed. The melanocytes within the scar tissue are unable to be produced and if they are, they are not able to distribute and rearrange themselves as they were before. This can be seen by the discoloration of a scar after the wound has healed. UV light is a leading cause of cancer, due to the lack of melanocytes within the scar tissue, the light is able to penetrate at a more aggressive level than typical skin would experience.

2.5. Bioengineering

The development of bioengineering has increased the search for new bioinspired materials, with tunable mechanical, chemical, optoelectronic properties. The newer materials are meant to replace metal or organic polymers that cause cytotoxicity and other adverse health effects. Natural melanin is difficult to manipulate because of its ability to easily crosslink. When put into a PEG (polyethylene glycol) solution to form a hydrogel, the PEG solution has a very strong hydrogen bonding affinity. This causes the crosslinking between PEG and naturally produced melanin.
2.6. *Melanin*

Melanin is a collection of small molecules forming a pigment naturally produced by many organisms. It presents itself in bird feathers, black mold, squid ink, and in both human hair and skin. Melanin is produced by melanosomes which are found within melanocytes. These melanocytes are located in the epidermis of the skin and in hair follicles. Humans produce two types of melanin; pheomelanin and eumelanin. Pheomelanin is a red/brownish color while eumelanin is black in color. Melanosomes are approximately 0.5 microns in diameter and 1 micron in length. Melanosomes are organelles that synthesize, store, and transport melanin. The synthesis of melanin is done through the process of melanogenesis where the amino acid tyrosinase is oxidized and then polymerizes into melanin.
Figure 5: A) Eumelanin and Pheomelanin Chemical Structures. B) Electrograph of melanocyte with melanosomes.

The molecular weight of eumelanin is 318.3 g/mol. The average size of the radius of gyration of the melanin particle is approximately 16.5 Angstroms.\textsuperscript{75}
Figure 6: FTIR range from 900-1800 with characteristic peaks between 1500-1800 for synthetic eumelanin.\textsuperscript{76}
Table 2: Synthetic Eumelanin FTIR characterization of peaks.\(^6\)

Melanin is a multifunctional material as it provides UV protection, allows for thermoregulation, can provide chemical protection, allows for metal binding, and provides mechanical protection.
Figure 7: Comparison of yeast cell one melanized and one albino.

Figure 7 shows an electronmicrograph of melanized and non-melanized Cryptococcus neoformans yeast cells. The melanized cells are more resistant to mamalian immune cell attack. Another example of melanin's additional mechanical strength can be seen in melanized fungal hyphae which when melanized are structurally harder and more pathogenic than its non-melanized counterpart.

2.7. Structural Color

The melanin structure found in birds and butterflies does not present itself as black or brown like the melanin found in other organisms. Instead they are very colorful and bright. This is called structural color. Structural color is created from micro or nanostructures which both reflect and scatter light so that constructive interference occurs, resulting in the bright colors. Color traditionally is seen in pigments or dyes, which absorb light and over time begin to fade. Structural color does not rely on either
pigmentation or dye and because it is composed of both micro and nanostructures, it is resistant to fading. The color can be caused by different methods such as diffraction and thin-film layering.

Thin-film layering is a structural coloration in which two or more semi-transparent surfaces are layered which causes construction and deconstructive interference. However, instead of grating thin-film layering; structural color is determined by the thickness of the transparent layers which determines the interference pattern seen.

Diffraction grating is when objects or slits are comparable in size to the wavelength of the wave that hits it. The light bends around the objects or is reflected in such a way that either constructive or destructive interference occurs. This allows for color to be amplified or canceled out. Naturally the Menelaus blue morpho butterfly has diffraction gratings within its wings. The morpho butterfly wings are actually brown but appears bright blue to our eyes. The true brown color of the butterfly wings is melanin.

2.8. Melanin in Bioengineering

There are two types of melanin found in humans; eumelanin and pheomelanin. The main difference between the two melanin structures is the presence of sulfur within the composition, pheomelanin containing sulfur. Melanin is effective at absorbing light which extends close to the near infrared regions. Melanin has been used as a possible photothermal agent (PTA) because it provides good photothermal conversion efficiency and degradability without subjecting toxicity in-vivo. There was a study done on mice
using poloxamer solution containing melanin. The solution was injected into the tumor cite of the mouse and after exposure to light, the tumor disappeared without causing damage to the mouse. The mouse was irradiated with 808nm NIR laser at 1.5W cm\(^{-2}\) for 3 minutes. The photothermal conversion of the Pol-Mel was enough to kill the cancer cells within the mouse.\(^{15}\)

**Figure 8:** Mouse with tumor treated with Pol-Mel, subjected to 808 nm NIR laser at 1.5W cm\(^{-2}\) for 3 minutes with results.\(^{15}\)

