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Optimization of Conditions for Production of Mechanically Strong Suckerin Protein Hydrogels

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Honors Research Project

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Honors Abstract Addendum

Hydrogels are polymer networks with a large water content. Uses include biomedical applications like drug delivery, wound dressing, and more. Current natural hydrogels lack mechanical robustness. Squid ringed teeth (completely protein) have a modulus comparable to thermoplastics; adding this to hydrogels could improve modulus while maintaining biocompatibility.

Three experiments were performed: Hofmeister solubility study, SRT powder additive, and SRT aqueous solution. The first showed that LiCl solution without acetic acid dissolved the most SRT; overall LiCl dissolved more than all others. SRT as a powder additive increased the modulus of the hydrogels, however the error in the measurements make it difficult to make definitive conclusions. The addition of powdered SRT increases the modulus of gelatin hydrogels ~6 to ~8 kPa. The SRT solution studies showed that increasing the SRT content and longer setting time increase modulus of the gels, however the error is large and definite conclusions cannot be made.

Multiple things could be investigated to draw stronger conclusions. Investigation of LiCl and other salts to increase SRT solubility may be of interest. Redesigning the indentation method for determining modulus would be advantageous. The current method involves large error; an automated method may provide more consistent results.

Executive Summary

Purpose

Hydrogels are synthetic and/or natural polymer networks with a large water content. Hydrogels have many uses, including biomedical applications such as drug delivery, wound dressing, tissue engineering and many more. Natural hydrogels (i.e. protein hydrogels) are of interest for their biocompatibility and biodegradability. However, these materials lack mechanical robustness. This study investigates adding a type of protein found in the squid ring teeth (suckerin proteins) and its effect on the elastic modulus of hydrogels. Previous studies have shown that squid ring teeth (SRT) proteins have unique properties that may be useful for this application. The suckerin proteins exhibit thermoplastic properties, meaning they can be melted, molded, and harden without losing mechanical strength. SRT are also completely compromised of proteins, without mineral phases or other substances that strengthen similar biological structures. Additionally, SRT have an elastic modulus that rivals current synthetic thermoplastics. Because of the unique properties SRT have, they make a great candidate for an additive to gelatin hydrogels to increase mechanical strength while maintaining biocompatibility.

Experiment Descriptions and Results

Three types of experiments were performed in this study. First, the solubility of SRT in aqueous acetic acid solution with the addition of salts (CsCl and LiCl) was investigated. This experiment aimed to utilize the Hofmeister effect, which describes how the addition of salts to protein solutions can modify the solubility of the proteins. The second type of experiment involved adding SRT as a powder additive to the aqueous gelatin solution. Third, SRT was dissolved in an aqueous acetic acid solution and then mixed with an aqueous gelatin solution to form the gels.

The Hofmeister solubility experiment showed that the addition of LiCl helped to improve the solubility of SRT in all concentrations of acetic acid (0, 5, and 10wt%) compared to CsCl and no salt. The solution that dissolved the greatest portion of SRT was the 100 mM LiCl solution with no acetic acid,

which was not expected; acetic acid generally helps dissolve SRT. It should be noted, however, that the trials with no acetic acid were run only once compared to three times with the others. All samples using 100 mM CsCl showed less SRT dissolved in solution even compared ti SRT in the solution with no acetic acid or salt.

This study also showed that SRT as a powder additive does increase the modulus of the hydrogels, however the error in the measurements is too great to draw any other trends from the data. The control had an average elastic modulus of 11.35 kPa, while the 1.0wt% and 2.3wt% SRT gels' average moduli were 17.88 kPa and 21.46 kPa, respectively. The issue comes with the error; both the 1.0wt% and 2.3wt% SRT gels have a standard deviation of about 4 kPa, while that of the control is only about 1 kPa. While is does appear that SRT increases the modulus of the hydrogels, it cannot be said that more SRT means a higher elastic modulus from these results. It can be said with moderate certainty that the addition of SRT has the ability to increase the modulus of gelatin hydrogels roughly 6 to 8 kPa.

The experiments involving mixing two solutions (one SRT, one gelatin) showed similar results as the powder additive experiment. For one study, the addition of SRT to the gels did increase the modulus; the control's average modulus was 11.35 kPa, 1.0wt% SRT was 18.37 kPa, and 2.3wt% SRT was 21.61 kPa. However, the error of the 2.3wt% modulus measurements was high: about 5 kPa. Because of this, the errors of the 1.0wt% and 2.3wt% moduli overlap so it cannot be concluded that more SRT increases elastic modulus. Later, a study was done testing the hydrogels twice after two and nine days of gelation to determine the relationship between time and modulus. The modulus of all the samples increased from two to nine days, but not all increased the same amount. The control's modulus increased by 29.4%, 1.0wt% by 75.1%, and 2.3wt% by 12.6%. After two days, the 2.3wt% sample had the highest average modulus with 12.28 kPa, but after nine days the 1.0wt% SRT samples had the highest with 18.88 kPa. As with the other experiments, the error is too high to make definitive conclusions about the effect of SRT wt% in the hydrogels and modulus.

Recommendations

The results from this project show that the addition of SRT (as a powder or solution) to gelatin hydrogels does increase the elastic modulus, but the error in the measurements prevents one from correlating amount of SRT to modulus. The main issues that still remain include low solubility of SRT in solution, error in elastic modulus experiments, and homogeneity of the gels. More work could be completed using LiCl or other salts to further increase the solubility of SRT, with more emphasis on samples without acetic acid. Also, the current method of measuring elastic modulus could use improvement to try and decrease the error of the measurements. This could be done a number of ways, including designing a sturdier indenter set-up, mechanically operating the indenter with automation for more consistency, and/or using a larger sample size to obtain a flatter surface to perform the test on (See more in the Error Discussion portion of the Analysis and Discussion section). Homogeneity will likely be improved once SRT solubility is improved, but more should be investigated to overcome this issue.

Broader Implications

This project allowed me, as an undergraduate student, to learn more about what goes into technical research, gain confidence in drawing conclusions and analyzing data, and learn new lab and imaging skills. I also learned how to better manage my time and how to push a project forward when things do not go as planned. This SRT hydrogel project also allowed me to contribute to finding better products and solutions for people receiving medical treatments in the future. Investigating using a natural polymer to increase the mechanical strength of hydrogels could result in less invasive and less harmful devices and products for the biomedical market. If the body can recognize and break down all the materials used in the hydrogels, it would likely reduce the risk of rejection, infection and speed recovery time.

Introduction

Hydrogels are polymer networks that contain a large amount of water [1], put simply. These networks do not dissolve in the water because of chemical and/or physical crosslinks among the polymer chains [1, 2]. Hydrogels are becoming more of interest because of their potential in the medical field, especially since traditional synthetic polymers are usually hydrophobic, making them more difficult for use in the body [1]. Hydrogels have the potential to be used in many biomedical applications, including drug delivery, implants, biosensors, tissue engineering scaffolds, wound healing, and regenerative medicine and even non-medical applications such as additives to foods [1, 2, 3].

