THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF ENGINEERING SCIENCE AND MECHANICS

PRODUCTION OF SQUID PROTEIN BIOPLASTICS USING 3D PRINTERS

ANDREW BLAKE TUCKER

SPRING 2016

A thesis
submitted in partial fulfillment
of the requirements
for baccalaureate degrees
in Engineering Science and Biochemistry and Molecular Biology
with honors in Engineering Science

Reviewed and approved* by the following:

Melik C. Demirel
Professor of Engineering Science
Thesis Supervisor and Honors Adviser

Reginald F. Hamilton
Professor of Engineering Science
Faculty Reader

* Signatures are on file in the Schreyer Honors College.

ABSTRACT

Plastic pollution is becoming epidemic that is getting more and more difficult to ignore. Each year, Americans discard more than 30 million tons of plastic, only 8% of which gets recycled. It is time to find an alternative that will significantly reduce the plastic footprint humans are leaving on the Earth. With their natural origins, squid sucker ring teeth (SRT) proteins are highly biodegradable in comparison to their traditional plastic counterparts. This, along with their high mechanical strength and biocompatibility, makes them an attractive plastic alternative. These proteins are thermoplastic and moldable into any geometry by thermal processing techniques. However, the need for these shaping techniques greatly limits the applications of these proteins. In this study, squid SRT proteins were formed into a gel that is moldable at room temperatures and was subsequently 3D printed into a scaffold. The formation of this gel opens a number of potential applications for these SRT proteins, and the 3D printing of this protein based biocompatible scaffold shows potential for these SRT proteins as advanced materials in medicine. This is the first time that a thermoplastic protein has been bio-printed at room temperature.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my honors and research advisor, Dr. Melik Demirel, for allowing me to be a part of his research group and work in his lab. His support and guidance have allowed me to enjoy a rewarding undergraduate research experience and have given me a wealth of opportunities that I never would have thought possible.

I would like to thank all the members of the Demirel Research Group for creating a pleasant and lively research environment, for helping me grow accustom to working in the lab, and for offering advice when it was needed.

I would also like to thank the College of Engineering Research Initiative and the Schreyer Honors College for financial support.

Chapter 1

Introduction

A Brief History of Plastics

Plastic is everywhere. It holds the water that we drink, makes up the keyboards that we type with, and even the clothes that we wear. Today, one cannot go about their business without somehow interacting with plastic. Amazingly, this prevalence has only come about over the past half century [1-3] due to the incredibly versatile physical properties that plastics possess. They are highly moldable, are inexpensive and easy to produce, and are lightweight. Additionally, the mechanical properties of a certain plastic can vary greatly based on processing, which only expands their already wide range of applications. It took very little time for humankind to recognize and exploit the benefits that plastics have to offer. It has taken even less time for the repugnant drawbacks of these unnatural polymers to show themselves.

Today, the internet offers a plethora of pictures displaying sea creatures trapped in plastic confines, small islands of plastic floating in the oceans, and mounds of plastic waste resting in landfills near our back yards. [1,3] It is increasingly apparent that the unnatural creation of these polymers from petroleum and natural gas have made them nearly impossible for the environment to degrade. These harsh pictures and realizations have prompted reform in the plastics industry as the public has become more environmentally aware. However, this reform has only occurred in half measures that do not truly solve the issues related to the use of plastics. Measures such as recycling and “degradable” plastics seem like plausible solutions in theory, but fall considerably

short when put into practice. In fact, a study conducted in 2008 found that about 86% of plastic waste finds its way into landfills while a mere 6% is recycled. [4] The Plastic Pollution Coalition gives similar numbers, stating that Americans discard 30 million tons of plastic each year, only 8% of which gets recycled. [5]

Degradable plastics are a similarly poor solution. Degradable plastic formation involves reacting the fossil-fuel-based monomer that traditionally make up plastics with another chemical. The product molecules then polymerize, similar to traditional plastics [6]. The difference is that this new plastic will react with something in the environment (sunlight, bacteria, etc.) and will break apart. [2] This seems like a viable solution. The plastic that once polluted the environment will be broken down and will no longer pile up in the ocean or landfills and will no longer ensnare animals. However, these degradable often do not breakdown fully, leaving behind small, nearly microscopic plastic particles. In the case that they do fully break down, they can often leave a buildup of the monomers that originally formed the plastic. The environmental effects of these small plastic particle and monomer deposits remain unknown, but it is quite clear that these degradable plastics are not the great solution that everyone desires. [2] There remains a significant need for the discovery of plastic alternatives.

Fortunately, recent developments in the fields of material synthesis and biotechnology provide hope in this respect. Plastic alternatives made from natural polymers produced by plants and animals are inherently biodegradable yet share many properties with traditional plastics. Some examples of these new plastics are polylactic acid (PLA), derived from sugarcane, and hydroxyalkanoate polyesters (PHAs), which are derived from bacteria. Relatively recent discoveries have also found that squid contain another natural plastic alternative. The proteins that make up the sucker ring teeth found on squid tentacles have shown remarkable thermoplastic

and adhesive properties. Even with these discoveries, there continues to be an unmet need for the development of biomaterials that can match the performance of synthetic materials in a more sustainable way. With more research, these proteins could become the basis for plastics of the future and could be the solution to the problem of plastic pollution that has plagued the Earth for the past half-century.

Squid Sucker Ring Teeth Proteins

Everyone knows that suction cups cover a squid's tentacles, but what many may not know is that each suction cup contains a ring of sharp teeth that help the squid latch onto prey. These sucker ring teeth (SRT) are composed entirely of structural "suckerin" proteins. SRT have a ring structure with internally aligned nanoscale pores. [7] The SRT proteins themselves have a structure unique from other strong protein polymers in that it does not derive its strength from chain entanglements, covalent crosslinking, or mineral and metal presence. [8,9] Instead, strength of SRT proteins, which at 6-8 GPa in dry conditions and 2-4 GPa in hydrated conditions [8] is similar to that of strong synthetic polymers such as PMMA, PEEK, or polyamides [9], comes from its β-sheet secondary structure. The β-sheet structure of these squid proteins is extremely similar to that of spider silk. Silk crystal is made of highly ordered β-sheet stacks formed by repeating units of amino acids aligning and forming a hydrogen-bonded network. In silk these β-sheet stacks are neatly oriented and separated by amorphous regions of proteins. In SRT protein, these β-sheet stacks are also present due to repeating units, but they are

oriented randomly, as seen in Figure 1. [10] The nanoconfined β-sheets, connected by amorphous regions, reinforce the supramolecular network and give the SRT their strength [8,9].