Melanin is also known for other features other than improving mechanical strength, UV-protection, and ionizing blocker. It can also be used as an antimicrobial agent.\(^{17}\)
2.9. Melanin binding

Ocular eumelanin has been reviewed and studied because melanin binds to different drugs in unexpected ways. It has been shown that melanin is hydrophobic, and its binding actions have been described as a combination of electrostatic and hydrophobic interactions. There does not seem to be an exact answer as to why the melanin binds and cross-links, although there are speculations that it could be: electrostatic and hydrophobic interactions, hydrogen bonding, charge transfer interactions, or some combination of the above. The phenomena has been researched in the medial field for drug delivery systems and implantable devices. A prediction as to why eumelanin bonds rapidly is due to its characterization for protection, especially within the eye where if something enters it could be detrimental. 23

2.10. Rule of Mixtures

The rule of mixtures or composites with homogeneously distributed particles can be applied on to conventional composites, nanocomposites, and nanocrystalline material. 78 The rule of mixtures is used to correlate the mechanical properties of a composite. 78 The rule of mixtures uses the Reuss and Voigt models to find the upper and lower bounds, which rely on the equal stress and strain assumption respectively. The rule of mixtures can be considered inaccurate if the material has hard particles, specifically plastic properties. If the material has plastic properties the best way to correlate the material properties would be to use finite element analysis. 78 In this study, the material in question is a hydrogel. The rule of mixtures is a much faster and allows for a simplified
model because the material has more elastic properties than plastic. The following equations are used to find the upper and lower bound of a material. The lower bound equation: \( H_{\text{up}} = f_h H_h + f_s H_s \). The upper bound equation is: \( H_{\text{low}} = \frac{f_h}{H_h} + \frac{f_s}{H_s^{-1}} \). \( H_s \) and \( H_h \) are the hardness values of the hard and soft phases, respectively. The low and upper scripts are the lower and upper bound of hardness, respectively. The upper and lower bound are graphed and the area between the two equations is the predicted area for the hardness of the material after a compression test is performed.

2.11. Thesis Statement

Silk hyrdrogels are at the forefront of tissue engineering due to their biocompatibility, bioreactivity, and biodegradability. However they are not able to be used for practical applications due to their mechanical strength.

First aim: Melanin is known to improve mechanical strength and is also biocompatible and biodegradable. By understanding the material properties of silk hydrogels incorporated with melanin, there would be conclusive data showing whether or not the addition of melanin improved the structural integrity of the hydrogels.

Second Aim: When in the presence of silk solution, the melanin causes the hydrogel to crosslink without the presence of a catalyst or oxidator. Finding the reasoning behind this phenomina will allow for further investigation as to how the structure of melanin influences the mechanics of the hydrogel.
3. **Materials and Methods**

As discussed in the previous chapter the specific aims of this research are: 1) Improve the mechanical strength of a silk hydrogel. 2) Investigate the mechanics of silk hydrogels incorporated with melanin.

3.1. **Materials**

The Bombyx Mori silk worm cocoons were collected and obtained naturally after the silkworm was done making the cocoon. The cocoons were then processed, boiled, dried, washed, rinsed, and suspended in an aqueous solution to allow hydrogel to form within a mold. The cocoons used in this experiment were procured by Wright Patterson Air Force Base (WPAFB). The sodium carbonate, LiBr, CuSO_{4}, M9 salt, MgSO_{4}, glucose, horseradish peroxidase, and CaCl_{2} were all purchased from Sigma Aldrich. Purified DI water (18.2 MΩ resistance), *E. coli* with engineered tyrosinase, and LB were procured by WPAFB.

3.2. **M9 Minimal Media**

A 1000mL solution of M9 minimal medium was created by mixing: 100mL of 10xM9 salt, 2mL 1M MgSO_{4}\(\bullet\)7H_{2}O, 40 mL of 0.5M glucose, 1 mL 0.1M CaCl_{2}\(\bullet\)2H_{2}O, and 860 mL dH_{2}O. After the solution was thoroughly stirred without precipitate it was sterilized through filtration. This solution was used for the induction of tyrosinase.