Hydrogels may be compromised of synthetic polymers, natural polymers, or a combination of both [1]. Using natural polymers, like proteins, is advantageous because of the inherent biocompatibility, biodegradability, and biologically recognizability [1]. One of the issues with current hydrogels made of natural polymers is that they do not have adequate mechanical strength to handle some of the desired applications [1]. Finding a protein to use in the polymer matrix in these hydrogels is advantageous because of its biodegradability, biocompatibility, natural biochemical and mechanical properties, along with ease of large-scale production through recombinant DNA synthesis. [3] Additionally, proteins undergo “hierarchical self-organization” and can be mimicked outside a cellular environment. They also often contain domains that assist living cells in signaling their interactions among other proteins and ligands. [3] It has not yet been proven, but the hope is that the proteins in hydrogel form would retain this function when mixed with other types of proteins and synthetic polymers to enhance the properties of the gel. [3]

One such protein that has promising properties and has been in the spotlight of this area of research recently is a protein found in a surprising place: the ringed teeth lining the tentacles of squid and cuttlefish. Squid ring teeth (SRT) are found in the tentacles and arms of Decapodiform cephalopods. Each sucker on each tentacle contains one embedded SRT, helpful for capturing and handling prey. [4] These teeth must be able to withstand large shearing and compressive forces [4], which on its own is not surprising. The interesting part is that the SRT are made up entirely of proteins, with no mineral phases, covalent cross links, or chitin as often found in similar biological structures. [4,5] Since the SRT are entirely compromised of proteins, known as “suckerins”, it makes a great candidate for an additive to hydrogels to increase the mechanical strength.

This work investigates the addition of SRT in various ways to gelatin hydrogels. The hydrogels are tested for mechanical modulus via contact mechanics using the Johnson-Kendall-Roberts model [13]. To do this, an indenter is brought down to contact the hydrogel to apply pressure. During the procedure, the force applied to the gel and the distance the gel is indented is recorded. The force is measured by performing the test on a balance and the distance is recorded by taking photos throughout the test. The mathematical models will be explained later in this paper. Some gels are freeze dried in order to perform SEM imaging to determine any structural differences among the samples and investigate morphology.

This work proceeds other work done in this laboratory involving photo crosslinking and pH experiments. [6,7] The findings from those projects helped guide and influence the work done here. It is the hope of the research team that these findings will help us increase the strength of hydrogels while maintaining biocompatibility for the benefit of medical applications. The following includes a

background of the work done, methodology and procedures, data and results, analysis of the results, and conclusions and recommendations.

Background

Hydrogels

Hydrogels are defined in various ways and are often easier to identify rather than define. They are neither completely solid nor liquid, but somewhere in between. [8] Like a solid, they do not flow, but molecules can diffuse through it, like a liquid. [8] Hydrogels are 3-dimentional, crosslinked, hydrophilic polymer networks that absorb and retain large amounts of water, because of hydrophilic groups on the backbone of the polymer, without dissolving. [7, 8] Another definition from [7] is "two or multi-component systems consisting of a 3D network of polymer chains and water that fills the space between macromolecules." Crosslinking among the chains provides mechanical strength and aids in keeping the polymer matrix insoluble in water. [8] One of the main types of natural hydrogels, which is investigated in this project, is made from collagen protein. Collagen protein can be found in the skin, bones, and connective tissue of animals and humans.

Hydrogels may be compromised of physical and/or chemical bonds. Physical interactions include hydrogen bonds, electrostatic forces, hydrophobic interactions, and chain entanglements. [8] Physical interacts are weaker than chemical bonds and reversible. [8] An example of a hydrogel compromised of physical interactions is Jell-O. [8] Chemical interactions are permanent, covalent bonds among the polymer chains that are not physically reversible. [8] Hydrogels may also be made up of natural and/or synthetic polymers. [8] Natural polymers include proteins and polysaccharides, which are biocompatible and biodegradable. [8] Synthetic polymers and monomers may also be used in hydrogels to tune and control specific properties, have longer functional life, higher water absorption, and a wide variety of chemical resources. [7, 8] However, synthetic polymers run the risk of not being biocompatible/biodegradable and could be toxic or have other adverse effects to the body. [8]

Hydrogels made from natural polymers have many advantages, including biocompatibility, biodegradability, and being bioabsorbable, may be injected in vivo as liquid to gel in the body, adequate transport properties, time release of medication or nutrients, easily modified [8], and a degree of flexibility similar to natural tissue. [7] Current and potential uses of hydrogels are seemingly endless and include such things as contact lenses, disposable diapers, drug delivery, wound dressing, part of EEG and ECG medical electrodes, blood compatible surface for medical devices, scaffolds in tissue engineering [8], hygienic products, agriculture, sealing, coal dewatering, artificial snow, food additives, pharmaceuticals, various biomedical applications, tissue engineering and regenerative medicines, diagnostics, separation of biomolecules or cells, barrier materials to regulate biological adhesions, and biosensors. [7]

Although hydrogels have great potential, there are still issues that need overcome. Some of the current issues is the high cost of manufacturing, difficulty loading with nutrients or medications, difficulty sterilizing, non-adherence, and low mechanical strength. [8] These and other problems must be overcome before hydrogels may be used to their full potential.

Squid Ring Teeth

An interesting source of possible additives comes from an unexpected place: the sucker ring teeth lining the tentacles of squid and cuttlefish. [10] These teeth all have a basal ring with a series of pointed dentitions, which is why they are referred to as “teeth”. In each sucker along the tentacles and arms there is one ringed tooth structure, usually a few millimeters in size. The sucker ring teeth (SRT) are used for grappling and handling prey, so the teeth undergo relatively large sheer and compression forces. [9] SRT are sharp; they are sharp enough to lacerate a human arm. [9] Because of the design of the teeth, the more the prey struggles, the more shear force is applied from the teeth so they have little chance of escape. [9]

Figure 1: Image of the muscular arms and tentacles of a squid whose ringed teeth were harvested for this study.

Figure 2: Image of the sucker along the tentacles of a squid, each of which have a ringed tooth embedded.

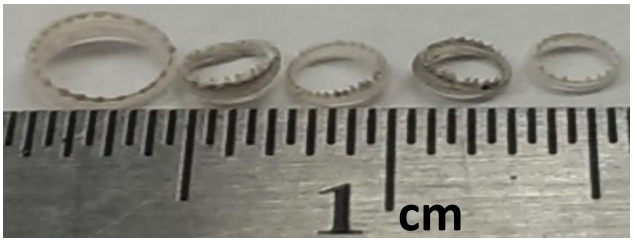

Figure 3: Squid ring teeth used for this study; ruler shown for scale.

SRT are unique in their structure and composition. They are naturally occurring block-copolymers, and they are solely compromised of protein. Normally, tough biological structures like these have mineral phases or chitin which helps to strengthen the network and provide increased modulus. However, SRT do not have any mineral phases. Mineral phases can be found in structures like bones, mollusk shells, sponge spicules, and echinoderm ossicles. [9] Instead, the SRT are completely compromised of protein. Natural and synthetic polymers (protein is a natural polymer) are normally stabilized by chain entanglements (amorphous thermoplastic polymers), dense covalent interchain cross-linking (like in thermoset resins, insect exoskeletons, and squid beaks) or the addition of stiffer reinforcement phases like minerals in bone and other biomaterials. [10] Again, SRT's make up does not involve any of these. Instead, the suckerin proteins include nanoconformed beta-sheet-reinforced* polymer networks, found in other biological materials for robustness. [10]

The presence and arrangement of the beta-sheets in the SRT are a result of the primary amino acid sequence. The amino acid composition of the suckerin proteins are mostly Gly (37 mol%), Tyr (14%), His (13%), and smaller amounts of Leu, Ala, Thr, Ser, and Val. [9, 10] It is estimated that the beta-sheets are roughly 5 strands wide and 8-10 amino acids in length, equating about 2.4-2.6 nm in the H-bond direction and 3-3.5 nm along the protein sequence. The beta-sheets of the SRT appear to have a regulated size, yet they seem to be randomly placed throughout the amorphous domain. [10] Other studies have shown that beta-sheets of similar dimensions provide strength and toughness from the presence of H-bonds. [10] Other things noted from x-ray spectroscopy analysis include: uniform distribution of Cl and S, no metals, no mineralization, and no semi-crystalline organic constituents (including chitin). [10] According to Guerette, an "amorphous halo" was detected, which suggests an overall semicrystalline structure of amorphous domains strengthened by seemingly randomly oriented beta-sheets.