The impressive strength of this entirely proteinaceous polymer is one of the qualities that give it great potential as a future material. The polymer's other unique qualities include thermoplasticity, adhesive properties, and self-healing propeties. Due to their reversible aggregation mechanism, SRT are an ideal thermoplastic material. This means that the material becomes moldable when heated above a certain temperature and solidifies upon cooling. While most biopolymers exhibiting thermoplastic behavior lose their mechanical properties upon cooling, SRT do not. This property allows shaping of SRT into any 3D geometry (including fibers, colloids, and thin films) while retaining the significant strength seen in SRT. [10,11]

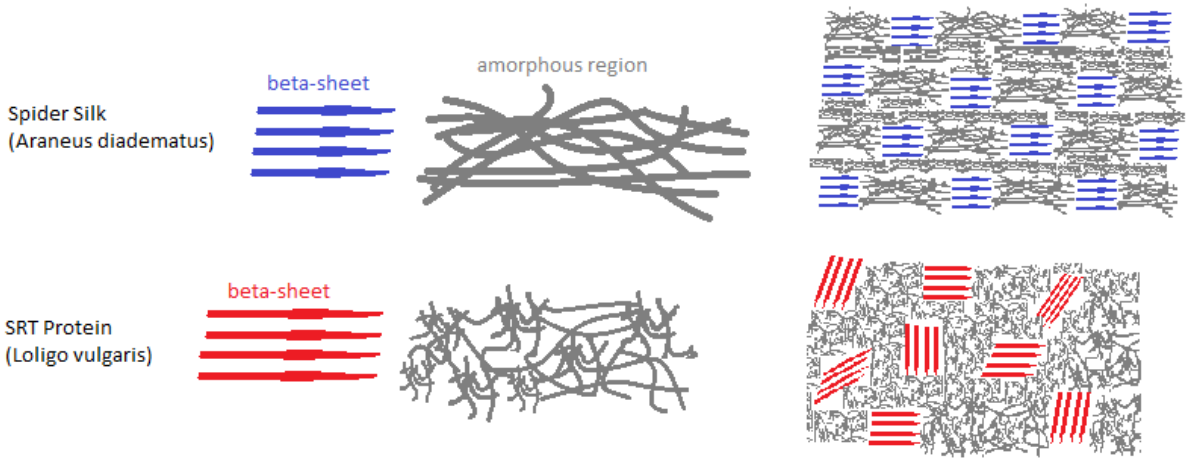


Figure 1. Models of two semi-crystalline bioelastomer: (i) spider silk and (ii) SRT Protein

Though not exhibited in nature, the adhesive properties of SRT are quite remarkable. SRT is a thermoplastic hot melt pressure sensitive adhesive (PSA), meaning it can adhere to a variety of surfaces with pressure contact when in its hot melt form. Typically, PSAs are made from petroleum-based chemicals, though some natural elastomers have also been used as adhesives. The issue with these natural elastomer adhesives is that they are often display weak

shear strength in comparison to their synthetic counterparts. SRT, however, have a shear strength higher than most natural and synthetic polymers in both wet and dry conditions. Additionally, SRT are not cytotoxic like synthetic PSA's. Thus, SRT proteins could be a valuable adhesive in the medical and other fields. [7]

A third unique property of SRT is the ability of the proteins to self-heal. In other words, if a structure made out of SRT proteins breaks, more SRT proteins can fix the break. This is done by first heating SRT proteins above their glass transition temperature, causing them to transition into their melt phase. Application of the melt to the broken SRT structure along with pressure and cooling results in repair of the initial SRT structure. In addition, the repaired structure retains the mechanical properties seen in the initial structure before breakage. [12]

With their unique strength, thermoplastic nature, adhesive properties, and self-healing abilities, SRT are a material with great potential in a wide range of applications. In fact, many have equated the potential of SRT to silk, which has found use in wide range of fields including biomaterials coatings, drug delivery, and membrane filtration. [10] Others have stated that SRT-based materials could find use as artificial limbs, scaffolds in tissue engineering, or simply as a packaging material. [8] Before they can reach these glorious heights, SRT must first overcome a number of hurdles, one of which is processing techniques. Thermal processing techniques, such as extrusion or injection molding, can fashion this thermoplastic protein into any geometry, but the high thermal expansion coefficient of proteins greatly limits precise control of these proteins during manufacturing. The alternative, solution processing, also has limitations in large-scale manufacturing and long-term stability. Therefore, there remains a significant need for a room temperature processing technique. An ability to 3D print this material is also highly desirable and has a number of applications, specifically in the medical field where a biodegradable

material that can be produced in any geometry or shape is highly desirable. [13] The approach outlined in this thesis was successful in producing a protein gel and conducting the first ever bio-printing of a proteinaceous material, bringing SRT one step closer to the expansive list of potential applications detailed above.

Chapter 2

General Materials and Methods

Squid sucker ring teeth (SRT) used in this study were isolated from both Longfin Inshore Squid (*Loligo Pealei*) and European Common Squid (*Loligo Vulgaris*). SRT were isolated by physically prying the SRT from the squid suction cups using a toothpick.

In many of the studies conducted, SRT were dissolved in DMSO at certain concentrations. A predetermined amount of SRT was weighed out in a 2 mL glass vial followed by addition of a specified volume of DMSO to achieve the desired concentration. Dissolution of SRT in DMSO occurred by water bath sonication using an Aquasonic model 150D. Sonication lasted for 1 hour to ensure the entirety of the SRT had dissolved. Unless otherwise specified, all solutions were prepared in 2 mL glass vials, 20 mL glass vials, or glass culture tubes.

Chapter 3

Gel Formation Mechanism

Introduction

It was discovered that an SRT gel could be made repeatedly by dissolving SRT in DMSO and mixing with 2,3-dimethyl-1,3-butadiene. Following this discovery, the gel's formation mechanism was investigated in order to gain a better understanding of the origin of the gels properties. Before using the gel in various applications, we needed to learn more about the gel and about how we could manipulate the gel's properties.

Materials and Methods

To form the gel, SRT was dissolved in DMSO at the desired concentration. This solution was then added to 2,3-dimethyl-1,3-butadiene at the desired volume ratio. A magnetic stir bar and a stir plate were then used to mix the solutions at 600 rpm for 10 minutes, resulting in gel formation. If heating during gel formation was required, the stir plate was also used to heat the solutions at the specified temperature.

Gel was formed as described in three separate 20 mL glass vials, labeled 1, 2, and 3. The excess liquid in Vial 1 was decanted off and 2 mL of water added in its place. The excess solution in Vial 2 was decanted and 2 mL of DMBD was added in its place. The same process was repeated with Vial 3, this time replacing the decanted solvent with 2 mL of DMSO.

Gel formation was also attempted with HFIP. SRT was dissolved in HFIP at a concentration of 100 mg/mL in a glass 2 mL vial. This solution was then added to DMBD at a

ratio of 4:1 DMBD to SRT-HFIP solution in a 20 mL glass vial. The mixture was ten stirred at 600 rpm for 10 minutes.

A systematic study investigating the effects of varying SRT concentration was run in a similar manner. SRT was dissolved in DMSO at concentrations of 10 mg/mL, 25 mg/mL, 50 mg/mL and 100 mg/mL, each in a 2 mL glass vial. Each DMSO-SRT solution was then added to DMBD in a separate 20 mL glass vials at a ratio of 2:1 DMBD to DMSO-SRT. Stirring at 600 rpm mixed the solutions and caused gel formation. This process was then repeated, this time heating the mixture at 60 °C while stirring.

Gels formed both with and without heating were also analyzed using FTIR. Gels were formed as previously described using a concentration of 100 mg/mL SRT in DMSO at 2:1 DMBD to DMSO. One gel was formed by stirring at room temperature while the second was formed while stirring at 60 °C. The IR spectrum of each gel was obtained and analyzed using a ThermoFischer Nicolet 6700 FTIR spectrometer and the computer software OMNIC. Spectra of pure DMSO and pure DMBD were also obtained for comparison. This process was then repeated, this time forming gels in a solution of 1:1 DMBD to DMSO.