3.3. **Melanin Induction**

The melanin used in this experiment was produced by engineered tyrosinase which was secreted outside of *E. Coli* bacterial cells. The procedure for melanin production
happened over a three day period. On the first day the 3 mL of LB broth and 100 µg/mL Ampeciline culture 1269 (NEB5α/pZR011 c.1) was incubated at 37°C, at 200 RPM. After a day the overnight culture was diluted 1:100 into a mixture of LB and 100 µg/mL Ampeciline. This was then incubated at 37°C, shaking at 150 RPMs. After 3 hours the optical density was tested at 600nm. The optical density needs to be between 0.8-1.2. Once this optical density was achieved, the solution was spun down at 5000RPM for 10 minutes in sterile bottles and the liquid was poured off the top. The induction took place in the M9 induction solution. The inducer and additional CuSO₄ were added into the culture 50 mM IPTG (5µL 1M IPTG), 100 µL of 0.2M CuSO₄, and 1 mL of 200 mM L-tyrosine Na salt in mpH₂O. Then incubated overnight at 37°C at 150RPM.
Culturing:

IPTG (induction): + +
CuSO₄: + +

Post-culturing addition:

Tyrosine: - +

Figure 9: Presence of Tyrosinase in M9 solution. Source: Dr. Chia Hung

On the third day the culture was spun down for 10 minutes at 5000 RPM to separate the cells. The top layer of liquid was poured off leaving the cells behind. This solution was black in color indicating the presence of melanin.

3.4. Concentration of Melanin

The melanin was washed and concentrated. This was done by pouring the solution into a spin cell with a 5 kDa filter with 45psi of Nitrogen. After all of the M9 solution was added to the spin cell, it was then washed with water. This diluted the M9 solution and allowed the melanin particles to be suspended in water. The first concentration was from 500mL to 125mL, then the addition of water up to 300mL. Once the solution...
dropped below 100mL, it was then filled back up to 300mL a total of 8 times. This process can be seen in Figures 10-12.

Figure 10: Spin cell with melanin and M9 solution.
Figure 11: Set up of spin cell on hot plate with waste beaker.

Figure 12: Waste beakers from 3rd flow through on left and 1st and 2nd flow through on right.
By the last flow through adding water to wash out the M9 salt solution, the waste beaker was almost completely clear. The final amount of melanin solution was 5mL. The concentration of melanin in the water was 2000u/mL and 22.5mg/mL. This melanin solution was then added to the silk solution to make the silk hydrogels.

3.5 Silk Solution

3.6.1 Cocoon Processing

The general silk solution was made from dissolving silk cocoons into a liquid solution. The B. Mori (Bombyx Mori) cocoons first needed to be processed. The cocoons were cut open with a small pair of scissors. Once opened the catipilar remains and dust were removed. Any badly stained section of the cocoon was removed. The cocoon was cut into smaller pieces, and then delaminated-seperating into 2-3 layers. Thourough delamination is important for efficient degumming (removal of sericin).

3.6.2 Cocoon Degumming

3L of boiling 18.2 MΩ water was added to a 4L beaker and covered with aluminium foil. Once the water boiled, 6.36g of sodium carbonate (Na$_2$CO$_3$) was added along with 7.5g of processed silkworm cocoons. The solution was continualy stirred while boiling for exactly 30 minutes. At this step the silk molecular weight degraded as while sericin (glue) is removed. After the 30 minutes the degummed silk fibers (fibroin) was removed and placed into aluminium foil, using gloves and silicon hot hands to squeeze the remaining solution out of the silk, the aqueous waste was discarded. The silk fibers were then rinsed with distilled water and placed into 2L of 18.2 MΩ water, stirred,
and left to sit for 20 minutes. The silk was then removed and hand wrung then placed into
a new 2L of 18.2 MΩ water for 20 minutes, wrung again, and the process was repeated
again for a total of 3x 20 minute rinses. After the third rinse, the silk was wrung out and
placed in a large weigh boat and left under a fume hood overnight. After drying the
weight was measured, the degumming process takes approximately 25% wt. of the silk,
yielding 5.63g of of fibers (frequently recover 5.5g). Figure 13 shows an example of
degummed silk. This silk still needed the fibers to be hand pulled apart and seperated
finely for a better dissolution.

Figure 13: Dried silk fibrin after degumming. Source: Dr. Chia Hung
3.6.3. *Fibroin Dissolution*

Purified fibroin should be dissolved at 20% wt/vol in 9.3M LiBr (Lithium Bromide).