The microstructure of the natural SRT is also something of interest and plays a role in their strength and rigidity. SEM images show that the proteins form tubular structures that run parallel to the teeth protrusions. This structure helps contribute to the bending stiffness of the teeth, important when there are large bending and shearing forces. The channels are roughly 215 nm in diameter and run through the tooth and basal ring sections. The outer surfaces of the teeth have filled channels and lack any detectable porosity. The channels do not appear to be arranged in any spatial order, such as hexagonally. Also, the pore fraction is not constant throughout the tooth; some areas have a denser protein concentration than others. There is a decrease in pore fraction from tooth core to the outer surface. Along with that comes a decrease in channel diameter and increase in channel spacing. This phenomenon is seen in other natural materials, like wood, trabecular bone, and the stems of plants. However, the pore fraction is much lower in SRT than these other materials (20% compared to 80-90%). [9] Guerette believes that the SRT's impressive characteristics come from their microstructure (channels, porosity gradient) rather than the biochemical or elemental gradients. [9]

The elastic modulus and hardness of the SRT vary from dry to hydrated and from tooth periphery to core. When dry, the peak modulus is 7-7.5 GPa and hardness is 0.7 GPa, near the denser tooth periphery. The minimum modulus is 4.5-5 GPa and hardness is 0.4 GPa at the core of the teeth. Those values are lowered when the SRT are in a hydrated state. The hydrated tooth periphery has a modulus of 2.75 GPa and hardness of 0.25 GPa, while the hydrated core has a modulus of 1.75 GPa and 0.15 GPa hardness. There is roughly a three-fold decrease in modulus and hardness between the dry and hydrated states. [9] Similarly, Guerette reports the modulus of dry SRT is 6-8 GPa and hydrated is 2-4

GPa. Guerette also investigated the modulus over time and with ethanol and urea treated samples. If the modulus is lowered by the presence of ethanol, that suggests that much of the interactions in the SRT are hydrophobic, and if urea decreases the modulus, that suggests H-bonds are heavily involved in the strength and stability of the SRT. Ethanol treatment did not significantly affect the modulus, however the urea treatment lowered the modulus to about 20 MPa. This suggests that hydrogen bonds play a very large role in the structure of the SRT and hydrophobic interactions are minimal. [10]

SRT are unique and have properties not found in other natural, mechanically robust materials. Since they are completely made of proteins, production of the suckerin proteins would be relatively easy with recombinant protein production, an already well-known method. Along with that, SRT would likely be biocompatible, meaning the body would recognize the proteins. The suckerin proteins would also be biodegradable and the manufacturing process would be environmentally friendly. Beyond that, SRT has strength and thermoplastic behavior that rivals those of current thermoset polymers. The thermoplastic behavior means that the SRT can be melted, reshaped, and hardened again with losing only minimal physical properties. [10] The addition of SRT (from the natural source or recombinant production) to polymers could help the environment by providing a cleaner, biodegradable product and be safe for use in medical devices and implants.

*Beta-sheets are a secondary structure of proteins. This means that the side chains along the amino acid sequence of the protein interact with each other, in this case through hydrogen bonds. Beta-sheets involve segments of protein that weave back and forth, interacting with hydrogen bonds, to form a sheet-like structure for reinforcement. [11]

Experimental Methods

This protein hydrogel research project can be broken up into 3 sections: harvesting and grinding the SRT, preparing the hydrogels, and determining the elastic modulus of the gels. The first of these involves purchasing squid tentacles, carefully removing the ring teeth, placing them in an ethanol solution for storage, and then grinding them into a powder and forming at SRT solution once the hydrogel is about to be prepared. The second portion is the most complex and where the variables will be adjusted during this project. A gelatin solution is prepared, the SRT solution is prepared with the ground up SRT, any additional additives incorporated into the gel, pH adjusted, and then placed into a well to set. Third, after the gels have set for a set period of time, the elastic modulus of the gels are determined by applying the JKR model, explained shortly.

Harvesting of the SRT from the tentacles of squid is done manually with tweezers. The SRT are dried and any excess tissue on the teeth are removed. Then, they are placed in an aqueous 30% ethanol solution for storage. Once the SRT solution is ready to be prepared, the SRT must be dried and ground up. Initially, the SRT would be patted dry with a paper towel and then ground with a mortar and pestle. This process was long and tiresome, and resulted in a large range of particle sizes of SRT. A better method was found: drying the SRT in a laboratory oven first, then grinding with a mortar and pestle. This resulted in a much finer powder and in less time. This method was used from then on for the SRT hydrogel experiments. Lastly, the SRT are dissolved in 5 wt% acetic acid (AA), as determined by Benekos [6]. The target concentration for this solution is usually 5 wt% SRT, however the solubility of the suckerin

protein varies from batch to batch, so the concentration of this solution is checked before each experiment.

Preparing the hydrogels is where much more manipulation comes into play. First, a total protein percent much be chosen for the gels in an experiment. This includes both the bovine/porcine gelatin and suckerin proteins. Then, the ratio of gelatin to suckerin proteins must be determined. From this, the desired concentration of gelatin can be prepared and warmed to melt the solid gelatin pellets into solution. Then, the pre-prepared suckerin protein solution and gelatin are added into a centrifuge tube, along with any other additives being investigated. One common additive is NaOH, used to adjust the pH. The pH may be monitored by using a pH probe before the gel sets. During this entire process, the centrifuge tubes are kept at 37°C in a warmer to avoid premature setting. Once all of the components have been added, the tubes are quickly vortexed to help maintain homogeneity and then pipetted into the wells that the gels will form in. The setting conditions of the gels vary; one includes putting a lid on the flat bottom wells and placing in a refrigerator or another involves placing the tray in ice water to set the gels quickly (again, for homogeneity) and then placing them in a refrigerator to preserve. For storage, another method used is vacuum sealing the gels and then putting them into the refrigerator to keep the moisture in the gels.

Lastly, the mechanical strength of the gels were characterized. Since the size of the hydrogels are relatively small, contact mechanics was chosen as the approach to measure the elastic modulus of the samples. The indentation approach was employed for this project, which involves using an indenter to apply force to the hydrogel, measuring the pressure from the applied force and the distance the sample is compressed. The Johnson-Kendall-Roberts (JKR) model was used.