Results

The addition of water to the gel in Vial 1 caused the gel to change in color from slightly opaque with a yellow tint to an entirely opaque white. This change is most likely the result of SRT protein crosslinking that occurs upon interaction with water. Doubling the concentration of DMBD in Vial 2 caused the gel to harden. Doubling the concentration of DMSO in Vial 3

appeared to soften the gel slightly. Additionally, attempted gel formation using HFIP resulted in initial gel formation followed quickly by disappearance of the gel (Figure 2).

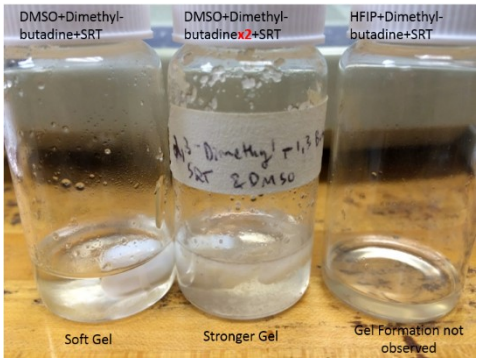


Figure 2. Gel Formation in Various Solvents

Following these observations, a systematic study whereby DMBD was mixed with SRT/DMSO solution of varying concentrations was carried out. The results can be seen in Table 1 below. Out of the concentrations tested, gel formation only occurred in the 100 mg SRT per mL DMSO sample. However, upon heating, gel formation occurred in all concentrations tested.

Table 1. Concentraion of SRT in DMSO and Resulting Gel Formation

Concentration (mg SRT/mL DMSO)	Gel Formation Before Heating	Gel Formation After Heating
10	No	Yes
25	No	Yes
50	No	Yes
100	Yes	Yes

The gels formed in the 100 mg/mL samples of 2:1 DMBD to DMSO, both with and without heating, were then analyzed using FTIR spectrometry. The spectra of the heated and room temperature DMSO-DMBD solutions were nearly identical with the only difference being

a small peak at 1600 cm^{-1} seen in the room temperature sample, but not in the in the heated sample (Figure 3, Appendix A).

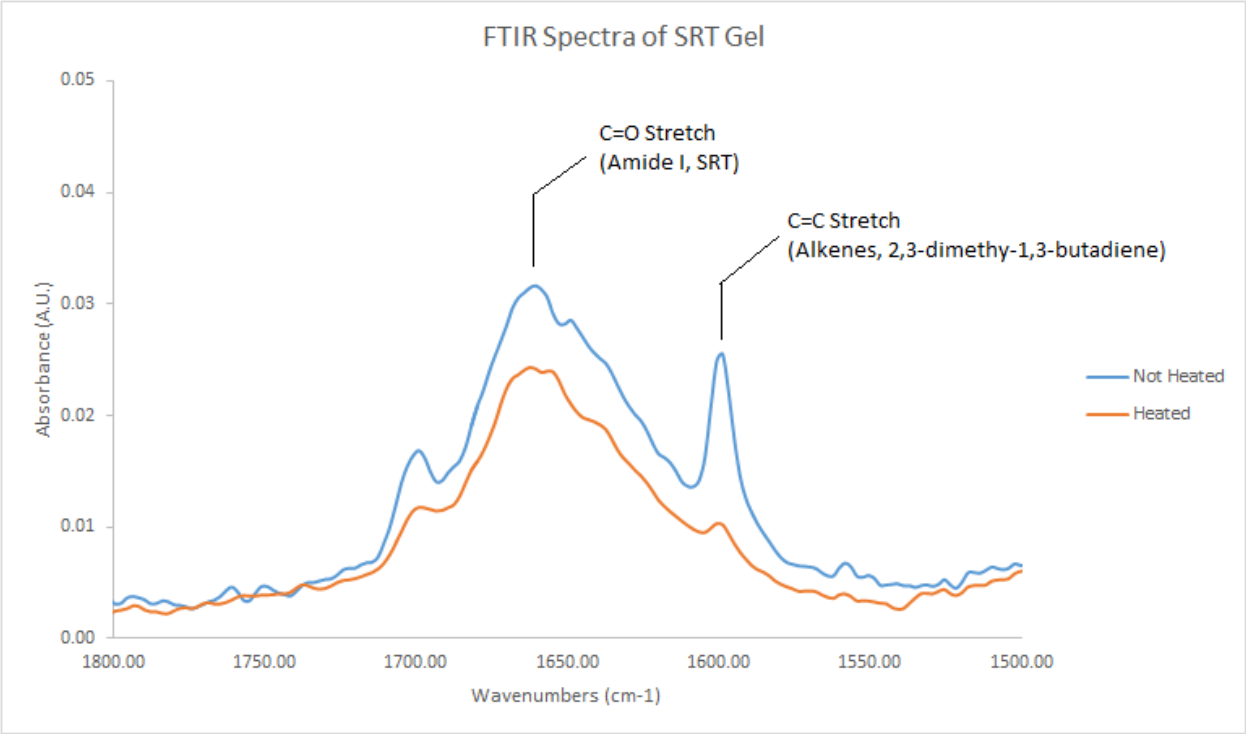


Figure 3. FTIR Spectra of SRT gels made with 2,3-dimethyl-1,3-butadiene and DMSO at a ratio of 2:1 both with and without heat

Discussion

Based on these results, a number of hypotheses emerged. Since formation did not occur with HFIP but did occur with DMSO, the formation mechanism may be related to some difference between the two solvents. Since they have very different dipole moment (DMSO = 5.86, HFIP = 3.73 calculated at http://www.molcalc.org), it was hypothesized that the mechanism could be related to the dipole moments. DMSO is also aprotic, while HFIP is protic. Therefore, the mechanism could involve the fact that DMSO does not form hydrogen bonds while HFIP does. Additionally, it was noted that DMBD contained a stabilizing agent, butylated

hydroxytoluene (BHT), at a concentration of 100 ppm, which could be initiating gel formation. A final hypothesis was that the DMBD reacted with itself to form a cyclic compound. This has been seen in a similar hydrocarbon, isoprene. It was observed that these cyclic dimers occur naturally in small amounts, but their presence greatly increases upon heating. [14] This cyclic compound, we hypothesized, would fit between the β-sheets of the SRT proteins and cause gel formation by making the SRT proteins less soluble.

To test these theories, a systematic study was set up whereby DMBD was mixed with SRT/DMSO solution as before, but the concentration of SRT was varied. If the gel formation mechanism was due to any of the previously proposed theories, some amount of gel should form no matter what concentration of SRT protein is used.

As can be seen in Table 1, only the concentration of 100 mg/mL resulted in gel formation before heating. This result disagrees with each of the hypotheses above. Changing the concentration of SRT will have no effect on the dipole moment of DMSO, nor will it change the fact that DMSO is aprotic. Additionally, it does not have an effect on the concentration and presence of BHT, meaning that this, too, is not an aspect of the mechanism of gel formation. The cyclic compound hypothesis also seems to dispel by these results. After all, DMBD, and presumably the cyclic structures it naturally forms, are present in the same amount no matter the concentration of SRT. However, the fact that there is so little cyclic compound formation without heating could mean that, at lower concentrations of SRT, the limited number of cyclic compounds have a more difficult time finding a stack of β-sheets to fit between and cause gel formation. This would explain the absence of gel formation at lower concentrations in unheated solutions. The results of gel formation upon heating also supports this theory, as heating causes gel formation at all tested SRT concentrations. Since heating induces formation of these cyclic

compounds, it logically follows that a rise in cyclic compounds results in increased cyclic compound-SRT β-sheet interactions and greater gel formation.