The 5.63g of fibroin solution required at least 28mL of LiBr solution, so 30mL was used. The 9.3M LiBr solution required 24.2g of LiBr powder in a 100mL beaker with a stir bar. The 100mL beaker was placed in a circulating water bath at 60°C with the addition of 18.2MΩ DI water into the 100mL beaker up to 30mL. The solution was stirred until clarified. The solution was then poured into a graduated cylinder to validate that it was 30mL, then returned back to the same beaker. 10mL of LiBr solution was added to a 50mL centrifuge tube, a small amount of the degummed silk was added, then push down the with a spatula to saturate the silk. After fully saturated, more LiBr solution and silk were added until all the silk in the tube was completely saturate. This tube was placed into the 60°C water bath and left to incubate for 2 hours and was checked at 30 minutes and 60 minutes. After two hours the solution looked similar to that which is seen in Figure 13, and had a concentration of about 3%.
3.6.4. Dialysis

A 12kDa MWCO (Molecular weight cut off) dialysis tubing cut to an appropriate length was used and rehydrated by soaking in 18.2MΩ water for approximately 5 minutes. One end of the tubing was secured with a clamp. The tubing was then gently inflated with air using a pipette boy and 10mL serological pipette. The LiBr solution was removed from the water bath and while it was still warm, transferred using the pipette into the tubing. Once it was around 2/3 of the way filled, the open end was secured. The dialysis tubing was then placed into 4L of 18.2MΩ water with a stir bar, on a stir plate in a 4°C refrigerator. The dialysis water was changed a total of 6 times; the first hour, fourth hour, and twice a day on the following days (morning and evening). On the
morning of the fourth day the aqueous silk was removed from dialysis tubing by squeezing into 50mL centrifuge tube. The silk was then spun in the centrifuge at 10k rpm for 10 minutes. This solution was then decanted into a new tube leaving behind the pelleted impurities, this was then repeated. The fibroin solution concentration was checked at this point using 1.5mL tubes of known mass with 100µL of silk solution (weighed), then placed in the oven to evaporate the water and reweighed. The solution was stored at 4°C and labeled. This solution has a shelf life of approximately 1 month. The dialysis setup is seen in Figure 15.

![Figure 15: 12kDa Dialysis tubing filled with silk solution. Source: Dr. Chia Hung.](image)
3.6.5. Silk Concentration

In order to concentrate the silk further, reverse dialysis was necessary. First a dialysis cassette of 3500 MWCO (needs to be well below that of polyethylene glycol). A 10% weight by volume solution of polyethylene glycol (PEG) 8000 in 18.2MΩ water. The dialysis cassette was rehydrated in 18.2MΩ water then filled with silk solution. The filled dialysis cassette was put into the PEG solution with a stir bar and stirred overnight at 4°C. The silk solution used in this experiment was 7% silk by volume.

3.7. Silk Hydrogel Incorporated with Melanin

After the silk solution and melanin solution are made, the silk hydrogels were injected into cylindrical molds. Each hydrogel was 100µL total composed of 25µL of water and 75µL of silk solution, this ratio was decided based on the speed at which the hydrogel solidified. This ratio allows for control over the amount of melanin added to the hydrogel solution. 2µL of 1% H₂O₂ was added to 50µL of the 75:25 solution. The other 50µL had 4µL of HRP (horseraddish peroxidase) added to it. The solution with H₂O₂ was gently pipetted and mixed into the solution with the HRP. This mixture should be thoroughly mixed however the cross-linking occurs almost immediately. This means that the full 100µL of solution should be quickly and gently pipetted into the molds. The melanin solution replaced the 25µL of water in different concentrations and the same process took place. The ratio of water and melanin was varied by adding 12.5µL of both water and melanin solution to the silk solution. This was done so the gradient effect
within the hydrogels could be analyzed and put into quantitative perspective. 25µL of melanin was added to the silk solution without HRP or H₂O₂ to allow for a control. This same process was also done with a solution of gold particles suspended in water. The hydrogels were left in a closed moist enviroment to reduce the amount of water evaporated for at least 36 hours. After the hydrogels are solidifed they are demolded and tested.

**Figure 16: Diagram of silk solutions seperated then mixed into the hydrogel mold.**

3.8. Characterization Methods

3.8.1. FTIR

The fourier transform infaired is used to understand the different bonds within the hydrogel. This quantitative analysis allows visual and numerical identification for the
differences between the hydrogels to differentiate the bonds occurring within them. This allows us to see if the melanin acts as both the catalyst and oxidator in the solution or if the crosslinking was due to hydrogen bonding or something unknown. This technique helps with understanding the phenomena of the melanin silk mixture.

3.8.2. X-Ray Diffraction

X-Ray Diffraction determines the crystallographic structure of a material by irradiating it with x-rays and then measuring the intensities and angles of the scattering after the x-ray hits the material. The intensities and angles are recorded then able to be plotted after the noise is reduced. The slope of the plotted data allows for the degree of crystalization to be seen. This method was used to test the distance between the beta-sheets and indicates if there are differences between the structure of the hydrogels with HRP and melanin combination and the hydrogels without HRP. In order to test this the Xeuss 3.0 was used, it can be seen in Figure 17.
This test allowed for the indication of hydrogen bonding or if the mechanical properties were different between the samples. While the hyrogel was still in a liquid state it was pipetted into a small capillary tube. This was then tested using WAX and SAX (wide angle x-ray and small-angle x-ray) in which the times were varried in order to reduce the amount of noise seen in the results.
3.8.3. *BioSoft*

BioSoft is a nanoindenter which tests the material on the nano-level. The Bruker BioSoft uses a point or a hole punch to puncture the material. This instrument allows us to measure the regional mechanical strength of the material.