The JKR model accounts for adhesion in the contact area between the indenter and the sample being compressed, which the Hertz model does not. The JKR model also relates the contact radius formed at the interface of the indenter and the sample (a), the radius of curvature of the indenter (R), the elastic modulus of the system (K), the applied load (P) and the energy release rate (W) in

(1) $a^3 = \frac{R}{K}\{P + 3W\pi R + [6W\pi RP + (3W\pi R)^2]^{1/2}\}$

The displacement of the hydrogel by the indenter, δ, and R can be related to a with the equation:

(2)$a_{Hertz}^2 = \delta R$

Equation (2) is a simplification based on the Hertz model: assuming that at a load of zero, the contact area, a, is zero. This approximation is the only part derived from the Hertz model. This assumption is then used in the JKR model. Hence, the parameter a can be related to P and δ via equation (3):

(3) $a = \left\{1 + \frac{3}{\frac{P}{\pi WR}} + \left[\frac{6}{\frac{P}{\pi WR}} + \left(\frac{3}{\frac{P}{\pi WR}}\right)^2\right]^{1/2}\right\}^{1/3} (\delta R)^{1/2}$

With algebraic manipulation, this equation can be arranged to:

(4) $\frac{a^{3/2}}{R} = \frac{1}{K}\left(\frac{P}{a^{3/2}}\right) + \left(\frac{6\pi W}{K}\right)^{1/2}$

Equation (4) is in slope-intercept form, so a linear plot of $\frac{a^{3/2}}{R}$ vs $\frac{P}{a^{3/2}}$ can be used to determine the value of 1/K, or simply K. Additionally, K is defined in equation (5), where v_s and v_i is the Poisson ratios of the surface of the hydrogel and the indenter, respectively, and E_s and E_i is the modulus of the surface of the hydrogel and the indenter, respectively.

(5) $\frac{1}{K} = \frac{3}{4}\left(\frac{1-v_s^2}{E_s} + \frac{1-v_i^2}{E_i}\right)$

The indenter to be used is made of glass, so the assumption can be made that E_i will be much greater than E_s, so the second term on the right-hand side of equation (5) can be neglected, resulting in an equation solving for E_s after some algebraic manipulation:

(6) $E_s = \frac{3K(1-v_s^2)}{4}$

This data is collected by placing the hydrogel sample on an analytical scale and slowly bringing down the indenter to contact the gel. Meanwhile, the applied load is measured by the scale and tracked on a computer and photos are taken with a camera set-up aimed at the gel to measure the δ at each applied force. This data is then plotted ($\frac{a^{3/2}}{R}$ vs $\frac{P}{a^{3/2}}$), a linear trendline calculated, average slope determined, and that slope is used to find K, then plugged into equation (6) to find the elastic modulus of the hydrogel (E_s). This process is repeated for each hydrogel for each experiment completed.

Data and Results

Solubility Experiment: Hoffmeister Effect

One of the issues from previous SRT hydrogel experiments in the lab was the poor solubility of the SRT in the aqueous acetic acid solution. In an attempt to increase the solubility of the proteins in solution, various salts were added to the SRT solutions at different concentrations, utilizing the properties of the Hoffmeister effect. The Hoffmeister effect describes the effects of ions on the solubility of proteins in solution; some salts (cations and anions paired) work to increase or decrease the solubility of proteins. [12] Proteins, including SRT, should be able to be "salted in" or "salted out" of solution with various salts. The salts chosen for this experiment were CsCl and LiCl.

100 mM solutions of each salt solution were created with various acetic acid concentrations. The solutions were prepared in centrifuge tubes, centrifuged at 4,000 RPM for 2 minutes twice, and the supernatant and left-over precipitate were used to quantify the SRT dissolved. **Figure 4** below shows the undissolved percentage of SRT for each salt solution tested, including a control with neither acetic acid nor a salt.

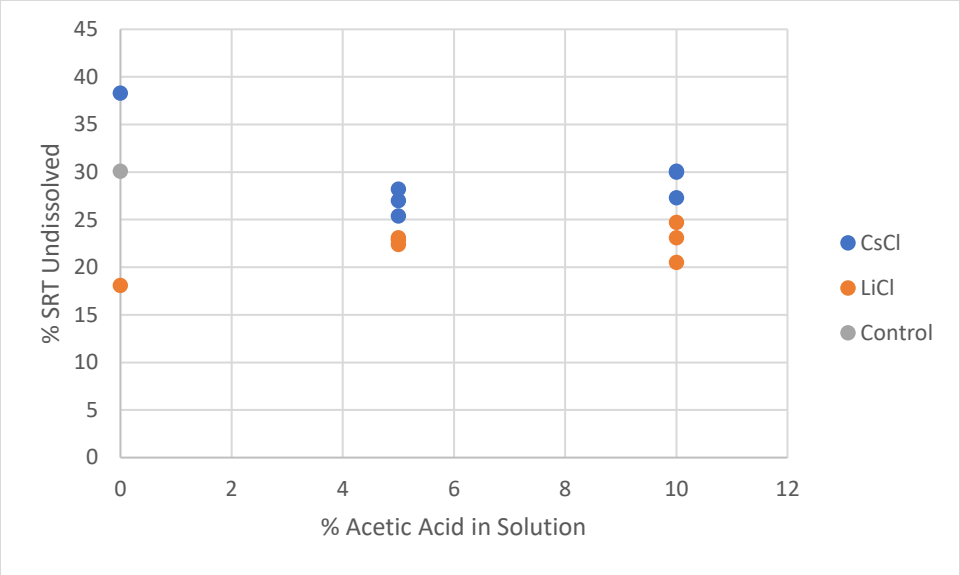

Figure 4: Results from Hofmeister ion experiment. All salt solutions are 100 mM. All solutions with LiCl show less SRT left undissolved.

SRT Concentration Study: Modulus Effect

The first experiment investigating the elastic modulus of hydrogels with SRT involved repeating a few of the samples from previous experiments: S31 and S34 from Ahmed Hussien. S31 is made up of a 50/50 by mass solution of 10wt% gelatin and ~6wt% SRT solutions. S34 is 75/25 of those solutions, respectively. S31 is about 2.3wt% SRT and S34 is about 1.0wt% SRT. These samples are reproduced throughout this project. **Figure 5** below shows the initial modulus data from this experiment. Note the standard deviation represented by the error bars in the graph. Here, the v value is set to ½.

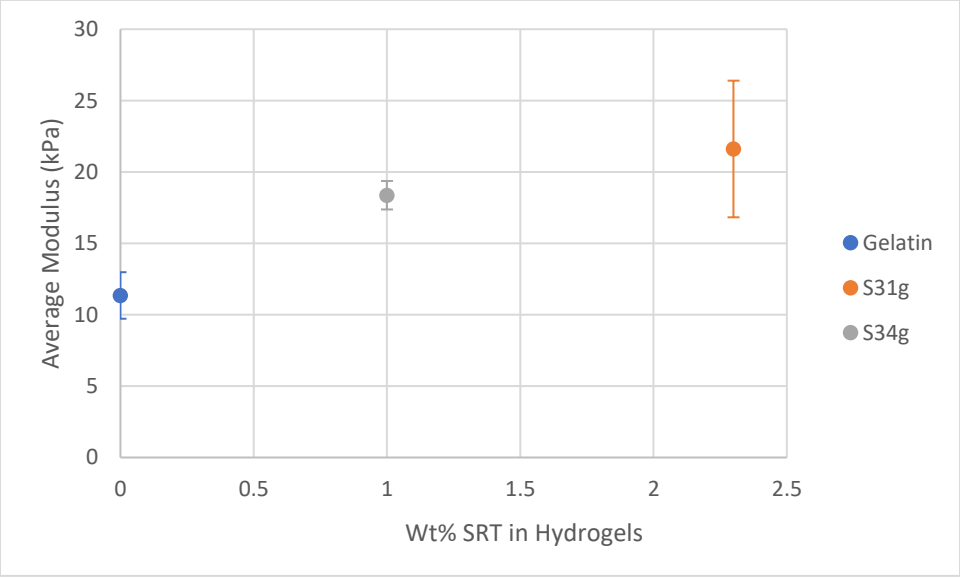

Figure 5: Elastic modulus data from JKR indenter method. The average elastic modulus increases as the amount of SRT in the gels increase, however the error of the 2.3 wt% SRT gels have a large error and definite correlations cannot be drawn.