Further confirmation of this hypothesis was obtained using FTIR analysis. The spectra of the heated and room temperature DMSO-DMBD solutions were nearly identical with the only difference a small peak at 1600 cm^{-1} that was seen in the room temperature sample, but not in the heated sample (Figure 3). This wavenumber corresponds to a C=C bond, indicating that upon heating this bond disappears or at least decreases greatly in presence. Since cyclization of DMBD similar to that of isoprene involves the elimination of two C=C bonds [14], this change in IR spectra indicates that cyclization is occurring upon heating.

Lastly, this mechanism was confirmed by reacting SRT-DMSO solution with benzene to form a gel. Since benzene is already a cyclic compound, gel formation would occur without any heating if the proposed mechanism of gel formation was correct. A solution of 100 mg/mL SRT in DMSO was mixed with benzene in a 2:1 ratio of benzene to DMSO-SRT solution. The result was immediate formation of a gel. This indicated that the proposed solubility mechanism for gel formation was correct.

Chapter 4

Gel Properties

Introduction

With the formation mechanism determined, attention shifted towards gaining a better understanding of the properties of the SRT gel. Specifically, our interests lay in determining the concentration dependence of the gel formation and the solubility of the SRT protein itself. Developing an understanding of the concentration dependence of gel formation would allow for the prediction of how much time it would take for a gel to form under certain conditions.

Materials and Methods

A systematic study was conducted to determine the concentration dependence of gel formation. SRT concentrations of 10 mg/mL, 25 mg/mL, 50 mg/mL, and 100 mg/mL in DMSO were prepared in 2 mL glass vials. Each SRT-DMSO solution was then added to benzene at a 2:1 ratio of benzene to SRT-DMSO solution. The solutions were mixed at 300 rpm and room temperature and the amount of time that elapsed before gel formation was measured using a stopwatch. This process was then repeated using a concentration of 1 mg/mL of SRT in DMSO to determine if the relation found in the previous study held true for extremely low concentrations

In order to determine solubility, SRT was dissolved in DMSO at a concentration of 100 mg/mL. This solution was then combined with DMSO and a co-solvent (benzene, dichloromethane, or tert-amyl alcohol) in varying proportions as seen in Table 2. These

solutions were left on a shake plate for 12 hours overnight to allow the gel to equilibrate with the dissolved SRT. Following shaking, the solution and any gel formed were centrifuged at 10,000 rpm for 30 min to separate out the solid gel. The supernatant from each sample was removed and a Lowry assay was run to determine the concentration of the SRT in each supernatant, which was equivalent to the solubility of SRT in the supernatant's respective binary solvent mixture.

Table 2. Sample Preparation for Solubility Determination

Sample	% co-solvent	Total Volume (µL)	Volume co-solvent (µL)	Volume DMSO/SRT solution (µL)	Volume DMSO Added (µL)	Final Protein Concentration (mg/mL)
1	10	150	15	125	10	83.3
2	15	200	30	50	120	25
3	25	200	50	40	110	20
4	35	200	70	20	110	10
5	50	200	100	15	85	7.5

Results

The results of the systematic study used to determine the concentration dependence of gel formation can be seen in Table 3 and Figure 4 below.

Table 3. Protein Concentration and Time until Gel Formation

Concentration (mg SRT/mL DMSO)	Gel Formation	Time (seconds)
100	Yes	0.01
50	Yes	3
25	Yes	60
10	Yes	300

Gel formed at every concentration tested, similar to the results seen when forming the gel using DMBD and heat. Gel formation also occurred more quickly as SRT concentration increased. Figure 4 reveals that gel formation time is exponentially related to the concentration of SRT in DMSO that is added to the benzene. Gel formation was also observed when repeating

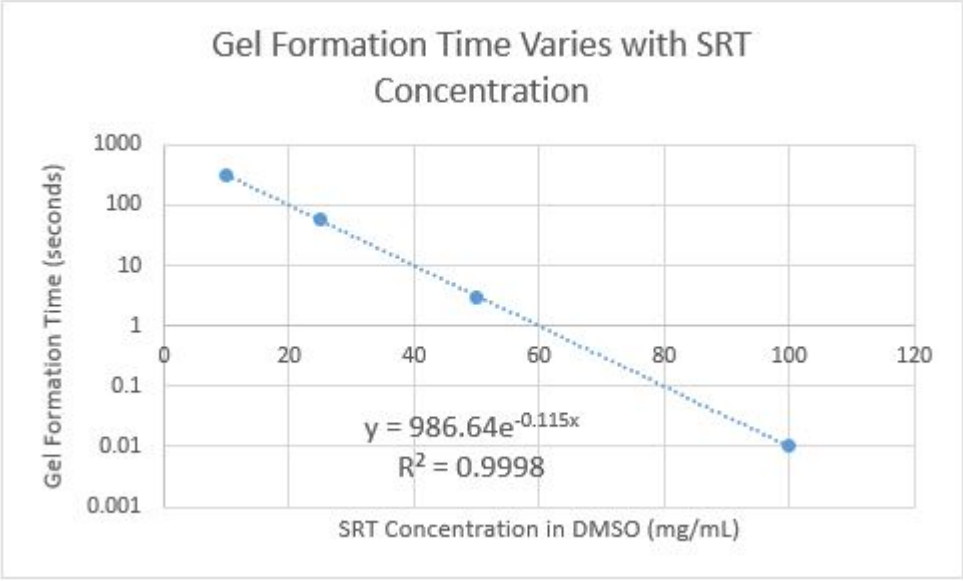


Figure 4. Gel formation time varies with SRT concentration in DMSO. Note that the y-axis is on a logarithmic base 10 scale.

the experiment using a concentration of 1 mg/mL of SRT in DMSO, though the time of formation was difficult to estimate due to the very small amount of gel that formed.

The results of the SRT solubility study are displayed in Figure 5 below. SRT solubility seems to decrease severely once the co-solvent concentration increases above 10%. Protein Concentrations were determined using the standard curve generated during the Lowry assay (Appendix B).