The nano-indenter acts similarly to a compression test; a material is placed on a plate and a metal beam pushes into the material. The difference between a macro compression test and the nanoindenter is the side of the beam. In this experiment a titanium beam with a diamond tip was used. Because it is a compression test, the machine
uses relative positions so it is not necessary to calibrate, however multiple tests on the same material are run in similar areas to get an accurate result. This equipment was used with a larger hydrogel without the presences of the catalyst or oxidator. The melanin solution was injected in a singular point within the hydrogel while in a mold in the liquid state. This was not mixed and was left to set for 2 days allowing the hydrogel to become a solid after cross-linking. The hydrogel was put onto the indenter and tested in multiple different areas across the hydrogel to see the differences between where the melanin is present and where it was not. The hydrogel is in Figure 18 was made by adding the melanin solution (66µL) to 200µL of silk solution. This hydrogel was made without HRP or H₂O₂.

Figure 19: Silk incorporated with melanin hydrogel to be tested on BioSoft.
3.8.4. Micro-Tester (Micro-Squisher):

The micro-squisher was used to find the mechanical strength over the hydrogel as a whole. The mico-tester works similarly to the nano-indenter in the sense that it is a compression test.

Figure 20: CellScale squishing test being run on a silk hydrogel without melanin.

Figure 21: CellScale squishing test running on a silk hydrogel incorporated with melanin.
The CellScale Microsquisher uses a beam with a plate on the end in order to perform a compression test on the material. The hydrogels were loaded onto the microsquisher and then compressed for 10 seconds, with a 2 second hold, and then recovered for 10 seconds until the beam was back to the starting position. The force and the distance the beam moved were recorded along with the diameter of the hydrogel.
4. Results and Discussion

4.1. FTIR

As stated before, the FTIR provides a quantitative analysis allowing visual and numerical identification for the differences between the hydrogels to differentiate the bonds occurring within them. This technique helps to understand the phenomena of the melanin silk mixture. And proves whether or not the melanin is changing the chemical structure of the hydrogels.

Figure 22: FTIR of silk hydrogel composed of 75% silk solution made with HRP and $\text{H}_2\text{O}_2$. 
The FTIR of the control (75% silk solution) hydrogel showed that there are many peaks which were further investigated such as the two peaks noted in the figure and the two characteristic peaks between 1300-1500 cm\(^{-1}\) which supply the background for differentiating between hydrogels and the cross-linking causation from \(\beta\)-sheet formation consistent with the presence of HRP and H\(_2\)O\(_2\), or if there is another reason the hydrogels with melanin formed hydrogels.

Figure 23: FTIR of 12% melanin solution hydrogel made the standard way with HRP and H\(_2\)O\(_2\).
Figure 24: FTIR of 25% melanin solution hydrogel made in the presence of HRP and H$_2$O$_2$. 
Figure 25: FTIR of 25% melanin solution hydrogel without the presence of HRP
and H₂O₂.

Figure 26: FTIR of 25% heated melanin solution hydrogel without the presence of
HRP and H₂O₂.
Figure 27: Comparison of the FTIR from each sample of hydrogel.

In the comparison of the FTIR data, each peak starts at a different spot, however there are some peaks that exist in the same place. We can see the important peaks at certain wavelengths in Figure 27. The peaks further discussed are those at: 3300, 3060, 2400, 1650, 1530, and 1250 cm\(^{-1}\). When the data is shown on the same graph, there were
differences seen. One noticeable difference is between the 2000-2500 cm\(^{-1}\) region where typically triple bonds appear. An inverted peak in this area means CO\(_2\) is present and this explains why it is inverted because instead of transmittance, it shows as absorbance. This is typically due to the atmosphere around the FTIR and could simply be from someone breathing on it.