Table 1: Analysis of variables for the initial SRT hydrogel experiment. Since the P-value is below alpha (0.05) and F is greater than F critical, there is a statistical evidence that increasing the SRT content increases the modulus of the hydrogels, however the range of the modulus measurements should not be ignored.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1025.034	1	1025.034	29.84029	5.21E-05	4.493998
Within Groups	549.6108	16	34.35067			
Total	1574.645	17				

SRT Powder Additive

Next, SRT was investigated as a powder additive rather than dissolving in solution first. This removes the need for acetic acid (used in the SRT solution to help solubility), so it eliminates the need for pH adjustment with NaOH solution. In order to easily compare this data to the SRT solution data, the amount of SRT added to the gels were ~2.3 and ~1.0 wt% SRT. The modulus calculations were done with v as 1/2 and 1/3, since the v of these hydrogels has not been tested. **Figure 6** shows the average modulus data for this experiment. Raw data can be found in the appendix.

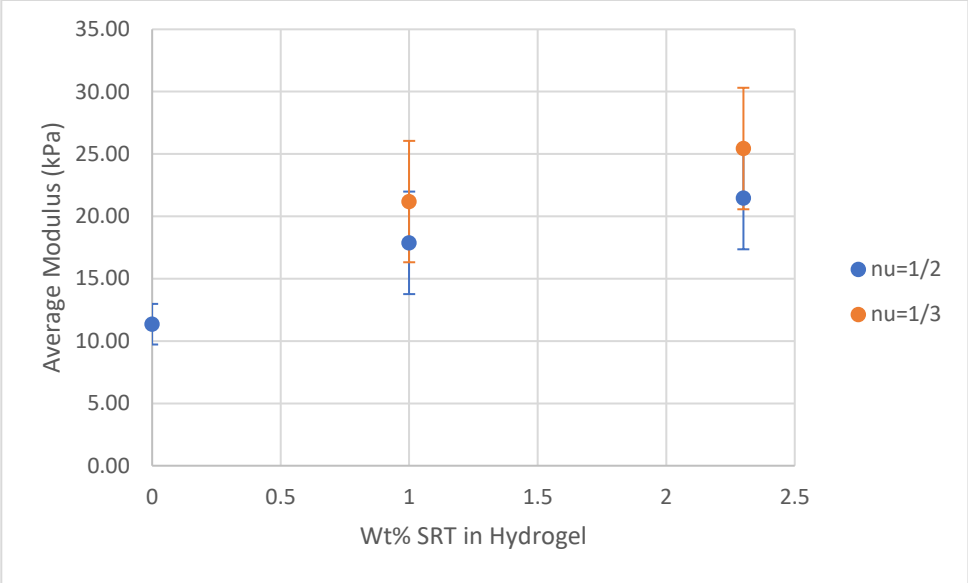

Figure 6: Modulus data for SRT as powder additive. Modulus increases as a SRT content increases, but the error is too large to draw a definite correlation.

Table 2: Analysis of variables for the SRT as a powder additive hydrogel experiment. Since the P-value is much lower than alpha (0.05) and F is greater than F critical, there is a statistical evidence that increasing the SRT powder content increases the modulus of the hydrogels, however the range of the modulus measurements should not be ignored.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1548.735	1	1548.735	116.9081	2.86E-10	4.30095
Within Groups	291.4442	22	13.24747			
Total	1840.18	23				

SRT Hydrogels: Time Study

After performing other modulus experiments and from other journal articles, it became apparent that the modulus of the hydrogels changes over time. It was then decided to test SRT hydrogels multiple times over time. The first test was done two days after the gels were made, and the second was completed nine days after. The tests done after two days were difficult; the indenter pierced through the gel during most tests, so the modulus results found may be incorrect. **Figure 7** shows the time study data with v equal to 1/2, and **Figure 8** with v as 1/3.

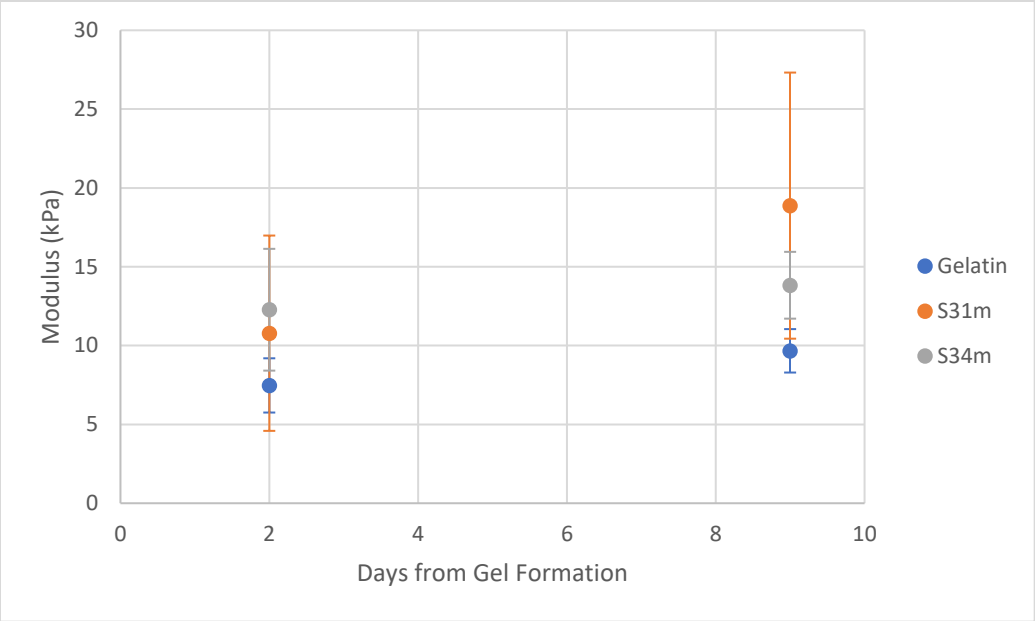

Figure 7: SRT hydrogel modulus over time, v as 1/2. Average modulus increases over time, as expected. Error in modulus remains high, making it difficult to determine correlations.