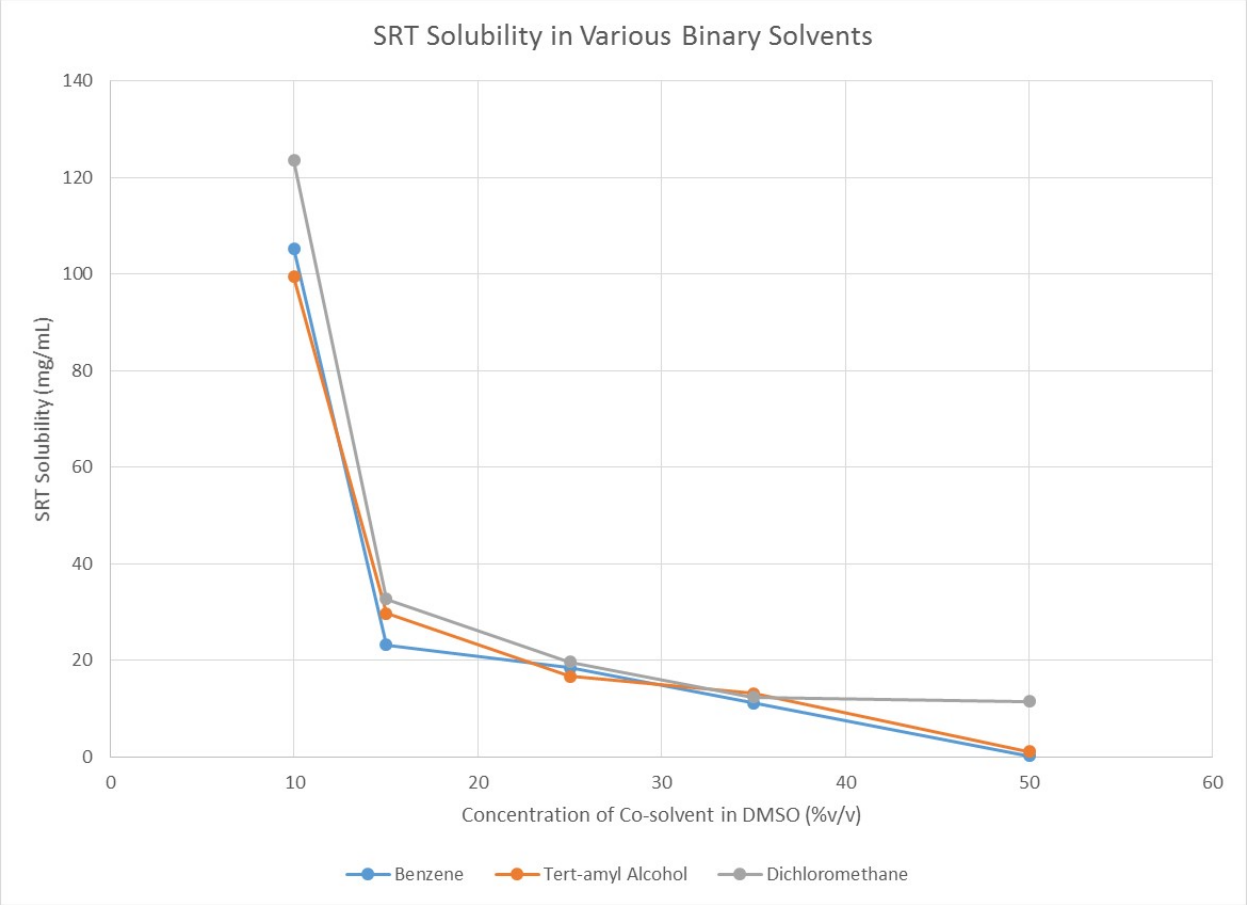


Figure 5. Depiction of the solubility of SRT protein in various binary mixtures of DMSO and another solvent.

Discussion

The systematic study of concentration dependence revealed that the time elapsed before gel formation was exponentially related to the concentration of SRT used during formation. Such a result is somewhat expected due to the solubility mechanism of gel formation.

At higher concentrations of SRT, there will be more protein in solution and more protein will quickly interact with the cyclic hydrocarbon benzene upon its addition. Thus the gel will quickly form as the benzene is able to interact with SRT protein so quickly. In lower concentrations, the gel will form more slowly as it takes longer for the benzene molecules to run into the SRT protein in the solution. Determining the mathematical relation between time until gel formation and SRT concentration is especially important from an industry perspective. If a company desires to make the SRT gel in bulk, they will need to know how long gel formation will take to occur at the specific SRT concentration being used. This way, they can remove the SRT gel immediately after it has formed and begin the process again. This allows the efficiency of gel formation to be maximized along with the productivity of the gel formation process.

Determination of SRT solubility in various binary solvent mixtures is also very important. As expected, the SRT was more soluble at lower concentrations of co-solvent in DMSO. This is because there is less co-solvent to interact with the dissolved SRT protein. Thus, once the hydrocarbon has caused initial gel formation, re-dissolving of gel SRT by DMSO will likely counteract any additional gel formation, establishing an equilibrium. As co-solvent concentration increase, this equilibrium shifts in favor of gel formation. Interestingly, SRT solubility drops off significantly once co-solvent concentration increases above 10%. Determining this SRT solubility is any important step in better understanding the properties of the gel. The sharp drop in solubility indicates that formation of the gel at a co-solvent concentration slightly higher than 10% will be most efficient in terms of using the least amount of co-solvent to bring about gel formation. Again, this has large implications on the industrial production of the SRT gel. Using a smaller amount of hydrocarbons in the process means it will be more efficient and economically viable.

Chapter 5

3D Printing

Introduction

The ability to 3D print the SRT protein is the penultimate goal of room temperature processing. If the SRT protein gel could be 3D printed, it could be shaped into any geometry very easily. 3D printing is also a low energy process, meaning it is an environmentally friendly manufacturing process for these SRT proteins.

Materials and Methods

Upon formation of the first SRT gel using 2,3-dimethyl-1,3-butadiene, printing was attempted. Gel samples were sent to the lab of Dr. Ibrahim Ozbolat to be printed using a 3D bioprinter made by his lab. Upon printing the gel was treated with water to crosslink the SRT proteins and harden the printed matrix.

The Ozbolat group also seeded cells in the printed structure to conduct cell viability studies. The process was repeated using SRT gel formed with benzene.

Results

The gel was successfully printed into a matrix that could be used in tissue engineering applications (Figure 6). Treating with water caused the printed gel to harden by initiating crosslinking among SRT proteins. Unfortunately, cells seeded on the SRT-butadiene gel did not proliferate and died.

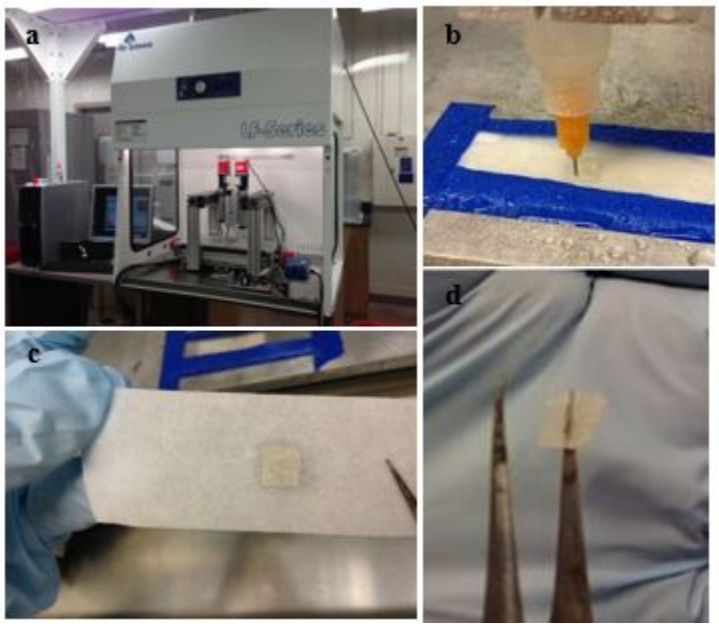


Figure 6. A tissue-engineering scaffold was printed using SRT protein gel: (a) 3D bioprinter (b) Scaffold being printed from SRT gel (c) Bioprinted scaffold (d) Scaffold held by tweezers after crosslinking

Seeding of the cells was successful on unprinted benzene gels. Issues arose when printing the benzene gel due to its stiffness and inconsistency. Currently work is being done to manipulate the stiffness and consistency of the gel so that it can be 3D printed more successfully

Future Directions

Future research will aim at further characterization of the gels properties and obtaining a better understanding of how to alter gel properties by adjusting preparation procedures. Studies of the gel using rheology, FTIR, and XDR will allow for better characterization of the gel. Once 3D printing of the benzene gel is successful, cell viability studies will be run on the 3D printed matrix. Success with these experiments will lead to *in vivo* osteogenesis animal studies with the printed SRT matrix.