The differences between the FTIR peaks can be explained by instrument testing. Most of the peaks appear to be in the same position as the control hydrogel. The two large peaks between 1700-1500 cm\(^{-1}\) show the \(\beta\)-sheet formation in silk fibroin. The right shift seen in the first of the two peaks in the same area is due to the tyrosinase, this was why the heated melanin was shifted back over to the left because the enzyme is no longer active. The heated melanin peaks between 1700-1500 cm\(^{-1}\) look similar to the control. A peak at 1250 cm\(^{-1}\) was seen which shows an \(\alpha\)-bond. An \(\alpha\)-bond is an indication of a gaussian chain/coil or a structural shift in the molecular bonding.
Figure 28: FTIR of Silk Hydrogels wavelength between 1400 and 1000.
Figure 29: FTIR 25% melanin with presence of HRP, with a wavelength between 1400 and 1000.
There were three samples for this test; a hydrogel without melanin, a hydrogel with HRP, and a hydrogel with just silk solution and melanin. The results from the Xeuss 3.0 WAX was unable to detect much of a change. The only detection that occurred in all three samples was that between 1-5 Angstroms there was indication of β-sheet formations.
formed in all three samples. However there were differing SAX results.

Figure 31: All three samples of silk hydrogel plotted in order to see the slope.
Figure 32: Comparison of all SAX slopes.

From Figure 32, it is seen that the slope changed from -3 to -2 for the hydrogels with melanin present. All the tubes were the same diameter so they are able to be compared over the x-axis. This indicates the presence of a gaussian chain/coil. This is not present in liquid melanin nor in silk solution. These gaussian chains form from the hydrogen bonding and the eumelanin structures folding back onto themselves and continuing to fold. This can also be seen in the results from the FTIR at the 1250 wavelength.

4.3. Nano-indenter

The Nano-indenter measures the regional mechanical strength of the material. The regional strength is tested to identify if the silk hydrogel incorporated with melanin has different mechanical strength depending on the concentration of melanin within the
The results give us a better understanding as to if the material is not only stronger on the macro-level, but also stronger on the nano-level. The results from the BioSoft Nano-indenter showed that although the entire hydrogel gelled from the rapid progression of β-sheet formations which cascades through the hydrogel once they are formed. There was a difference seen throughout the hydrogel. The areas with melanin had a modulus higher than the areas which are more white as seen in Figure 33. In order to run a test on the nano-indenter a thicker hydrogel was necessary, because the light from the microscope produced too much heat; causing the hydrogels to dry and crack preventing them from being tested. Nevertheless, result shows that the regional strength of the material was increased with the addition of melanin.

Figure 33: Load and test times for multiple tests run on the nano-indenter.
Table 3: Tests run on nano-indenteter with elastic modules, stiffness, and uncertainty.

<table>
<thead>
<tr>
<th>Test File Name</th>
<th>Elastic Modulus (kPa)</th>
<th>Elastic Modulus Uncertainty (kPa)</th>
<th>Adjusted R Squared</th>
<th>Stiffness (µN/µm)</th>
<th>Stiffness Uncertainty (µN/µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>low melanine.tdm</td>
<td>6.156</td>
<td>0.03324</td>
<td>0.9983</td>
<td>79.27</td>
<td>0.428</td>
</tr>
<tr>
<td>increased melanine 1.tdm</td>
<td>7.789</td>
<td>0.07827</td>
<td>0.9944</td>
<td>100.3</td>
<td>1.008</td>
</tr>
<tr>
<td>increased melanine 2.tdm</td>
<td>13.48</td>
<td>0.5371</td>
<td>0.9893</td>
<td>173.6</td>
<td>6.915</td>
</tr>
<tr>
<td>increased melanine 3.tdm</td>
<td>7.734</td>
<td>0.173</td>
<td>0.9947</td>
<td>99.59</td>
<td>2.227</td>
</tr>
<tr>
<td>increased melanine 6.tdm</td>
<td>22.37</td>
<td>0.1072</td>
<td>0.9987</td>
<td>288.1</td>
<td>1.381</td>
</tr>
<tr>
<td>increased melanine 7.tdm</td>
<td>13.98</td>
<td>0.1666</td>
<td>0.9921</td>
<td>180</td>
<td>2.145</td>
</tr>
<tr>
<td>increased melanine 8.tdm</td>
<td>13.41</td>
<td>0.178</td>
<td>0.9987</td>
<td>172.6</td>
<td>2.292</td>
</tr>
<tr>
<td>increased melanine 9.tdm</td>
<td>5.504</td>
<td>0.0485</td>
<td>0.9958</td>
<td>70.87</td>
<td>0.6244</td>
</tr>
<tr>
<td>increased melanine 10.tdm</td>
<td>1.962</td>
<td>0.04764</td>
<td>0.9672</td>
<td>25.27</td>
<td>0.6159</td>
</tr>
</tbody>
</table>

The results were interpreted and the stiffness and elastic modulus were similar in all of the areas tested. Then the assumption could be made that the beta sheets with and without melanin were the same. In this test the melanin concentration was changing and so was the regional mechanical strength. This means that depending on the concentration of melanin in the area, the regional mechanical strength varies.