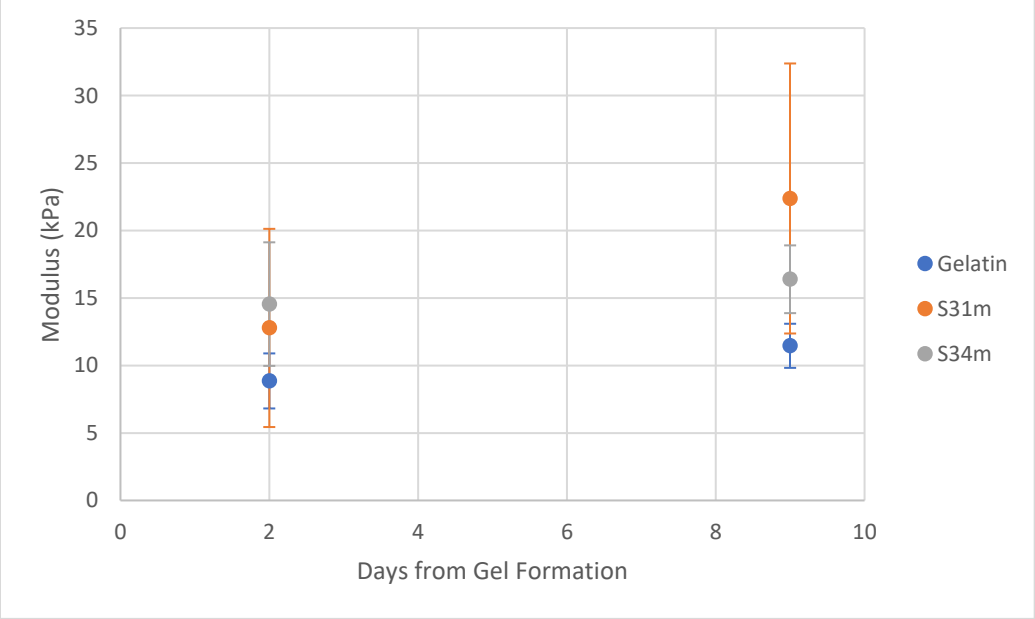

Figure 8: SRT hydrogel modulus over time, v as 1/3. Average modulus increases over time, as expected. Error in modulus remains high, making it difficult to determine correlations.

Table 3: Analysis of variables for the hydrogels with SRT modulus over time experiment, v=1/2. Since the P-value is much lower than alpha (0.05) and F is greater than F critical, there is a statistical evidence that increasing the SRT powder content increases the modulus of the hydrogels, however the range of the modulus measurements should not be ignored.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	132.7067	1	132.7067	8.758998	0.014299	4.964603
Within Groups	151.509	10	15.1509			
Total	284.2158	11				

Table 4: Analysis of variables for the hydrogels with SRT modulus over time experiment, v=1/3. Since the P-value is much lower than alpha (0.05) and F is greater than F critical, there is a statistical evidence that increasing the SRT powder content increases the modulus of the hydrogels, however the range of the modulus measurements should not be ignored.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	366.3727	1	366.3727	43.96699	5.85E-05	4.964603
Within Groups	83.32903	10	8.332903			
Total	449.7017	11				

SEM Images

The Hitachi TM3030 Scanning Electron Microscope (SEM) at the University of Akron's NCERCAMP lab to determine any structural differences among the hydrogels. The gels for SEM imaging were prepared using the method previous described, but they are kept in the centrifuge tubes and placed in the ice bath rather than being transferred into the small wells used for modulus testing samples. Once the gels have set (at least 24 hours), the samples are put in a deep freezer around -30°C for at least 24 hours, then placed in a vacuum chamber. Then the samples are sliced with a razer blade and placed in the SEM for imaging.

Samples of gelatin only, 1 wt% SRT, and 2.3 wt% SRT were prepared, freeze-dried, and imaged. Images were taken from the top, middle, and bottom of the centrifuge tube samples to determine if the

samples are homogeneous and if the structure changes from top to bottom. **Figure 9** shows these samples less magnified, and **Figure 10** shows the samples with greater magnification.

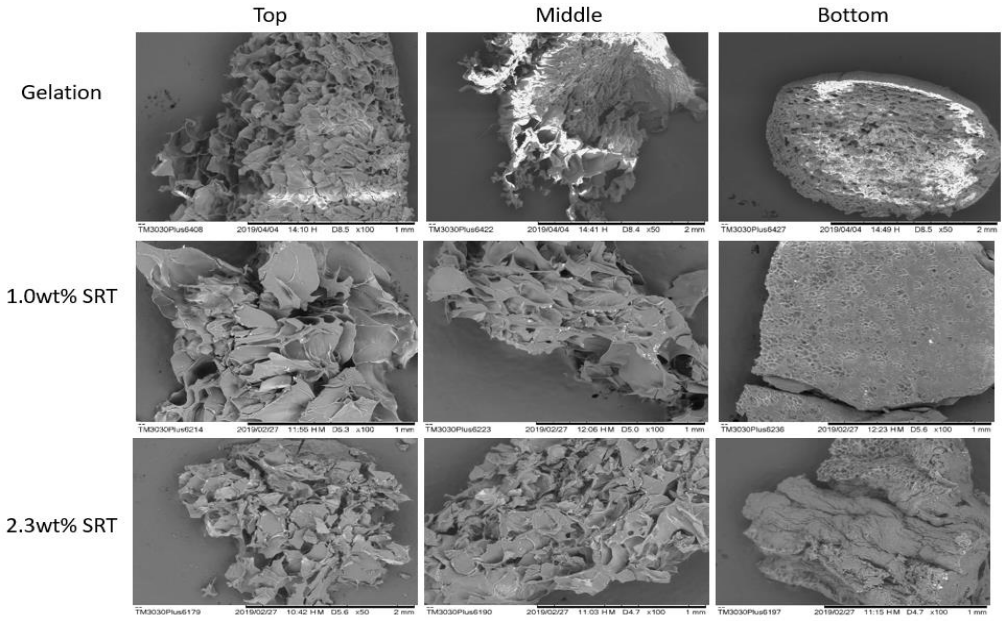

Figure 9: SEM images of freeze-dried hydrogels with various amounts of SRT. Samples from the top, middle, and bottom of the samples were imaged separately. These images are lower magnification than Figure 7.

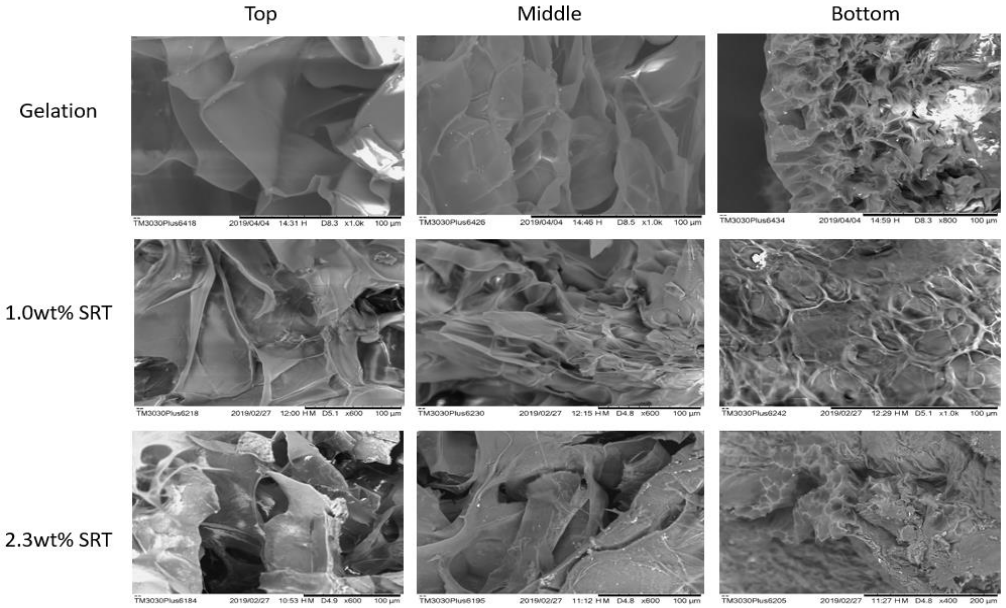

Figure 10: SEM images of freeze-dried hydrogels with various amounts of SRT. Samples from the top, middle, and bottom of the samples were imaged separately. These images are higher magnification than Figure 6.