Chapter 6

Colloids

Introduction

In order to facilitate processing of SRT proteins at room temperature, it was also proposed that the SRT proteins be made into a gel form by creating of SRT coated colloid particles. A colloid is a suspension of small particles of one substance, called the dispersed phase, in another immiscible substance, called the dispersion medium. These particles are so small that they are unaffected by gravity and thus remain suspended. Colloid creation occurs when mixing two immiscible substance together. During mixing, one substance disseminates into the other substance and forms the previously described small particles.

For the formation of the SRT gel, we hypothesized that dissolved SRT protein would spontaneously precipitate and polymerize in a thin coat around these small, dispersed colloidal particles, so long as the dispersed phase could not dissolve the protein. These SRT-coated colloids (Figure 7) would then be isolated and mixed into a neutral, unreactive gel commonly used in 3D printing. A 3D bioprinter could then print the gel-colloid mixture into any geometry, in this case a small scaffold. Heating of the scaffold would then cause the neutral, unreactive gel to melt away and cause the hydrocarbon within the SRT coated colloids to expand. Since SRT are thermoplastic, the proteins would transform from their solid phase to their melt phase and expand along with the hydrocarbon. The expanding SRT shells would contact and combine with one another due to the self-healing properties of SRT, forming the initially printed structure without the presence of the neutral gel. Upon cooling the SRT protein shells would harden and retain their expanded shape while the hydrocarbons contract back to their original volume. The

result would be an SRT proteinaceous scaffold that could be used for various biomedical applications.

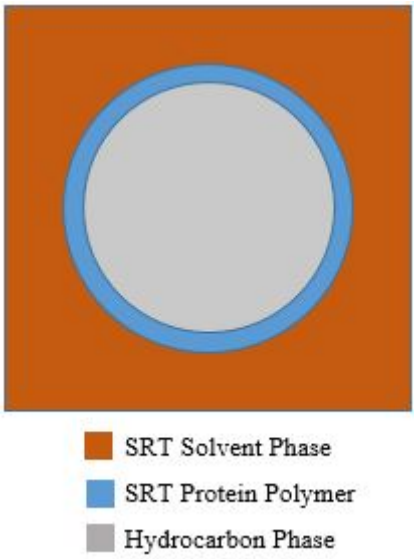


Figure 7. Cross Section of an SRT-coated Colloid Particle

Materials and Methods

To make the colloids, a hydrocarbon phase was chosen along with a second immiscible phase that could dissolve SRT. The two most commonly used substances to dissolve SRT are dimethyl sulfoxide (DMSO) and hexafluoro-2-propanol (HFIP). HFIP is highly corrosive and difficult to work with, so DMSO was chosen as the solvent for SRT. The hydrocarbon initially chosen was isopentane (Figure 8) because it had a boiling point (28 °C)[15] close to the SRT glass transition temperature (32 °C) [16], its surface tension (15 mN/m)[15] is significantly lower than that of DMSO (43.5 mN/m)[17], and because it is relatively inexpensive. Due to its differences in density and surface tension, the isopentane would form droplets in the DMSO that the SRT proteins could then coat.

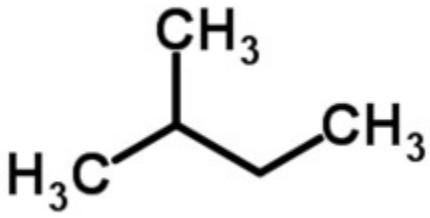


Figure 8. Structural Formula of Isopentane (2-methyl Butane)

SRT was dissolved in DMSO at a concentration of 100 mg/mL to ensure that enough SRT would be present to coat the colloid particles. This SRT/DMSO solution was then added to isopentane in a volume ratio of 4:1 isopentane to DMSO. The mixture was then stirred with a magnetic stir bar at a speed of 600 rpm for 10 minutes in order to form colloids. The coated colloid particles congregated in between the DMSO and isopentane phases once stirring ceased. The coated colloid particles were then isolated from DMSO and isopentane using a separation funnel. The separated isopentane and DMSO were combined and mixed again at 600 rpm for ten minutes, and any colloid particles that formed were again isolated using the separation funnel. Images of the colloids were captured using a microscope (Figure 9).

Results

The experimental design consisted of repeating this procedure while first varying the ratio of DMSO to isopentane, then varying the stirring speed alone, and then varying the concentration of SRT protein, all while observing the effects that these variances had on colloid properties. Following this, the process was repeated using a different hydrocarbon.

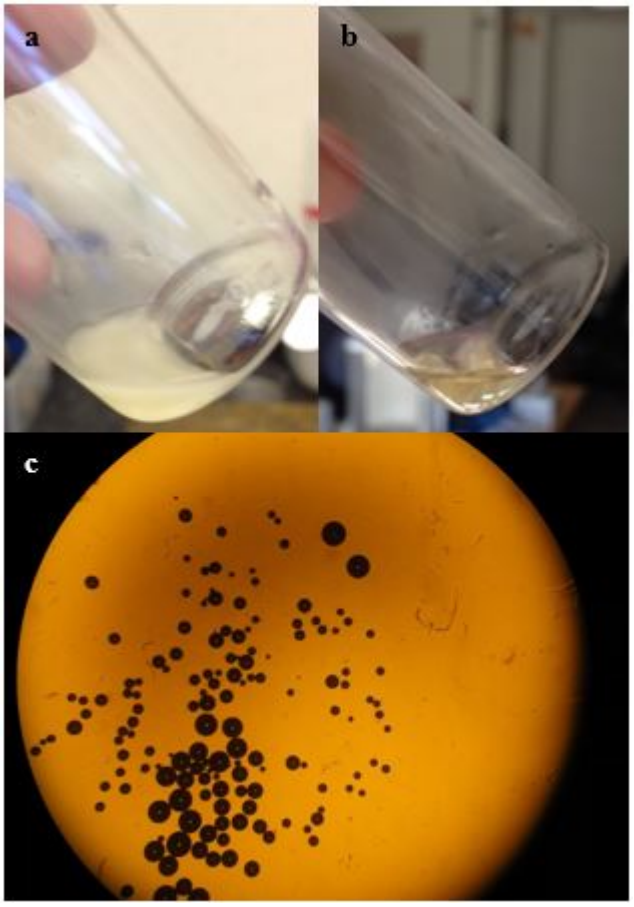


Figure 9. Colloid Formation: a) Isolated colloids b) Isolated colloids after 18 hours c) Colloids under the microscope

These variances never occurred as issues arose during the first formation of colloids. Though creation of the colloids was successful, allowing them to sit overnight seemed to cause the colloids to disappear (Figure 9). However, upon further investigation, it appeared that these SRT-coated colloids interacted with one another to form a crystalline network. These results are similar to those seen by Li *et al.*, who grew nanosized, polymer single-crystal-like capsules using poly(L-lactic acid) as a polymer and mixing *p*-xylene and water to form miniemulsions. [18]

Discussion

Though more research must be done on these SRT-coated colloids, the results are promising. The similarity in the results of SRT colloid formation to the single-crystal-like capsules produced by Li *et al*. indicated that consistent, successful creation of these SRT-colloid crystalline structures could have a number of applications. One exciting potential application is in drug delivery. Due to the biodegradable properties of SRT and the fluid nature and weak mechanical properties of colloid particles, a capsule made from SRT-colloid crystalline structures would degrade easily and harmlessly in the body to release a desired drug.