4.4. Micro-Squisher

After the test is run, the data files were saved and imported into an excel file to calculate both stress and strain. The stress is calculated by using the equation:

$$\sigma = \left(\frac{\text{current size}}{106}\right) \left(\frac{\text{diameter}}{1000}\right)^2 \times 2 \times \pi \times P_i.$$
The Strain is calculated by the equation: \( \varepsilon = \frac{\text{current size} - \text{starting size}}{\text{starting size}} \). From these values the overall Young’s modulus was calculated by using the least squares method for both the stress and strain. In excel it is a function called LINEST(), highlighting the linear region up until the hold, determining the Young’s Modulus for the hydrogel. This was then put into JMP(SAS Institute) for statistical analysis.

4.5. Mathematical Model

The mathematical model is a linear representation of what would be expected if more melanin was added. This prediction is assuming that there is no interference between

![Figure 34: Model of projections of Young’s Modulus with the addition of melanin.](image)
the HRP and the melanin. However, one of the reasons that the model is skewed is due to the presence of both HRP and melanin in the solution. The upper bound is found by using the Kevin-Voigt Model which is the upper bound elastic modulus prediction for the law of mixtures. In order to predict this behavior, the material type that was chosen most similarly to the hydrogel was that of a foam. This is because when the hydrogel is formed the mixtures are fully incorporated and mixed together and cross-linked to one another. By using the area of a cylinder, and knowing that the material is acting as a solid throughout with pours, the equation $F = E \varepsilon A$ is used. Where $F$ is the force applied, $E$ is the elastic modulus, $\varepsilon$ is strain, and $A$ is the area of the cylinder. By taking the average force, strain, and area of the test we can predict the upper bound Young’s Modulus for the mathematical model.

4.6. Statistical Analysis

All the statistical analysis was run on JMP software using a 95% confidence interval with at least 5 samples run for each variable.
4.6.1. Concentration of Melanin

Figure 35: One Way Analysis of Young’s Modulus and Concentration of Melanin

The One Way Analysis seen in Figure 35 shows that the silk solution without melanin has a much lower Young’s Modulus.

![Figure 35](image)

Figure 36: Analysis of Varience of Concentration of Melanin and Young’s Modulus.

The Analysis of varience can be seen in Figure 36, showing that one of the three levels was not the same as the other. The p-value was less than .001 showing significant statistical differences. In Figure 36 it can be seen that the 75% silk solution has a
different letter next to it, meaning that it was significantly different than the other two.

Figure 37: Connecting Letters Report and Order Differences Report for Concentration of Melanin and Young’s Modulus.

The Order Difference Report adds to the confirmation that the silk without melanin had a much lower Young’s Modulus after interpretation. Both p-values comparing the 75% silk solution to both the 75% silk solution with 25% and 12.5% melanin solution were less than .05, however there was no difference between the 12.5% and 25% melanin solutions as seen by the connecting letters report (represented by the same letter) and the p-Value greater than .05 in the order difference report.
4.6.2. Presence of HRP and $H_2O_2$

As seen in Figure 38 the presence of HRP and $H_2O_2$ affects the Young’s Modulus. This can be further analyzed by looking into Figure 39 which shows the analysis of variance.
Figure 39: Analysis of Variance for presence of HRP and H$_2$O$_2$ with Young’s Modulus.

The analysis of variance shows that there is a difference in the Young’s Modulus, which means that the presence of HRP and H$_2$O$_2$ have an effect on the strength of the hydrogel.

Figure 40: Connection Letters Report and Ordered Differences Report for presence of HRP and H$_2$O$_2$.

As seen in Figure 40 the connecting letters report shows that the presence of HRP and H$_2$O$_2$ again was significantly different. This was represented by both the different connecting letters and the p-Value were less than .05. Interpreted this means that the presence of both HRP and H$_2$O$_2$ reduced the Young’s Modulus. The result however
included the control which is the silk hydrogel without melanin as well. In order to investigate further, the hydrogels without the addition was reanalyzed.

Figure 41: One Way Analysis of the presence of HRP and H$_2$O$_2$ and Young’s Modulus without control.

As seen in Figure 41, there was a difference between the hydrogels in the presence of HRP and H$_2$O$_2$. This was further analyzed by the analysis of variance seen in Figure 42.
The analysis of variance shows again that there was a significant difference between the hydrogels that were made in the presence of HRP and H$_2$O$_2$. The conclusion can be made because the p-Value was less than .05.