Analysis and Discussion

Solubility Experiment: Hoffmeister Effect

This project investigated the addition of squid ring teeth (SRT) proteins (or suckerin proteins) to gelatin hydrogels in hopes of increasing the elastic modulus of the gels for more mechanically strong gels. Previous work has indicated the pH must be within in a small range (5-6.5) [Ahmed] and laid the groundwork and provided methods to improve upon for this project. The solubility of SRT in aqueous acetic acid solutions with salts were investigated using the Hofmeister effect, SRT as a powder additive was investigated, and various experiments involving mixing solutions of SRT and gelatin were completed.

The Hofmeister experiment did prove to have an effect on the solubility of the SRT in solution. **Figure 4** in the Data and Results section shows that the LiCl solutions at all acetic acid concentrations dissolved a larger portion of SRT than CsCl. Interestingly, the control (with no salt or acetic acid) dissolved a larger portion of SRT than the CsCl solution without acetic acid. It should be noted that the trials run with no acetic acid were only tested once unlike the other trials which were performed three times. The solution that dissolved the most SRT was the LiCl solution with no acetic acid with only 18.1% of SRT not dissolved, which was not expected. Again, this trial was only run once, so more testing should be completed to confirm this finding. The solution that dissolved the least SRT was the CsCl solution without acetic acid (38.3% undissolved) followed by the control with no acetic acid nor salt (30.1%). The CsCl trials with 5% and 10% left 26.87% and 29.13% SRT undissolved, respectively. The three LiCl solutions tested had the three lowest amounts of undissolved SRT. Moving forward, LiCl could be investigated further as it appears to help the solubility of SRT. More work on the LiCl without acetic acid should be conducted to confirm the current results that it dissolves more SRT than the LiCl solutions with acetic acid. If acetic acid is not needed, pH modification with NaOH would not be needed, simplifying the process of forming the hydrogels.

SRT Concentration Study: Modulus Effect

This initial modulus study did not show that the addition of SRT had a large effect of the mechanical properties of the hydrogels. There was an increase in modulus from the control (no SRT) versus the samples with SRT, however. It appears in this study, the addition of 1.0 wt% SRT increased the modulus roughly 6 kPa (11.35 kPa to 18.37 kPa, standard deviations about 1 kPa for both). The average modulus of the 2.3 wt% gels were 21.61 kPa, however the standard deviation of those trials was 4.79 kPa. The error of the 2.3 wt% SRT gels overlaps that of the 1.0 wt% gels, so it is inconclusive from this experiment whether more SRT increases the elastic modulus. Although this experiment was inconclusive, it did have some mechanically stronger hydrogels at higher SRT concentrations, so more testing was done to try and draw clearer conclusions. There is not a distinctive difference in the structure of the freeze-dried hydrogels as captured by the SEM images.

SRT Powder Additive

The addition of SRT as a powder additive to gelatin hydrogels did show an increase in modulus versus the control. The control (no SRT) had an average modulus of 11.35 kPa, while that of the 1.0 wt% and 2.3 wt% SRT were 17.88 kPa and 21.46 kPa, respectively. (These values were calculated setting v to 1/2.) However, the standard deviations of the modulus for the gels with SRT is large, about 4.11 kPa for

both 1.0 and 2.3 wt% SRT. The errors overlap the averages of these moduli, so it is inconclusive whether the amount of SRT in the hydrogels changes the elastic modulus. All that can be said is that the average modulus increases when more SRT is added to the hydrogels, but the standard deviation is too large to say for sure that more SRT increases the modulus of gelatin hydrogels. It can be said with moderate certainty that the addition of SRT increases the elastic modulus about 6 to 8 kPa.

SRT Hydrogels: Time Study

The investigation of modulus of gelation hydrogels with and without SRT over time gave some expected and some unexpected results. The moduli of the gels increased over time from the first test after two days to the second test after nine days, as expected. However, the gels did not increase in modulus by the same amount. The control hydrogels increased about 29.4% (7.47 to 9.67 kPa), the 1.0 wt% SRT hydrogels increased about 75.1% (10.78 to 18.88 kPa), and the 2.3 wt% SRT hydrogels increased by only 12.6% (12.28 to 13.83 kPa). These values are for v=1/2. The modulus of the 1.0 wt% SRT gels increased the most, by far, while that of the 2.3 wt% increased the least. These findings may be of interest when investigating more on the modulus of these hydrogels over time.

It should also be noted that many of the modulus tests performed two days after formation did not go as planned; the indenter often pierced through the hydrogel, giving less accurate results. The moduli measured are most likely less than the true moduli because of this issue. Care should be taken in the future to not pierce through the gels when only a short amount of time has passed since the preparation of the gels.

SEM Images

Figures 9 and 10 in the Data and Results section show some of the SEM images taken of the freeze-dried hydrogel samples. **Figure 9** shows that the bottom of all of the samples (gelatin, 1 wt% SRT, and 2.3 wt% SRT) are more compact and has fewer and smaller voids than the top and middle of the samples. The bottom section of the gelatin sample looks to be more porous than both the samples with SRT, however. The gelatin only sample also appears to have smaller ridges than the samples with SRT; the SRT may play a role in “smoothing out” some of the ridges, apparent from analyzing **Figure 9**. However, the higher magnification images of **Figure 10** show that the samples with SRT are rougher and more porous closer up. Overall, the samples with SRT appear to be homogenous. The 2.3 wt% top and middle images in **Figure 10** do have some small deposits, but it is unclear whether that is from the addition of SRT or a result of cutting the samples with a razor blade.

Error Discussion

There are many sources of error involved in this project that must be acknowledged. The main sources come from the current modulus testing procedure, variations in the solubility and homogeneity of the hydrogel samples, and issues of evaporation of water from the gels while in storage. Some other issues involve human error, such as small variations in actual amounts of SRT solution and gelation solution used to form the gels, and instrument error (mainly from balances) that could effect the true concentration of the SRT solutions and hydrogels themselves.

One of the main issues that could be improved upon is the current indenter method used to find the elastic modulus of the gels. The variation of modulus among similar or the same samples started and remained relatively large throughout the project. Things that may be considered to improve this process

include: using a larger sample gel size, using a standardized indenter/probe designed for this test, lowering the indenter the exact same amount throughout each test and among all tests (would likely require a device be built), and determining a way to not pierce through the gels when testing. The current indenter is held to the stage with tape and often slips and turns sideways. This could have an effect on the results. A larger gel size may also help with quantifying the distance the probe is indented along with giving more places to test the modulus. The current wells (about 0.5 cm in diameter) cause the top of the gels to be concave down, so the actual contact area may not be what has been calculated. A larger sample size may help the top of the gel be flatter and give a more accurate modulus.