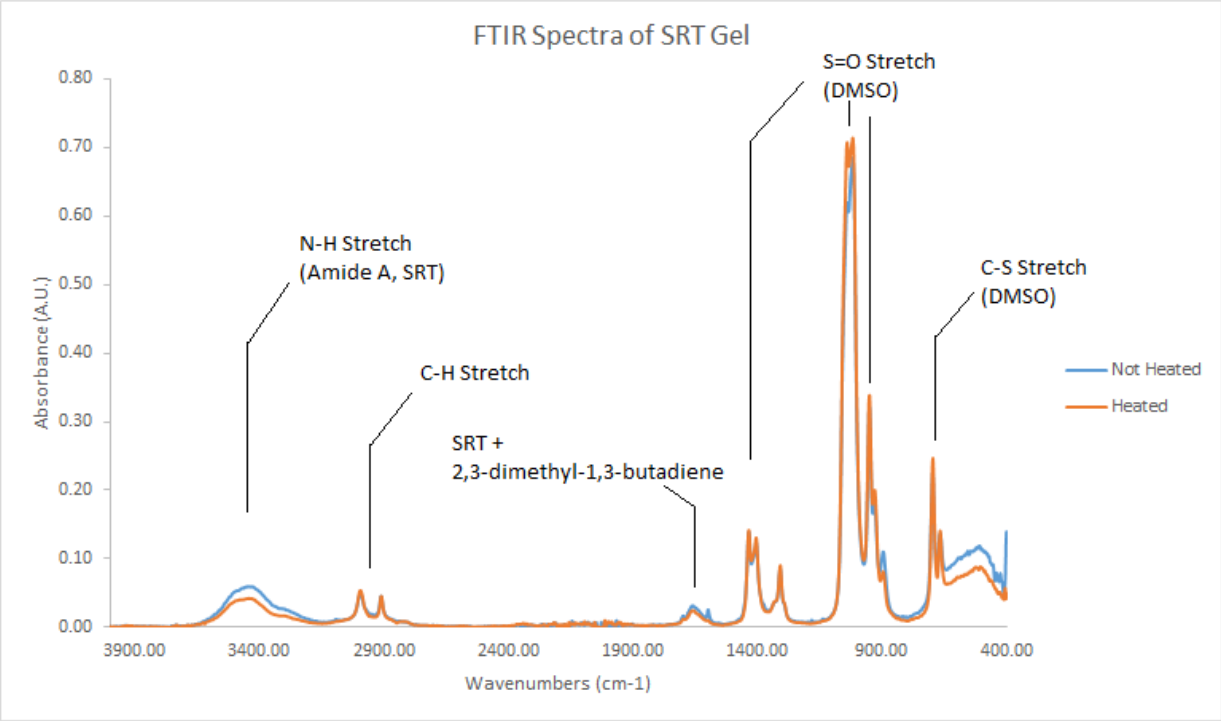
Chapter 7

Conclusion

It has become increasingly apparent that traditional polymers and plastics are not a sustainable resource for the future. Due to their unnatural origins, they do not degrade in nature and have an adverse effect on the environment and the other species within our ecosystem. In addition, their origins in petroleum and natural gas, along with the energy required to process these synthetics, makes them an inefficient resource. New, natural polymers offer a solution to this problem, and there is perhaps no better plastic alternative than squid sucker ring teeth (SRT) proteins. SRT protein polymers show remarkable strength along with unique reversible thermoplastic properties and strong adhesive properties. However, thermal processing techniques are imprecise and solution processing of SRT has a number of limitations. Thus, processing of these proteins at room temperature is desirable. Such processing was achieved in this work by forming an SRT protein gel. The gel formation mechanism was characterized as solubility dependent. The concentration dependence of the gel, along with the solubility of SRT proteins was studied in order to determine the feasibility of producing the gel at an industrial level. Finally, the SRT protein gel was 3D printed in what is the first example of 3D printing using proteinaceous material. This indicates the potential of SRT proteins in applications as advanced medical materials including scaffolds, implants, bone reattachment adhesive, and more. Early results in colloid preparation with dissolved SRT protein also indicated a potential use in drug delivery.

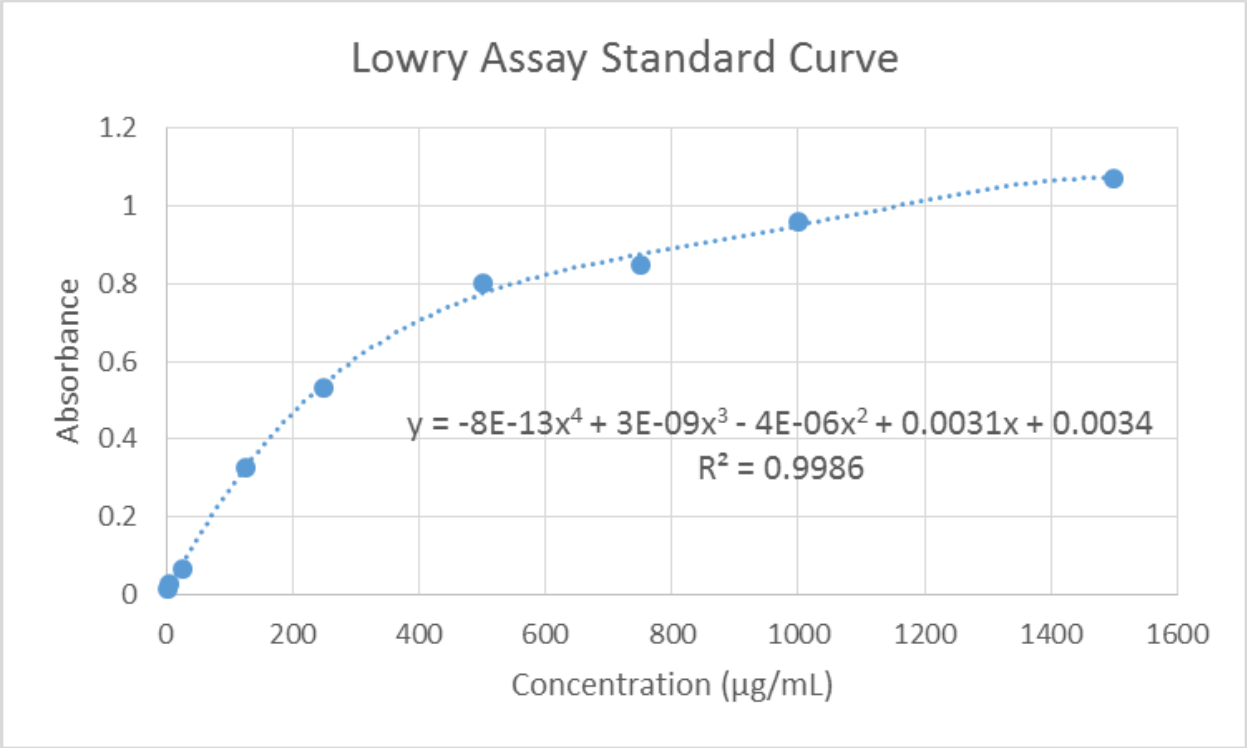
Appendix A

Full FTIR Spectra of Gels Formed using 100 mg/mL SRT in DMSO and Solutions of 2:1 DMBD to DMSO-SRT



Appendix B

Lowry Assay Standard Curve



BIBLIOGRAPHY

[1] Ryan, P. G. (1993). Monitoring the abundance of plastic debris in the marine environment. *Nature*, *361*, 23.