As seen in Figure 43, the connecting letters report shows that the presence of HRP and H$_2$O$_2$ was significantly different represented by two different letters. This was also confirmed by the ordered differences report with the p-Value being less than .05. Interpreted this means that the melanin incorporated hydrogels are proven to have a higher young’s modulus when the solution consists of the melanin solution and the silk
solution without the presence of HRP and \( \text{H}_2\text{O}_2 \) which is typically necessary to crosslink the silk hydrogels. A hypothesis was that the melanin particles were causing shear stress while being pipetted. In order to test this a solution of 75% silk solution and 25% gold particle suspended in water solution was tested. The gold particle solution did not gel in the presence of the silk concluding that the particles were not causing shear stress on the silk solution when it was pipetted.

4.6.3. Heated Melanin Solution

In order to make sure that the tyrosinase present in the melanin was not causing a reaction to form the hydrogels, the melanin solution was heated. Figure 43 shows the one-way analysis of heated melanin and young’s modulus.

![Figure 44: One Way Analysis of the heated melanin and Young’s Modulus.](image)
The one-way analysis looks as though there could have been a difference, however this needs to be further investigated by looking into the analysis of variance which can be seen in Figure 45.

![Analysis of Variance Table]

**Figure 45: Analysis of Variance for heated and non-heated melanin solution with Young’s Modulus without control.**

The analysis of variance shows that the probability is 0.0637 which is higher than .05, which means that it is not significantly different. This can also be seen in the connecting letters report and ordered difference report.

![Connecting Letters Report]

**Figure 46: Connection Letters Report and Ordered Differences Report for heated melanin and young’s modulus.**

The connecting letters report was represented by the same letter meaning that there is no significant difference between if the melanin solution is heated or not heated. Interpreted this means that although heating the melanin solution does inactivate the
enzyme, it cannot be stated that the enzyme is affecting the strength of the hydrogel.

Although the strength of the hydrogel was not significantly different between the heated and non-heated young’s modulus, there was a significant change in the optical characterization. The hydrogels that have been heated appear to be shiny and reflective while the melanized hydrogels that were not heat treated were dull. This characterization can be seen in Figure 47.

![Figure 47: Melanin hydrogels, the hydrogel on the right is incorporated with heat treated melanin solution while the one on the left is melanin solution.](image)

The initial thought was that the melanin particles were blocking the pores forcing the melanin hydrogels to retain their water and because water is incompressable it was
allowing the hydrogel to be stiffer than the non-melanin silk hydorgels. More hydrogels were made and instead of the addition of the melanin water solution, a gold water solution was added instead without the addition of HRP. The gold-silk solution did not gel which means that particle blocking was not the cause of the strengthened hydrogel or the reasoning for the formation of hydrogels without the presence of the cataylst. Another explaination that the melanin solution and the silk solution were geling without the catalyst could be that the enzyme tyrosinase that is still leftover in the melanin solution might be reacting with the silk solution. In order to rule this out, some of the melanin solution was heated to 90°C for 10 minutes. This denatures the enzyme which caused it to be inactive. The heated melanin solution was added to the silk solution and pipetted into the mold, and after two days it gelled. This was also tested on the microindenter and was just as strong.
5. Conclusion

This research was done to understand and test if the melanin would be able to enhance the properties and characteristics of a silk hydrogel. The result clearly show that the melanin was able to increase the mechanical strength of the hydrogels. This confirms that our initial hypothesis was true that melanin improves the mechanical strength of the material. However the statistical analysis showed that the presence of HRP in the hydrogels that contained melanin resulted in a lower Young’s Modulus compared to the ones without HRP. The silk solution was mixed without the HRP in order to have a control, but it ended up cross-linking and creating a hydrogel. This hydrogel was also compressed using the microsquisher. The hydrogel without HRP had a higher young’s modulus, than the hydrogel with HRP, meaning that interference or weaker chemical bonds were occurring before stronger ones could.

The interpretation is that the melanin must be causing the cross-linking to occur. The explanation as to why the hydrogels with melanin are stronger is due to the melanin hydrogen bonding with the silk solution. Hydrogen bonds are known to be very strong and due to the structural changes within the silk solution, the cascade of β-sheet formations begins. However when the HRP and melanin are in the same hydrogel, the HRP catalyzes β-sheet formations so quickly that the hydrogen bonds are unable to occur, resulting in a weaker hydrogel comparatively.

In conclusion, we can see from the FTIR results that hydrogen bonds are formed from the melanin and in the presence of HRP the melanin bonding happens slower than
the HRP binding preventing the stronger bonds from forming. Both the Nano-indenter and the micro-squisher show that the melanin improves the mechanical strength on both the micro and nano level compared to the silk hydrogel without melanin. Lastly, the melanin solution that was heated had denatured tyrosinase that proves that although it did not have a change in mechanical strength, its presence changes the qualitative data.

Further examination into the UV blocking properties, crystalization, and interactions with cells found in the skin is recommended.
6. References


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