Homogeneity of the gels has also been an issue throughout this project. Some progress has been made, including vortexing the samples right before making the gels, using an ice bath to set the gels faster and immediately after vortexing, and grinding the SRT into a finer powder by oven drying the SRT first (helping the solubility). However, there could still be improvements. More work could be done with LiCl or other salts using the Hofmeister series to increase the solubility of proteins, ultimately improving the homogeneity of the gels. Separating the ground SRT by grind size and using only the finest powder to form the gels may also be helpful in future experiments.

The issue of water evaporation from the hydrogels during storage remains a large issue to overcome. Before this project was completed, the hydrogels were simply capped with a plastic top (not sealed or enclosed) and set in a lab refrigerator in another plastic container with some DI water in it. The DI water in the larger plastic container was meant to keep the gels hydrated. That method was difficult to keep up with since one would have to replenish the water every few days, and there is no guarantee the gels would still contain the same amount of water as when they were formed. During this project, the gels were vacuum sealed and placed in the refrigerator for storage in hopes that the water would remain in the gels. It turns out that some water still evaporates from the gels and collects on the plastic film above the gel. There is currently no way to quantify how much of the water evaporated and the actual protein content of the gels during testing. This likely results in inaccurate moduli.

Overall Results and Conclusions

The addition of squid ring teeth proteins to hydrogels does seem to have an effect on the elastic modulus from looking at the results of this study. Both as a powder additive and in a solution, the addition of SRT does work to increase the modulus of the gelatin hydrogels, however it is difficult to determine how the amount of SRT in the hydrogel effects the modulus. The range of moduli gathered during this project was too large to determine if a greater amount of SRT actually increases the modulus of the gels. Overall, the average modulus is higher for the greater amount of SRT in the gels, but a definite correlation between amount of SRT and modulus has not been found. SEM images did not show any significant changes to the structure of the hydrogels, but it was shown that the bottom of the hydrogel samples are more compact than the rest of the sample. Improvements to storage of samples, the solubility of SRT in solution, and contact mechanics testing would be advantageous and may lead to more conclusive results in the future.

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Appendix

Table A1: Percent SRT undissolved in various aqueous acetic acid solutions with and without the addition of a salt. (Averages and standard deviation)

Salt	% Acetic Acid	Average % Undissolved	Std Dev
CsCl	0	38.30	N/A
CsCl	5	26.87	1.40
CsCl	10	29.13	1.59
LiCl	0	18.10	N/A
LiCl	5	22.80	0.36
LiCl	10	22.77	2.12
None	0	30.10	N/A

Table A2: Percent SRT undissolved in various aqueous acetic acid solutions with and without the addition of a salt. (Raw data)

Salt	Solution	% Undissolved
CsCl	DI	38.3
CsCl	5% AA	28.2
CsCl	5% AA	27
CsCl	5% AA	25.4
CsCl	10% AA	27.3
CsCl	10% AA	30
CsCl	10% AA	30.1
LiCl	DI	18.1
LiCl	5% AA	23.1
LiCl	5% AA	22.4
LiCl	5% AA	22.9
LiCl	10% AA	24.7
LiCl	10% AA	23.1
LiCl	10% AA	20.5

Table A3: Raw data, average and standard deviation modulus data from initial testing of hydrogels, with and without SRT.

E (kPa)	**S31g (higher SRT concentration, ~2.3 wt%)**	**S34g (lower SRT concentration~1.0 wt%)**	**0 wt.% SRT**
E (trial 1)	18.227102	29.52705653	11.89
E (trial 2)	24.9954253	19.07609355	9.52
E (trial 3)	2.190808506	17.66654185	12.64
average	21.61126365	18.3713177	11.35
stdev	4.785927303	0.996703572	1.628588

Table A4: Average modulus and standard deviation data for SRT as powder additive experiment.

Wt% SRT	**v**	**Average Modulus (kPa)**	**Standard Deviation (kPa)**
0.0	1/2	11.35	1.63
1.0	1/2	17.88	4.11
2.3	1/2	21.46	4.11
1.0	1/3	21.19	4.87
2.3	1/3	25.44	4.87

Table A4: Raw data for SRT as powder additive experiment. (v=1/2)

SRT wt%	**Modulus (kPa)**		
2.3	26.18633		
2.3	18.71646	ave	21.46472
2.3	19.49136	sd	4.107354
1	10.499		
1	18.95881		
1	17.36	ave	17.87503
1	20.84397	sd	4.110305
1	17.29956		
1	22.28884		

Table A5: Raw data for SRT as powder additive experiment. (v=1/3)

SRT wt%	Modulus (kPa)		
2.3	31.03565		
2.3	22.18247	ave	25.43966
2.3	23.10087	sd	4.867975
1	12.44326		
1	22.4697		
1	20.57481	ave	21.18522
1	24.70396	sd	4.871473
1	20.50318		
1	26.4164		

Table A6: Average and standard deviation of SRT hydrogel modulus over time, v as 1/2.

Wt% SRT	Days Past	Average Modulus (kPa)	Standard Deviation (kPa)
0.0	2	7.47	1.72
1.0	2	10.78	6.19
2.3	2	12.28	3.86
0.0	9	9.67	1.38
1.0	9	18.88	8.44
2.3	9	13.83	2.12

Table A7: Average and standard deviation of SRT hydrogel modulus over time, v as 1/3.

Wt% SRT	Days Past	Average Modulus (kPa)	Standard Deviation (kPa)
0.0	2	8.86	2.04
1.0	2	12.78	7.34
2.3	2	14.55	4.58
0.0	9	11.46	1.63
1.0	9	22.37	10.00
2.3	9	16.39	2.51

Table A8: Raw data, average and standard deviation of SRT hydrogel modulus over time study two days after gel preparation, v as 1/2.

Trial	Gel A	31m	34m
1	6.584184	19.71925	17.12903
2	6.801864	11.10019	8.82826
3	7.078078	9.522566	9.499737
4	6.383527	11.26686	10.16571
5	10.51502	2.314233	15.75265
Average	7.472534	10.78462	12.27508
Std Dev	1.720262	6.194755	3.862863

Table A9: Raw data, average and standard deviation of SRT hydrogel modulus over time study two days after gel preparation, v as 1/3.

Trial	Gel A	31m	34m
1	7.803477	23.37096	20.30107
2	8.061468	13.15578	10.46312
3	8.388833	11.286	11.25895
4	7.565662	13.35332	12.04825
5	12.46224	2.742794	18.6698
Average	8.856337	12.78177	14.54824
Std Dev	2.038829	7.341932	4.578208

Table A10: Raw data, average and standard deviation of SRT hydrogel modulus over time study nine days after gel preparation, v as 1/2.

Trial	Gel A	31m	34m
1	9.652483	16.8283	14.22425
2	7.773322	24.25479	17.09777
3	10.04037	15.94705	11.50991
4	9.275909	7.654387	13.79144
5	11.59551	29.70823	12.51461
Average	9.66752	18.87855	13.82759
Std Dev	1.378201	8.440454	2.11827

Table A11: Raw data, average and standard deviation of SRT hydrogel modulus over time study two days after gel preparation, ν as 1/3.

Trial	**Gel A**	**31m**	**34m**
1	11.43998	19.94465	16.85837
2	9.212826	28.74642	20.26402
3	11.8997	18.90021	13.64137
4	10.99367	9.071866	16.34541
5	13.74283	35.20976	14.83213
Average	11.4578	22.37458	16.38826
Std Dev	1.633424	10.0035	2.510542