[2] Klemchuk, P. P. (1990). Degradable plastics: A critical review. *Polymer Degradation and Stability*, *27*(2), 183–202. http://doi.org/10.1016/01413910(90)90108J

[3] Green, D. S., Boots, B., Blockley, D. J., Rocha, C., & Thompson, R. (2015). Impacts of Discarded Plastic Bags on Marine Assemblages and Ecosystem Functioning. *Environmental Science & Technology*, *49*(9), 5380–5389. http://doi.org/10.1021/acs.est.5b00277

[4] North, E. J., & Halden, R. U. (2013). Plastics and environmental health: the road ahead. *Reviews on Environmental Health*, *28*(1), 1–8. http://doi.org/10.1515/reveh2012-0030

[5] Plastic Pollution Coalition (2016). The Movement. Retrieved from http://www.plasticpollutioncoalition.org/the-movement/

[6] Freinkel, S. (2011). Plastics: A Toxic Love Story. New York, NY: Houghton Mifflin Harcourt.

[7] Pena-Francesch, A.; Akgun, B.; Miserez, A.; Zhu, W.; Gao, H.; Demirel, M. C. (2014). Pressure Sensitive Adhesion of an Elastomeric Protein Comples Extracted From Squid Ring Teeth. *Advanced Functional Materials*, *24*(39), 6227-6233.

[8] Guerette, P.A.; Hoon, S.; Ding, D.; Amini, S.; Masic, A.; Ravi, V.; Venkatesh, B.; Weaver, J. C.; Miserez, A. (2014). Nanoconfined β-Sheets Mechanically Reinforce the

Supra-Biomolecular Network of Robust Squid Sucker Ring Teeth. *ACS Nano*, *8*(7), 7170-7179.

[9] Ding, D.; Guerette, P. A.; Hoon, S.; Kong, K. W.; Cornvik, T.; Nilsson, M.; Kumar, A.; Lescar, J.; Miserez, A. (2014). Biomimetic Production of Silk-Like Recombinant Squid Sucker Ring Teeth Proteins. *Biomacromolecules*, *15*, 3278-3289.

[10] Demirel, M.C.; Cetinkaya, M.; Pena-Francesch, A.; Jung, H. (2014). Recent Advances in Nanoscale Bioinspired Materials. *Macromol. Biosci.*, *15*(3), 300-311.

[11] Latyza, V.; Guerette, P.A.; Ding, D.; Amini, S.; Kumar, A.; Schmidt, I.; Keating, S.; Oxman, N.; Weaver, J. C.; Fratzl, P.; Miserez, A.; Masic, A. (2015). Multi-scale thermal stability of a hard thermoplastic protein-based material. *Nature Communications*, *6*, http://doi.org/10.1038/ncomms9313

[12] Sariola, V.; Pena-Francesch, A.; Jung, H.; Cetinkaya, M.; Pacheco, C.; Sitti, M.; Demirel, M. C. (2015). Segmented Molecular Design of Self-Healing Proteinaceous Materials. *Scientific Reports*, *5*, 13482 http://doi.org/10.1038/srep13482

[13] Ventola, C. L. (2014). Medical Application for 3D Printing: Current and Projected Uses. *Pharmacy and Therapeutics*, *39*(10), 704-711.

[14] Binder, J. L.; Eberly, K. C.; Smith, G. E. P., Jr. (1959). The Dimers of Isoprene. *Journal of Polymer Science*, *38*, 229-239.

[15] Shell Chemicals. (21-Nov-2007). *Isopentane* [Safety Data Sheet]. Retrieved from http://www.shell.com/business-customers/chemicals/our-products/solvents-hydrocarbon/pentanes.html Accessed November 15, 2015.

[16] Demirel, Melik C., and Abdon Pena-Francesch. Compositions and Methods Related to Proteins Capable of Reversible Transitiobn to a Melt. Patent WO2014160131 A1. 02 Oct. 2014.

[17] Adamson, A. W.; Gast, A. P.; Physical Chemistry of Surfaces; 6 Ed, Wiley , 1997.

ACADEMIC VITA

Academic Vita of Andrew Blake Tucker
Abtucker363@gmail.com

Education

B.S. in Engineering Science, B.S. in Biochemistry and Molecular Biology
Honors: Engineering Science and

Thesis Title: Production of Squid Protein Bioplastics Using 3D Printers
Thesis Supervisor: Melik Demirel

Work Experience

Pennsylvania State University State College, PA
Teaching Assistant for Physical Chemistry Fall 2014

- Attended and assisted with class twice a week, held office hours to answer questions and tutor students, communicated with students via email

Milton S. Hershey Medical Center Hershey, PA
MD/PhD Exposure Program Summer 2014 & 2015

- Investigated the role of Transient Receptor Potential Vanilloid (TRPV1) channels in osmotically induced secretion of vasopressin
- Studied brain stem neurons in rats using electrophysiological techniques
- Performed the daily tasks of the research from surgically inserting chronic catheters into rats to analyzing blood samples for electrolyte levels
- Presented research findings at the annual Experimental Biology Conference in 2015

Presentations

Presented poster titled "Osmotically-induced Vasopressin Secretion is Not Altered in Novel Knock-Out Rats Lacking Transient Receptor Potential Vanilloid (TRPV1) Channel" at Experimental Biology conference in 2015.

Awards and Achievements

American Physiological Society Excellence in Undergraduate Research Awardee 2015
American Physiological Society Outstanding Undergraduate Abstract Awardee 2015
American Heart Association Summer Undergraduate Research Fellowship Awardee 2015
American Heart Association Summer Undergraduate Research Fellowship Awardee 2014
Schreyer Honors College Academic Excellence Scholarship Awardee 2012-2016
Achieved the rank of Eagle Scout in the Boy Scouts of America
Dean's List - Fall 2012, Spring 2013, and Fall 2013, Spring 2014, Fall 2014, Spring 2015

Community Service Involvement

Member of Global Medical Brigades

- Travelled to Honduras for a week in 2016
    - Set up and ran a medical aid clinics in several underserved community to provide medical treatment to hundreds of disadvantaged Hondurans
    - Helped to build a clean water system in a rural community
- Travelled to Nicaragua for ten days in 2015
    - Set up and ran a medical aid clinic in an underserved community to provide medical treatment to hundreds of disadvantaged Nicaraguans
    - Participated in a public health project to build latrines, showers, and wash basin for a very rural Nicaraguan community